

From Microbiology and Tumor Biology Center  
Karolinska Institutet, Stockholm, Sweden

# **Serodiagnostics of schistosomiasis using keyhole limpet hemocyanin (KLH) as antigen**

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## ABSTRACT

The diagnostics of invasive helminth infections is often indirect and based on antibody detection in serum specimens from infected individuals. This is the case in schistosomiasis (bilharzia) where no excreted eggs can be found in light, often asymptomatic, infection. Travellers, especially adventure tourists, and immigrants to endemic areas run a high risk of becoming infected. If the diagnosis is missed, infected individuals may carry intravascular worms for more than 30 years and thereby run a risk for tissue injury due to egg deposition.

Early during infection antibodies against gut associated schistosome antigens (GAA) are detected by an indirect immunofluorescent assay where sections of adult worms are used as antigen. We observed a similar, previously unrecognised staining pattern in a significant proportion of GAA-positive sera, which subsequently was shown to be due to antibodies, which may cross-react with keyhole limpet hemocyanin (KLH). This cross-reacting glycoprotein, from the mollusk (*Megathura crenulata*), has been introduced as diagnostic antigen for the detection of antibodies against schistosomes. However, in the literature the limited experience using KLH in diagnostics of early infection shows discrepant results.

The aim of this study was to understand why antibodies, reacting with KLH, are produced during schistosomiasis and to evaluate the diagnostic potential of such antibodies. This was done by localisation of KLH-cross-reactive epitopes in the different life stages of the parasite and by partial identification by immunoblotting of electrophoretically separated schistosome egg components.

We found that shared antigens are expressed in different life stages of the parasite: at the surface and in the secretions of the cercaria, at the schistosomula surface, in the excretory/secretory (E/S) ducts of the adult worms, in reproductive tissues, at the surface of the miracidia, in and around eggs in granulomas, and in Kupffer cells in the liver.

These observations explain why an immune response against KLH-cross-reactive schistosome components is readily induced in infected individuals. However, when we evaluated a simple dot-ELISA for the detection of anti-KLH antibodies, it had poor specificity and the sensitivity varied when different patient groups were tested. A significant finding was that trichinellosis patients had anti-KLH antibodies and also reacted with schistosome ducts. We conclude that KLH is a potentially useful target antigen for serodiagnostics. However, multiple antigenic epitopes in KLH may explain reported discrepant results and specificity and sensitivity problems.

*Festina lente*  
(Aldus Manutius 1450-1515)

## LIST OF PUBLICATIONS

- I. Cross reacting antibodies against keyhole limpet haemocyanin may interfere with the diagnostics of acute schistosomiasis  
Cecilia Thors & Ewert Linder. *Parasite Immunology* 1998, 20, 489-96
- II. Localization and identification of *Schistosoma mansoni*/KLH-crossreactive components in infected mice  
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## LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CAA	Circulating anodic antigen
CCA	Circulating cathionic antigen
CDIFA	Colloidal dye immunofiltration assay
CFT	Complement-fixation test
CHR	Cercarienhüllen reaction
COPT	Circumoval precipitation test
DDIA	Dipstick dye immunoassay
DIG-TIA	Diffusion-in-gel thin layer immunoassay
E/S	Excretory/secretory
ELISA	Enzyme-linked immunosorbent assay
FAST	Falcon assay screening test system
FITC	Fluorescein isothiocyanate
Fuc	Fucose
GAA	Gut associated antigen
Gal	Galactos
GalNAc	N-acetyl galactosamine
GlcNAc	N-acetyl glucosamine
GASP	Gut-associated proteoglycan
GST	Glutathione-S-transferase
HRP	Horseradish peroxidase
IFA	Indirect immunofluorescent (antibody) assay
IHA	Indirect hemagglutination
Lac	Lactose
KLH	Keyhole limpet hemocyanin
MAMA	Microsomal adult membrane antigen
NMRI	Naval Medical Research Institute
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PZQ	Praziquantel
RIA	Radioimmunoassay
RT	Room temperature
SA	Somatic antigen
SBL	Statens Bakteriologiska Laboratorium
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamid gel electrophoresis
SEA	Soluble egg antigens
Sh	<i>Schistosoma hematobium</i>
Sj	<i>Schistosoma japonicum</i>
Sm	<i>Schistosoma mansoni</i>
Th	T helper cells
TIA	Thin layer immunoassay
TRITC	Tetramethylrhodamine isothiocyanate
TR-IFMA	Time-resolved immunofluorometric assay
TSP	Transferable solid phase
WB	Western blot





## INTRODUCTION

Human schistosomiasis is an infection caused by trematodes (blood flukes) mainly belonging to three species of the genus *Schistosoma*: *S. mansoni*, *S. japonicum*, and *S. hematobium*. Less widely distributed species are *S. intercalatum* and *S. mekongi*. More than 200 million people in 74 countries with tropical or subtropical climate are infected of which 85% live in Africa, south of Sahara (Figure 1). Among them, 120 million are symptomatic and 20 million exhibit severe disease. Six hundred million people are at risk of being infected (Chitsulo *et al.*, 2000; Engels *et al.*, 2002). Transmission of the infection takes place via water and infection may occur after brief water contact in travellers and immigrants to endemic areas. In the latter patient groups infection is often asymptomatic which gives rise to diagnostic problems.

Schistosomes are successful parasites, apparently as the result of prolonged co-evolution with their hosts. In order to develop methods for schistosomiasis control we need to identify different aspects of the host – parasite relationship. The present study on the early antibody response in schistosomiasis should be regarded as a contribution to achieve such goals.

## HISTORY

The German parasitologist, Theodor Bilharz, working in Cairo, described the worm “*Distoma haematobium*” (subsequently *S. hematobium*) in 1851 and published the finding together with his teacher, professor Siebold in Breslau (Bilharz and von Siebold, 1852-53). The connection to urinary disease (hematuria) with presence of eggs in urine was made a year later.

In 1902, Manson described a patient with lateral spined eggs while Harley patients with urinary schistosomiasis having terminal spined eggs only (Manson, 1902). It took half a century for the controversy regarding terminal and lateral spined eggs to resolve when Leipner showed that two species occur which have different intermediate hosts (Leipner, 1916). Manson in England found only one kind of eggs in stools “in this case, as so often happens, in bilharzia ova from the alimentary canal, the spine is placed laterally” (Manson, 1902). The reason for this prolonged controversy was that double infections with *S. hematobium* and *S. mansoni* were so common in the Nile delta, a situation persisting into this day (Figure 1). Mansons patient, being a British citizen, who had travelled in the Caribbean, can be regarded as the first case of “travel-

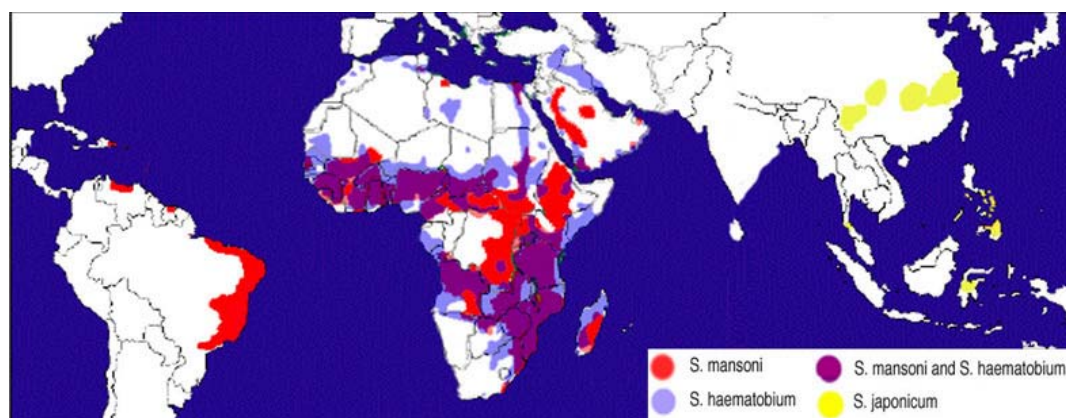
associated” schistosomiasis described! At the same time, this finding was the first case of schistosomiasis outside Africa and a consequence of the slave trade.

There is evidence that schistosomiasis was present in ancient Egypt: two observations of *S. haematobium* eggs in the kidney of Egyptian mummies have been described in the literature (Reyman *et al.*, 1977; Ruffer, 1910b). Also circulating schistosome antigen has been demonstrated in mummies (Deelder *et al.*, 1990; Miller *et al.*, 1992).

Archeological studies in China have shown that schistosomiasis japonica has been present for more than 2200 years (Mao and Shao, 1982). The first detailed description of Katayama disease (acute schistosomiasis) in rice farmers was made by a Japanese physician Dario Fujii in 1847. He also noted that cattle and horses were affected. The connection of the disease to occurrence of schistosome eggs was made more than 60 years later, when schistosome eggs were found in a patient with Katayama disease (Kean *et al.*, 1978). The worm was detected and described in 1904 (Katsurada, 1904; Kean *et al.*, 1978).

## GEOGRAPHICAL DISTRIBUTION OF SCHISTOSOMIASIS

*S. haematobium* is the most prevalent of the species and is endemic in Africa and the Middle East (Figure 1 blue & violet). *S. mansoni* occurs in Africa, South America (Brazil, Surinam and Venezuela), Caribbean (Puerto Rico, St Lucia, Guadeloupe, Martinique, Dominicans Republic, Antigua and Monserrat) and areas of the Middle East (Figure 1 red & violet), while *S. japonicum* exists in East Asia (parts of China: Changjiang-river, Sichuan and Yunnan, and the Philippines) (Figure 1 yellow)



**Figure 1.** Distribution of schistosomiasis. (Source: WHO)

## SCHISTOSOME-HOST INTERACTIONS

### The life cycle of schistosomes (see appendix)

The life cycle of schistosomes involves both invertebrate and vertebrate hosts. The vertebrate (definitive host) becomes infected when coming in contact with fresh water containing a free-swimming larval form (cercariae) derived from the intermediate snail host. The cercariae are attracted to skin lipids and attach to and penetrate the skin of the host (Linder, 1990). The bifurcated tail is lost during the penetration process and the anterior body part transforms into a schistosomulum. At the transformation the trilaminar tegument is transformed to become heptalaminar. The schistosomulum migrates through the tissues and reaches the lungs via the blood circulation or the lymphatic system. The final habitat in the host is the liver and intestinal mesenteric veins (*S. mansoni*, *S. japonicum*) or pelvic venules (vesical plexus) (*S. hematobium*), where the schistosomules mature to adult worms. The “homing” of the developing schistosomes is a fascinating recognition phenomenon of which we have little or no knowledge. In contrast to other trematodes the schistosomes have different sexes. The flat body of the male worm forms a “gynacophoric canal” that surrounds the female during a prolonged mating process, which results in continuous egg production. Eggs penetrate the terminal branches of blood vessels and pass through the tissues of the intestinal or bladder wall to become excreted in the faeces (*S. mansoni*, *S. japonicum*) or urine (*S. hematobium*). However, many eggs (roughly 50%) are not excreted from the vertebrate host, but are lodged in the liver, bladder, ureters or other tissues. The host inflammatory response to the eggs in tissues is the main cause of pathology in all forms of schistosomiasis.

The life cycle is completed when the excreted eggs hatch in fresh water and release another free-swimming larval form, the miracidia. The miracidia infect a receptive fresh water snail, which becomes the intermediate host (*S. hematobium*: *Planorbis* spp, *S. mansoni*: *Biomphalaria* spp, *S. japonicum*: *Oncomelania* spp). In the snail miracidia transform into primary sporocysts, which produce secondary sporocysts that migrate to the snail hepatopancreas and gonads. Asexual divisions take place in the secondary sporocysts and, after about one month, swarms of free-swimming cercariae emerge from the snail. From one miracidium thousands of unisexual cercariae emerge. The cercariae are motile and equipped with a bifurcated tail, which acts as a propeller. They are infective for about two days after release.

## Parasite survival in the definitive host

To become successful parasites, schistosomes have evolved several ways to evade the host immune system and other host defences. The adult schistosomes are dependant on the host for nutrition. In the blood vessels they are surrounded by soluble nutrients but at the same time a target for defence attacks by the host. The schistosomula, which have lost their protective surface coat, and especially the adult worms, are exposed to the immune system, but the features of their tegument offer protection from immune attack. The tegument consists of a double lipid bilayer. The outer bilayer is covered by mucins, has few exposed antigens and it can be rapidly replaced if damaged. Host components, e.g. blood group antigens as well as major histocompatibility complex antigens, complement and IgG, can be incorporated into the tegument (Clegg *et al.*, 1971; McLaren, 1984; Smithers *et al.*, 1969; Torpier *et al.*, 1979). This uptake of host antigens has been regarded as “antigenic disguise”. Inactivation of the complement attack complex seems to occur by production of the complement inhibitor known as protectin (CD59) (Fishelson, 1995; Morgan *et al.*, 2000; Parizade *et al.*, 1994). Peptides, potentially capable of interfering with endocrine hormones of the host, have been identified in the different life stages of the parasite. Such peptides may inhibit the host immune system by interfering with cell mobility (Salzet *et al.*, 2000; Thompson, 2001). However, the complex interactions between parasite and host are incompletely understood. Uptake of host components may serve several purposes such as inactivation of effector molecules and uptake as part of a metabolic process. Thereby the metabolism of the parasite can at the same time be regarded as evasion of attack by the host defences. This is clearly demonstrated in the case of immunoglobulin uptake where immunoglobulin molecules seem to be taken up and degraded of the parasite (Thors *et al.*, 2006b).

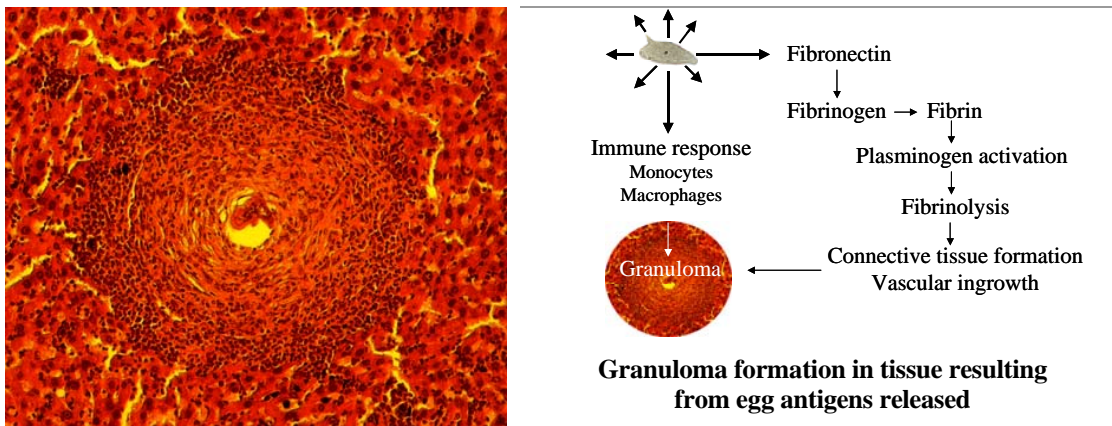
For disposal of waste products and regulation of internal fluids there is an excretory (protonephridial) system. It is a branched tubular system with flagellated flame cells at the tip of the branches. The flame cells cause influx of extracellular fluids into the tubes, the contents of which are excreted via the excretory pore at the posterior end of the body (Fried and Haseeb, 1991). By using monoclonal antibodies in confocal laser scanning microscopy (Bogers *et al.*, 1994) and in immunoelectron microscopy (de Water *et al.*, 1987) the excretory system of adult *S. mansoni* worms has been visualized. The details are not known but uptake and release of substances apparently takes place both at the surface and in the gut.

## The response to *Schistosoma*-infection in the definitive host

After the penetration of cercaria and before the onset of egg deposition the immune response is primarily of Th1 type (promoting cellular immunity) and directed against worm antigens. At the onset of egg production, the immune response switches to Th2 type, promoting an antibody response directed preferentially against highly immunogenic egg glycans. At the same time IgE levels raise and eosinophilia is seen (Pearce *et al.*, 2004). The secretions from tissue-trapped eggs in the liver are hepatotoxic (Dunne *et al.*, 1991), but the immunoglobulins and granuloma formation around the eggs prevent these toxins from damaging the surrounding hepatocytes (Doenhoff *et al.*, 1986; Hassounah and Doenhoff, 1993). This correlates well with the findings in human schistosomiasis patients: a severe hepatosplenic disease is seen in patients with a Th1 response, while a Th2 response gives a mild intestinal disease (Mwatha *et al.*, 1998). Notable is that egg antigens cannot induce Th2 responses if they are deglycosylated (Okano *et al.*, 1999).

The granuloma is a peculiar host response to mainly carbohydrate antigens and seen e.g. in tuberculosis and leprosy. Schistosome granulomas in infected individuals are of different composition reflecting their different age. The granulomatous lesions surrounding the tissue-trapped eggs consist of macrophages, eosinophils, CD4+ T-cells, fibronectin, fibrin, plasmin and collagen fibres. The granulomas resolve as the eggs die and leave fibrotic nodules (Figure 2).

Already infected individuals are less susceptible to new infections (concomitant immunity) (Smithers and Terry, 1967; Smithers and Terry, 1969). One hypothesis is that newly transformed schistosomula may be more sensitive to immune attack than the adult worms that provoked the partly protective immunity. Another explanation is the hepatic shunt theory: eggs clog the intrahepatic circulatory system and force the schistosomula to bypass the liver via a collateral circulation resulting from hepatic damage and fibrosis. Thereby the maturation of schistosomula into adult worms, which appears to take place in the hepatic environment, is inhibited (McHugh *et al.*, 1987).



**Figure 2.** Histological appearance of granuloma in liver caused by *S. mansoni* egg and outlines of events involved.

## Schistosomiasis

Eggs excreted in the urine (*S. hematobium*) and stools (*S. mansoni* and *S. japonicum*) cause mucosal damage leading to haematuria or blood in the stool. The main pathophysiology in schistosomiasis is due to the host immune response against eggs, which are lodged in the tissues, but several other conditions are seen at different stages of infection (Table 1).

**Table 1.** Symptoms and signs caused by different schistosome life stages

<i>Parasite stage</i>	<i>Site</i>	<i>Time after infection</i>	<i>Symptoms/signs</i>
Cercariae	Skin	0-2 weeks	Cercarial dermatitis
Schistosomula	Lungs	4-7 days	Cough, fever, eosinophilia
Eggs	Circulation/tissues	3-9 weeks	Katayama fever

### *Acute infection*

Cercarial dermatitis may occur at the site of penetration of the cercaria within a few hours to one week after infection. (This condition also occurs in swimmer's itch, caused by bird schistosomes). Katayama fever is a reaction to heavy infection and occurs in the acute phase, usually 1-2 months after contact with contaminated water. It is caused by circulating immune complexes, mainly in *S. japonicum*- and *S. mansoni*-infections and often associated with abdominal pain, hepatosplenomegaly, and diarrhoea.

### *Chronic infection*

Chronic infection is the rule in many endemic areas. Because of the high morbidity, manifestations of infection are regarded as normal in many societies in tropical Africa. During chronic infection the pathology is caused by eggs lodged in the tissue, which cause inflammation, fibrosis and calcification. In chronic *S. hematobium*-infection the

genitourinary tract is affected and lesions in the bladder lead to severe fibrotic damage, and predispose to the development of bladder stones and squamous carcinoma of the bladder. Infertility in women from areas endemic for *S. hematobium* is often a result of egg granulomas in the oviducts.

In *S. mansoni*- and *S. japonicum*-infection, the intestine and the liver are damaged. A life threatening state is Symmers'periortal fibrosis of the liver that may lead to rupture of submucosal esophageal varices. The central nervous system may also be affected at early stage of the infection, with severe consequences like paralysis due to tissue damage caused by deposition of eggs (Ferrari, 2004; Kirchhoff and Nash, 1984; Nascimento-Carvalho and Moreno-Carvalho, 2005) (Table 2).

**Table 2.** Sites of pathology in chronic schistosomiasis

<b>Schistosoma sp</b>	<b>Sites of pathology</b>
<i>S. hematobium</i>	Genitourinary tract CNS Cutaneous
<i>S. mansoni</i>	Hepatic/intestinal tract Spleen Lungs & heart (cor pulmonale 5%) Kidney (glomerulonephritis) Spinal cord CNS
<i>S. japonicum</i>	Hepatic/intestinal tract CNS

***Schistosomiasis in travellers and immigrants to endemic areas*** (Table 3)

Even if schistosomiasis in travellers to endemic areas is a minor problem in comparison to the gigantic morbidity seen in endemic areas, research on this group of infected individuals has some relevance also for the situation in endemic areas. Adult populations may encounter schistosomiasis for the first time as a consequence of population movements, e.g. due to irrigation projects, building of dams, famine, poverty and war.

There are many reasons for travelling: job and business related, tourism, visiting friends and relatives, education and research, missionary commissions etc.

A network, European Network on Imported Infectious Diseases Surveillance (TropNetEurop) a group of tropical medicine institutions in Europe, was founded in 1999 to collect epidemiological and clinical data on imported infectious diseases to Europe (Grobusch *et al.*, 2003).

In travellers and immigrants, newly infected with low infective dose, the symptoms may be diffuse or absent. In a study, describing 1107 British cases, more than fifty percent were asymptomatic after travel in Africa (Whitty *et al.*, 2000). In a group of six Finnish hunters in the Central African Republic one *S. mansoni* egg in one index case resulted in adequate diagnosis and treatment for schistosomiasis (Pitkanen *et al.*, 1990). The asymptomatic cases in industrialized countries suggest underdiagnosis and thereby that travel-associated schistosomiasis is an underestimated problem (Hatz, 2005).

Tissue damage due to deposited eggs has been described in various organs, but it is hard to evaluate the risk for such complications (Crump *et al.*, 2000; Davies and Davis, 2001; Greenwald, 2005; Leman *et al.*, 2001; Murdoch, 2003; Schwartz *et al.*, 2002; Sheorey *et al.*, 2004; Weber *et al.*, 1998).

**Table 3.** Reports of imported cases of schistosomiasis after visits in endemic area

<i>Nationality</i>	<i>Visited region</i>	<i>Reference</i>
Australia	Africa	(Harangozo <i>et al.</i> , 1995)
Denmark	Africa	(Tarp and Andersen, 1996)
Finland	Central African Republic	(Pitkanen <i>et al.</i> , 1990)
Great Britain	Africa	(Whitty <i>et al.</i> , 2000)
	Africa (sub-Saharan)	(Day <i>et al.</i> , 1996; Jordan and Webbe, 1996)
	Malawi	(Moore and Doherty, 2005)
Germany	Africa (Niger, Mali, Lake Malawi, Lake Victoria) South America Iraq Mekong river	(Jelinek <i>et al.</i> , 1996; Jelinek <i>et al.</i> , 1997)
Israel	Lake Malawi	(Potasman <i>et al.</i> , 1996)
Italy	Cameroon	(Raglio <i>et al.</i> , 1995; Raglio <i>et al.</i> , 2002)
	South Tunisia	(Bianchi and De Carneri, 1970)
Netherlands	Southern Africa 47%	(Bierman <i>et al.</i> , 2005)
	Mali	(van Lieshout <i>et al.</i> , 1997)
	Ethiopia (Omo river)	(Zuidema, 1981)
	Lake Malawi	(Kager and Schipper, 2001)
New Zealand	Africa	(Ellis-Pegler, 1996)
Northern Ireland	Africa	(Ingram <i>et al.</i> , 1996)
Spain (various)	Africa: Mali, (Dogon country), Uganda	(Bou <i>et al.</i> , 2001; Colebunders <i>et al.</i> , 1995; Corachan <i>et al.</i> , 1997; Corachan <i>et al.</i> , 1992; Roca <i>et al.</i> , 2002)
Sweden	Africa	(Evengard, 1990)
US	Ethiopia (Omo river) Kenya Malawi Tanzania	(Centers for Disease Control, 1984; Centers for Disease Control, 1993; Istre <i>et al.</i> , 1984; Outwater and Mpangala, 2005; Schwartz <i>et al.</i> , 2005)
various (expatriates)	Tanzania (Lake Malawi)	(Trachtenberg <i>et al.</i> , 2002)



## DIAGNOSTICS AND DISEASE CONTROL

Eosinophilia and increased levels of IgE are hallmarks for helminth infections and frequently a reason for further laboratory examination. The diagnosis of schistosomiasis can be performed by direct morphological methods and immunological methods (antibody and/or antigen detection).

### Morphological (microscopical) methods

The detection of excreted ova in faeces (Katz *et al.*, 1972) and urine is the most widely used diagnostic method of schistosome infections (Table 4). The specificity is high, but the sensitivity is low due to large day-to-day variations in excretion of eggs (Ruiz-Tiben *et al.*, 1979; Utzinger *et al.*, 2001; Yu *et al.*, 1998) and uneven distribution of eggs in excreta (Engels *et al.*, 1997; Ye *et al.*, 1998). The sensitivity of faeces examination may be improved by taking rectal snip biopsies, however, increased number of examined faecal samples make the two methods equally reliable (Rabello, 1992). In patients with low intensity of infection there are no detectable eggs before specific antibodies are present.

**Table 4.** Assays for the diagnosis of schistosomiasis: Microscopical methods

Kato-Katz thick smear (faeces)	<i>S. mansoni</i> / <i>S. japonicum</i> eggs	(Kato and Miura, 1954; Katz <i>et al.</i> , 1972; Martin and Beaver, 1968)
Formol-ether/ formol ethyl acetate concentration of faeces	<i>S. mansoni</i> / <i>S. japonicum</i> / <i>S. intercalatum</i> / <i>S. mekongi</i> eggs	(Ridley and Hawgood, 1956; Young <i>et al.</i> , 1979)
Sedimentation and/or filtration of urine	<i>S. hematobium</i> eggs	(Braun-Munzinger, 1986; Dazo and Bilas, 1974; Peters <i>et al.</i> , 1976)

### Antibody detection methods

There are many variations of antibody detection methods. Commonly used methods, ELISA (Engvall, 2005; Engvall and Perlman, 1971; Lequin, 2005) and IFA, employ antigens from all life stages of the schistosome including live parasites (eggs, cercaria), crude extracts, purified and recombinant antigens. The different antigen matrices include different plastics, nitrocellulose, agar and blood cells. Some of the assays are summarized in Table 5.

**Table 5.** Assays for the diagnosis of schistosomiasis: Antibody detection

<i>Method</i>	<i>Antigen</i>	<i>Reference</i>
CFT		(Yoshimoto, 1910) (Dunston and Pepler, 1965)
Intradermal test		(Kan, 1936) (Pesigan <i>et al.</i> , 1951; Sherif, 1956; Zhu, 2005)
IFA	Larval, worms GAA (CCA+CAA)	(Nash, 1978) (Tarp <i>et al.</i> , 2000)
IHA	Cercariae, worms	(Oelerich <i>et al.</i> , 1975; van Gool <i>et al.</i> , 2002)
RIA	Eggs	(Pelley <i>et al.</i> , 1977)
Flocculation tests	Cercariae	(Anderson, 1960)
COPT	Live eggs	(Fraga de Azevedo and Rombert, 1964; Hillyer <i>et al.</i> , 1979; Oliver-Gonzalez, 1954)
CHR	Live cercariae	(Ahmed <i>et al.</i> , 1993; Fraga de Azevedo and Rombert, 1964)
ELISA	Eggs and worms (all species)	(Hillyer and Gomez de Rios, 1979; Huldts <i>et al.</i> , 1975)
	CEF6 (egg fraction)	(Doenhoff <i>et al.</i> , 1993; Doenhoff <i>et al.</i> , 1985)
	P28 glutathione S-transferase	(Auriault <i>et al.</i> , 1990)
	Serine proteinase inhibitor-like molecule	(Li <i>et al.</i> , 1995)
	Tropomyosin	(Xu <i>et al.</i> , 1991)
	Sm cathepsins B and L	(Grogan <i>et al.</i> , 1997)
	Sm22.3	(Hancock <i>et al.</i> , 1997)
SmE16 (egg)	(Moser <i>et al.</i> , 1992)	
Dot ELISA		(Boctor <i>et al.</i> , 1987)
DIG- ELISA	Eggs and worms (all species)	(Elwing and Nygren, 1979; Ismail <i>et al.</i> , 1989)
FAST-ELISA	MAMA	(Tsang and Wilkins, 1991) (Tsang and Wilkins, 1997)
TSP-ELISA	heat shock protein 70, Sm31, Sm32	(Moser <i>et al.</i> , 1990)
Dot-DIA	SEA, KLH	(Rabello <i>et al.</i> , 1992; Rabello <i>et al.</i> , 1993)
WB	MAMA	(Tsang <i>et al.</i> , 1983a; Tsang <i>et al.</i> , 1983b) (Tsang and Wilkins, 1991) (Tsang and Wilkins, 1997)
	Sm31/Sm 32	(Ruppel <i>et al.</i> , 1985)
	Worms (crude)	(Sulahian <i>et al.</i> , 2005)
CDIFA	SEA (Sj)	(Xiang <i>et al.</i> , 2003; Xiao <i>et al.</i> , 2005)
TIA, DIG-TIA	Worms	(Elwing <i>et al.</i> , 1977; Nilsson <i>et al.</i> , 1980) (Elwing and Nilsson, 1980)
DDIA	SEA (Sj)	(Zhu <i>et al.</i> , 2002; Zhu <i>et al.</i> , 2005)

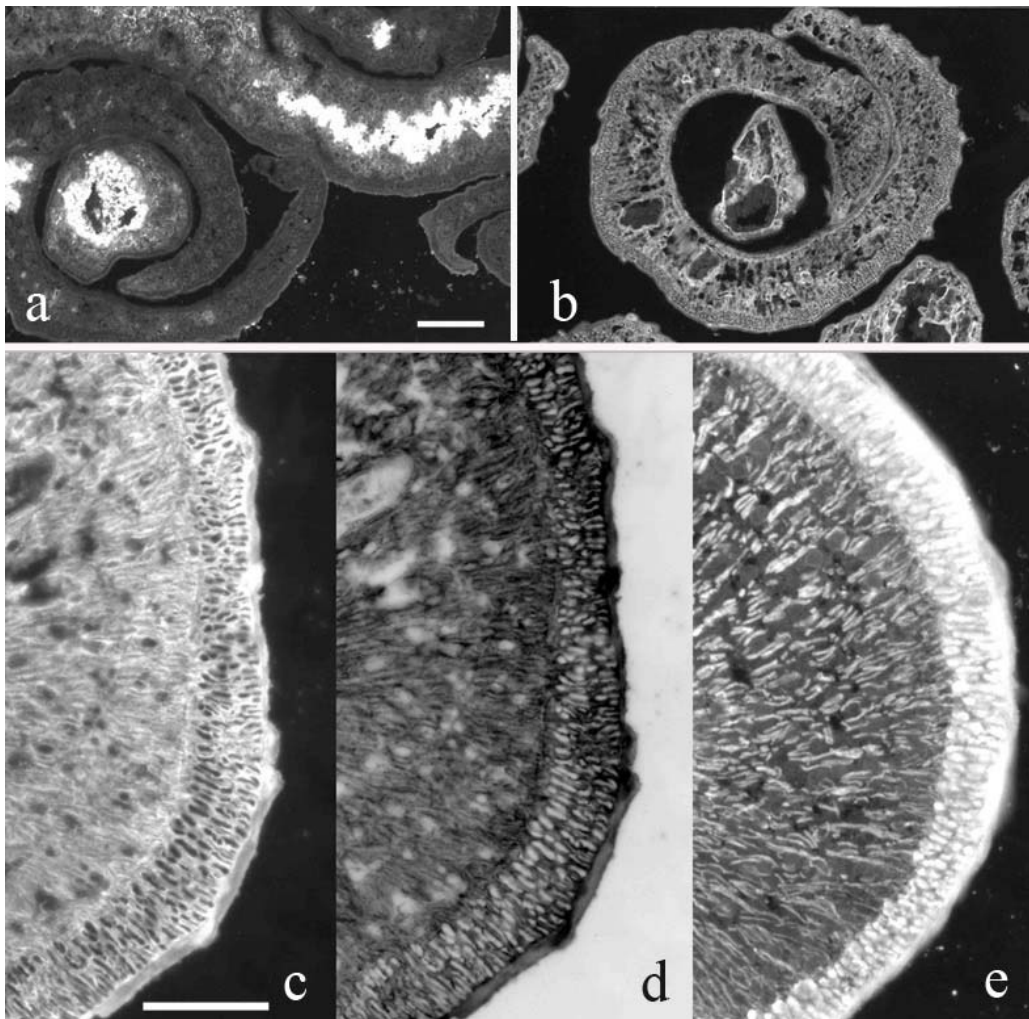
## ***Gut associated antigens (GAA) and somatic antigens (SA) in diagnosis of schistosomiasis***

### **Gut associated antigens (GAA)**

The indirect immunofluorescent assay (IFA), using worm sections, for diagnosis of schistosomiasis was introduced 1969 (Vernes *et al.*, 1969). The typical and distinct reaction of patient serum antibodies with the gut region of both male and female worms was easy to recognize (Figure 3a). A report that circulating schistosome antigens appeared in serum of mice 26 days after infection (Berggren and Weller, 1967) was followed by an extensive and ongoing research regarding their purification, characterization, localization and antigenicity (Nash, 1974; Nash *et al.*, 1977; Nash *et al.*, 1974; von Lichtenberg *et al.*, 1974). Released schistosome antigens are glycoproteins. The main ones are termed circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) and can be demonstrated using an antigen capture assay (Deelder *et al.*, 1994). Antibodies against both CAA and CCA, which occur early during infection (Okot-Kotber, 1978; van Helden *et al.*, 1975), are responsible for the GAA reaction in adult worms in IFA (Deelder *et al.*, 1980). GASP (gut-associated proteoglycan) and GASCAP (gut-associated circulating anodic proteoglycan) are synonyms to CAA (Nash and Deelder, 1985).

### **Somatic antigen (SA)**

Occasionally sera from schistosomiasis patients give another type of staining pattern in IFA on adult worms. It was described as “diffuse” parenchymal pattern (van Helden *et al.*, 1975). The term “diffuse” can be misleading because the staining pattern in the parenchyma is very distinct (Figures 3b and 3c). However, the term “diffuse” can be interpreted as adequately pointing out the distinction towards the “focal” staining pattern of anti-GAA. Antibodies against SA occur, in contrast anti-GAA antibodies, later in infection and are probably reflecting release of structural worm components of dead worms. The SA pattern may be difficult to separate from “non-specific” or unwanted reactions commonly seen in uninfected patients. One such reaction is caused by antibodies against muscle antigens, such as actin and myosin, which are found in chronic inflammatory disorders (Figure 3e).



**Figure 3.** Staining patterns seen in IFA using adult worms as antigen and schistosomiasis patient sera and a control serum. a) acute infection (GAA), b) chronic infection (SA), c) SA, d) inverted SA, e) muscle. Bars 150  $\mu\text{m}$ .

### Other diagnostic detection methods

There are assays for measuring circulating antigens, both in serum, urine, saliva and milk (Table 6). Two proteoglycans used, CAA and CCA (Deelder *et al.*, 1976), have been partially characterized in their chemical structure (see section “Schistosome glycans...”). The advantage with an antigen detection assay is to prove an ongoing infection and it also gives the possibility to follow treatment effects. The sensitivity of detection methods used (IHA, ELISA, WB) as similar to that of morphological methods (de Jonge *et al.*, 1988; Deelder *et al.*, 1989a) and not sensitive enough for diagnosing individuals with light infections (Doenhoff *et al.*, 2004; van Lieshout *et al.*, 2000).

Recently, PCR methods for detecting of schistosome DNA in faeces were developed (Gobert *et al.*, 2005; Pontes *et al.*, 2003).

**Table 6.** Assays for the diagnosis of schistosomiasis: Antigen/DNA detection

<i>Method</i>	<i>Antigen/DNA</i>	<i>Reference</i>
IHA	CAA (GASP) CCA (=M antigen) E/S	(Deelder <i>et al.</i> , 1989b) (Carrier <i>et al.</i> , 1978)
ELISA (sandwich)	CAA	(Gundersen <i>et al.</i> , 1992)
	31/32 kDa (worms)	(Li <i>et al.</i> , 1994b)
	70 kDa (worms)	(Fu and Carter, 1990)
	A repetitive carbohydrate epitope Sm	(Hassan <i>et al.</i> , 1992)
	26 kDa Sj GST (worms)	(Davern <i>et al.</i> , 1990)
	Sj (whole blood)	(Hirose <i>et al.</i> , 2005)
Reagent strip	CCA	(van Etten <i>et al.</i> , 1994)
TR-IFMA	CAA	(de Jonge <i>et al.</i> , 1989)
Lectin blot	Eggs	(Linder, 1986b)
PCR	<i>S. mansoni</i> DNA <i>S. japonicum</i> DNA	(Pontes <i>et al.</i> , 2003); (Gobert <i>et al.</i> , 2005)

### Diagnostic use of KLH in schistosomiasis

KLH has been used as antigen in the serodiagnosis of *S. mansoni* (Alves-Brito *et al.*, 1992; Mansour *et al.*, 1989; Rabello, 1992), *S. hematobium* (Xue *et al.*, 1993) and *S. japonicum* (Li *et al.*, 1994a). Demonstration of anti-KLH has been used mainly to differentiate acute from chronic schistosomiasis (Table 7). The methods used were ELISA dot-ELISA and dot-DIA. But a report by Verweij claimed that in a group of Dutch travellers, returning from Mali with acute schistosomiasis, the sensitivity in an anti-KLH ELISA was low. The authors suggested that egg deposition was a prerequisite for the anti-KLH response (Verweij *et al.*, 1995).

**Table 7.** Comparison of anti-KLH antibodies in patients with acute and chronic schistosomiasis

<i>Reference</i>	<i>Method</i>	<i>Anti KLH antibodies in acute schistosomiasis</i>		<i>Anti KLH antibodies in chronic schistosomiasis</i>	
(Mansour <i>et al.</i> , 1989)	ELISA	28/30	93%	0/30	0%
(Alves-Brito <i>et al.</i> , 1992)	ELISA	51/53	96%	5/180	3%
(Rabello <i>et al.</i> , 1993)	Dot-ELISA	22/25	88%	5/37	14%
	Dot-DIA	22/25	88%	6/25	24%
	ELISA	23/25	92%	4/37	12%
(Li <i>et al.</i> , 1994a)	ELISA	35/42 IgM	83%	0/40	0%
		41/42 IgG	98%	1/40	2%
(Verweij <i>et al.</i> , 1995)	ELISA	20/27 IgM	74%		
		17/27 IgG	63%		

### **KLH**

Hemocyanin is a high molecular weight respiratory metalloprotein in the hemolymph of many mollusks (including *Biomphalaria glabrata*) and crustaceans. KLH is isolated from the hemolymph of the giant keyhole limpet *Megathura crenulata* in large-scale production. It possesses remarkable immunostimulatory properties in man and animals (Curtis *et al.*, 1970; Herscowitz *et al.*, 1972) and is one of the most commonly used carriers for haptens. It is also used as an immunotherapeutic agent in bladder carcinoma (Wishahi *et al.*, 1995) (Jurincic-Winkler *et al.*, 2000; Lamm *et al.*, 2000).

The carbohydrate content has been estimated to 4% (Harris and Markl, 1999). There are two subunits of KLH (KLH1 and KLH2). Mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine are present in both subunits, while fucose just exists in KLH2. By using lectins, the saccharide components and carbohydrate-peptide linkages of KLH were shown to be N-linked in several functional units in KLH1 and KLH2. One functional unit in KLH2 binds to PNA, but not to ACA, indicating O-linked  $\beta$ -anomer of Gal( $\beta$ 1 $\rightarrow$ 3)GalNAc- (Stoeva *et al.*, 1999).

### **Common epitopes in KLH and schistosomes**

In 1985 Rivera-Marrero *et al* showed that the mollusk *Biomphalaria glabrata* and *S. mansoni* shared antigens when human immune serum precipitated with a hepatopancreas antigen extract from *B. glabrata* (Rivera-Marrero and Hillyer, 1985). The antigenic identity between *B. glabrata* and *S. mansoni* was shown by competitive inhibition to be associated with a strongly immunogenic 38 kDa surface carbohydrate component of schistosomula (Dissous *et al.*, 1986). In an immunization experiment to

evaluate the protective role of this 38 kDa antigen, the control animals, which were injected with the hapten carrier KLH, developed antibodies against a 38 kDa antigen in *S. mansoni* schistosomula (Grzych *et al.*, 1987). As a consequence of this finding, KLH was used as antigen in ELISA and dot-ELISA to measure antibodies in schistosomiasis patients (Alves-Brito *et al.*, 1992; Li *et al.*, 1994a; Mansour *et al.*, 1989; Markl *et al.*, 1991; Rabello *et al.*, 1993; Verweij *et al.*, 1995; Xue *et al.*, 1993; Yi *et al.*, 1991) (Table 6).

One immunodominant epitope present on *S. mansoni* glycolipids, responsible for the cross-reactivity of *S. mansoni* and *S. hematobium* infection sera with KLH has been characterized as a terminal fucose residue linked in the ( $\alpha 1 \rightarrow 3$ ) position to N-acetylgalactosamine [Fuc( $\alpha 1 \rightarrow 3$ )GalNAc-] (Kantelhardt *et al.*, 2002a). Recently, detailed structural analysis of the KLH carbohydrate entities responsible for cross-reactivity with glycoconjugates from *S. mansoni*, using a hyperimmune rabbit anti-SEA serum, showed novel types of N-glycans with a Fuc( $\alpha \rightarrow 3$ )GalNAc( $\beta 1 \rightarrow 4$ )[Fuc( $\alpha 1 \rightarrow 3$ )]GlcNAc motif (Geyer *et al.*, 2005).

### **Schistosomiasis control**

WHO has recently initiated a massive treatment campaign in the South parts of Africa (Chitsulo *et al.*, 2000). Despite many successful control programs taking place during the last 20 years, the distribution of the disease is constant or increasing. People movements, increasing population, sanitary problem and newly built irrigation plants and dams result in new regions of schistosomiasis (Crompton *et al.*, 2003; Fenwick *et al.*, 2003).

In theory schistosomiasis could be eradicated if excreted egg were prevented from reaching fresh water harbouring the intermediate snail host. This is probably the case even if man is not the only definitive host. Thus education, installation of latrines, improvement of water supply, biological control of snails are, in combination with treatment, important control methods.

### **Chemotherapy**

Praziquantel is the drug of choice for treatment (Cioli and Pica-Mattocchia, 2003) and morbidity control (Colley and Evan Secor, 2004) of schistosomiasis. It acts on all human species of *Schistosoma* (James *et al.*, 1977; Webbe and James, 1977) but is stage specific: cercariae, young schistosomula and adult worms are susceptible, but juvenile schistosomes are not (Andrews, 1985; Gonnert and Andrews, 1977; Sabah *et*

*al.*, 1986; Xiao *et al.*, 1987). The effects of praziquantel are associated with Ca<sup>2+</sup> influx into the worm (Pax *et al.*, 1978) that leads to strong muscular contraction and tegumental damage (Becker *et al.*, 1981; Mehlhorn *et al.*, 1981). As a consequence of the tegumental injury, schistosome antigens are exposed at the surface (Harnett and Kusel, 1986), and, in the mouse model, it has been shown that host immune response/antibodies are necessary to accomplish the effect of praziquantel (Brindley *et al.*, 1989; Doenhoff *et al.*, 1987). The cure rate of praziquantel was better in mice with heavy as compared to mild infection (Nessim and Demerdash, 2000).

The exact mechanism of action of praziquantel is not known, but a recent report claimed that schistosome calcium channels might be the molecular targets of praziquantel (Kohn *et al.*, 2001; Kohn *et al.*, 2003).

Derivates of artemisinin (arteether, artemether, artesunate, dihydroartemisinin), better known as anti-malarial drugs, also have anti-schistosomal properties. Artemisinin kills schistosomula (1 to 3 weeks old liver stages). Several clinical trials with artemether showed its efficacy in reducing the incidence and intensity of infection (N'Goran *et al.*, 2003; Utzinger *et al.*, 2000; Xiao *et al.*, 2000). The risk of inducing resistance in malaria-parasites has limited the use of this drug to malaria-free regions.

Oxamniquine is an alternative drug, but it is only effective on cercariae, young schistosomula and adult worms of *S. mansoni* (Cioli *et al.*, 1995; Katz, 1980) and its use is declining (Chitsulo *et al.*, 2000).

### **Resistance**

Reports from Egypt (1992) (Ismail *et al.*, 1999; Ismail *et al.*, 1996; Ismail *et al.*, 2002) and Senegal (1997) (Gryseels *et al.*, 1994; Stelma *et al.*, 1995) on suspected resistance (or tolerance) to praziquantel gathered the world's expertise of the issue. Fortunately Egypt some years ago reported no more signs of resistant worms and that all patients were susceptible to treatment. In Senegal the treatment failure could partly be explained by the extreme high load of egg excretion before treatment or due to re-infection (Gryseels *et al.*, 2001). The possibility of resistance has lead to more intense work to develop alternative drugs, especially new variants of praziquantel och artemesinin (Doenhoff *et al.*, 2002; Fenwick *et al.*, 2003).

### ***Treatment failure in travellers***

Several reports on treatment failure in returning travellers with *S. hematobium* have been recorded (Alonso *et al.*, 2006; Lawn *et al.*, 2003; Wolfe, 2003). Some authors think that the standard dose (40 mg per kg) may be insufficient and observed that



treatment had to be repeated for three consecutive days (40 mg/kg/day) to clear the infection (Alonso *et al.*, 2006; Lawn *et al.*, 2003; Wolfe, 2003).

### ***Vaccination***

So far no vaccine against schistosomiasis is available, but several candidates like Sm97 paramyosin, Sh28-gluthathione S-transferase (GST) and Sm28-triose phosphate isomerase (TPI) (MAP4) are under investigation. Sh28-GST which appeared to be a prostaglandin D2 synthase produced by cercariae, become ready for phase III vaccination trial (Bergquist *et al.*, 2002). Prostaglandin D2 inhibits the migration of Langerhans cells to the lymph nodes (Angeli *et al.*, 2001).

## **SCHISTOSOME GLYCANS OF DIAGNOSTIC INTEREST**

Schistosomes are rich in membrane-bound and circulating fucosylated carbohydrate structures, but like other helminths, lack sialic acid and sialyltransferase. Detailed structures of schistosomal glycoconjugates have been defined (Cummings and Nyame, 1999; Nyame *et al.*, 2004) and some schistosome glycans of diagnostic importance include Lewis x (Le<sup>x</sup>) antigen, LacdiNAc (LDN), fucosylated LacdiNAc (FLDN, LDNF) and LDN-DF (Kantelhardt *et al.*, 2002b; Ko *et al.*, 1990; Naus *et al.*, 2003; Srivatsan *et al.*, 1992a; Srivatsan *et al.*, 1992b; van Remoortere *et al.*, 2000; Wuhler *et al.*, 1999). Le<sup>x</sup>, a common mammalian leucocyte marker (CD15), is present in oligosaccharides on many membrane-bound glycoproteins of the adult schistosomes and on CCA (van Dam *et al.*, 1994). Among helminths only schistosomes synthesize Le<sup>x</sup> antigen.

The circulating anodic antigen (CAA) (Deelder *et al.*, 1976) is a negatively charged glycoprotein. The carbohydrate chains consist of multiple disaccharide units of N-acetyl-galactosamine and glucuronic acid (Bergwerff *et al.*, 1994).

# AIMS

## GENERAL AIM

Study the basis for using KLH as a diagnostic antigen in schistosomiasis and evaluate the potential for KLH in diagnostics in comparison with currently used routine method at the Swedish Institute for Infectious Disease Control (SMI) \*.

## SPECIFIC AIMS

- ② Show the expression of KLH cross-reacting immunogens in the different life stages of the parasite.
- ② Identify SDS-PAGE separated parasite eggs (SEA) components carrying KLH-cross-reactive epitopes.
- ② Identify the possible basis for immunogenicity of parasite components cross-reacting with KLH in the infected host.
- ② Determine the relative sensitivity of anti-KLH antibody detection in cases of early schistosomiasis.

## Ethical considerations

The experimental infection has been performed adhering to ethical considerations as stated in the Swedish Animal Welfare Agency.

The patient material was studied as part of a methods development program aiming at improved serological diagnostics of invasive worm infections.

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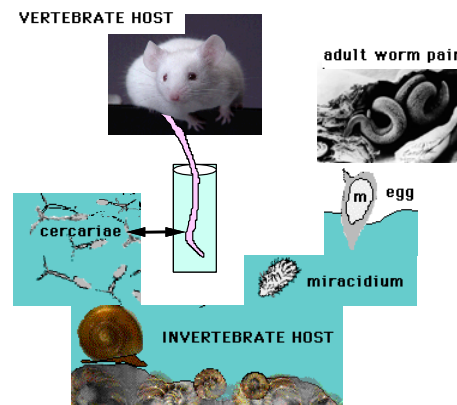
\* SMI functions as a reference laboratory for Sweden and its neighbouring countries Norway and Finland

# MATERIALS AND METHODS

## THE PARASITE

### *Schistosoma mansoni* infection in mice

*S. mansoni* (Puerto Rican strain) was maintained in NMRI mice. Eight-week-old mice were infected percutaneously with about 100 *S. mansoni* cercariae. After 8-10 weeks of infection the mice were sacrificed and intravascular worms were recovered in RPMI-1640 medium (Sigma) supplemented with 2% PEST (penicillin 10000 IU/ml, streptomycin 10 mg/ml, Sigma) and heparin (1000 IE/ml) (Smithers and Terry, 1965). The livers were removed and put into in 0.9% NaCl and kept in +4°C over night. To release the eggs the livers were mashed through a net and the cold physiological medium was gradually changed to room tempered water. When the eggs had hatched, the miracidia were picked with a Pasteur pipette and counted and 8 to 10 of them were allowed to infect one *Biomphalaria glabrata* snail. The infected snails were kept in aquarium for 4 weeks before shedding cercariae.



## ANTIGENS

### Adult *S. mansoni* worms

#### *Frozen worms*

The worms were washed three times in 0.9% NaCl after recovery from mice and immediately frozen in a gelatin capsule with Tissuetek™ in liquid nitrogen. They were kept at -80°C until cut in 5 µm sections and placed on glass slides. Before use, the antigen slides were fixed in acetone for 10 minutes.

#### *Paraformaldehyde treated worms*

The worms were fixed in 4% paraformaldehyde, washed in phosphate buffered saline pH 7.4 (PBS) before embedded in paraffin and sectioned. After deparaffination, IFA was performed.

#### *Worms in liver tissue*

Both frozen and paraffin sections of liver tissue containing worms were used in IFA.

### Cercariae

1. Live cercariae were added as aliquots of 10 µl on microscope slides coated with human skin lipid, dried at RT and fixed in acetone for 10 minutes or
2. Cercariae were fixed in 4% paraformaldehyde and washed three times in PBS before use in IFA.

## Schistosomula

Schistosomula were prepared as described earlier (James and Taylor, 1976). Briefly, cercariae were passed 14 times through a 20 gauge needle, put at 37°C for 3 hours until the tails are dropped.

## Eggs and Miracidia

Isolated eggs were allowed to hatch in water, dried on glass slides and then fixed in acetone.

Frozen and paraffin sections of liver tissue with egg granulomas were used in IFA.

## Soluble antigens

### *SEA*

Schistosome egg antigens (SEA) are complex host-immunogenic glycoproteins, consisting of at least 30 components with apparent molecular masses ranging from 10 to >200 kDa (Carter and Colley, 1978). Eggs were prepared from mouse livers (Smithers, 1960). After trypsin treatment, passing through sieves and washing steps the eggs were put in PBS-Dulbecco pH 7.3 with 0.1 mM PMSF/ml, homogenized on ice and ultracentrifugated (100000 g) for 90 minutes at 4°C. The supernatant was dialyzed in 0,1 mol/L Tris/HCl pH 8,9 over night and centrifuged at 1000 g. The protein concentration was measured by the BCA (bicinchonimic acid) method (Pierce 23225) and supernatant kept at -80°C until used.

### *KLH*

The commercially available KLH (H-7017) was purchased from Sigma, St. Louis, MO. In dot-ELISA each dot consisted of 20 µg of KLH and in WB each gel contained 20 µg of KLH.

### *Trichinella* antigen

Sections of *T. spiralis* larvae (Ruitenbergh *et al.*, 1975) were used in IFA. (Paper I)

## ANTIBODIES

### Patient sera

Sera from 198 individuals, who traveled to endemic areas in Africa, were examined for suspected schistosomiasis at the diagnostic laboratory of SMI, during 1996 and 1997. For comparison between anti-duct reactions in IFA and anti-KLH reactions in dot-ELISA, 44 patient sera with antibodies against GAA or both GAA and SA and 9 sera from patients with suspected schistosomiasis but without anti-GAA and anti-SA antibodies were tested. Ten sera from patients with trichinellosis, serum from a chronically *S. hematobium* infected patient and 34 sera from hospital patients sent for clinical chemistry examinations, were used as controls. (Paper I)

Sera from 83 Swedish adventure tourists, traveling in endemic areas in Africa for 1 to 2 months, were examined for suspected schistosomiasis at the diagnostic laboratory of SMI during 1997 and 2002. After treatment with praziquantel consecutive sera were available from 24 out of 42 patients, who tested positive for anti-schistosome antibodies. (Paper III)

In IFA sera were tested in 3-fold serial dilutions starting at 1:10. The serum dilution in the dot-ELISA was 1:100. (Paper I)

The “antibody profiles” (antibodies against GAA, SA in IFA and SEA in ELISA) in the two groups of schistosomiasis patients differ. This probably reflects the heterogenicity of patient material in paper I (see Results and discussion, Figures 5 and 6 left).

#### Absorption of serum specimens with KLH

To each dilution of serum 1 mg/ml (Paper I) or 5 mg/ml of KLH (Paper III) was added for 1 hour at RT followed by +4°C over night.

#### Rabbit sera

For the localization of anti-KLH-reactive epitopes in the different life stages of *S. mansoni*, commercially available rabbit anti-KLH antibodies (H-0892, Sigma, St. Louis, MO) were used in a 1:40 dilution for IFA staining. Rabbit anti-KLH and rabbit anti-SEA antibodies (raised by immunisation with three monthly doses of 0.1 and 1 mg of SEA) were used in a 1:100 dilution for WB. (Paper II). In the KLH dot-ELISA, rabbit anti-KLH antibodies, diluted 1:100, were used as control (Paper I).

#### Mouse sera

Anti-*S. mansoni* gp50 monoclonal antibodies (Linder *et al.*, 1991) were used as control in IFA. (Paper I). A mouse monoclonal antibody against paramyosin (Pearce *et al.*, 1988) was used as a marker of intact parasite tegument. (Paper II)

### **ANTI-IG CONJUGATES & LECTIN CONJUGATES**

In IFA, FITC (SBL SH8039301-1, Sweden) and TRITC (Dakopatts AB, Denmark R320) conjugated sheep anti human Ig antibodies were used in dilutions 1:10 and 1:30.

In dot-ELISA horseradish peroxidase (HRP) conjugated anti-human Ig antibodies (SBL mol HRP/Ig 2.6) and anti-rabbit Ig antibodies (Amersham NA9340) were used in dilutions 1:10000 and 1:1000 respectively. The same dilution of the Amersham conjugate was used in WB.

Fluorescein isothiocyanate (FITC)-labeled *Arachis hypogaea lectin* (PNA) (Sigma L-7381), specific for terminal  $\beta$ -D-galactose residues (Goldstein and Poretz, 1986), was used as reference to show parasite surface carbohydrates, cercarial “kissing marks” and perioval hatch fluid material as described previously (Linder, 1986a; Linder *et al.*, 1991).

**Table 8.** Antigens, antibodies and conjugates used in IFA, dot-ELISA and WB

<b>Assay</b>	<b>Antigen</b>	<b>Antibody</b>	<b>Conjugate/Lectin</b>
IFA	Worms	Patient sera*	Anti-human Ig-FITC
			Anti-human Ig-TRITC
		Rabbit anti-KLH	Anti-rabbit-Ig-FITC
		Mouse anti-gp50	Anti-mouse Ig-FITC
	Cercaria		PNA-FITC
		Rabbit anti-KLH	Anti-rabbit-Ig-FITC
	Schistosomula	Mouse anti-paramyosin	Anti-mouse Ig-FITC
		Rabbit anti-KLH	Anti-rabbit-Ig-FITC
	Eggs and Miracidia		
	Eggs in liver tissue		
Worms in liver tissue			
	<i>Trichinella</i> larvae	Human anti-trichinella	Anti-human Ig-FITC
Dot-ELISA	KLH	Patient sera*	Anti-human Ig-HRP
		Rabbit anti-KLH	Anti-rabbit Ig-HRP
WB	KLH	Rabbit anti-KLH	
		Rabbit anti-SEA	
SEA	Rabbit anti-KLH		
	Rabbit anti-SEA		

\* See Materials and Methods, Antibodies

## INDIRECT IMMUNOFLOUORESCENCE ASSAY (IFA)

The IFA was carried out on different *S. mansoni* life stages and *Trichinella* larvae attached to glass slides (see section “Antigens” and Table 8). After fixation in acetone for 10 minutes or deparaffination, the antigen slides were incubated with antibodies for 30 minutes, washed 3 times 5 minutes in PBS and then incubated with the corresponding conjugate for 30 minutes. After repeated washing steps in PBS the slides were mounted in Vectashield mounting media (Vector Laboratories, H-1000). Lectin conjugate was diluted in PBS-Dulbecco and polyvinylalcohol was used as mounting medium for lectin stained preparations. All incubations were done in a humid chamber at RT.

### Deglycosylation

For deglycosylation (Paper III) the antigen slides were treated with 20 mM sodium periodate in 0.1 M sodium acetate buffer pH 4.5, for 1.5 hour in the dark. The reaction was stopped, by adding equal amount of 50 mM sodium borohydride for 30 minutes.

### Microscopy & photography

The microscopy was performed using a Leica DMRB fluorescence microscope equipped with filter for FITC.

For photography and image processing an AxioCam digital camera (Carl Zeiss, Oberkochen, Germany), Openlab software (Improvision, Coventry, UK) and Photoshop 6 (Adobe, San Jose, CA) were used.

## **DOT-ELISA**

In dot-ELISA the antigen (KLH), diluted in PBS, was applied to nitrocellulose membrane by using a microfiltration apparatus from BioRad laboratories (170-6545, Hercules, CA) according to the instruction manual. After washing in PBS and blocking with 5% non-fat milk, the serum specimens were tested in a dilution of 1:100 in PBS, containing 0.1% Tween 20 (PBS-T). Following three washes in PBS-T, the conjugates were added (see section: “Anti-Ig conjugates...”). After three additional washes the membrane was removed from the apparatus and placed in a vessel with PBS to remove excess of Tween 20 and the substrate and chromogen solution (0.0125% H<sub>2</sub>O<sub>2</sub> and 0.5 mg/ml 4-chloro-1-naphthol in triethanolamine-buffered saline, pH 7.5) were added. The reaction was stopped after approximately 10 minutes by immersing the membrane in distilled water. The colour reactivity was monitored virtually using three gradings of positive reactions. All steps were done at RT.

## **PAGE-SDS AND WESTERN BLOTTING (WB)**

The antigens (SEA and KLH) were separated on 10.6% SDS-PAGE with a 3% stacking gel under reducing or non-reducing conditions and transferred to nitrocellulose membranes (Laemmli and Favre, 1973). The membranes were cut to strips and blocked in PBS with 5% non-fat milk for 30 minutes. The serum specimens (see section: “Antibodies”) were diluted in PBS-T with 1% bovine serum albumin (BSA) and incubated with the strips for 1 hour. Following three washings in PBS-T-BSA, the conjugate was added (see section: “Anti-Ig conjugates...”) for 1 hour. The substrate/chromogen solution (see section “dot-ELISA”) was added after three washings in PBS-T-BSA. The reaction was stopped after approximately 10 minutes by immersing the strips in distilled water. Molecular weight standards were from BioRad (161-0324, Hercules, CA).

### **Deglycosylation**

The nitrocellulose strips, containing KLH or SEA, were equilibrated for 30 minutes at RT in 0.1 M Na-acetate buffer, pH 4.5, before periodate treatment. Incubation with 20 mM Na-periodate in 0.1 M Na-acetate buffer, was performed over night in the dark at +4°C and followed by washing in 0.1 M Na-acetate buffer. The strips were incubated in 50 mM Na-borohydride in PBS for 30 minutes and subsequently washed in PBS-T-BSA (Woodward *et al.*, 1985).

## RESULTS AND DISCUSSION

This study on KLH as antigen in the serological diagnostics of schistosomiasis was motivated by:

- The observation of a previously unrecognised IFA staining pattern obtained with patient sera suspected for schistosomiasis using sections of adult worms as antigen
- A similar IFA staining pattern seen on sections of adult worms with anti-KLH antibodies
- Discrepant results reported in the literature regarding the usefulness of KLH as antigen in serodiagnostic assays for acute schistosomiasis

Diagnostics for schistosomiasis in travellers and immigrants, into an endemic area, relies on serology in the absence of sufficiently sensitive morphological “parasitological” diagnostic methods. Serology, using adult schistosomes as antigen in an IFA, to detect anti-GAA antibodies, is regarded as highly sensitive and specific. The observed novel “duct” staining pattern resembles the diagnostic GAA pattern (Figures 4a and b). We found that the two staining patterns usually occur together in most of the studied schistosomiasis patient sera. This circumstance and the morphological similarity between GAA and duct (both patterns located to tubular structures) explains why it has not been previously described. The duct staining pattern – without any GAA reactivity – was seen with sera from trichinellosis patients (Figure 4c). The duct staining pattern, seen with patient sera, was indistinguishable from the reaction seen with rabbit anti-KLH antibodies. These observations formed the basis for subsequent studies aiming at understanding why anti-KLH cross-reactive antibodies are seen in schistosomiasis patients.

### EXPRESSION OF KLH-CROSS-REACTIVE IMMUNOGENS IN THE SCHISTOSOME

By IFA, commercial rabbit anti-KLH antibodies localised KLH cross-reacting antigens in different life stages of the schistosome: the secretions produced by the cercaria upon attachment, at the surface of cercaria and schistosomula, in E/S products of the adult worms, in reproductive tissues of both male and female worms, at the surface of the miracidia, around eggs in granulomas, and in Kupffer cells.



KLH-cross-reactive immunogens occur in all schistosome life stages and apparently in secreted products. Thus it is likely that such E/S products should be readily recognized by the host immune system. The cross-reactive epitopes appear to be carbohydrates based on the capacity of periodate to abolish the binding of antibodies, both in IFA and WB (Paper II, Figures 4j, 5 and unpublished observations).

## **HETEROGENICITY OF ANTIBODIES REACTING WITH KLH AND WITH SCHISTOSOME DUCTS**

The observed reaction of anti-KLH antibodies with ducts raised the question: To what extent is the observed duct-reactivity in patient sera due to antibodies cross-reacting with KLH?

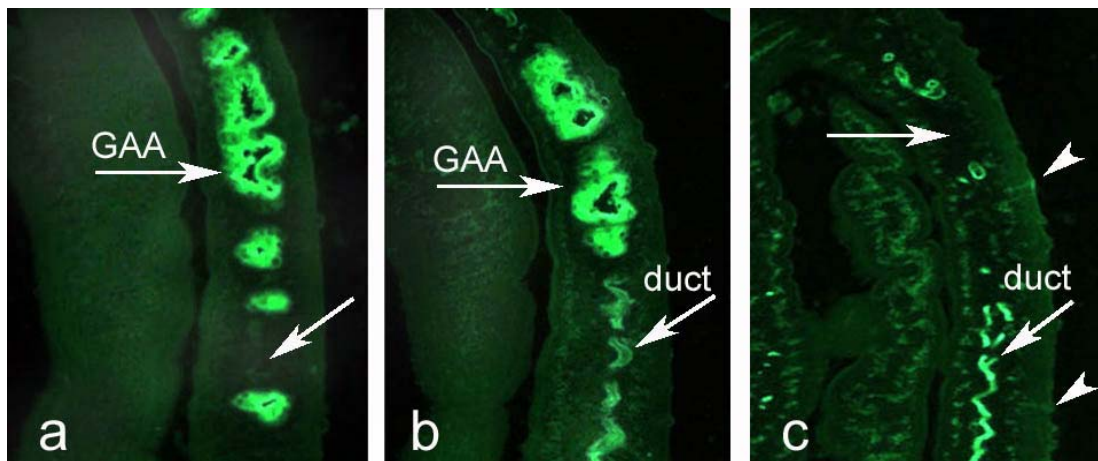
Two possibilities exist: anti-duct antibodies may or may not react with KLH. Also the reverse is possible: anti-KLH antibodies may not react with ducts. These questions were approached in two ways: prevalence data and absorption experiments using KLH to abolish anti-duct antibody reactivity.

Prevalence data showed that out of 51 sera, 15 had only anti-duct antibodies (in IFA), 13 had only anti-KLH antibodies (in dot-ELISA), and 23 had both duct and KLH antibodies. This suggests that in addition to common antigens, there seem to be duct specific, non-KLH-cross-reactive antigens.

Absorption of sera with KLH (paper I) was done to find out to what extent the duct-reactivity is due to antibodies cross-reacting with KLH. The reactivity against duct in all (6/6) trichinellosis patient sera and 3 out of 6 sera of unknown origin disappeared after absorption. This is consistent with results of KLH absorption experiments performed in Paper III. Pooled duct-reactive sera lost their ability to stain ducts after absorption with KLH. However, 7 out of 9 sera from patients with schistosomiasis were unaffected by KLH absorption (Paper I). The absorption experiments also support the conclusion that duct reactive antibodies may be both duct specific and KLH cross-reacting.

Our results further suggest that some anti-KLH antibodies may not react with schistosomes. The presence of anti-KLH antibodies in control sera, seen in dot-ELISA, is an important finding which restricts the use of this method for diagnostic purposes. The poor specificity of dot-ELISA, as a diagnostic method for schistosomiasis, may be due to antibodies induced by other helminths such as *Trichinella*, but hemocyanins, similar to KLH, are present in mollusks and cross-reacting carbohydrate antigens may occur even in plants (Hoglund *et al.*, 2002). Thus individuals may generate anti-KLH

antibodies by ingesting KLH-cross-reactive antigens present in food. Such antibodies, however, do not necessarily react with schistosome ducts, as 13 out of 36 sera, positive in KLH dot-ELISA, were duct negative (Paper I). Apparently the dot-ELISA detects antibodies directed against various different antigenic epitopes present on KLH. This is consistent with our observation made by WB (Paper II) and the known complexity of the KLH molecule (Geyer *et al.*, 2005; Harris and Markl, 1999). Furthermore, our recent observation of anti-Thomsen-Friedenreich (TF) antibodies in experimental *S. mansoni*-infected mice is an example of anti-duct reactive antibodies, which show some reactivity with KLH (Dahlenborg *et al.*, 1997; Geyer *et al.*, 2005; Thors *et al.*, 2006a).



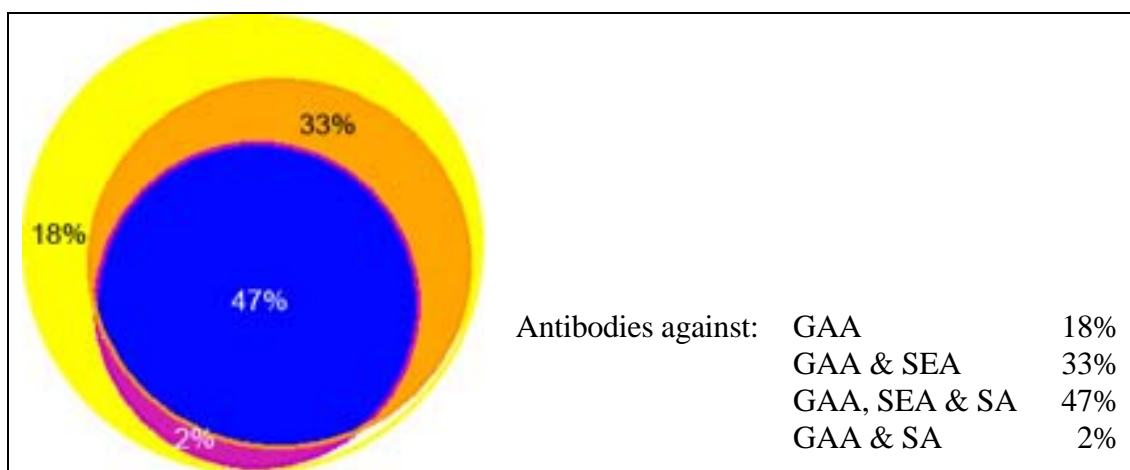
**Figure 4.** Gut and duct: Two distinct staining patterns in IFA on parallel sections of adult worms. a) anti-GAA antibodies; b) anti-GAA antibodies and anti-duct antibodies; c) anti-*Trichinella* antibodies (anti-duct antibodies), Arrow-heads suggest delicate connection of the duct system to the surface at tubercles.

## ANTI-DUCT REACTIVITY IN SCHISTOSOMIASIS PATIENTS

The proportion of schistosomiasis patient sera (defined as anti-GAA positive) reacting with ducts varied from about half (Paper I) to 95% (Paper III). The reason for this difference is not evident, but could be explained by data suggesting that antibodies responsible for the two distinct patterns (GAA and duct) have different kinetics. It appears that both antibodies occur early in the infection, as seen in the one case, where sero-conversion was demonstrated for both anti-GAA and anti-duct antibodies, whereas it is possible that anti-GAA antibodies remain after anti-duct antibodies have declined. This possibility is supported by published report showing that anti-KLH antibodies decline 6 months after treatment (Li *et al.*, 1994a), but Verweij could not see such decline after 1 year (Verweij *et al.*, 1995). This discrepant results showing that a

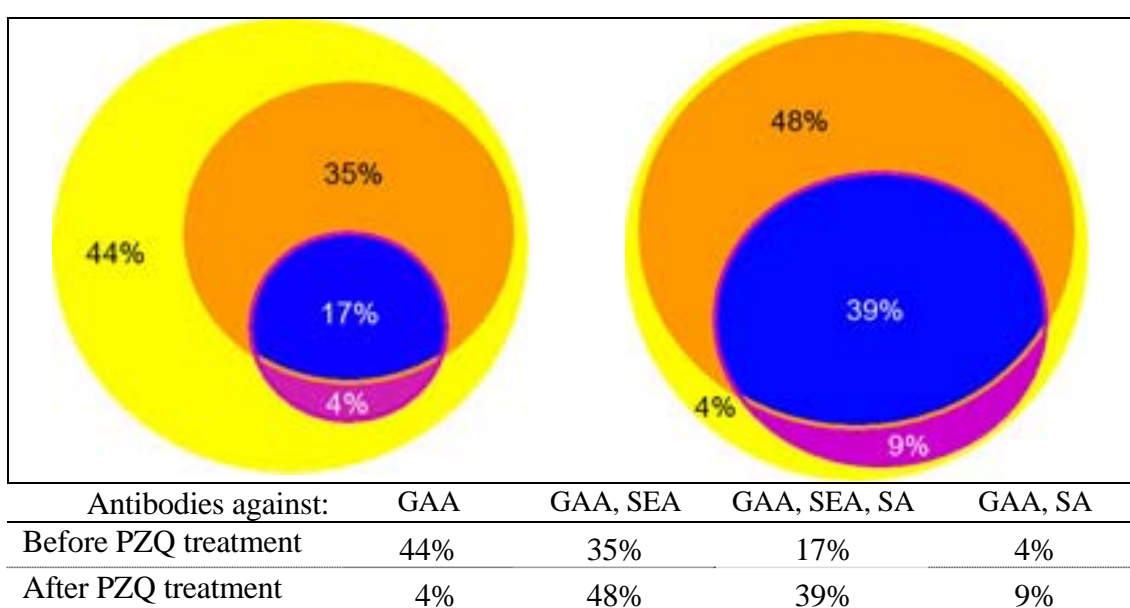
decline in anti-KLH antibodies after treatment may or may not be demonstrable stress the importance of developing assays using well defined target antigens. There are indications however, that the immune response changes with time and this may be an effect of treatment.

The “antibody profile” in schistosomiasis patient sera, described in paper I (Figure 5), differs from the “antibody profile” in patient sera tested in paper III (Figure 6 left), suggesting that the patient group in paper I is more heterogenous regarding to stage of infection.



**Figure 5.** Antibody “profile” against different schistosome antigens in schistosomiasis patient sera (Paper I).

The “antibody profile” in schistosomiasis patient sera changed after PZQ treatment. The proportion of patients with only anti-GAA antibodies decreased (from 44% to 4%), while antibodies against SA and SEA (in ELISA) increased (Figure 6).



**Figure 6.** Antibody “profile” against different schistosome antigens in schistosomiasis patient sera (24) before and after treatment with praziquantel (Paper III)

## UNRESOLVED QUESTIONS

The low specificity of anti-KLH dot-ELISA may not rule out the use of a KLH based screening test for schistosomiasis in travellers and adventure tourists. However, additional information is needed in order to answer the following questions:

1. Are “false positive” anti-KLH antibodies in health control sera directed against different epitopes (e.g. protein epitopes) than anti-duct antibodies present in schistosomiasis patients – and trichinellosis patients?
2. Can a modified anti-KLH antibody assay be designed, which takes into consideration the complexity of the KLH molecule and the expression of multiple antigenic epitopes?
3. What proportion of duct-reactive sera in paper III contains cross-reactive anti-KLH antibodies? What is the specificity of KLH-non-cross-reactive anti-duct antibodies?
4. Can the “false negative” anti-KLH results among schistosomiasis patients be due to a more rapid decline in the KLH-cross-reactive antibodies as compared to anti-GAA antibodies?

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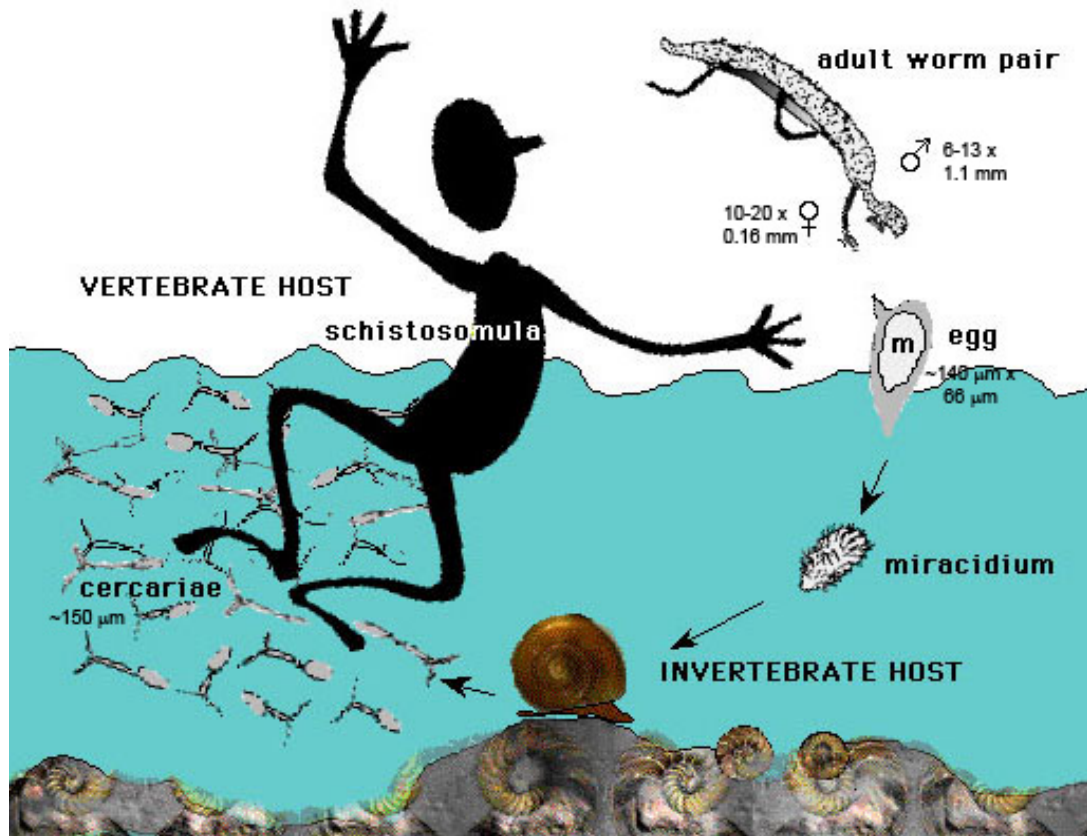
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## The life cycle of *Schistosoma mansoni*



Life cycle tape



The infection of the definitive host occurs by penetration of the skin by cercariae. The cercariae transform to schistosomula, which leave the skin via the blood vessels and draining lymphatics and reach the lungs. Several days later the schistosomula leave the lungs and migrate to the hepatic portal system, where they mature into adult worms and pair up. After about 5 to 7 weeks eggs are discharged with faeces. In fresh water miracidia hatch from the eggs and penetrate the intermediate host – a snail. One miracidium develops into many sporocysts and, in about 4 weeks, thousands of cercariae are produced and released into the water.

