

Molecular diagnosis of low intensity *Schistosoma japonicum* infections

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by

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“When I read in the Renmin Ribao of June 30, 1958 that schistosomiasis had been wiped out in Yukiang County, thoughts thronged my mind and I could not sleep. In the warm morning breeze next day, as sunlight falls on my window, I look towards the distant southern sky and in my happiness pen the following lines.”

Mao Zedong, July 1, 1958

FAREWELL TO THE GOD OF PLAGUE

(to the tune of lüshi poems)

I

So many green streams and blue hills, but to what avail?
This tiny creature left even Hua To powerless!
Hundreds of villages choked with weeds, men wasted away;
Thousands of homes deserted, ghosts chanted mournfully.
Motionless, by earth I travel eighty thousand li a day,
Surveying the sky I see a myriad Milky Ways from afar.
Should the Cowherd ask tidings of the God of Plague,
Say the same grieves flow down the stream of time.

II

The spring wind blows amid profuse willow wands,
Six hundred million in this land all equal Yao and Shun.
Crimson rain swirls in waves under our will,
Green mountains turn to bridges at our wish.
Gleaming mattocks fall on the Five Ridges heaven-high;
Mighty arms move to rock the earth round the Triple River.
We ask the God of Plague: "Where are you bound?"
Paper barges aflame and candle-light illuminate the sky.

Explanatory notes:

God of Plague: from Taoism, which often explain disease through ghosts and spirits. Sending away the God of Plague is an important healing ritual with strong folk color, usually performed by setting fire to paper boats, as seen in the last line of the poem.

lüshi: formal form of Chinese poetry with eight lines per verse. Flourished in the Tang dynasty

Hua To (or Hua Tuo): legendary physician, allegedly living year 110-207 A.D.

li: traditional Chinese unit of distance, now standardized to 500 metres, 80 000 li equals the Earth's circumference or the distance a person can "travel" a day, standing motionless as the Earth rotates

cowherd: character in Chinese myths, married to Heaven Emperor's daughter, who weaves the cloud brocade and heaven clothes

Yao and Shun: emperors of China 2200-2300 years B.C., both glorified for their virtues. Yao chose the common man Shun as his successor instead of his sons

Five Ridges: places in Jiangxi, Hunan, Guangdong and Guangxi provinces

Triple River: ancient name for places in Shaanxi, Hebei and Henan provinces

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PREFACE

Schistosoma japonicum is the “Asian brother” in the group of clinically important *Schistosoma* worms that infects humans, having the lowest number of human cases. Hence it has not received the same amount of interest in the international medical literature as schistosomiasis in sub-Saharan Africa, which carries approximately 90% of the schistosomiasis burden. Many factors differ greatly between schistosomiasis in Africa and Asia, and some of them have been discussed in more detail in this thesis, like the predominance of low intensity infections and the animal reservoir in Asian schistosomiasis. There are other differences that are important when thinking about the feasibility of control measures and diagnostic strategies. This includes long lasting political commitment, an outstanding economical development and increased technological standard. The everyday life for many people in rural China has perhaps not changed dramatically over the last few decades, but for the country as a whole it has now come to the stage where they send people out in space. Measures that are unrealistic or unnecessary in Africa might be possible and wanted in Asia. The situation for *S. japonicum* has a resemblance to *S. mansoni* in South America, and indeed some of the thoughts on diagnostic strategies in this thesis have a background from that area.

Important topics like immunity, vaccines, use of ultrasound and choice of treatment have not been covered in this thesis. Even though they can have important consequences for diagnosis and infection control, I feel that they are beyond the scope of this thesis.

Diagnostic tests are used in many different settings. My primary focus has been diagnostic strategies in groups of people, be it as part of a control programme, for surveillance, epidemiological purpose or for research, more than diagnostics in individual patients in hospitals and clinics. This focus has perhaps not been clearly expressed in my papers. However, the results and discussions can also be of interest for the latter.

Some readers may accuse me of putting too much focus on China at the sacrifice of the Philippines in the introduction and discussion part of this thesis. There are several reasons for this imbalance. Firstly, information regarding the different aspects of the infection, including estimated numbers of human cases, is more easily accessible for China compared to the Philippines. The number of international papers dealing with epidemiology, diagnostic

strategies and discussion of control measures clearly goes in China's favour. The fact that my "field study" was done in China and the contact to my good Chinese colleagues contribute to this bias. However, the situation is very similar in the two countries and the results presented here are equally relevant to both.

Hope you enjoy the reading!

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I would start by thanking my crew of supervisors. Maria V Johansen has been my main anchor in questions regarding *S. japonicum*. She has been one of the “working horses”, deeply involved in planning, being among the first to read new drafts and always responding quickly to bigger and smaller requests. Her contagious enthusiasm convinced me that it had to be nothing else but *S. japonicum*. Gunnar S Simonsen has likewise been a horse, often doing the tedious work of going through early drafts and has in addition countless times given me a clear answer to general questions regarding the maze of research and PhD. He has also been my boss for most of my PhD-time, giving me more support and time than I could fairly demand. Hanne H Haukland has given me valuable scientific input and not the least pointed out linguistic vagueness in my manuscripts. She was also my boss for a period during this project. Birgitte J Vennervald, always supportive, gave useful contributions both to planning and in revising manuscripts and served as my second column in schistosomiasis matters.

I’m most grateful to my good colleagues at Department of Microbiology and Infection Control, who without complaining did all the work I should have done instead of writing a PhD. This includes Lars Vorland, my former boss, who tempted me to start on this PhD. Thanks to our “PCR specialists” Håkon Haaheim, Stig Ove Hjelmvoll and Joachim Hegstad, without whom I would have been in very deep waters. Thanks to Girum Tadesse for sedimentation technique microscopy.

Thank you to my colleagues at Department of Medical Biology for bearing with me the countless PCR runs and (with mild, curious disbelief) the spiking of stool samples with eggs.

Thanks to the staff at DBL- Centre for Health Research and Development, and especially Susanne Kronborg and Bente Larsen Jensen, for making me feel most welcome and for providing practical help and worms and eggs and other necessities.

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I would like to thank my friend Rasmus Goll for solid advice within PCR and epidemiology in addition to help reminding me of the other important things in life, like giant insects from outer space.

My wife Anne Mette Asfeldt has with her own PhD fresh from the press been a most useful discussion partner on topics like epidemiology, writing manuscripts and the formalities of a PhD. She has together with our boys, Aslak and Eskil, directly or subtly helped me putting the priorities in life straight when things like an ailing PCR run felt like a small catastrophe.

University Hospital of North Norway has most kindly provided most of the funds necessary.



Figure 1. Collection of samples, Anhui province, China (Paper three and four).

ABBREVIATIONS

The following abbreviations are commonly used in the thesis:

<i>S. japonicum:</i>	<i>Schistosoma japonicum</i>
<i>S. mansoni:</i>	<i>Schistosoma mansoni</i>
<i>S. haematobium:</i>	<i>Schistosoma haematobium</i>
PCR:	Polymerase chain reaction
ROSE:	Rapid one-step extraction
MGB probe:	Minor groove binding probe
FEC:	Formol-ethyl acetate sedimentation concentration
IHA:	Indirect hemagglutination assay
ELISA:	Enzyme-linked immunosorbent assay
SEA:	Soluble egg antigen
COPT:	Circumoval precipitin test
DDIA:	Dipstick dye immunoassay
CAA:	Circulating anodic antigen
CCA:	Circulating cathodic antigen
Nucleotides:	
A:	Adenine
C:	Cytosine
G:	Guanine
T:	Thymine
U:	Uracil
Mixed nucleotides, also known as degenerate or wobble nucleotides:	
R:	Either adenine or guanine (half the primers containing each)

LIST OF PAPERS

Paper I

Novel real-time PCR for detection of *Schistosoma japonicum* in stool.

Lier T, Simonsen GS, Haaheim H, Hjelmevoll SO, Vennervald BJ, Johansen MV. Southeast Asian J Trop Med Public Health 2006 Mar;37(2):257-64.

Paper II

Real-time PCR for detection of low intensity *Schistosoma japonicum* infections in a pig model.

Lier T, Johansen MV, Hjelmevoll SO, Vennervald BJ, Simonsen GS. Acta Trop 2008 Jan;105(1):74-80.

Paper III

Real-time PCR for detection of low intensity *Schistosoma japonicum* infections in China.

Lier T, Simonsen GS, Wang T, Lu D, Haukland HH, Vennervald BJ, Hegstad J, Johansen MV. Am J Trop Med Hyg 2009 Sep;81(3):428-32.

Paper IV

Low sensitivity of the formol-ethyl acetate sedimentation concentration technique in low-intensity *Schistosoma japonicum* infections.

Lier T, Simonsen GS, Wang T, Lu D, Haukland HH, Vennervald BJ, Johansen MV. PLoS Negl Trop Dis 2009;3(2):e386.

SUMMARY

Introduction:

Schistosoma japonicum is a parasitic fluke. The adult worms live as a pair in the small mesenteric blood vessels along the intestines. The eggs are excreted in the stool. It is endemic mainly in parts of China and the Philippines with the estimated prevalence in humans exceeding a million cases. Successful control programmes have run for several decades with the result that most remaining infections are of low intensity. This is a challenging diagnostic situation and has reduced the predictive values of the diagnostic tests commonly used so far. Antibody detection results in many false positive cases due to past infections and cross reactions with other infections. Kato Katz thick smear and hatching test, which both detect eggs in stool, miss a substantial number of the infected cases.

Aim:

Our aim for this project was to look for an alternative diagnostic strategy. Our emphasis was on real-time PCR, since this is a method with potentially high sensitivity and specificity.

Results:

The results of our investigations are presented in four papers:

1. In the first paper we developed and evaluated a PCR which targets the mitochondrial NADH dehydrogenase I gene from *S. japonicum*. SYBR Green was used for detection. We also compared different modifications of DNA extraction methods and found two methods to be equally efficient; the non-commercial ROSE extraction and the commercial QIAamp DNA Stool Mini Kit. The PCR had high sensitivity in stool samples artificially spiked with eggs, even in samples containing a single egg. The PCR was specific for *S. japonicum* when it was tested on different *Schistosoma* species and other worms commonly found in stool.
2. In Paper two we compared the PCR (using both extraction methods) with a sensitive stool microscopy test and antibody detection in an animal model using twelve pigs with a low intensity *S. japonicum* infection and three uninfected controls. PCR with either extraction method were equally sensitive as microscopy. However, both the faecal PCR and microscopy results were mostly negative when faecal egg output almost reached nil in the chronic last stage of the trial, despite persistent worm burdens. In this stage the PCR gave higher proportion positive samples than microscopy. Antibody titers remained high throughout the study. PCR was consistently negative in serum and urine samples.

3. In Paper three we used clinical samples from 1727 persons from Anhui province, China, to compare PCR with tests commonly used in China. We developed a new PCR which targeted the same gene as the PCR described in Paper one and two, now using ROSE extraction and a MGB probe for detection. Detection of antibodies in serum was done by IHA. Kato-Katz thick smear microscopy, hatching test and PCR was done on the same stool sample. The prevalence was very high when IHA was used (26.1%). PCR resulted in a higher prevalence (5.3%) than hatching (3.2%) or Kato-Katz (3.0%). It was of some concern that most of the stool samples were only positive in one or two of the three stool based tests. Possible reasons for this disagreement are discussed. PCR displayed better agreement with IHA than the other two stool-based tests. A commonly used diagnostic algorithm with initial screening for antibodies and subsequent testing with Kato-Katz of the seropositive would have resulted in treatment of 22 people, compared to 50 people if PCR replaced Kato-Katz. We also showed that it is possible to do a cheap, non-commercial DNA extraction in a local laboratory with quite basic equipment and do the amplification itself in a larger laboratory.
4. Formol-ethyl acetate sedimentation concentration technique (FEC) is preferred by many clinical microbiology laboratories for the detection of parasites in stool samples, but there are no previously published results for *S. japonicum*. A sub-set of clinical samples from 106 Chinese persons were selected from the sample collection in Paper three. A person was considered positive by the 'reference standard' if antibody detection (IHA) was positive together with Kato-Katz positive and/or hatching test positive. This reference standard resulted in a disappointingly low FEC sensitivity of 28.6% and a specificity of 97.4%.

Conclusion:

PCR seems to be a diagnostic alternative with high sensitivity and specificity and could have a role in a diagnostic algorithm. However, it is still an expensive alternative.

CHAPTER 1 GENERAL INTRODUCTION

1.1. Introduction to *S. japonicum*

Human schistosomiasis, earlier known as bilharziasis, is a parasitic disease caused by blood-dwelling flukes (flatworms, trematodes) of the genus *Schistosoma*. '*Schistosoma*' is derived from the Greek 'skhistos' for split or cleft and 'soma' for body, and it refers to the groove in the adult male's body which holds the female [1]. In contrast to other trematode parasite species, which are hermaphroditic, the schistosome worms have separate male and female sexes. The three major species in humans are *S. haematobium*, *S. mansoni* and *S. japonicum*. The first species causes urinary schistosomiasis and the two latter intestinal schistosomiasis [2]. *Schistosoma* uses freshwater snails as an intermediate host and humans are infected in freshwater by skin-penetrating larvae. The adult *S. japonicum* worms inhabit the small venules along the intestines, in the mesenteric venous system, and the eggs are excreted in the stool [3].

Schistosoma japonicum differ from the other *Schistosoma* in humans by being a zoonosis. In addition to humans, a large number of mammals can act as a definite host.

Eggs of *S. japonicum* have been identified in two ancient corpses, both more than 2000 years old, from two different provinces in central China. Descriptions resembling acute schistosomiasis can be found in old volumes of traditional Chinese medicine dating back to 400 B.C. The first reported diagnosed case in modern China was by the American physician Logan in 1905 [4;5] and by the American scientist Wooley in the Philippines in 1906 [6]. At the time of the founding of the People's Republic of China in 1949 there were 10-12 million cases, and the effect on public health was devastating in the endemic areas. The new Chinese government was very concerned about the situation (see "Farwell to the God of Plague" by Mao Zedong) and soon launched a huge and still ongoing control programme.

Schistosoma japonicum was once endemic in Japan, with the last new human case found in 1977. In 1847 a local practitioner of Chinese medicine in Katayama District in Japan, Yoshinao Fujii, described in his "Katayama memoirs" quite accurately the cercarial dermatitis and the acute and chronic symptoms of a curious endemic disease. The disease was well known since the old times by the local farmers, and was called Katayama disease after the local Kata Yama ('mountain') that rise out of the rice fields. The serum sickness-like syndrome of acute schistosomiasis is still named Katayama fever. The hunt for the causative agent was close around the turn of the century in Japan, but the credit for discovering the new

fluke species has been attributed to Fujiro Katsurada, who described the adult worms from a dead cat in 1904 and proposed the name *Schistosomum japonicum*. There was likewise a dispute of whether the route of infection was through the mouth (supported by many scientists) or through the skin (the general opinion of farmers in endemic areas). Fujinami and Nakamura conducted experiments in 1909 with calves wearing protective boots or not and others receiving suspected polluted water and grass or not. The conclusion was a percutaneous route only. Miyari and Suzuki described the intermediate snail host in 1913. These early discoveries are described by Tanaka and Tsuji [7].



Figure 3. Male (outermost) and female adult worms. From [8].

1.2. The lifecycle of *S. japonicum*

When egg-containing stool is deposited into freshwater, the egg hatches and releases a free-swimming larva called a miracidium. The miracidium finds and infects a freshwater snail of the genus *Oncomelania*. Inside the snail the miracidium divides and transforms into a number of sporocysts which again divide into hundreds of cercariae. The cercariae escape from the snail and swim to find and penetrate the skin of a mammalian host. Once inside, the larva follows the circulation and ends up as a young adult in the portal vessels in the liver. Here male and female worms pair up. The male worm “embraces” the female worm which from now on lives inside the longitudinal ‘schist’ or gynaecophoric canal of the male. Together they migrate downstream in the portal veins and lodge in small venules along the large intestine (in large mammals) or small intestine (in small mammals). The eggs pass through the gut wall and are excreted in the stool.

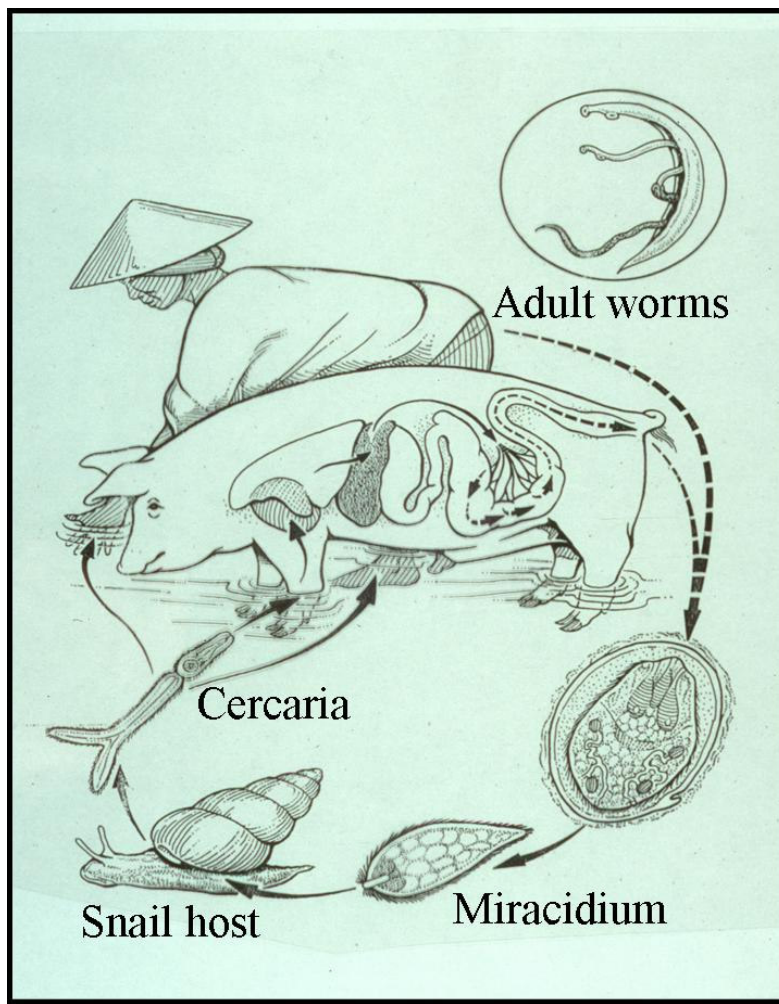


Figure 2. The lifecycle of *S. japonicum*. Illustration by P. Hamilton.

The *S. japonicum* egg is ovoid, 70-100 µm long with a thin, clear, non-operculated shell with a small inconspicuous spine. Inside the shell the embryo develops into a miracidium over a 6-day period. Passage of the egg through the vein wall and gut wall is aided by histolytic enzymes produced by the miracidium inside the egg. The eggs use 6-10 days or more to pass through the tissue and into the stool. Eggs may remain viable in stool for a week or more before eventually reaching freshwater, as long as they are not exposed to heat or desiccation. Exposure to freshwater stimulates hatching. The miracidium will swim freely looking for *Oncomelania* snails. It does not feed, and remains active up to 8-12 hours until its food store is exhausted. Once a snail is found, the miracidium penetrates the soft parts of it within minutes. Inside the snail the parasite develops into first primary, then secondary sporocysts and eventually into cercariae. This development includes division, so that a single miracidium can give rise to hundreds or thousands of cercariae which are shed from the snail over a period of months. The period from miracidium penetration to cercarial shedding varies from 17 days to several months or more, depending on the temperature [9]. The one-millimetre long, free-living cercaria is swimming, looking for its mammalian host. It does not feed and can survive up to 2-3 days, even though the proportion capable of penetrating and surviving the initial time in the mammalian host decreases rapidly within this timeframe. Vibrations and sudden changes of light as a potential host enters the water stimulate cercarial activity. It adheres to the skin and can within few minutes penetrate undamaged skin by the help of enzymes and movement of the tail. Once inside, it sheds its tail and transforms into a schistosomulum. The schistosomulum follows the circulation, transverses the pulmonary capillary bed and if necessary also other capillary beds to reach the hepatic portal veins where it grows to reach adulthood and sexual maturity. The 10-20 mm long, muscular male embrace the longer, cylindrical female with the lateral flaps of the male. Together they migrate to their destined portal venules. The adult worms are covered by a continuously changing, acellular, bilipid membrane called the tegument. Host antigens are incorporated into the tegument to help evading the immune system. The adult worms feed on nutrition from the blood, which is either ingested through the mouth or taken up through the tegument. The time between cercarial penetration until the first eggs are passed in stool is usually 30-38 days. The female *S. japonicum* worm is particularly productive and can lay up to 3500 eggs/day.

1.3. The epidemiology of *Schistosoma japonicum*

1.3.1 Geographical distribution

Schistosoma japonicum is endemic in parts of mainland China, parts of the Philippines and in two small foci in Indonesia.

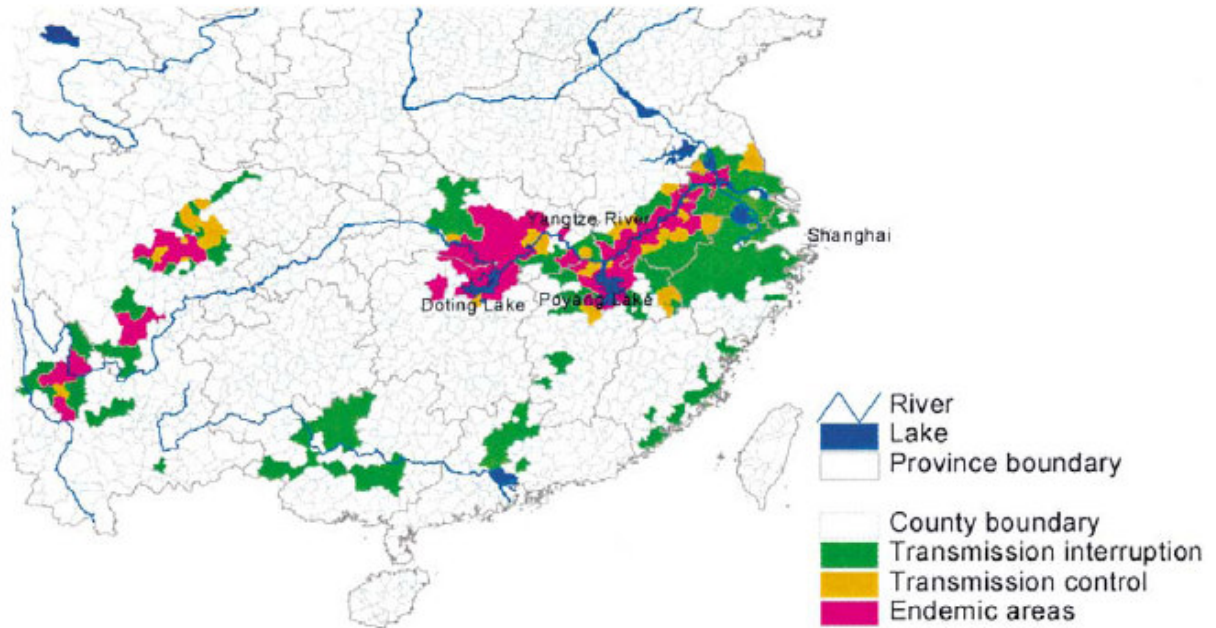


Figure 4. Areas endemic for *S. japonicum* in China. From [10].

China has been very successful in combating the parasite. During the last 50 years the number of human cases has been reduced by >90%, and >60% of previously endemic counties have reached the criteria for transmission interruption [4;11;12]. However, since the turn of the century the number of human cases has been stabilized or even shown a slight increase [11-13]. Reasons for this are discussed in chapter 1.5. The areas still endemic are mainly along the Yangtze River and can be divided into three categories, based on geography and ecological characteristics, each with different challenges in terms of control; i) Mountainous regions, mainly provinces to the west (Sichuan and Yunnan). ii) Swamp and lake regions (including Poyang and Doting lakes south of Yangtze), mainly Hubei, Hunan, Jiangxi, Anhui and Jiangsu province. iii) Plain regions in the Yangtze delta, mainly part of Anhui and Jiangsu province [5]. A large, nationwide cluster sampling survey in 2004 estimated the number of human cases in China to be 726 000 [12], which is somewhat lower than other estimates [11;13;14]. The prevalence varied from 0.3% to 4.2% between the endemic provinces, with a

prevalence of 2.5% in the villages classified as endemic in the nation as a whole. The prevalence was <1% in more than half of the endemic villages. The highest prevalence was found in swamp and lake regions and the lowest in plain regions. The geometric mean of infection intensity (based on three Kato-Katz slides) for all the endemic villages was 33 eggs per gram (epg) stool.

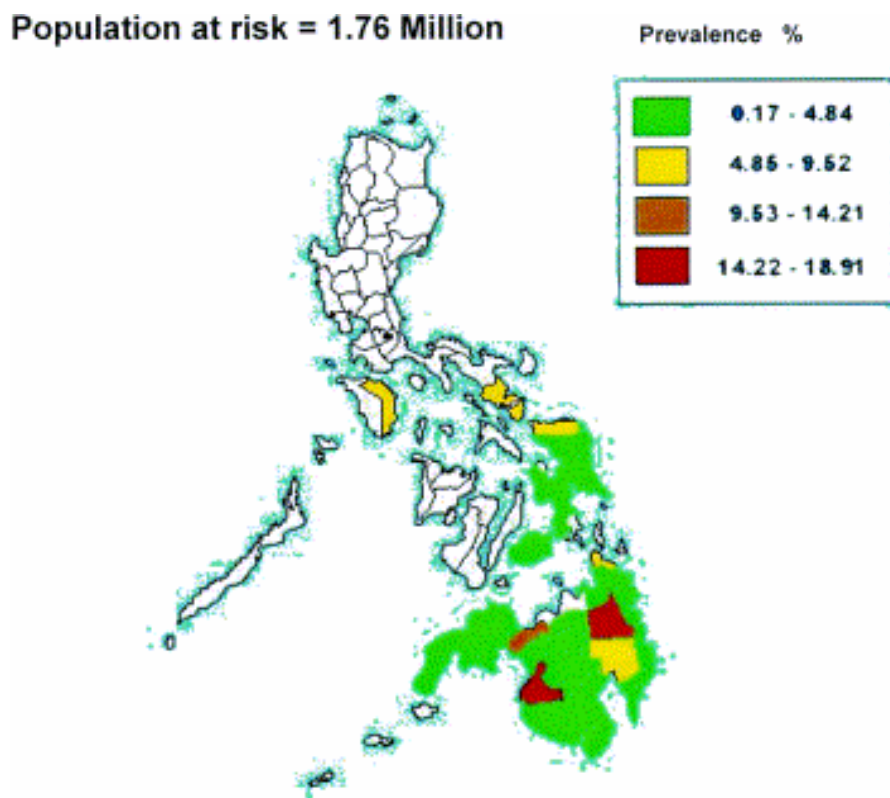


Figure 5. Areas endemic for *S. japonicum* in the Philippines. From [15].

The history of schistosomiasis in the Philippines has resemblances to the Chinese history. The mean prevalence decreased from 35-40% in the 1950's until the beginning of the 1990's when it stabilized. During the 1990's the mean prevalence in the endemic areas was 4-5% [6;15].

There are few published results with estimates of the number of human cases in the Philippines, but a paper from WHO in 2000 estimates 430 000 cases [14].

In Indonesia schistosomiasis is endemic in two isolated areas, Lindu and Napu valley in Central Sulawesi. The prevalence is around 1% or less, with a few thousands human cases [16;17].

A close relative of *S. japonicum*, *S. mekongi*, is a public health problem in Laos and Cambodia [18].

1.3.2. Transmission

Some elements that are important for transmission are unique to *S. japonicum* among the *Schistosoma* species of relevance to humans. One of these is the intermediate snail host, *Oncomelania* sp., which is amphibious and not aquatic as the other snail hosts. For the most part the very young snails live in the water while the elder live out of the water, usually on the mud amongst dense vegetation close to water, where they find humidity and shade. This amphibious nature restricts the Philippine *Oncomelania* snails to those islands where rainfall occurs throughout the year (mainly in the eastern part) [9]. In China there are different ecological habitats which differ in i) whether they are affected by flooding or not, ii) the definitive mammalian host primarily responsible for maintaining transmission and iii) in part with different *Oncomelania hupensis* subspecies [4]. This has to be taken into account when transmission reducing measures are considered. In the great river plains, marshland and big lakes in the lower stretches of the Yangtze River, the annual change in water level and occasional flooding is vital to schistosomiasis transmission. Flooding drowns many of the adult snails, but can increase the snail habitat and the number of snails considerably. Wu and colleagues collected data retrospectively for a 22-year period and found that potential *Oncomelania* habitats were 2.6 times larger in years with flooding. The snail density and the proportion of infected snails dropped in the two first years after a flood, but increased significantly in the third year. There were on average 2.8 times more cases of acute schistosomiasis in years with flooding [19]. Another factor likely to influence transmission is the large water management projects in China, especially the enormous Three Gorges Dam, one of the world's largest hydroelectric power projects, located in the upper part of the Yangtze and soon to be completed. The effects on the river ecology are large and complex, and the effect on schistosomiasis transmission is difficult to predict. It will secure better control with the (occasional disastrous) flooding, but will provide a huge potential snail habitat. The Three Gorges area is presently not schistosomiasis endemic, but there are endemic areas both upstream and downstream. Zhu *et al.* have made a comprehensive review of these potential effects [20]. For decades marshland has been drained, embankments built and canals lined with concrete in order to increase farmland and to destroy snail breeding grounds. However, this has reduced the buffer or water storage capacity and has probably resulted in more frequent and more serious flooding. "Return Land to Lake Programmes" and other similar programmes aim to reverse these measures, but may also provide increased habitat for snails [21].



Figure 6. Typical environment for *S. japonicum*, Anhui province, China.

Another factor that separates *S. japonicum* from the other main *Schistosoma* species, and which is important to transmission, is the animal reservoir. More than 40 wild and domesticated mammals have been shown to be natural definitive hosts, but only a few are important for transmission to humans [22]. To what extent each species contributes in transmission to humans is not easy to assess as there are many factors involved, such as life span of the animal, whether it excretes viable *S. japonicum* eggs, the total egg output of the species in a given area (number of animals, stool volume, eggs per gram), hatchability and whether the faeces reaches water that is used by humans or animals. Recently a new tool which hopefully can provide new data into this field has been developed. Microsatellite markers (mutations in small, repetitive gene elements) have been used in a few population genetic studies to examine relationship between *Schistosoma* from different host species in a given area. Knowledge about transmission from animals is important in order to implement effective control measures. Without control of the animal reservoir, transmission control can be hard to obtain. Cattle, buffaloes, pigs, goats and rats are mentioned as important species [5]. In China bovines (cattle and water buffaloes) are traditionally considered the most important animals in transmission, and recent microsatellite data support this [22-25]. Studies from the Philippines suggest that cats and dogs may be important, while the importance of rats is not resolved [22;26-28]. However, previous studies in the Philippines estimate that humans are the source for 75% of the transmission [6;9]. The relative contribution from

different mammals is likely to differ between countries and between provinces, but local factors may also be important. Wang *et al.* calculated a relative transmission index for two villages within Anhui province and found that water buffaloes were the most important contributor to transmission (90%) in one village and humans (80%) in the other [29]. Knowledge of the animal reservoir is important in order to treat the right animals and for estimating the effect of changes in agricultural practice (tractors instead of buffaloes, keeping animals in fences or restricting grazing in transmission sites, introducing new species like goats) [30].

1.4. The pathology and morbidity associated with *S. japonicum* infection

The main pathology and morbidity in schistosomiasis are not associated with the adult worms, but with the eggs that are trapped in the tissue, or more precisely the immunological response to these eggs. The pathology is a result of the balance between cellular and humoral immune responses, which are influenced by factors like host and parasite genetics, host nutritional and immune status, location of egg deposition and presence of co-infections [31;32]. The immune response also changes or modulates during the course of the infection [33]. The details of this immune response are complex and not fully understood and are not within the scope of this introduction.

Even though the pathological process and morbidity are similar between the different *Schistosoma* species, there are some differences. Although not definitely proven, *S. japonicum* is generally considered to be more pathogenic than the other species and *S. japonicum* infections results in more cases of acute disease. This may be because *S. japonicum*, being a zoonosis, is less well adapted to man or because of the very high egg productivity of the female *S. japonicum* worm [9;34]. The level of infection intensity correlates with the level of morbidity, and this is part of the rationale behind the morbidity control strategy. However, due to the complex and individual immune response, this is not always the case. Other data suggest that this correlation is less pronounced in *S. japonicum*, resulting in cases with severe morbidity even in low intensity infections [34].

Schistosomiasis can be divided into acute schistosomiasis ('Katayama fever') and chronic schistosomiasis. Acute schistosomiasis is an immunological response to the infection ('serum sickness'), probably relating to onset of egg production and appears on average 40 days after infection. Symptoms are fever, cough, muscle pain and a large, tender liver. As opposed to infections due to the other species, acute *S. japonicum* infection does not only occur in

persons infected for the first time, but also in previously infected persons, especially if they are exposed to a heavy infection. Acute *S. japonicum* infections are still quite common, especially after severe flooding, and are used to monitor progress in schistosomiasis control in China. Acute *S. japonicum* infections are generally more severe than acute infections caused by the other species. Before praziquantel was available, the mortality reported from different hospitals was between 2% and 20%. In a historic record from China from 1950, 4000 villagers out of 7000 acquired acute infection after a flooding episode, and 1300 of them died [4;9;34].

In chronic *S. japonicum* infections the organs mainly affected are the large intestine and the liver and spleen. In the large intestine inflammation and granuloma formation around the eggs and later fibrosis may lead to ulceration, microabscesses and pseudopolyposis. This may result in abdominal pain and changing bowel movement with episodes of diarrhoea, sometimes bloody. The most serious pathology in intestinal schistosomiasis is due to eggs that are swept up in the portal blood flow and trapped in the presinusoidal venules within the portal triads in the liver. Granulomatous inflammation arises around the eggs. Eventually the eggs will be reabsorbed or calcify and the tissue damage will turn into fibrosis. In the early inflammatory phase, the liver may be enlarged, especially the left lobe. Years later, if the infection continues and advanced fibrosis has occurred, the liver may become smaller, hard and nodular. Massive fibrosis in the portal space creates a pathognomonic histological pattern called ‘Symmer’s pipestem fibrosis’. Another hepatic effect seen in *S. japonicum* hepatic disease is widespread septal fibrosis and calcification that produces parenchymal abnormalities (“network pattern”) detectable with ultrasound [4;35]. The progressive occlusions of portal veins lead to portal hypertension, splenomegaly, ascites, portocaval shunting and gastrointestinal varices. This process takes years. In contrast to liver cirrhosis, the function of the hepatocytes and hence much of the liver function is preserved for a long time. In general early pathology is reversible after successful treatment and in absence of re-infections, while advanced fibrosis is not. Massive bleeding from gastrointestinal varices and hepatic coma are the two main causes of mortality [4;9;34]. Cerebral schistosomiasis as a host reaction to schistosome eggs is a well-known clinical picture with various cerebral symptoms, including convulsions [4;9]. Even though most of the *S. japonicum* infected persons are regarded as asymptomatic, there is still a substantial morbidity with impact on the growth of children, quality of life and working capacity. Several authors argue that the burden of schistosomiasis, often presented as ‘Disability-adjusted life years’ (DALY) rankings, has been significantly underestimated because the disease has many non-specific symptoms and signs [36-39].

1.4.1 Animal models for *S. japonicum* infection

Animals can serve different purposes in schistosomiasis research; keeping a transmission circle going in the laboratory, for research on the animal reservoir of *S. japonicum* and as a model for human infection. In vaccine development animals are both useful as a model and are at the same time the primary target for some vaccines. Different animals can serve as models, each with its own advantages and disadvantages. Primates are difficult and expensive to keep, and hence are rare in schistosomiasis research. Rodents, especially mice, are abundant and easy to keep, and have been used in most of the studies involving an animal model. However, there are huge differences to humans in terms of anatomy, physiology and immunology. Problems using mice as a model include size; it has been estimated that a single worm pair in a mouse is equivalent to 4000 worm pairs in a human (a very heavy infection) [40]. The average lifespan of schistosomes is longer than that of the mouse, making long term studies difficult. The adult worms lodge in the small intestines rather than in the large intestines. Differences in immunology and drug metabolism may reduce the relevance of some studies on vaccines and drugs [40]. Willingham and Hurst have presented numerous arguments why pigs may be the preferred animal in many *S. japonicum* studies requiring an animal model [40]. Besides being a natural host for *S. japonicum*, the many similarities with humans when it comes to size, anatomy, immunology, metabolism, nutrition and physiology have made pigs a popular animal model in a wide range of biomedical research and they have even served as donors for transplant organs. They have a short reproduction cycle, large litter size, are relatively easy and cost-effective to house and can be handled in an ethically acceptable way. Small studies using pigs as an animal model in schistosomiasis have been published previously, but by far the most comprehensive work has been done on the Danish *S. japonicum* pig model. *Johansen et al.* have published a thorough review which outlines the methodology and major results of this model [41]. In the following I will briefly outline some of the results; references are found in the review. The model has overall a low worm establishment rate, and after studying several routes of infection, intramuscular injection of cercariae was found to have the highest establishment rate in addition to being easy to administer. Worm establishment was enhanced when fodder with reduced protein content was used. Studies showed that a 5-gram sample from either left median or left lateral lobe provides a fairly reasonable estimate of the total liver tissue egg count. Due to patchy egg distribution in the large intestine, the whole mucosa should be scraped off before examination. Adult worms can be collected by perfusion of the portal veins and passing the fluid through a sieve. Later the mesenterium can be manually searched for residual worms and worm nodules.

In order to detect and count eggs in stool, a new technique was developed, the DCEP/DBL technique. This is presented in more detail in section 1.6.1 'Stool diagnosis'. The prepatent period of *S. japonicum* in this model was around 6 weeks. There was a peak in egg excretion around weeks 8-10, especially in heavy and moderate infections. The following decrease in egg excretion with increasing age of infection was presumably due to a reduction in worm burden, a reduction in egg production per worm and possibly an increased retention of eggs in tissues. Since most studies have been on host/parasite relations, the infections have generally been kept at a subclinical level. Pathology observed includes peteccial haemorrhages and hyperemic foci in the intestines and septal and periportal fibrosis in the liver. An animal model can be no more than a model, but experience with the Danish pig model shows that pigs can be very valuable in studies of *S. japonicum*. Even though there are some differences to human infections, there are many similarities. In the before mentioned paper Willingham and Hurst quote Phillips and Tumbleson that states: "...pigs are like people. They tend to be diurnal, sedentary, meal eaters with a fondness for alcohol and are prone to obesity and cardiovascular disease. Yet, with training and diet restriction they become fit and passable athletes...As swine become used more widely, they continue to be characterized in more detail and to be recognized as the exemplary nonprimate model of the human situation." [42]

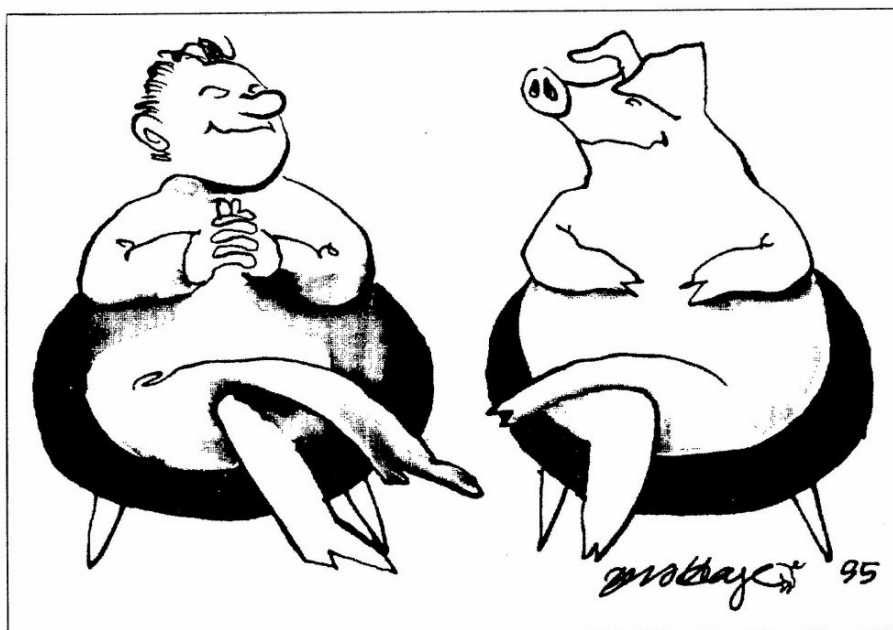


Figure 7. Cartoon by Jens Hage.

1.5. Strategies for control

As previously mentioned, the schistosomiasis situation was very serious at the time when the People's Republic of China was founded in 1949. Villages were deserted or inhabited only by a few surviving widows. A large proportion of the rural population lost their working capacity due to wasting, weakness and ascites before suffering a premature death 30-40 years old. Thousands of soldiers became incapacitated after acquiring the infection through military manoeuvres or flood relief work [4;5]. The huge effect on health, social development and economy was recognised by the new Chinese government which gave it top priority in public health. The longstanding political commitment through the national schistosomiasis control programme has been a success with a 90% reduction in the number of human cases from the 1950's until today. The control strategy in China has moved through different stages, influenced by international "trends" in schistosomiasis control, but with clear national adaptations [13;43]. From the 1950's until mid-1980's the main strategy was, besides free diagnosis and treatment and health education, an emphasis on snail control through a long array of measures, resulting in a reduction of the snail-infested areas by more than two-thirds [13]. In the 1980's praziquantel came on the market; a safe, highly efficacious and easy to administer oral drug. The global strategy changed to morbidity control through mass treatment with praziquantel, with or without diagnostic testing. China imported this strategy, but kept some emphasis on snail control and treatment of animals. A huge ten-year long World Bank Loan Project from 1992 enhanced this strategy, resulting in the prevalence in humans and bovines being cut further in half [44]. However, since then the control seems to have stagnated and there may even have been an increase in the number of infected people [11;30]. The reason for this is complex. Many authors emphasize that transmission control can not be achieved based on praziquantel alone, even though it is effective in reducing prevalence and morbidity [44-47]. It is worth remembering that *S. japonicum* was eliminated from Japan before praziquantel came on the market, mainly through snail control, changes in agricultural practice and socioeconomic development [48]. Problems to further success of the control programme include the factors discussed under 'Transmission'. Snail control is difficult in most of the remaining endemic areas, and it seems not to be universal agreement on the feasibility and necessity of snail control in order to be further successful. Snail control has been a part of the Chinese criteria for transmission control and elimination, but this does not seem to be an important part of the new national control strategy [5;30;49]. Another possible threat to further success is decreasing participation of the population for testing and

treatment, especially when symptoms and signs of the disease are getting scarcer [50;51]. Due to economic market reforms the schistosomiasis control stations tend to shift their emphasis from prevention to handling of individual patients [52].

In short, a lesson is learnt from twenty years with a control strategy largely focusing on using praziquantel to treat humans and bovines. It seems that this strategy reduces the prevalence of schistosomiasis in humans in the endemic areas, often down to 1-5%, but fails to reach transmission control or to eliminate the infections. This is mainly due to frequent re-infections, which in turn probably is due to the animal reservoir and the persisting snail population [49]. Currently we may see a change in the Chinese control strategy. Wang *et al.* have published a very interesting study, using two intervention and two control villages, all previously subjected to annual praziquantel treatment. They added very comprehensive new interventions which included removing cattle from snail-infested grassland, providing farmers with mechanized farm equipment, improving sanitation (providing tap water and building lavatories on land and in boats) and implementing an intensive health education programme. After three transmission seasons the prevalence dropped from 11% and 4% to less than 1% in the intervention villages, but remained at baseline in the control villages [30]. According to the authors these interventions have been adopted as the national strategy for controlling schistosomiasis in China.

The schistosomiasis control programme in the Philippines has largely followed the same stages as in China. Early efforts focused on snail control with a shift towards case detection and mass treatment when praziquantel became available. Current problems include low disease awareness, lack of funds, an aggravated security problem and general poverty in the endemic areas [6;15]. Engels and colleagues have published a paper where the step-wise theoretical control approach from morbidity control through transmission control to elimination of schistosomiasis is nicely illustrated [53].

1.6. Diagnosis of schistosomiasis

Diagnosis is central to all aspects of schistosomiasis. Decisions on treatment on individual and community levels, assessment of morbidity and prognosis, evaluation of chemotherapy and other control measures all build on the results from diagnostic tests. The different tests vary considerably in terms of sensitivity and specificity, ease of use and cost. Selection of which test to use must hence correspond to the type of information sought. Individual diagnosis in a hospital has other demands than large scale screening programmes [54;55]. A given test may perform differently with different *Schistosoma* species, host factors, diet, ethnic groups, sex and age and not the least with changing schistosomiasis prevalence and infection intensity. Hence should a test be evaluated under conditions similar to those where the test will be used and re-evaluated when the conditions change [54;56].

Direct diagnostic methods detect ova, schistosome antigens or schistosome DNA in urine, stool, tissue or blood. Indirect methods rely on clinical, biochemical or immunological disease markers (including antibody detection).

When the performance and predictive values of a diagnostic test are evaluated, it is often compared to a set of samples previously classified as ‘positive’ or ‘negative’. This classification is done by using a different test, combination of tests or other diagnostic criteria, often referred to as the ‘gold standard’ or ‘reference method’. Hence, the perceived performance of the test being evaluated will rely heavily on the quality of the gold standard. If for example the gold standard has low sensitivity, it will wrongly classify true positive samples as being ‘negative’. If the evaluated test finds these samples ‘positive’ (which they truly are) the evaluated test will wrongly be judged as having poor specificity. This is an important aspect when diagnosis of low intensity schistosomiasis infections is concerned, because such a high quality ‘gold standard’ is lacking.

1.6.1 Stool diagnosis

Variations of Kato-Katz thick smear is the dominating stool diagnostic method in the schistosomiasis endemic countries, especially in field conditions. Except for a microscope, only basic equipment (mostly single-use) and basic chemicals are needed. It is cheap and quick and easy to perform. Glycerol in the overlaying cellophane “coverslip” will clear up the stool material. Hookworm eggs clear rapidly and will be invisible within few hours. The content of schistosome eggs also clear, but the egg shell does not collapse, so the eggs are

recognizable for a longer time. *Ascaris* and *Trichuris* eggs have thick shells and remain visible for many months [57-59]. The size of the hole in the template used for smear preparation decides the approximate weight of the examined stool sample. The most common size equals 41.7 mg stool, but other sizes are in use. Hence Kato-Katz is one of few quantitative tests. The number of eggs on the slide is counted and multiplied with a factor to get the number of eggs per gram stool (example with one 41.7 mg slide: 5 eggs x 24 = 120 epg). Quantification allows an estimate of infection intensity, which is useful for stratification of infected individuals, as an indirect indicator of morbidity, in transmission analyses, and in evaluation of interventions and diagnostic tests. During the last decade, the following new classification has been adopted in China: <40 epg; light infection, 40-99 epg; moderate and \geq 100 epg; heavy infection [13]. The common WHO classification (for *S. mansoni*) has 100 epg and 400 epg as the limits for moderate and heavy infection [60]. However, with the Kato-Katz technique the examined amount of stool is small, and the main criticism against this method is the low sensitivity in low intensity infections. Yu *et al.* examined duplicate smears from seven consecutive stool samples in two villages (n = 570). The proportion of individuals with at least one smear positive for *S. japonicum* increased from 42% to 68% and from 17% to 36% when one and seven stool samples were used, respectively. The relative sensitivity was lowest in infections with \leq 100 epg. For these infections the proportion of positive cases increased steadily even up to seven examined stool samples. No extra positive individuals were found after four examined stool samples in infections with > 100 epg [61]. Lin *et al.* examined a village for two consecutive years (total n = 1780), each year using three smears from two stool samples (six smears in total) as the gold standard. The majority of the infections had < 100 epg. The prevalence increased without a clear levelling-off from one to six smears. If a single smear or three smears had been used, the prevalence would have been underestimated by 55% and 25%, respectively. Again the underestimation was highest in those with lowest infection intensity [51]. Zhang and colleagues used a Bayesian modelling approach and calculated that up to 83% of the infections were missed by using a single slide [62]. Kato-Katz is less suitable for stool samples from animals because of high content of plant fibres and soil material.

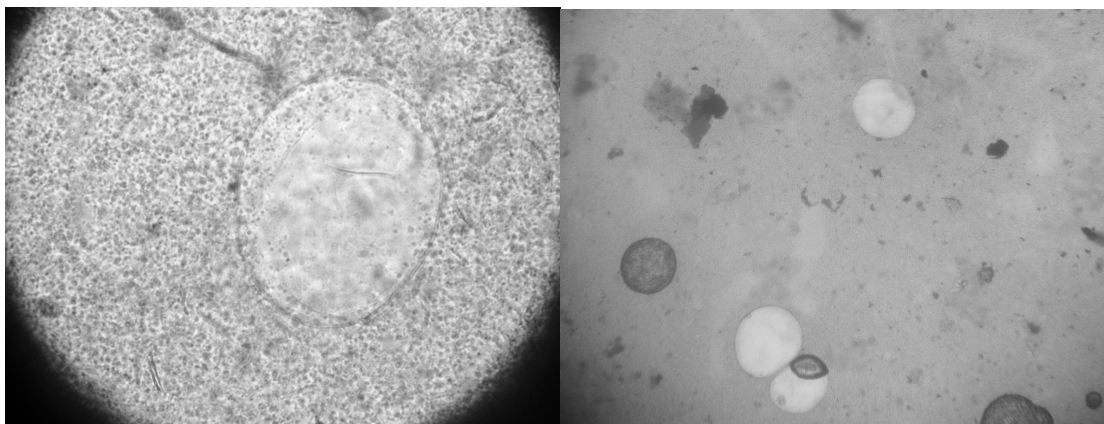


Figure 8. Kato-Katz thick smear. Egg of *S. japonicum* (left) and artefacts (no eggs, right).

Hatching test is used for diagnosis in both humans and animals, but has almost exclusively been used in China. It utilizes a large stool sample, usually about 30g. The most commonly used method is variations of the 'nylon bag' method. The stool sample is flushed with water through a mesh container into a nylon bag with a dense mesh. The content of the bag is transferred into a bottle with a long neck and filled with fresh water. The bottle is kept at 25-30 °C and in strong light. It is regularly checked for swimming miracidia [63;64]. The method has potentially high sensitivity since a large sample is used. The main problem is that the method seems difficult to standardize. There are probably many environmental factors affecting the hatchability and hatching time. Factors such as temperature, light and ionic content of the water have been considered as important, but there is no universal agreement on the importance even for these factors [65-67]. Immature eggs are not likely to hatch. Variations in such factors may in part be the reason for the inconsistency in diagnostic performance of hatching in different studies [63]. Yu *et al.* even found a large difference in the sensitivity of hatching test when compared to Kato-Katz between two villages, using the same method and the same team. In one village prevalence was higher with hatching than duplicate Kato-Katz smears from a single stool specimen (31% versus 24%), in the other village the hatching prevalence was half that of Kato-Katz (33% versus 68%) [63]. Hubbard *et al.* found a *S. japonicum* prevalence of 25% in a population of 4000 using hatching test on three consecutive stool samples together with three Kato-Katz smears from a single sample to assess infection status. Only five percent of the positive samples were only positive with the hatching test [64]. In a study by Zhou and others they defined an individual as a positive case if any of the following was positive: Kato-Katz (three smears), hatching test or soluble egg

antigen (SEA) ELISA (if the individual had not received treatment the previous two years). By these criteria hatching achieved a sensitivity of only 17% [68]. Hatching test is also demanding and time consuming to perform.



Figure 9. Hatching test. Flushing stool into a nylon bag sieve, Anhui province, China.

Several concentration techniques for parasite microscopy exist, but the most common is variations of the formol (or formalin)-ether sedimentation concentration technique (FEC). In recent years, ethyl acetate has largely replaced ether due to safety reasons [69]. Ridley-Allen technique and Ritchie technique are other names associated with the technique or variations thereof [70;71]. FEC is probably the most commonly used technique for parasite examination of stool samples in clinical microbiology laboratories in Europe and North America. It is the recommended method in larger textbooks and in well known diagnostic guidelines [72-74]. It

is used to a lesser extent in less affluent countries, at least in field conditions and in larger surveys for helminth infections, where Kato-Katz is dominating. However, FEC is a recommended method in several textbooks aiming at district laboratories in tropical countries [75;76]. Several variations of the technique are used, but generally a stool sample (1-4 gram) is mixed with formalin and sieved (through gauze, metal or plastic mesh) into a tube. Ethyl acetate is added and the tube centrifuged. The three top layers (ethyl acetate, debris and formalin) are removed, leaving a sediment for microscopy. FEC utilizes a larger stool sample than Kato-Katz, but usually only a single slide is looked at, representing only a fraction of the original stool sample. Since the amount examined is not usually measured, FEC gives at best only a semi-quantitative result. Several authors have evaluated FEC in the diagnosis of *S. mansoni*, usually compared with Kato-Katz. In the four most comprehensive comparisons the sensitivity of FEC is surpassed by the sensitivity of 1-3 Kato-Katz slides [77-80]. In one of these studies Ebrahim *et al.* found that there was little difference between the methods when infection intensity was >100 epg, and even a single Kato-Katz smear then had a sensitivity of >95%. The same authors also used a quantitative FEC technique and interestingly found 48 positive cases (out of totally 576 positive) excreting < 30 epg that were positive with FEC, but negative in 4 Kato-Katz smears [80]. In two smaller studies a modified FEC technique was superior to a single and triplicate Kato smears, respectively (Kato technique includes weighing the stool sample instead of using template) [71;81]. The latter of these studies calculated that in samples with >100 epg, Kato recovered twice the number of eggs as the FEC technique, indicating a large loss of eggs in the FEC process. Ebrahim *et al.* came to the same conclusion in their study with FEC and Kato-Katz [80]. Parasep Faecal Parasite Concentrator (DiaSys Europe Ltd., Berkshire, United Kingdom) is a commercial kit for the FEC technique. It consists of an enclosed, disposable tube system with built-in sieve and pre-filled with the necessary chemicals. The system is amongst others used at the laboratory at Hospital for Tropical Diseases, London, and for preparing samples to the parasite part of the United Kingdom National External Quality Assessment Service (UK NEQAS). Dacombe *et al.* used Parasep for demonstrating hookworm and *S. mansoni* eggs when comparing FEC and Kato-Katz [77]. There are no peer-published results comparing Parasep with conventional FEC technique. An evaluation report from Hospital for Tropical Diseases by Kettelhut and others doing this comparison on 100 samples is available as supportive information through the electronic version of Paper four. The results were comparable.

As discussed in Paper four the performance of the FEC technique compared to other methods in detecting *S. mansoni* eggs (or other helminth eggs) must be extrapolated to *S. japonicum*

with great care. *Schistosoma japonicum* eggs are round and lack conspicuous characteristics and hence may be easier to overlook in methods requiring microscopy. To our knowledge Paper four is the first to evaluate FEC technique for diagnosing *S. japonicum*.

FEC technique is labour intensive, even though with the Parasep tube system much less so. The FEC slides contain a lot of faecal debris and is time consuming to examine, more so than for example the “cleaner” Kato-Katz slide. To what extent the different variations of the technique (different sieves, centrifuge conditions etc) does influence the performance is not fully known. A huge advantage with the FEC technique is that it is equally well suited for demonstrating both helminth eggs and most protozoa. The importance of this depends on in which situation the test is used.

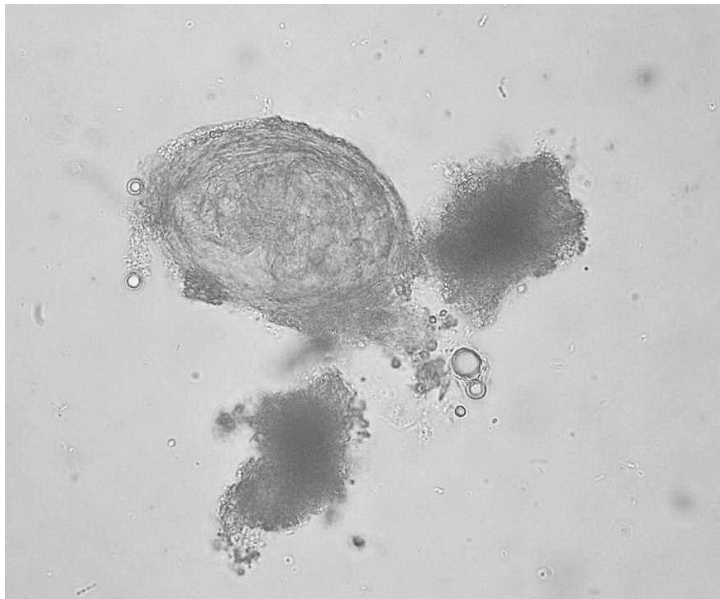


Figure 10. FEC microscopy. *Schistosoma japonicum* egg, partly covered by debris.

During the development of the Danish schistosomiasis pig model (see chapter 1.4.1.) a new technique for demonstrating schistosome eggs in animal stool was developed; the DCEP (Danish Centre for Experimental Parasitology) or DBL filtration/sedimentation technique (DBL technique) [82]. There are several advantages to this method. It is regarded as quantitative (even though the number of eggs lost in the process is not known) and uses no hazardous chemicals. The eggs remain alive and egg viability can be evaluated by looking for flame cell activity in the microscope, and it allows hatching from the remaining sediment. Even stool containing much vegetable fibres can be processed, which for example is difficult

with Kato-Katz. It allows differentiation between *S. japonicum* eggs and the ciliate *Balantidium coli*, which is common in pig stool and has similar shape and size as the eggs. The technique was significantly better at recovering eggs than the modified Bell filtration technique [82]. Carabin and others have used this technique to demonstrate *S. japonicum* eggs in stool from different animals in a series of articles from the Philippines [28]. To my knowledge it has not been used in humans. The technique is very labour intensive, both the sample preparation and the microscopic examination.

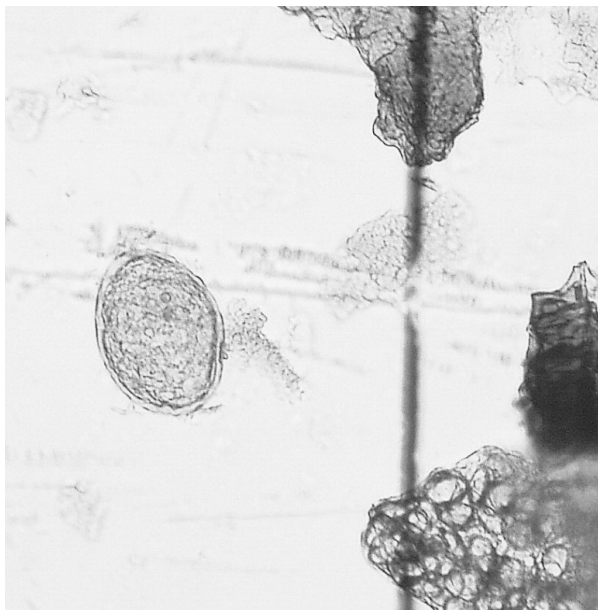


Figure 11. DBL filtration/sedimentation technique. *Schistosoma japonicum* egg.

1.6.2 Immunodiagnosis

Detection of antibodies and detection of antigens represent two different principles and are hence discussed separately.

Antibody detection has mainly been used in non-endemic countries to diagnose imported schistosomiasis and in endemic countries with predominantly low intensity infections; that is *S. mansoni* in South America and *S. japonicum* in China and the Philippines. Antibody detection is a very valuable supplement to egg microscopy in travellers from non-endemic countries with suspected schistosomiasis [83-85]. The difficulties with persisting antibodies and cross reactions discussed below are much less pronounced in this group. Some of the problems encountered when it is used in endemic countries are to some degree intrinsic to antibody detection and are hence common for most assays. Antibodies persist for some time

even after successful treatment or disposal of the infection for other reasons. The rate of seroconversion back to negative after treatment varies between studies, even with a given assay [83;86-88]. Antibody assays may cross react with other infections, especially other fluke infections, and hence give a false positive result. The antibody response is an indirect measure of infections and is dependent on immunological factors of the infected person. The correlation between antibody level and infection intensity is therefore usually poor. Despite these shortcomings antibody assays have been used extensively, especially in China, since they are perceived as having high sensitivity even in low intensity infections. There is an overwhelming number of different antibody assays that has been studied. They differ depending on what lifecycle stage the antigen originates from (worm, cercariae, egg), purity of the antigen (crude, purified, recombinant) and choice of assay system (complement fixation, indirect immunofluorescence, indirect hemagglutination, ELISA and many more) [89;90]. Some authors argue that several of the before mentioned shortcomings can be minimized by the use of certain purified antigens or detection of certain immunoglobulin isotypes [89;91]. So far however none of these assays have made it into routine use. The reason may be that they did not perform as well as initially hoped for, that the manufacturing of the assay is too expensive and technologically difficult, or that more testing is required [92]. The assays that are presently in large scale use include the circumoval precipitin test (COPT) and IHA and ELISA with soluble egg antigen (SEA).

COPT was shown by Alarcón de Noya *et al.* to have high sensitivity even in low intensity *S. mansoni* infections in Venezuela, but Chinese reports have shown only 70-80% sensitivity [86;88]. Despite that COPT has been used for 50 years, the method still seems to lack agreed standardizations and positive cut off level [86;93]. It is quite complicated and time consuming to perform. Few papers have been published with the method in recent years, and it is probably being surpassed by IHA and ELISA assays.

IHA has been used extensively in China for several decades. Some variations in the kind of erythrocytes (sheep, human, lyophilized etc) and the antigens used do occur. Even though some studies have indicated that fractioned egg antigens give higher specificity than the more commonly used SEA, the latter antigen is used in most of the papers published in English over the last few years [63;86;94;95]. Of these studies, Yu *et al.* used the most comprehensive gold standard; positive with either duplicate Kato-Katz slides from seven different stools or hatching test. The prevalence was high in this study (68%). By this gold standard they found IHA sensitivity to be 80% and the specificity 48%. They found a positive correlation between infection intensity and degree of positive IHA. They concluded that IHA is unsuitable for

individual screening, but may be used for community diagnosis [63]. Zhou and co-workers used three Kato-Katz slides from two stool samples as gold standard in two villages; A (prevalence 19%) and B (prevalence 7%). This resulted in IHA sensitivity of 84% and 92%, and specificity of 56% and 67% in village A and B, respectively. They also noted increasing IHA sensitivity with increasing infection intensity, but also up to 18% false negative IHA in light infections. The agreement between the Kato-Katz results and IHA results was also poor (κ 0.11-0.23) [94]. Later Zhou *et al.* did a longitudinal study comparing Kato-Katz (three slides from a single stool) and IHA by collecting samples in a village each year for six years. This time the overall IHA sensitivity was calculated to almost 100% and the same figure for the negative predictive value. The overall specificity was 73% and positive predictive value 25%. The IHA prevalence was 2-9 times that of the Kato-Katz prevalence, which was 3-14%. Again the agreement between IHA and Kato-Katz results was quite low (κ 0.25-0.35) [95].

ELISA exists with a large number of modifications in terms of assay design and antigen used [89-91]. Some modifications have in smaller studies shown promising results with less cross reactivity towards other parasite species and faster seroconversion after successful chemotherapy [86]. However, a standard ELISA with SEA is the most commonly used version, both within and outside the endemic countries. In the study from village A and B referred to previously, ELISA was also compared to the Kato-Katz gold standard. The ELISA sensitivity was slightly higher than that for IHA (88% and 96% in village A and B, respectively), but the specificity was lower (38% in both villages) [94]. Lin and co-worker studied ELISA and Kato-Katz (three smears from each of two stools) for two consecutive years. The Kato-Katz prevalence was 19% and 13%, the ELISA prevalence 66% and 51%, and geometric mean of infection intensity was 17 epg and 22 epg for the two years, respectively. Hence, this was a population with relatively high prevalence, but low infection intensity. With Kato-Katz as the gold standard they found ELISA to have sensitivity of 79% and 87%, specificity of 39% and 54%, positive predictive value of 21% and 25% and negative predictive value of 93% and 94%. There was a poor correlation between antibody levels and infection intensity levels (epg levels). They studied the changes in ELISA results from one year to the next and with some concern found that ELISA, when compared to Kato-Katz, had limited ability to detect new infections (people Kato-Katz negative first year, positive the next) and to discriminate active from past infections (people Kato-Katz positive first year, negative the next). The authors suggest that ELISA alone must be used with caution for both individual level diagnosis and for population-based targeting. However, due to the high negative predictive value, ELISA might be used as the first test in a diagnostic algorithm

where seropositive cases are being further tested with for example Kato-Katz on multiple stools [96].

A few rapid assays for antibody detection using dipsticks or test cards have been developed. They have the advantage of easily being field-applicable and can contribute to quick decisions regarding further diagnosis and treatment. Since these assays make use of the same antigens (primarily SEA) as the conventional assays, they are prone to have the same problems with cross reactivity and persisting antibodies. Available kits include dipstick dye immunoassay (DDIA), colloidal dye immunofiltration assay (CDIFA) and dot immuno-gold filtration assay (DIGFA) [97-101].

Detection of antigens secreted from the parasite is in principle an interesting diagnostic alternative, as it demonstrates the presence of the parasite directly, not an indirect immune response. Hence the level of antigen might correspond to the number of worms present and should also decrease sooner than antibodies after successful treatment. The best studied antigens have been circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum or urine. These antigens are associated with the parasite gut [102;103]. CAA has even been detected in Egyptian mummies [104]. Especially during the 1990's there were many studies using different assay variations with CAA and CCA in order to determine sensitivity and specificity, to determine relationship to infection intensity and change after treatment [105-112]. In recent years most of the studies have been on rapid diagnosis by detecting CCA in urine with a dipstick type assay [113-116]. The overall results from these studies are that the sensitivity of the assays unfortunately is not superior to that of Kato-Katz. The circulating antigen assays also contain monoclonal antibodies, which may be a technical and economical challenge if the assays were to be used on a large scale. The available literature on circulating antigens and *S. japonicum* is much scarcer than for the other two main *Schistosoma* species, even though there were some encouraging results from studies on mice [117;118]. Van t' Wout and colleagues studied CAA in serum in *S. japonicum* infected patients before and after praziquantel treatment. They concluded that there was a positive correlation between counted epg and antigen level, and there was a significant drop in CAA levels within 5 days after treatment. This was based on few measurements only. The sensitivity was 73% in egg-excreting patients (n=48) and 62% in the subgroup with 100 epg or less [119]. In China they have also tried other antigens like Sj31/32, but the problem of low sensitivity in low intensity infections seems to be the same [90;120;121]. The reason for the almost absence of published studies on antigen detection in *S. japonicum* may in part be due to the results of a Chinese collaborative study from 1996. Here 13 antigen detection systems

(including several CAA and CCA assays) were tested in parallel on 448 sera. This included 50 negative controls from non-endemic areas, 100 egg-excreting persons (of whom 82 had ≤ 10 epg) and the rest was from post-treatment cases, other parasitic infections and persons with hepatitis B. The specificity was 90% or higher for nine of the assays (determined by using the 50 negative controls). However, only three of the assays had sensitivity above 60%, with the highest being 81% (determined by using the 100 egg-excreting persons) [90;122].



Figure 12. IHA kit used in Paper three and four.

1.6.3 Molecular diagnosis

Molecular based diagnostics and PCR-techniques in particular, are among the most important developments in diagnostic techniques over the last 20 years. Gradually PCR has also been used to detect a number of different parasitic infections [123;124]. Hamburger and colleagues described a highly repeated sequence that comprises at least 12% of the *S. mansoni* genome [125]. This sequence was then used by Pontes *et al.* in a PCR to detect *S. mansoni* first in a small trial with a few stool and serum samples and later in the first real clinical trial with a *Schistosoma* PCR [126;127]. In the latter study they found a higher prevalence using PCR on a single stool sample than duplicate Kato-Katz slides in three stool samples (38.1% versus 30.9%). This paper was an important source of inspiration for me to start on this PhD. Several publications on PCR using this and other sequences to detect *S. mansoni* or *S. haematobium* in snails, water samples, animal models and DNA extracts from adult worms have been

published [128-135]. The number of clinical trials are less numerous. Obeng *et al.* used real-time PCR to detect *S. haematobium* in urine and Hove and colleagues used multiplex real-time PCR to detect both *S. haematobium* and *S. mansoni* in stool samples [136;137]. In the latter study they used a mitochondrial gene (*cox1*) as the PCR target, and in the case of *S. mansoni* they compared the PCR with Kato-Katz (duplicate 25mg smears from each sample) in two stool samples. The prevalence, using both tests on both samples, was slightly higher for Kato-Katz than for PCR (80% versus 73%). Both prevalence and egg count were quite high in this study (54% of egg positive persons had >100 epg). Allam *et al.* examined 77 samples that were Kato-Katz negative with a PCR targeting the mentioned Hamburger sequence and found 23% to be PCR positive for *S. mansoni* [138]. They also used the Percoll stool microscopy technique which is discussed further in chapter four.

PCR assays aimed at detecting *S. japonicum* are few. Two published assays claim to be able to detect all three main human *Schistosoma* species, but in the case of *S. japonicum* they have only been tested on extracts from adult worms and a single clinical sample, respectively [139;140]. Interestingly the latter of these assays used serum samples. Three further PCR papers have been published which all target the repetitive retrotransposon Sjr2 in *S. japonicum*; in one paper they detected cercariae in *Oncomelania* snails, in another cercariae in water and in the last paper they detected parasite DNA in stool and serum in a rabbit model [141-143]. In the latter they could detect DNA in serum the first week post-infection and it became negative 10 weeks after praziquantel treatment. Gobert and colleagues designed a multiplex PCR with two primer sets each for *S. mansoni* and *S. japonicum*, all targeting the mitochondrial genome and tested mainly on stool from mice and on two human stool samples [144]. Even though the assay could detect DNA from as little as 1/3 of an egg, the overall detection threshold in mouse stool was found to be ≥ 60 epg. If the detection threshold is that high, it will limit the utility of the test. However, this threshold was determined from a very limited number of samples.

As described above, several different PCR assays for detection of one or more of the three main human *Schistosoma* species have been published, some targeting the nuclear genome and others the mitochondrial genome. Very few assays have been tried on more than a few clinical samples and hence the diagnostic value of PCR in different epidemiological situations is still not clarified.

Very recently, Xu and colleagues presented results using a PCR related technique for amplification of *S. japonicum* DNA: the loop-mediated isothermal amplification (LAMP) technique [145]. This technique makes it possible to amplify and detect DNA without the use

of a thermal cycler and has since the first report in 2000 been rolled out as an alternative to PCR, especially in resource-limited laboratories [146]. Xu and colleagues used the retrotransposon SjR2 as the LAMP target. The assay was positive in rabbit sera one week post-infection and turned negative 12 weeks post-treatment. It was also positive in 29/30 human serum samples from persons with < 50 epg stool as determined by Kato-Katz.

1.6.4 Clinical diagnosis

I will briefly discuss other diagnostic alternatives: Asking for clinical symptoms/signs and examining rectal biopsies.

The use of questionnaires for schistosomiasis screening has been evaluated quite thoroughly in sub-Saharan Africa [147]. While the use of questionnaires generally are perceived as useful, even though not without problems, for *S. haematobium* screening, the diagnostic performance is weaker and the usefulness more debated for intestinal schistosomiasis [9;147-149]. Especially those with light infections might lack or only have slight symptoms/signs. The classical symptoms/signs like abdominal pain, colicky cramps, blood in stool, diarrhoea, hepato- or splenomegaly are not pathognomonic or characteristic only for intestinal schistosomiasis. Attempts have been made to increase the diagnostic performance of questionnaires in areas with low schistosomiasis prevalence by including additional risk factors such as migratory status, water contact patterns and previous schistosomiasis treatment [147;150]. The utility of questionnaires in low prevalence areas must be validated locally and the value of questionnaires in such areas is not yet established [148]. The main advantages of using questionnaires are low costs and no need for invasive diagnostic procedures. Two studies using questionnaires have been performed in China. Zhou *et al.* applied 15 multiple-choice questions on schoolchildren and used a gold standard of positive Kato-Katz, hatching test or SEA ELISA. The latter provided that the child had not been treated within the last two years. The survey resulted in a prevalence of 30%. When six of the questions were used; diarrhoea, frequency of water contact, school grade attained, weakness, past history of *S. japonicum* infection and previous treatment for schistosomiasis, the questionnaire resulted in a sensitivity of 94% and a specificity of 92%. A simplified version used a “yes/no” answer to three questions. Here a “yes” to either one major (water contact) or two minor questions (diarrhoea and weakness) resulted in a sensitivity and specificity of 86% and 98%, respectively [68]. In this study the prevalence of infection was quite high. Tan and colleagues developed a questionnaire for detection of new infections in children and adults after a

flooding event. They used a case-control study design and six variables: Knowledge of *Schistosoma* transmission, years of education, annual income and duration and intensity of water contact due to different activities. The study found the sensitivity, specificity and positive and negative predictive values to be 82%, 82%, 70% and 91%, respectively [151]. The prevalence before the flooding was 15% and 8% in the two studied villages.

Schistosomiasis can be diagnosed by examining a biopsy ('snip') from the rectal mucosa. The biopsy is crushed between two glass slides and examined in a microscope directly or after adding a few drops of glycerol-malachite solution. By this method the viability of the eggs can be assessed. Even though most studies evaluating the use of rectal biopsies are small and/or old, there are indications that the method has superior sensitivity compared to a stool microscopy test. However, due to the invasive nature of the technique it is usually limited to assessment of selected patients in a hospital or large clinic setting and not used on a routine basis or in screening programmes. The utility of the technique must be weighed against the alternative of performing repetitive stool microscopy tests or a combination of different diagnostic tests. The technique has been used in studies for assessment of cure after treatment [9;81;152-154].

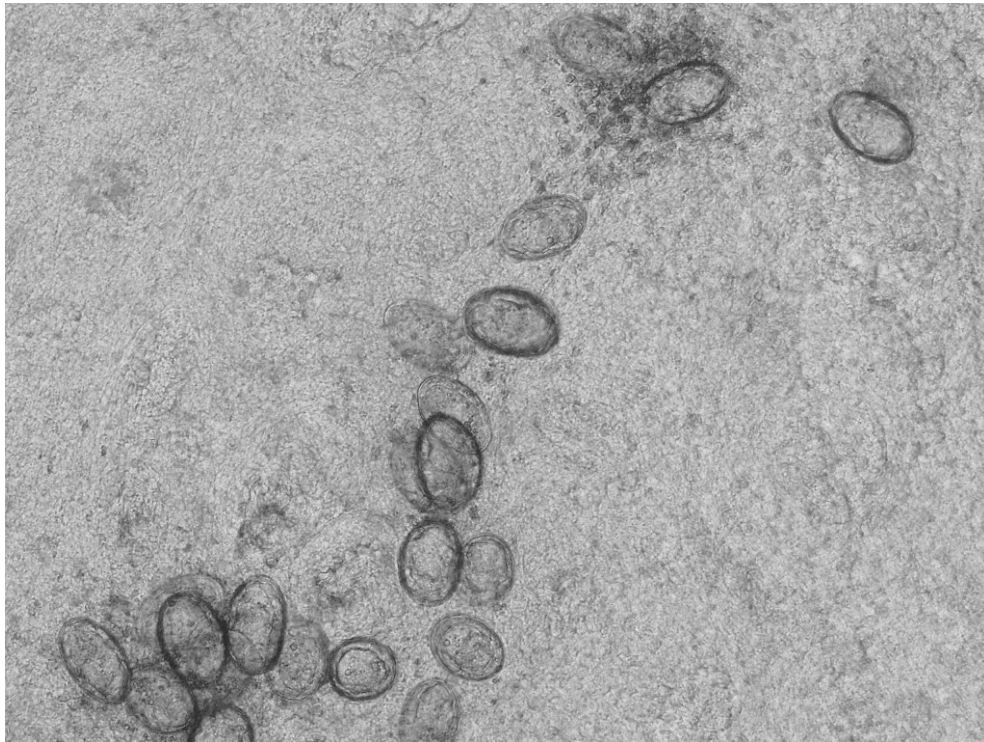


Figure 13. *Schistosoma japonicum* eggs in caecal mucosa from pig. Biopsy crushed between two glass slides. From Paper two.

1.6.5 Combining tests

Combining different tests is a common strategy to increase the likelihood of detecting a schistosomiasis infection when assessing individual patients. Combining tests is less common in screening programmes, probably because it increases the cost and complicates the logistics of the screening. Primary screening with detection of antibodies and subsequent testing with Kato-Katz of those who are positive have been used in the Chinese control programme, especially when the anticipated prevalence is low [86]. This strategy was used in the large, nationwide survey on the epidemiology of schistosomiasis in China in 2004, where the participants were first screened for antibodies with an ELISA assay. Those seropositive were subsequently examined with three Kato-Katz slides from a single stool specimen [12]. In Venezuela *S. mansoni* infections are predominately of low intensity. In a comprehensive study Alarcón de Noya and colleagues tested the study population (n = 1495) with three Kato-Katz smears from one stool and three serologic tests: COPT, a variation of SEA ELISA (SMP-ELISA) and alkaline phosphatase immunoassay (APIA). They suggested three criteria for the definition of a 'schistosomiasis case': i) positive Kato-Katz, ii) negative Kato-Katz, but positive COPT and without schistosomiasis treatment the previous year, iii) negative Kato-Katz and COPT, but positive SMP-ELISA and APIA and with no previous treatment. By these criteria the prevalence was 18%, of which 34% were positive by criterion i), 45% by criterion ii) and 21% by iii). Those positive by criterion i) were mainly young adults, those positive by ii) were more evenly spread over the age groups, while criterion iii) was mainly positive among older adults [155;156].

1.7. Objectives

Most of the remaining *S. japonicum* infections in the endemic areas are now of low intensity. It has become increasingly difficult to reliably diagnose these infections with the diagnostic tests commonly used so far. Our aim was to search for a diagnostic alternative. The main emphasis was put on real-time PCR, since this is a method with potentially high sensitivity and specificity.

We wanted to:

1. Develop, optimize and assess a real-time PCR and an associated extraction method for detection of *S. japonicum*.
2. Assess sensitivity and specificity of the PCR in an animal model where the true infection status was known.
3. Compare the PCR with diagnostic tests commonly used on clinical samples from an endemic area.
4. Investigate whether the formol-ethyl acetate sedimentation concentration technique (FEC) could be a valuable diagnostic alternative.

CHAPTER 2 METHODS

The following is an introduction to the main methods used. Please see the ‘Materials and methods’ sections of each paper for more details.

2.1. Real-time PCR

2.1.1. Extraction methods

The purpose of the extraction methods is to release DNA by disrupting membranes and to destroy as many potential PCR inhibitors, especially proteins, as possible. Mainly two extraction methods have been used in the present work.

The non-commercial ROSE (Rapid One-Step Extraction) uses chemicals (‘ROSE buffer’) and heat for extraction, and for denaturation of inhibitors. When first published by Steiner *et al.* the method was used without subsequent ethanol precipitation. They just mixed the resulting eluate 1:170 with water prior to PCR amplification to dilute inhibitors [157]. When Pontes and colleagues used this extraction for their *S. mansoni* PCR, they added a final step where DNA was precipitated with ethanol [126]. This contributes to wash away inhibitors. The final eluate was mixed 1:100 with water prior to PCR amplification. Chelex 100 is a chelating resin sometimes used for DNA extraction as the resin beads bind polar cellular components after breaking open cells, while DNA and RNA remain in water solution above chelex. Chelex was added to ROSE buffer and called ROSEX buffer [158]. Several authors have suggested adding bovine serum albumin to the PCR mixture to reduce the effect of inhibitors, especially when complex biological samples like stool are to be examined [159]. In the first paper all combinations of ROSE or ROSEX buffer, with or without ethanol precipitation and with or without adding BSA were tested. The necessity of diluting the final eluate with water (1:170 or 1:100, respectively) was also tested out for all the different protocols.

The other extraction method used was the commercial QIAamp DNA Stool Mini Kit from Qiagen. Chemical compounds added to the stool lyse the cells and bind inhibitors. DNA is purified in spin columns, where DNA is bound to a silica-gel membrane at the bottom of small tubes, washed and then released. From the kit handbook we used the protocols “DNA Isolation from Larger Amounts of Stool” followed by the protocol “Isolation of DNA from Stool for Pathogen Detection”. The first of these protocols allowed us to use one gram stool. This does not increase the DNA yield, since after the initial lysis a volume equivalent to 220

mg stool are pipetted and used in the second protocol. However, this might decrease the effect of unevenly dispersed eggs in stool. In the first paper we tested an increased lysis temperature of 90° C, but it offered no advantage compared to the standard of 70° C when tested on a small number of samples. We also tried with and without adding BSA to the eluate, the first recommended by the manufacturer.

2.1.2. Primers

Two different primer sets have been used, one set in Paper one and two and the second set in Paper three. Both were designed to target the mitochondrial NADH dehydrogenase I gene from *S. japonicum*, which codes for an enzyme involved in the electron transport. Mitochondrial genes are among others used to study phylogenetic relationships and to recognise different *Schistosoma* strains. The consensus sequences from alignment of nine and ten sequences (first and second primer set, respectively) were used, all published in GeneBank [160-162]. Three of the sequences originated from laboratory strains, six were from wild type isolates and one was of unknown origin. Together they represented seven different geographical areas in China and the Philippines. Primers were designed using Primer Express software.

The primer set chosen for the first two papers was SjND1FW (forward: 5'TGR TTT AGA TGA TTT GGG TGT GC3') and SjND1RV (reverse: 5'AAC CCC CAC AGT CAC TAG CAT AA3'). The resulting PCR product (amplicon) was 82 base-pair (bp) long. SjND1FW was designed as a degenerated primer, with half of the primers containing A and half G in position 3, since one strain (*S. japonicum*-Sichuan) had a C→T replacement at the primer binding site. Later it was also discovered a 3 bp mismatch between this strain and the very end of the forward primer. This has probably no practical consequence, even for detection of this particular strain.

For the third paper we chose the primer set F-SJ-ND1 (forward: 5`-ACT GGT TAT GGT TTG TTG ATG TTA GGT-3`) and R-SJ-ND1 (reverse: 5`-AGC CAC ACG AAC AGC ACT AAT C-3`), which synthesises a 75 base pair long amplicon.

2.1.3. Real-time PCR amplification and detection

When the temperature in the PCR mixture is raised to 95 °C, the two complementary strands in the DNA separate (denaturation). When the temperature is lowered, the forward and reverse primers anneal to each of the two now single-stranded DNA templates. The TaqMan polymerase binds to the primer-template hybrid and starts synthesizing a new strand,

complementary to the template strand, through so-called elongation. This cycle is then repeated. Each cycle will theoretically double the number of amplicons, assuming 100% efficiency. The cycling process is controlled by an instrument, a thermal cycler. If the thermal cycler has real-time format it will simultaneously detect the generation of amplicons/PCR products during the cycling process. The temperature cycling protocol was the same in Papers one, two and three: 2 min at 50 °C, 10 min at 95 °C, followed by cycles of 15 sec at 95 °C and 1 min at 60 °C. In Papers one and two (using SYBR Green) we used 40 cycles and in Paper three (using probe) 50 cycles.

We have used the enzyme uracil N-glycosylase (UNG) to prevent carry-over contamination of previous PCR products. With this system the nucleotide uracil (U) replaces thymine (T) in the PCR products. In the first step of the cycling protocol at 50 °C the UNG enzyme will cut any carry-over PCR products from previous runs at the U binding sites. The next step at 95 °C deactivates the enzyme.

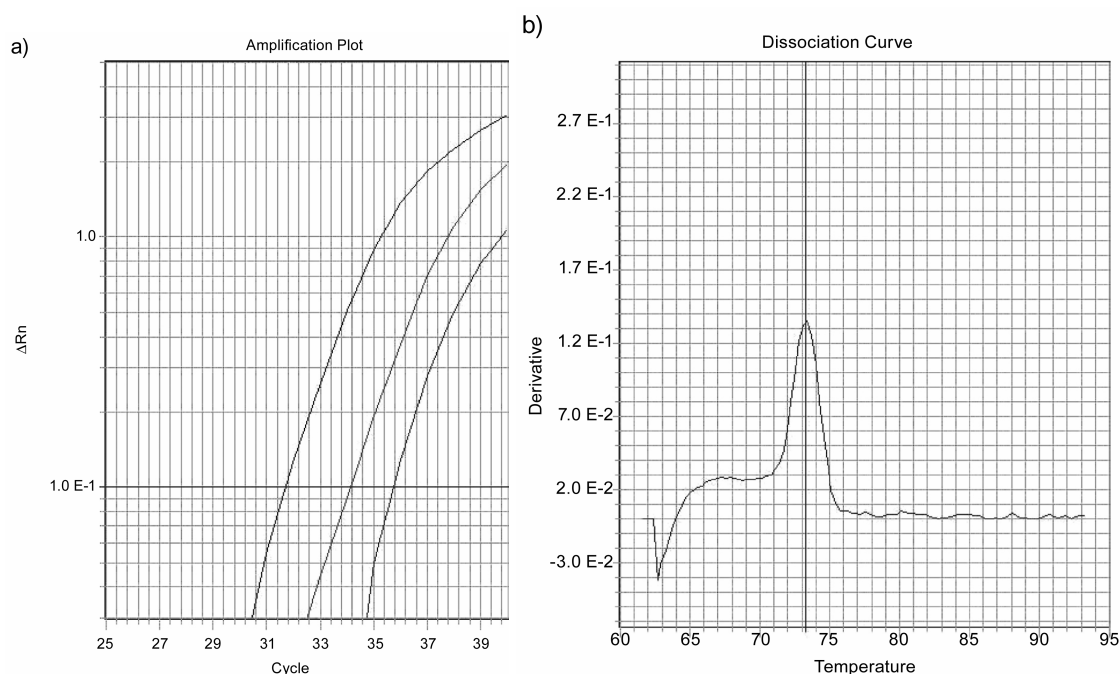


Figure 14. Real-time PCR results as they are presented by the thermal cycler. Plot a) depicts a section of an amplification plot showing increasing fluorescence in three samples containing (from left) 100, 10 and 1 epg. Cycle number on x-axis, fluorescence on y-axis. This plot is similar whether SYBR Green or probes are used for detection. Plot b) shows melting point analysis ('dissociation curve') for SYBR Green. The amplicon has a melting point of 73° C. Both plots from Paper one.

We have used two different systems to detect generated amplicons. In the first two papers we used SYBR Green I, a dye that binds to double-stranded DNA only. SYBR Green emits green light when subjected to blue light. The emission is very weak when the dye is free in solution, but very strong when it is bound to DNA. Hence the fluorescence will increase if there is an exponential increase in amplicons. Since SYBR Green binds to all double-stranded DNA, including spurious amplicons and primer-dimers, a melting point analysis is done after the last PCR cycle in order to increase specificity. When the mixture is heated, the double-stranded amplicons will separate at a certain temperature and the fluorescence disappears. At which temperature this happens, depends on the length of the amplicon and the content of G and C nucleotides, which binds stronger than A and T. If the melting point is at the expected temperature is it likely that the desired amplicon has been produced.

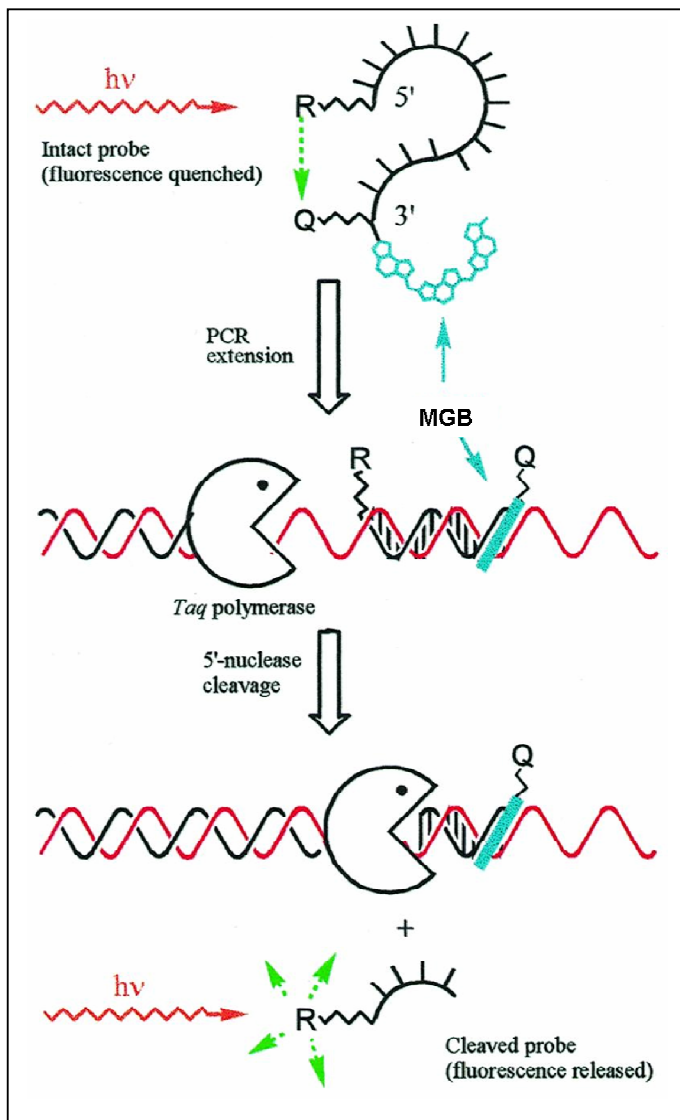


Figure 15. MGB probe. The probe has a reporter molecule in one end which emits a fluorescence signal when subjected to light. In the other end is a quencher molecule which absorbs the reporter signal when the molecules are in proximity to each other. The MGB molecule binds in the minor groove of the DNA and results in a stronger binding between the amplicon and the probe. When the polymerase elongates the complementary strand, it simultaneously breaks up the probe. The reporter and quencher molecules are separated and the reporter will emit light detectable by the thermal cycler. Illustration from [163].

In Paper three we used a Minor Groove Binding (MGB) probe, MGB-SJ-ND1, with the sequence 6-FAM-5'-AGG TTC TTG GAA AAA GTA T-3'MGBNFQ (MGBNFQ = Minor groove binder/Non-fluorescent quencher). The probe binds to the target sequence/amplicon in a similar way as the primers, but in-between these. The use of probes for detection increases the specificity of the assay.

In Paper one and two the samples were run in triplicate. At least one parallel had to be positive for the sample to be regarded as positive. In Paper three PCR reactions were carried out in duplicate from every sample. Samples that were positive in only one parallel were re-run in triplicate, and the test was regarded as positive when at least one of these triplicates was positive.

2.2. Stool microscopy and hatching test

2.2.1. DBL-filtration/sedimentation technique

The technique is sometimes also referred to as the DCEP (Danish Centre for Experimental Parasitology) technique. It was used to detect and count *Schistosoma* eggs in pig stool in Paper two.

In brief, a five-gram sample is taken from a larger, manually homogenized stool sample. The five-gram sample is suspended in 1.2% saline and washed through a series of sieves (400, 100 and 45 µm). The material from the 45 µm sieve is washed with saline into a sedimentation glass and left to sediment twice in the dark. Each time the supernatant is decanted. After centrifugation the sediment is re-suspended to a total volume of 2.25 ml and one-fifth of the sediment is examined with a microscope and chamber slides. The examined volume corresponds to one gram stool, and hence the result is recorded as number of eggs per gram stool (epg) [82].

2.2.2. Formol-ethyl acetate sedimentation concentration technique (FEC)

The technique was used on human stool samples in Paper four. Several variations of this technique exist. We used a commercial kit. Parasep Midi Faecal Parasite Concentrators are closed, single use tubes with built-in filter. They were pre-filled with 6 ml 10% buffered formalin, one drop of surfactant (Triton X-100) and 2 ml ethyl acetate. They

were used in accordance with the manufacturer's instruction sheet (DiaSys Europe Ltd., Berkshire, United Kingdom). A teaspoon was used to measure an amount of stool equivalent to 1 g.

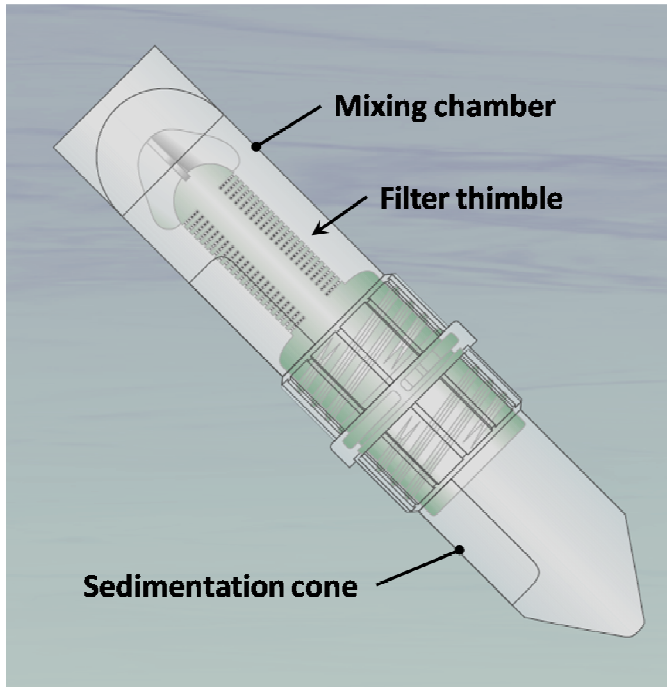


Illustration from manufacturer (modified).

Figure 16. Parasep tube. The stool was added to the chemicals in the mixing chamber, the tube system closed and vortexed. The tubes were then inverted and centrifuged. After removal of the upper layers (ethyl acetate, fatty plug and formol) in the sedimentation cone the sediment is ready for microscopy. A single slide was examined per sample, using a large coverslip (24 x 50 mm), going through the whole slide at 100x magnification.

2.2.3. Kato-Katz thick smear

Kato-Katz was used on human stool samples in Paper three and four.

It required a microscope slide, a screen (piece of nylon mesh), a small plastic spatula, a template (a plastic card with a hole of known volume in it) and hydrophilic cellophane soaked in glycerol. The volume of the hole in the template corresponded to 41.7 mg stool. The template was placed on top of the slide. The screen was placed on top of the stool sample. The spatula was scraped on top of the screen to press stool through the screen. Again using the spatula the hole in the template was filled with sieved stool. The template was removed, leaving a “tablet” of stool on the slide. The “tablet” was covered with a strip of cellophane, the slide inverted and gently pressed to evenly spread the stool sample between the slide and the cellophane [57]. Glycerol clears the stool material and even more so the *S. japonicum* eggs, which were seen in the microscope as holes in a

background “carpet” of stool. Three slides were made from a single stool sample per person and examined within a week. The total number of eggs in all of the three slides was multiplied by eight to give the result as epg.

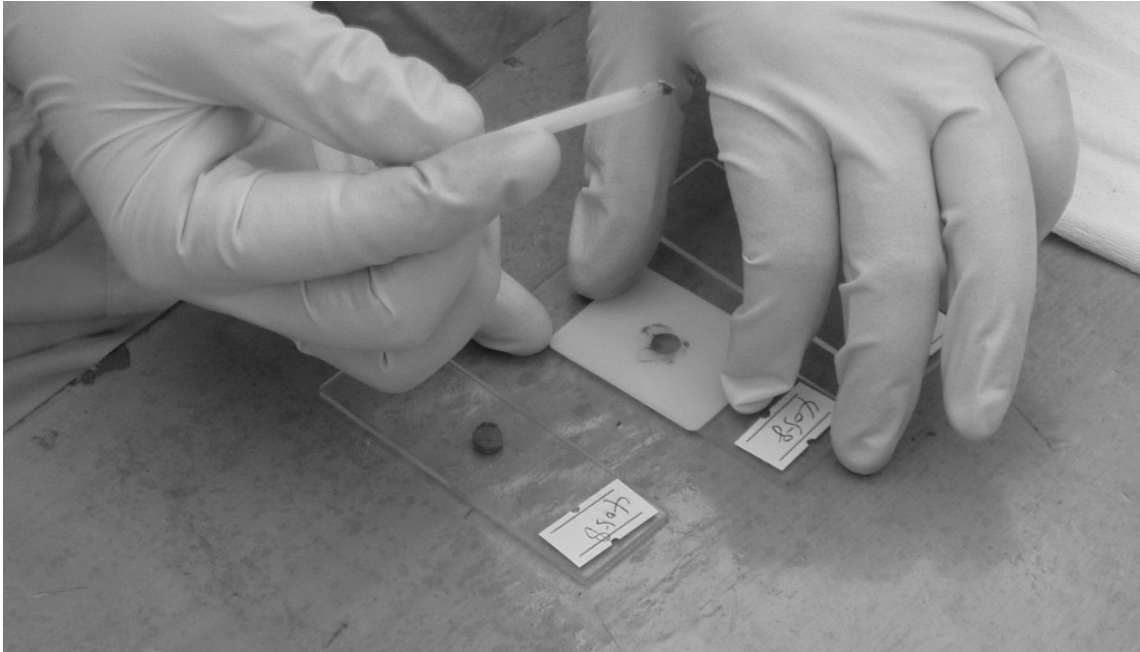


Figure 17. Preparation of Kato-Katz thick smear using a plastic template (white), Anhui province, China.

2.2.4. Hatching test

Hatching test was done by the ‘nylon bag’ method. Approximately 30 gram stool (“size of a dove’s egg”) was put into a metal container with a coarse metal mesh. The fine material was flushed into a fine-meshed nylon bag using flowing water and a stirring stick. Furthermore, the sediment was washed by flowing water and stroking the bag on the outside with chopsticks until the water was clear. The content of the bag was then transferred to an Erlenmeyer flask containing 300 ml water. The water used in the process was non-chlorinated tap water originating from the Yangtze River and stored for a minimum of one day prior to use. The flasks were left in a well-lit room at approximately 28 °C. With a strong light from the back or side of the flasks, they were checked by two of the staff for the presence of swimming miracidia after 4, 6, 8 and 24 hours.

2.3. Antibody detection

2.3.1. Soluble egg antigen (SEA) ELISA

This method was used to measure antibodies in pig serum in Paper two. The method used was a classical ELISA using *S. japonicum* soluble egg antigen as the antigen and HRPO (horseradish peroxidase) and OPD (*o*-Phenylenediamine) for detection.

ELISA plates were coated with SEA (produced at DBL-Centre for Health Research and Development) at 4 °C overnight and then washed. After adding blocking buffer and BSA the plates were incubated at room temperature for 1 hour and washed. Serum was diluted 1:250 with blocking buffer, added to each well and the plates were incubated at room temperature for 2 hours and then washed. HRPO conjugated goat anti-pig IgG-h+1 was diluted 1:4000 with blocking buffer and added to each well and incubated at room temperature for 1 hour before the plates were washed. The plates were developed with OPD substrate and the reaction stopped by adding H₂SO₄ after 18 minutes. The plates were read at 490 nm, using an ELISA reader. Each serum sample was tested in duplicate. A serum sample from one of the infected pigs in the acute stage of *S. japonicum* infection served as a positive control whereas a sample from an uninfected pig was used as a negative control.

All samples were tested during a three days period. Most samples from each pig were tested on the same ELISA plate.

2.3.2. Dipstick dye immunoassay (DDIA)

This method was also used on pig serum in Paper two. The DDIA kit was produced in China [97]. The kit contained a colloidal dye solution with dye labelled SEA and nitrocellulose strips. The control line on the strip consisted of anti-SEA IgG. The test line in the modified version used for animal samples, which was the version used in our paper, consisted of staphylococcal protein A. In the version made for testing human serum, the test line consists of sheep anti-human IgG. If there are antibodies in serum, they will bind to the SEA (which is already bound to a dye) in the solution. When the antibody/SEA/dye complex moves up the strip, the antibody will bind to the

staphylococcal protein A in the test line. The control line is just a control of flow as it will bind directly to the dye labelled SEA. The analysis was performed according to the kit instructions: 50 µl dye solution and 10 µl serum were mixed in the enclosed cup. The strip was inserted into the cup and the result read when all of the solution was absorbed. A very faint purple-blue test line was interpreted as inconclusive and an evident line as positive.

2.3.3. Indirect hemagglutination assay (IHA)

The method was used to measure antibodies in human sera in Papers three and four. We used a kit commercially manufactured at Anhui Provincial Institute of Parasitic Diseases, Wuhu, China. The kit contains human erythrocytes coated or “sensitized” with SEA on the surface. The SEA came from infected rabbits. Serial dilutions of a serum sample were made in a microtiter plate. Sensitized erythrocytes were added to each well, the plate shaken and left in room temperature for an hour. The plates were then checked by the naked eye. If there are antibodies present in the serum sample they will bind to SEA on the erythrocyte surface. The antibody/erythrocyte complex will agglutinate to each other and adhere to the wall of the well, creating a diffuse “carpet” in the well. In a negative sample the erythrocytes will slide down the conical walls of the well and gather as a small, well defined button at the bottom. The test was considered positive when the positive reaction appeared at a titer $\geq 1:10$.

CHAPTER 3 GENERAL RESULTS

This chapter gives a brief summary of the main findings in the different papers.

Paper 1: Novel real-time PCR for detection of *Schistosoma japonicum* in stool.

Lier T, Simonsen GS, Haaheim H, Hjelmevoll SO, Vennervald BJ, Johansen MV. Southeast Asian J Trop Med Public Health 2006 Mar;37(2):257-64.

Aim of study: The aim was to evaluate our novel real-time PCR method for detection of *S. japonicum* in stool samples. Further, we wanted to evaluate different modifications of an inexpensive, non-commercial extraction method, ROSE, as well as the commercial extraction method QIAamp DNA Stool Mini Kit. We wanted to assess sensitivity in stool samples spiked with *S. japonicum* eggs and specificity towards other *Schistosoma* species and helminths commonly found in stool.

Materials and methods: *Schistosoma japonicum* eggs from mouse livers were mixed with one gram non-infected stool. Different combinations of modifications of ROSE extraction were tested against each other: ROSE or ROSEX buffer, with or without ethanol precipitation, dilute or not dilute the eluate, and with or without adding BSA to the eluate. QIAamp extraction was evaluated using two lysis temperatures: 70 and 90 °C. Sensitivity was tested on samples spiked with known numbers of eggs (most samples had <10 epg). Specificity was tested against adult worms of *S. japonicum* (two strains), *S. mansoni*, *S. haematobium* and *S. bovis* and eggs from *S. mansoni*, hookworm, *Trichuris trichiura* and *Taenia sp.* SYBR Green was used for detection of the PCR amplicons.

Results: The ROSE modification yielding the best result was ROSE buffer with ethanol precipitation, BSA and diluting the final eluate 1:100. Using 90 °C yielded no better result than 70 °C in QIAamp extraction and the latter was chosen as the preferred temp. Using these modifications, 29 samples of 30 and 23 samples of 25 were positive with ROSE and QIAamp extraction, respectively. The two *S. japonicum* strains were PCR positive, while the other adult *Schistosoma* worms were negative. The samples with eggs from other helminths were also negative.

Conclusions: The preferred modifications of ROSE extraction and QIAamp DNA Stool Mini Kit extraction were found. There were no obvious differences in sensitivity between these two extraction methods, and both are used in Paper two. The PCR had high sensitivity, even in samples spiked with a single egg. The PCR was also specific for *S. japonicum* when tested against other *Schistosoma* species and helminths.

Paper 2: Real-time PCR for detection of low intensity *Schistosoma japonicum* infections in a pig model. Lier T, Johansen MV, Hjelmevoll SO, Vennervald BJ, Simonsen GS. Acta Trop 2008 Jan;105(1):74-80.

Aim of study: The aim was to use an animal model with low intensity infections to compare PCR, using two different extraction methods, with a sensitive stool microscopy test. Antibody detection should also be done. Finally, we wanted to test the usefulness of PCR in serum, urine and caecal biopsies.

Materials and methods: Fifteen pigs were allocated into two groups of six pigs each, all infected, and an uninfected control group of three pigs. The pigs in one of the infected groups were treated with praziquantel eight weeks post-infection (p.i.), and all pigs followed for 16 weeks p.i. Stool samples were collected from each pig at 16 time points. PCR, using both QIAamp and ROSE extraction, as well as DBL technique microscopy was done on all stool samples. Serum samples were collected at 9 time points and antibody detection was done by SEA ELISA and DDIA. PCR was done on 54 serum samples and 14 urine samples. At the end of the trial, adult *Schistosoma* worms and eggs in liver and caecal mucosa were counted. PCR was done on three caecal biopsies from each pig, taken with a pincher intended for rectal biopsy. We also measured hands-on time spent for each method.

Results: Microscopy examination of 185 stool samples resulted in 35% positive samples, of which 77% contained 1-4 epg. The proportion of positive samples was 40% of 190 samples and 33% of 178 samples for PCR with ROSE and QIAamp extraction, respectively. Egg excretion was highest in the acute stage of infection (day 40-64 p.i.) and with very few positive samples in the subsequent chronic stage, irrespectively of

treatment. In the latter stage PCR gave a higher proportion of positive samples than microscopy: ROSE PCR 17% positive versus microscopy 7% and QIAAMP PCR 15% positive versus microscopy 5%.

Conclusions: It was generally a very low egg output in this study. PCR with either extraction method were as equally sensitive as microscopy. Hence, PCR is highly sensitive, but when faecal egg output almost reaches nil in the chronic stage despite persistent worm burden, both faecal PCR and microscopy results were mostly negative. SEA ELISA titers remained high throughout the study in both the treated and the non-treated group. PCR was consistently negative in serum and urine samples and negative in most of the caecal biopsies. Real-time PCR is less labour intensive than most microscopy methods. Since ROSE extraction was equally sensitive as and cheaper than QIAamp extraction, ROSE was our preferred method to use in Paper three.

Paper 3: Real-time PCR for detection of low intensity *Schistosoma japonicum* infections in China. Lier T, Simonsen GS, Wang T, Lu D, Haukland HH, Vennervald BJ, Hegstad J, Johansen MV. Am J Trop Med Hyg 2009 Sep;81(3):428-32.

Aim of study: We wanted to use clinical samples to compare PCR in stool samples with diagnostic tests commonly used in China.

Materials and methods: A new real-time PCR with MGB probe was designed and evaluated with samples spiked with *S. japonicum* eggs, with adult *Schistosoma* and *Opisthorchis* worms and clinical samples containing *Ascaris* and *Trichuris* eggs. ROSE extraction was used. A single stool sample and a serum sample were collected from 1727 individuals from Anhui province, China. Detection of antibodies in serum was done by IHA. Kato-Katz, hatching test and PCR was done on the same stool sample. Three Kato-Katz slides per sample were examined.

Results: The prevalence using the different tests were 26.1%, 5.3%, 3.2% and 3.0% for IHA, PCR, hatching and Kato-Katz, respectively. The average egg count was low with 57% of the Kato-Katz positive samples having < 40 epg. The degree of agreement between the three stool based tests was low. In fact, 52% of the PCR positive samples

were only positive by PCR. The corresponding figures for Kato-Katz and hatching test were 49% and 33%, respectively. PCR displayed better agreement with IHA than the other two stool-based tests. A commonly used diagnostic algorithm with initial screening for antibodies and subsequent testing with Kato-Katz of the seropositives would have resulted in treatment of 22 persons, compared to 50 persons if PCR replaced Kato-Katz.

Conclusions: The prevalence was very high when IHA was used. PCR resulted in a higher prevalence than hatching or Kato-Katz. However, the low agreement of the results between the three stool based tests suggest that the diagnostic problem is more serious than just the difference in prevalence between the three tests. Possible reasons for this disagreement are discussed. We also showed that it is possible to do a cheap, non-commercial DNA extraction in a local laboratory with quite basic equipment and do the amplification itself in a larger laboratory.

Paper 4: Low sensitivity of the formol-ethyl acetate sedimentation concentration technique in low-intensity *Schistosoma japonicum* infections. Lier T, Simonsen GS, Wang T, Lu D, Haukland HH, Vennervald BJ, Johansen MV. PLoS Negl Trop Dis 2009;3(2):e386.

Aim of study: The formol-ethyl acetate sedimentation concentration technique (FEC) is preferred by many clinical microbiology laboratories for the detection of parasites in stool samples. It was potentially more sensitive than the diagnostic methods traditionally used for detecting low intensity *S. japonicum* infection, but no studies had been published. We wanted to compare FEC with diagnostic tests commonly used in endemic areas, using clinical samples.

Materials and methods: A sub-set of samples from the study in Paper three was selected based on the result of Kato-Katz thick smear and hatching test (approximately one-third positive). Kato-Katz, hatching and FEC were done on the same stool sample. Detection of antibodies in serum was done by IHA. A person was considered positive by the 'reference standard' if IHA was positive together with Kato-Katz positive and/or hatching test positive.

Results: Out of a total of 106 samples there were 47 (44%) positive for IHA, 19 (18%) for Kato-Katz thick smear, 27 (26%) for hatching test and 10 (9%) for FEC. A total of 28 persons (26%) were reference standard positive, and this resulted in a sensitivity of 28.6% and a specificity of 97.4% for FEC. Two samples were FEC positive, but reference standard negative. As in Paper three the average egg count was low and the agreement of the result between the different tests was also quite low.

Conclusions: Irrespective of the problems with finding a reliable reference standard, FEC seems to have low sensitivity in low intensity *S. japonicum* infections.



Figure 17. Basic equipment for DNA extraction, Anhui province, China.

CHAPTER 4 DISCUSSION

There are several situations where a reliable diagnosis of *S. japonicum* infections is important; assessment before treatment, evaluating efficacy of control interventions, detection of resurgent infections before and after elimination etc. Diagnostic tests are applied in many different scenarios, ranging from care for the individual patient in a hospital to the different stages of a control programme and in scientific trials. In the papers and in this thesis I have tried to emphasize that different diagnostic scenarios demand different diagnostic strategies. When the control of schistosomiasis in an area progresses, the endemicity of the parasite as well as the objectives of the control programme changes. When endemicity is high, reduction of infection intensity and morbidity control is usually the goal, and mass administration of efficacious drugs the mean to reach this goal. In this situation, infections are usually of high intensity and hence “easier” to diagnose. The focus is also more on simplicity and cost than on sensitivity of the diagnostic test. As endemicity decreases, reduction in prevalence and later transmission control becomes the goal. The change in objectives and even more so the change in endemicity will demand more of the diagnostic tests. The need for a dynamic view on diagnostic strategies has been discussed thoroughly in an opinion paper by Bergquist and colleagues [56]. The shortcomings of the conventional methods for diagnosing *S. japonicum* in the endemic areas today have been discussed in the papers and previously in this thesis. At the moment I cannot see an obvious solution to this diagnostic dilemma. In this chapter I will discuss pros and cons, limitations and possible future research needs for PCR and other diagnostic strategies.

Direct detection of eggs in stool and detection of antibodies in serum represent two different approaches, each with certain advantages and disadvantages, some of which have been discussed in chapter 1.6. One advantage of methods detecting eggs in stool is that they are perceived as having high specificity. Whether this is true for Kato-Katz when *S. japonicum* eggs are scarce is discussed in Paper three. Probably the main disadvantage with these methods is low sensitivity in low intensity infections. Only a small amount of stool is processed, often just a fraction of a gram. This is especially true for Kato-Katz. We processed a full gram of stool for the FEC technique, but only a minor

portion of the resulting sediment is examined. Even a full gram of stool represents only a few percent of the daily stool output in a human [29]. In the animal study in Paper two we did indeed experience that egg output can be very low. In the chronic phase almost all stool samples were negative despite persisting worms. All methods also have a varying degree of eggs lost during processing. In addition there is the problem of uneven egg distribution within the sample and day to day variation in egg excretion [61;164]. Several authors have suggested increasing Kato-Katz sensitivity by examining multiple smears, although it is not universally agreed whether or not they have to be from different, consecutive samples [51;61;63]. Examination of two or more samples represents of course a larger logistic challenge to a control programme than a single sample. I am not sure if examining more Kato-Katz slides is sufficient to solve this diagnostic dilemma when using a realistic number of smears. Yu *et al.* examined duplicate smears from seven consecutive stool samples per person without seeing a flattening out of the prevalence from the first to the last smear [61]. It should be noted that 24 smears (41.7 mg) are needed to amount to one gram stool. In addition, if false positive Kato-Katz really is a problem when eggs are scarce, this problem will not decrease with increasing number of smears.

The study in Paper four did not indicate that the FEC technique was a better diagnostic alternative. However, as a sidetrack, I was impressed by the Parasep tubes used. They were clean and easy to use, requiring very little processing hands-on time compared to a traditional open FEC assay. From a practical standpoint I can recommend these if FEC is to be used in a field study. The price is however higher.

It would be very useful to have a sensitive test for direct detection of *S. japonicum* (i.e. eggs in stool), even if it is more labour intensive than Kato-Katz. Two techniques, Percoll and FLOTAC, have been presented as sensitive microscopy based methods for detecting helminth eggs in stool [138;165-167]. Allam *et al.* compared Percoll, PCR and Kato-Katz for detecting *S. mansoni* in stool. Even though PCR detected more cases than Percoll, the authors preferred the latter due to simplicity, cost and better agreement with Kato-Katz, since a few ‘certainly positive’ samples were negative with PCR [138]. FLOTAC detected more cases of *Ascaris*, *Trichuris* and hookworm when compared to Kato-Katz, and in one case also FEC [166;167]. Neither of these have been tested for *S. japonicum*.

The Percoll technique processes only 220 mg stool (FLOTAC up to 1 gram) and FLOTAC seems to result in considerably lower egg counts than Kato-Katz. Both facts can become a problem if the techniques are used on low intensity *S. japonicum* infections. However, it would be interesting to see their performance in clinical samples from such infections.

A potential problem to all stool based tests is that stool is an “unpopular” sample specimen. Over time this dissatisfaction may lead to lower compliance to control programmes and even “cheating”, like passing a single stool off as coming from several people [51;155]. Collecting serum samples is probably more accepted in the population. In light of this, to detect schistosomiasis by PCR in serum is an interesting idea, as suggested by Wichmann and colleagues [140]. The usefulness of this in low intensity *S. japonicum* infections is however far from proven.

The problem with false positives when assays like IHA and SEA ELISA are used for antibody detection has been discussed in the papers and in chapter 1.6.2. Despite this, several authors argue for the use of serology in control programmes when prevalence and intensity is low [56;89;90]. Indeed there are several advantages with this strategy; it is simple, relatively cheap, has higher sensitivity than available stool based tests etc. Moreover, a certain degree of false positives and subsequent treatment of not infected people may be justified by the very few serious side-effects and the low cost of praziquantel. However, those responsible for a control programme must define at which point this practice is not economically or ethically acceptable. As prevalence falls there is a risk of treating far more people that are not infected than those who are. It is possible that implementing new antibody assays with purified antigens could reduce some of the problems related to the present assays [86;91]. Some of these alternative assays have shown promising results, but very few have been tested in large trials. This is an area in need of more research. These alternative assays would come at a higher cost than the presently used IHA or SEA ELISA. Since the assays would be used on predominantly negative samples, a possible way of keeping cost down could be to test on pooled sera; that is mixing serum from several people and run the test on the mixture. If the mixture is positive, one has to test the individual components of the mixture. A study to investigate the use of pooled sera to diagnose *S. japonicum* with IHA has been done by Jia and

colleagues [168]. It would of course be necessary to investigate whether such pooling is feasible in order to maintain an acceptable sensitivity and what would be the ideal number of sera pooled.

Combination of tests is another interesting diagnostic strategy which can be useful in low prevalent settings. It can be carried out in two principally different ways. You can make a definition of a positive case which involves tests on equal footing. A person is considered positive if positive with either test A or test B. In principle this means testing with test B those who are negative with test A. This has been done by de Noya and colleagues in Venezuela (see chapter 1.6.5. for more details) [155]. A different approach is to do testing in series by first testing with test A and subsequently testing those positive with test B. This is a common strategy for example in HIV testing in Europe and North America, where prevalence is low and reliability of the test result is important. It has also been used for schistosomiasis in China by using IHA or SEA ELISA as the first test and Kato-Katz as the second, either on the general endemic population or selectively in areas with especially low prevalence (often <1%) [12;86]. In such a strategy it is essential that test A has high sensitivity, while high specificity is desirable, but not critical. Preferably, test A should also be simple, cheap and easy to perform. Antibody detection in serum is often a natural choice. Whether IHA or SEA ELISA is suitable as test A is debatable. Even though low specificity is considered the largest drawback of these tests, there is also some controversy regarding sensitivity. As mentioned previously, the lack of a reliable 'gold standard' has resulted in various figures for test properties. If the figures for sensitivity of around 80% as reported by some (see chapter 1.6.2.) are indeed true, it will mean that one of five positive will be "lost" even in the first testing round. At the present time there are not many well tried out alternatives to these two. The "new" test population (those positive in test A) will have a higher prevalence and hence result in a higher positive predictive value for test B. Even so, the selection of test B is even more difficult, since this test ideally should have both quite high sensitivity and specificity. If some false positives are acceptable, sensitivity should be prioritized. Since the number needed to test has been significantly reduced, a more expensive and more labour intensive test can often be tolerated. Test B can be a stool based test or a different serological test. A limited number (i.e. 1-3) of Kato-Katz slides has in my opinion

probably not sufficient sensitivity to be used as the second test when infection intensity is low. This was demonstrated in Paper three. More than twice as many people would have received treatment if PCR had been used as test B compared to three Kato-Katz smears. What could be an acceptable test B is not established; whether multiple Kato-Katz smears from a single or multiple samples will suffice or an entirely different test.

Presently it is uncertain if PCR will play a role in a future *S. japonicum* diagnostic strategy. Whether a certain test has a role to play depends only in part on the accuracy of the test itself. Cost, simplicity, availability of equipment, knowledge and policy priorities are among other factors determining if a diagnostic test will be used. There is yet limited experience with the use of *S. japonicum* PCR in clinical samples, and more research is needed. Choosing the best PCR target is one example. When we first embarked upon this project, there were no *S. japonicum* PCR published. By now there are several, and with different primer targets. There are specific reasons why we chose the mitochondrial NADH dehydrogenase I gene as the target. Firstly, several sequences of the gene, representing different geographical locations, were published. This made it more likely that our target was conserved and that our PCR could be used in different locations. Secondly, as there are many mitochondria and hence several copies of the target in each cell, we hoped this would increase the sensitivity. Lastly, since the gene codes for an essential enzyme, we assumed that mutations would be less likely than in a non-coding region.

We also chose the real-time format, mainly because it makes it much easier to process a large number of samples compared to using agarose gel for detection. The downside to real-time PCR is that it requires a more expensive thermal cycler. We tried to outline a possible solution to this in Paper three by showing the feasibility of doing a cheap DNA extraction at the local lab and the amplification and detection of the product in a centralized lab where a real-time cycler may be available. Prices on PCR equipment and consumables are becoming more and more acceptable. An increasing number of laboratories in low income countries now have or will have real-time PCR technology available for HIV viral load determination and other assays. Another advantage with the real-time format is the possibility of a quantitative test. This was very briefly explored in Paper one, but would need a more thorough investigation. A quantitative test is

advantageous, but it usually suffices to be able to categorize into intensity groups (for example <40, 40-99 and ≥ 100 epg). The specificity of the PCR assay should also be explored further. We tested specificity towards the closest relatives, the other *Schistosoma* species, and towards a few other helminths and bacterial species. However, other helminths and especially other flukes present in endemic areas, such as *Paragonimus*, could with advantage be included. Specificity with stool samples not containing *S. japonicum* is another idea. Ideally these non-infected stool samples should come from within or close to the endemic areas, as there is presumably quite a difference in the intestinal flora in for example China and Northern Norway.

Even though our PCR detected more positive samples than Kato-Katz and hatching, it does of course not rule out false negative PCR samples. As mentioned previously in this chapter, Allam *et al.* noticed a few samples that probably contained *S. mansoni* eggs, but were negative by PCR [138]. The same was noticed by Pontes *et al.* and Hove *et al.* [127]. In figure one in Paper three about agreement between the stool based tests, three samples are positive for both Kato-Katz and hatching, but PCR negative. This could be because there by chance simply were no eggs in the sample used for PCR. It could also be due to substances in the sample inhibiting the PCR or degradation of target DNA. It would be interesting to see whether inhibition is a problem. This could be done by an internal control strategy; a known DNA sequence/target is added to the samples and amplified by a different set of primers. If the internal control is negative, inhibition might be present in the sample. However, this strategy will not tell the whole story, as only one of the PCR could be inhibited and not the other. Presence of inhibition does of course not necessarily mean a false negative sample, as there might not be *S. japonicum* present either. However, it would give the opportunity to resolve the issue by trying to dilute the sample more, ask for a new sample for PCR or demonstrate the infection by other means. Using such internal controls in a routine PCR is probably not realistic, as it will add cost and complexity to the assay, but it would be feasible as a study. In the already mentioned study by Hove *et al.*, they used this strategy on stool samples from 88 individuals. They found no sign of inhibition, but it is difficult to extrapolate these results due to differences between their assay and the ones used in the present thesis.

One valid objection to the possible strategies discussed in this chapter is that costs have not been sufficiently emphasized, and high costs are perhaps the biggest disadvantages of PCR assays. On the other hand, if the diagnostic strategy in an area does not make the diagnosis accurate enough to help controlling the parasite, then that would be a waste of money, too. Sadly, it seems that most improvements of the currently used diagnostic strategies will lead to an increase in cost. The cost issue, combined with the possibility of testing serum samples, makes the LAMP technique described in chapter 1.6.3 a most interesting line of research. The promising initial results by Xu *et al.* must be confirmed in larger trials [145].

Even though diagnostics are important for several purposes, it is just a piece in the puzzle to achieve efficient control of *S. japonicum*. Morbidity control, mainly through mass administration of praziquantel, has been cost-effective for reducing the number of infected persons. However, it now seems like the time has come to implement new control measures at least in part of the endemic areas. This has been nicely outlined in two papers by Wang and colleagues [30;169]. Unfortunately, increased costs seem unavoidable to achieve further progress in *S. japonicum* control programmes.

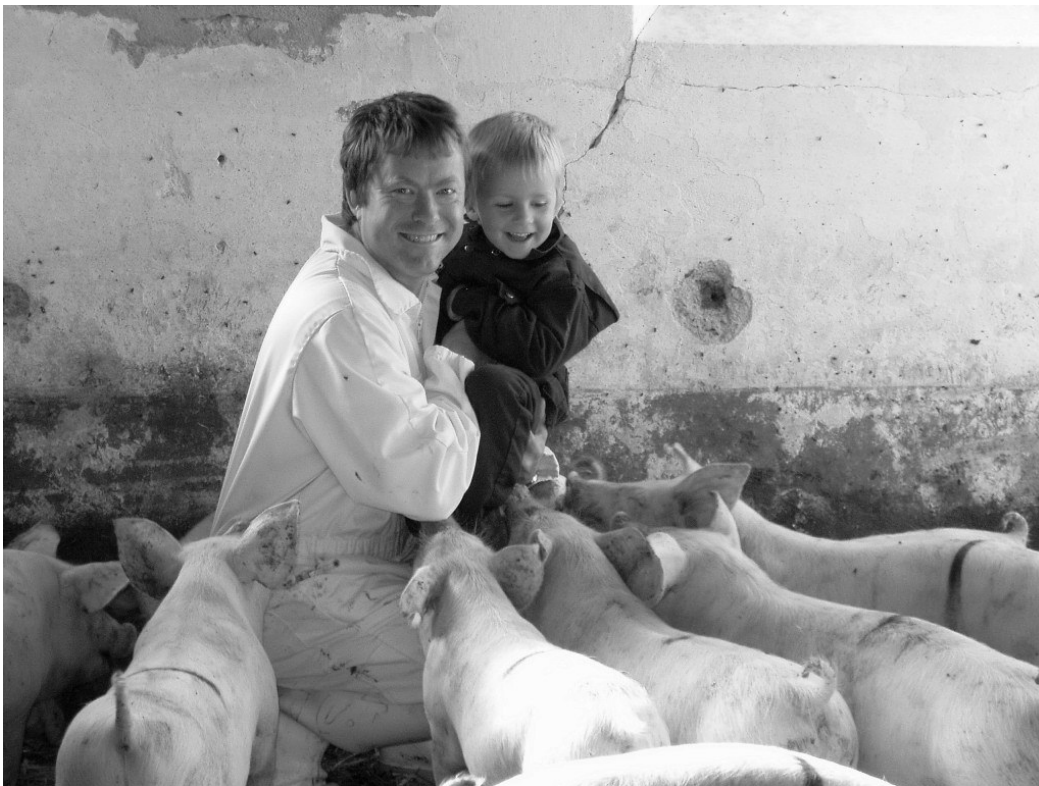


Figure 18. Early in the animal study (Paper two).

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Paper I

Paper II

Paper III
+ Appendix

Paper IV

