



# Hepatic Fibrosis in Experimental *Schistosoma* *japonicum* Infection in Pigs

*A Histopathological and Immunohistochemical Study*

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Master of Science Programme in Veterinary Medicine  
for International Students  
Faculty of Veterinary Medicine and Animal Science  
Swedish University of Agricultural Sciences

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The present thesis is a partial fulfilment of the requirements for  
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To my parents (**Dr. B. Linga Reddy** and **Smt. B. Premala**)

## Abstract of thesis

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*Schistosomiasis japonica* is a zoonotic, parasitic disease caused by the trematode *Schistosoma japonicum*, with *Oncomelania* snails serving as the intermediate hosts. *Schistosomiasis japonica* is endemic in China, the Philippines, and Indonesia and is a major public health problem. The inflammatory response to the schistosome eggs in host tissues leads to formation of perioval granulomas, especially in the liver and intestine. In the liver, this eventually results in chronic portal fibrosis referred to as pipe-stem fibrosis. The pig has many biological similarities with man and is also a natural host for *S. japonicum*, which has led to exploration of this species as a large animal model of human schistosomiasis japonica. In pigs, there is marked portal and septal fibrosis of the liver in the early stage of patent infection, whereafter the fibrosis gradually resolves due to spontaneous recovery from the infection. The pig can thus serve as a model of the development and regression of schistosomal liver fibrosis.

In the present study, twenty-six pigs were divided into six groups, A to F. Groups A to D (n = 5/group) were infected with *Schistosoma japonicum* cercariae and groups E and F (n = 3/group) served as uninfected controls. The degree of fibrosis was assessed at 3 different time-points upto 21 weeks after a single infection and after a primary infection followed by a challenge infection, using semi-quantitative histopathological scores as well as quantitative image analysis on liver sections and the two methods were compared. A possible correlation between fibrosis and liver tissue egg counts (TEC) was investigated and the fibrotic lesion was characterised by immunohistochemical detection of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), desmin and collagen type-1.

Major histopathological lesions were egg granulomas, diffuse portal and septal inflammatory cell infiltration, and fibrosis. Granulomatous obstruction of portal veins was common. The degree of fibrosis varied from mild to marked in all four groups of infected pigs and there were no significant differences in liver fibrosis scores or area of fibrosis between the groups. The measurement of the area of fibrosis by image analysis was found to be well correlated with the semi-quantitative histopathological scores for total fibrosis *i.e.* the sum of portal and septal fibrosis scores, as well as for portal and septal fibrosis respectively. There was a positive correlation between liver tissue egg counts (TEC) and septal and total fibrosis scores as well as the area of fibrosis.

Expression of  $\alpha$ -SMA was detected in connective tissue cells in portal and septal areas of normal and infected livers, but the numbers were increased proportionally to increases of the degree of fibrosis in infected pigs. Similar expression was observed in thickened portal veins of infected pigs and in hepatic stellate cells (HSC), smooth muscle cells of vessel walls, pericytes, and sub-epithelial cells of bile ducts of all pigs. Desmin expression was detected in HSC and in smooth muscle cells of portal veins, hepatic arteries and bile ducts in both normal and infected pigs. In areas with portal vein destruction, scattered desmin-positive cells identified as smooth muscle cells were often found. Collagen type-1 was present in increased amounts in portal and septal areas in infected pigs. In granulomas, there was a peripheral network of  $\alpha$ -SMA-positive flattened, fibroblast-like cells and concentric collagen type-1-positive fibres, whereas desmin-positive cells were not observed.

In conclusion, this study demonstrated a correlation between liver fibrosis and liver TEC. Good correlation was also found between the area of fibrosis as measured by image analysis and the semi-quantitative histopathological scores, making image analysis a useful additional tool for assessment of liver fibrosis in the pig model. In fibrotic portal and septal areas  $\alpha$ -SMA-expressing connective tissue cells and collagen type-1 were increased. Collagen type-1 and  $\alpha$ -SMA, but not desmin, were also detected in granulomas. Desmin could be used as a marker of portal vein destruction.

Key words:

*Schistosoma japonicum*, pig, liver fibrosis, histopathological scores, image analysis, tissue egg counts, granuloma,  $\alpha$ -SMA, desmin, collagen type-1.

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## INTRODUCTION

Schistosomiasis is a parasitic disease causing chronic ill health in humans with serious consequences for socio-economic development in tropical and subtropical regions (Hirst and Stapley, 2000). It is caused by several species of blood flukes of genus *Schistosoma* belonging to class Trematoda of phylum Platyhelminthes. Schistosomiasis is also known as bilharzia after Theodor Bilharz, who first discovered schistosomes in 1851 in Egypt. It is endemic in many less developed countries in tropical and subtropical regions of Africa, Asia, the Caribbean and South America (Wiest and Olds, 1992). Due to variations in the prevalence of the intermediate snail host species, patterns of water exposure and other socio-cultural factors, schistosomiasis is not uniformly distributed in endemic areas (Review by Bica *et al.*, 2000). The three major schistosome species which infect humans are *S. haematobium*, *S. mansoni* and *S. japonicum*. *Schistosoma haematobium* inhabits venules of the urinary tract and damages mainly the bladder and ureters. The latter two species live in intestinal venules and affect mainly the liver and gut (Cheever and Yap, 1997, Kojima, 1997).

In humans, schistosomiasis is the second most common parasitic infection after malaria with approximately 200 million infected, and in sub-Saharan Africa alone, it is estimated that 200,000 die each year as a result of the infection (Review by Bica *et al.*, 2000; WHO, Report, 2002). In China, results from the 1995 nationwide sampling survey indicate that 865,000 humans are infected by *S. japonicum* (Ross *et al.*, 2001). Schistosomiasis also affects animals and some schistosome species, e.g. *S. japonicum*, are of zoonotic importance with domestic animals, especially water buffaloes, cattle and pigs, serving as reservoir hosts (McGarvey *et al.*, 1999; He *et al.*, 2001; Ross *et al.*, 2001).

Most disease caused by schistosome infection is related to the host's immunological response to eggs laid in the veins of the host rather than to the parasite itself (Cheever and Yap, 1997). The eggs retained in the tissues induce a granulomatous reaction leading to tissue damage and eventually fibrosis (Chen and Fu, 1989). Chronic infections with *S. japonicum* and *S. mansoni* cause significant morbidity and mortality due to granuloma formation in the intestine and liver. The resulting fibrosis of the liver leads to portal hypertension and its complications splenomegaly, esophageal varices, haematemesis and death due to gastrointestinal bleeding (Review by Bica *et al.*, 2000). The development of fibrosis is related to the intensity of the schistosome infection (Cheever *et al.*, 1980). *Schistosoma japonicum* infection also causes decreased food intake and increased nutrient losses and is associated with stunting of child growth and development (Stephenson, 1993; Olds, 1996). *Schistosoma japonicum* is considered to cause more severe disease than *S. mansoni* due to higher egg output by the female worms, about 3500 eggs /day for *S. japonicum* compared to 300

eggs /day for *S. mansoni* (Review by Cheever, 1985; Wiest and Olds, 1992; Olds *et al.*, 1996).

This study concerns hepatic fibrosis in experimental *S. japonicum* infection in pigs, with special reference to methods for evaluation of the degree of fibrosis and immunohistochemical identification of connective tissue cells and extra-cellular matrix (ECM) components.

## LITERATURE REVIEW

### The parasite and its life cycle

*Schistosoma japonicum* is a digenetic trematode with a life cycle that comprises four stages: one stage within a definitive mammalian host for sexual reproduction, another stage within an intermediate snail host for asexual reproduction, and two free-living stages, the miracidium and the cercaria (Sturrock, 1993). Snails belonging to the genus *Oncomelania*, which are operculated fresh water snails, serve as intermediate hosts for *S. japonicum* (Soulsby, 1965). Man and domestic animals e.g. cattle, buffaloes, sheep, goats, pigs, dogs and cats as well as several wild mammalian species serve as its definitive hosts (He, 1993). Non-human primates, rodents and rabbits are also final hosts and have been used in experimental infections (Warren, 1979; Cheever *et al.*, 1980). The schistosome sexes are separate; the *S. japonicum* male is 10-20 mm long and 0.55 mm wide, whereas the female is 20-30 mm long and 0.3 mm wide. The adult male and female worms form permanent pairs for sexual reproduction with the slender female lodging in the gynaecophoric canal of the stouter male (Georgi, 1999). The thus formed adult worm pairs live in the mesenteric and intestinal veins of the definitive host, where the female worms start laying eggs (Sturrock, 1993). The female is able to produce 1000-3500 eggs per day, which are laid in clusters in small venules of the intestinal submucosa and mucosa. Within 9-12 days the embryo contained within the egg matures into a miracidium. Along with extravasated blood, tissue debris and inflammatory cells, *S. japonicum* eggs are expelled into the intestinal lumen and excreted via faeces by peristaltic movements of the bowel. Once excreted from the host the eggs will hatch when they come into contact with water to release the miracidia.

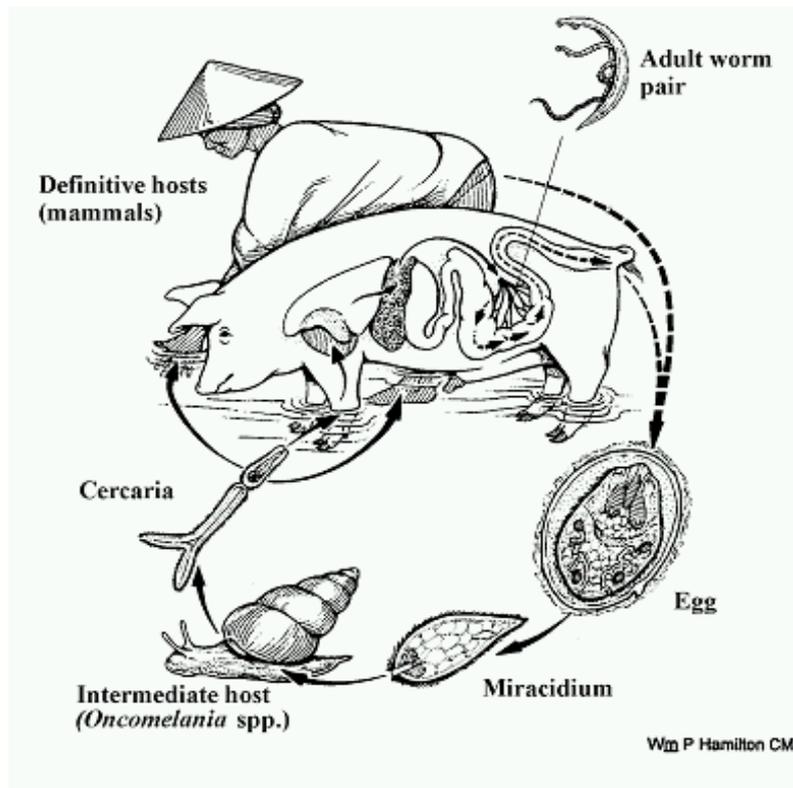


Fig.1. The Life cycle of *Schistosoma japonicum*

The free-living miracidium has to find a suitable host within few hours after hatching. The miracidium penetrates the snail and changes into a primary sporocyst. After 8-10 days several secondary sporocysts break out through the wall of primary sporocysts and migrate actively through the connective tissue and blood sinuses of the snail to the hepatopancreas and gonads (Sturrock, 1993). From secondary sporocysts, cercariae develop. A single miracidium gives rise to thousands of cercariae. The cercaria is the second free-living larval stage and the infective stage for the mammalian final host. All the cercariae derived from one miracidium are of the same sex. The length of the prepatent period in the snail host from penetration to cercarial shedding varies with ambient temperature. It ranges from 17-18 days at +30-35°C to several months or more at lower temperatures. At temperatures below +15°C development may be suspended (Sturrock, 1993). At temperature of +5°C or less snail survival is high, but reduced breeding occurs. Cercariae usually die within hours after escaping from the snail, but may remain viable for up to 2 days. As the cercaria penetrates the skin it sheds its tail and is transformed into the next larval stage, the schistosomulum. It migrates through tissues, penetrates a vessel and is carried to the lungs where it remains for approximately 1 week. Thereafter, the schistosomulum is carried by the blood flow passively to the right heart and on to the pulmonary capillaries. From pulmonary capillaries it reaches the left heart and then the systemic circulation. It

reaches the liver via the splanchnic vasculature (the mesenteric artery and capillaries and hepatic portal vein). In portal venules it matures into an adult worm in about 4-5 weeks. The adult male and female worms form permanent pairs for sexual reproduction and inhabit in mesenteric and intestinal veins, where the females start laying eggs (Cheever and Neafie, 2000). The average prepatent period in humans is 42 days, whereas in cattle it is 36 days, in buffaloes 42 days, in pigs 27-42 days, and in dogs 29-35 days (Yason and Novilla, 1984; Basch, 1991; He *et al.*, 1992).

## **Disease syndromes and complications**

### *A. Cercarial dermatitis (Paddy field itch)*

Skin penetration with *S. japonicum* cercariae is associated with local pruritis, erythema and papules (Chen, 1993). About one third of the affected individuals develop local dermatitis after contact with cercariae in infested water (Chen and Fu, 1989).

### *B. Acute schistosomiasis (Katayama fever)*

Acute schistosomiasis may occur at the time of first exposure to *S. japonicum* both in persons living in endemic areas and in previously uninfected persons from non-endemic areas (Review by Chen and Mott, 1988; Chen, 1993). Persons who have an active chronic infection and those who have a recent history of infection with documented treatment and cure may also develop the acute form of the disease (Chen, 1993).

The acute syndrome is due to massive exposure to *S. japonicum* cercariae in a short period of time and tends to occur during the rainy season, especially in flooded areas where the population of *Oncomelania* snails is dense, and prevention and control activities are limited (Chen, 1993). The incubation period i.e. the interval between exposure to infection and the onset of clinical disease is 41.5 days (range 14-84 days) after a single day's exposure to infested water in persons without previous history of *S. japonicum* infection (Chen, 1993). The main symptom of acute infection is fever, which is intermittent or remittent, peaking in late evening and returning to normal or below 38°C in early morning. Rigor, sweating, headache and general muscular pain usually occur in association with fever. Gastrointestinal disturbances, consisting of loss of appetite, nausea, abdominal pain and distension, and diarrhoea with mucus and blood, may be prominent. The liver is usually enlarged and tender on physical examination and in about one third of patients a slightly enlarged spleen can be palpated (Review by Chen and Mott, 1988).

### *C. Chronic hepatosplenic schistosomiasis*

Chronic cases of schistosomiasis are often asymptomatic. General symptoms of heavily infected persons include weakness, fatigue, abdominal pain, irregular bowel movements and bloody stools (Review by Chen and Mott, 1988). In most of the patients with advanced schistosomiasis japonica, portal hypertension is

observed. Splenomegaly is frequent and the left lobe of liver may be disproportionately enlarged. On palpation, the liver is smooth, firm and without tenderness. As the disease advances, the hardness of liver increases and the surface becomes nodular or irregular in severe cases. Advanced hepatosplenic disease is usually associated with dilatation of abdominal collateral veins, often forming a so called Medusa head, and oesophagogastric varices. Haematemesis and melaena occur frequently and blood loss due to bleeding varices is the major cause of death (Review by Chen and Mott, 1988). Ascites is common and occurs as a result of portal hypertension and hypoalbuminaemia (Chen, 1993).

#### *D. Other clinical forms of schistosomiasis*

##### Cerebral schistosomiasis

*Schistosoma japonicum* may affect the brain, causing meningoencephalitis in the acute stage and epileptic seizures in the chronic phase (Review by Chen and Mott, 1988).

##### Pulmonary schistosomiasis

Clinical pulmonary schistosomiasis due to inflammation induced by newly deposited eggs is frequently seen in the acute phase of infection. Coughing is a predominant symptom, usually associated with fever, and dry or moist rales can be heard on auscultation. Chronic pulmonary schistosomiasis is rarely seen (Chen, 1993).

### **The pig as an animal model of human disease**

In biomedical research the pig has been paid extensive attention as a model of human disease due to anatomic, physiologic, metabolic and nutritional similarities with man (Review by Johansen *et al.*, 2000). Pigs are relatively hairless and their integumental system is very similar to that of humans from physiological and histological standpoints (Swindle, 1986; Swindle *et al.*, 1988). Pigs and humans are both monogastric omnivores and have physiological and digestive functions and splanchnic blood flow analogous to that of humans (Willingham and Hurst, 1996). The livers of pigs and humans have similar gross anatomy, physiology and metabolic functions (Willingham and Hurst, 1996). Growing pigs can be regarded as appropriate models for growing children due to their comparable gastrointestinal tract. Pigs reach sexual maturity early, have a short reproductive cycle and are highly prolific as compared to other large animals. They are also readily available, easy to work with and are relatively cost effective to house and maintain (Review by Johansen *et al.*, 2000). Rodents are easy to maintain and inexpensive, but their gut physiology and their biological and immunological responses to *S. japonicum* deviate much from those of humans (Basch, 1991). In addition, the life span of the worm exceeds that of the mouse (Basch, 1991). Use of primates as models for humans is restricted for a number of reasons, e.g. ethical concerns, limited supply, high cost, slow reproduction, maintenance difficulties, and the risk of them harboring viruses communicable to humans (Willingham and Hurst, 1996; Review by Johansen *et al.*, 2000).

## Schistosomiasis in pigs

Pigs serve as natural definitive hosts for *S. japonicum* and are important reservoir hosts and transmitters of the parasite (Review by Johansen *et al.*, 2000). The main target organs in the pig are the same as those in man, the liver and intestine (Yason and Novilla, 1984; Willingham *et al.*, 1994). Information regarding schistosomiasis japonica that may be of comparative interest to humans can thus be obtained by experimental studies in the pig model (Willingham *et al.*, 1998).

### *Clinical signs and pathology*

Except for diarrhoea pigs usually do not develop any clinical signs in experimental infections (Willingham *et al.*, 1998). However, pigs infected with massive doses (5000 to 6000 cercariae) may show listlessness, depression, cough, nasal discharge and poor appetite (Yason and Novilla, 1984). In natural infections, the main clinical signs in pigs are anorexia, fever, weakness, depression, coughing, nasal discharges, diarrhoea and voiding of faeces stained with blood, and the growth rate of young pigs may be reduced (Dumag *et al.*, 1980; Zhong and Fang, 1981; Hurst *et al.*, 2000).

Pathological changes are produced mainly in the liver and intestine (Willingham *et al.*, 1998). Gross pathological lesions of the liver include disseminated small, firm, gray-white nodules on the liver surface and within the parenchyma and a generalised increase in the interlobular connective tissue network (septal fibrosis) (Johansen, 1998; Willingham *et al.*, 1998) (Fig. 2). Portal lymph nodes are often enlarged. Microscopic lesions include egg granulomas in portal triads, interlobular septa and intralobular areas. Egg granulomas often cause obstruction of portal veins and bile duct hyperplasia is commonly observed (Yason and Novilla, 1984; Hurst *et al.*, 2000). Portal and septal infiltration of inflammatory cells and fibrosis are prominent in the early stages of infection after which these changes are gradually reduced with time (Hurst *et al.*, 2000). Hepatic fibrosis in pigs has been shown to be proportional to the intensity of infection and to liver TEC and granuloma numbers (Willingham *et al.*, 1998; Hurst *et al.*, 2000). Praziquantel treatment also reduces portal and septal fibrosis (Johansen *et al.*, 1998). For the study of development and resolution of schistosomal hepatic fibrosis the pig is thus a useful model.

In the intestine, schistosome eggs or lesions related to eggs are observed (Dumag *et al.*, 1980). Focally distributed petechial or echymotic haemorrhages and small hyperaemic foci are found in the mucosa of the large intestine (Yason and Novilla, 1984; Willingham *et al.*, 1998) (Fig. 3). In massive infections, the intestinal mucosa may be congested and covered with blood-tinged mucus, and mucosal thickenings are noted (Yason and Novilla, 1984) (Fig. 4). Fibrosis of the gut wall is noted focally in areas with high granuloma numbers (Hurst *et al.*, 2000).

*Fig 2-4, Gross pathology of the liver and large intestine*

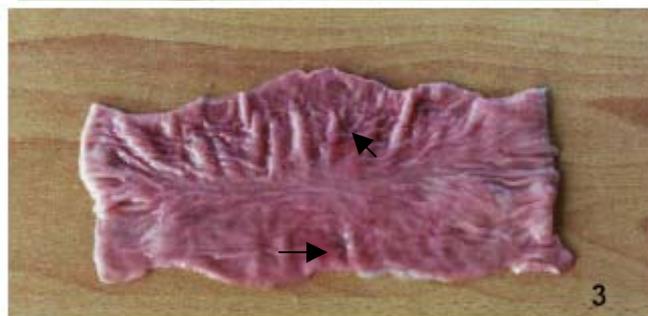
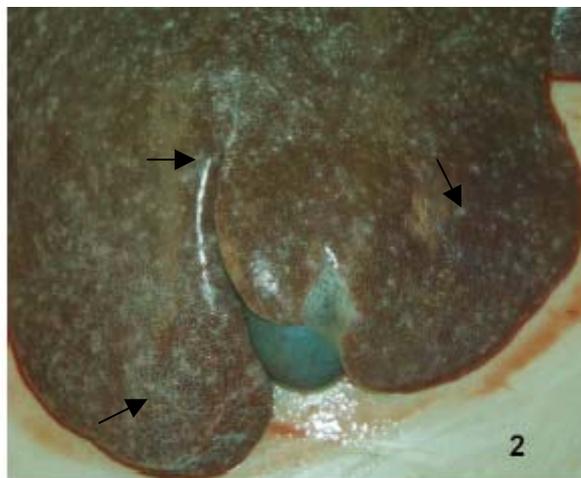


Fig.2 Liver with small firm gray white nodules and increased network pattern (arrows).

Fig.3 Large intestine with haemorrhages.

Fig.4. Large intestine with mucosal thickenings (arrows).

### **The schistosome egg (perioval) granuloma**

According to Weinstock (1992) granulomas are chronic, usually focal, inflammatory responses to persistent irritants. Granulomas contain macrophages in various stages of differentiation and other inflammatory cell types. These lesions resolve, leaving residual fibrosis. Granulomatous responses are complex, employing a vast array of immunologic mechanisms. Granulomas of various disease states may have different immunoregulatory mechanisms governing their formation and resolution.

Granulomas may be induced by infectious agents like bacteria, fungi and parasitic ova or by non-infectious agents like barium (Weinstock, 1992). In schistosomiasis, granulomas develop around the ova, containing living embryos, that imbed in different tissues of the host. The egg shell protects the ova from host destruction. The ova release a variety of substances of which some are toxic to host tissues whereas others are antigenic, leading to antigen-specific humoral and cell-mediated immune responses. The formation of granulomas in the liver is a manifestation of delayed type hypersensitivity to soluble egg antigens, which are released by the trapped ova (Warren *et al.*, 1967). The reaction is mediated by CD4+ T helper (Th) lymphocytes with a predominantly Th2-like cytokine profile characterised by the production of interleukin (IL)-4, IL-5 and IL-10 (Cheever *et al.*, 1998). Granuloma formation is a protective phenomenon for the host as it sequesters toxic and antigenic substances and eventually it destroys the egg and removes residual debris. Deleterious effects of granulomas include focal tissue injury and induction of appreciable fibrosis. Granulomas contain macrophages, epithelioid cells, giant cells, eosinophils, lymphocytes (T and B cells) and a few mast cells. The inflammatory cells rest on collagenous matrix produced by fibroblasts. This matrix displaces normal organ parenchyma (Weinstock, 1992).

Granulomas decrease in size when they enter the involutinal stage and become less cellular, lose their cellular halo and form laminar collagenization (Weinstock, 1992). A decrease in the intensity of the granulomatous response to newly deposited eggs, termed immunomodulation, occurs in the chronic or late stage of infection in the murine *S. mansoni* model. T cells are of prime importance for the formation of large *S. japonicum* granulomas, whereas modulation of the size of granulomas is primarily antibody mediated (Review by Cheever, 1985).

### **Hepatic fibrosis in schistosomiasis**

Hepatic fibrosis can be defined as an increase in the amount of fibrous connective tissue in relation to the parenchyma of the liver (Pauly and Ruebner, 1987). Fibrosis results when the rate of collagen synthesis is higher than that of collagen degradation (Chen *et al.*, 2002).

The fibrotic changes of the liver in chronic human schistosomiasis are often referred to as Symmers' clay pipe-stem fibrosis after Symmers' first description of the typical lesions in 1904. Symmers' pipe-stem fibrosis is portal fibrosis without

bridging, nodular formation or significant hepatocellular destruction (Lambertucci, 1993). It is a result of egg-induced chronic presinusoidal inflammation leading to deposition of fibrous material and vascular destruction. These fibrotic changes produce a stellate portal fibrosis all along the intrahepatic branches of the portal vein, so that they are seen in anatomic cross-section to resemble a clay 'pipe stem' (Lambertucci, 1993). Symmers' used the term cirrhosis, but as the liver parenchymal cells essentially are unharmed, the terms periportal or portal fibrosis have later been preferred by other authors (Review by Warren, 1979).

In humans development of portal fibrosis is related to the intensity of infection (Review by Warren, 1979). Differences in the pathological lesions produced by major schistosome species e.g. *S. mansoni* and *S. japonicum* are due to differences in the number of eggs produced and the pattern of egg laying (Review by Bica *et al.*, 2000). Eggs released into the portal circulation reach the liver via the blood flow. Pylephlebitis, periphlebitis and portal fibrosis are caused by granuloma formation around the eggs (Review by Bica *et al.*, 2000). The main branches of the portal vein may be partially or completely destroyed, whereas the lobular architecture of the hepatic parenchyma is maintained (Review by Bica *et al.*, 2000). Portal blood flow obstruction owing to granulomatous inflammation and fibrosis leads to portal hypertension, splenomegaly and development of a porto-systemic collateral circulation (Chen, 1993). If unresolved, hepatic involvement may manifest as bleeding esophageal varices (Olds and Stavitsky, 1986; Olds and Kresina, 1989). A general correlation between fibrosis and the number of eggs in the liver tissue, recovered by tissue digestion, is found (WHO report, 1985).

## **Experimental models of hepatic fibrosis due to schistosomiasis**

### *Primates*

According to von Lichtenberg and Sadun (1968), clinical evolution of pipe-stem fibrosis in the chimpanzee is similar to that of humans. After an acute stage, a compensated stage of pipe-stem fibrosis develops characterised by extensive pathological lesions in the liver, splenomegaly and portal collateral formation with minor hepatocellular change. In capuchin monkeys, livers show granulomas of 0.1 cm on capsular surface and streaking of terminal portal triads on cut surface (Cheever *et al.*, 1974). Lesions are more marked at the early stage of infection than at later stages.

### *Rabbits*

Hepatic fibrosis in rabbits is correlated to the intensity of infection (Review by Cheever, 1985). In heavily *S. japonicum*-infected rabbits, typical Symmers' fibrosis develops in the acute stage of infection (Cheever *et al.*, 1980). Microscopically, fibrosis is confined to portal areas around patent and occluded veins in rabbits at 10-12 weeks post infection (PI). In the chronic stage, in addition to Symmers' fibrosis, septal fibrosis develops as bands of fibrous tissue linking portal tracts 10-30 weeks PI.

### *Mice*

Mice infected with *S. mansoni* develop a clinical syndrome, which is similar to that of human schistosomiasis, with hepatomegaly, splenomegaly, portal hypertension and oesophageal varices (Warren, 1979). The lesions developed in mice infected with *S. mansoni* are similar to pipe-stem fibrosis seen in humans, i.e. portal and septal fibrosis due to massive and concentrated deposition of eggs in portal areas, whereas the liver parenchyma maintains its normal architecture (Andrade, 1987). After 5 weeks of infection, the collagen content of the liver rises rapidly and reaches a steady state by 16 weeks (Warren, 1966). Eight weeks PI the liver weight is 70 % greater than normal, the spleen weight is more than doubled and the portal pressure is twice as high as normal. Microscopically groups of eggs are found in tissue sections with an intense fibrotic reaction around them and infiltration of lymphocytes, eosinophils and histiocytes. During 12-14 weeks PI, portal vessel walls are thickened and bands of fibrosis are seen intersecting the liver lobules (Warren and Moore, 1966). In *S. mansoni* infection, granulomatous inflammation has a potential role in hepatic fibrogenesis (Wyler, 1992). In mice as in humans, liver granulomas contain predominantly collagen type-1 and type-3 (Biempica *et al.*, 1983; Grimaud *et al.*, 1987). Collagens are secreted by fibroblasts in response to egg antigens and granuloma derived cytokines (Wyler *et al.*, 1987). Modulation of the size of granulomas is observed in mice, i.e. the size of granulomas formed late (10-12 weeks PI) are smaller than those formed at 6-8 weeks PI (Review by Cheever, 1985).

### *Pigs*

Marked portal and septal fibrosis develop early and later on decline spontaneously as the pigs undergo self-cure, which makes pigs useful in studies of the development and resolution of hepatic fibrosis (Hurst *et al.*, 2000). Portal fibrosis correlates with the ultrasound measurements of the portal vein trunk diameter and the diameters of the right and left portal vein branch, and the mean hepatic collagen content in infected pigs is higher than in uninfected pigs (Kardorff *et al.*, 2003).

## **Cytokines involved in hepatic fibrosis**

Due to injury or insult IL-1, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), and IL-6 mediate changes in hepatic metabolism (Review by Kresina *et al.*, 1992). Generally in hepatic fibrosis, the lack of liver clearance function results in increased serum cytokine levels. In schistosomiasis, mechanical obstruction of portal blood flow occurs, but the liver parenchyma remains largely intact and liver synthetic function is maintained (Review by Dunn and Kamel, 1981). The production of IL-4 and IL-5 in the granuloma is stimulated by soluble egg antigens (Review by Kresina *et al.*, 1992). IL-5 is responsible for the generation of eosinophils and IL-4 for the IgE response (Coffman *et al.*, 1989). For the maintenance of the granulomatous response, IL-1 and TNF- $\alpha$  play a role (Review by Kresina *et al.*, 1992). Hepatic granuloma cells secrete a variety of fibrogenic cytokines that initiate the scarring process (Wyler, 1992). Fibroblast-stimulating Factor (FsF)-1 is a lymphokine that

stimulates matrix synthesis and fibroblast growth. Together with other granuloma derived cytokines, F $\beta$ F-1 is down-regulated in the chronic stage of infection, which leads to reduced scar formation. Due to immunomodulation in mice, granuloma size decreases in late stages of infection compared to the early stages, but the reduced inflammatory response does not result in less fibrosis, as there is no reduction of hepatic collagen deposition (Olds and Kresina, 1989). Granuloma size and hepatic fibrosis thus appear to be independently regulated (Prakash *et al.*, 1991; Cheever, 1997). The regulation is affected by the balance of cytokines, affecting the reaction size and fibrosis to different degrees or in different ways.

### **Extra-cellular matrix (ECM) and connective tissue cells in hepatic fibrosis**

Fibrous tissue is composed of ECM and connective tissue cells. Collagen types-1, 3, 4, and 5, procollagen type-3, fibronectin and laminin are major components of fibrous tissue in the liver in schistosomiasis mansoni (Andrade *et al.*, 1992). In the dynamic process of deposition and resorption in liver fibrosis in experimental *S. mansoni* infection, glycosaminoglycans and collagens are two major ECM components (el-Meneza *et al.*, 1989). Fibroblasts are the most important connective tissue cells involved in ECM production in normal livers. In liver fibrosis, a major proportion of the ECM is probably produced by mesenchymal fibroblast-like subpopulations (Abdel-Aziz *et al.*, 1991; Bhunchet and Wake, 1992; Friedman, 1993; Gressner, 1994). In response to injury, fibroblasts and smooth muscle cells start proliferating and produce an extensive collagen network (Review by Kovacs, 1991). Hepatic stellate cells, also called perisinusoidal cells, fat storing cells or Ito cells, are highly specialised cells in the wall of liver sinusoids. In their quiescent stage, HSC are storage cells for vitamin A (Review by Ramadori and Saile, 2002). In normal human adult liver, only some of the HSC express  $\alpha$ -SMA, but the expression is prominent in liver injury and fibrogenesis (Schmitt-Gräff *et al.*, 1991). The first evidence that liver damage leads to activation and proliferation of HSC was produced in the rat/CCL4 model (Kent *et al.*, 1976). In their *in vivo* studies, Mak and Lieber (1988) showed that in baboon and human livers, damage induced by alcohol administration leads to a process of activation, during which HSC lose their lipid droplets and show resemblance to liver myofibroblasts (MF). The HSC first transdifferentiate to transitional cells (intermediate between HSC and MF and then to a MF-like phenotype, i.e. a cell intermediate between a fibroblast and a smooth muscle cell. Myofibroblasts show elevated collagen and lack vitamin A (Blomhoff *et al.*, 1991; Knittel *et al.*, 1992). An imbalance between ECM production and degradation by HSC, transitional cells and perisinusoidal MF leads to perisinusoidal and pericellular fibrosis (Kent *et al.*, 1976; Mak and Lieber, 1988; Schmitt-Gräff *et al.*, 1991). Studies done in rats indicate that there is an increase in the number of HSC in acute liver injury, whereas in chronically injured livers not only HSC, but also MF are involved in scar formation (Knittel *et al.*, 1999).

Hepatic stellate cells express  $\alpha$ -SMA during activation in the rat (Review by Ramadori and Saile, 2002). In chronically damaged liver, the expression of  $\alpha$ -SMA increases (Review by Ramadori and Saile, 2002). In CCL<sub>4</sub>-induced liver

fibrosis  $\alpha$ -SMA is expressed by MF around perifibrotic areas and along fine collagen bundles (Tanaka *et al.*, 1991). In normal rat livers, desmin is expressed by HSC and smooth muscle cells of blood vessels. In early rat liver fibrosis induced by injections of pig serum, MF show a strong expression of actin and desmin (Ballardini *et al.*, 1988). Pericytes, mesenchymal cells with long cytoplasmic processes that surround the endothelial cells along capillaries and small venules, express  $\alpha$ -SMA as well as myosin and tropomyosin giving them a contractile function (Junqueira *et al.*, 1986). Myofibroblasts express the fibulin-2 gene and produce IL-6, whereas they do not undergo apoptosis nor express the CD-95 ligand gene (Review by Ramadori and Saile, 2002). However, the transdifferentiation of HSC to MF has been questioned by some authors since in contrast to MF, HSC express CD95L in primary cultures (Review by Ramadori and Saile, 2002). Thus HSC and MF may be similar but not identical cell populations.

In rabbits, MF play a predominant role for the formation and development of schistosomal hepatic fibrosis (Yang *et al.*, 1999). In hepatic granulomas of mice infected with *S. mansoni*, there is a strong induction of the expression of the desmin gene in fibroblastic cells, which is correlated with increases of types-1 and 3 collagen gene expression (Bolmont *et al.*, 1991). Collagen type-3 mRNA increases until 20 weeks PI whereas collagen type-1 mRNA increases up to 18 weeks and is maintained till 20 weeks PI (Bolmont *et al.*, 1991).

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## INTRODUCTION TO RESEARCH REPORT

Mice infected with *S. mansoni* develop lesions similar to pipe stem fibrosis of humans (Andrade, 1987). In *S. japonicum* infected mice, portal inflammation is observed in the early stage of infection and portal fibrosis develops in the late stage, when inflammation decreases (Warren and Berry, 1972). However, rodents deviate much from humans in their gut physiology, and biological and immunological responses (Basch, 1991; Review by Johansen *et al.*, 2000). The life span of the schistosome worm exceeds that of the mouse and due to their small size, even a single worm pair represents a heavy infection leading to extensive pathological lesions of the liver, making studies of schistosome infections of duration and intensity more relevant to man impossible (Cheever, 1969; Basch, 1991). The murine model is thus not ideal for comparative pathological research on schistosomiasis. A number of recent studies have highlighted the use of the pig as a large animal model of human schistosomiasis japonica (Willingham and Hurst, 1996; Hurst *et al.*, 2000; Review by Johansen *et al.*, 2000). In the pig, marked hepatic fibrosis develops in the acute stage of *S. japonicum* infection and then diminishes as the infection progresses into the chronic stage. This makes the pig a useful model for studies of both the development and resolution of hepatic fibrosis. The present study is based on tissue material collected from an earlier experiment in pigs infected with *S. japonicum* (Willingham *et al.*, 1997).

### **The aims of the present study of experimentally *S. japonicum*-infected pigs were the following**

- To assess, by a semi-quantitative histopathological scoring system, the degree of hepatic fibrosis at different time-points after a single infection and after a primary infection followed by a challenge infection.
- To compare the results of the semi-quantitative scoring with quantitative measurement of the area of fibrosis by image analysis.
- To investigate possible correlations between liver fibrosis and liver tissue egg counts.
- To further characterise the fibrotic lesion by investigating the cellular expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and desmin and the presence of collagen type-1 in the extracellular matrix (ECM) using immunohistochemistry.

## RESEARCH REPORT

### A Histopathological and Immunohistochemical Study of Hepatic Fibrosis in Experimental *Schistosoma japonicum* Infection in Pigs

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#### ABSTRACT

Schistosomiasis is an important cause of hepatic fibrosis in man. In the present study, we investigated the development of hepatic fibrosis in the pig model of human schistosomiasis japonica. Twenty-six pigs were divided into six groups, A to F. Groups A to D (n = 5/group) were infected with *S. japonicum* cercariae and groups E and F (n = 3/group) served as uninfected controls. The degree of fibrosis was studied in 20 pigs at three different time-points upto 21 weeks after a single infection and after a primary infection followed by a challenge infection, using semi-quantitative histopathological scores as well as quantitative image analysis on liver sections, and the two methods were compared. Possible correlation between fibrosis and liver tissue egg counts (TEC) was investigated, and immunohistochemistry was used to further characterise the fibrotic lesion. Major histopathological lesions were egg granulomas, often obstructing portal veins, diffuse portal and septal inflammatory cell infiltration, and fibrosis. The degree of fibrosis varied from mild to marked in all four groups of infected pigs with no significant differences between the groups. The area of fibrosis as measured by image analysis was well correlated with the semi-quantitative histopathological scores for total fibrosis i.e. the sum of portal and septal fibrosis scores, and for portal and septal fibrosis respectively. There was positive correlation between liver TEC and septal and total fibrosis scores as well as the area of fibrosis. The number of connective tissue cells expressing  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in portal and septal areas was increased proportionally to increases of the degree of fibrosis in infected pigs. Expression of  $\alpha$ -SMA was also observed in thickened portal veins of infected pigs, and in smooth muscle cells of vessel walls, pericytes, and sub-epithelial cells of bile ducts, and hepatic stellate cells (HSC) of all pigs. Desmin expression was detected in smooth muscle cells of vessels and bile ducts, and in HSC of all pigs. In areas with portal vein destruction, scattered desmin-positive smooth muscle cells were often found. Collagen type-1 was present in increased amounts in portal and septal areas in infected pigs. In granulomas, there were concentric layers of  $\alpha$ -SMA-positive cells and collagen type-1-positive fibres at the periphery, whereas desmin expression was not observed.

In conclusion, this study demonstrated a good correlation between the area of fibrosis as measured by image analysis and the semi-quantitative histopathological scores, making image analysis a useful tool for assessment of liver fibrosis in the pig model. There was also a positive correlation between the degree of liver fibrosis and liver TEC. Expression of  $\alpha$ -SMA and collagen type-1 was increased in fibrotic portal and septal areas and was also detected in granulomas, whereas desmin expression could be a useful a marker of portal vein destruction.

**Key words:**

*Schistosoma japonicum*, pig, liver fibrosis, histopathological scores, image analysis, tissue egg counts, granuloma,  $\alpha$ -SMA, desmin, collagen type-1.

**INTRODUCTION**

Schistosomiasis is one of the oldest known infections of humans (Olds and Dasarathy, 2000). *Schistosoma japonicum* is endemic in China, the Philippines, and Indonesia and is unique among the schistosomes infecting humans in that it has a zoonotic transmission with domestic animals, especially buffaloes, cattle and pigs, serving as the main reservoir hosts (McGarvey *et al.*, 1999; WHO Report, 2002). Schistosomiasis is one of the most important causes of liver fibrosis in man (Warren, 1980). The fibrosis is usually limited to portal areas, whereas hepatic architecture is well preserved and liver synthetic function is maintained (Andrade, 1965; Review by Dunn and Kamel, 1981). The most serious clinical consequence is portal hypertension caused by presinusoidal obstruction of the portal blood flow (Chen, 1993). Ultrasonography and pathological examination demonstrate that in human hepatosplenic schistosomiasis, resorption of portal fibrosis occurs and portal hypertension is reduced after treatment, except in very advanced cases (Ohmae *et al.*, 1992; Andrade, 1994). Inflammatory responses to schistosome egg antigens lead to the formation of granulomas, which is a protective phenomenon aimed at encapsulating and destroying the egg, but which also leads to tissue destruction and fibrosis (Weinstock, 1992). Fibrous tissue is composed of extracellular matrix (ECM), including collagen, and connective tissue cells (Andrade *et al.*, 1992). Fibrosis develops as a result of proliferation of collagen synthesizing cells, increased biosynthesis of collagen by existing cells and/or deficient degradation of collagen (Review by Dunn and Kamel, 1981). In liver fibrosis, portal fibroblasts and hepatic stellate cells (HSC) become activated and differentiate into cells with a myofibroblast phenotype, which express the intermediate filament  $\alpha$ -SMA and are involved in the production of collagen (Review by Ramadori and Saile, 2002). In the rat model of liver fibrosis, both HSC and myofibroblasts have been shown to express the intermediate filament desmin, a marker for muscle cells, and desmin expression has also been detected in fibroblasts of the periovular granuloma in the murine model of schistosomiasis mansoni (Ballardini *et al.*, 1988; Bolmont *et al.*, 1991). The pig is used as an experimental large animal model of human diseases due to anatomic, physiologic, metabolic and nutritional similarities with man (Willingham and Hurst, 1996; Review by Johansen *et al.*, 2000). Pigs develop typical acute schistosomiasis japonica similar to that of humans, the most severely infected organs being the liver and large intestine (Yason and Novilla, 1984; Willingham *et al.*, 1998). Assessment of the degree of liver fibrosis in humans is commonly done by semi-quantitative histopathological scoring, but quantitative analysis of fibrosis by image analysis has also been used (Chevallier *et al.*, 1994; Pilette *et al.*, 1998). Semi-quantitative scores, but not image analysis, have previously been used to assess liver fibrosis in the pig model of human schistosomiasis japonica (Hurst *et*

*al.*, 2000). In the present study we investigated liver fibrosis in tissue samples obtained at necropsy from different groups of *S. japonicum*-infected pigs by semi-quantitative histopathological scores as well as quantitative image analysis. The two methods were compared and correlation of liver fibrosis with liver TEC was investigated. Very little is known about qualitative aspects of the fibrotic lesion in schistosomal liver fibrosis in the pig, and therefore we also used immunohistochemistry to detect collagen and connective tissue cells involved in ECM production.

## MATERIALS AND METHODS

### *Animals and experimental design*

Twenty-six helminth naive, specific pathogen free Danish Landrace / Yorkshire / Duroc crossbred pigs, initially 8 to 12 weeks of age, were used for the experiment. Twenty of the pigs were allocated into four groups (A-D) containing five pigs each and were infected by intramuscular injection with Iscoves medium-suspended cercariae of a *S. japonicum* isolate originating from Zhejiang (Chekiang) province, People's Republic of China, and maintained in *Oncomelania hupensis hupensis* snails at the Danish Bilharziasis Laboratory, Charlottenlund, Denmark. Each pig received a single primary infection dose of 850 cercariae and the pigs of group C also received a challenge infection of 850 cercariae. The remaining six pigs served as uninfected controls (groups E and F). The experimental design is presented in Table 1. The pigs were treated in accordance with animal ethics laws of Denmark. For further experimental details see Willingham *et al.* (1997).

### *Liver tissue egg counts*

The eggs per g liver tissue were obtained by KOH digestion of 5 g liver samples taken from the left lateral liver lobe of each pig as previously described (Bøgh *et al.*, 1996).

Table. 1. Experimental design

Group	Number of pigs	Primary infection week	Challenge infection week	Necropsy week	Duration of infection weeks
A	5	0	-	12	12
B	5	0	-	21	21
C	5	0	12	21	21
D	5	12	-	21	9
E	3	-	-	12	-
F	3	-	-	21	-

### *Tissue sampling procedure*

The pigs were killed by an overdose (30 mg/kg) of pentobarbital intravenously. Pieces of liver tissue were collected from all pigs, fixed in 10% neutral-buffered

formalin, trimmed, processed conventionally and embedded in paraffin. Serial sections of 4µm were cut onto SuperFrost® Plus glass slides (Menzel-Gläser, Germany) and stained with haematoxylin and eosin or Masson's trichrome for histopathological evaluation and image analysis. Unstained sections of each series were used for immunohistochemical studies. The slides were given coded labels to keep the investigators unaware of the group affiliation of the pigs.

### *Histopathology*

Histopathological evaluation included assessment of the degree of diffuse portal and septal inflammatory cell infiltration and the degree of portal and septal fibrosis. Fibrosis was assessed in the Masson's trichrome stained sections, using the following semi-quantitative scoring system: score 0 = none, score 1 = mild, score 2 = mild to moderate, score 3 = moderate, score 4 = moderate to marked, and score 5 = marked fibrosis (Fig. 1, 2, 3). The sum of the scores for portal and septal fibrosis were used as a score for total fibrosis. The scoring of the hepatic fibrosis was done by two researchers (A.B. and M.H.) independently, after which the results were compared. The scores given by the two researchers was found to be mostly in agreement, but in case of difference of opinion, the sections were re-examined and discussed over multihead microscope until a consensus was reached. Portal and septal inflammatory infiltration was graded as mild, moderate or marked.

Fig. 1-3. Semi-quantitative histopathological scoring of portal and septal fibrosis on Masson's trichrome stained liver sections from *Schistosoma japonicum*-infected pigs.

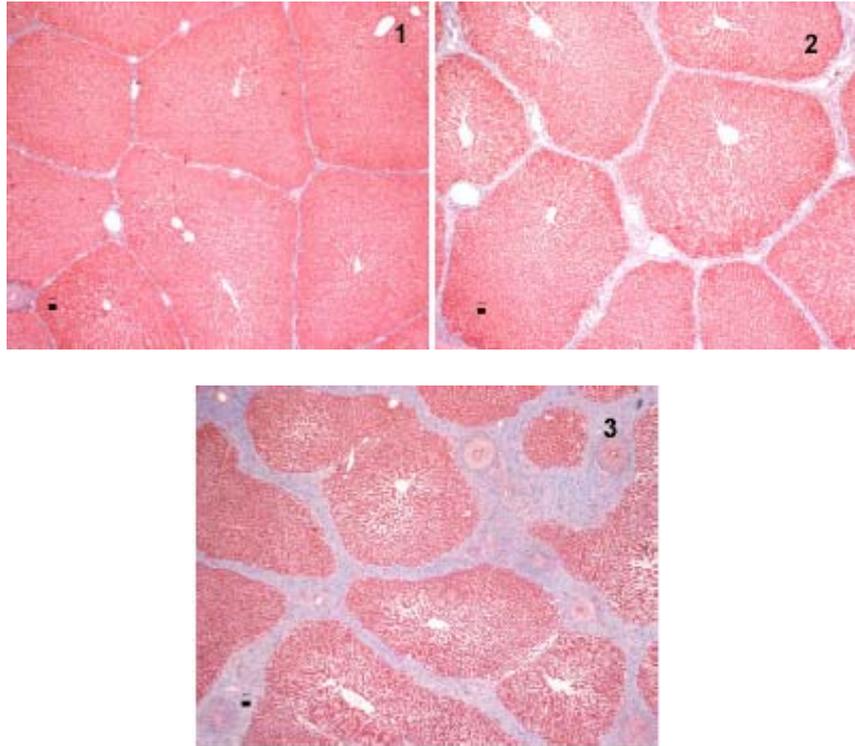


Figure-1. Score 1 portal and septal fibrosis. Pig from group C.  
Figure-2. Score 3 portal and septal fibrosis. Pig from group B.  
Figure-3. Score 5 portal and septal fibrosis. Pig from group A.

## Image analysis

Portal and septal fibrosis were also evaluated in the Masson's trichrome stained sections using a quantitative image analysis system (Easy Image Analysis 2000, Tekno optic AB, Stockholm, Sweden). Twenty images at objective 4X were obtained from each pig by a Nikon Digital Still camera DXM 1200 connected to a Nikon Eclipse E 600 microscope. This camera can digitize images in 1280 X 1024 pixels. Two categories, termed Blue and Red, respectively, were analysed. Category Blue represented the fibrosis in portal and septal areas stained blue in the sections. Category Red represented the liver parenchyma and other structures that were stained red. The non-fibrotic central parts of granulomas, which were usually stained pink or pale red in the sections, were deleted from the images by an interactive procedure before analysis to avoid inclusion in category Red. The area of each category was measured in each image and the mean area fraction of the fixed image area was calculated for each pig. The threshold values used for hue for category Blue were 29-218 and for category Red 220-252. For both categories, the threshold values for lightness were 0-240 and for saturation 0-255.

### *Immunohistochemistry*

Three sections of the series from each pig were employed for immunohistochemical procedures using the Streptavidin Biotin Complex/Horse Radish Peroxidase (StreptABC /HRP) method (DAKO A/S, Glostrup, Denmark). The primary antibodies used, their specificity, isotype and optimal dilution are presented in Table 2. Tris-Buffered Saline (TBS) of 0.05M, pH 7.6 was used for all dilutions and rinses.

After deparaffinization, the sections were given pretreatment for antigen retrieval in the following way: For detection of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) the sections were pretreated with target retrieval solution (TRS, DAKO A/S, Glostrup, Denmark) for 20 min at 97°C. For the anti-desmin antibody the sections were first pretreated with trypsin (1mg/ml, Sigma<sup>®</sup>, Steinheim, Germany) for 15 min at 37°C and then with TRS at 97°C for 20 min. For the anti-collagen type-1 antibody the sections were pretreated with pepsin (4 mg/ml) for 2 hrs at 37°C followed by trypsin (1mg/ml) for 15 min at 37°C.

After pretreatment the following procedure was employed for all the sections: Endogenous peroxidase was quenched by using 1.5% H<sub>2</sub>O<sub>2</sub> (3% H<sub>2</sub>O<sub>2</sub> for collagen type-1) for 10 min. Normal horse serum of 1:10 dilution was applied for 60 min to avoid non-specific protein binding. Endogenous avidin and biotin were blocked for 30 min each by using an avidin biotin blocking kit (Vector laboratories, Burlingame, CA, USA). This step was omitted for the collagen type-1 antibody; since it produced a marked background staining that interfered with the immunostaining.

The optimally diluted primary antibody was applied for 16-18 hrs at 4°C. Subsequently a biotinylated, pan-specific secondary antibody (anti-mouse / rabbit /

goat IgG) (Vector laboratories, Burlingame, CA, USA) and StreptABC/HRP were applied for 30 min each. For specificity control, the primary antibody was replaced by normal mouse IgG2a, normal mouse IgG1 and normal rabbit Ig (DAKO), respectively, each diluted to the same protein concentration as the primary antibody. Immunoreactivity was detected by incubation with a chromogen solution containing 0.6mg/ml diaminobenzidine (DAB) and 0.3% H<sub>2</sub>O<sub>2</sub> in 0.05M TBS for 5-6 min, producing a brown immunostaining. The sections were counterstained with haematoxylin for 30 s, mounted with xylene-soluble medium and examined with light microscopy.

Table 2. The primary antibodies used, their target specificity and optimal dilution employed in the immunohistochemical procedure.

Antibody	Specificity	Isotype	Dilution
M0851 <sup>1</sup> Monoclonal (mouse)	Human $\alpha$ -smooth muscle actin	IgG2a, Kappa	1:500
M0724 <sup>1</sup> Monoclonal (mouse)	Desmin	IgG1, kappa	1:1000
2150-0020 <sup>2</sup> Polyclonal (rabbit)	Collagen type -1	-	1:20

<sup>1</sup>DAKO A/S, Glostrup, Denmark

<sup>2</sup>Biogenesis Ltd, Poole, UK

### *Statistical analysis*

The non-parametric Mann Whitney test was used to analyse differences between the groups in liver TEC and the degree of fibrosis. Before statistical analysis, the values obtained by image analysis of Blue and Red categories were recalculated so that they made up a sum of one hundred using Microsoft<sup>®</sup> Excel. The relationship between liver TEC and fibrosis, assessed by either semi-quantitative histopathological scoring or image analysis, was investigated by Pearson's correlation test. For all tests, p values <0.05 were considered significant.

## RESULTS

### *Liver TEC*

The results of the liver TEC at the time of necropsy are presented in Table 3. The liver TEC were higher in group B than in any of the other groups of infected pigs, but the result was significantly different only from that of group D. Detailed parasitological results are presented elsewhere (Willingham *et al.*, 1997).

Table 3. Results of liver tissue egg counts (TEC) in the different groups of pigs (group means  $\pm$  SD).

Groups	TEC (eggs /g liver)
A	612 $\pm$ 836.0
B	1718.6 $\pm$ 1471.4 <sup>a</sup>
C	1141.4 $\pm$ 877.0
D	196 $\pm$ 132.5 <sup>a</sup>

<sup>a</sup>  $p < 0.05$

### *Histopathology*

The major histopathological lesions were variable numbers of perioval granulomas, diffuse infiltration of inflammatory cells, mainly eosinophils and small mononuclear cells, and fibrosis of portal areas and interlobular septa. Granulomatous obstruction of portal veins with destruction of the vein wall was also frequently observed. The diffuse portal and septal inflammatory cell infiltration was moderate to marked in all groups of infected pigs, with no prominent differences between the groups.

### *Semi-quantitative histopathological scoring of liver fibrosis*

The scores for portal, septal and total liver fibrosis are presented in Table 4. The highest fibrosis scores were found in group B and the lowest in group C, but the degree of fibrosis was variable in all four groups of infected pigs and the differences between the groups were not statistically significant.

Table 4. Histopathological fibrosis scores and area of fibrosis as measured by image analysis in the different groups of pigs (group means  $\pm$  SD).

Groups	Portal fibrosis scores	Septal fibrosis scores	Total fibrosis scores	Area of fibrosis (arbitrary units)
A	2.8 $\pm$ 1.4	2.6 $\pm$ 1.5	5.4 $\pm$ 2.9	8.408 $\pm$ 6.192 <sup>a</sup>
B	3.0 $\pm$ 1.2	3.0 $\pm$ 1.2	6.0 $\pm$ 2.4	11.186 $\pm$ 5.501 <sup>b</sup>
C	1.8 $\pm$ 0.8	1.8 $\pm$ 0.8	3.6 $\pm$ 1.6	7.786 $\pm$ 4.135 <sup>b</sup>
D	2.8 $\pm$ 1.1	2.4 $\pm$ 0.5	5.2 $\pm$ 1.6	8.776 $\pm$ 4.296 <sup>b</sup>
E	-	-	-	3.765 $\pm$ 1.215
F	-	-	-	3.098 $\pm$ 0.530

<sup>a</sup>-No statistically significant difference from controls (group E).

<sup>b</sup>-Statistically significant difference from controls (group F) ( $p < 0.05$ ).

#### *Quantitative measurement of liver fibrosis with image analysis*

The results of the image analysis of the area of liver fibrosis are presented in Table 4. In similarity with the histopathological scores, the highest values were observed in group B and the lowest in group C, but the differences were not statistically significant. The values for infected pigs were higher than those found in the uninfected controls and this difference was statistically significant for groups B-D versus group F.

#### *Relationship between liver TEC and liver fibrosis*

There was a positive correlation between liver TEC and septal fibrosis ( $r=0.491$ ,  $p < 0.05$ ) as well as total fibrosis ( $r=0.446$ ,  $p < 0.05$ ). There was also a positive correlation between liver TEC and the area of fibrosis as measured by image analysis ( $r=0.503$ ,  $p < 0.05$ ).

#### *Relationship between histopathological grading and image analysis*

There was a strong positive correlation between the semi-quantitative total fibrosis scores and the quantitative image analysis measurements ( $r=0.721$ ;  $p < 0.001$ ). There was also a positive correlation between image analysis measurements and the two variables included in the total fibrosis scores, i.e. portal fibrosis ( $r=0.699$ ;  $p < 0.01$ ) and septal fibrosis ( $r=0.728$ ;  $p < 0.001$ ).

#### *Immunohistochemistry*

Sections incubated with normal mouse IgG2a, normal mouse IgG1 and normal rabbit Ig were negative.

##### *Control pigs*

In the uninfected controls,  $\alpha$ -SMA was expressed by HSC in a dotted line pattern. The expression was more pronounced in the centrilobular than in the periportal area of the hepatic lobules. In portal vein walls, smooth muscle cells and fibroblast-like cells expressed  $\alpha$ -SMA. Expression was also detected in smooth muscle cells of hepatic veins and arteries, pericytes around capillaries and sub-epithelial (myoepithelial) cells of bile ducts. In the connective tissue of portal areas and interlobular septa, scattered cells with fibroblast morphology showed

expression of  $\alpha$ -SMA. Desmin was expressed by HSC in a dotted line pattern along the sinusoids similar to that observed for  $\alpha$ -SMA. However, the expression was more prominent in the periportal than in the centrilobular area. Smooth muscle cells of the portal vein and hepatic artery as well as scattered periductal smooth muscle cells of the bile duct expressed desmin. The anti-collagen type-1 antibody stained extracellular fibres in the connective tissue of portal areas, interlobular septa and around the central vein.

#### Infected pigs

The staining pattern for the three antibodies used was similar in the four different groups of infected pigs. Compared to the controls, the number of cells expressing  $\alpha$ -SMA in portal areas and interlobular septa was increased in infected livers in a manner that was proportional to the increase of the degree of fibrosis. In addition to the staining observed in the controls, expression of  $\alpha$ -SMA was detected in most cell layers of thickened portal vein walls in infected pigs (Fig. 4). Desmin expression in the portal areas of infected livers was noted only in smooth muscle cells of portal vein walls and also in smooth muscle cells of hepatic veins and hepatic arteries similar to the pattern found in the controls (Fig. 5). In areas with portal vein destruction scattered desmin-positive smooth muscle cells were often detected (Fig. 6). In the infected pigs, collagen type-1 positive extracellular fibres were found in increased numbers in portal areas and interlobular septa, proportionally to increases of the degree of fibrosis (Fig. 7).

In granulomas,  $\alpha$ -SMA was expressed by flattened fibroblast-like cells forming a concentric network pattern in the peripheral part of the granuloma, whereas the granuloma centres did not show any  $\alpha$ -SMA-positive cells (Fig. 8). Concentrically arranged fibres positive for collagen type-1 were detected to various degrees at the periphery of most granulomas (Fig. 9). Desmin-positive cells were not observed in any granulomas.

Fig. 4-9, Immunoperoxidase staining of liver sections from *Schistosoma japonicum* infected pigs.

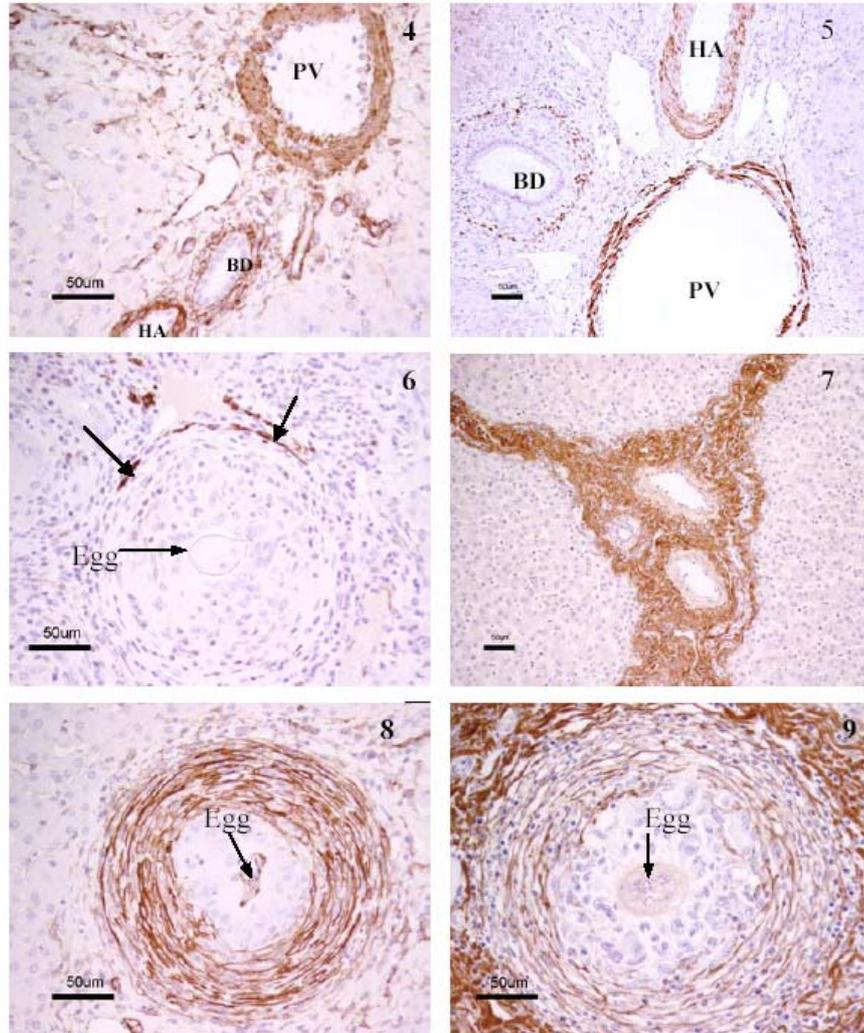


Figure-4

Portal area showing expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in smooth muscle cells and fibroblast-like cells in the thickened portal vein (PV) wall, smooth muscle cells of the hepatic artery (HA), pericytes around capillaries and sub-epithelial cells of the bile duct (BD). Pig from group B.

Figure-5

Portal area showing desmin expression in smooth muscle cells of the portal vein, hepatic artery and periductal cells of bile duct. Pig from group C.

Figure-6

Granuloma with egg in the centre causing obstruction of a portal vein. Note the desmin-positive smooth muscle cells (arrows) outlining the partly destroyed portal vein wall. Pig from group C.

Figure-7

Portal area and interlobular septa showing increased amounts of fibres positively stained with the anti-collagen type-1 antibody. Pig from group A.

Figure-8

Granuloma with egg in the centre showing  $\alpha$ -SMA-positive, flattened fibroblast-like cells forming a concentric pattern in the peripheral part. Pig from group B.

Figure-9

Granuloma with egg in the centre showing a fine concentric network of collagen type-1-positive fibres. Pig from group A.

## DISCUSSION

Previous experimental studies in the pig model of human schistosomiasis japonica have shown that pigs develop marked hepatic fibrosis in heavy infections (Willingham *et al.*, 1998; Hurst *et al.*, 2000). In the present histopathological study we investigated the degree of liver fibrosis in four different groups of pigs in an experiment designed to evaluate the effect of a patent primary infection on a challenge infection (Table 1). We used a semi-quantitative liver fibrosis scoring system and found that portal and septal fibrosis were present to various degrees in all four groups without any statistically significant differences between the groups. It thus appears that in this experiment neither the duration of infection (9, 12 and 21 weeks post infection, PI) nor a challenge infection superimposed on a primary infection had any significant effects on the degree of fibrosis. This is in contrast to a previous experimental study in the pig model, which showed prominent fibrosis in the acute phase of a single dose infection (11 weeks PI) and significantly less fibrosis in the chronic phase (24 weeks PI) (Willingham *et al.*, 1998; Hurst *et al.*, 2000). Another study of pigs investigated the effects of a low or high level of primary infection upon a challenge infection, using grossly observed septal fibrosis and liver collagen content as indicators of liver fibrosis (Pedersen *et al.*, 2003). Similar to our findings, fibrosis was not affected by the duration of infection or by the challenge infection, with the exception of the challenge control group, examined at 8 weeks PI, which in contrast to the challenge control group (D) in our study, showed significantly more fibrosis than any of the other groups.

Liver fibrosis in schistosomiasis is associated with the presence of eggs and host responses to them (Cheever and Yap, 1997). When all the groups of the present study were analyzed together, a correlation between liver tissue egg counts (TEC) and septal as well as total fibrosis scores was demonstrated. A similar relationship between liver TEC and fibrosis scores has previously been reported in the pig (Hurst *et al.*, 2000). The lack of significant differences between the groups for liver TEC (with the exception of the higher values observed in group B compared to group D) and liver fibrosis scores may be due to the small number of pigs in each group (n = 5) and the high variability within the groups.

Semi-quantitative histopathological fibrosis scores are commonly employed to assess the degree of fibrosis in human liver biopsies and has previously been used in the pig model as well (Chevallier *et al.*, 1994; Pilette *et al.*, 1998; Helal *et al.*, 1998; Hurst *et al.*, 2000). However, there are certain disadvantages with this

method, e.g. it is time consuming and there may be considerable inter- and intra-observer variability, which has led to the exploration of image analysis as an alternative method to assess liver fibrosis (Pilette *et al.*, 1998). Image analysis has been found to be rapid and the risk of inter- and intra-observer variability is reduced. The quantitative data obtained via image analysis are also more suitable for correlations with quantitative parasitological data, such as tissue and faecal egg numbers, than the semi-quantitative scores. In the present study, we therefore compared the semi-quantitative fibrosis scoring system with the area of fibrosis as measured by quantitative image analysis and found very good correlation between the two methods. Positive correlation was noticed between the area of fibrosis and portal fibrosis, septal fibrosis and total fibrosis, respectively. Our results are in agreement with the work done by Pilette *et al.* (1998) on liver biopsies from human patients with chronic liver disease due to alcoholism or chronic hepatitis B or C infection, which showed a correlation between semi-quantitative scores and the area of liver fibrosis as determined by image analysis. As demonstrated by these authors, an additional advantage of using image analysis to assess liver fibrosis is that it can be adapted for various kinds of fibrosis.

In human and rat liver fibrosis due to a variety of causes, activated hepatic stellate cells (HSC), also referred to as perisinusoidal cells, Ito cells or fat storing cells, are believed to be responsible for the increased collagen production (Levy *et al.*, 2002). These activated cells can be differentiated from quiescent HSC by their  $\alpha$ -smooth muscle actin (SMA) immunoreactivity. In the present pigs,  $\alpha$ -SMA was expressed in vessel walls of normal liver, similar to what has been described in another study of pigs (Costa *et al.*, 2001). In contrast to that study, we also found that HSC in normal livers were positive for  $\alpha$ -SMA. A possible explanation for this difference is that our pigs were killed with an overdose of pentobarbital and the liver samples from both controls and infected pigs were collected after death, whereas Costa *et al.* used liver biopsies from anaesthetised pigs as normal controls. These authors found a very rapid modification of HSC, as indicated by the expression of  $\alpha$ -SMA, in response to *ex-vivo* liver perfusion and suggest that these modifications may be a result of alteration of the blood pressure in the livers. Expression of  $\alpha$ -SMA by HSC has also been noted in postmortem liver specimens from human patients dying from shock of different causes (Schmitt-Gräff *et al.*, 1991). Our study thus does not provide any answer to the question of whether or not HSC are activated in schistosomal hepatic fibrosis in pigs.

Alpha-SMA is also a good marker for detection of myofibroblast-like cells in reactive fibrosis in rats and humans (Nouchi *et al.*, 1991). Myofibroblasts, as identified by their positivity for  $\alpha$ -SMA, were detected in as early as 4 weeks PI in the livers of rabbits infected with *S. japonicum*, whereafter they gradually increased in numbers as fibrosis progressively aggravated (Yang *et al.*, 1999). We found expression  $\alpha$ -SMA in cells of fibroblast morphology in portal and septal areas in normal and infected livers, but the numbers were increased in the infected livers proportionally to the degree of fibrosis. This suggests that these cells are myofibroblasts or myofibroblast-like cells and that they are involved in the increased production of extracellular matrix (ECM) in response to *S. japonicum* infection. However, in chronic human hepatosplenic schistosomiasis *mansoni* only

a transient role for myofibroblasts in the development of pipe-stem fibrosis has been proposed (Andrade *et al.*, 1999).

In normal adult human liver, desmin is only rarely expressed by HSC (Schmitt-Gräff *et al.*, 1991). However, desmin is expressed by HSC in normal rats (Ballardini *et al.*, 1988). We found that the HSC were positive for desmin, especially in the periportal area, which is in agreement with previous findings in pigs (Wake and Sato, 1993). Desmin was expressed by smooth muscle cells in portal veins, both in intact veins and in scattered cells in those disrupted by granulomatous inflammation and obstruction, similar to findings in chronic human hepatosplenic schistosomiasis (Andrade *et al.*, 1992). In the present pigs more cells of the portal vein walls expressed  $\alpha$ -SMA than desmin, which is in agreement with what has been reported in normal and diseased human livers (Schmitt Gräff *et al.*, 1991).

Collagen type-1 is a major constituent of the ECM in liver fibrosis in chronic human and murine schistosomiasis mansoni (Biempica *et al.*, 1983; Grimaud *et al.*, 1987; Andrade *et al.*, 1992). In our study, collagen type-1 was present in normal liver in connective tissue of portal and septal areas and vessel walls, which is in accordance with the results obtained in pigs by Costa *et al.* (2001). In fibrotic livers, collagen was increased in proportion to the increase of the degree of portal and septal fibrosis.

In murine *S. mansoni* infection, connective tissue cells in periportal granulomas have been identified as myofibroblasts based on morphological criteria (Boloukhere *et al.*, 1993). It seems likely that the  $\alpha$ -SMA positive cells that we found in the outer part of granulomas in the pigs also are myofibroblasts, but further studies including ultrastructure are required to better characterise these cells. Fibroblasts in hepatic granulomas in murine schistosomiasis mansoni express desmin (Bolmont *et al.*, 1991). In contrast, no desmin expression was found in granulomas in the present experiment, suggesting that the pig differs from the mouse in this respect. Granulomas contained concentric layers of collagen type-1, which is in accordance with descriptions of the murine *S. mansoni* model and of man (Grimaud *et al.*, 1987; Andrade *et al.*, 1992; Jacobs *et al.*, 1997).

In conclusion, this study of pigs experimentally infected with *S. japonicum* confirmed previous observations that there is a correlation between liver fibrosis and liver TEC in this model. Good correlation was also found between the area of fibrosis as measured by image analysis and the semi-quantitative histopathological scores, making image analysis a useful tool for assessment of liver fibrosis in the pig model. Collagen type-1 is a major component of the ECM and  $\alpha$ -SMA-expressing connective tissue cells are present in increased numbers in fibrotic portal and septal areas. *Schistosoma japonicum*-induced granulomas in the pig contain collagen type-1 fibres and  $\alpha$ -SMA-, but not desmin-expressing cells. Desmin expression was detected in smooth muscle cells in vessel walls in portal and septal areas and could therefore be used as a marker of portal vein destruction. Further studies in the pig model should include better characterisation of the

connective tissue cells involved in extracellular matrix production, especially myofibroblasts. Quantitative data on liver fibrosis obtained by image analysis can be used for correlations with other parasitological variables as well, e.g. faecal egg counts.

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