2. LITERATURE REVIEW

2.1 Taxonomy of *Dicrocoelium* spp.

The taxonomy of *Dicrocoelium* spp. (LA RUE, 1957) is as follows:

Phylum	**Plathelminthes**
Superclass	**Trematoda**
Class	**Digenea**
Superorder	**Epitheliocystida**
Order	**Plagiorchiida**
Suborder	**Plagiorchiata**
Superfamily	**Plagiorchioidea**
Family	**Dicrocoeliidae**
Subfamily	**Dicrocoeliinae**
Genus	***Dicrocoelium***

2.2 Species

Dicrocoelium dendriticum (RUDOLPHI, 1819) is the species of *Dicrocoelium* with the widest distribution, found in a range from Portugal to central Asia and also in North America. *D. hospes* (LOOSS, 1907) is present in western, central and eastern Africa, while *D. chinensis* (TANG *et al.*, 1978) is distributed in China, east Siberia and Japan. Further, *D. suppereri* (HINAIDY, 1983) and *D. orientalis,* (SUDARIKOV AND RYJIKOV, 1951) are morphologically identical with *D. chinensis* and are considered to be synonyms. Interestingly, *D. suppereri* has been recently found in a mufflon in Austria, far from localities where *D. chinensis* is usually reported. Possibly it was imported, and came to Europe with infected sika deer (*Cervus nippon*) in the 19th century.

Other members of the genus *Dicrocoelium* are avian parasites.

Dicrocoelium species differ in some morphological characteristics, geographic distribution and ecological features.

2.3 Morphology

Dicrocoelium spp. (δικροσ: bifid; κοιλια: gut) are characterised by a lancet shaped body, with an oral and a ventral sucker. The body size is 5–10 mm in length and 2–3 mm in width, semitransparent and pied, with a black uterus and white vitellaria visible to the naked eye. The eggs are oval, dark brown, typically operculate, small (38–45 μm x 22–30 μm), with two characteristic dark points (so called "eye spots"), and contain a miracidium (EUZÉBY, 1971). The species differ in the position of testis, the length of the vitellaria and the position of the ventral sucker in relation to the cirrus sack (SCHUSTER, 2002).

For long, this parasite was thought to be an immature form of *Fasciola hepatica,* as both trematodes are frequently found together in the liver of ruminants.
D. dendriticum was first described by Rudolphi in 1803, who classified a trematode, isolated by Buchholz in 1790 from a man in Weimar, with the name of *F. lanceolata.* Few years later it was classified as *Distoma hepaticum* by Rudolphi himself.
The synonymy of this parasite is complex, due to the different generic and specific denominations given (MAPES, 1951; SCHUSTER, 1987).

2.4 Life cycle

The life cycle of *D. dendriticum* is extremely complex with land molluscs and ants required as first and second intermediate hosts, respectively. Numerous studies were carried out, over more than a century, to try to elucidate its life cycle (MAPES, 1951; DEL RIO, 1967). For many years snails were considered to be the only intermediate hosts, an assumption thought to be definitive by some (MATTES, 1936; NEUHAUS, 1936). But, since KRULL and MAPES (1952) demonstrated the role of *Formica* spp. as the second intermediate host, several studies have confirmed this and much information is now available on the life cycle of *D. dendriticum* (HOHORST and LAMMLER, 1962).

The adults of *D. dendriticum* live in the liver and bile ducts of the definitive hosts (mainly ruminants, equids and lagomorphs), where they lay their embryonated eggs which pass through the intestine to be eliminated in the faeces. Egg hatching and miracidium liberation only occurs in the intestine of land molluscs of numerous species that act as first intermediate hosts. The miracidium penetrates the intestinal wall of the

mollusc and settles in the hepatopancreas, where it becomes a mother sporocyst, taking the shape of the spaces between the hepatopancreatic lobules, because it has no wall itself. This larval stage produces daughter sporocysts with their own wall in which cercariae form when they are well developed. The cercariae leave the sporocysts when they are mature and migrate to the respiratory chamber of the mollusc where they are covered in slime. The so called “slimeballs” are eliminated through the pneumostoma by respiratory movements of the snail. When these slimeballs are ingested by different species of ants, which act as second intermediate hosts, the cercariae cross the craw of the ants, lose their tail and one of them (sometimes two or three), called the "brainworm", settles in the suboesophageal ganglion of the ant, while the rest become metacercariae in the abdomen. When the temperature falls, the brainworm alters the behaviour of the ant by causing a cramp of its mandibular muscles. Due to this, the ant remains temporarily attached to grass and this can promote ingestion by the definitive host. The mature abdominal metacercariae excyst in the intestine, the young flukes migrate to the liver through the common bile duct and become adult worms in the bile ducts. When these are mature, they lay eggs which exit in the faeces of the host and this allows the life cycle to begin again.

2.5 Epidemiology

The worldwide distribution of *D. dendriticum* is due to several factors, mainly:

- Environmental factors: eggs are extremely resistant in the environment;
- Intermediate hosts**:** widespread and do not require moist land to develop as others flukes do(eg. *Fasciola*) (MANGA-GONZÀLEZ *et al*., 2001);
- Definitive hosts**:** wide range of definitive hosts - mainly ruminants, but also equids, lagomorphs, rodents, humans.

2.5.1. Environmental factors

The dispersal of eggs of *D. dendriticum* and contamination of pastures can be by domestic and wild ruminants but also by rabbits and hares (BORAY, 1985). Eggs that have passed in the faeces are highly resistant to temperature variations, can over-winter and remain infective for up to 20 months on pastures. Under field conditions it has been demonstrated that egg survival is not an age-dependent but a seasonal phenomenon (85% in winter and minimal values in summer) and that there is no relationship between

infectivity of the eggs and their age. Others have reported no loss in infectivity during a period of study of 15 months (ALUNDA and ROJO-VAZQUEZ, 1983).

2.5.2 Intermediate hosts

More than 100 mollusc species can act as the first intermediate host of *D. dendriticum* (MANGA-GONZÀLEZ *et al*., 2001).

The role played by molluscs in the epidemiology of dicrocoeliosis is very important as *D. dendriticum* egg hatching and miracidium liberation only occurs in the intestine of the molluscs that act as intermediate hosts. Moreover, the parasite multiplies enormously by asexual reproduction inside molluscs (numerous cercariae can be formed from one ingested egg). This increases the likelihood of parasite transmission.

The life history of the mollusc intermediate hosts is of great epidemiological interest, as regards both the intake of *D. dendriticum* eggs, dependent on the molluscs' activity, and the survival of the parasite in them. Species, age, nutritional state of the molluscs, infective dose, ambient temperature and relative humidity influence the development of larval stages in the first intermediate hosts (MANGA-GONZÀLEZ *et al*., 2001).

In the snail *Helicella obvia*, the infection rate for *D. dendriticum* was accurately measured over four grazing seasons in Germany (SCHUSTER, 1993). The population structure of *H. obvia* showed fluctuations, with small snails predominating from April to June, medium sized snails from July to September, and the largest snails in the spring of the following year (SCHUSTER, 1993).

Comparison between infection rate and snail age suggested that young snails in the first year were less involved in the epidemiology of dicrocoeliosis than medium-shell-diameter snails, especially in the spring of the second year (SCHUSTER, 1993). The largest snails seem to be more susceptible to *D. dendriticum*, because of their active metabolism and good nutritional conditions for developing sporocysts (ALUNDA and ROJO-VÀZQUEZ, 1983). Conversely, a decrease in infection rate during the summer of the second year can be accounted for by the death of heavily infected snails in which sporocysts cause disruption of the hepatopancreas (important for glycogen storage), then impairment of reproductive activity and shortening of life expectancy (SCHUSTER, 1992).

Based on the findings of Schuster, the development of first and second generation of sporocysts requires two years and sporocyst maturation follows the snail's life cycle, becoming active more often in spring than in summer and autumn (OTRANTO and TRAVERSA, 2002).

The importance of ants in the epidemiology of dicrocoeliosis is mainly due to their abundance, wide distribution and the fact that the alteration in their behaviour, caused by the presence of the parasite in the brain, facilitates their ingestion by definitive hosts when the infected ants are *in tetania* on plants. At least 21 ant species, mainly from the *Formica* genus, have been described as receptive to this parasite in different countries (MANGA-GONZÀLEZ *et al*., 2001).

In the second intermediate host, the size and numbers of metacercariae have been correlated to the ant species and size; the larger the ant specimens examined, the greater the number of metacercariae retrieved (SCHUSTER, 1991). Moreover, the number of *D. dendriticum* metacercariae per ant varied among the different species and even within the same one. This variability could be due to the season of year, as it is higher in summer (PARASCHIVESCU *et al.*, 1976), to the different affinity of the ant species for slimeballs (LOOS-FRANK, 1978), and to the type of vegetation and the species of ant (PARASCHIVESCU, 1978).

"Plant-topping" by ants *in tetania* is caused by the “brainworm”, a metacercaria encysted in the ant's suboesophageal ganglion, whereas the other metacercariae mature in the ant's gaster. This behaviour of the infected ants is mainly regulated by fluctuations in the ambient temperature, as a result of which the availability of metacercariae to the grazing animais has a circadian rhythm. The *tetania* of the infected ants normally occurs when solar intensity and temperature decrease at the end of the afternoon and disappears in the morning when sunshine and temperature increase. The alteration in ant behaviour favours ingestion of the parasite by the definitive host.

2.5.3 Definitive host

The ecology of *D. dendriticum* suggests that the main definitive hosts are herbivores. According to TVERDOCHLEBOV and AJUPOV (1988) the definitive host spectrum includes 19 Families from nine mammalian orders, including herbivores, carnivores and omnivores. Most common definitive hosts are ruminants, equids and lagomorphs, whereas canids, rodents, suids, primates including humans are occasional

hosts (SCHUSTER, 2002). Human infection could be related to consumption of fresh vegetables where infected ants are attached, or to particular Asian food habits involving the consumption of ants (AZIMOVA *et al*., 1988). In 2004 a German patient with gastrointestinal symptoms has been found positive to dicrocoeliosis at coprological examination, and treated with triclabendazole (RACK *et al*., 2004). Also recently, a case of biliary obstruction caused by *D. dendriticum* has been reported in a 65-year-old woman (KARADAG *et al*., 2005).

Regarding small ruminants, the most frequent definitive hosts, sheep seem to be more susceptible to *D. dendriticum* than goats, although no substantial differences have been registered in terms of worm burden and EPG count (JITHENDRAN and BHAT, 1996).

Animal age and relative susceptibility to the parasite have not yet been fully elucidated. Some authors recorded a higher mean parasitic burden in lambs than in adults (MANGA-GONZÀLEZ *et al*., 1991) and in cattle aged up to two years than in older animals. By contrast, another investigation indicated an increase in prevalence depending on animal age, from 24.2% in calves younger than 18 months, to more than 70% in animals older than six years (DUCOMMUN and PFISTER, 1991). These results may indicate that the age of infected animals influences the egg output rate.

Stress-inducing factors, such as animal transportation and confinement, enhanced *Dicrocoelium* egg production, probably by inducing immune depression in animals (SOTIRAKI *et al*., 1999).

Recently, the relation between parasite burden and egg output was assessed by necropsy and faecal analysis in two groups of lambs experimentally infected with different doses of metacercariae (i.e. 1000 and 3000 metacercariae). Egg elimination, which started about two months after the infection, was demonstrated to be higher in lambs infected with 3000 metacercariae than in those infected with 1000 metacercariae and the mean number of parasites recovered at necropsy (at 120–180 days post infection) from the first group was higher than from the second group (CAMPO *et al.*, 2000).

Sex has also been linked to the susceptibility of animals to the small liver fluke. In different investigations the sex of cattle was related to the effect on the incidence of dicrocoeliosis by *D. hospes* (ASANJI and WILLIAMS, 1984) and *D. dendriticum*

(DUCOMMUN and PFISTER, 1991). Necroscopic examination of 2033 livers from cattle, naturally infected with *D. dendriticum*, revealed a prevalence of dicrocoeliosis of 62.5% in females and 9.8% in males. The higher rate in females may be because dairy heifers and cows graze for several seasons, acquiring infection, while the steers and oxen spend a considerable period of their lives in fattening units (DUCOMMUN and PFISTER, 1991). Seasonality of infection is favoured by movement of animals from lowland to mountain pastures where they become infected by the ants and then bring the infection back to the valley during the winter. Some species of intermediate hosts (the snails *H. obvia* and *Cionella lubrica* and the ants *F. pratensis* and *F. cunicularia*) are present in mountain pastures up to altitudes of 1800–2600 m.a.s.l. in Europe, e.g. Switzerland, Spain and Austria and in India (ECKERT and HERTZBERG, 1994; JITHENDRAN and BHAT, 1996). The transhumance seems to predispose animals to infection, not only because of the presence of intermediate hosts, but also because of the high stress induced by the transhumance on pasture-grazing nomadic sheep and goats.

2.6 Pathogenesis, clinical findings and lesions

Compared with other flukes the hepatic lesions caused by *D. dendriticum* often remain undetected. In fact, it is relatively difficult to produce experimental infections to investigate the pathogenesis of *D. dendriticum,* and field infections are mostly mixed infections of various and more pathogenic helminths that mask the merely *Dicrocoelium*-induced symptoms and lesions. The behaviour of young flukes may be the cause of lack of evident lesions and symptoms, in fact the juvenile flukes migrate directly up the biliary duct system of the liver without penetrating the gut wall, liver capsule, or liver parenchyma as in the case of fasciolosis (THEODORIDIS *et al.*, 1991).

Other authors remark that in very heavy infections the pathological changes of the liver result in thickened main hepatic ducts combined with enlargement of their mucosa, glandular proliferation, increase of connective and muscle tissue, cellular infiltration, and proliferation of small bile ducts (WOLFF *et al.*, 1984; CAMARA *et al.*, 1996). Due to its buccal stilets, the small liver fluke may also irritate the bile duct surfaces, causing proliferation and changes in the septal bile ducts of the lobular hepatic edges. In prolonged infection, fibrosis and cirrhosis of the parenchyma are reported to have occurred (WOLFF *et al.*, 1984).

A direct correlation was observed between worm burden and lesion scores in infected animals. Five different degrees of macroscopic liver lesions were classified, from normal liver to indurated liver with scarring, markedly distended liver ducts thickened with severe fibrosis and heavy worm burden (JITHENDRAN and BHAT, 1996). Modifications of bile duct surfaces and fibrotic lesions of the liver were observed to increase with changes in the level of infection up to 300 *D. dendriticum* while, above this value up to 600 flukes, a decrease was observed, perhaps due to the host's reaction (CAMARA *et al*., 1996). In heavy infections a large number of worms are detectable inside the bile ducts and gall bladder, the liver is swollen, with thickened ducts, cholangitis, whitish spots on the surface, marked scarring and cirrhosis resulting in liver impairment (JITHENDRAN and BHAT, 1996).

In experimentally infected hamsters, parasite-induced pathological changes were characterised by bile duct proliferation, enlargement of the surface area, infiltration by lymphocytes, macrophages and eosinophils into the portal tracts, collagen infiltration of portal tracts and interlobular septa, liver atrophy (SANCHEZ-CAMPOS *et al*., 2000). Chronic inflammation may be related to oxidative alterations. A decrease in superoxide dismutase activity in both the cytosol and mitochondria was registered, indicating inefficient scavenging of reactive oxygen, leading to oxidative liver damage and an increase in alanine transaminase and aspartate transaminase activities (SANCHEZ-CAMPOS *et al*., 1999).

Besides necropsy findings, some studies have been conducted to evaluate the loss of important body constituents (i.e. plasma proteins) caused by dicrocoeliosis. These were detected by radioisotopic techniques used to evaluate the pathophysiological patterns in animals naturally infected by *D. dendriticum* (DARGIE, 1975). After injecting sheep with ^{51}Cr-labelled red cells, ^{125}I-labelled albumin and ^{59}Fe-citrate (red cells iron incorporation rates), no statistically significant reduction in red cell survival times and plasma albumin was detected (THEODORIDIS *et al.*, 1991). It was concluded that burdens of up to 4000 *D. dendriticum* do not cause significant blood or plasma protein losses in sheep and that the alteration in those parameters registered during infection with *F. hepatica* (HOLMES *et al.*, 1968) and *Haemonchus contortus* (DARGIE and ALLONBY, 1975) may be due to the greater pathogenicity of the latter two helminths.

2.7 Diagnosis

Dicrocoeliosis often remains clinically undetected or undiagnosed, probably because of its usually subclinical manifestation (DUCOMMUN and PFISTER, 1991). Its diagnosis is mainly due to recovering adults in the liver at necropsy or detecting eggs at coprological examination.

Trials on indirect diagnostic tests have been done but validation is still needed.

2.7.1 Direct methods

The most commonly used technique for the diagnosis of dicrocoeliosis is coprological examination. This can reveal the presence of small (40 mm×25 mm), thick-walled, yellowish-brown eggs, which contain a miracidium. Usually sedimentation techniques are used, but recent studies have proved that sedimentation technique has a very low *Dicrocoelium* recovery rate (42.2±1.5%), compared with the flotation method (REHBEIN *et al.*, 1999). Rehbein tested three different high density solutions, $ZnSO_4$ (sp.g. 1.3–1.45), K_2CO_3 (sp.g. 1.45) and HgI_2/Kl (sp.g. 1.44), as modified McMaster methods for faecal egg counts in sheep faeces (REHBEIN *et al.*, 1999). The HgI_2/Kl solution gave the highest percentage rate of egg recovery from the faeces (91.2 ± 9.4%) and there was no significant influence of flotation time on the egg count.

Recently some Italian researchers (CRINGOLI *et al*., 2004) conducted a study to evaluate the influence of flotation solution, sample dilution, and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of *D. dendriticum* in a composite sample of faeces from naturally infected sheep. The findings of this study showed that the highest reliability of the McMaster technique for estimating *D. dendriticum* egg counts in faeces from pastured sheep is obtained when using flotation solutions based on HgI_2/Kl, dilutions which do not exceed 1:15, and the McMaster slide area (volume) of 1.0 ml.

In lambs experimentally infected with different doses of metacercariae (i.e. 1000 and 3000 metacercariae) egg elimination started 49–79 days post infection(d.p.i.) (mean = 59 d.p.i.), indicating that a negative coprological examination cannot assure true parasitological negativity (ROJO *et al*., 1981; CAMPO *et al.*, 2000); these data are in agreement with the prepatent period reported by another author (TARRY, 1969). By

comparing the results of faecal examination to the liver necropsy of sheep and goats, the first procedure proved to detect the presence of dicrocoeliosis only in one out of three cases (JITHENDRAN and BHAT, 1996).

2.7.2 Indirect methods

Indirect diagnostic methods of dicrocoeliosis still need validation. Studies on immunological methods for detection of antibodies against *D. dendriticum* are scant and relatively recent, developed only in the last 30 years (OTRANTO and TRAVERSA, 2002). Humoral immune response to experimental *D. dendriticum* infection was first studied by RENZ (1972) who investigated complement fixing antibodies in rabbits. Later CALAMEL (1977) used immuno-fluorescence to follow the humoral immune response in lambs entering *D. dendriticum*-infected pastures for the first time (CALAMEL, 1977). The results obtained are difficult to evaluate, because that was not an experimental infection and the exact moment of infection is not known. In addition, the presence of antibodies in the lambs could be due to passive immunity from infected mothers, as indicated by studies carried out by BALDELLI *et al.* (1981) using ELISA on lambs born to naturally infected mothers. An ELISA technique for diagnosis was also used by SAVITSKII and PONOMAREVA (1984), and HARALAMPIDIS (1987).

Other authors have used immunological techniques to diagnose dicrocoeliosis in naturally infected flocks of sheep, such as MAÑAS-ALMENDROS (1980) who used latex test, obtaining high sensitivity, although low specificity because of cross-reactivity with *F. hepatica.*

The formation of humoral antibodies has also been studied in golden hamsters naturally infected with *D. dendriticum* using precipitation, haemagglutination, complement fixation and ELISA tests (SCHRÖDER and GEYER 1976; SCHRÖDER 1979, BODE and GEYER 1981). The kinetics of antibodies in the serum of rabbits experimentally infected with this parasite has been followed using several techniques, although ELISA and passive haemagglutination were the most specific (SAVITSKII, 1983).

Regarding the use of different antigens to evaluate the immune response in dicrocoeliosis, most of the immunodiagnostic tests used by the different authors employed somatic extracts of adult flukes (PIERGILI FIORETTI *et al*. 1980; BALDELLI *et al.* 1981; JITHENDRAN *et al.* 1996); others used aqueous crude extract of eggs as antigen

(BODE and GEYER, 1981). WEDRYCHOWICZ (1995) studied the bile antibody responses of naturally infected cattle to somatic (So.), excretory-secretory (E/S) and surface antigens of adult *D. dendriticum* and defined the spectra of these products. Moreover, WEDRYCHOWICZ (1984) studied the bile, faecal and serum antibody responses of sheep naturally infected by *D. dendriticum* both against somatic proteins, surface proteins and glycoproteins and against excretions and secretions of adult *D. dendriticum.*

Recently the study by Gonzàlez-Lanza (GONZÀLEZ-LANZA *et al.*, 2000) well clarified some aspects of the immunological diagnosis of dicrocoeliosis. The *D. dendriticum* diagnosis by ELISA test in experimentally infected lambs was 20 days earlier than coprological diagnosis using the classical sedimentation method. Two different antigens were used: excretory/secretory antigen and somatic antigen. The antibody titres peaked 60 days post infection (p.i.) in both cases and remained high until the end of the trial (180 days p.i.). No correlation between parasitic burden and antibody response was observed (GONZÀLEZ-LANZA *et al.*, 2000).

Recently somatic and excretory-secretory antigens of *D. dendriticum* were analyzed by SDS-PAGE and Western blot analysis (REVILLA-NUIN *et al.*, 2005). Western blot analysis using sera of ovine infected with *D. dendriticum* revealed eight main antigenic polypeptides ranging from 24 to 205 kDa for somatic antigen and seven for excretory/secretory antigens with apparent molecular mass in the range of 26-205 kDa. A specific parasite protein with an approximate molecular weight of 130kDa in SDS-PAGE gels was detected, that could be used for *D. dendriticum* infection diagnosis.

2.7.2.1 The indirect ELISA - Enzyme-Linked Immunosorbent Assay

Among the indirect diagnostic methods, the indirect ELISA is a sensitive and simple technique for quantitative antibody investigation. (ENGVALL and PERLMANN, 1972b). According to KEMENY and CHANTLER (1988) test parameters as implementation, robustness, specificity and reproducibility are more important than sensitivity and quantitative measurement of many samples using high concentration of antibody and antigen.

The indirect ELISA, also called Sandwich-ELISA is the simplest form of

enzyme-linked immune test (ENGVALL and PERLMANN, 1972a). As first step the antigen is produced, in the case of excretory/secretory antigen of *D. dendriticum*, by means of purified products from worm culture. The somatic antigen, on the other hand, is produced by sonication of whole worm suspension, and then filtration by cellulose membranes. As second step, the purified antigen is bound to the microtiter plate through incubation. Then antibodies of serum samples are allowed to react with the antigen. After each coating process the microtiter plate is washed carefully, in order to eliminate unbound material (KEMENY and CHANTLER, 1988). The specificity of antigen-antibody reaction can be improved through antigen purification. As indicator for the antigen-antibody reaction, enzyme-linked antibody solutions (conjugate) are used, depending on animal species. After adding the proper substrate for the enzyme (e.g. peroxidase or alkaline phosphatase), the colourimetric reaction takes place, it is proportional to the amount of antigen-antibody complexes, and can be quantitatively read by a photometer (KEMENY and CHANTLER; 1988; ROLLE and MAYR, 1993).

2.7.2.2 Enzyme-Assisted Immunoelectroblotting (IEB or Western Blotting)

This diagnostic method is composed of three main phases: protein separation by Polyacrylamide Gel Electrophoresis (SDS PAGE); transfer of protein bands onto proper membrane (Blotting); immunoassay to detect antibody-antigen reaction.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the nett charge, size and shape of the molecules and on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used to study the properties of a single charged species, and as a separation technique.

Support Matrices

The most commonly used support matrices, agarose and polyacrylamide, provide a means of separating molecules by size, in that they are porous gels. A porous gel may act as a sieve by retarding, or in some cases completely obstructing, the movement of large macromolecules while allowing smaller molecules to migrate freely. Polyacrylamide, which is easy to handle and to make at higher concentrations, is used to

separate most proteins and small oligonucleotides that require a small gel pore size for retardation.

Separation of Proteins

Proteins are amphoteric compounds; their charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The charge carried by a protein is in addition independent of its size, ie: the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecules differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both the size and the charge of the molecules.

Separation of Proteins under denaturing conditions

Sodium dodecyl sulphate (SDS) is an anionic detergent, which denatures proteins by wrapping around the polypeptide backbone; SDS binds to proteins specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size. This is done with 2-mercaptoethanol or dithiothreitol, both strong reducing compounds. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

Continuous and Discontinuous Buffer Systems

There are two types of buffer systems in electrophoresis, continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system.

Electroblotting

Electroblotting has been carried out by many laboratories for some time, and there are a large number of different instruments that efficiently transfer proteins (or other macromolecules) transversely from gel to membrane. Most of these, however, are based on the design of TOWBIN *et al*. (1979), a system with electrodes dipped in a large tank.

"Semi-dry" or "horizontal" blotting has been recently introduced. With this technique, one uses two plate electrodes (stainless steel or graphite/carbon) to produce a uniform electrical field over a short distance, and sandwiches between these of up to six gel/membrane/filter paper assemblies, all well soaked in transfer buffer. The assembly is clamped, and electrophoretic transfer effected in this position, using as transfer buffer only the liquid contained in the gel and filter papers or other pads in the assembly.

There are a number of advantages to this procedure over the conventional up-right protocol, not the least of which is that as little as a couple of hundred millilitres of buffer are all that is needed for electroblotting several gels, compared to as much as five litres for some commercial kits. In addition, several gels can be blotted simultaneously, electrodes can be cheap carbon blocks and less power is required for transfer.

Immunoassay

The reason for transferring proteins to membranes from gels is so as to be able to access them more efficiently with various probes, as polyacrylamide is not particularly amenable to the diffusion of large molecules. The most popular types of probes for immobilised proteins are antibodies of one type or another: the attachment of specific antibodies to specific immobilised antigens can be readily visualised by indirect enzyme immunoassay techniques, usually using a chromogenic substrate, which produces an insoluble product. Probes for the detection of antibody binding can be conjugated anti-immunoglobulins.

The immunoassay is normally done by blocking the transfer membrane with a concentrated protein solution (e.g. 10% foetal calf serum, 5% non-fat milk powder) to prevent further non-specific binding of proteins. This is followed by incubation of the membrane in a diluted antiserum/antibody solution, washing of the membrane, incubation in diluted conjugated probe antibody or other detecting reagent, further washing, and the colorimetric/autoradiographic/chemiluminescent detection.

The power of the technique lies in its ability to detect simultaneously a specific protein by means of its antigenicity, and its molecular mass; proteins are first separated by mass in the SDS-PAGE, and then specifically detected in the immunoassay step (RYBICKI and PURVES, 1996).

2.8 Treatment and control of dicrocoeliosis

Control of the small liver fluke is difficult and unsatisfactory mainly because of the complexity of its biological life cycle and epidemiology.

Control has been based mainly on husbandry practices (avoid grazing early in the morning or late in the evening, when infection by ants is most likely), on controlling the first and second intermediate hosts, and on the treatment of animals.

As in the case of lungworms and liver fluke (CABARET and GALKIN-CABARET, 1985), soil maps should be assessed to detect the presence of calcareous soil, which are suitable habitats for *D. Dendriticum* intermediate hosts.

Methods to control the intermediate hosts may be feasible in small but their high cost prevents large-scale use. Control of intermediate hosts by chemicals is not advisable for ecological and economic reasons. One control strategy might be the use of turkeys, chickens, geese and ducks into small pastures to eat snails and ants. In small areas, covering ant nests with branches of trees and bushes may prevent or reduce the infection of livestock since ants affected by metacercariae-induced cataleptic cramp are found mainly within 30–50 cm from the nest base (BADIE and RODELAUD, 1988).

In practical terms, prophylaxis in endemic areas is based on strategic treatment of all animals exposed directly (by free grazing) to infection. Among the anthelmintic drugs, many benzimidazole (albendazole, triclabendazole, fenbendazole, mebendazole, cambendazole, thiabendazole) and pro-benzimidazole (thiophanate) derivatives have been used against *D. dendriticum*, at higher doses than against tapeworms, flukes, lung and gastrointestinal nematodes (ONAR, 1990).

Albendazole, administered at dose rates of 15 and 20 mg/kg *per os* or intraruminally, caused a mean burden reduction of *D. dendriticum* in livers of 98.2% and 99.6%, respectively (HIMONAS and LIAKOS, 1980; SCHUSTER, 1992) while a single dose of 10 mg/kg *per os* decreased parasitic burdens by 92.2% (CORDERO, 1980).

The efficacy of intraruminal slow-release capsules of albendazole against sheep gastrointestinal nematodes and small liver fluke and their effects on productivity

(cheese and wool production) were tested on 300 ewes (ČORBA *et al*., 1991). Treated animals had negative coprological tests for ten weeks after the administration of capsules and an efficacy of 96.2–99.2% was recorded at necropsies, against gastrointestinal nematodes (*Nematodirus* spp., *Oesophagostomum* spp., *Cooperia* spp., *Trichostrongylus* spp. and *Trichuris ovis*), and of 88.5% against *D. dendriticum*. An increase in production levels of treated animals was also observed, together with a reduction of pasture contamination with nematode larvae. The efficacy of these intraruminal albendazole capsules was then assessed specifically against heavy *D. dendriticum* infections; a significant decrease in EPG was reported during the first two weeks with an efficacy of 91.8% at liver dissection (ČORBA and KRUPICER, 1992). The absence, in some countries, of an albendazole withdrawal period for milk and meat production, is important for the management and safety of sheep products; the danger of fostering resistance of the parasites to anthelmintics must also be taken into consideration, when using such capsules and boluses.

Triclabendazole, another benzimidazole derivative, proved to be a very effective fasciolicide, against mature and immature liver flukes at single oral doses of 5 and 10 mg/kg, although no efficacy was found against dicrocoeliosis and paramphistomatosis (GÜRALP and TINAR, 1983).

A single oral dose (40 mg/kg) of thiophanate in cattle, goats and sheep was effective against the small liver fluke especially in sheep. The rate of metabolic change of the drug into ethyl-benzimidazol carbamate, its main metabolite, was 57% for cattle, 52% for goats and 34% for sheep (DELATOUR *et al*., 1988).

The efficacy of thiophanate (50 mg/kg) and albendazole (5 mg/kg, repeated after one week) against *D. dendriticum,* was compared in heavily infected sheep. On day 28 treated and control animals were slaughtered and *Dicrocoelium* numbers were observed to have decreased by 74.4% and 12.7% in the thiophanate and albendazole treated groups relative to the untreated control animals (ONAR, 1990). Both drugs were highly effective against gastrointestinal nematodes, although albendazole used at the currently recommended dosage, was not effective against *D. dendriticum*. The choice between these two drugs also depends on the concomitant presence or absence of other endoparasites, such as cestodes or *F. hepatica* against which albendazole, unlike thiophanate, is effective.

A combination of thiophanate and brotianide (50.0 mg and 5.6 mg/kg, respectively) was demonstrated to have a higher efficacy against mature *D. dendriticum* (100%) and *F. hepatica* (99.8%) than tetramisole and oxyclozanide (15 mg/kg for both) (TINAR *et al*., 1988).

The activity of a single oral treatment (7.5, 10.0 and 12.5 mg/kg) of luxabendazole was tested against gastrointestinal and lung nematodes and liver flukes in naturally infected sheep. Although at all the doses employed, luxabendazole was 100% effective against gastrointestinal and lung nematodes (with the exception of *Strongyloides papillosus*, efficacy of 79.7–87.6%), the flukicidal efficacy was less satisfactory against *D. dendriticum* (efficacy of 64.6%, 63.2% and 83.8% at the doses of 7.5, 10.0 and 12.5 mg/kg, respectively). Doses of 2×12.5 mg/kg one week apart produced satisfactory results against *D. dendriticum* infection (KASSAI *et al*., 1988).

Netobimin (a nitrophenyllunidine compound) has been used for the treatment of dicrocoeliosis in sheep both at a dose of 20 mg/kg (SANZ *et al*., 1987) and 15 mg/kg (ROJO-VAZQUEZ *et al*., 1989) with good results. After a single treatment with netobimin (15 mg/kg), a decrease was observed in faecal egg counts on days 2 and 4 with altered egg morphology (integrity, absence of germinal cell masses, etc.). The mean fluke burden was significantly different ($p<0.05$) at necropsy in treated (98) and untreated (1215) sheep, with an efficacy of 91.9% (ROJO-VAZQUEZ *et al*., 1989).

Based on the epidemiology of *Dicrocoelium* and of its intermediate hosts, treatments are suggested two or three times a year at the beginning of spring and before housing the animals in autumn. Transhumant sheep and goat flocks should be treated once they reach the valley (JITHENDRAN and BHAT, 1996).

To date, no vaccination strategies have been studied against dicrocoeliosis even though an antibody-dependent response against *D. dendriticum* has been reported.