Evolution of Liver Fibrosis During Long-term Experimental *Schistosoma japonicum* Infection in Pigs

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To my late parents (**Pa D. C. Ngwa** and **Mami Elisabeth Ghang**)

Quotable quotes

Joy comes by putting Jesus first, others second and yourself last.

The seed we sow today determine
The kind of fruit we'll reap tomorrow.

Man says that seeing is believing
But God says that believing is seeing.

It's better to give others a piece of your heart
Than a piece of your mind.

Sin is the disease
Christ is the cure.

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Abstract of Thesis

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Schistosomiasis japonica, caused by the zoonotic trematode *Schistosoma japonicum*, is a highly debilitating parasitic disease endemic in China, the Philippines and Indonesia. The disease is a serious threat to public health and a major cause of liver fibrosis in humans. The tissue damage caused by the host tissue reaction to schistosome eggs trapped in the portal system of the liver leads to portal fibrosis and hypertension. The fibrosis is characterised by excessive deposition of extra-cellular matrix (ECM), especially collagen types 1 and 3 in various proportions, in portal areas. The pig is a natural host for *S. japonicum* and has several anatomical, physiological and immunological similarities with man, which has led to the exploration of the pig as a large animal model of human schistosomiasis japonica. In pigs, pronounced portal and septal fibrosis develops at the early stage of infection, when egg excretion is high and then gradually regresses over time as the pigs undergo self-cure. This makes the pig a useful animal model for studies of the pathogenesis of the development and resolution of liver fibrosis, including any qualitative changes in the ECM that may occur during the infection period.

In the present study, liver fibrosis during the course of long-term *S. japonicum* infection in pigs was investigated. Three groups of pigs were infected with 1000 *S. japonicum* cercariae and necropsied at 8, 16 and 24 weeks post infection (p.i.). Parasitological variables included faecal egg and miracidial counts and liver tissue egg counts (TEC). The degree of fibrosis was assessed in Masson's trichrome-stained liver sections, using both semi-quantitative histopathological scoring and quantitative area measurement by image analysis. The number of perioval granulomas per area unit in the same sections was determined. Collagen type 1 was detected by immunohistochemistry and the area fraction in selected areas of interlobular septa was measured by image analysis. The relationship between fibrosis and parasitological variables was investigated.

Faecal egg and miracidial counts peaked at 8 weeks p.i. and declined rapidly thereafter to low levels at 24 weeks p.i. Liver TEC and granuloma density were also highest at 8 weeks p.i. and decreased at the later time points. The liver lesions were characterised by perioval granulomas, diffuse inflammatory cell infiltration, and portal and septal fibrosis. Scores for both portal and septal fibrosis were highest at 8 weeks p.i. and were reduced at the later time points, and similar results were obtained for the area of fibrosis. Collagen type 1 was present in portal and septal areas in proportion to the degree of fibrosis in the infected pigs. The area fraction for collagen type 1 in septa was significantly higher in infected than in control pigs, but no difference was found between the different time points in

infected pigs. There was a correlation between the area of fibrosis and faecal miracidial counts and between granuloma density and faecal egg counts. Fibrosis was strongly correlated with granuloma density, but not with liver TEC. The two methods used for assessment of liver fibrosis were found to be well correlated.

In conclusion, this study confirmed the results from other studies that marked liver fibrosis develops at the acute stage and is reduced at later stages of *S. japonicum* infection in pigs, and that the degree of fibrosis is related to granuloma density in the liver. The results suggest that faecal egg excretion could be used as a marker of liver pathology. Quantitative image analysis gave comparable results to semi-quantitative histopathological scoring and is thus a useful, additional tool for assessment of the degree of liver fibrosis in this animal model. Finally, the study showed that the area fraction of collagen type 1 in fibrous septa was increased in the infected pigs, but did not change in connection with the resolution of fibrosis that occurred during the course of infection.

Key words:
Schistosoma japonicum, liver fibrosis, pig, granuloma density, histopathological scores, image analysis, collagen type 1.

Contents

INTRODUCTION

Schistosomiasis, also known as bilharziasis, is a major chronic, debilitating, parasitic disease affecting man and animals in tropical and subtropical countries. It is caused by infection with schistosomes (blood flukes), which are trematodes of the phylum Platyhelminthes. It is estimated that over 200 million people are infected by schistosomes world wide (WHO, 2002). Five schistosome species that infect humans have been described (Michael and Refaat, 1981). Of these five species, *Schistosoma mansoni, S. haematobium* and *S. japonicum* are the most prominent with devastating effects. *S. intercalatum* and *S. mekongi* are the other species that infect humans.

S. mansoni and *S. haematobium* are endemic in Africa and the Middle East, *S. mansoni* also in Central and South America, mainly in Brazil (Michael and Refaat, 1981). *Schistosoma japonicum*, the major Asian schistosome, is endemic in mainland China, on some islands in the Philippines and in two small foci in Indonesia (Chen, 1993). The parasite was formerly endemic also in Japan, but there it has now been eradicated (Tanaka *et al.,* 1984). In Southeast Asia, *S. mekongi* occurs endemically in Cambodia and Laos. *Schistosoma intercalatum*, finally, is endemic in the Central African sub-region, particularly in Cameroon, Congo Democratic Republic and Gabon (Corachan *et al.,* 1988).

The adult worms of *S. mansoni*, *S. japonicum* and *S. mekongi* inhabit intestinal and mesenteric veins and cause hepato-intestinal or hepatosplenic schistosomiasis, characterised by hepatosplenomegaly, bloody diarrhoea and severe abdominal pain in heavy infections (Kojima, 1997). Adult *S. intercalatum* worms also commonly inhabit the mesenteric veins, but this parasite mainly causes severe colonic disease with less prominent liver involvement (Van Wijk, 1969). Clinical manifestations include bloody diarrhoea, haematuria and anaemia. In contrast, the adult worms of *S. haematobium* inhabit the venous plexus of the urinary bladder and ureters and cause the urinary form of schistosomiasis, with haematuria and dysuria as the most important clinical symptoms (Cheever *et al.,* 1977). This infection is also known to be associated with bladder cancer and inflammation of genital organs.

It has been demonstrated that *S. japonicum* infection results in a more severe hepatic disease and growth stunting than *S. mansoni* infection (McGarvey *et al*., 1993; Olds *et al.,* 1996). Furthermore, among the schistosomes pathogenic to man, *S. japonicum* is the only one of zoonotic importance. Several species of domestic and wild mammals are infected, with cattle, water buffaloes and pigs serving as its main reservoir hosts (Mc Garvey *et al.,* 1999; He *et al.,* 2001).

The schistosome egg, and not the adult worm itself, is the major pathogenic factor in schistosomiasis (Warren, 1973; Cheever and Yap, 1997). The trapped eggs initiate tissue injury that results in granuloma formation and fibrosis (Chen and Fu, 1989). In the liver, fibrosis is localised around the intrahepatic branches of the

portal vein, a lesion referred to as pipe-stem fibrosis (Andrade, 1965). The degree of fibrosis is generally related to the infection intensity, especially the schistosome egg density in the liver (Cheever *et al.,* 1980).

Experimental studies in rodents and non-human primates have provided important knowledge of the pathogenesis of schistosomiasis. However, the small size of rodents and their short life-span are serious drawbacks of using them as models of human schistosomiasis as it makes it impossible to study infection intensities and durations relevant to man and to evaluate the effect of immunisation or treatment in the long term. Non-human primates don't suffer these drawbacks, but their use in biomedical research is problematical for both ethical and economical reasons. Thus, in this study, the pig model of human schistosomiasis was used. Pigs are biologically similar to man in several ways and are natural hosts for *S. japonicum* (Willingham and Hurst, 1996; Johansen *et al.,* 2000). Infection with *S. japonicum* in pig is associated with clinical disease, marked pathological lesions, including liver fibrosis, and reduced growth (Yason and Novilla, 1984; Willingham *et al.,* 1998; Hurst *et al.,* 2000b). The pig develops marked liver fibrosis at an early stage of infection with *S. japonicum* after which the fibrosis spontaneously regresses in a few months, making the pig a useful animal model for studies of the development and resolution of this lesion (Hurst *et al.,* 2000a).

This study concerns liver fibrosis in experimental *S. japonicum* infection in pigs, with special reference to methods of evaluating the degree of fibrosis during long-term infection. Image analysis was used to measure the area of fibrosis for comparison with histopathological scoring and for quantitation of a major component of the extra-cellular matrix (ECM), collagen type 1, as detected by immunohistochemistry in tissue sections. The relationship between fibrosis and different parasitological variables was also investigated.

LITERATURE REVIEW

The parasite and its life cycle

General

Schistosomes have an indirect life cycle that comprises four stages: One stage within a definitive mammalian host for sexual reproduction, an asexual multiplication stage within an intermediate fresh water snail host, and two free-living stages, the miracidium and the cercaria (Sturrock, 1993). The presence of schistosomes in an area is greatly determined by the distribution of the intermediate freshwater snail host (Sturrock, 1993). Various *Bulinus* species of fresh water snails serve as intermediate hosts for *S. mansoni*, *S. haematobium* and *S. intercalatum,* whereas *S. mekongi* is transmitted by snails of the genus *Tricula.*

S. japonicum

Small amphibious fresh water snails of the genus *Oncomelania* serve as intermediate hosts for the different geographic strains of *S. japonicum,* and

humans, domestic and wild mammals including pigs, water buffalo, cattle, sheep, goats, dogs, cats, and field rats are definitive hosts (Warren, 1979; Cheever *et al.,* 1980). Non-human primates including gorillas and monkeys as well as laboratory animals, such as rodents and rabbits, can also serve as definitive hosts for *S. japonicum* and have been used in experimental infection studies (Warren, 1979; Cheever *et al.,* 1980).

The mature worms live in pairs in the mesenteric veins of the definitive host, where they may survive for a considerable period of time (Sturrock, 1993). The dorso-ventrally flattened adult male schistosome, which is 10 - 20mm in length and 0.55mm in width, embraces the cylindrical and much longer female schistosome, which is 20 - 30mm long and 0.3mm wide, in a gynaecophoric canal (Sturrock, 1993). In a day, the *S. japonicum* female is capable of producing 1000 to 3500 eggs, which are laid in clusters in small venules of the intestinal mucosa and sub-mucosa. Each egg contains an embryo that develops into a miracidium within a period of 9 - 12 days. The eggs find their way into the lumen of the gut through the wall of the blood vessels and surrounding tissues with the aid of histolytic enzymes secreted by the miracidium through the egg shell. The enzyme secretions induce an inflammatory reaction that promotes further tissue destruction. The schistosome eggs are voided together with extravasated blood, tissue debris and inflammatory cells through a process believed to be aided by peristaltic bowel movements (He, 1993). Schistosome eggs excreted from a definitive host hatch to release the miracidium when they reach fresh water. The hatching process, which is temperature-dependent with an optimal range of 10 - 30 ºC, is influenced by changes in osmotic pressure and light (Sturrock, 1993).

Newly hatched miracidia initially swim rapidly at a speed of close to 2mm per second in straight lines, scanning the environment for snails. They finally tend to become stationary in areas inhabited by snails, for example along the margins of water bodies or where there are submerged leaves of aquatic plants (Chernin and Dunaven, 1962). When contact is established with the right intermediate host, the free-living miracidium gets attached to exposed parts of the body of the snail through the aid of secretions from apical gland cells. The miracidium then penetrates the snail and develops into an elongated sac-like structure called the primary sporocyst (Sturrock, 1993).

The primary sporocyst matures and gives rise to the secondary sporocyst, a process which usually takes 10 - 12 days. Cells budding from the germinal epithelium within the secondary sporocysts develop into cercariae. The cercariae leave the sporocysts and migrate to the exposed surface of the snail from where they are shed. The pre-patent period from penetration to cercarial shedding is influenced by ambient temperature, and varies from 17 - 18 days at 30 – 35 ºC to several months at lower temperatures (Sturrock, 1993).

The cercaria, which is free-living and adapted to life in fresh water, is the infective stage for the definitive mammalian host. Upon shedding, the cercaria remains quite active in fresh water for about 12 hours, and may still be viable for up to three days. When in contact with the skin of the definitive host, the cercaria penetrates the skin with the aid of its secretion of proteolytic enzymes. During this

process, it sheds its tail and is transformed to the next larval stage called the schistosomulum. The newly transformed schistosomulum may remain unchanged in the skin for some days, but more commonly it enters a lymphatic vessel and eventually reaches the venous system through the thoracic duct. The schistosomulum finally reaches the portal system of the liver after some few days, and matures into an adult worm in a period of about four weeks. Some of the schistosomulum reaches the lungs via the venous circulation and then further to the systemic circulation. In the lungs, the migrating schistosomula cause marked inflammatory responses (Chen and Mott, 1988). When sexual maturity is attained, the muscular male schistosomes clasp the slender females to form permanent pairs, and these pairs migrate through the hepatic portal vein to veins around the intestines, specifically the mesenteric and intestinal veins, which are their final habitat and area for oviposition (Cheever and Neafie, 2000).

On average, the pre-patent period for *S. japonicum* in humans is close to 42 days, and based on results from experimental studies, it has been found to be 27 - 42 days in pigs, 29 - 35 days in dogs, 42 days in buffaloes and 36 days in cattle (Yason and Novilla, 1984; Basch, 1991; He *et al.,* 1992).

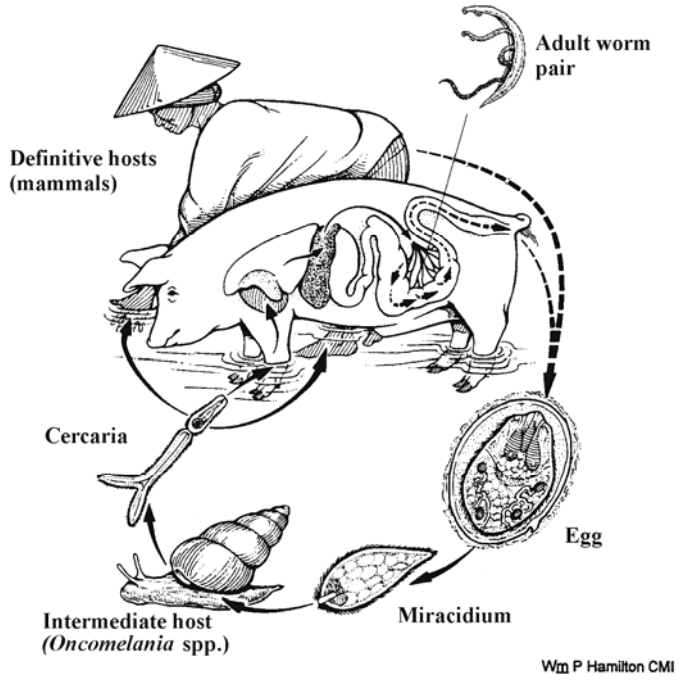


Fig. 1: The life cycle of *Schistosoma japonicum*

Disease syndromes and clinical manifestations

The severity of the disease schistosomiasis is influenced by the intensity and duration of infection, location of egg deposition, immune status of the host and concomitant infections (Cheever *et al.,* 1980).

Cercarial dermatitis

Cercarial penetration of the skin may be associated with local pruritus, erythema and papules. This clinical manifestation is commonly referred to as Paddy Field Itch (Chen and Fu, 1989), and is rather uncommon in endemic populations (Chen, 1993).

Acute schistosomiasis japonica

Acute schistosomiasis japonica, also known as Katayama fever or acute serum sickness-like illness, tends to be more severe than the acute disease caused by the other major schistosomes (Sleigh and Mott, 1986). It is commonly noticed after heavy infections, and the clinical manifestations usually coincide with the onset of oviposition. Common clinical signs include fever, chills, headache and muscular pain, inappetence, nausea, abdominal pain and dysentery (Olveda and Domingo, 1987). Physical examination reveals an enlarged and tender liver (Chen, 1993).This form of the disease is common in persons living in endemic areas after their first exposure to *S. japonicum* and in persons who enter an endemic region for the first time (Chen, 1993). Clinical signs of meningoencephalitis may also be observed at the acute phase of the disease (Garcia, 1976; Chen and Mott, 1988).

Chronic schistosomiasis japonica

There are two major forms of chronic schistosomiasis japonica: the intestinal and the hepatosplenic forms. Lethargy, lower abdominal pain and diarrhoea are commonly observed in patients with the intestinal form of the disease. At times, diarrhoea alternates with constipation, giving rise to thin, cord-like stools (Chen, 1993). The hepatosplenic form is characterised by fatigue, weakness, abdominal pain and diarrhoea (Ross *et al*, 2000). At the early stage of hepatosplenic *S. japonicum* infection, the left lobe of the liver is usually enlarged, smooth, firm and tender upon palpation. This enlargement of the left liver lobe is very characteristic of the disease (Chen and Mott, 1988). Splenomegaly is also a common clinical feature especially in heavy infections. Other clinical features of hepatosplenic schistosomiasis japonica are oedema, loss of libido, impotence in men, and sterility in women.

In advanced cases of hepatosplenic schistosomiasis japonica, there is usually a dilation of the collateral veins of the abdomen and oesophago-gastric varices (Chen and Mott, 1988). Rupture of oesophageal varices is the major cause of death in affected persons due to blood loss through internal bleeding. Ascites and epileptic seizures are other common clinical features of chronic schistosomasis japonica and the ascites may persist for several years (Chen and Mott, 1988).

In severe *S. japonicum* infection in children, normal growth may be hindered, sometimes resulting in dwarfism (Chen and Mott, 1988). Schistosomiasis japonica has been observed to be one of the major causes of retarded growth and

development in children in China and the Philippines (McGarvey *et al.,* 1996). Dwarfism, however, is uncommon nowadays due to the availability of effective chemotherapy and the existence of a more efficient control scheme to minimize infections (Mao and Shao, 1982).

Immunopathological manifestations of schistosomiasis japonica

Invasion by schistosomula and adult schistosomes

The schistosomula larvae are prone to attack by the host immune mechanism and thus may be eliminated through antibody-dependent cellular cytotoxicity (ADCC) mediated by immunoglobulin E (IgE) and eosinophil granulocytes (Pearce *et al*, 1986). Adult schistosomes, on the other hand, evade the immune system of their host by incorporating host derived macromolecules into their tegument membrane, thereby masking the expression of their own antigens (Smithers and Terry, 1969).

Immune reactions caused by the schistosome egg

The schistosome egg is the major pathogenic factor in schistosomiasis, as in contrast to the schistosomulum and the adult worm; it elicits a strong immune reaction and immunopathology. The immune response is elicited by antigenic substances secreted by the miracidium present within the egg (Weinstock, 1992). Since the body initially cannot destroy the egg shell, it serves as a nidus around which macrophages, epithelioid cells, multinucleated giant cells, eosinophils, lymphocytes, neutrophils, mast cells and fibroblasts accumulate and become organised to form a granuloma. The granuloma develops around the schistosome eggs that imbed in the different tissues of the host. The egg shell protects the egg from host destruction, and the egg secretes a variety of antigenic substances leading to antigen-specific humoral and cell-mediated immune responses. The process of granuloma formation is dynamic and complex and usually lasts for many weeks, during which the granuloma, develops, matures, involutes and heals leaving a fibrous scar as the egg is destroyed (Warren and Domingo, 1970; Weinstock, 1992). The granuloma protects the host from the antigenic secretions of the schistosome egg and finally eliminates the egg, an effect that is beneficial to the host, but also has detrimental consequences in that its formation leads to considerable tissue damage and fibrosis (Warren, 1973).

The egg-induced granulomas of *S. japonicum* are T-cell mediated delayed – hypersensitivity responses (Garb *et al*, 1981; Stavitsky, 1987). The anti-egg inflammatory response is initiated by CD4+ T-helper (Th) lymphocytes with a predominantly Th2-like cytokine profile characterised by the production of interleukins (IL)-4 and IL-10 (Cheever *et al.,* 1998). The granuloma contains CD4+ and CD8+ T lymphocytes as well as B lymphocytes. There is usually an increased cytokine production and low humoral reactions at the peak of granulomatous response in the acute phase of the infection (Boros, 1994). As the infection reaches the chronic stage, humoral responses with anti-egg IgG remain high, whereas the cell-mediated immune reactions become depressed (Yang *et al.,* 1999).

Liver fibrosis in schistosomiasis japonica

The wound healing process

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers. The process constitutes an array of interrelated and sequential events that culminate in the development of scar tissue to replace the tissue that has been injured or lost (Aukhil, 2000). In general, the significant presence of collagen types 1 and 3, and several inflammatory cells, including fibroblasts and endothelial cells, are characteristics of scar formation. The thick fibrils of collagen type 1 entwine with the fine network of collagen type 3 to regulate the tensile strength and distensibility of the scar tissue (Burgeson, 1998).The wound healing process includes haemostasis, inflammation, granulation, and remodelling (Cho, 1998).

In the haemostasis phase, platelets adhere to damaged endothelium and discharge adenosine diphosphate (ADP) thereby promoting thrombocyte clumping, which dams off the wound. The framework for completion of the coagulation process is formed at this initial phase, with fibrin providing the structural support for cellular constituents of inflammation (Cho, 1998).
The inflammatory phase is initiated by the release of numerous cytokines from platelets. During this phase, polymorphonuclear leukocytes engorge the wound, cleanse it and clear it of debris. As the process continues, monocytes (macrophages) exude from the vessels, continue with the cleansing process and secrete many factors that influence the wound healing process (Tanenbaum, 1995; Cho, 1998). In the granulation phase, fibroblasts migrate into the wound, lay down new collagen of types 1 and 3, and also produce fibronectin. Collagen type 3 predominates initially, but is later replaced by type 1 collagen (Cho, 1998). Angiogenesis and re-epithelialization also occur in this phase. In the remodelling phase, the wound undergoes constant alterations, a process that can last for years (Cho, 1998). Collagen is degraded and deposited during this phase in an equilibrium-producing fashion that results in no change in the amount present in the wound (Cho, 1998; Aukhi, 2000).

Wound healing in liver fibrosis

When liver tissue is injured, e.g. by granuloma formation in schistosomiasis japonica, the wound healing process is initiated (Weinstock, 1992). The major ECM proteins deposited during this process in the liver are collagen types 1 and 3 (Dunn *et al.*, 1977; Burgeson, 1988; Kershenobich and Weissbrod, 2003). Proteoglycans, fibronectin and laminin are other important ECM constituents. Fibronectin and laminin appear in the granulation stage of wound healing in *S. japonicum* infection. Both proteins have influence on the function of neutrophils and macrophages, which is mainly phagocytosis of necrotic hepatocytes. They also promote vascular development, and form the matrix for the deposition of collagen fibrils at later stages. The deposition of ECM proteins around the schistosome egg subsequently interferes with circulation in the liver.

Development of liver fibrosis and portal hypertension are the principal causes of mortality in chronic schistosomiasis japonica (Chen and Mott, 1988). In advanced cases, severe fibrotic changes known as Symmers' pipe-stem fibrosis occur. Symmers' pipestem fibrosis is a stellate portal fibrosis along the large intra-hepatic branches of the portal vein, which resemble clay pipe-stems when viewed in anatomic cross-section (Symmers, 1904; Lambertucci, 1993). It is an essentially portal fibrosis without the bridging, nodular formation and significant hepatocellular destruction that are characteristic of liver cirrhosis (Lambertucci, 1993). This pathological condition has been shown to be induced by the granulomatous response to the eggs that are deposited in the portal areas of the liver (Cheever, 1997). The eggs are swept from the intestine into the portal system of the liver via the venous circulation. They get trapped in the portal venules, causing a pre-sinusoidal obstruction of portal blood flow, which results in portal hypertension.

The eggs of *S. japonicum* induce a strong Th2 cytokine response, leading to granuloma formation and excessive deposition of newly synthesised ECM constituents and subsequently liver fibrosis (Cheever *et al.,* 1980). Fibrosis occurs when the dynamic equilibrium between collagen synthesis and degradation is altered (Chen *et al,* 2002). It has been demonstrated that the immuno-regulatory mechanism of fibrosis in schistosomiasis is partly independent from that of granulomatous inflammation (Kresina *et al.,* 1992; Cheever, 1997). When the initiating cause of the fibrosis is eliminated, immature fibrous tissues degrades rapidly, whereas mature collagen is more resistant to degradation, being stabilised by cross-linking molecules that block collagenolytic enzyme activity (Andrade, 1994). Experimentally, liver fibrosis has been demonstrated to regress slowly but steadily after elimination of the schistosome infection by chemotherapy, and the use of ultrasound examination has shown that this occurs in humans as well (Andrade *et al.,* 1993; Cai *et al.,* 1997).

However, in advanced Symmers' pipe-stem fibrosis, chemotherapy may be of little value, since it's the end result of host responses to schistosome eggs in the liver and the portal tracts are greatly expanded with dense deposits of collagen (Ohmae *et al.,* 1992). There is often no active infection that could be cured by chemotherapy at this stage.

Diagnosis, treatment and control

Diagnostic methods

Various diagnostic methods are employed in the diagnosis of *S. japonicum* infection. The most commonly applied method, which is highly specific, is the direct parasitological examination of faeces, tissues and organs (especially liver biopsies) for schistosome eggs (Chen and Mott, 1988). The modified Kato-Katz thick-smear technique, which is quantitative and suitable for surveying the prevalence and intensity of infection in the population, is the most commonly used method for stool examination (Ross *et al.,* 2001). The disadvantage of this method is that the sensitivity is low, so that eggs may not be detected at light infection.

Schistosomiasis japonica can also be diagnosed indirectly through observation of pathological changes initiated by the infection, such as hepatosplenomegaly detected by clinical examination (Chen and Mott, 1988). Also, computer tomographic scanning and other imaging techniques such as ultrasound and magnetic resonance imaging are employed to diagnose typical liver lesions of schistosomiasis japonica (Cai *et al.,* 1992; Monzawa *et al.,* 1994).

Finally, schistosomiasis japonica can be diagnosed serologically. This involves the application of test systems and antigens such as ELISA, IHA and indirect immunofluorescent assay to determine the immune response to schistosome egg and worm antigens (Chen and Mott, 1988). These test systems are highly sensitive, but do not differentiate past infections from present ones, since the antibody response may remain long after the infection has ceased. Circulating schistosome antigens, in contrast, can be used to detect an ongoing infection, and also for the evaluation of the effect of chemotherapy (Ross *et al,* 2001).

Treatment

Many different drugs, including antimonials and furapromidials, have been used in the chemotherapy of schistosomiasis japonica in the past (Chen, 1985). However, due to their immense toxicity and the long duration of treatment required, they have been discarded in favour of the more recently developed drug praziquantel, which has low toxicity, great efficacy and is easy to administer (Cioli, 1998; Sleigh *et al.,* 1998). A disadvantage of praziquantel is that it has little or no effect on the developing larvae of 3 – 21 days post infection (Ross *et al.,* 2001). The standard single applicable dose is 40mg/kg for humans and pigs, and 25mg/kg for cattle (Johansen, 1998; Li *et al.,* 2000).

Of recent, an antimalarial drug called artemether has been demonstrated to have a strong effect on the schistosomula (or juvenile) stage of the parasite with an applicable dose of 6mg/kg administered orally (Xiao *et al.,* 2002). Thus, a combination of praziquantel and artemether would have a greater effect than each drug administered on its own. However, in areas where both schistosomiasis and malaria occur, artemether should be reserved for malaria in order to reduce the risk of drug resistance.

Control

The public health significance of schistosomiasis japonica is large in endemic regions of China and the Philippines, although established control programmes have greatly reduced the level of infection in certain areas (Ross *et al.,* 1997; Sleigh *et al.,* 1998). The control programmes, which are aimed at reducing transmission and morbidity of *S. japonicum* infection, involve chemotherapy in humans and reservoir hosts, health education, improved sanitation and control of the intermediate host population (Olveda *et al.,* 1996; Ross *et al.,* 1997; Sleigh *et al.,* 1998).

Schistosomiasis japonica in pigs

Clinical manifestations

The pig is naturally infected with *S. japonicum* in endemic regions in China and the Philippines (Dumag *et al.,* 1980; Yuan *et al.,* 1993). The major clinical signs in the pig following natural infection include anorexia, fever, lethargy, depression, coughing, nasal discharges, diarrhoea and voiding of blood stained faeces (Dumag *et al.,* 1980; Hurst *et al.,* 2000b). In experimental studies, pigs have been reported to develop only diarrhoea at the onset of egg excretion when infected with up to 2000 *S. japonicum* cercariae (Willingham *et al.,* 1998), and acute schistosomiasis with severe clinical signs when infected with massive doses, 5000 – 6000 *S. japonicum* cercariae (Yason and Novilla, 1984).

Pathology

The pathological manifestations are associated with tissue reactions around the trapped eggs mainly in the intestines and liver (Willingham *et al.,* 1998; Hurst *et al.,* 2000a). Macroscopically, the intestinal mucosa is thickened, congested and covered with blood-tinged mucus in heavy infections (Yason and Novilla, 1984). Also, petechial and echymotic haemorrhages are multifocally distributed in the mucosa of the large intestine (Willingham *et al.,* 1998; Hurst *et al.,* 2000b) (Fig. 2). In the liver, disseminated small, firm, gray-white nodules are observed on the liver surface and within the parenchyma, with a generalised increase in the interlobular connective tissue network (septal fibrosis) (Willingham *et al.,* 1998; Johansen, 1998) (Fig. 3). Enlargement of the portal lymph nodes is a common feature. The predominant microscopic lesions in the liver are granulomas, diffuse portal and septal infiltration of eosinophils and small mononuclear cells and fibrosis (Yason and Novilla, 1984; Hurst *et al.,* 2000a). In the intestine, egg associated non-granulomatous inflammatory foci are observed in the mucosa, whereas egg granulomas are found mainly in the sub-mucosa of the intestines (Hurst *et al.,* 2000a). There is fibrosis of the gut wall in areas containing several granulomas.

Immune response

The pig has been observed to elicit a Th2 response with marked IL-4 and IL-10 expression in the liver and intestinal tissues at 10-weeks p.i. with *S. japonicum* (Oswald *et al.,* 2001). The Th2 response also results in marked blood and tissue esosinophilia in the pig. Antibody titres against *S. japonicum* antigens where found in the prepatent phase of infection in naturally infected pigs, suggesting an early onset of the immune response (Hurst *et al.,* 2000b). The titres had increased further around the time of patency and remained persistently high for several weeks after that.

The S. japonicum egg granuloma in pigs

A typical *S. japonicum* granuloma in the pig contains eosinophils, macrophages, epithelioid cells, giant cells, lymphocytes (T and B cells), some mast cells and fibroblasts. Immunophenotyping has been used to characterise the different stages of *S. japonicum* granuloma in the pig liver (Hurst *et al.,* 2002). All the stages show

marked major histocompatibility complex (MHC) class II expression in epithelioid cells, and a CD4+ and CD8+ T cell dominance. The presence of B-cell-rich, small lymphoid nodules apposed to the granulomatous reaction possibly supplying the granuloma with plasma cells, is a unique feature in the pig (Hurst *et al.,* 2002).

The pig as an animal model

In the research world, an animal model is a living organism in which biological activities or behaviour can be studied or in which an induced or spontaneous pathological process, which occurs similarly in humans or in other animal species, can be investigated (Held, 1983; Johansen *et al.,* 2000). For an assumption or hypothesis to be properly investigated, an appropriate animal model needs to be chosen. The pig has anatomical, physiological, immunological, nutritional, metabolic and circulatory functions which are quite similar to those of humans. Furthermore, the pig is an omnivorous monogastric animal having dietary preferences similar to those of humans, and its integumental system is also quite similar from the histological and physiological view points (Swindle, 1984; Willingham and Hurst, 1996). These similarities make the pig an appropriate animal model for the study of several conditions affecting humans and have lead to an extensive use of this animal species in biomedical research (Willingham and Hurst, 1996). It should be noted that organ similarities between man and pig have made it possible for these vital structures to be transplanted from pigs to humans (Luisa, 1996). In addition to the biological similarities, pigs have certain other advantages as experimental animals: they attain sexual maturity early in life and have a relatively high reproductive rate when compared to other farm animals; they are easy to handle and are cost-effective (Willingham and Hurst, 1996; Johansen *et al.,* 2000).

Animal models of human schistosomiasis japonica

In the case of human schistosomiasis japonica, the pig, stands tall as an excellent large animal model for the better comprehension of this condition. Experimental studies have shown that pigs develop typical schistosomiasis with lesions similar to those of humans, the liver and intestines being the most severely affected organs (Yason and Novilla, 1984; Willingham *et al.,* 1994, 1998; Hurst *et al.,* 2000a). Marked hepatic fibrosis develops early during the infection and declines spontaneously later on as the pigs undergo self-cure (Willingham *et al.,* 1998; Zhang *et al.,* 1998). In addition, the pig's liver is similar to that of humans in terms of gross anatomy, physiology and metabolic functions (Swindle, 1984; Phillips and Tumbleson, 1986). The pig may thus be particularly useful for studies of the development and resolution of hepatic fibrosis in schistosomiasis (Hurst *et al.,* 2000a). In addition, due to their comparable size with humans, pigs are suitable for investigation of pathological effects of different levels of intensity of infection.

Non-human primates have also been found to be good models (von Lichtenberg *et al.,* 1971). However, the use of primates is restricted, primarily for ethical reasons,

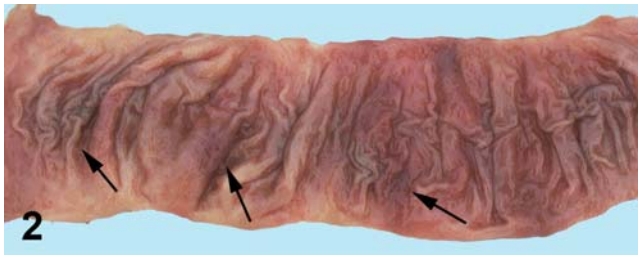
but also because they are very costly and difficult to maintain, reproduce slowly and may have viruses that are dangerous to humans (Nyindo and Farah, 1999; Johansen *et al.,* 2000). As for rodents, they are inexpensive and easy to maintain, but their gastro-intestinal physiology and their biological and immunological responses to *S. japonicum* infections deviate much from those of humans and the life-span of the schistosome may exceed that of the mouse (Basch, 1991; Johansen *et al.,* 2000). Furthermore, some rat species are resistant to schistosome infection.

Liver fibrosis in schistosomiasis japonica in the pig

Liver fibrosis induced by *S. japonicum* infection in the pig has been found to be correlated with both eggs per g liver and granuloma density (Hurst *et al.,* 2000a). Two patterns of fibrosis have been described in pigs: Portal fibrosis, characterised by the thickening of the fibrous tissue surrounding central and peripheral portal branches, that resembles the classical human pipe stem fibrosis (Symmers, 1904), and septal fibrosis, characterised by an increase of the normal fibrous interlobular (porto-portal) septa (Kardorff *et al.,* 2003) (Fig. 4). Septal fibrosis is not a common feature in humans. Liver fibrosis occurs at an early stage of infection in pigs and chronic liver fibrosis due to *S. japonicum* has not been described. Use of ultrasonography has shown that the early hepatic lesions in pigs, especially septal fibrosis, are correlated with markers of hepatic morbidity, such as liver size and hepatic collagen content (Kardorff *et al.,* 2003).

Histopathologically, egg granulomas are found mainly in portal fields but also in interlobular septa. Granulomatous obstruction and phlebitis of portal veins are common and in both portal areas and septa there are diffuse inflammatory cell infiltration dominated by eosinophils, lymphocytes and plasma cells. Fibrosis is confined to portal fields and septa and the lobular architecture of the liver is largely preserved (Hurst *et al.,* 2000a). In the pig, it has been shown that one major component of the ECM, collagen type 1, increases proportionally to the amount of fibrosis in portal and septal areas in response to *S. japonicum* infection (Baddamwar *et al.,* 2004). Myofibroblasts, i.e. fibroblast-like cells expressing α-smooth muscle actin, occur in increased numbers in the fibrosed portal areas and interlobular septa.

Various methods have been employed in evaluating liver fibrosis in the pig *S. japonicum* model including ultrasonography (Kardorff *et al.,* 2003), histopathological fibrosis scoring (Hurst *et al.,* 2000; Baddamwar *et al.,* 2004) and quantitative image analysis (Baddamwar *et al.,* 2004).


2

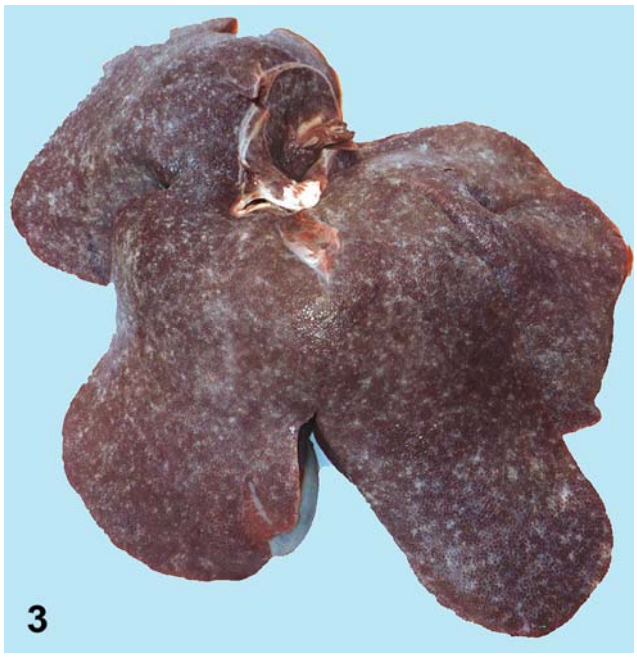

3


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Fig. 2-4: Organs from pigs experimentally infected with *Schistosoma japonicum*.

Fig. 2: Large intestine (colon) with haemorrhagic lesions and mucosal thickenings (arrows), 24 weeks post infection (p.i.).
Fig. 3: Enlarged liver with small, gray- white nodules and interlobular septa fibrosis (septal fibrosis), 8 weeks p.i.

Fig. 4: Transverse section of a liver lobe showing fibrotic thickening of a large portal vein branch (arrow), 16 weeks p.i.

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INTRODUCTION TO RESEARCH REPORT

A number of studies have emphasised the use of the pig as a large animal model of human schistosomiasis japonica (Willingham and Hurst, 1996; Johansen *et al.,* 2000). The pig develops marked liver fibrosis at an early stage of infection with *Schistosoma japonicum* after which the fibrosis spontaneously gradually regresses as the pig undergoes self cure. This makes the pig a useful animal model for studies of the development and resolution of this lesion. The pathogenesis of liver fibrosis in pig schistosomiasis is incompletely understood, but the degree of fibrosis has previously been shown to be correlated to liver tissue egg counts and granuloma density (Hurst *et al.,* 2000). A method for quantitation of fibrosis in liver tissue sections using image analysis has previously been developed and found to correlate well with conventional semi-quantitative histopathological scoring (Baddamwar *et al.,* 2004). In liver fibrosis induced by schistosome infection in man and laboratory animals, major constituents of the extra-cellular matrix (ECM) are collagen types 1 and 3, often at various levels at different stages of infection (Biempica *et al.,* 1983, Grimaud *et al.,* 1987, Chen F *et al.,* 2002, Xiong *et al.,* 2003). Information about possible changes in the ECM in liver fibrosis in pigs is scant, although collagen type 1 has been shown to be present in portal and septal areas proportionally to the degree of fibrosis there in *S. japonicum*-infected pigs. (Baddamwar *et al.,* 2004)

The aims of the present study were as follows:

- To obtain basic information about liver fibrosis in the pig by assessment of changes in its degree and composition during the course of long-term *S. japonicum* infection.
- To investigate the relationship between fibrosis and parasitological variables.
- To compare the use of semi-quantitative histopathological scores and quantitative measurement of the area of fibrosis with image analysis for assessment of the degree of fibrosis.
- To test the use of image analysis for quantitation of collagen type 1, as detected by immunohistochemistry in liver tissue sections.

RESEARCH REPORT

Evolution of Liver Fibrosis During Long-term Experimental *Schistosoma japonicum* Infection in Pigs

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ABSTRACT

The parasitic disease schistosomiasis is a major cause of liver fibrosis in man. In the present study, liver fibrosis during the course of long-term infection was investigated in the pig model of human schistosomiasis japonica. Three groups of pigs were infected with 1000 *Schistosoma japonicum* cercariae and necropsied at 8, 16 and 24 weeks post infection (p.i.). Parasitological variables included faecal egg and miracidial counts and liver tissue egg counts (TEC). The degree of fibrosis was assessed in Masson's trichrome-stained liver sections, using both semi-quantitative histopathological scoring and quantitative area measurement by image analysis. The number of perioval granulomas per area unit in the same sections was determined. Collagen type 1 was detected by immunohistochemistry and the area fraction in selected areas of interlobular septa was measured by image analysis. The relationship between fibrosis and parasitological variables was investigated.

Faecal egg and miracidial counts peaked at 8 weeks p.i. and declined rapidly thereafter to low levels at 24 weeks p.i. Liver TEC and granuloma density were also highest at 8 weeks p.i. and decreased at the later time points. The liver lesions were mainly characterised by perioval granulomas, diffuse inflammatory cell infiltration, and portal and septal fibrosis. Scores for both portal and septal fibrosis were highest at 8 weeks p.i. and were reduced at the later time points, and similar results were obtained for the the area of fibrosis. Collagen type 1 was present in portal and septal areas in proportion to the degree of fibrosis in the infected pigs. The area fraction for collagen type 1 in septa was significantly higher in infected than in control pigs, but no difference was found between the different time points in infected pigs. There was a correlation between the area of fibrosis and faecal miracidial counts and between granuloma density and faecal egg counts. Fibrosis was strongly correlated with granuloma density, but not with liver TEC. The two methods used for assessment of liver fibrosis were well correlated.
In conclusion, this study confirmed results from other studies that marked liver fibrosis develops at the acute stage and is reduced at later stages of *S. japonicum*

infection in pigs, and that the degree of liver fibrosis is related to granuloma density. The results suggest that faecal egg excretion could be used as a marker of liver pathology in pigs. Quantitative image analysis gave comparable results to semi-quantitative histopathological scoring and is thus a useful, additional tool for assessment of the degree of liver fibrosis in this animal model. The area fraction of collagen type 1 in fibrous septa was increased in infected pigs, but did not change in connection with the resolution of fibrosis that occurred during the course of infection.

Key words: *Schistosoma japonicum*, liver fibrosis, pig, granuloma density, histopathological scores, image analysis, immunohistochemistry, collagen type 1.

INTRODUCTION

Schistosomiasis is a highly debilitating, parasitic disease affecting millions of people around the world (WHO, 2002). Schistosomiasis japonica, caused by the blood fluke *Schistosoma japonicum,* is endemic in China and the Philippines, where it is a serious threat to public health (Zhou *et al.,* 2005). The parasite also infects a wide range of mammalian hosts, including cattle, water buffaloes and pigs, which serve as important reservoir hosts.

Schistosomiasis is one of the most common causes of liver fibrosis in humans (WHO, 2002). The primary lesion is the host tissue reaction to schistosome eggs trapped in the portal system of the liver, leading to portal fibrosis and hypertension (Cheever, 1985). Fibrosis is generally related to infection intensity, especially the schistosome egg density in the liver, and occurs when the deposition of extracellular matrix (ECM) exceeds its degradation (Meneza *et al.,* 1989; Cheever and Yap, 1997). Major components of the ECM in fibrotic livers are collagen type 1 and 3, laminin and fibronectin (Andrade *et al.,* 1992; Chen F *et al.,* 2002). During the course of infection, the ECM does not only change quantitatively but there are also qualitative changes in its composition (Grimaud *et al.,* 1987; Biempica *et al.,* 1983; Chen JL *et al.,* 2003). The application of ultrasonography and liver biopsy indicates that fibrosis is partially reversible after chemotherapy (Ohmae *et al.,* 1992). However, reversibility decreases with increased severity of fibrosis, and may not occur in advanced chronic cases. The mechanisms involved in liver fibrogenesis and fibrosis resolution in schistosomiasis are still incompletely understood.

There are several anatomical, physiological and immunological similarities between man and pig and the pig is also a natural host for *S. japonicum*, which has led to the exploration of the pig as a large animal model of human schistosomiasis japonica (Willingham and Hurst, 1996; Johansen *et al.,* 2000). An experimental, single *S. japonicum* infection has been shown to induce liver fibrosis correlated with the density of eggs and granuloma in pigs (Hurst *et al.,* 2000). The liver lesion resembles human schistosomal fibrosis in several ways. Portal fibrosis is regularly found at the microscopic level and is also occasionally visible grossly (Hurst *et al.,* 2000; Kardorff *et al.,* 2003). In pigs, unlike in man, there is usually prominent fibrosis of the interlobular septa (septal fibrosis) as well. Liver fibrosis

in pigs is pronounced at the early stage of infection, when egg excretion is high and then gradually regresses over the following weeks as the pig undergoes self-cure (Willingham *et al.,* 1998). Use of ultrasonography has shown that the early hepatic lesions, especially septal fibrosis, in pigs are correlated with markers of hepatic morbidity, such as liver size and hepatic collagen content (Kardorff *et al.,* 2003). At six months post infection (p.i.) liver fibrosis is slight or absent (Willingham *et al.,* 1998). The pig thus appears to be a useful animal model for studies of the pathogenesis of early development of liver fibrosis and its resolution at later stages, including any qualitative changes in the ECM that may occur during these events.

Assessment of the degree of liver fibrosis in man is commonly done by semi-quantitative histopathological scoring (Chevallier *et al.,* 1994; Helal *et al.,* 1998). Methods for quantitative analysis of human liver fibrosis by image analysis have also been developed (Chevallier *et al.,* 1994; Pillete *et al.,* 1998). Both semi-quantitative scoring and image analysis have previously been employed to assess liver fibrosis in the pig (Hurst *et al.,* 2000; Baddamwar *et al.,* 2004).

The present study was based on tissues and parasitological data from pigs up to six months after experimental *S. japonicum* infection. The objectives of the study were to assess changes in the degree of liver fibrosis during the course of long-term infection and to investigate the relationship between fibrosis and several parasitological variables. Two methods for assessment of liver fibrosis, semi-quantitative histopathological scoring and quantitative image analysis were compared. Image analysis was also used for assessment of possible changes over time of one component of the ECM, collagen type 1, as detected by immunohistochemistry in tissue sections.

MATERIALS AND METHODS

Experimental animals and study design

Thirty Chinese Landrace pigs initially aged 8 to 11 weeks and weighing 16 to 22 kg were included in the experiment. The pigs had been kept in pens since birth and were previously unexposed to *S. japonicum*. Twenty-one pigs were allocated into three groups (A, B, C) of seven pigs each and infected by direct skin penetration with a single dose of 1000 cercariae of *S. japonicum* obtained from *Oncomelania hupensis hupensis* snails purchased from the Institute of Schistosomiasis Control, Jiangsu province, China. The other nine pigs, divided into three groups, (A_c, B_c and C_c) served as uninfected controls. Albendazole (20mg/kg body weight) was administered orally to all the pigs to eliminate gastrointestinal parasites prior to the commencement of the experiment. The pigs were housed in pens, fed a standard commercial pig feed and drinking water was provided *ad lib*. The design was approved by the Experimental Animal Centre, Huazhong University, Wuhan, Hubei province. Faecal samples were collected from all pigs immediately before infection (week 0) and then at 2, 4, 6, 8, 12, 16, 20 and 24 weeks post infection (p.i.), respectively, for faecal egg counts and detection and counting of viable eggs via miracidial hatching. Groups A, B and C and their corresponding control

groups were killed at weeks 8, 16 and 24 weeks p.i., respectively, for collection of tissue samples for liver tissue egg counts and histopathology.

Faecal egg counts and faecal miracidial counts

Faecal eggs counts were obtained by a combined filtration and sedimentation / centrifugation
method (Willingham *et al.,* 1998). Briefly, a 20g sample of faeces was washed with water through a two-layer nylon net (the mesh size of the inside layer was 120 holes/square inch and of the outside 260 holes/square inch). The material collected in the outside layer was washed, allowed to sediment and the resulting sediment was centrifuged and then re-suspended in 10ml saline. Of the suspension, 5ml was used for egg counts and 5ml for miracidial counts. For egg counts, 0.1ml suspension was smeared onto a glass slide and examined microscopically and schistosome eggs were counted manually. Three such slides were examined and the average number of eggs per slide was calculated and then multiplied by 5 to get the number of eggs per g faeces. The remaining 5ml of the sediment suspension was added into flask of chlorine-free water for detection of miracidial hatching at 20 - 30ºC. The supernatant containing miracidia was collected from the neck of the flask at 3 and 6 hours, respectively, and put in a Petri dish. Five drops of 3% potassium iodide solution was added to kill the miracidia, which were then counted manually in a microscope. The result was divided by 10 and expressed as miracidia per g faeces.

Liver tissue eggs counts (TEC)

The number of schistosome eggs in the liver was determined by digesting a 5g sample of liver tissue recovered at necropsy in 20ml of 5% KOH overnight. Three 0.1ml aliquots of the digestion fluid were smeared onto a glass section and used to count the eggs microscopically. The mean of the three counts was multiplied by 40 to get the number of eggs per g liver.

Tissue sampling and preparation procedure

The pigs were killed by electric shock and bleeding. Tissue samples from the liver of each pig were collected, fixed in 10% neutral-buffered formalin overnight, routinely processed, and embedded in paraffin for histopathological studies and immunohistochemistry (IHC). From each pig, 4µm thick liver sections were cut onto Super Frost® Plus glass slides (Menzel-Gläser, Germany) and stained with haematoxylin and eosin (H&E) and Masson's trichrome (MT) for histopathology and image analysis. Unstained sections of each series were employed for immunohistochemical studies. The slides were coded to prevent the investigator knowing the group affiliation of the pigs.

Semi-quantitative scoring of liver fibrosis

The degree of portal and septal fibrosis was assessed in MT stained sections using the following semi-quantitative scoring system: score 0 = none, score 1= mild, score 2 = mixed mild and moderate, score 3= moderate, score 4 = mixed moderate and marked, and score 5 = marked fibrosis. Examples of liver sections with the

different fibrosis scores are presented in Figure 1. The sum of the scores for portal and septal fibrosis for each pig was used as a score for total fibrosis.

Liver granuloma density

The number of granulomas per area unit of the liver was assessed in images obtained from the MT-stained sections at 4x for image analysis (see section on image analysis below). The number of granulomas per 10 such images was counted in each infected pig. To avoid double-counting of granulomas that were only partially seen in the image, such granulomas were included if they were on the upper and right edge but excluded if they were on the lower and left edge. The results were used a measurement of liver granuloma density.

Immunohistochemistry

Three liver sections of each pig were used for detection of collagen type 1, with the Streptavidin Biotin Complex / Horse Radish Peroxidase (Strep ABC / HRP) method (DAKO A/S, Glostrop, Denmark). All dilutions and rinses were performed with Tris-Buffered saline (TBS) of 0.05M and pH 7.6. Following de-paraffinisation, the sections were pre-treated with pepsin (1mg/ml) in 0.5M acetic acid for 90 min at 37ºC. After the pre-treatment procedure, endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 10 min. Normal goat serum of 1:50 dilution was applied for 30 min to prevent non-specific protein binding. Endogenous avidin and biotin were blocked for 30 min each by employing an Avidin-Biotin blocking kit (Vector laboratories, Burlingame, CA, USA). The primary antibody used was rabbit anti-human collagen type 1 (polyclonal antiserum, DAKO, diluted 1:50). Sections were incubated with the primary antibody at 4ºC over night, after which a biotinylated secondary antibody (goat anti-rabbit IgG, Vector laboratories, diluted 1:200) and Strept ABC / HRP were applied sequentially for 30 min each. For specificity control, the primary antibody was replaced by normal rabbit Ig (DAKO), diluted to the same protein concentration as the primary antibody.

Immunoreactivity was revealed by incubation with a chromogen solution containing 0.6mg/ml diaminobenzidine (DAB) and 0.3% hydrogen peroxide in 0.05M TBS for 4–5 min, producing a brown immunostaining. The sections were counterstained with haematoxylin for 1 min, rinsed in tap water, mounted with a xylene-soluble medium and examined with light microscopy.

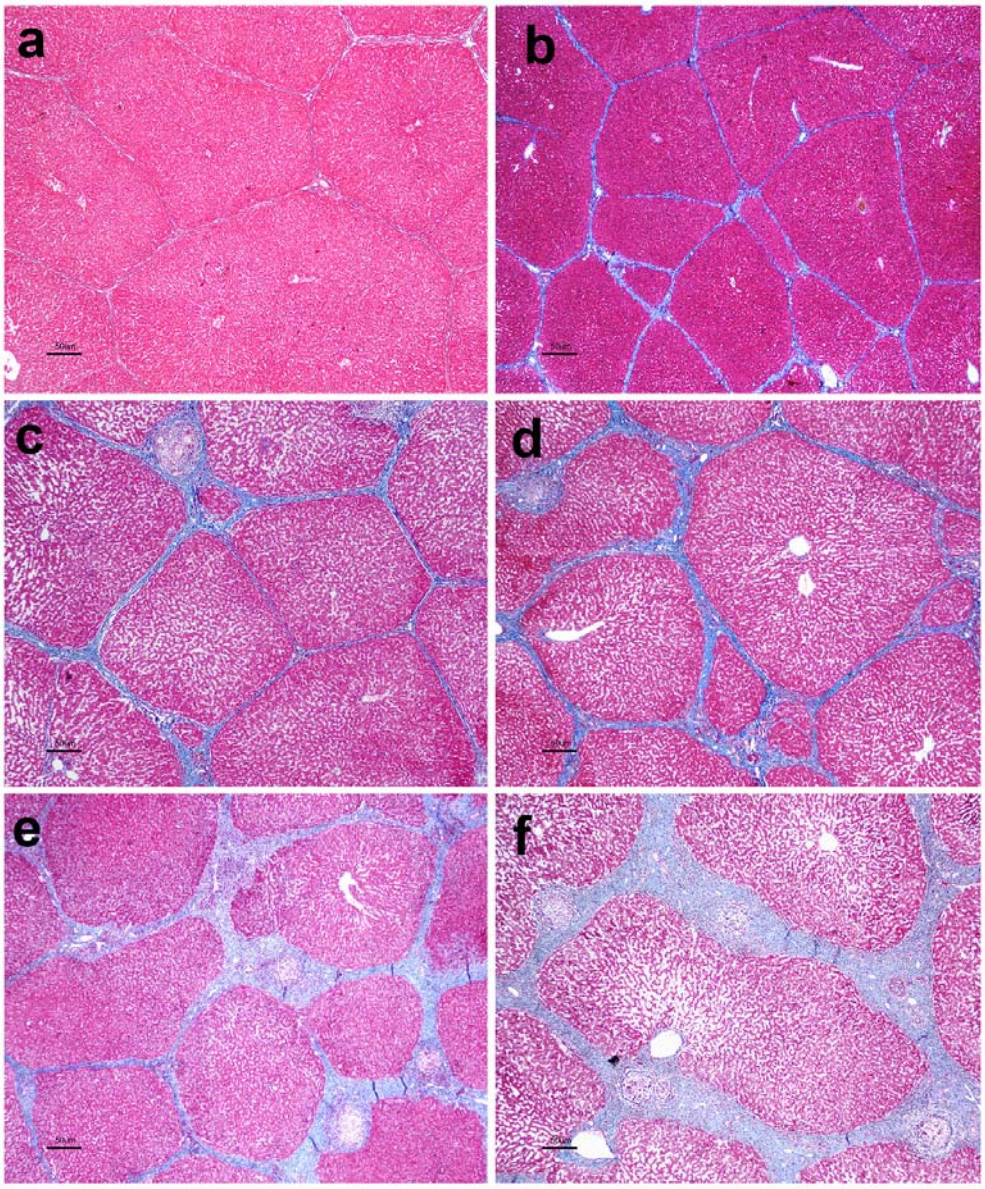

Figure 1. Semi-quantitative histopathological scoring of portal fibrosis (PF) and septal fibrosis (SF) on Masson's trichrome stained sections from an uninfected pig (a**)** and *Schistosoma japonicum* infected pigs (b – f).

a = PF and SF score 0
b = PF and SF score 1
c = PF and SF score 2
d = PF and SF score 3
e = PF and SF score 4
f = PF and SF score 5

Image analysis

A quantitative image analysis system (Easy Image Analysis 2000, Tekno optic AB, Stockholm, Sweden) was used for quantitative evaluation of the area of portal and septal fibrosis in the MT-stained sections and the area occupied by collagen type 1 in septa in the immunostained sections. A Nikon Digital Still camera DXM 1200 connected to a Nikon Eclipse E 600 microscope was used to obtain images for the quantitative evaluation process.

MT-stained sections

Ten different images of MT-stained liver sections at the 4X objective were obtained from each pig. Fibrosis in portal and septal areas that stained blue in the sections, referred to as the Dark Blue category, was measured. In each image, this category was measured three times and the mean area fraction of the fixed image area was calculated for each pig. The threshold values for hue, lightness and saturation were 142 – 232, 16 – 242, and 5 – 255 respectively

Sections immunostained for collagen type 1

Twenty different images of the immunostained sections at the 40X objective were obtained for each pig. To assess possible changes in the contents of collagen type 1 in the fibrous tissue of the liver, a septal area of approximately 5000 area units (range 4,000 – 5,999 area units) without the presence of granulomas was analysed in each image. Two categories, termed Brown and Blue, respectively, were measured within each selected area. Category Brown represented fibres immunostained for collagen type 1 and category Blue other tissue within the selected areas that stained blue with haematoxylin. The selected measurement area in each image was measured three times and the mean area fraction of the selected image area was calculated for each pig. For both categories, the threshold values for hue, lightness and saturation were 0 – 255, 16 – 235, and 0 – 255 respectively.

Statistical analysis

The differences between the groups of pigs regarding liver TEC, histopathological fibrosis scores, granuloma density, area of fibrosis and area of collagen type 1 were analysed by the non-parametric Mann Whitney test. Pearson's correlation test was used to investigate relationships between parasitological variables, between fibrosis and parasitological variables and granuloma density, respectively, as well as between the two methods for assessment of fibrosis, i.e. histopathological scores and area of fibrosis obtained by image analysis. For faecal egg excretion, the data obtained at 8, 16 and 24 weeks p.i. were used in the correlation analyses. For all tests, P values < 0.05 were regarded as statistically significant.

RESULTS

Faecal egg excretion (egg and miracidial counts)

Patent infections were obtained in all of the infected pigs. Faecal egg excretion was first detected at 6 weeks p.i. and had increased at 8 weeks p.i. in all three groups. At 12 weeks p.i. it had decreased markedly and was further decreased at 16 weeks p.i. in group B and C. It remained low until the end of the experiment at 24 weeks p.i. in group C, the only group followed for the duration of the experiment. The pattern was similar for the miracidial counts, although the number of miracidia per g was generally much lower than the total number of eggs per g. The results from group C are presented in Figure 2.

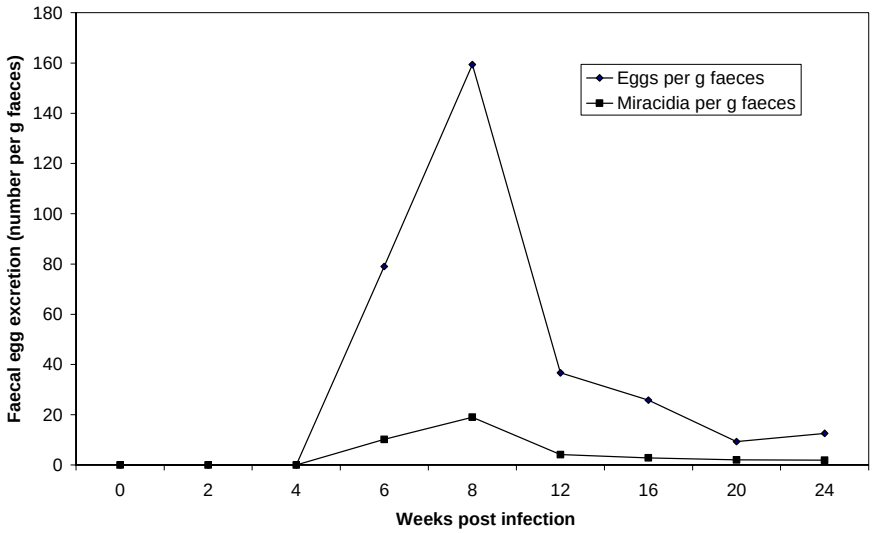

Figure 2. Feacal egg excretion expressed as eggs and miracidia per g faeces (geometric means) for group C throughout the experimental period.

Liver TEC

The number of eggs per g liver tissue (group median values) was higher in group A (2520) than in group B (640) and group C (400), respectively, ($p<0.05$). No statistically significant difference between groups B and C was observed.

Histopathology

The liver lesions were characterised by perioval granulomas, diffuse inflammatory cell infiltration, mostly by eosinophils, lymphocytes, plasma cells and macrophages, and fibrosis of the portal areas and interlobular septa.
Granulomatous endophlebitis and obstruction of portal veins with obliteration of the vessel wall were also observed.

Semi-quantitative histopathological scoring of liver fibrosis

The scores for portal, septal and total liver fibrosis (group median values) are presented in Figure 3. All the scores for fibrosis were higher in group A than in group B and C, respectively, ($p<0.05$), whereas no differences were observed between group B and C for any of the scores.

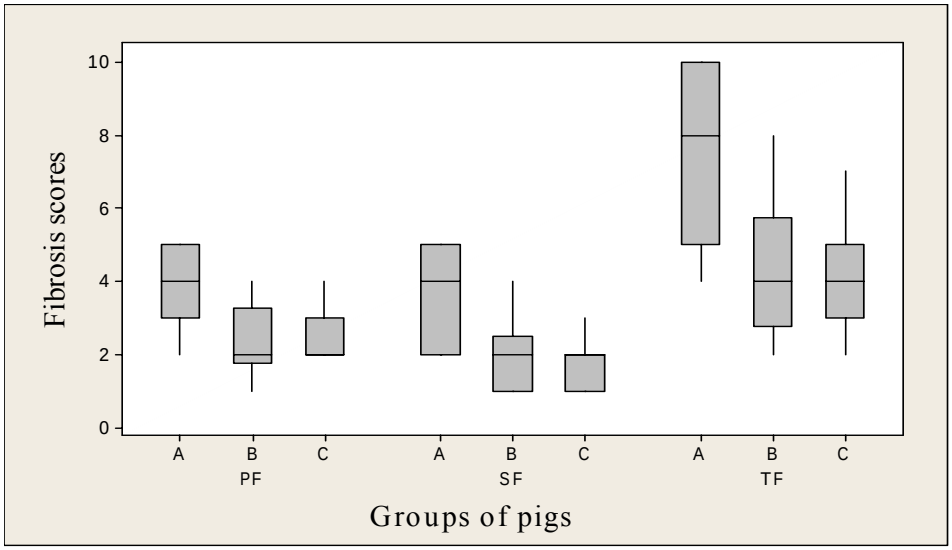

Figure 3. Histopathological scores of portal (PF), septal (SF) and total fibrosis (TF) in the different groups of pigs. [Group A = 8 weeks post infection (p.i.); group B =16 weeks p.i. and group C = 24 weeks p.i.]

Liver granuloma density

The granuloma density (group median values) was 39.0 in group A, 22.5 in group B and 5.0 in group C. The observed difference between group A and C was statistically significant ($p<0.05$).

Quantitative measurement of liver fibrosis with image analysis

The results of the measurement of the area of liver fibrosis in the MT-stained sections (Category Dark Blue) are presented in Table 1. The area of fibrosis was significantly higher in the infected pigs than the uninfected controls for all the three groups ($p<0.05$). Among the infected pigs, group A showed significantly higher values than group B and C, whereas no difference was observed between the two latter groups.

Immunohistochemical detection of collagen type 1 in the liver

In liver sections from the uninfected pigs, the anti-collagen type 1 antibody stained extra-cellular fibres in the connective tissue of the portal triads, interlobular septa and around the central veins. In the infected pigs, immunostained fibres were found in increased numbers in the portal areas and interlobular septa proportional to the observed increase of the degree of fibrosis (Figure 4.a). Also, concentrically arranged fibres positive for collagen type 1 were detected to a variable degree at the periphery of most granulomas. Figure 4.b.

Quantitative measurement of components of septal fibrosis with image analysis

The results of the measurement of the area fraction of fibres immunostained for collagen type 1 (Category Brown) and other tissue (Category Blue) in interlobular septa are shown in Table 1. The values obtained for category Brown in the infected pigs were significantly higher than those in the uninfected controls for all three groups ($p<0.05$), whereas the opposite was true for the category Blue values. However, no difference was observed between the groups of infected pigs.

Table 1: Area of fibrosis and area of components of septal fibrosis as measured by image analysis in the different groups of pigs (group median values).

Group	Area of fibrosis (fraction of fixed image area)	Area of components in septal fibrosis (fraction of selected image area)	
	(Category Dark Blue)	Collagen type 1 (Category Brown)	Other tissue in septa (Category Blue)
Infected			
A	14.7 [a,b]	66.5 [b]	32.9 [b]
B	6.5 [a,c]	73.4 [c]	27.6 [c]
C	5.2 [a,d]	66.9 [d]	33.6 [d]
Controls			
Ac	2.3 [b]	29.8 [b]	61.2 [b]
Bc	1.5 [c]	39.7 [c]	64.7 [c]
Cc	2.6 [d]	30.4 [d]	68.4 [d]

a: Group A vs group B and C, respectively, $p<0.05$.
b, c, d: Infected vs controls, $p<0.05$.

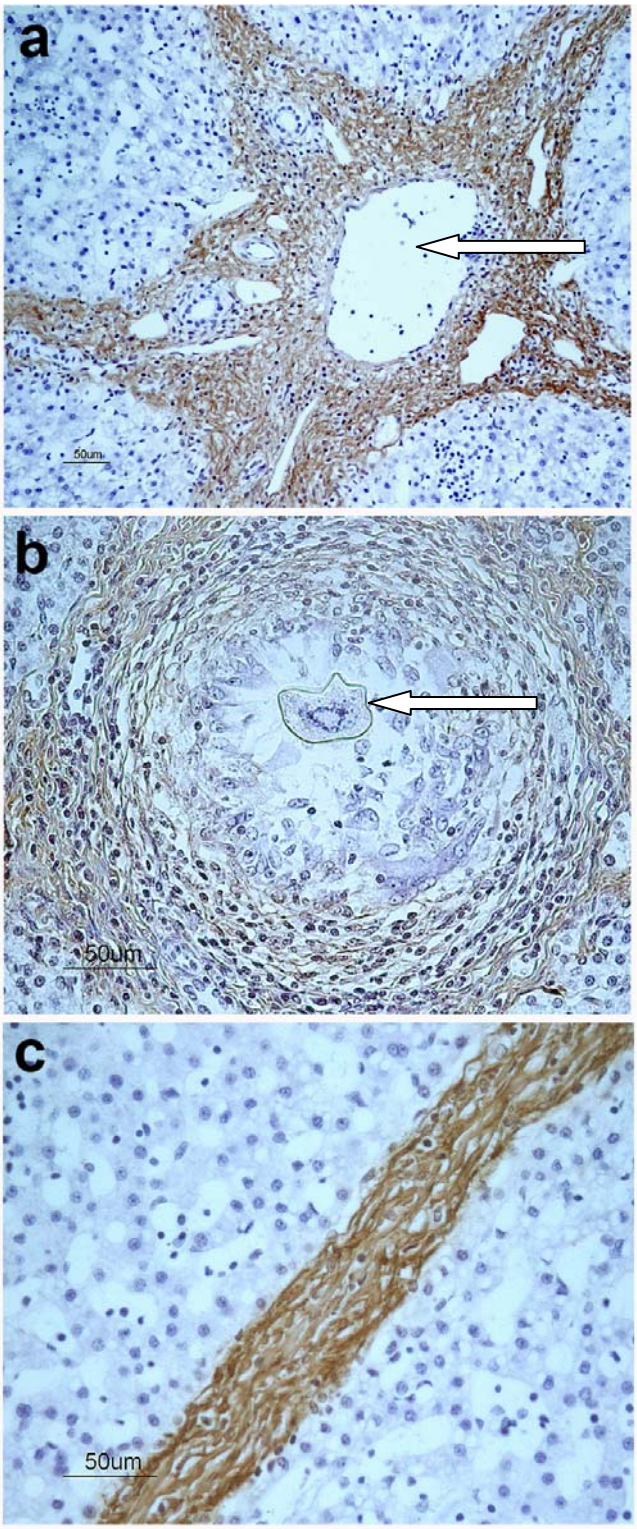
a
50um
b
50um
c
50um

Figure 4. Liver sections of *Schistosoma japonicum* infected pigs immunostained for collagen type 1 (brown stain).

a: Enlarged portal area and interlobular septa with numerous collagen type 1 fibres. The portal vein is dilated (arrow).

b: Granuloma with an *S. japonicum* egg in the centre (arrow) and concentric layers of collagen type 1 fibres at the periphery.

c: Broadened interlobular septum with densely packed collagen type 1 fibres. The image magnification is the same as that used for image analysis.

Relationship between faecal egg excretion and other variables

A strong positive correlation was found between the number of eggs per g faeces and liver TEC ($r=0.740$, $p<0.001$). There was also a correlation between the number of miracidia per g faeces and liver TEC ($r=0.483$, $p<0.05$) and the area of fibrosis as measured by image analysis ($r=0.452$, $p<0.05$), respectively. The number of eggs per g faeces was also correlated with liver granuloma density ($r=0.450$, $p<0.05$).

Relationship between liver TEC and liver fibrosis

No correlation was observed between liver TEC and liver fibrosis.

Relationship between granuloma density and liver fibrosis

There was a positive correlation between granuloma density and portal fibrosis ($r=0.638$, $p<0.05$), septal fibrosis ($r=0.700$, $p<0.01$), total fibrosis ($r=0.684$, $p<0.001$), and the area of fibrosis as measured by image analysis ($r=0.626$, $p<0.01$).

Relationship between semi-quantitative histopathological scoring and quantitative image analysis

There was a strong positive correlation between the results of the two methods used for assessing liver fibrosis, the semi-quantitative histopathological scores and the quantitative image analysis measurements of the area of fibrosis. The correlation coefficients (r) for portal, septal and total fibrosis scores, respectively, and the area of fibrosis were 0.888, 0.834 and 0.917, respectively, ($p<0.001$).

DISCUSSION

In the present histopathological study, the degree of liver fibrosis was investigated and correlated with parasitological variables in groups of pigs at three different stages of long-term experimental *Schistosoma japonicum* infection. We found that liver fibrosis was marked at 8 weeks post infection (p.i.) (group A), was reduced at 16 weeks p.i. (group B) and remained low at 24 weeks p.i. (group C). Our finding that prominent liver fibrosis occurs at the early stage of *S. japonicum* infection is in agreement with a number of previous studies in pigs (Johansen, 1998; Willingham *et al.,* 1998; Hurst *et al.,* 2000; Kardorff *et al.,* 2003; Pedersen *et al.,* 2003). Mild fibrosis at later stages of *S. japonicum* infection in pigs has also been described previously (Willingham *et al.,* 1998; Hurst *et al.,* 2000; Watanabe *et al.,* 2004).

We used both semi-quantitative histopathological scores and quantitative measurement of the area of fibrosis with image analysis to assess the degree of

fibrosis. Histopathological scoring is commonly used for evaluation of liver fibrosis in humans and methods involving the use of image analysis have also been developed (Chevallier *et al.,* 1994; Helal *et al.,* 1998; Rozario and Ramakrishna, 2003). Semi-quantitative fibrosis scores and the area of fibrosis as measured by image analysis have been found to be well correlated in a previous study based on human liver biopsies (Pilette *et al.,* 1998). Both methods have been employed in earlier studies of liver fibrosis in *S. japonicum*-infected pigs (Hurst *et al.,* 2000; Baddamwar *et al.,* 2004). In line with the results by Baddamwar *et a*l (2004), we found a very good correlation between the two methods, suggesting that both histopathological scores and quantitative measurement of the area of fibrosis with image analysis are valuable methods for investigations of liver fibrosis in the pig *S. japonicum* model. A major advantage of image analysis over histopathological scoring is that it reduces the risk of inter- and intra-observer variability, which are serious drawbacks with scoring systems (Pilette *et al.,* 1998).

In the present study of pigs infected with a single dose of 1000 *S. japonicum* cercariae, the faecal egg excretion showed a peak at 8 weeks p.i., was markedly reduced at 12 weeks p.i. and then further reduced to low levels at the end of the experiment (24 weeks p.i.). A similar pattern of faecal egg excretion, with a peak at 7 - 8 weeks p.i and a reduction thereafter has been reported from other studies of pigs experimentally infected with relatively high single doses of *S. japonicum* cercariae (Willingham *et al.,* 1998; Pedersen *et al.,* 2003). It has been shown in humans infected with *S. mansoni* that there is a correlation between faecal egg excretion and the degree of portal fibrosis as assessed by ultrasound (Doehring-Schwerdtfeger *et al.,* 1990). In the present study we found a correlation between the faecal egg counts and granuloma density ($r=0.450$, $p<0.05$), but not between faecal egg counts and liver fibrosis. However, the excretion of viable eggs (faecal miracidial counts) was found to be correlated with the area of fibrosis ($r=0.452$, $p<0.05$). No such relationship have prevoiusly been demonstrated in pigs, and our findings suggest that faecal egg excretion could be used as a marker of liver pathology in the pig model of schistosomiasis japonica.

In the murine model of *S. japonicum*, liver fibrosis in schistosomiasis is related to the presence of schistosome eggs and immune responses to them (Cheever and Yap, 1997). Previous experimental studies in *S. japonicum* infections in the pig model have shown a correlation between liver fibrosis scores and liver TEC (Hurst *et al.,* 2000). A similar relationship has been described in other experimental models of human schistosomiasis as well (WHO report, 1985; Bica *et al.,* 2000). In the present study, liver TEC were highest at 8 weeks p.i. and had decreased at the two later time points, a pattern that was very similar to that observed for liver fibrosis, but no statistically significant relationship could be established between liver TEC and fibrosis as assessed by either of the two methods used. A possible explanation for the lack of correlation might be the high variability in liver TEC observed in individual pigs. However, our study provided evidence of a relationship between liver granuloma density and the portal, septal and total fibrosis scores and the area of fibrosis, respectively. There are no earlier reports on a relationship between granuloma density and area of fibrosis in pigs, but a

correlation with histopathological fibrosis scores has been reported (Hurst *et al.,* 2000).

The extra cellular matrix (ECM) of liver fibrosis is composed of various proteins, including several types of collagen, laminin and fibronectin, and the composition has been shown to change between different stages of fibrosis (Dunn *et al.,* 1977; Kershenobich, Stalnikowitz and Weissbrod, 2003). In human schistosomiasis mansoni, collagen types 1 and 3, as detected by immunohistochemistry, have been found to be present in increased amounts in fibrotic livers (Biempica *et al.,* 1983; Andrade *et al.,* 1992). Prominent deposition of collagen types 1 and 3 has been detected immunohistochemically at 10 weeks p.i. in *S. japonicum*-infected mice (Xiong *et al.,* 2003). In *S. japonicum*-infected rabbits, marked fibrosis at 12 weeks p.i. was preceded by a 12 and 11-fold increase in mRNA expression for collagen types 1 and 3, respectively (Chen F *et al.,* 2002).

Very little is known about the composition of the ECM in liver fibrosis induced by *S. japonicum* in pigs. Baddamwar *et al* (2004) showed that collagen type 1 was increased in portal and septal areas proportional to the increase of fibrosis in those areas. In the present study, the amount of collagen type 1, upon histological examination, appeared to be proportional to the amount of fibrous tissue in the livers. To further investigate this, we developed a method by which we were able to measure collagen type 1 in immunostained liver tissue sections using image analysis.

We found that the area fraction of collagen type 1 was significantly higher in the septa in the infected pigs than in the controls at the three time points examined. However, no difference was observed between the different groups of infected pigs. This suggests that the proportion of collagen type 1 is increased in fibrotic compared to normal septa, but that it remains relatively constant in the fibrotic septa during the course of *S. japonicum* infection in pigs, despite the observed decreases in scores for septal fibrosis and in area of fibrosis. The resolution of fibrous tissue that occurs between the acute and chronic stage the infection in pigs thus does not seem to influence the density of collagen type 1 in the septa, although the total amount, as assessed in the immunostained sections, appears to be reduced. Further investigations are necessary to find out if the proportions of other constituents of the ECM, especially collagen type 3, might change during fibrosis resolution.

In conclusion, this study confirmed results from other studies that marked liver fibrosis develops at the acute stage and is reduced at later stages of *S. japonicum* infection in pigs, and that the degree of liver fibrosis is related to granuloma density. The results also suggest that faecal egg excretion could be used as a marker of liver pathology in pigs. Quantitative image analysis gave comparable results to semi-quantitative histopathological scoring and is thus a useful, additional tool for assessment of the degree of liver fibrosis in this animal model. Finally, this study showed that the area fraction of collagen type 1 in fibrous septa was increased in infected pigs, but did not change in connection with the resolution of fibrosis that occurred during the course of infection.

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