CHAPTER III
LITERATURES REVIEW

1. Biology of *Fasciola* spp.

1.1 Definition and Classification of *Fasciola* spp.

Fasciolosis, caused by *Fasciola* spp., is an economically important disease of domestic livestock, in particular cattle and sheep. Human are accidental host. *Fasciola* spp. are desinated to kingdom Animalia, phylum Plathyhelminthes, class Trematoda, subclass Digenea, family Fasciolidae and genus *Fasciola*. The two species most commonly implicated as the etiological agents of fasciolosis are *Fasciola hepatica* and *Fasciola gigantica*.

The parasites in the subclass Digenea are characterized by a complex life cycle in which one or more intermediate hosts are involved. This differentiates them from the subclass Aspidogastrea. Several variations of the life cycle exist, but generally require a molluscan primary or intermediate host in which larval multiplication occurs and vertebrate final or definitive host in which sexual reproduction occurs. Members of the family Fasciolidae are hermaphroditic and self-fertilization may occur, although sexual reproduction is normally achieved by cross-fertilization in the final host.

1.2 Geographic distribution of *Fasciola* spp.

The study of the epidemiology of fasciolosis in livestock encompasses the factors that affect the prevalence and intensity of infection and how these impact the animals both in terms of clinical disease and the economic effects of productivity losses.

Fasciola spp. can be found worldwide but the two mentioned species differ in their geographic distribution. *F. hepatica* predominates in the temperate zone (Table 1 and Figure 1) while *F. gigantica* is restricted to tropical regions and has been recorded in Africa, the Middle East, Eastern Europe and South, East, and Southeast Asia including Laos, Cambodia, and Thailand (Table 2 and Figure 1).

Table 1 Recorded prevalence of *Fasciola hepatica*

Country/region	Types of animal	Prevalence (%)	Reference
Africa			
Morocco	Cattle	10.4	Moukrim and Rondelaud
	Goats	17.1-23.8	(1991), Khallaayoune *et al*.
			(1991)
Americas			
Canada (Quebec)	Dairy cows	Up to 68	Bouvry and Rau (1986)
Brazil (state of Parana)	Cattle	0.95	Luz *et al*. (1992)
	Buffalo	19.8	
Chile	Cattle	Up to 94	Alcano (1985)
Jamaica	Cattle	22.2	Bundy *et al*. (1983)
	Goats	17.2	
	Sheep	0.72	
Mexico	Cattle	5.2	Encinas-Garcia *et al*. (1989)
Peru	Cattle	29	Leguía *et al*. (1989)
USA			
California	Beef cattle	52.7	Briskey *et al*. (1994)
Colorado		5.9	
Idaho		36.7	
Nebraska		19	
Texas		15.6-17.3	
Florida		68	Kaplan (1994)
Montana		17.2	Knapp *et al*. (1992)
Asia and Australasia			
Kursk region of the	Cattle	30.5	Bausov *et al*. (1981)
former USSR	Sheep	16.7	
Iraq	Sheep	14.4	A-Al-Bayati *et al*. (1991)
Jammu and Kashmir,	Sheep	30	Pandit *et al*. (1989)
India			
New Zealand	Cattle	8.5	Mitchell (1995)
	Sheep	4.4	

Country/region	Types of animal	Prevalence (%)	Reference
Queensland, Australia	Cattle	1.1	Baldock and Arthur (1985)
Turkey	Cattle, buffalo and Sheep	29.3	Celeb and Ultav (1988)
Europe			
Belgium	Double muscled beef cattle	12.5	Genicot *et al*. (1991)
Germany	Cattle	10.7	Simmank (1987)
Ireland	Cattle	45	Department of Agriculture (Ireland) figure (unpublished)
Italy	Cattle	5	Poglayen *et al*. (1995)
Poland	Cattle	7.2	Konopka (1993)
	Sheep	3.7	
Spain	Cattle	29.5	González *et al*. (1989)
	Sheep	14.7	Manga *et al*. (1990)
United Kingdom	Cattle	10	Taylor (1989)

Source: Torgerson and Claxton, 1999

Table 2 Recorded prevalence of *Fasciola gigantica*

Country/region	Types of animal	Prevalence (%)	Reference
Africa			
Egypt	Cattle	4-6	Lofti *et al*. (1995)
	Buffalo	2-5	
	Sheep	1-2	
	Goats	Up to 1	
	Donkeys	4.5	Hasslinger and Elseify (1996)
Gambia	Cattle	20	Ndao *et al*. (1995)
Kenya	Cattle	12	Waruiru *et al*. (1993)
Nigeria	Cattle, sheep and goats	43	Nwosu and Srivastava (1993)
Zimbabwe	Cattle	65.2	Vassilev (1994)
Asia			
Bangladesh	Cattle	44	Chowdhury *et al*. (1994)
India			
Uttar Pradesh	Buffalo	39.0	Bhatia *et al*. (1989)
	Goats	57.3	
	Sheep	81.4	
Maharashtra	Buffalo	7.4	Ratnaparkhi *et al*. (1993)
	Cattle	18.18	
	Sheep	8.87-11.32	
	Goats	25.76	
Iraq	Sheep	3	A-Al-Bayati *et al*. (1991)
Nepal	Buffalo	33.3-71.4	Shrestha *et al*. (1992)
	Cattle	24.7-28.6	
Thailand	Buffalo	47.1	Sukhapesna *et al*. (1990)

Source: Torgerson and Claxton, 1999

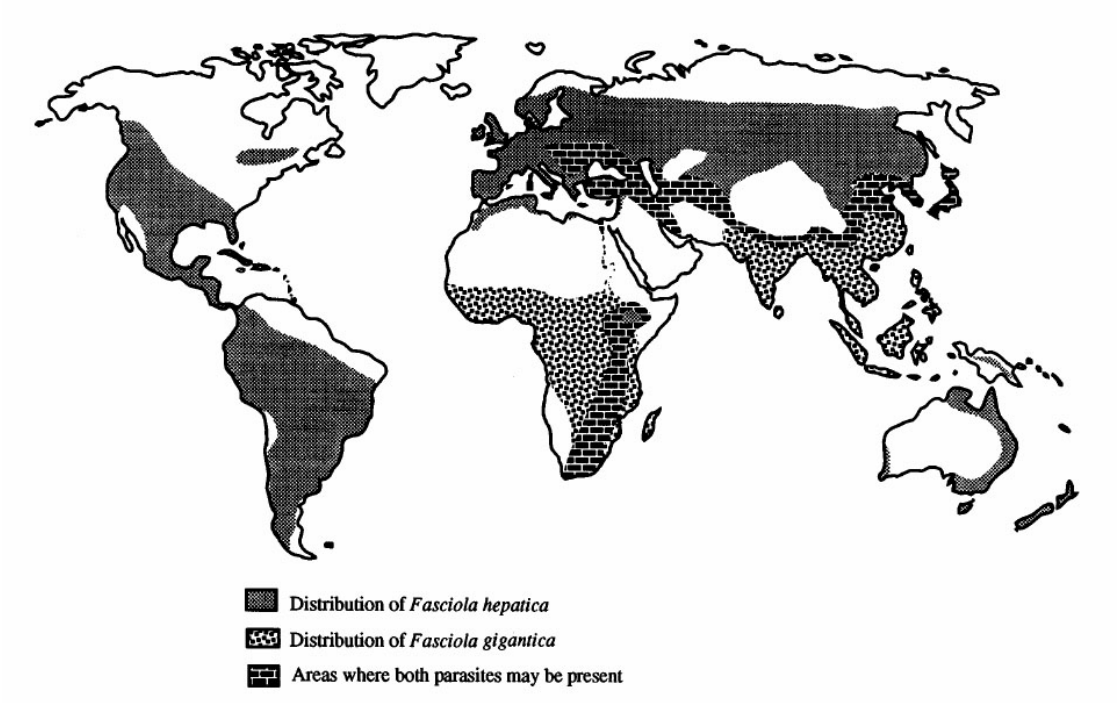


Figure 1 The geographical distribution of *Fasciola hepatica* and *F. gigantica*
Source: Torgerson and Claxton, 1999

1.3 Life cycle and Morphology of *Fasciola* spp. (Figure 2)

The adult parasites are hermaphrodites and have a transparent, flat leaf-like body. The smaller species, *F. hepatica*, is 20 to 30 mm long and 8 to 15 mm wide. It has an anterior elongation (a cephalic cone) on which the oral and ventral suckers are located, these being approximately of equal size. The intestine of the adult parasite is highly branched, with numerous diverticulae extending from the extreme anterior to the extreme posterior of the parasite body. The two testes, also highly branched, are located in tandem in the posterior half of the body. The smaller branched ovary is located just above the testes and is linked to a short convoluted uterus opening to a genital pore above the ventral sucker. The vitellaria are highly diffuse and branched in the lateral and posterior region of the body.

F. gigantica is similar to *F. hepatica*, but much larger, reaching 25 to 75 mm body length and 15 mm body width. In addition the cephalic cone is proportionally shorter than that of *F. hepatica*, the body is even more leaf-like in shape (Figure 3*a*). The egg is also very similar to that of *F. hepatica*, but again is larger in size. Both species live in liver bile of mammalian definitive hosts (cattle, sheep and occasional human).

The eggs, laid by the adult parasites, are passed from the common bile duct into the duodenum and subsequently into the feces. The egg, which is covered by a rigid eggshell, contains a fertilized oocyte surrounded by vitelline cells and a large number of yolk granules. Eggs are yellowish brown in color, oval in shape, 130-145 μm long and 70-90 μm wide, and have an indistinct operculum (Figure 3*b*). The eggs are released with the feces in natural water resources. The embryogenesis of the parasite is influenced by environmental conditions, *e.g.* temperature, humidity, oxygen concentration and pH.

The miracidia develop within 2 to 3 weeks at 23-26°C, pH 7.0 and enough moisture and oxygen (Thomas, 1883a, 1883b; Al-Habbib, 1974). Light and temperature stimulate hatching of fully embryonated eggs (Jepps, 1993).

The miracidium is about 130 μm in length, broad at the anterior end and tapering posteriorly to a blunt end. The cuticle is ciliated, and there is an anterior papilliform protrusion and a pair of darkly stained eye spots visible near the anterior end of the body (Figure 3*c*). After hatching from the egg the miracidium immediately starts to

swim and tries to find the first intermediate host, a snail of the genus *Lymnaea* (Figure 3*d*). If it cannot infect a snail within 24 hours it will die (Thomas, 1883a, 1883b; Hop Cowdery *et al*., 1978).

The miracidia develop to sporocysts inside the snail. The young sporocyst migrates *via* the blood vessels or lymph channels primarily to the digestive gland (often referred to as the liver). A sporocyst consists initially of a tiny ball of tightly packed germinal cells in which remnants of the eye spots can be seen, each germinal cell gives rise to a ball of new germinal cells from which the next larval stages, the rediae, develop.

The redia is roughly cylindrical in shape with a length of 1-3 mm and possesses a pair of marginal lappets (bulging projections) at the posterior end and a raised collar-like structure just behind the anterior end of the body. There is a mouth which leads into a muscular pharynx and posteriorly to a simple unbranched intestine (Figure 3*e*). The body of the redia contains numerous germinal cells which multiply to form germinal balls from which the larval stage, the cercariae, are produced (Thomas, 1883a, 1883b). Each redia produces 16 to 20 cercariae.

The cercaria is tadpole-like with a discoidal body and a long tail. The body measures 250-350 μm in length, the tail is twice as long (~500 μm) and an oral and a ventral sucker in the centre of the body (as in the adult parasite) are present (Figure 3*f*).

After 4 to 7 weeks after infection, the cercariae emerge from the snail. They swim freely in the water and settle on various objects, *e.g*. blades of grass, watercress or other water plants and encyst to metacercariae.

The metacercaria has a round shape and is composed of an outer cyst and an inner cyst (Figure. 3*g*). The outer cyst consists of tanned protein and an underlying fibrous layer of mucoprotein. The inner cyst has a complex mucopolysaccharide layer. The outer cyst is essential for attachment to the substrate and probably acts as well as a barrier against bacterial and fungal infection. The inner cyst is crucial in survival of the metacercaria (Boray, 1963).

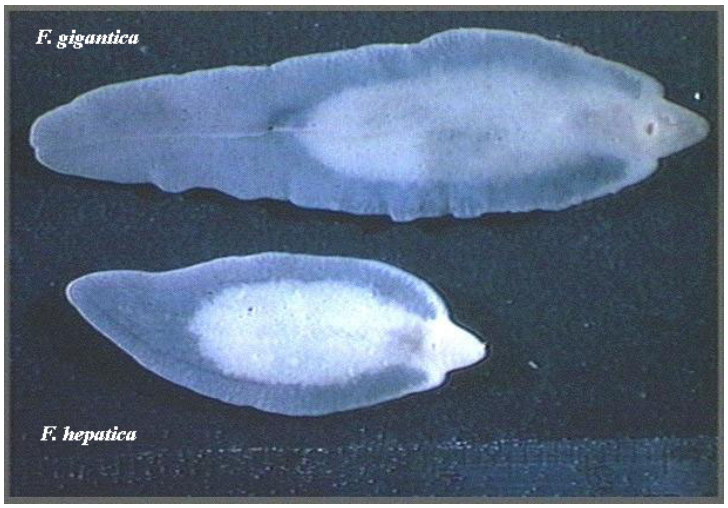
When the definitive host ingests the infected plant, the metacercariae excyst in the small intestine. The newly excysted juvenile parasites immediately penetrate the intestinal wall and enter the abdominal cavity. From there they migrate over a period

of approximately seven days directly to the liver. The young parasites then penetrate the liver parenchyma and migrate through it, feeding mainly on blood, for about six weeks. After this period the parasites enter the bile duct, maturing to the adult about three months after the initial infection. Egg production then commences, completing the life cycle.

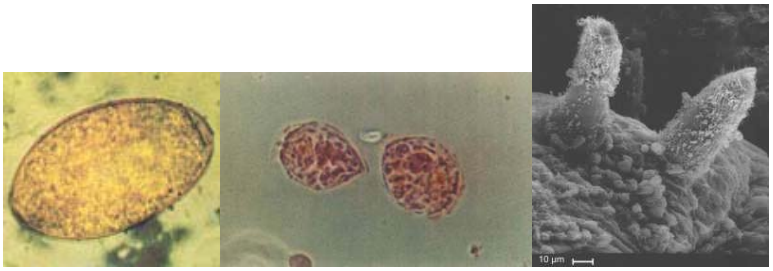
Figure 2 The life cycle of *Fasciola* spp.

Source: http://www.itg.be/.../images/prevs/CD_1078_037c.jpg

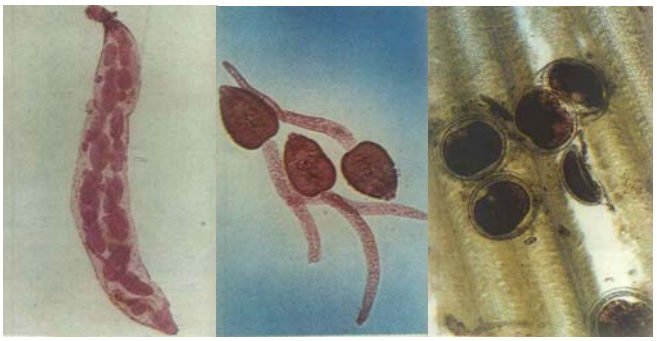
(accessed on 15 October 2006)



(*a*)



(*b*) (*c*) (*d*)



(*e*) (*f*) (*g*)

Figure 3 The developmental stages of *Fasciola* spp.

(*a*) Adult *Fasciola* spp., upper: *F. gigantica*, lower: *F. hepatica*, (*b*) Egg, (*c*) Miracidia, (*d*) Penetrating miracidia, (*e*) Redia, (*f*) Cercariae, (*g*) Metacercariae

Source: http://www2.mf.uni-lj.si/~mil/helm1/jpeg

(accessed on 15 October 2006)

2. Pathology, pathophysiology and clinical manifestation

The pathology of the disease in cattle and sheep is characterized by weight loss, anaemia, hypoproteinaemia and (after infection with 10,000 metacercariae) death (Boray, 1969). Resistance develops with age so that adult cattle are quite resistant to infection. Parasites in the liver bile ducts are confronted with a hostile inflammatory reaction. The bile ducts thicken due to epithelial hypertrophy and subsequent fibrosis of the walls of the duct. Calcium deposits start to form in the duct walls after 16 to 20 weeks of infection. As a result the ducts enlarge up to three cm in diameter and become prominent on the surface of the liver. The lumina of the ducts are variously dilated and stenosed and the epithelium shows ulceration and haemorrhage.

The severity of disease varies depending on the level of infection, the nutritional plane of the animals and also varies between animals in a group. Acute fasciolosis may cause sudden death of stock, especially sheep and goats. Often there is no warning, but there may be abdominal pain and ascites. Because it generally occurs as a result of a large intake of larvae over a short period its occurrence may be predicted by a study of climatic conditions. Deaths two months after the start of grazing on swampy pasture are typical scenario. Subacute disease is haemorrhagic anaemia which is slightly more protracted than the acute disease. Losses of up to 25% of flock are possible in outbreaks of acute fasciolosis. Acute haemonchosis also causes a rapidly fatal anaemia. Calves may suffer from acute fasciolosis in heavy infestations. Chronic disease is accompanied by weight loss, pallor of mucous membranes, ventral edema and wool break. Sheep die with obvious signs including the presence of typical eggs in faeces. A history of grazing fluke-prone areas is usually found. In cattle, the chronic disease is uncommon and often manifests as a small production loss. Reductions of 13 kg carcass weight over 124 days for cattle have been reported (Marley *et al.*, 1996). Reductions in milk production are equivocal. Fasciolosis may be involved in a complex with bovine ostertagiosis and be associated with parasitic gastroenteritis.

3. Immunology of the host-parasite relationship in fasciolosis

3.1 Immune response of the host against parasite during *Fasciola* spp. infection

An effective immune response against *Fasciola* spp. infection depends on a dynamic interplay between the host's effector responses and the parasite's defense and immunomodulatory systems. Evidence suggests that the juvenile or immature parasite is the target of protective host immune responses (van Milligen *et al*., 2000) but the effector mechanisms employed vary between hosts. Mice have a functional dichotomy in the T-helper (CD4+) response. Th1 cells secrete gamma-interferon, IL-2 and IL-12 which stimulate inflammatory immune responses, such as delayed type hypersensitivity (DTH) and the production of complement fixing antibodies. Additionally the Th1 cytokines promote the ability of macrophages to be activated, macrophages that effectively phagocytose and destroy microbes. Th2 cells secrete IL-4, IL-5 and IL-6 which regulate the humoral immune response (amount and isotype of antibody); Th2 cells promote B cell switching to IgE production, eosinophil activation and the production of non-complement fixing IgG isotypes (Sher and Coffman, 1992; Abbas *et al*., 1996).

Rats develop immunological resistance to reinfection with *F. hepatica* (Hughes, 1987) but whether the mechanism of this resistance of rats to *F. gigantica* is mediated by an immune response is unknown. The level of resistance varies considerably and is dependent on several factors including the strain, age, and sex of the rats (Hayes *et al*., 1974a; Hughes *et al*. 1976; Rajasekariah and Howell, 1977a, 1981). Additionally, the level of resistance in rat is the same whether they are challenged with homologous or heterologous clones of the parasite (Chapman *et al*., 1981). "A clone is defined as the metacercariae produced from a single snail infected with a single miracidia".

In absence of a further challenge infection, rats harboring a primary infection for about six months become susceptible to reinfection with *F. hepatica*; interestingly, this correlates with decline in antibody levels suggesting a potential role of anti-*Fasciola* spp. in determining resistance. If chronically infected rats are subsequently reinfected, the ability to kill a further challenge infection is restored within 2 weeks (Hughes *et al*., 1977). Resistance to *F. hepatica* infection in rat appears to involve both antibodies

and lymphoid cells. Immunity can be transferred from infected animals by both sera and lymphoid cells (Armour and Dargie, 1974; Hayes *et al*., 1974b, 1974c; Rajasekariah and Howell, 1979; Mitchell *et al*., 1981).

The resistance in rat involves two basic mechanisms (Hayes and Mitrovic, 1977; Rajasekariah and Howell, 1977b; Doy *et al*., 1978). The first, juvenile parasites coated with secreted antibodies in the gut, are unable to penetrate the gut wall (Burden *et al*., 1983; van Milligen *et al*., 1998). The second mechanism which determines resistance in rat acts through the cell mediated-immune response including macrophages, eosinophils, neutrophils, and mast cells (Hughes, 1987).

In sheep, a large infiltration of white blood cells into the liver and production of antibodies to the parasite have been observed (Sinclair, 1962, 1971; Boray, 1967, 1969; Ross *et al*., 1967; Movsesijan *et al*., 1975; Knight, 1980; Sandeman and Howell, 1980a, 1980b; Wedrychowicz *et al*., 1984; Boyce *et al*., 1987). The severity of disease due to *Fasciola* spp. infection may, however, be reduced in exposed sheep. A challenge infection with *F. hepatica* after a sensitizing primary infection can result in decreased worm size, reduced egg production by adult worms, delayed onset of anaemia, an earlier rise and a greater number of eosinophils, and earlier lymphocyte infiltration into the liver (Boray, 1969; Sinclair, 1962, 1971, 1973, 1975; Meeusen *et al*., 1995). Boray (1967, 1969) suggested that the reduction in the severity of the pathological changes in the liver of challenged sheep was due to the fibrosis caused by the primary infection and this fibrotic response appears to be a result of the immunological response of sheep to *F. hepatica* infection (Sinclair, 1968, 1970). The resistance to *F. gigantica* infection is well described in sheep (Boyce *et al*., 1987; Wiedosari and Copeman, 1990; Spithill *et al*., 1999b). The comparison of cellular response between Bellilois sheep infected with *F. hepatica* and *F. gigantica* suggested eosinophil numbers increased more quickly and strongly in *F. gigantica*-infected sheep than in *F. hepatica*-infected sheep. In both groups, peripheral blood mononuclear cell (PBMC) proliferation in response to parasite excretory-secretory products (ESP) showed a similar kinetics. IL-10 production in *F. gigantica*-infected sheep was significantly lower than in *F. hepatica*-infected sheep during infection. The lower susceptibility to *F. gigantica* infection in sheep could be explained by the more

intense cellular response induced by the parasite and the weaker capacity of *F. gigantica* to evade the immune response (Zhang *et al*., 2005).

In cattle, resistance to infection with *F. hepatica* has been observed and the animals are able to expel both primary and challenge infections (Boray, 1969; Dawes and Hughes, 1970; Doyle, 1971, 1972, 1973a; Anderson *et al*., 1978; Kendall *et al*., 1978; Doy and Hughes, 1984). The development of resistance to reinfection with *F. hepatica* is dependent on the duration of primary infection (Doyle 1973a). A long period of infection causes higher resistance to reinfection than a short infection period. The physical barriers including fibrosis of the liver and calcification of the bile ducts, due to migration and feeding activities of parasites from a primary infection are responsible for the resistance to challenge infections with *F. hepatica* in cattle (Boray, 1969; Hughes, 1987).

Elimination of a primary infection in cattle coincided with the highest level of homocytotrophic antibodies at 20-28 weeks post-infection (Doyle, 1973b). However, whether these antibodies play any functional role in the expulsion of the parasite is unknown. The cellular immune mechanisms play an important role in the resistance to *Fasciola* spp. (Corba *et al*., 1971). In natural infection of cattle by *F. hepatica*, Th1 responses are down-regulated in cattle infected by *Fasciola* spp. and antigen-specific T cell clones isolated from chronically infected cattle expressed only Th0 or Th2 phenotypes (Brown *et al*., 1994). A specific T cell clone isolated from immune cattle also expressed a Th0 response (Shoda *et al*., 1999). The lymphocytes that were isolated from cattle chronically infected with *F. hepatica*, also showed that proliferation in response to *F. hepatica* antigens failed to produce IFN-γ (Clery *et al*., 1996). These results suggest an inverse correlation between chronic *F. hepatica* infection and induction of parasite-specific type 1 T cells. The IgG2 response in cattle is positively regulated by IFN-γ, whereas the IgG1 response is positively regulated by IL-4 (Estes and Brown, 2002). Type 0 helper T cell clones specific for *F. hepatica* predominantly stimulated IgG1 production *in vitro* (Brown *et al*., 1999). Thus, the observation of elevated parasite-specific IgG1 levels but low IgG2 levels in infected cattle is consistent with these observations (Clery *et al*., 1996; Spithill *et al*., 1997; Brown *et al*., 1999; Estes and Brown, 2002). The humoral and cellular immune responses to *F. gigantica* were studied in infected experimental buffalo (Zhang *et al*.,

2006). The result showed that 33.4 ± 9.1% of the infection dose was recovered as adult parasites. The IgG level against excretory-secretory product (ESP) significantly increased from 3 weeks post-infection (WPI) and displayed a peak at 13 WPI. Eosinophil numbers increased significantly from three WPI in *F. gigantica*-infected buffaloes and displayed a peak at eight WPI. PBMC proliferation induced by *F. gigantica* ESP (FgESP) increased from two WPI with a peak at five WPI. IFNγ secretion by FgESP-stimulated PBMC appeared early from one WPI with three peaks at two, five and eight WPI, respectively. IL-10 production was observed from 2 WPI with two peaks at 4 and 9 WPI, respectively. Based on these results, Zhang *et al*. (2006) suggested that buffaloes were highly susceptible to *F. gigantica* infection, and that this susceptibility could be associated with the late and weak cellular immune response in the early phase of infection and the Th0-like response throughout the infection.

3.2 Mechanism of immune evasion/ modulations strategies of *Fasciola* spp.

The potential of parasites to survive in hosts reflects evolutionary adaptations that permit these organisms to evade or resist immune effector mechanisms. The anatomical sequestration is one mechanism for *Fasciola* and other parasites, the final residence of the parasites is in the immunological privileged environment of the bile ducts. The parasite must first face off the immune system as they migrate through the intestinal wall and liver tissue on their way to this site. Additionally, they have developed better means of counteracting the immune system, such as enhanced expression of antioxidants or immune evasion strategies (secretion of antibody-cleaving enzymes, immunomodulating molecules and anti-inflammatory agents).

3.2.1 Production of antioxidant defense enzymes

Several studies have provided direct and indirect evidences of a role for antioxidant defense (AOD) enzymes in protecting *Fasciola* spp. against attacks by free radicals, both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Baeza *et al*., 1993; Jefferies *et al*., 1997; Cervi *et al*., 1998, 1999; El Ghaysh *et al*., 1999; Piedrafita *et al*., 2000, 2001). The increase of ESPs released by *F. hepatica* and *F. gigantica in vitro* correlated with an increasing suppression of $O_2^{\cdot}$ and H_2O_2 production by sheep neutrophils (Jefferies *et al*., 1997; El Ghaysh *et al*., 1999). The

researchers proposed that both observations were linked to superoxide dismutase (SOD); however, both reports measured little or no SOD among the respective *Fasciola* spp. ESPs.

The majority of *F. hepatica* AOD enzyme work has focused on glutathione S-transferases (GSTs). FhGSTs exists as a mix of homo- and hetero-dimers with subunit sizes ranging from 24-29 kDa (Howell *et al*., 1988; Hillyer *et al*., 1992; Wijffels *et al*., 1992). FhGSTs and FgGSTs are expressed in a range of tissues including the parenchyma, gut, and tegument (Howell *et al*., 1988; Wijffels *et al*., 1992; Creaney *et al*., 1995, Khawsuk *et al*., 2002). The level of GST expression is lower in recently excysted juvenile (REJ) liver fluke than in immature and adult *F. hepatica* (Piedrafita *et al*., 2000). FhGSTs are proposed to play three roles. Firstly, FhGSTs are involved in detoxifying cytotoxic aldehydes produced during lipid peroxidation (Brophy *et al*., 1990); secondly, FhGSTs are involved in the absorptive function of the adult parasite's gut (Creaney *et al*., 1995); and thirdly, FhGSTs interact with haematin and this is proposed to prevent blockadge of the parasite's gut by haematin crystal formation (Brophy *et al*., 1990). Other AOD enzymes [SOD, catalase, glutathione peroxidase (GSH-Px) and Peroxiredoxin] have been reported (Sanchez-Moreno *et al*., 1987; Piedrafita *et al*., 2000; Brophy *et al*., 1990, McGonigle *et al*., 1997).

In *F. gigantica*, GST and SOD activities have been described in adult flukes (Estuningsih *et al*., 1997; Paykari *et al*., 2002; El Ghaysh *et al*., 1999). Adult *F. gigantica* released two products (less than 10 kDa and greater than 50 kDa) that were able to suppress the release of toxic oxygen intermediates by neutrophils (El Ghaysh *et al*., 1999). This result implies the presence of factors, possible AOD enzymes, in *F. gigantica* which are effective against reactive oxygen intermediates.

3.2.2 Glycocalyx turnover

The surface of *Fasciola* spp. is covered by a syncytial epithelium called tegument. The surface glycocalyx may contribute to immune evasion by three mechanisms. Firstly, the composition of glycocalyx changes during the development of the parasite as the parasite matures (Threadgold, 1963, 1967; Bennett and Threadgold, 1973, 1975). The glycocalyx of newly excysted juveniles (NEJ) is

derived from T0-type tegument cells which transform immediately into T1-type tegumental cells after the parasites enter the liver tissue. Prior to entry into the bile ducts T2-type tegument cells differentiate in the parenchyma, make contact with the tegumental syncytium and take over the function of glycocalyx synthesis (Figure 5). This changing glycocalyx composition is reflected in changes in the humoral immune responses of the host. Secondly, the glycocalyx is continuously sloughed off and replaced by the secretory vesicles. In the juvenile flukes this continuous turnover replaces the glycocalyx approximately every three hours (Hanna, 1980a, 1980b). Thus, it has been proposed that antibody-bound immune effector cells, such as eosinophils and neotrophils do not make sufficiently tight contact with the parasite to allow degranulation and damage to the surface but are shed with the glycocalyx (Duffus and Franks, 1980; Hanna, 1980a). Thirdly, shed products of the glycocalyx may simply trap and remove circulating anti-fluke antibodies preventing their participation in potential lethal ADCC reactions (Duffus and Franks, 1980).

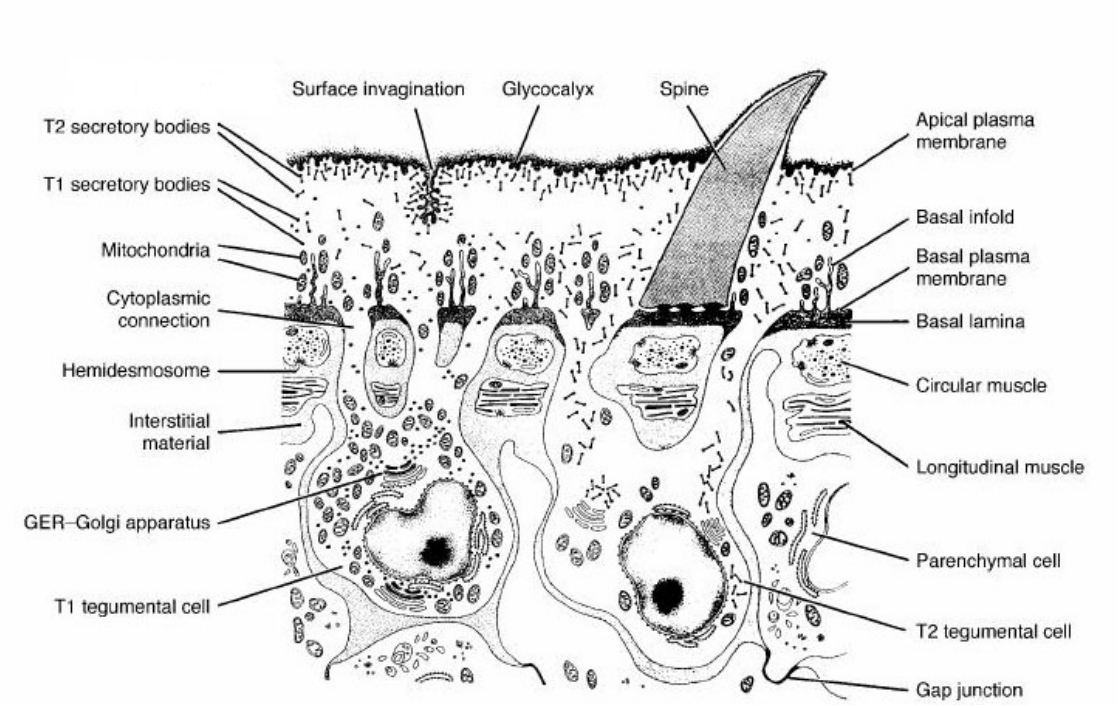


Figure 4 Diagram showing the fine structure organization of the tegument and related structures in *F. hepatica*

Source: Fairweather *et al*., 1999

3.2.3 Immunomodulating molecules

Parasites release a variety of molecules that help them to penetrate the defensive barriers and avoid the immune attack of the host. Not only proteases, but also inhibitors of these enzymes (serpins, aspins, and cystatins) to inhibit proteases, both of the host and their own. Additionally, helminthes homologues of cytokines and molecules containing phosphorylcholine, influence the immune response of the host biasing it towards the anti-inflammatory Th2 type. Nucleotide-metabolizing enzymes and cholinesterase are secreted by the worms to reduce inflammation and to expel the parasites from the gastrointestinal tract.

Recently, molecules responsible for immune modulation in *Fasciola* spp. have been identified and characterized. Glycoconjugates released from the sloughed glycocalyx and/or phosphorylcholine-enriched antigens secreted by the parasite may interfere with antigen processing by macrophages or suppress T lymphocyte responses (Sloan *et al*., 1991). A Kunitz-type (Fh-KTM) serine proteinase inhibitor expressed in the gut, parenchyma and tegument of adult *F. hepatica* was reported (Bozas *et al*., 1995) and may inhibit the activity of elastases released by neutrophils or interfere with cytokine production by lymphocytes.

3.2.4 Proteases

Like other organisms, parasites require proteases in many biological roles (nutrition uptake, tissue/ cell invasion, excystment/ encystment, exsheathment and hatching, protein processing and activation and immunoevasion). In *Fasciola*, the cysteine proteases are crucial enzymes and many cysteine proteases have been identified and characterized. *F. hepatica* cathepsin L proteases are purported to play key roles in tissue invasion and immunoevasion (Mulcahy and Dalton, 2001). Cathepsin L proteases can degrade both the extracellular matrix (fibrillar collagen, types I and II) and basement membrane (type IV collagen) (Berasain *et al*., 1997). A cathepsin B protease, temporally secreted by juvenile and immature parasites of *F. hepatica*, represents the major protein found in juvenile excretory-secretory products ESPs (Tkalcevic *et al*., 1995; Creaney *et al*., 1996; Wilson *et al*., 1998) and this protein is also found in somatic extract of juvenile parasites (Tkacevic *et al*., 1995). RNA transcripts for cathepsin B were localized to the caecal epithelium of both

metacercariae and juvenile forms of *F. gigantica* (Heussler and Dobbelaere, 1994; Meemon *et al*., 2004). Cathepsin L proteases also degrade haemoglobin *in vitro*, pointing to the possible digestion of host haemoglobin for nutritional purposes (Dalton and Heffernan, 1989; Wilson *et al*., 1998). The haemoglobin degradation activity of *S. mansoni*: cathepsin B, cathepsin L, asparaginyl endopeptidase (legumain), cathepsin C and an aspartyl protease have been identified and also play a role in haemoglobin hydrolysis *via* a protease cascade. It is likely that an analogous haemoglobinolytic protease system also exists in *F. hepatica*, where a number of cysteine proteases including cathepsin B-like, cathepsin L-like, dipeptidylpeptidase I and asparaginyl-endopeptidase activities have been identified. Cathepsin L proteases have been shown to cleave immunoglobulins from mice and humans (all IgG subclasses) in the hinge region of the heavy chain *in vitro* (Carmona *et al*., 1993; Smith *et al*., 1993; Berasain *et al*., 2000). The potential importance of this cleavage was demonstrated when the addition of adult *F. hepatica* ESPs along with *Fasciola* spp. immune sera prevented the antibody-mediated attachment of eosinophils to REJ liver flukes *in vitro* (Carmona *et al*., 1993; Smith *et al*., 1993; Berasain *et al*., 2000). This was reversed when the cysteine protease inhibitor leupeptin was added to the incubation. This is an important potential evasion mechanism, considering *in vitro* work that showed the high toxicity of the major basic protein (at micromolar concentrations) released by bovine eosinophils to REJ *F. hepatica* (Duffus *et al*., 1980). However, there is no direct evidence that the eosinophils actually kill REJ *F. hepatica*. It should be noted that in this work by Carmona *et al*. (1993) there was no cytotoxicity mechanism shown in the absence of cathepsin L and the ESPs used were from adult flukes; since the antibody-mediated effector responses against juvenile flukes, a direct role of adult ESPs in perturbing an effector response against juvenile parasites is unclear given that these parasites occupy different niches during infection. Such a mechanism is relevant if cathepsin L proteases from immature parasites have properties similar to those of adult cathepsin L proteases. Cysteine proteases isolated from *F. gigantica* worm extracts have been shown to digest both bovine IgG and bovine globin (derived from bovine haemoglobin) *in vitro* (Fagbemi and Hillyer, 1991, 1992). These findings imply that *F. gigantica* may evoke evasion strategies similar to those evoked by *F. hepatica*.

Nowadays, there is direct evidence for a role for cathepsin L proteases in immunomodulation of the host during *F. hepatica* infection. Down-regulation of type 1 response in *F. hepatica*-infected mice was shown to be mediated by cathepsin L proteases present in ESPs (O'Neill *et al*., 2001). Injection of mice with purified cathepsin L suppressed the type 1 response to the *B. pertussis* vaccine and to keyhole limpet haemocyanin. These results show that fluke cathepsin L proteases exert a profound effect on the ability of mice to mount type 1-like responses, implying that the proteases have a direct effect on the pathways determining type 1 responses. Whether these effects also operate in ruminants during fluke infection awaits determination.

Cathepsin L proteases have been also demonstrated to play a role in immunomodulation of host T cell responses (Prowse *et al*., 2002). Cathepsin was shown to be a major component of ESPs that suppressed sheep T cell proliferation . Analysis of the effect of ESPs and recombinant *F. hepatica* cathepsin L on the expression of 22 different sheep T cell surface markers by flow cytometry showed that ESPs or two recombinant cathepsin L proteases significantly reduced surface CD4 expression. A similar effect on CD4 of human T lymphocytes was also observed. These results show that *F. hepatica* cathepsin L has a direct effect on at least one major component of the T cell response (CD4), at least *in vitro*, and that this effect could be involved in the immunomodulation of sheep T cell proliferation. Similar effects were also observed with cathepsin L from *F. gigantica* (Piedrafita *et al*., 2004). However, further *in vivo* studies are required to determine whether such effects occur during the course of a fluke infection. Many studies provide strong evidence for the notions that cathepsin L proteases released by the parasite play a key role in this immunomodulatory activity (Brady *et al*., 1999; O'Neill *et al*., 2000, 2001; Prowse *et al*., 2002).

4. Control of fasciolosis

Many strategies have been proposed to eradicate fasciolosis. Specific aims of the control programme may be to prevent the build-up of parasites in the environment and to avoid areas of heavily contaminated pasture (Brunsdon, 1980). Reduction of pasture contamination may be accomplished through the use of anthelmintics,

management regimes, molluscicides and biological competition as components of an integrated control programme. In addition, the use of resistant animals to reduce the impact of infection may have potential, especially where treatment costs are relatively high (Roberts and Suhardono, 1996).

Effective strategies such as anthelmintic treatment, grazing management, application of molluscicide, and fencing off or draining swampy areas are used to control *F. gigantica*. However, their relevance is limited by the paucity of epidemiological information about *F. gigantica* on which application of such strategies is based, the restricted economic and agricultural options available to most farmers in areas where infection with *F. gigantica* is endemic and the lack of information about the benefits of implementing measures for control.

Chemotherapy is the principal method employed to control *F. hepatica* and *F. gigantica*. However, whereas anthelmintic is commonly the mainstay in programmes to control *F. hepatica*, it is not widely used against *F. gigantica*. Ideally, control measures for *F. gigantica* should be low cost, readily available and applicable with little disruption to existing agricultural practices.

4.1 Anthelmintics

Many control programmes have been proposed but the treatment of infected host with anthelmintics is the principal method employed to control *Fasciola* in livestock. A range of anthelmintics exists, including benzimidazoles, salicylanides, nitrophenols and halogenated hydrocarbons. They differ in availability, price, efficacy and safety. Most of the products which are listed have efficacy against both *F. hepatica* and *F. gigantica*. However, triclabendazole has reduced efficacy in buffalo as a result of achieving a lower serum concentration than in cattle (Sanyal, 1995).

In comparative efficacy trials, it has been shown that triclabendazole, rafoxanide and closantel exerted an appreciable retardation of the development of immature flukes in treated sheep. The level of retardation was directly related to the level of efficacy of the drugs against early immature flukes and considerably extended the prepatent period of the surviving fluke populations (Boray *et al*., 1997). This phenomenon has a great influence on success of strategic control programmes. Since triclabendazole is highly effective against flukes aged one week or older, the drug is

most suitable for reducing the pasture contamination for extended periods. Retarded development of flukes after treatment with closantel has been reported (Maes *et al*., 1985, 1990) and a lower degree of retardation has also been observed in cattle after treatment with clorsulon (Malone *et al*., 1984; Yazwinski *et al*., 1985).

Retardation of immature flukes which survived treatment appears to be applicable to all anthelmintics and the degree of retardation depends on the efficacy of the drugs against the immature stages. This phenomenon has a great advantage in strategic control by reducing early pasture contamination with eggs. Unfortunately, recent studies have demonstrated that resistance may develop to most anthelmintics, mainly affecting the flukes aged six weeks or younger (Boray, 1990). It appears that during the early immature stages selection for resistance will occur rapidly if eradication of *Fasciola* spp. is attempted with frequent treatments and this method of control is not desirable. Less frequent strategic treatments with a possible yearly rotation of anthelmintics or anthelmintic combinations which are effective against both immature and adult flukes will provide the best method of successful control of fasciolosis.

4.2 Alternative adjunct control strategies

The literature on alternatives or adjuncts to anthelmintics for control of *F. gigantica* has been reviewed by Roberts and Suhardono (1996). The available information is scanty despite the desirability for development of such options. Published recommendations include; use of grazing management (Schillhorn van Veen, 1980); molluscicides (Dinnik and Dinnik, 1963; Preston and Castelino, 1977; Mzembe and Chaudhry, 1981; Harrison *et al*., 1996); predator/competitor of snails (Nguma *et al*., 1982); predator with fish (Gupta *et al*., 1986) or duck (Touratier, 1988; Rai *et al*., 1996); and breeding resistant livestock (Roberts *et al*., 1997). However, none has been widely adopted for control.

5. Essential antigen for vaccine development against fasciolosis

As mentioned earlier, fasciolosis is considered the single most important helminth infection of cattle (Fabiyi, 1987) with high prevalence rates in tropical regions and developed countries. Although triclabendazole is an effective drug for controlling disease caused by both species of *Fasciola* (Boray *et al*., 1983; Estuningsih

et al., 1990; Suhardono *et al*., 1991), the cost of treatment with this drug is a barrier to its wide adoption by rural producers in developing countries. Furthermore, resistance to triclabendazole has been reported in sheep infected with *F. hepatica* (Overend and Bowen, 1995) suggesting that the effectiveness of this drug may eventually be compromised by the selection of resistant parasites in the field. The development of vaccine will provide producers with an alternative, environmental friendly, cost effective and sustainable strategy for the control of fasciolosis.

The immunization of host species against *Fasciola* using irradiation-attenuated metacercariae was one of earliest strategies tested in an attempt to identify host-protective antigens. Protection with irradiation-attenuated vaccines has been successfully induced against fasciolosis (Dargie *et al*., 1974; Nansen, 1975; Haroun and Hillyer, 1986; Acosta *et al*., 1989). The results of many recent studies of the use of defined antigens as vaccines against *Fasciola* have been published (Rickard and Howell, 1982; Haroun and Hillyer, 1986; Estuningsih *et al*., 1997). Several antigens from *Fascila* have been defined including fatty acid binding protein (FABP), glutathione S-transferase (GST), cathepsin L (CatL), hemoglobin, paramyosin, Kunitz-type serine proteinase inhibitor (KTM), saposin-like proteins (SAPLIPs), leucyl aminopeptidase (LAP) and asparaginyl endopeptidase or legumain (LGMN).

5.1 Fatty acid binding protein (FABP)

FABPs were the first defined, purified antigen fraction to be tested as a vaccine against fasciolosis. FABPs are the major component of the FhSmIII(M) complex that was further fractionated to obtain a purified protein of 12 kDa size (termed Fh12). Fh12 was demonstrated to significantly reduce (55%) the mean worm burdens in cattle and in mice (69-78%; Hillyer, 1985; Hillyer *et al*., 1987). The Fh12 cDNA sequence encodes a protein with high similarity to the family of FABPs (Rodriguez-Perez *et al*., 1992) suggesting that Fh12 may play a role in the intracellular transport of long-chain fatty acids and their acyl-CoA esters. The FABP fraction from *F. gigantica*, formulated with Freund's adjuvant, was tested as a vaccine in cattle and a low but significant 31% reduction in worm burdens was observed (Estuningsih *et al*., 1997): however, a recombinant *F. gigantica* FABP (rFABP) expressed in *E. coli* (Smooker *et al*., 1997) did not elicit protection. Since there appears to be a family of FABPs in

Fasciola (Bozas and Spithill, 1996; Smooker *et al*., 1997), it is possible that the immune responses induced by the native FABP mixture differ from that induced by a single rFABP, due to subtle conformational differences, or that the particular FABP molecule which is target of the immune response differs from the sequence which was cloned. Alternatively, it is feasible that the use of the N-terminal polyhistidine tag which was used to allow purification of the rFABP protein may interfere with the induction of the protective immune response. A corollary of this hypothesis is that the protective epitope(s) on FABP may be encoded in N-terminal amino acid sequence. The mechanism of protection induced by the FABP vaccine in cattle remains to be determined.

5.2 Glutathione S-transferase (GST)

The glutathione S-transferases (GST) comprise a family of isoenzymes involved in the cellular detoxification of a broad range of chemical substrates. Neutralization of the substrates, through the conjugation of glutathione, renders the product more water soluble, less toxic and more readily excreted from the host. Purification of GST from adult *F. hepatica* by glutathione affinity chromatography yielded two closely migrating species at approximately 23-26.5 kDa as defined by SDS-PAGE (Howell *et al*., 1988; Brophy *et al*., 1990; Wijffels *et al*., 1992). An early vaccine study had described the failure of GST in Freund's complete adjuvant (FCA) to protect rats from infection with *F. hepatica* (Howell *et al*., 1988). In contrast, sheep which received multiple vaccinations with native *F. hepatica* GST in FCA showed a 57% reduction in worm burden, marking the first demonstration of protection in sheep against *F. hepatica* using a defined antigen (Sexton *et al*., 1990). The efficacy of GST from *F. gigantica* was assessed in Brahman-cross cattle. No significant reduction in worm burdens or faecal egg counts was observed despite the use of the same adjuvants previously shown to induce protection against *F. hepatica* with GST (Estuningsih *et al*., 1997). These results suggest that vaccine formulations protective for *F. hepatica* may not be effective against *F. gigantica* and the possible basis for this difference in efficacy has been discussed (Estuningsih *et al*., 1997).

5.3 Cathepsin L (CatL)

Fasciola contains an abundance of proteolytic enzymes, in particular in the excretory-secretory (ES) material, which can be easily collected from the adult parasites *in vitro*. Immunization with cathepsin L of *Fasciola*, formulated in Freund's complete adjuvant, has been firstly reported to induce a high >70% reduction in worm burdens and the output of eggs by parasites in vaccinated sheep (Wijffels *et al*., 1994). In cattle, Dalton's laboratory has demonstrated that vaccination with two different FhCatL proteins (CatL1 and CatL2), together with Freund's complete adjuvant could reduce both worm burdens (up to 69%) and egg production and viability (up to 65%), particularly in concert with a large haeme-containing protein complex (Haemoglobin) (Dalton *et al*., 1996). CatL proteases from *F. gigantica* have also been tested as vaccine in cattle, using DEAE Dextran/SM as adjuvant, no protection or reduction in faecal egg count was observed despite the induction of high total antibody titres (Estuningsih *et al*., 1997). These results suggest that this may be due to the use of a different adjuvant and it may be that Freund's adjuvant induces critical immune effector responses required to inhibit parasite infection and fecundity.

5.4 Haemoglobin

In 1996, haeme containing protein was first isolated and characterized from the ES material of adult *F. hepatica* (McGonigle and Dalton, 1995). This protein, with an apparent molecular weight greater than 200 kDa, was shown to have an absorption spectrum similar to haemoglobins. This protein was highly immunogenic in cattle after liver fluke infection; anti-haemoprotein antibodies were generated within a week of infection. Dalton *et al*. (1996) have performed a set of vaccination trials using FhCatL1, FhCatL2 and the haemoprotein. As mentioned above, FhCatL1 alone yielded up to 69% protection, haemoprotein alone gave 44% protection and combination of the two antigens gave 52% protection. However, immunizing with the haemoprotein and FhCatL2 increased efficacy to give 72% reduction in worm burdens. Importantly, this combination also resulted in 98% decrease in fecundity, perhaps due to a reduction in oxygen delivery by the haemoprotein.

5.5 Paramyosin

Several studies have reported the use of the subtegumental protein paramyosin of *Schistosoma* as a vaccine in mice (Pearce *et al*., 1988; Flanigan *et al*., 1989; Ramirez *et al*., 1996). Paramyosin with a size of 94 kDa was extracted from whole adult fluke homogenates. In a sheep vaccination trial, two groups of six animals were vaccinated subcutaneously 4 weeks apart with a dose each time of 100 μg paramyosin emulsified either in FCA or SM containing 5% w/v DEAE-dextran, and challenged 2 weeks after the booster immunization. Sera were analyzed by ELISA for their specific antibody content to paramyosin. Titres rose rapidly but were significantly higher in the FCA group (>1:100,000) than in the DEAE-dextran group (1:30,000). There was the evidence of a marked reduction in the mean worm burden compared to controls (45%) for the FCA group. In the cattle trial, a similar protocol was followed using only QA/SM as adjuvant. The reduction of mean worm burdens in the liver was 47% and this was significant ($P<0.05$) (Spithill *et al*., 1999a). Paramyosin of *F. gigantica* has also been tested in cattle but no protection was observed (Estuningsih *et al*., 1997). The potential of paramyosin as a vaccine candidate for *Fasciola* remains to be confirmed.

5.6 Kunitz-type serine proteinase inhibitor (KTM)

A Kunitz-type serine proteinase inhibitor (termed Fh-KTM) of 6751 Da belonging to the bovine pancreatic trypsin inhibitor (Kunitz) family has been isolated from *F. hepatica* (Bozas *et al.*, 1995). Fh-KTM is a weak inhibitor of trypsin but the specific target enzyme of Fh-KTM within adult *F. hepatica* has yet to be identified. Immunofluorescent and immunogold localization studies on adult *F. hepatica* revealed that Fh-KTM is an abundant molecule localized to the luminal surface of the gut, the entire outer tegument which includes the tegument tissue surrounding the oral sucker and specific subcellular organelles of parenchymal cells.

The abundance of Fh-KTM and the gut and tegumental localization pattern suggested that this molecule could be used as a vaccine target since gut-associated molecules have successfully been used as vaccines against other blood-sucking parasites such as *Boophilus microplus* and *Haemonchus contortus* (Willadsen *et al*., 1995; Munn, 1997). In the vaccine trials in sheep and cattle, high antibody titres were

observed following vaccination but no reduction in worm burden was observed in any of the vaccinated infected animals.

5.7 Saposin-like proteins (SAPLIPs)

The Saposin-like proteins (SAPLIPs) was previously described as saposins. They are a group of four water-soluble cofactors for specific sphingolipid glycohydrolases and they are processed from a single precursor protein named prosaposin (Kondoh *et al*., 1991). The saposins A-D (molecular weight about 10 kDa) are derived by proteolytic cleavage from a 554 amino acid precursor, prosaposin. They are found in membrane-bound as well as extracellular form. Their gene expression during development is under tissue- and cell-specific control. *In vitro*, prosaposin has most of catalysis-enhancing activities of the four individual saposins. Prosaposin and all of the saposins bind *in vitro* to gangliosides, cerebrosides, sulfatides, and ceramide and can facilitate glycolipid insertion into erythrocyte ghosts or brain microsomes, suggesting a potential role in membrane biogenesis. Saposins are found principally in lysosomes, where they facilitate the catabolism of glycosphingolipids (Kolzer *et al*., 2004).

In *F. hepatica*, two saposin-like protein encoding genes were isolated and molecular characterized. The immunization trial of recombinant FhSAP-2 showed high protection efficiency in infected rabbits. A reduction in worm burden and egg release of more than 80% was observed. The recombinant FhSAP-2 protein is also a *Fasciola-Schistosoma* cross-reactive antigen, but its protection-inducing activity against *Schistosoma mansoni* has not been determined yet (Espino and Hillyer, 2003).

5.8 Leucine amoinopeptidase (LAP)

Leucine aminopeptidase (LAP) is an exoproteinase that has been isolated from an *F. hepatica* detergent-soluble extract and characterized. LAP cleaves the substrate leucine-7-amino-4-methylcoumarin (NHMec). Histochemical methods showed that the protein is located in the epithelial cells that line the digestive tract of parasite. This enzyme most likely functions in the final stages of the catabolism of peptides that are generated by the degradation of host tissue by endoproteases, such as the cathepsin L proteases, and are absorbed by the epithelial cell (Acosta *et al*., 1998). Vaccine trials with LAP were done in sheep, the set of vaccines also contained CatL1 and CatL2. In

the first trial, sheep were immunized with CatL1 or CatL2 and the mean protection levels obtained were 33% and 34%, respectively. Furthermore, a significant reduction in egg output was observed in sheep vaccinated either with CatL1 (71%) or with CatL2 (81%). The second trial was performed to determine the protective potential of the two CatL assayed together, as well as in combination with LAP, and of LAP alone. The combination of CatL1 and CatL2 induced higher levels of protection (60%) than those produced when these enzymes were administered separately. Those sheep that received the cocktail vaccine including CatL1, CatL2, and LAP were significantly protected (78%) against metacercarial challenge, but vaccination with LAP alone elicited the highest level of protection (89%). All vaccine preparation induced high immunoglobulin G titers which were boosted after challenge infection, but no correlations between antibody titers and worm burdens were found. However, the sera of those animals vaccinated with LAP contained LAP-neutralization antibodies. Reduced liver damage, as assessed by the level of the liver enzyme gamma-glutamyl transferase, was observed in the groups vaccinated with CatL1, CatL2, and LAP or with LAP alone (Lucía *et al*., 1999).

6. Legumain-like-family

Conventionally, proteases are assigned to clans and families depending on a number of characteristics including sequence similarity, possession of inserted peptide loops, and biochemical specificity to small peptide substrates. Alternatively, Robust classification relies on sequence homology directly spanning the catalytic cysteine and histidine and where known, the glutamine of oxyanion hole. Cysteine proteases of parasitic organisms are divided into two main groups referred to clans, CA and CD (Barrett, 1994; Rawling and Barrett, 1993).

Family C13; legumain-like proteases belong in clan CD and are among the important proteases found in parasites. The peptidase family C13 contains asparaginyl endopeptidases and glycosylphosphatidylinositol:protein transamidase. The active site residues are in the order His, Cys in the protein sequence. The histidine occurs within the motif Xaa-Xaa-Xaa-Xbb-Xbb-**His**-Gly-Xbb, in which Xaa is a hydrophobic amino acid and Xbb is a small amino acid (Ala, Asp, Glu, Gly, Ser or Thr). The cysteine occurs in a similar motif, Xaa-Xaa-Xaa-Xbb-Xbb-**Cys**. Most members of the family

are endopeptidases with a restricted specificity for asparaginyl bonds, although only a minority of such bonds are cleaved in proteins. One of the best synthetic substrates is Z-Ala-Ala-Asn-NHMec. Maximal activity is seen at pH 4-6; at higher pH legumain is denatured. At pH 4.0, cleavage at aspartyl bonds has been observed. The strict specificity for the P1 residue of the substrate is a characteristic shown also by other peptidases in clan CD. Peptidases in family C13 are inhibited by thiol-blocking reagents such as iodoacetamide and *N*-ethylmaleimide, but are unaffected by E-64 and leupeptin, potent inhibitors of peptidases in family C1. Members of family C13 react more rapidly with iodoacetamide than with iodoacetate, the reverse of what is found for peptidases in family C1. Aza-peptide epoxides are also potent inhibitors of legumains (James *et al*., 2003). Like many peptidases in family C1, legumain is inhibited by ovocystatin, but by a different reactive site (Alvarez-Fernandez *et al*., 1999). In keeping with its lysosomal location, mammalian legumain is glycosylated. Some plant legumains also are glycosylated, although not that of castor bean. No tertiary structure has been reported, but similarities in sequence and predicted secondary structure around the active site residues lead to assignment of family C13 in clan CD, and suggesting that the fold is similar to that of the caspases in family C14 (Chen *et al*., 1998) (Figure 5).

Figure 5 Tertiary structure of CASPASE-1, catalytic residues are shown in ball and stick representation: His237 in purple and Cys285 in yellow. Ac-Tyr-Val-Asp is shown in grey in ball-and-stick representation

Source: http://merops.sanger.ac.uk, structure for peptidase C14.001 (CASPASE-1) (accessed on 2 November 2006)

6.1 Asparaginyl endopeptidases

Asparaginyl endopeptidases exclusively hydrolyse peptides and proteins on the carboxyl side of asparaginyl residues. These enzymes are often referred to as 'legumain-like' as the template proteases was first identified and characterized from the plant legume, *Canavalia ensiformis*, the jack bean (Shutov and Vaintraub, 1987). Legumain-like proteases have been identified in many plants and mammals including, human, mouse, rat and pig. The hepatic trematodes, *S. mansoni* and *F. hepatica* both contain these proteases (Dalton *et al*., 1995; Caffrey *et al*., 2000). Legumains are thought to localize to lysosome compartments. In *S. mansoni* the legumain is detected in the gut gastrodermis and the parasite caecal lumen. This fluke enzyme is expressed in two forms, the native active protein has been biochemically characterized and contains the active site Cys^{197} (*S. mansoni* legumain numbering) (Caffrey *et al*., 2000). In addition to this protease there is a distinct transcript which has an asparagine residue in place of the active site cysteine (Caffrey *et al*., 2000). Substitution of the cysteine in place of the asparagine in the inactive form of the schistosome legumain (Asn197Cys) restores enzymatic activity with a similar substrate profile to the wild type enzyme when using combinatorial peptide libraries (Sajid and McKerrow, 2002). Why the parasite maintains a proteolytically inactive enzyme with apparently intact subsites is unclear. In plant and mammal, legumains are involved in a variety of defined processing actions such as converting pro-proteins and zymogens to their mature biologically active forms. For example, a *Vigna mungo* (mung bean) legumain can process an associated Clan CA vacuolar cysteine peptidase zymogen to its catalytically mature form *in vitro* (Okamoto and Minamikawa, 1999). Also, mammalian legumains have been shown to process tetanus toxin antigen for presentation to T cells by the major histocompatibility complex (MHC) class II (Manoury *et al*., 1998) and convert lysosomal cathepsins from their single-chain to two-chain forms (Shirahama-Noda *et al*., 2003). Li *et al*. (2003) demonstrated activation of LGMN by using purified recombinant mammalian proenzyme both *in vitro* and *in vivo*. The activation is autocatalytic, requires sequential removal of C- and N-terminal propeptide at different pH threshold (high activity exhibited at pH 3.5-4.5), and is bimolecular. Removal of the N-terminal propeptide requires cleavage after aspartic acid rather than asparagines. Cellular processing, either of exogenously

added AEP precursor or pulse-labeled precursor, introduces at least one further cleavage to yield the final mature lysosomal enzyme. They also showed that human monocyte-derived dendritic cells harbour inactive proforms of LGMN that become activated upon maturation of dendritic cells with lipopolysaccharide (Li *et al*., 2003). Therefore, consistent with this function as a 'protein processor', *S. mansoni* legumain had been hypothesized to *trans*-activate other gut associated zymogens (*e.g.* SmCatB1 and SmCatL1) to their mature catalytic forms (Dalton and Brindley, 1996). Support for this hypothesis has been demonstrated recently whereby the zymogen of SmCatB1, which was unable to autocatalyze its own activation, was rapidly converted to its mature catalytic form by *S. mansoni* legumain *in vitro* (Sajid *et al*., 2003) (Figure 6).

One of the characteristics of the legumain-like class of cysteine proteases is their insensitivity to generic cysteine protease inhibitors such as E64 and leupeptin. Proteolytic activity is inhibited by the general thiol-blocking reagents iodoacetamide and iodoacetic acid as well as the broad specificity macromolecular protease inhibitors, α_2-macroglobulin and chicken ovamucoid cystatin. This lack of selective inhibition has hampered detailed biochemical characterization. Recently Aza-peptide epoxides are a new class of irreversible cysteine protease inhibitors. Derivatives containing a P1 aza-asparagine residue are specific for *S. mansoni* and pig kidney legumains (James *et al*, 2003).

The possibility that *S. mansoni* legumain (Sm32), or subunits thereof, might represent a useful vaccine is being actively pursued. Mice have been shown to respond with selective antibody to *S. mansoni* legumain after injection into the ear pinna with the Sm32-encoding DNA construct (Chlichlia *et al*., 2002). However, after infection with *S. mansoni*, no significant decrease in worm burden was elicited. There was a modest anti-fecundity effect nonetheless: egg production was decreased by 37% using recombinant vector compared with 23% with vector alone (Chliclia *et al*., 2002). In addition, progress has been made towards defining protective B- and T-cell epitopes on *S. mansoni* legumain that might form part of unit vaccine (Chacón *et al*., 2003). The study used 22 discontinuous peptides, covering the entire sequence of *S. mansoni* legumain, to immunize inbred and outbred strains of mice. Peptides with high homology to host legumain, and thus at risk of causing autoimmune responses, were poorly immunogenic. However, other peptides with less homology and of greater

hydrophobicity elicited antibodies that reacted with the parent antigen. Such peptides were proposed as useful starting points for further trials (Chacón *et al*., 2003).

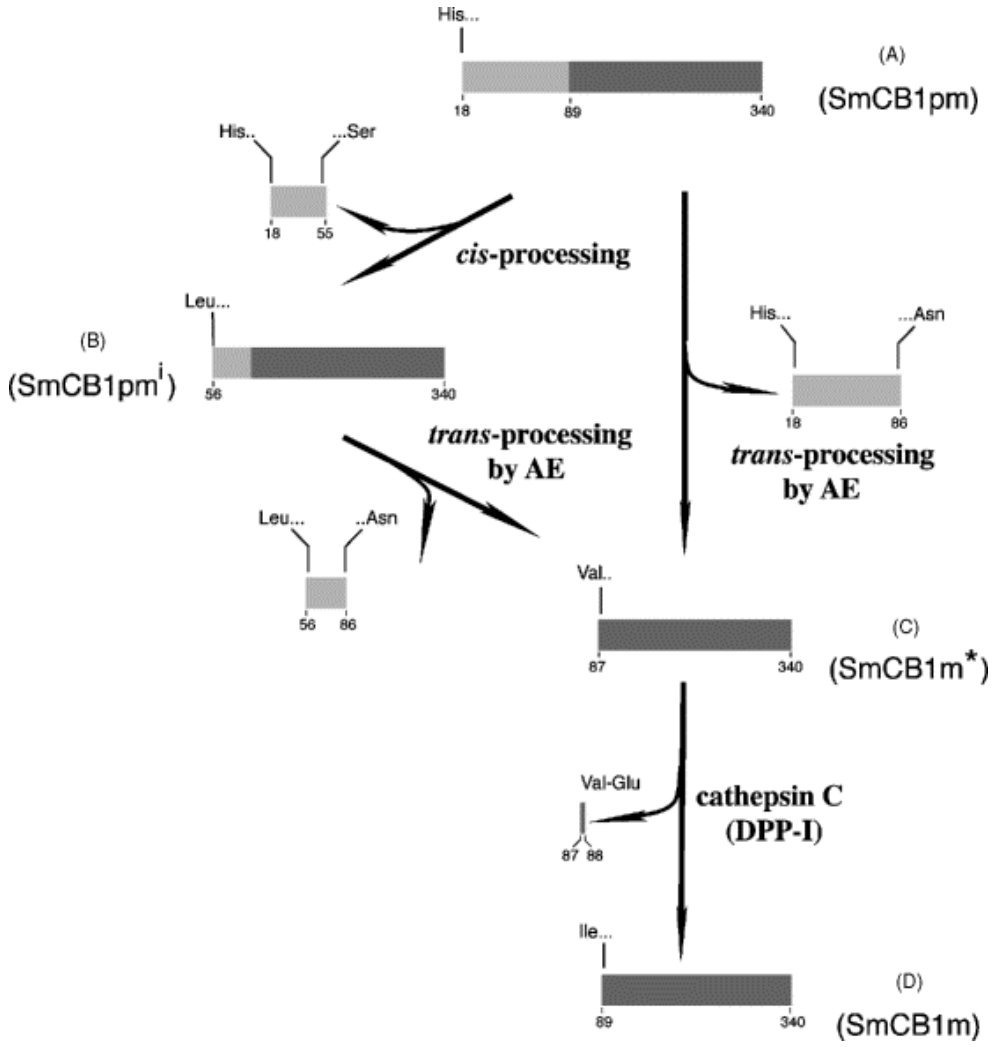


Figure 6 Scheme of processing and activation of SmCB1pm. (A) SmCB1pm undergoes *cis*-processing in the pro-region at Phe-His-Ser^{55}-↓-Leu-Asp-Asp to yield SmCB1pm^{i}; (B) SmCB1pm^{i} or SmCB1pm is *trans*-processed at Asp-Trp-Asn^{86}-↓-Val-Glu-Ile by the SmAE to yield activated SmCB1m*; (C) SmCB1m* undergoes further maturation by a cathepsin C-like activity [a.k.a. dipeptidyl peptidase I (DPP I)] that removes the N-terminal dipeptide, Val-Glu to yield SmCB1m; and (D) both SmCB1m* and SmCB1m are catalytically indistinguishable. Numbers refer to the amino acid residue position with respect to the start methionine (no. 1).

Source: Sajid *et al*., 2003

6.2 GPI: protein transamidases

DNA sequences have been identified from *Caenorhabditis elegans*, *Drosophila melanogaster*, *Trypanosoma brucei* and *Plasmodium falciparum* (and a fragment from *Cryptosporidium*) that show striking similarities at the amino acid level to legumain (Figure 7A). However, these proteins are assigned to sub-family within the legumain-like proteins called glycosylphosphatidylinositol (GPI): protein transamidases. In human, *Saccharomyces* and *Leishmania mexicana* (Hilley *et al*., 2000; Sharma *et al*., 2000) these proteins have been shown to play a role in the attachment of pre-formed GPI anchors to precursor proteins in the endoplasmic recticulum (ER) (Sajid and McKerrow, 2002). The two distinct clades of the legumain-like family, the transamidase-like clade and the asparaginyl-endopeptidase like clade, represent the different functions of these two classes of C13 proteases within the phylogenetic relationship (Figure 7B). It is likely that the *D. melanogaster*, *C. elegans* and *P. falciparum* proteases may function in GPI: protein transamidation. There is significant sequence similarity within the asparaginyl endopeptidase-clade around the active site His/Cys; whereas, there is little sequence homology around the His/Cys within the transamidase clade, suggesting an extremely early evolutionary divergence.

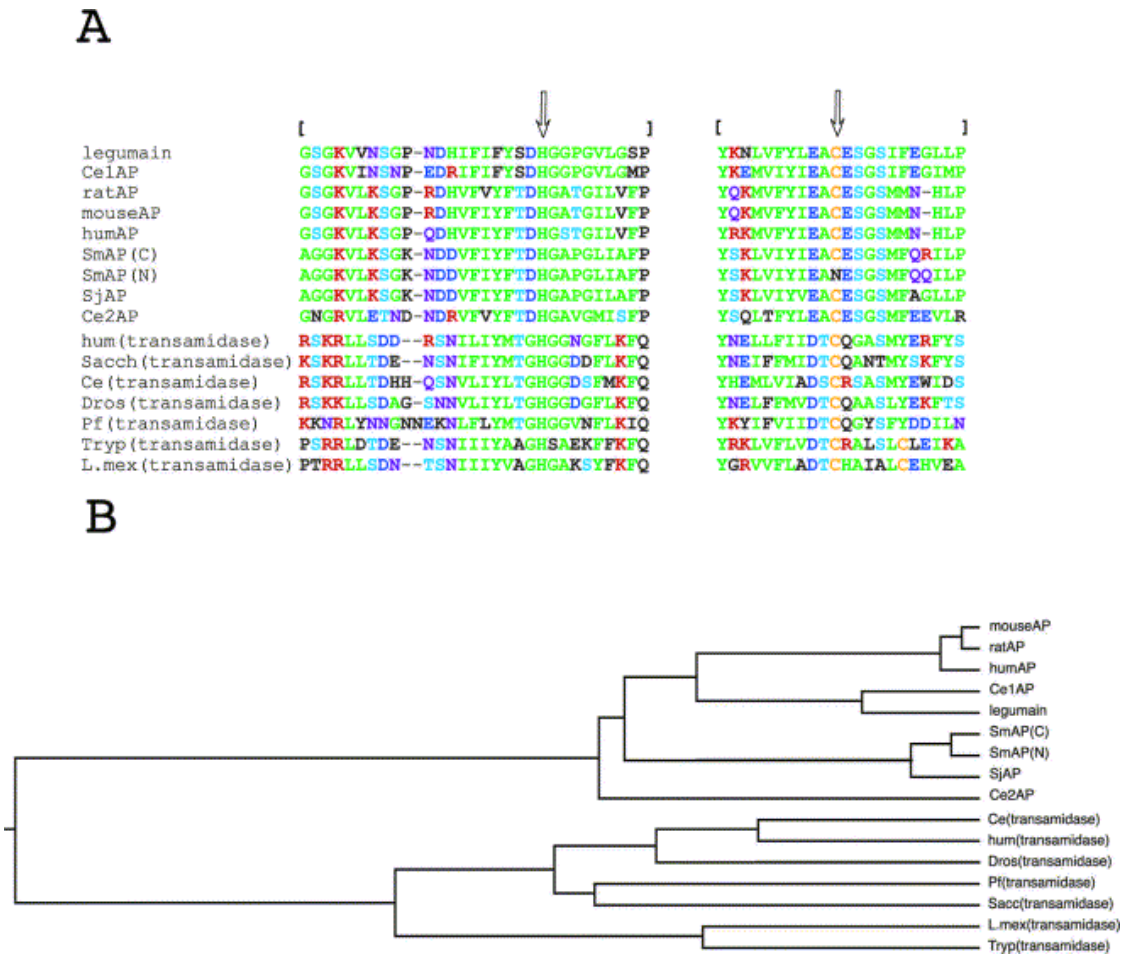


Figure 7 Comparison of the legumain-like superfamily showing (A) sequence alignment spanning the active site histidine and cysteine were complied using ClustalW at http://www.sacs.ucsf.edu. Active site residues are helmint with arrows. (B) Phylogenetic tree using the sequences shown in the alignment was generated using the ClustalW method. Sequences were obtained either from GenBank or EMBL, SmAP(N) was from Caffrey *et al*., 2000 and the *T. brucei* sequence was from Simon Lillico. Abbreviations: AP, asparaginyl endopeptidase; Ce, *Caenorhabditis elegans*; hum, human; Dros, *Drosophila*; Sm, *Schistosome mansoni*; Sj, *S. japonicum*; Sacc, *Sacchromyces*; Pf, *Plasmodium falciparum*; L. mex, *Leishmania mexicana*; Tryp, *Trypanosoma brucei*; all alignments are available on request.

Souce: review of Sajid and McKerrow, 2002