DIAGNOSTIC POTENTIAL OF EXTANT ANTI-*SCHISTOSOMA* GENUS-SPECIFIC MONOCLONAL ANTIBODIES

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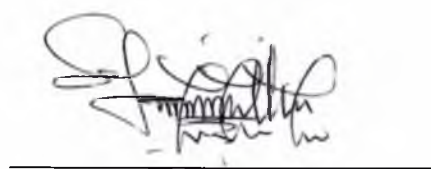
DECLARATION

I hereby declare that except for the references cited from the work of other researchers, which I have duly acknowledged, this work was as a result of my own original research and this thesis, either in whole, or in part has not been presented for another degree elsewhere.



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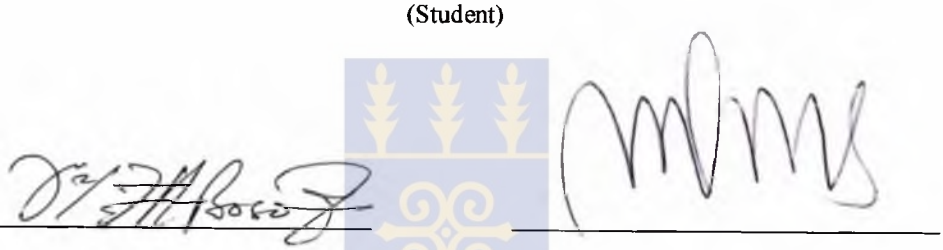

Dr. K. M Bosompem
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DEDICATION

To my wife
And
Children

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ABBREVIATIONS

ABTS	Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
DAB	Diaminobenzidine tetrahydrochloride
DMSO	Diamthylsulfoxide
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FITC	flourescein isothiocynate
Hr	Hour
HRPO	Horseradish peroxidase
IFAT	Indirect immunoflourescent antibody test
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
l	Litre
MoAb	Monoclonal antibody
ml	Millilitre(s)
min	Minutes
Mr	Molecular weight
µl	Microlititre
µg	Microgramme
NMIMR	Noguchi Memorial Institute for Medical Research
PBS	Phosphate buffered saline

PH	Negative logarithm base of hydrogen ion concentration
TBS	Tris buffered saline
Xg	Times gravitational force
ABTS	2,2-azo-bis-3-ethylbenzthiazoline-6-Sulphonic acid
OPD	O-phenylenediamine
TMB	3,3' 5,5'-tetramethylbenzidine base
pNPP	p-nitro phenylphosphate
IMDM	Iscove's Modified Dulbecco's Medium

ABSTRACT

The suitability of five *Schistosoma* genus-specific monoclonal antibodies (MoAbs) (Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10) in detecting schistosome antigens in infected human and cattle was evaluated. These antibodies were employed in various diagnostic assays to diagnose human and animal schistosomiasis. Extant MoAb secreting hybridoma cells were first propagated in culture to produce the MoAbs. The culture supernatants containing secreted antibodies were analysed by immunodiffusion and the isotypes of the immunoglobulins shown to be IgM (Sh3/34.10, Sh3/38.2, Sh5/32.30 and Sh5/34.10) and IgG1 (Sh4/14.3). Gel purified fractions of the MoAbs were utilised in developing dipstick ELISA and micro-plate ELISA in diagnosing schistosomiasis. Also, the suitability of the indirect immunoflourescent Antibody Test (IFAT) was employed to demonstrate anti-*schistosoma* antibodies in the blood of infected cattle.

Three of the antibodies (Sh3/38.2, Sh4/14.3, Sh5/32.30) showed no cross-reactivity with *Plasmodium falciparum* circum-sporozoite protein (CSP) and crude antigen extract of *P. falciparum.* However Sh3/34.10 and Sh5/34.10 reacted with the crude antigen extract of *P. falciparum* at high antibody concentrations even though the reactivity was abrogated at higher antibody titres.

In human schistosomiasis, the diagnostic potential of the MoAbs in detecting schistosome antigens were assessed alongside microscopy and the standard Sh2/15.F urinary schistosomiasis dipstick ELISA developed by researchers at the NMIMR. Out of 74 human subjects from a schistosomiasis endemic area screened for urinary schistosomiasis, 81.1% were microscopically positive for *S. haematobium* eggs whilst the standard dipstick assay gave prevalence estimate of 87.3%. The sensitivity of this assay was 100% whereas the specificity was 64.7% compared with microscopy as the gold standard test. Dipstick assays utilizing the individual *Schistosoma* genus-specific monoclonal antibodies estimated prevalences of urinary schistosomiasis between 48.6 and

58.1%. The sensitivities of the assays were however lower (60.0-71.1%) compared with microscopy as the gold standard test. Nonetheless, each of the antibodies showed a high specificity of 100% in detecting *S. haematobium* urinary antigens. The *Schistosoma* genus-specific antibodies performed similarly when utilized in dipstick to detect *S. mansoni* infections.

Two assays (IFAT and MoAb-based plate ELISA) were developed to diagnose animal schistosomiasis in cattle. The sensitivities of these assays compared well with microscopy. In determining the prevalence of *S. bovis*, microscopy gave a prevalence of 53.1% compared with 50.0% determined by IFAT. Micro- plate based ELISA utilizing different *Schistosoma* genus-specific MoAbs estimated prevalence of *S. bovis* between 46.9% and 50.0%. These assays were sensitive (ranging from 88.2-94.1%) compared with microscopy as the gold standard test and the specificity was each 100%. In view of the limitations of the microscopical approach the assays provided alternative diagnostic tool in detecting animal schistosomiasis in cattle.

The study also demonstrated 3.1% (1/32) prevalence of *S. indicum* in mixed infection with *S. bovis* by microscopy. Eleven other cattle parasites eggs were demonstrated by microscopy with prevalence ranging from 3.1-21.9%. There was no evidence of cross-reactivity between the antigens of these parasites and the *Schistosoma* genus-specific MoAbs utilised.

This study revealed the diagnostic potentials of the *Schistosoma* genus-specific MoAbs in detecting schistosome antigens in both humans and cattle. The development of *Schistosoma* genus-specific MoAb-based dipstick ELISA for diagnosing schistosomiasis is promising.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Schistosomiasis is the name given to the group of human and animal diseases caused by blood-dwelling helminth of the genus *Schistosoma.* Schistosomiasis infections are normally chronic and take many years. Despite the availability of chemotherapy, more than 200 million people and millions of domestic animals are infected with schistosomes in 73 countries that harbour the intermediate snail host of the family Planorbidae (Manson-Bahr and Bell, 1991). In addition, 600 million people are at risk of infection. The affected areas include Africa, South America, India, South-East Asia, the Far East, part of the Middle East and the Mediterranean countries including a small focus in Portugal (Rollinson and Southgate, 1987). The World Health Organisation (W.H.O) reports that schistosomiasis is second most prevalent parasitic disease in the tropics after malaria (Atlas, 1995).

Three main species of schistosomes (*Schistosoma mansoni, S. japonicum* and *S. haematobium*) are responsible for infection in humans (Boothroyd and Komuniecki, 1995). However the contributions of other species such as *S. mekongi* and *S. intercalatum* cannot be underestimated. A number of species are also known to infect livestock. These include: *S. matheei* (commonly in cattle, sheep and goat); *S. bovis* (cattle); *S leiperi* (herbivores); *S indicum* (variety of domesticated animals including sheep camel goat and cattle) and *S. spindale* (ruminants) (Rollinson and Southgate, 1987). Schistosomiasis is primarily a rural disease whose transmission depends on a variety of factors including; (1) contamination of fresh water with human/animal urine or faeces containing viable *Schistosoma* eggs, (2) the presence of schistosomiasis host snails and in the case of aquatic species, water temperature, rate of flow, acidity or alkalinity and the content of organic matter conducive to snail growth are important factors and (3) human contact with water

containing infective cercariae (Manson-Bahr and Bell, 1991). Infection with schistosomiasis is initiated when cercariae penetrate the skin and transform into schistosomula, which subsequently enter the vasculature and migrate via the lungs to the hepatic portal system (*S. mansoni* and *S. japonicum*) or the urogenital venules (*S. haematobium)* (Boothroyd and Komuniecki, 1995). In some parts of Africa, where *S. haematobium* and *S. mansoni* are prevalent, mixed infections are common. Manson-Bahr and Bell (1991) reported 60% mixed infections in parts of the Nile Delta and 22% in European patients in Zimbabwe.

Typically in areas endemic for Schistosomiasis, the distribution of infection intensity is overdispersed, such that younger people (less than 18 years old) excrete more eggs and therefore are more heavily infected than older individuals (Boothroyd and Komuniecki, 1995). Following chemotherapy, re-exposure to infective cercariae leads to rapid re-infection in young people who may regain heavy infection whereas older individuals tend to be less severely affected.

A definitive diagnosis of schistosomiasis may be achieved by examination of the urine, stool, rectal or bladder biopsy for eggs through microscopy and also by serological tests. The microscopical technique, which is the most commonly used, is very specific (100%) but not sensitive enough. Moreover it is time consuming, tedious and not field applicable making its dependency limited. There is therefore the need for differential diagnosis. The Serological tests (based on the detection of host antibodies directed against schistosome antigen) on the other hand are useful in diagnosis of both apparent and inapparent infections. Virtually all the well-established assays including Enzyme-linked immunosorbent assay (ELISA), Indirect Immunofluorescence assay, Slide flocculation, Plasma card, Precipitin, Indirect Haemagglutination, Miracidial immobilization, Circumoval precipitin test (COPT) and Complement fixation test (CFT) (Manson- Bahr and Bell, 1991), have been applied to identification of schistosome species and the diagnosis of schistosomiasis, however, very few have been advocated for large-scale use in

diagnosis due to lack of sensitivity. Among the most promising alternative diagnosis for urinary schistosomiasis are the *Schistosoma* genus- specific circulating anodic and cathodic antigen (CAA and CCA) detection assay (Kremsner *et al.*, 1994; De Jonge *et al.*, 1989) and the recently developed *S. haematobium* species-specific dipstick ELISA (Bosompem *et al.*, 1996a). These are more sensitive and detect circulatory and or urinary antigens utilizing non-invasive membrane-based assays, which are more suited for field use in endemic areas. The CAA and CCA detection assays are yet to be adopted for use. On the other hand, the urinary schistosomiasis dipstick has been tested in some parts of Ghana and successfully adopted for application in routine diagnosis of the disease in the field. The specific detection of *S. mansoni* is yet to be developed.

The incidence of mixed infections limits the extent of the applicability of the urinary *Schistosoma* dipstick method in diagnosis because positive test results are silent on the mixed infections of *S. haematobium* with other schistosome species. There is thus the need to develop field applicable assays, which are either *S. mansoni* species-specific or *Schistosoma* genus-specific so that other schistosome infections and or mixed infections could be diagnosed. In circumstances where the urinary *Schistosoma* dipstick is used alongside a *Schistosoma* genus-specific assay in humans, intestinal schistosomiasis may be diagnosed by exclusion. It was for these reasons that the present study was conducted to determine the diagnostic potential of existing *Schistosoma* genus-specific monoclonal antibodies and further utilised in the development of membrane/micro-plate based ELISA's for the diagnosis of both human, and animal schistosomiasis, which has little attention.

Objectives of the study

(i) To produce monoclonal antibodies *in vitro* by propagating existing hybridoma cells and purifying the antibodies for use in diagnosis.

(ii) To determine the specificity of purified monoclonal antibodies in cross-reactivity studies using crude antigen extracts of *S. haematobium*, *S. mansoni, S. japonicum,* and *Plasmodium falciparum*..

(iii) To utilize *Schistosoma* genus-specific monoclonal antibodies in developing membrane or micro-plate based ELISA for diagnosis of schistosomiasis.

(iv) To diagnose *S. mansoni* infection in humans by exclusion using the developed *Schistosoma* genus-specific ELISA and the existing *S. haematobium* species-specific dipstick method. ELISA.

(v) To utilize the developed *Schistosoma* genus-specific monoclonal antibodies in detection of schistosome antigens in infected animals using blood specimens.

(vi) To determine the specificity and sensitivity of the *Schistosoma* genus- specific monoclonal antibody-based assays by comparison with the microscopical detection of parasite ova in urine and stool specimens.

Justification for the study

The determination of the diagnostic potential of *Schistosoma* genus-specific monoclonal antibodies would promote accurate diagnosis of mixed schistosome infections in humans. Also the establishment of field applicable *Schistosoma* genus-specific diagnostic assays would enable investigations into the occurrence and spread of animal schistosomiasis in Ghana on which currently there is little, if any, information available.

LITERATURE REVIEW

Schistosomiasis and Schistosomes

Schistosomes are trematodes belonging to the family Schitosomatidae. Members of this family show morphological and physiological peculiarities, which set them apart from other trematodes. They are dioecious digenia. The male worm is flat and leaf-like and is folded to form a gynaeocophoric canal formed by ventrally flexed lateral outgrowths where the slender female is held (Manson-Bahr and Bell, 1991; Rollinson and Southgate, 1987).

Schistosomes live in the blood stream of warm-blooded hosts, being the only trematodes to do so. The specific location of the parasite in the host depends on which schistosome species is involved. For instance *S. haematobium* localises in the vesical plexus around the urinogenital system whilst *S. mansoni, S. japonicum, S. intercalatum* and *S. mekongi* are normally found in the hepatic portal system around the gastro-intestinal tract (Sturrock, 1993).

The life cycle of schistosomes consists of alternate generations, each with its own host. The adult worms infect vertebrates and the two larval stages miracidia and cercaria occur in susceptible snail. Miracidia hatch from the eggs and invade the snails specific to each schistosome species whilst the cercaria leave the snail to invade definitive host. A feature of the life cycle of schistosomes is that multiplication takes place in each of the different hosts so that it is very difficult to break the cycle (Manson-Bahr and Bell, 1991).

The family schistosomatidae is divided into three subfamilies, the Schistosomatinae, Bilharziallinae and Gigantobilharzianae (Rollinson and Southgate, 1987), consisting of twelve genera in all. Seven out of these genera are confined to birds and the rest to mammals. However, only the genus *Schistosoma* is associated with man (Rollinson and Southgate, 1987). This genus has achieved the greatest geographical distribution and diversification in terms of numbers of recognised parasite species and

different hosts parasitised. The overall distributional range of the different schistosome species is primarily influenced by the presence or absence of suitable mammalian and intermediate snail host (reviewed by Sturrock 1987). Some schistosomes easily infect snail of one geographical area but fail to infect or infect with difficulty the same snail from a different area (Manson-Bahr and Bell, 1991). The genus *Schistosoma* is recognized by eighteen species placed in four different groups also called species complexes by Kuntz (1955). This was based on the common relationships of the parasite species within particular snail host genera and on zoo-geographical distribution pattern as well as the morphology of the parasite eggs. The groups are the *S. haematobium, S. mansoni, S. japonicum* and *S. indicum* species complexes.

The S. haematobium group

Schistosoma haematobium was the first schistosome to be described. Bilharz identified the parasites in 1852 and in 1864 Harley reported urinary schistosomiasis in people in South Africa (Rollinson and Southgate, 1987; Sturrock, 1993). The *S. haematobium* group is widely distributed in Africa and the Eastern mediterranean region including Iran, Islamic Republic of Iraq, Jordan, Lebanon, Oman, Saudi Arabia, Syria and Yemen (Sturrock, 1993). The species complex consists of seven species. These are *S. haematobium, S. intercalatum, S. metheei, S. bovis, S. carassoni, S. margrebowiei* and *S. leperi*. Members within the group show substantial dissimilarities in the morphology of the egg as well as the definitive host specificity. There is thus evidence for geographical variation in many characters associated with the *S. haematobium* group of species. For example, studies by Wright and Knowlers (1972) on laboratory hamsters showed that differences occur between geographical strains of the parasite in many biological features such as intermediate host specificity, infectivity of cercariae, growth rates and maturation times of adult worms, fecundity rate and host-organ distribution of eggs. In general the intermediate host of *S. haematobium* species complexes are the *Bulinus* species. *S.*

haematobium in the Afrotropical region is transmitted by snails of the *B. africanus* group, whilst in the Mediterranean area and S. W. Asia it is by tetraploid members of the *B. truncatus/tropicus* complex. In Arabia and Mauritius transmission is by members of the *B. forskalii* group. In West Africa, however, all the three snail groups are known to act as intermediate hosts for *S. haematobium* (Frendson, 1979). Of particular significance is the division between *S. haematobium* from North Africa and the Middle East, and the parasite from the Afrotropical region. With few exceptions, neither of the parasites can develop in the snail host of the other.

Though there are marked differences between members of the *S. haematobium* group, natural hybridisation has been reported to occur. For example a Camerounian strain of *S. haematobium* and *S. intercalatum* hybrid was reported from Loum, in a previously 'pure' *S. haematobium* area (Jordan and Webbe, 1993). Again, ample evidence has accumulated from different sources with various reports of the occurrence of natural hybrids of *S. haematobium* and the cattle parasite *S. metheei* in humans (Van Wyk, 1983).

The *S. mansoni* group

Intestinal schistosomiasis in man is mainly caused by *S. mansoni*, the schistosome species with considerable amount of information (Rollinson and Southgate, 1987). The early stages of chronic infections are free of obvious symptoms and signs but the later stages, with liver and spleen enlargement, are more easily detected by clinical examination (Jordan and Webbe, 1993). The parasite is common in Libya, Oman, Saudi Arabia, People's Democratic Republic of Yemen, Madagascar and over greater part of Africa south of the Sahara. In the Carribbean it is endermic in Puerto Rico, Saint Lucia, Antigua, Dominican Republic, Martinique and Montserrat. Several reports of infection with *S. mansoni* in a wide array of mammalian hosts have been made. The role of animal hosts in maintaining the parasite has also been reported. For instance, Fenwick (1969) recovered *S.*

mansoni from all seven baboons examined in an area of Northern Tanzania, particularly uninhibited by man.

Experimental evidence has shown that *S. mansoni* strains from different geographical areas may display differences in their biological characteristics and in their infectivity or pathogenicity. For instance, de Lima e Costa and Katz (1982) observed that in mice, strains of *S. mansoni* originating from the same locality may show statistical differences in infectivity, effect on leucocyte count, numbers of eggs per female worm and response to treatment. Other members of the species complex include *S. edwardiense* and *S. hippopotami*, both of hippopotamus (Rollinson and Southgate, 1987), and *S. rodhaini* found in dogs, civic cat as well as a variety of rodents. The major intermediate hosts are the group of snails of the genus *Biomphalaria*. Almost all known species are susceptible to *S. mansoni*.

The *S. indicum* group

Members of this species complex are mostly of veterinary importance. *S. indicum,* first reported in equines by Montgomery (1906) is known to infect a variety of domesticated animals on the Indian subcontinent including sheep, camel, goat, cattle and buffalo. Another member *S. spindale* is found in the portal and mesenteric veins of the small and large intestine of ruminants. The parasite has been reported in many parts of India, Sri Lanka, Indonesia, Malaysia, Thailand and Vietnam. It is also thought to be one of the causes of schistosomal dermatitis in man, especially in rice paddy fields where *S. indicum* infected buffaloes coexist with the intermediate host *Indoplanobia exustus*. The rodent *Bandicota bengalensis* has been found naturally infected with *S. indicum* (Rollinson and Southgate, 1987).

Another member, of the *S. indicum* group, *Schistosoma nasale* is responsible for nasal schistosomiasis, or snoring disease in cattle, sheep and goats. The adult worms are found in the veins of the nasal mucosa. Also, *S. incognitum*, is reported in Thailand, Java

and Sulamesi primarily in rodent species of the genera *Bandicota* and *Rattus,* and in wild deer in Indonesia. In India, it is commonly found in pigs. In Thailand and Indonesia, *Radix auricularia* appears to be the intermediate host of *S. incognitum* (Rollinson and Southgate, 1987).

The *S. japonicum* group

Schistosoma japonicum differs from the other schistosome species affecting man in many ways. Probably, the greatest significance is the acute stage of its infection, a well recognised and frequently fatal condition that can be of epidemic proportion (Jordan and Webbe, 1993). It is responsible for a grave, debilitating and chronic form of intestinal schistosomiasis, which affects both man and domestic animals. It has a reputation of being the most serious of the schistosomes affecting man, generally because of the large number of eggs produced by the adult female worms. The disease in the Far East is endemic in parts of China (Hunan, Hubei, Jiangxi, Anhui and Jiangsu Provinces) (Yuan Hong, 1993), Phillipine, Japan (Kufu, Katayama and Chikugo River Basin), Taiwan and Indonesia. *Schistosoma japonicum* occurs as a natural parasite of a large number of mammalian species, which play an important role in the epidemiology of the disease. In some endemic areas in Mainland China, 31 species of wild animals including carnivores, rodents, primates, insectivores and artiodactyls have been found with natural infections of *S. japonicum* (Mao and Shao, 1982). All the strains of *S. japonicum* are transmitted by populations of the amphibious polytypic snail *Oncomelania hupensis* which consists of six subspecies. These are *O. h. chiui* and *O. h. formosana* in Taiwan, *O. h. hupensis* in Mainland China, *O. h. lindoensis* in Sulamesi, *O. h. quadrasi* in the Philippines and *O.h. nosophora* in Japan (Davis, 1980). Other group members include *S. mekongi* and *S. sinensium*. *S. mekongi* is responsible for human schistosomiasis in Khong Island in the Mekong River in Southern Laos and in many parts of northern and central Kampuchea

(Cambodia) (Voge *et al.*, 1978). The intermediate host is *Tricula aperta* (Liang and Kiticoon, 1980).

The life cycle of schistosomes

Though the first schistosome was described in 1851 by the German pathologist Theodor Bilharz, it was not until 1915 that the life cycle of the schistosomes was unraveled. This revealed the connection between the diseases and the fresh water planorbid snail which act as intermediate host to the parasites (Rollinson and Southgate, 1987). The life cycle comprises of the following stages; egg, miracidium, first and second stage sporocysts, cercaria, schistosomulum and adult worm. The first and second stage sporocysts reproduce asexually within the snail whilst the adult worms reproduce sexually in the definitive mammalian host.

The eggs laid by schistosome worms in the definitive mammalian host are passed out into fresh water via the urine or stool depending on the species of schistosome. In water, the eggs hatch into the first larval stage, the miracidium (Manson-Bahr and Bell, 1991). For instance *S. haematobium* eggs are mostly passed through urine (though not exclusive) whilst *S. mansoni*, S. *japonicum, S. matheei* and *S. intercalatum* eggs are released mostly through faeces. There is a wide range of size and shape of the schistosome eggs and the shape has been found to vary with the parasite species and diet of the definitive host (Rollinson and Southgate, 1987). The eggs are non-operculate, round or oval with one spiny appendage (Manson-Bahr and Bell, 1991), found in lateral or terminal position. This character of the eggshell is a very important distinguishing feature of one schistosome species from another on morphological basis. Schistosome eggs secrete histolytic enzymes through micropores in the shells, which help passage through the endothelium of the blood vessels. It is the soluble egg antigens, mostly the charbohydrate component) which is responsible for much of the pathology (Manson-Bahr and Bell, 1991;

Sturrock, 1993). The embryo within the egg develops into the next larval stage, the miracidium.

The miracidium has a complex array of sensory receptors, which are considered to aid in locating a snail host. It is thought that miracidia are attracted to intermediate snail host by undefined complex of water-soluble substances (miraxone) secreted by the snails. This has been confirmed by the attraction of *S. mansoni* miracidia to *B. glabata* (Chernin, 1970) and *S. haematobium* miracidia to *B. globosus* (Shiff and Kriel, 1970). Miracidium swims actively by means of its ciliated epidermis, which beat rhythmically to propel it to a compatible snail host. It must be emphasized that miracidia do not discriminate between species of snails and will sometimes enter the incorrect host thereby preventing further development (Manson-Bahr and Bell, 1991). However, if a miracidium enters a compatible intermediate host, it metamorphosis into the next stage, the mother sporocyst.

The penetration occurs by a mechanical action of the apical papilla breaking the snail's epithelium while the numerous membrane folds of the terebratorium act as a sucker to facilitate fixation as revealed by light and electron microscopy (Kinoti, 1971; Brooker, 1972; Lo Verde, 1975). The ciliated epidermis of the mother (primary) sporocyst is cast off and is replaced by a syncytial tegument. Within the elongated sac of the mother sporocyst, germinal cells develop into a number of daughter sporocysts. These leave the mother sporocyst after about 8 days and migrate to the digestive gland (liver) of the snail host (Manson-Bahr and Bell, 1991; Sturrock, 1993). Within them further germinal cells develop asexually into numerous biforked-tailed free-swimming cercariae. The mature cercaria escapes from the daughter sporocyst, migrates through the tissue of the snail and finally emerges to swim freely in the water by means of its muscular, bifurcated tail. Thousands of cercariae of the same sex may be developed from one miracidium. Cercariae are unisexual, non-feeding, fish-like with a pear-shaped head. They depend entirely on their glycogen reserves (Wilson, 1987) and are influenced by light, gravity agitation, touch and temperature. They are therefore short lived (Manson-Bahr and Bell, 1991). It is the

second free-living infective schistosome larva. Cercariae of all human schistosomes are grossly similar, about 1mm in length. The oral head has an anterior sucker (Cousin and Dorsey, 1991) and a prominent, muscular ventral sucker or acetabulum, a mouth, oesophagus and a pair of short caeca. It also has an excretory system of flame cells, tubules and excretory ducts leading into an excretory bladder at the posterior end of the body. Electron microscopy has revealed that the body is covered by a tegument bounded by a lipid bilayer, which appears trilaminate, usually covered by an amorphous glycocalyx (Sturrock, 1993). They tend to accumulate in the area of the mantle collar of the snail, that is, close to the head of the snail in the region where escape to the outside is simple.

The pattern of the release of cercariae differs somewhat between the three main human schistosomes. Normally *S. mansoni* cercarial shedding starts within 1 to 2 hours after stimulation. The process is more prolonged for *S. haematobium*, which takes 2 to 4 hours. Pesigan *et al.* (1958) reported that the process was even slower for *S. japonicum* requiring many hours of stimulation before shedding.

The life cycle is continued if a cercaria penetrates the intact skin of man or subcutaneous tissue into the blood circulatory system (Sturrock, 1993). Three distinct phases have been described by Haas and Schmidt (1982) to be involved in penetration. These are (1) attachment; (2) creeping over surface exploring for an entry site and (3) penetration into the epidermis. Phases 1 and 2 can be stimulated by thermal and chemical stimuli whereas phase 3 is stimulated by chemical stimuli alone. Haas and Schmidt (1982) further demonstrated that only aliphatic hydrocarbon chains with both a polar and a non-polar head group were effective stimulants. Free fatty acids produced on human skin by the action of bacterial esterases on triglycerides are thought to be the probable natural stimulants (Rollinson and Southgate, 1987).

Penetration of the cercaria is the result of coordinated muscular and secretory processes in the cercaria, presumably under nervous control. Following entry, the cercaria transforms into a tailless worm-like schistosomulum before entering the blood system

directly (or rarely, indirectly via the lymphatics) to be carried passively via the right heart, lungs and left heart to the general systemic vessels. It then enters the splanchnic blood vessels en route to the liver and matures into adult worm (Sturrock, 1993). Most worms leave the liver when they are sexually mature and have mated. They then migrate to the veins of the vesical plexus (*S. haematobium*) or the mesenteric veins (*S. mansoni, S. japonicum, S. metheei* and *S. intercalatum*) where they begin to lay eggs. The period between penetration by the cercaria and egg laying may be 30-50 days or more (Manson-Bahr and Bell, 1991).

The transformation from cercariae to schistosomulum involves profound morphological and physiological changes. The cercarial glycocalyx is lost during this period and the single bilipid epithelial membrane surrounding the body is replaced by a double bilayer that appears as a heptalaminate membrane in electron micrographs. The two lipid bilayers are linked by larger protein molecules detected by freeze-fracture ultramicroscopy (McLaren, 1980). A fully transformed schistosomulum has obvious oral and ventral suckers and is elongated posteriorly compared with the cercarial body as revealed by ultrastructural studies on the transformation process by Basch and Samuelson, (1990).

Cercaria as well as young schistosomulum, of 3-4 hours are susceptible to killing by eosinophils, neutrophils and macrophages (McLaren and Ramalho-Pinto, 1979; McLaren and James, 1985). They are fully susceptible to complement (C) damage either by the alternate or by the classical pathways (Fishelson, 1989). However, during development, schistosomulum acquires resistance to complement-mediated damaged (Levi-Schaffer *et al* 1982; Marikovsky *et al*., 1986;). This conversion to C-resistance occurs both *in vitro* and *in vivo* upon incubation in artificial medium (Clegg and Smither, 1972; Fishelson, 1989; Horta *et al*., 1991). The parasite also adapts to the isotonic medium within the definitive host (Sturrock, 1993; Tarrab-Hazdai *et al*., 1997).

The adult worm (male and female) has a prehensile oral sucker, which surrounds the mouth anteriorly, and a ventral sucker on the ventral surface. The worm attaches itself to the wall of the vessel in which it lives with the ventral sucker. A most distinctive feature is the large ventral grove, the gynaecophoric canal, in which the female is retained during pairing. The external surface is characterized by an abundance of papillae (Erasmus, 1987).

Externally, the body is covered by a tegument, which may bear spines, tuberculations and/or hairs. The tegument is an acellular, syncitial layer bounded externally by a plasma membrane composed of two bilipid membranes. *In vitro* studies by Sturrock (1993) indicates that the outer bilipid membrane is shed continuously and replaced by the products of organelles in the deeper syncytium of the tegument. This might presumably happen *in vivo*. The tegument is a living tissue capable of taking up nutrients and other essential substances, especially inorganic ions (McLaren, 1980). Circular and longitudinal muscles below the tegument and other specialized muscles are coordinated by a network of fibres to allow body contractions and other movements. The remaining organs are embedded in mesenchymatous cells (Sturrock, 1993).

Waste products are excreted by the two longitudinal canals, which open posteriorly and are fed by connecting tubules. There are flame cells whose function is to fan fluid wastes into the tubules by means of the vibratile cilia with which they are equipped (Manson-Bahr and Bell, 1991).

Proteolytic enzymes secreted by cells lining the gut act on host blood ingested through the mouth, breaking down serum globulins and haemoglobin from RBC into tyrosine (Sturrock, 1993). Schistosomes feed onliquid material in the host in which the worm lives and obtain oxygen from the blood of the host (Manson-Bahr and Bell, 1991).

The life span of the schistosome worm has been the subject of controversy. On the basis of data obtained from persons who left an endemic area and who years later were found to be still passing eggs, a claim has been made that the worm can live up to 30 years

(Adel, 1990). The mean life span of the worm, however seems to be much shorter. Warren *et al*, 1974 reported of a study outcome where a group of Yemeni immigrants moved to a non-endemic country, the mean life span of *S. mansoni* worm was shown to be 5-10 years. In *S. haematobium* infections, investigations suggest that its life span may even be shorter than that of *S. mansoni* (Anderson, 1987).

Schistosomiasis in Ghana

Ghana is among the countries with the highest prevalence rates of urinary schistosomiasis (Berquist, 1987). It is widespread in most parts of country (Ashitey *et al.*, 1974). The snails responsible for the transmission of the urinary schistosomiasis in Ghana are the *Bulinus trancatus* and *B. globosus* (Odei, 1961; Onori *et al.*, 1963). *B. globosus* is reported however to be the most important and widely distributed in the country.

The construction of the Akosombo dam on the Volta River, and the subsequent formation of the Volta Lake altered the existing physical, biological and the socio-economic environment of the people. Although the lake and the head pond created a number of developmental possibilities in fisheries, transport, agriculture, wildlife and tourism, they also created a number of public health problems, such as the incidence of schistosomiasis. The formation of the lake led to an explosion of aquatic weeds to which snail vectors of schistosomiasis attach and feed. In addition, organic materials in the lake increased as a result of the submerged vegetations, providing source of nutrients for the snails. This led to an increase in the population of *B. truncatus* in the lake (Odei, 1973). These problems notwithstanding, there was an accompanying increase in fish population. This attracted a number of fishermen, especially from the area of the Volta Delta, which was highly endemic for schistosomiasis (Kalitsi, 1973). The lake, which is the world's largest artificial lake, has approximately 90% of the children in the neighboring villages' infected with schistosomes. They often contaminate their environment creating continual cycle that leads to poor School performance and growth. Most Africa's dams are known to

have similar problems. For instance after the construction of the Diama Dam on the Senegal River in Mauritania, transmission rates rose to new heights. This introduced schistosomiasis to both Senegal and Mauritania. Other Dams such as Kainji in Nigeria and Kariba in Zimbabwe have contributed to the prevalence and spread of schistosomiasis.

Today, urinary schistosomiasis has become the main problem associated with the Volta Lake. This is accompanied by the fact that the inhabitants depend completely on the lake water for their domestic use. There is no proper human waste disposal system and the inhabitants freely urinate and defeacate at the lakeshore, perpetuating the cycle of schstosomiasis transmission (Kalitsi, 1973). It is reported that villages located closer to the lake generally showed a higher schistosomiasis infection rate than those situated further away. In a survey of school children from the village of Mepe, it was shown that the prevalence of schistosomiasis in children who had never visited the Volta Lake was only 10.7%, whereas those children who had visited the lakeside at least once had a prevalence of 69.9% (Jones, 1973).

The intestinal form of schistosomiasis is also known to occur mostly in the northern and south-eastern parts of the country (Paperna, 1968). Though the intermediate snail host (*Biomphilaria. Pfeifferi*) is widely distributed in the entire country most of them are however uninfected (Wen, personal communication). The contributions of other species such as *S. mekongi* and *S. intercalatum* to human infection are not known. Furthermore, it is not documented if any of the animal schistosomes are present in Ghana. Yet some of these animal parasites such as *S. bovis* occasionally infect humans (Rollinson and Southgate, 1987).

Schistosomiasis in Ghana is an important occupational hazard associated with fishing and farming. However large numbers of children and women are also infected as a result of domestic and recreational activities. A stratified random samples of schools of the Suhum Kraboa Coaltar District for prevalence and intensity of urinary schistosomiasis by Ashitey *et al*, (1974) showed overall prevalence of 56.9% and that schools with high

prevalence above 60% were close to the three main rivers, Densu, Suhum and Kua. Interestingly, out of 23892 new attendants, only 161 cases of urinary schistosomiasis were recorded in Suhum hospital. In 1994, a prevalence of 67.7% for urinary schistosomaisis and 68,9% for intestinal schistosomiasis in the Kasene-Nankana District in the upper east region of Ghana was attributed to the Tono Irrigation Scheme. It was also reported by Aryeetey *et al*, (2000), an overall prevalence of *S. haematobium* infection varying between 54.8 and 60.0% in the Southern Ghana at three defined rural areas drained by the Densu River. This indicates that urinary schistosomiasis is a major public health problem in this area.

In recent times, both *S. mansoni* and *S haematobium* have been reported to spread in Ghana and overlap in hyperendermic areas where the schistosomiasis snail hosts coexist. This has resulted in mixed infections involving both *S. mansoni* and *S. haemaobium* with more complicating effects on children. In 1996, an *S. mansoni* prevalence of 10.3% and a 22.5% mixed infection rates were recorded in Sogakope, a town in southeastern Ghana. In the same year a urinary Schistosomiasis prevalence of 47.6% was recorded in Mayera, a village in the Greater Accra Region of Ghana (Bosompem *et al.*, 1996b). Furthermore, this disease has severel effects on economic development of the country as it impacts negatively on the vast tourist and irrigation potential of the Volta and other Lakes. Unfortunately the picture regarding animal schistosomiasis and its effect on human in Ghana is yet to be determined.

Animal schistosomiasis

Natural infections of domestic and wild animals with human schistosomes have been investigated by several workers (Barbosa *et al.*, 1953; Pesigan *et al.*, 1958; Nelson *et al.*, 1962; Fenwick, 1969; Mansour, 1973). Nelson, (1960) concluded that all schistosomes infecting man in Africa are zoonotic and that the disease is naturally transmitted between reserviour hosts and man. In some other parts of the world (such as China and

Philippines), where human schistosome infection and disease have been reduced, further reduction seemed difficult because of the continual transmission from infected animals (McGarvey *et al.*, 1998). Natural survey data in China indicated an *S. japonicum* prevalence of 7% among cattle and 9.6% in water buffalo. Although infection rates of 10-15% were found in pigs and in water buffalo, specific community studies in endemic Provinces showed higher prevalence rates in cattle. Studies of the spatial distribution of animal faeces in the lake and marsh regions, and the mountainous regions of China suggested that cattle dung contributes substantially to potential transmission of *S. japonicum* infection (McGarvey *et al.*, 1998).

An epidemiological study of *S. japonicum* in domestic animals in two municipalities of the eastern coastal plain of Leyte, Philippines, showed that pigs had the highest rates of prevalence. They found out that dogs had the highest mean 24-hour-egg output, and pigs had the highest proportion of hatchable eggs. However, dogs' served important role in maintaining the transmission of the parasite as indicated by a high transmission potential and the close habitual contact of the animal to humans (Fernandez *et al.*, 1982).

In Zambia, schistosomiasis is reported to be endemic with occurrence of *S. haematobium* and *S. mansoni* in man and *S. matheei, S. margrebowiei* and *S. leiperi* in domestic and wild animals. *Schistosoma haematobium* and *S. metheei* are the most widely distributed species and their transmission overlaps in rural areas where man and domestic animals utilize the same water bodies. It is significant to note that *B. globosus* is the intermediate host for both species (Vercruysse *et al.*, 1994). Similarly in a study in Senegal, Albaret *et al*, (1985), reported that *Bulinus umbilicatus* is the principal vector of *S. curassoni* (the dominant species in domestic ruminants and in man) and *S. haematobium* (existing in pure infections and in mixed infections with *S. curassoni* in man)

Studies based on the morphological characteristics of eggs in South Africa, Zimbabwe and Zambia have revealed incidental *S. matheei* infections in man. Wright,

Southgate and Ross (1979) demonstrated that *S. haematobium* and *S. matheei* readily hybridize in man and that F1 hybrids are both viable and fertile. Pitchford (1977) therefore suggested that in time the two species would be supplanted by a single species, which may infect man and cattle with equal ease. Jiang *et al*, (1997) conducted investigations in highly endemic areas of schistosomiasis japonica in Weishan and Aryans countries. It was observed that, the number of domestic animals was increasing annually and the proportion of animal husbandry gains in the total agriculture income had a yearly escalating tendency and that infection rate of inhabitants was upgrading as a result of the development of frequent irrigation of domestic animals. Owing to frequent irrigation of domestic animals, serious spread of infection sources and high prevalence of schistosomiasis japonica occurred.

Van Wyk (1983) reported that in Southern Africa schistosomiasis is practically as widely disseminated in animals as in humans. The prevalence in animals is very high in certain areas, for instance up to 90% in parts of the lowveld of the Transvaal. *S. matheei* was the only schistosome of importance in animals in South Africa, with exception of *S. mansoni*, which had been recovered from primates and rodents and a single waterbuck (Pitchford, 1977) and *S. haematobium* recovered from one buffalo (Pitchford, 1977). *S. matheei* was discovered in 1926 by Veglia and in 1929 Le Roux (Veglia and Le Roux, 1929) reported *S. matheei* in sheep near Humansdorp in the eastern Cape Province and recovered *S. matheei* ova from cattle faeces on the same farm. It shares the intermediate snail host, *Bulinus* (*Physopsis*) *sp.* with the human schistosome *S. haematobium.* Apart from South Africa, *S. matheei* occurs in most neighbouring territories and as far as parts of Tanzania, Chad and Nigeria (Pitchford, 1977). It has the ability to develop in humans as cattle schistosome ova have been demonstrated in both urinary and intestinal infections in humans in Southern Africa by numerous workers (Alves, 1949; Pitchford, 1959; Cawston, 1922; Blackie, 1932; Kisner *et al.*, 1953; Cruz, 1971).

In Ghana however, the contributions of other schistosome species such as *S.*

mekongi S. bovis and *S. intercalatum* to human infection is not known. Personal communication with researchers at Animal Research Institute and Dr. Wen of the Schistosomiasis Control Unit of the Ministry of Health, Ghana revealed that currently there is no documentation on animal schistosomiasis. Thus the picture regarding animal schistosomiasis and its effect on human in Ghana is yet to be determined. Consequently veterinary epidemiological studies are urgently needed to improve the understanding of the zoonotic implications of the disease and to create a basis for suitable control measures in domestic animals if any.

Schistosome antigens

Attention has been focused on the schistosome surface as a primary source of parasite antigens for protective purposes and as a major site of immune attack. A variety of antigens secreted or excreted by the different life stages (adult worm, miracidia hatching fluid of viable eggs) of schistosomes are present in the circulation and/ or the urine of the infected host (Hermann, 1993). These have different antigenic stimuli. Several of the schistosome surface antigens have now been identified and a number have been cloned; many are glycoprotein, with both the peptide and carbohydrate components acting as important epitopes. Some of these epitopes are shared between different life cycle stages, providing a molecular basis for phenomena such as concomitant immunity and generation of blocking antibodies. A variety of other antigens of internal origin are released as the worm feeds and metabolizes (Hermann, 1993).

Adult Worm Antigen (AWA)

A detailed analysis by Norden and Strand (1984) of adult worm antigens recognized by sera from patients infected with *S. mansoni, S. haematobium* or *S. japonicum* concentrated on antigens present in the glycoprotein fraction prepared by affinity chromatography using Concanavalin A. This fraction was used based on the

previous work by Strand *et al.* (1982) showing that it is the most antigenic. Twenty to thirty polypeptides out of about 50 from each schistosome species were found to be antigenic as revealed by immunoprecipitation with the homologous infection sera. Sera from *S. haematobium* precipitated all but 3 of the antigens recognised by *S. mansoni* sera amongst the glycoproteins. However, a lower degree of cross reactivity was observed using sera from patients infected with *S. japonicum*.

Kelly *et al.* (1987) described similar findings by comparing antigens immunoprecipitated from cell free translation product synthesized from adult worm mRNA of *S. mansoni* and *S. haematobium*. Approximately 40 strongly labelled polypeptide antigens were resolved following 2-dimensional polyacrylamide gel electrophoresis and immunoprecipitates obtained by using homologous human infection sera. Once again extensive cross-reaction was evident with only three *S. haematobium* antigenic polypeptide (Mr 45,000, 27,000 and 14,000) being recognized specifically by *S. mansoni* infection sera.

The significance of these studies is that they give an indication of the antigenic complexity of the schistosomes. Of the three major species which infect humans, it appears that *S mansoni* and *S. haematobium* may be more closely related antigenically to each other than to *S. japonicum*, although a relatively small proportion of the polypeptides antigens is specific to any one species (Kelly, 1987).

Schistosomula Surface Antigens

A preferred parasitic stage for generation of monoclonal antibody to schistosome antigens is the schistosomulum. During the transition from cercariae into schistosomula, the trilaminate surface membrane of cercariae matures into a double unit bilayer that covers the schistosomula (Hockley and Mclaren, 1973). This surface membrane contains stage-specific glycoproteins, which may represent targets for the host's immune response (Hockley and Mclaren,). Monoclonal antibody that recognise surface antigens of

schistosomula have therefore been sought.

By far, the majority of studies of schistosomulum surface antigens have concentrated on *S. mansoni*. The frequent technique used to identify surface antigens include radio-iodination of live, freshly transformed schistosomula, followed by the immunoprecipitation with a variety of antisera.

Numerous polypeptides generally of Mr 100,000 have been labelled using lactoperoxidase-catalysed iodination by Ramasay, (1979) and Snary *et al.* (1980). Dissous *et al.* (1981) also identified a set of antigens of Mr 32,000-40,000 by precipitation with immune rat sera. Schistosomula surface antigens of *S. haematobium* have also been identified using iodogen-catalysed labelling followed by immunoprecipitation with human infection sera (Simpson *et al.*, 1985). Major antigens of Mr 17,000 and a complex of Mr 24,000-30,000 have been observed. Similar techniques had been used to identify surface antigens of *S. japonicum*.

Most of the schistosomulum surface antigens are glycoxylated and both carbohydrate and polypeptide moieties contribute to their antigenicity. It is reported that surface polypeptide epitopes can induce protection (Kelly, 1987). Polypeptides antigens present on the schistosomulum surface are therefore strong candidates as antigens capable of mediating immunity. They are recognised either singly or in combination by monoclonal antibodies that passively transfer protection in experimental animals. Importantly, they also elicit an antibody response in human patients and do not appear to show significant antigenic diversity. A number of monoclonal antibodies against the Mr 20,000 antigen have been produced (Tavares *et al.*, 1984; Yi *et al.*, 1986b) and although to date, none has consistently transferred immunity, in some experiments statistically significant levels of protection have however been obtained (Kelly, 1987).

Substantial evidence for the involvement of surface antigen in immunity is provided by the observations that schistosomulum surface antigens of *S. mansoni, S. haematobium* and *S. japonicum* show species specificity, whilst the somatic antigens show

considerable cross reaction (Kelly, 1987). The first immunization studies using purified antigens were reported by Smith and Clegg (1985), who used the protective monoclonal antibody WP66.4, which recognized schistosomulum surface antigen of Mr 200,000 and 38,000 to purify a cross-reacting antigen of Mr 155,000 from adult worm. In addition, an antigen of Mr 53,000 was purified from schistosomula extracts using a further MoAb which had previously been shown to bind the schistosomulum surface but which was not protective.

Schistosome Egg Antigens (SEA)

Many of the eggs produced by schistosomes do not reach the external environment but are swept to the liver by the portal blood flow where they impact on the presinusoidal capillaries (Saad *et al.*, 1994). Proteolytic enzymes are considered important mediators in the egg migration process from the site of ovipositor to the lumen of the gut or the bladder. The mature eggs secrete through the micropore in the eggshell, a complex mixture of antigens that elicit both cellular and antibody-mediated immunologic response (Smither and Doenhoff, 1982; Saad *et al.*, 1994).

Soluble egg antigens contain multiple glycoproteins, polysaccharides and non-glycoconjugated proteins (Carter and Colley, 1978 and 1979). Warren (1972) reported that SEA extracted from the schistosome egg by homogenization and ultra centrifugation could trigger of granulomatous hypersensitivity and other immune reactions typical of intact eggs.

Egg antigens may vary considerably in concentration among schistosome eggs of different geographic origin. A major egg glycoprotein was shown to be four times abundant in a Puerto Rican strain of *S. mansoni* than in an Egyptian strain (Hamburger *et al.*, 1982). Also Pelley *et al.* (1976) noticed intraspecies differences at the protein level by comparing protein of SEA among *S. mansoni* strains from different geographical regions using high performance SDS-polyacrylamide gel capillary electrophoresis.

Three egg antigens (major serologic antigens- MSA1, MSA2 and MSA3) have been purified from *S. mansoni* (Hamburger *et al.*, 1976 and Pelley *et al.*, 1976) and serological studies suggested that only MSA1 (negligible amount in immature eggs, but abundant in mature ones) was egg stage and species specific.

Nibbeling *et al.* (1998) demonstrated that 16 monoclonal antibodies were reactive with *S. haematobium* SEA in addition to *S. mansoni* SEA. The MoAbs were tested as possible immunodiagnostic reagents in homologous sandwich ELISA format to detect circulatory SEAs in serum and urine samples of *S. mansoni* or *S. haematobium* infected individuals. In another study, De Brito *et al.* (1998) reported that adult worm antigen (AWA) and SEA were localized ultrastructurally by immunoelution microscopy using two MoAbs in the glomeruli of hamsters infected with *S. mansoni* cercariae or injected with *S. mansoni* eggs. Both SEA and AWA were present mainly in cytoplasm of mesangial cells, matrix and glomerular basement membrane. Their findings suggested that egg antigens contribute to the pathogenesis of experimental glomerulopathy in the hamster.

Detection of circulating SEA is very important in the sense that it may provide complementary data to those of circulating adult worm antigens. This may enable a more sensitive level of antigen detection in a similar way to that found in parallel testing for CAA and CCA (Van Lieshout *et al.*, 1991). The measurement of SEA levels may also provide a more accurate representation of tissue egg burdens since it is the eggs that produce a great deal of the morbidity associated with *S. mansoni* infection and assessment of SEA levels could become a more accurate index of morbidity than existing diagnostic test. Finally assessment of SEA could also be used to measure the effects of anti-fecundity thereby providing a tool in monitoring effect of new vaccines.

Cross- reactive schistosome antigens

There are some egg antigens, which cross-react with other stages of the parasite. Von Lichtenberg *et al.* (1963) showed that mice immunized with eggs developed

antibodies, which reacted with the cercarial surface. Harn *et al.* (1984) produced anti-egg monoclonal antibodies that bound to the schistosomulum surface. In another study, Yi *et al* (1986a) described four MoAbs, which recognized epitopes present in all stages of the schistosome life cycle. There is enough evidence that much of the cross-reaction between the schistosomulum surface and the egg is due to shared carbohydrate epitopes (Omer-Ali *et al.*, 1986). The epitopes, as indicated, are not species-specific and it is thus suggested that the stimulation of antibody recognizing such carbohydrate epitopes may be a factor in cross-specific concomitant immunity.

Attallah *et al.* (1998) reported a polypeptide (Mr 7,000) in antigenic extracts of the three developmental stages (egg, cercaria and adult worm) of *S. mansoni* by western blotting. This antigen was isolated and purified from crude soluble worm antigen preparation by immunoaffinity chromatography using CNBr-activated sepharose-4B beads coupled with the BRL4 MoAb.

Excretory-secretory Antigens

Excretory-secretory group of antigens consists of polypeptides, glycoproteins, proteoglycans and polysaccharides (Nash and Deelder, 1985; de Jonge, 1990). They are gut-associated antigens present in the vomitus and in excretory-secretory materials of adult schistosomes and are released into circulation of the host by the regular regurgitation of digested blood. These antigens were described by Bergren and Weller (1967) as circulatory by virtue of the fact that they are found constantly in the blood and other body fluids of infected individuals. Their presence in a patient therefore indicates an active infection with viable worms (Hermann, 1993).

Two proteoglycan gut-associated antigens (circulating anodic and circulating cathodic antigens, CAA, CCA) have been detected in serum, urine and breast milk of African and South American patients with schistosomiasis mansoni (Santoro *et al.*, 1980; Feldmeier *et al.*, 1986b; de Jonge *et al* 1990) in serum and urine of patients infected with

S. haematobium or *S. intercalatum* (Feldmeier *et al.*, 1986b; de Jonge *et al.*, 1989b) and in serum of Filipinos infected with *S. japonicum* (Vant Wout *et al.*, 1992). This supports the report by Bawden and Weller (1974) that the CAA was not species-specific by demonstrating its presence in *S. mansoni, S. haematobium* and *S. japonicum* adult worm homogenates and in the sera of mice and hamsters heavily infected with either *S. mansoni* or *S. japonicum.* CAA as described by Gold, Rosen and Weller (1969) are heat stable, dialyzable and anodic as determined by its immunoelectrophoretic mobility unlike the low molecular weight CCA, which is cathodic.

Other circulatory antigens have also been identified and characterized. Crabtree and Senft (1974) described several purine salvage enzymes (adenosine phosphorylase, adenosine kinase, adenosine deaminase and purine nucleotide phosphorylase) present in schistosome vomitus (excretory-secretory gut product). These enzymes could incite histaminic skin reactions (reagenic responses) in the host.

Enzyme-linked Immunosorbent Assay (ELISA)

One of the most widely used applications of MoAbs is enzyme-linked immunosorbent assay (ELISA). ELISA is based on antibody recognition of a particular antigenic epitope. It combines the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme that also possesses a high turnover number (Wilson and Walker, 1995).

ELISA is replacing radioimmunoassays because of the lack of radio hazards. It is also extremely economical in the use of reagents (Riott *et al.*, 1993); large numbers of tests can be performed in a relatively short time. Also the enzyme conjugates are more stable than radioactive isotypes (McMichael and Beverley, 1986).

For screening, an indirect method is invariably used in which an appropriate enzyme is covalently coupled to anti-mouse or rat immunoglobulin antibody (McMichael and Beverley, 1986). Binding enzymes are selected which show simple kinetics and can

be assayed by a simple procedure (normally spectrophotometric). The most commonly used enzymes are the alkaline phosphatase, B-D-galactosidase and horseradish peroxidase. Binding of anti-immunoglobulin is detected by conversion of enzyme substrate to a coloured reaction product or precipitate.

The putative antiserum is reacted with specific antigen attached to a solid phase. Solid phases commonly used in ELISA include cross-linked dextran or polyacrylamide beads, filter paper (cellulose) discs (membrane-based ELISAs) and disposable polystyrene microtitre plates (plate ELISAs), which are convenient for large numbers of samples. The appropriate antigen or antibody may be attached to the solid phase by passive adsorption or covalent coupling with cyanogen bromide.

Colourless substrates that are converted to a coloured product by the enzyme are popular. For instance, p-nitrophenylphosphate (pNPP) is converted to the yellow p-nitrophenol by alkaline phosphatase. Substrates used with peroxidase include 2,2-azo-bis 3-ethylbenzthiazoline-6-sulphonic acids (ABTS), O-phenylenediamine (OPD), and 3,3', 5,5'-tetramethylbenzidine base (TMB), diaminobenzidine tetrahydrochloride (DAB), yield green, orange, blue and brown colours respectively. In all ELISAs reference positive and negative samples must be included in each series of tests to ensure accurate and reproducible results.

ELISA can be used for the assay of virtually any antigen, hapten or antibody. It is used predominantly in clinical biochemistry in the study of infectious diseases including detection of bacterial toxins. It is also used to assay antibodies in infectious diseases including *Plasmodium, Schistosoma* and *Trypanosoma spp.*

Monoclonal Antibodies (MoAbs)

Antibodies or immunoglobulins (Ig) are a group of glycoproteins present in the blood serum and tissue fluids produced by B-lymphocytes. They are the soluble form of the B cells antigen receptor. All antibodies have the same basic structure but they are

diverse in the region that binds to the antigenic determinant site (epitope) of the antigen. The epitopes may be either peptide or carbohydrate in nature or more rarely lipid. One part (Fab) binds to antigen and the other parts (Fc) interact with other elements of the immune system. Antibodies allow the immune system to recognize specific pathogens and their products (Roitt *et al.*, 1993).

Many different antibodies (polyclonal antibodies) secreted against several antigen epitopes are found in the immune sera. As a result immune sera are non-specific to any one particular determinant and cross-react with antigens from various sources (Campbell, 1984). They are therefore not suitable for assays in which antibody specificity is crucial. Polyclonal antibodies are particularly valuable for immunoprecipitation and immunoblotting. Prior to 1975, it was possible to only produce polyclonal antibodies. Kohler and Milstein (1975) developed a procedure to produce monoclonal antibodies from hybridoma cells. These are cells formed by fusion of B-lymphocytes (antibody producing cells) from the spleen of an immunized animal, with a myloma cell that has the capacity for unlimited proliferation (immortal). The single fused cell possesses the property of the immortal cell and the specific antibody production. The hybridoma can then be injected into animals to induce antibody-secreting myloma cells to produce antibodies collected in ascites (*in vivo*) or they can be grown in mass culture to produce specific antibodies (*in vitro*).

Antibodies produced by monoclonal hybridomas have many outstanding advantages over the conventional antisera. Apart from their monospecificity, specific antibody is available in concentrations several orders of magnitude greater than in the conventional sera and can be derived for almost any purpose. It is possible to introduce stable internal labels into the antibody molecule by culturing the hybridoma cells with radiolabelled amino acid, a possibility not available with the conventional sera (Rosemarine, 1986). These advantages have made certain experimental techniques much simpler and more reliable and perhaps more importantly, have allowed the development of

powerful experimental approaches not possible with conventional antibodies.

Uses of Antibodies

Monoclonal antibodies are in effect homogeneous immunological reagents of defined specificity, high specific activity and selected isotype. They have been established as exceptionally powerful research agents and also with potential clinical uses. In immunochemistry, MoAbs have been utilized for determining the structure and biochemical characteristics of molecules. They have made possible monospecific reagents of very high specific activity to virtually any molecule of interest, bringing the possibility of using immunochemical techniques to almost every laboratory. Monoclonal antibody techniques have provided an opportunity to re-evaluate the role of immunological methods for the diagnosis of infectious diseases. Antibodies of diagnostic potential have therefore been prepared in research laboratories against a battery of viruses, bacteria fungi and parasites (Nowinski *et al.*, 1983).

In a study by Nowinski *et al*, (1983) the diagnostic utility of monoclonal antibodies for gonococcal, chlamydial and herpes virus infections were described. The antibodies used demonstrated sensitivity of 94-99% for culture confirmation and 85-90% for direct diagnosis of specimens smeared on microscope slides. They have significant impart on the characterization of parasite antigens in schistosomiasis (Harn *et al.*, 1985; Capron *et al.*, 1987). Advances toward antigen analysis using MoAbs are of particular importance in defining relevant antigens to be selected for possible vaccine development (Simpson and Coli, 1987).

Purification of Antibodies

Purification and characterization is a prerequisite to studying biological molecules. Purified antibodies are requires for a number of techniques.

MoAbs produced in culture supernatants and ascites may contain impurities that

can ultimately affect the reactivity and or the sensitivity of the antibody. Particularly, antibodies present in the form of ascites are generally not ideal for use without some form of purification to remove contaminants such as the oily solutions used to induce and promote ascites formation. Also the common media used for the growth of MoAb-producing cells *in vitro* and supplements with fetal bovine serum (FBS) or fetal calf serum (FCS), which may account for about 10% of the complete medium. These serum supplements (proteins) usually serve as contaminants and they interfere in some of the assays that utilize MoAbs. Notwithstanding, unpurified antibodies can be perfectly used in cytotoxicity studies if the concentration of the antibodies is not important.

There is a wide variety of methods to purify antibodies and the correct choice of purification will depend on a number of factors including (1) the manner in which the antibody will be used (2) the species in which the antibody was produced (3) the class and subclass and (4) the source (ascites or tissue culture supernatant).

The most commonly used method in the purification is ammonium sulphate precipitation. The concentration at which antibody is precipitated varies from species to species but most antibodies will precipitate at 50% saturation. A disadvantage of the ammonium sulphate method is that the resulting antibody will not be pure. They will be contaminated with other high molecular weight proteins, as well as proteins that are trapped in the large flocculant's precipitate. Therefore ammonium sulphate precipitation is not suitable for single step purification but must be combined with other methods if a pure antibody is needed. The antibody isotype is one of the most important factors that influence the choice of protocol for subsequent purification (Goding, 1986). For instance, IgG monoclonal antibodies are best purified by ion-exchange chromatography whilst IgM antibodies require gel filtration.

Diagnosis of human schistosomiasis

The control of schistosomiasis is an urgent task, which requires improved diagnosis and treatment as well as effective prevention (Han Xu *et al.*, 1989). Decisions on prognosis and assessment of morbidity, evaluation of chemotherapy and control measures all build on the results from diagnostic tests (Hermann, 1993). It is thus important to select a diagnostic tool that corresponds to the type of information sought by the clinician or the epidemiologist. There is therefore the need for more simple, rapid, sensitive, reproducible and cost effective diagnostic test for schistosomiasis.

There are three different approaches for the diagnosis of the disease. These are the (1) direct parasitological methods, which detect schistosome ova in urine, stool or the rectal mucosa and histological methods disclose schistosomula, adult worm or eggs in tissue biopsies, (2) indirect methods which rely on clinical, biochemical or immunological disease markers, detect pathology typically or frequently associated with schistosome infections and (3) immunological methods which measure the immune response to certain schistosome antigens or the concentration of parasite-derived antigens in blood or urine (Hermann, 1993).

Eggs in urine

The intensity of schistosome infection is measured by quantitative egg counts, which are highly variable (Deelder *et al.*, 1989) and may depend on the immune status of the host (Damian, 1987).

Urine samples may be examined under microscope for *S. haematobium* eggs. This method has been widely used in individuals and community diagnosis (WHO 1983c). The method is simple, rapid, specific but not very sensitive. The sensitivity can however be increased if (1) samples are collected at noon where egg secretion is maximum, (2) examination of the sediment from large volume of urine (Peters and Kazura, 1987). It is generally accepted that the excretion of eggs in urine follows a circadian rhythm with a

peak around noon. Since eggs are responsible for the presence of lower renal track pathology in schistosomiasis haematobium, measurement of haematuria, proteinuria and leucocyturia also varies with the time of the day (Doehring *et al* 1985a, b).

Urine sediments may be obtained by centrifugation or filtration. In filtration, paper, polycarbonate, polyamide or membranes derived from other synthetic fibres may be used. The membranes differ in properties (Bradley, 1965; Peters *et al.*, 1976; Pugh, 1978). Paper filters have to be stained in order to allow identification of schistosome ova. On polycarbonate membranes, ova are more easily identified (Hermann, 1993). Staining with 1.0% trypanblue facilitates visualization and allows discrimination between viable and non-viable ova (Feldmeier *et al.*, 1979). Other stains in use include 5-10% tincture of iodine or solutions of eosin, methylene blue or ninhydrine.

Centrifugation is a valuable alternative to filtration. Richards *et al*, (1984) emphasized that the sensitivity of centrifugation compares favourably to that of the usual filtration of 10ml in low intensity infection.

It is worth noting that egg detection in urine has drawbacks. It may be biased in urine of females of childbearing age. This is because the epithelial cells frequently in female urine tend to clog filter membranes, which may completely disguise schistosome eggs. Also when urine is contaminated with menstrual blood, lysis of erythrocytes is required to disclose ova in sediments or on filter membranes. This may usually be achieved by addition of 10% HCl to urine sample (Feldmeier *et al.*, 1979).

Eggs in Stool

The ideal field method for the detection of schistosome ova in stool is still lacking (Hermann, 1993). Currently, the technique described by Kato and Miura (1954) for diagnosing hookworm/Ascaris infections, which was amended by Katz *et al.* (1972) and modified by several workers (Teesdale and Amin, 1976; Peters *et al.*, 1980) has been widely accepted as a valuable compromise for fieldwork. A specified amount of stool specimen cast by a template is treated with 50% (v/v) glycerol in water containing 3%

malachite green before microscopic examination. The method is sensitive for the detection of light infection, quick and suitable for large epidemiological studies.

Biopsies

Infections with all human schistosomes can be diagnosed by microscopic examination of minute biopsies of the rectal mucosa. Snips are taken from suspicious lesion or from the plica transversalis recti. The efficiency of rectal biopsy has been shown in schistosomiasis mansoni (da Cunha, 1982), schistosomiasis intercalatum (Feldmeier *et al.*, 1981a) the oriental schistosomes (Maunoury *et al.*, 1990). Even in infection with *S. haematobium*, eggs are frequently detected in rectal snips (Badran *et al.*, 1955; Harries *et al.*, 1986). The biopsies are crushed between two thick cover slips and the surface examined. Quantitative results are obtained that significantly correlate to the number per gram of faeces. If few drops of glycerol-malachite solution are added to the biopsy before compression, viable and non-viable eggs are easily differentiated by morphological aspects (Cancado *et al.*, 1965). Rectal biopsies are especially meaningful in the assessment of cure rates after chemotherapy. However, the histological sectioning is invasive and therefore limited in application.

Immunodiagnosis

Schistosomiasis remains a serious worldwide public health problem with a still unfulfilled need for routine cost- effective method of diagnosis. Such methods are required not only for people in endemic areas but increasingly for tourists who may be infected during visits to such places.

The routine parasitological diagnosis of schistosomiasis though very specific, is beset with a number of limitations making its use unreliable. These limitations include the relative insensitivity in the detection of low intensity infectious patients; difficulty in achieving homogenous distribution of *S. haematobium* ova in urine (Braun-Munzinger and Southgate, 1992) as well as the day-day and circadian variation in egg excretion. This may

lead to incorrect estimates in prevalence and intensity of infection (Gryseels, 1994). It is also laborious, not field applicable and expensive.

There is therefore the need for differential diagnosis. This must be simple, rapid, sensitive, reproducible and cost-effective. The increasing demands for simple and reliable assays have been noted in field monitoring of schistosomiasis in the endemic areas where control measures have been taken for years (Qian-Li *et al.*, 1993). It is therefore suggested that due to the relative insensitivity of both the parasitological and antigen detection, antibody detection method could find increasingly use in situations of low infection intensity (Hamilton *et al.*, 1998).

Numerous schistosome serodiagnostic assays (based on the detection of host antibodies directed against schistosome antigen) with high sensitivity and specificity have been developed (Mott and Dixon, 1982; Qian-Li *et al.*, 1993). Detection of specific antibodies is a valuable tool for immunoepidemiological studies, but antibody levels are, in general, not related with the intensity of the infection nor are they indicative of a still active infection (Deelder *et al.*, 1994). This does not discriminate between previous exposure and current infection (Simpson and Smithers, 1985). Assays that could measure circulatory antigens secreted by live parasites and discriminate infections based on their intensity might be able to evaluate the efficacy of chemotherapy and the effectiveness of future vaccines (Barsoum *et al.*, 1991). All these attributes would be highly desirable. Therefore the detection of circulatory antigens in schistosomiasis is increasingly used as a diagnostic tool, because level of antigens is correlated with worm burden and antigen would disappear relatively rapidly from circulation after successful chemotherapy (Deelder *et al.*, 1994).

Although circulatory antigens may be produced by several life cycle stages, including eggs, the most important diagnostic antigens are the CAA and CCA, which are associated with the syncytium lining the gut of the adult worm (Deelder *et al.*, 1976). The CAA level in relation to intensity of infection has been studied in *S. mansoni*, *S.*

haematobium, S. japonicum and *S. intercalatum* infections (Deelder *et al.*, 1989; Deelder *et al.*, 1990; De Jonge *et al.*, 1988). It was reported that, in general, CAA levels correlated with the intensity of infection as determined by egg output. When CAA or CCA is measured quantitatively, antigen concentration in serum or urine significantly correlated to the number of eggs excreted in stool or urine (Santoro *et al.*, 1980; Feldmeier *et al.*, 1986b; de Jonge *et al.*, 1989b; Von't Wout *et al.*, 1992). These findings confirmed the notion that the concentration of gut-associated antigens in blood or in urine is directly linked to the number of adult worm present in mesenteric or prevesical plexus, which in turn determines the number of eggs passed into the intestine or the bladder respectively.

Following chemotherapy however, CAA levels in serum were found to decline rapidly with a half-life of 2.5 days (De Jonge *et al.*, 1989). Deelder *et al*, 1994 reported similar findings that in *S. haematobium* and *S. japonicum* infections, CCA and/or CAA serum levels decreased soon after treatment.

The introduction of ELISA and radioimmunoassays has led to improvement in the sensitivity of serodiagnostic techniques for schistosomiasis (Smither and Doenhoff, 1982). ELISA however is the preferred option, in that the assay is based on stable antigen-antibody complexes and does not require the use of harmful radioactive substances.

In a cross-sectional survey, Al-Sherbiny *et al.* (1999) used serum, urine and stool samples from patients in an area known to be endemic for *S. haematobium* in Egypt. Diagnosis was approached in two parallel ways; the physical examination of urine and stool microscopic analysis and two advanced immunodiagnostic assay systems, Falcon assay screening test (FAST)-ELISA and the enzyme-linked immuno electro transfer blot (EITB). Parasitologically, the overall prevalence of *S. haematobium* was 15.8%. The combination of urine CCA and serum CAA for detecting circulatory antigens and the combination of the *S. haematobium* adult worm microsomal antigens (HAMA) FAST-ELISA and HAMA EITB for detecting antibodies significantly improved the sensitivity of detecting *S. haematobium* circulatory antigens and antibodies.

Diagnosis of animal schistosomiasis

Diagnosis of animal schistosomiasis is mainly based on the clinico-pathological symptoms of diarrhoea, wasting and anaemia. The relatively persistent diarrhoea, often blood stained and containing mucus may help to differentiate this syndrome from faciolosis (Urquhart *et al.*, 1988). The routine method of diagnosis is the microscopical examination for parasite eggs in faecal samples. However the demonstration of the characteristic eggs in the faeces or in squash preparations of blood and mucus from the faeces is useful in the period following high infection but less useful as egg production drops in the later stages of infection. Generally when schistosomiasis is suspected, diagnosis is best confirmed by a detailed post-mortem examination which will reveal the lesion and if the mesentery is stretched, the presence of numerous schistosomes in the veins (Urquhart *et al.*, 1988). These methods are time consuming and there is therefore the need for alternative diagnosis such as serological diagnosis particularly in epidemiological surveys.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Study area

Two villages, Galilea and Agbekpotsepko, were chosen as study areas based upon epidemiological data on the distribution of schistosomiasis. Galilea is a village in the Ga District of the Greater Accra region. It is located along the Weija Dam, which serves as the main source of domestic water for the community. The weedy bank of the dam contains large amount of decomposing plants and twigs and is infested with *Bulinus* and *Biomphilaria* snails. The dam constitutes a principal source of water and transmission of schistosomiasis.

Agbekpotsepko is located in the Dangbe West District of the Greater Accra region. The vegetation is mainly of grass with few trees. The area is swampy and constitutes a suitable breeding site for schistosome intermediate snail host. The main occupation of the community is farming and free range cattle rearing. The sources of water for the community are streams, which also serve the cattle. It was therefore suitable to select the area to investigate the prevalence of animal schistosomiasis.

Resuscitation of Hybridoma Cells

Monoclonal antibody secreting hybridama cell lines cryopreserved in 7.5% dimethylsulfoxide (DMSO) in Iscove's Modified Dulbecco's Medium (IMDM) were retrieved from liquid nitrogen at –196°C. The content of a vial was thawed rapidly in a water bath at 37°C and immediately transferred into a 15ml centrifuge tube containing 10ml of sterile growth medium [IMDM supplemented with 10% (v/v) heat inactivated (56°C/30min) Foetal Calf Albumin (FCS)]. This was gently mixed and washed by centrifugation at 415Xg for 15 min at 37°C to remove the DMSO. The supernatant was

discarded and the cell pellet resuspended in 3ml fresh growth medium. Approximately 1ml of cell suspension was transferred into 48 well plates and incubated at 37°C in a carbon (IV) oxide (CO_2) incubator (MCO 175, Sanyo Japan) at 5% CO_2 concentration. The moabs produced were Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10

Cloning and Propagation of Hybridoma Cells

Resuscitated hybridoma cells in culture were cloned to ensure monoclonality and continuous secretion of monoclonal antibodies. The cells in suspension were diluted with growth medium containing trypanblue (counter stain) to give 0.1% final concentration of the stain. Ten microlitres of the stained cell suspension was dispensed into a Neubauer counting chamber and the life cells counted microscopically (X100 magnification) using an inverted microscope (Olympus, Tokyo, Japan). The cells were cloned by limiting dilution in growth medium to give 1 cell/ 50 µl/ wellin 96 well tissue culture plates. These were cultured at 37°C in a CO_2 incubator and left undisturbed for 10 days. The plates were then examined for viable cell colonies by viewing the bottom of the wells or by observing under an inverted microscope. Wells with single viable cell colonies were marked and the cells transferred into 0.5ml of growth medium in 24 well tissue culture plate. After incubation for 3 days, 200µl of culture supernatant from each well was examined for antibody activity by micro-plate ELISA. The cells in antibody positive wells were transferred into 25cm^2 tissue culture flasks and added fresh growth medium for expansion.

The cells were sub cultured by transferring up to 2ml of the cell suspension into larger flasks (75cm^2 and 125cm^2) and the MoAb containing medium harvested when it became acidic (yellowish). Fresh growth medium was added and more antibody containing culture supernatant harvested from time to time. The supernatants were centrifuged at 415Xg for 5min to remove cell debris and stored at –20°C.

Cryopreservation of Hybridoma Cells

Cloned MoAb secreting hybridoma cells in logarithmic phase of growth were centrifuged at 400Xg for 5min. The pelleted cells were resuspended in stabilating medium containing 7.5% (v/v) DMSO (E. MERK, Darmstadt, Germany) to make a $2x10^6$ cells/ ml suspension. One millilitre aliqouts of the cell preparation were pipetted into cryopreservation vials before freezing in liquid nitrogen using a cryo–controller version 2.01 (Dept. of Biomedical Engineering, University Hospital, Copenhapen, Denmark) programmed to freeze cells over a period of 1 hr during which time specimen were subjected to a temperature gradient from room temperature to -140°C. The frozen vials were removed and immediately transferred onto labelled stabilation rack and immersed in liquid nitrogen at –196°C.

Determination of Immunoglobulin Class and Subclass

The MoAb class and subclass were determined using the double Immunodiffusion method described by Ouchterlony (1967). Commercially prepared antisera against murine Immunoglobulin isotypes IgG1, IgG2a, IgG2b IgG3, IgGM were used. The Quchterlony plates were made using 1% (w/v) agarose gel prepared by melting solid agarose in phosphate-buffered saline (15mM NaCl, 1.1mM $NaHPO_4$ and 0.1mM KH_2PO_4, pH 7.4). Five millilitres of the molten agarose was gently poured onto a microscope slide and allowed to solidify. Five wells equally spaced were cut into the solid gel in a circular arrangement surrounding a central well. Each well could hold approximately 15 µl of reagent. Antiserum was placed in central well and culture supernatants, concentrated thirty fold by amicon filtration placed in the surrounding wells. The prepared slides were left in a moist chamber overnight and the developed precipitin lines were observed by viewing the gels against light. The slides were then pressed under a pile of filter papers to dry the gel, which was then stained with coomasie blue and photographed.

Purification of Monoclonal Antiboddies

Monoclonal antibodies in culture supernatants were first concentrated by Amicon filtration, followed by ammonium sulphate precipitation and further purified by gel filtration.

Amicon Filtration

MoAb containing culture supernatants stored at –20°C were retrieved and thawed at 37°C in a water bath. Pooled culture supernatant was transferred into an Amicon filtration chamber (Nucleopone U.S.A) fitted with 100,000 molecular weight cut-off ultra-filtration disk membrane (Sigma, U.S.A). The culture supernatant was concentrated forty-fold by filtration under pressure using Nitrogen gas. Concentrated MoAbs were transferred into storage vials and either stored at –20°C or purified.

Ammonium Sulphate Precipitation

Monoclonal antibodies in Amicon concentrated culture supernatant were precipitated by the slow addition of saturated ammonium sulphate solution while mixing. Ammonium sulphate was added to a final concentration of 50% (v/v) for IgG and 30% (v/v) for IgM. The precipitate formed was pelleted by centrifugation at 1200Xg for 30min at 4°C and dissolved in minimum amount of distilled water. The antibody solutions were transferred into 1000MW cut-off dialysis membrane bags (Spectro Medical Industries Incorporated USA) and dialysed over night on ice against phosphate buffered saline (9mM NaH_2PO_4, 0.9mM Na_2HPO_4, 15mM NaCl, pH 7.4) with three buffer changes to remove the excess salt.

Gel Filtration

Ammonium sulphate precipitated and dialysed IgM MoAbs were further purified by gel filtration on Sephadex G-200 (Pharmacisa, Uppsala, Sweden), pore size 40-120 µm. This was prepared according to the manufacturer s instructions. The gel was allowed to

swell for 5 hours at 100°C and degassed for 2 hours. A 2.6 x 100cm column was packed and washed with at least one column volume of elution buffer (PBS, 0.138M NaCl; 2.8mM KCl; 10.2mM Na_2HPO_4; 1.8mM KH_2PO_4, pH 7.4). Twenty millilitres of dialysed MoAb diluted to reduce viscosity was applied onto the column and eluted with PBS, at a flow rate of 40ml/ hour. The eluates were collected 5ml/ tube and assayed for antibody activity by microplate-based ELISA. Fractions containing antibodies were pooled, concentrated by Amicon filtration and stored at –20°C until needed.

Micro-Plate ELISA

Micro-plate ELISA was used for detection of MoAb in fractionated reagents and in culture supernatant. Polysterene microplates (Sumilon S type MS-8496F) were coated with either *S. haematobium* soluble egg antigen (SEA) or P_2J (*S. haematobium* urinary antigen) at 37°C overnight to dry. The P_2J antigen stock was obtained from the Parasitology Unit of the NMIMR and the SEA (from an Egyptian strain of *S. haematobium*) was donated by the WHO. Soluble egg antigen was coated at 1.0µg/well and P_2J at 0.5µg/well in carbonate coating buffer. The plates were rinsed once with washing buffer, PBS pH 7.4, consisting of 0.136M NaCl, 9.7mM Na_2HPO_4, 1.4mM $KHPO_4$, 2.7mM KCl, 0.6mM $C_7H_5O_2Na$ and 0.5% (v/v) Tween 80. They were then incubated with different fractions of prepared MoAbs in duplicate wells for 15min. Undiluted normal culture medium and infected mouse serum diluted at 1:100 were added as negative and positive controls, respectively. The plates were flipped empty and rinsed twice with washing buffer to remove excess unbound MoAbs. The wells were then incubated for 30min (50µl/ well) with either goat anti-mouse IgM horseradish peroxidase (HRPO) conjugate for detection of IgM MoAbs (Sh3/34.10, Sh3/38.2, Sh5/32.30 and Sh5/34.10) or goat anti-mouse IgG-HRPO for Sh4/14.3. The conjugates were each diluted at 1:500 in blocking buffer (0.5% BSA in washing buffer). The plates were emptied, rinsed again and washed further by flooding three times, each with 10min incubation to

remove unbound conjugates. The presence of the bound conjugate was revealed by the incubation of substrate solution (100µl/ well) in the dark for 30min. The substrate solution [40mM 2,2'azino-bis-3-ethylbenzethiozaline-6-sulphonic acid (ABTS) and 0.01% (v/v) H_2O_2 in 50mM citrate buffer, pH 4.0] changed to green in wells with bound enzyme conjugates. Optical densities were read at 415nm wavelength using an MTP-32 microplate reader (Corona Electric, Japan).

Cross-reactivity testing of MoAbs using schistosome and *P. falciparum* antigens in microplate ELISA

The micro-plate ELISA procedure described above was also used to test the selected MoAbs for cross-reactivity using antigens of *Schistosoma* species (*S. haematobium, S mansoni and S. japonicum*), and *P falciparum* circum-sporozoite protein (CSP) and crude antigen extracts obtained from the Immunology Unit of the NMIMR. Briefly, polystyrene microplates were coated separately with the different antigens 50µl/well at 37°C overnight to dry. The plates were rinsed once with PBS washing buffer and incubated 50µl/well for 15min with test MoAbs, starting from neat, 1:5 and then diluted up to 1:3200. The plates were rinsed twice with washing buffer and then incubated for 30min with 50µl/well of goat anti-mouse IgM or IgG conjugate. The plates were again washed before incubation with substrate solution.

Preparation of Horseradish Peroxidase Antibody Conjugates

Purified *Schistosoma* genus-specific MoAbs were conjugated to HRPO using the periodate method described by Wilson and Nakane (1978). The protein concentration of antibody fractions was estimated by Buiret Protein assay and HRPO enzyme (Sigma USA) conjugated to antibody protein at a ratio of 1:1 (enzyme:antibody) by molecular weight. Calculated enzyme was dissolved in 2ml of 50mM sodium acetate buffer, pH 4.0 and activated (oxidized) using sodium metaperiodate ($NaIO_4$) in the ratio of 1:1 by weight.

The required quantity of $NaIO_4$ was dissolved in 1ml of sodium-acetate buffer, and the $NaIO_4$ solution then added dropwise to the HRPO solution. The mixture was kept in the dark for 20min and 300µl ethylene glycol ($C_2H_6O_2$) added and stirred. The resulting mixture was fractionated through a sephadex G-25 column (1.0cm diameter x 30.0cm) and eluted with sodium-acetate buffer. The pH of the eluted enzyme and the antibody solution were adjusted to 9.6 by the addition of 1-2ml carbonate-bicarbonate buffer, pH 9.6 followed by dropwise addition of saturated Na_2CO_3. The antibody and enzyme solutions were then added together, covered with aluminium foil and stirred slowly for 1hr at room temperature. Three hundred milligrams of glycine ($NH_2.CH_2.COOH$) was then added, mixed and the pH adjusted to 8.0 using 1M HCl. The conjugate was kept at 4°C overnight, after which it was precipitated with 50% (v/v) saturated ammonium sulphate solution and then centrifuged for 2 min at 9,900 xg. The pellet was dissolved and made up to the original volume with glycine/Na_2EDTA buffer (0.4M glycine, 0.3M NaCl and 20mM Na_2EDTA, pH 8.0). Twenty milligramms of ovalbumin was added and mixed to dissolve, and the solution centrifuged once more to remove particulate matter. The conjugate solution was then passed through a 0.45µm, followed by a 0.22µm membrane filter (Millipore Products Division, Bedford, MA, USA) to remove polymerised conjugate. Finally an equal volume of glycerol was added, mixed and the reagent stored at –20°C until used.

Coating of the Test MoAbs on PVDF and Nitrocellulose Membranes by Dot-ELISA

The ability of the test MoAbs to coat onto PVDF membrane (Millipore Corporation, Bedford MA) and Nitrocellulose membrane (Sigma Chemical Co. USA) was assessed using PBS of different pH (7.0, 7.2 and 7.4). Each of the MoAbs was diluted separately in the three different PBS buffers and two microlitres volumes dotted on membrane strips measuring 1x6cm. PVDF membrane strips were first soaked with methanol, quickly immersed in distilled water and then placed on moist filter paper before

dotting the samples. Nitrocellulose membranes were used without prior processing. The strips were then air dried for 5 min and incubated in blocking solution (0.3% w/v casein) for 30 min with gentle shaking. To assess binding of MoAb to the membranes, the strips were incubated for 1hr in goat anti-mouse HRPO IgM or IgG depending on the isotype of the test MoAb. After incubation the strips were washed three times (10 min/wash) in washing buffer (0.1% Triton X-100 in PBS). This was followed by a 1min incubation in substrate solution (25ml TBS, pH 7.4; 0.005g DAB; 150µl cabaltous nitrite; 15µl H_2O_2), and extensive washing in excess distilled water. Positive reactions which, appeared bluish black were visually assessed and graded.

Coating of the Selected MoAbs to Micro-titre Plates

Coating of the test MoAbs to micro-titre plates was investigated using different polystyrene plates; Sumilon (Ms-8496F), Sero-wel (Cat. Cat No. 612F96) and Sero-wel (Cat. No. 611U96). Dulbecco's PBS (pH 7.4) was used as coating buffer. Fifty microlitres of each dilution of test MoAb (1:25 –1:3200) was dispensed into wells of micro titre plates and incubated for 30 min. The plates were flipped empty and washed twice with washing buffer [PBS, 0.136M NaCl, 9.7mM Na_2HPO_4, 1.4mM $KHPO_4$, 2.7mM KCl, 0.6mM $C_7H_5O_2Na$ and 0.5% (v/v) Tween 80, pH 7.4] to remove unbound MoAbs. They were then incubated for 30 min (50µl/ well) with goat anti-mouse IgM-HRPO conjugate or goat anti-mouse IgG-HRPO diluted at 1:500 in blocking solution (0.5% Bovine serum albumin in washing buffer). After incubation, wells were washed further by flooding three times (10min/wash) to remove unbound conjugates. The presence of the bound conjugate was revealed by the addition of ABTS substrate solution (100µl/well) for 30min in the dark and the optical densities read at 415nm wavelength using an MTP-32 microplate reader (Corona Electric, Japan).

Dipstick ELISA procedure

Polyvinylidene difluoride membrane was cut into strips of dimension 2mm by 15mm and numbered at one end. The strips were wetted halfway from the unlabelled end by immersion in absolute methanol for 15sec. Wetted strips were immediately transferred into distilled water and later stored in 1:256 dilution of methanol and distilled water.

Stored strips were retrieved and incubated in test urine samples for 30min at room temperature. The strips were rinsed in excess TBS (50mM Tris, 200mM NaCl, pH 7.0) and then blocked for 5min in 35% skimmed milk in TBS. Control strips not incubated in test urine were also blocked. After incubation the strips were washed three times in TBS and transferred into combined reagents consisting of MoAb and goat anti-mouse HRPO conjugate in TBS containing 0.1% skimmed milk for 1 hour with gentle rocking. They were then washed three times (10min/ wash) in excess TBS with gentle shaking on a rocker and then developed by incubation for 1min in substrate solution containing 0.025g diaminobenzidine tetrahydrochloride, 300µl cobaltous chloride and 30µl 35% H_2O_2 in 50µl of TBS. Developed strips were immediately rinsed three times with excess distilled water to remove substrate. A bluish-black reaction represented positive results while negative results remained colourless. Control strips were used to assess the background and the test strips graded based on colour intensity.

Microplate-based Sandwich ELISA

Flat-bottomed Sero-wel micro-titre plates previously coated with MoAbs were washed twice with washing buffer and incubated for 1hr with schistosome infected human urine samples. The plates were subsequently washed twice and the wells incubated (50µl/ well) for 30 min with HRPO-conjugated MoAbs diluted at 1:500 in blocking solution. After incubation they were washed further by flooding three times (10min/ wash) to remove unbound conjugates. The presence of the bound conjugate was revealed by the addition of 100µl of substrate solution in each well and the plates kept in the dark for

30min. Optical densities were read at 415nm wavelength using an MTP-32 micro plate reader (Corona Electric, Japan).

Membrane-based sandwich ELISA

The ability of the test MoAbs to capture schistosome antigen in membrane-based sandwich ELISA was assessed using PBS coating buffer at pH of 7.4. PVDF strips, each coated with 2µl of a test MoAb in dots was air dried. The strips were incubated in blocking solution (0.3% w/v casein) for 30 min with gentle shaking and then incubated in schistosome infected human urine for 1 hr. They were then washed twice in washing buffer (PBS pH 7.4, 0.1% Triton X-100) and incubated with MoAb-enzyme (HRPO) conjugate for 1 hr. After incubation the strips were further washed three times (10 min/wash) in washing buffer and incubated in substrate solution (25ml TBS, pH 7.4; 0.005g DAB; 150µl cobaltous nitrite; 15µl H_2O_2) for 1 min before they were transferred into distilled water.

Microscopical Diagnostic Methods

Collection and Analysis of Human Urine and Stool Specimen

Urine and stool specimens were collected from primary school pupils in Galilea between 11.00 and 14.00 hours. The samples were transported on ice to the laboratory. Ten millilitres of each urine sample was filtered through a 25mm nucleopore filter (12µm pore size) secured in a Swimnex support chamber. The filter membrane was removed with forceps and the side with trapped eggs laid face down on a labelled microscope slide. The urine filtrate was used for dipstick assay whilst excess unfiltered urine was centrifuged at 1,290Xg for 5 min to sediment any schistosome eggs. The prepared slide and urine sediments were examined separately for schistosome eggs by microscopy. A drop of saline was placed on the filter to improve the refractive index.

To detect parasitic infections by the Kato-Katz technique (Katz *et al.*, 1972), a stool sample was taken with wooden spatula and forced through a stainless steel mesh to remove particulate and fibrous materials. The specimen was applied to fill a hole in a template set on a glass microscope slide, and the template carefully lifted to leave an intact plug of faeces. This was then covered with a 25x2mm cellophane cover slip previously impregnated with 50% (v/v) glycerol in water containing 3% malachite green. The slide was subsequently turned face down on a flat surface and pressed gently but firmly to spread the stool specimen evenly. Prepared slides were left for 30min in the light to clear before microscopical examination for parasite eggs.

Collection and Analysis of Cattle Blood and Faecal Samples

Blood and faecal samples of cattle (West African short horn breed) were collected from Agbekotsekpo in the Dangbe West District of the Greater Accra region. After collection, the blood samples were allowed to stand for 4 hours at room temperature and then kept at 4°C overnight to allow for maximum clotting. The samples were spun at 2,500xg and the supernatant (serum) collected and stored at –20°C until needed.

The faecal samples were analysed by microscopy. Three grammes of faeces was weighed into a beaker and 10ml of distilled water added to the sample and homogenised. The homogenised faeces was passed through a series of 5 sedimentation sieves with stirring and the material left on the sieve washed with distilled water. The debris was discarded and the filtrate transferred into a conical flask and allowed to stand for 2 min. The supernatant was decanted and the remaining sample suspension transferred into a volumetric flask. This was allowed to sediment for 2min and the supernatant discarded. Few drops of 5% methylene blue was added to the suspension and then observed under a microscope at 20x magnification for parasite ova.

Fixation of miracidia with Karnovsky reagent

Miracidia were hatched from *S. haematobium* eggs obtained from urine of infected individuals. The positive urine samples were pooled and spun at 500xg for 5min. The pellets containing the eggs were resuspended with a small volume of water <5ml and transferred into filtered pond water in a volumetric flask using a pipette. Care was taken to deposit the egg suspension at the bottom of the flask, which was then covered with aluminium foil up to the neck region. The contents of the flask was topped with water up to a level above the covered portion and placed in front of a light source. The miracidia hatched from the eggs were attracted to the light and concentrated in a small volume of exposed water. These were harvested with Pasteur pipette, chilled to 4°C and spun at 2350xg for 10min to concentrate the miracidia. Equal volume of 4% Karnovsky reagent containing paraformaldehyde and glutaldehyde in PBS, pH 7.4 was added to the miracidia suspension to make a final concentration of 2% Karnovsky reagent. This was incubated at 4°C for 2 hours to fix the miracidia. The fixed miracidia was spun at 2,350xg for 10min at 4°C and washed 3 times with PBS by centrifugation. Miracidia were counted to estimate the concentration and stored in PBS 4°C until use.

IFAT Procedure

Twenty-four microlitres of miracidia suspension was pipetted into a 1.5ml eppendorf tube and 1µl bovine serum added to make a final concentration of 1:25 dilution of the serum. The mixture was incubated for 1 hour at room temperature with shaking at 5 min interval. Dulbecco's phosphate buffered saline (DPBS) was added and washed by centrifugation at 4,700xg for 10 min. It was then incubated for 30 min with 1:50 dilution of anti-bovine FITC conjugate containing 0.01% trypan blue as counter stain. After incubation the mixture was washed three times by centrifugation at 4700xg for 10 min. The pellet was resuspended in 20µl of DPBS and 10µl of the suspension observed using fluorescent microscope (Olympus Optical Co. Ltd, Japan) at x400 magnification.

CHAPTER 3

RESULTS

Prepared monoclonal antibody reagents

Five hybridoma cell lines secreting *Schistosoma* genus-specific monoclonal antibodies (Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/34.10 and Sh5/32.30) were selected from a panel of 200 MoAbs generated at the Parasitology Unit of the NMIMR. Table 1, summarizes data on the type of monoclonal antibody fractions used, their isotypes as determined by Double immunodiffusion, and the biochemical nature of the antigenic epitopes detected. With the exception of Sh4/14.3, which is an IgG1, all the other monoclonal antibodies were of IgM class.

Plate 1 presents a photograph showing a precipitin line used to identify the subclass of Sh4/14.3 in Double immunodiffusion. The isotypes of the other monoclonal antibodies were determined similarly as summarised in Table 1.

Table 1. Some biochemical characteristics of the selected monoclonal antibodies

Monoclonal antibody	Antibody fraction	Isotype	Nature of epitope [a]
Sh3/34.10	Purified [b]	IgM	Protein
Sh3/38.2	Purified [b]	IgM	Carbohydrate/protein
Sh4/14.3	Amicon conc. [c]	IgG1	Protein
Sh5/32.30	Purified [b]	IgM	Protein
Sh5/34.10	Purified [b]	IgM	Protein

[a] Biochemical characteristics of the epitope bound by monoclonal antibody [after, Yeboah (1998) M.Phil thesis University of Ghana, Legon].
[b] IgM monoclonal antibody fraction purified by gel filtration.
[c] Monoclonal antibody in culture supernatant concentrated 30 fold by amicon filtration.

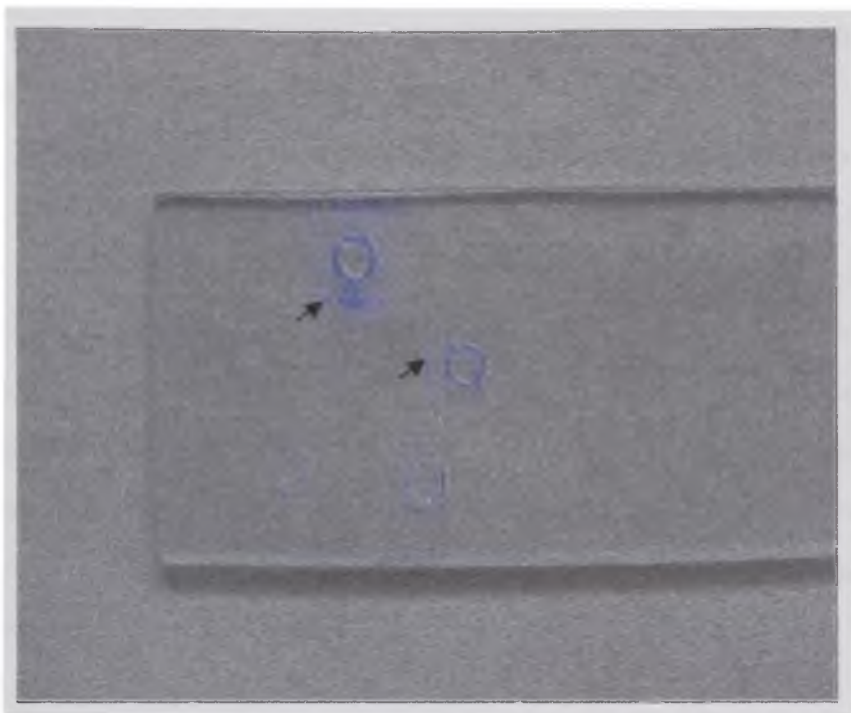

Plate 1 **Photograph showing precipitin lines (indicated by arrows) used to identify the subclass of Sh4/14.3 in double immunodiffusion**

Reactivity of selected monoclonal antibodies as determined by micro-plate ELISA

The suitability of the selected anti-schistosome monoclonal antibodies (Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/34.10 and Sh5/32.30) as diagnostic reagents was determined by their ability to specifically detect *Schistosoma* genus-specific antigens. As shown in Table 2, there was reactivity between the antibodies and the different schistosome antigens used. The genus-specificity of the MoAbs was confirmed using antigens of *S. haematobium, S. mansoni,* and *S. japonicum* by microplate ELISA.

Cross-reactivity analysis of the selected anti-schistosome monoclonal antibodies with *P. falciparum* crude antigen extract and circum-sporozoite antigen was determined. The results are also presented in Table 2. As shown, none of the test monoclonal antibodies could react with the circum-sporozoite antigens. However, two of the monoclonal antibodies (Sh3/34.10 and Sh5/34.10) reacted with the *P. falciparum* crude antigen extract at higher MoAb concentrations (Figures 1-5) even though the reactivity declined sharply to OD $\leq$ 0.1 at lower concentrations (dilution $\geq$1:100). On the other hand, the reactivity of Sh3/34.10 and Sh5/34.10 with the schistosome antigens remained high at all the dilutions tested (Figures 1 and 5).

Table 2. **Reactivity of the selected monoclonal antibodies with *Schistosoma* species and *Plasmodium falciparum* antigens**

MoAb	Reactivity of monoclonal antibodies with antigens of different schistosome species			Reactivity of monoclonal antibodies with *P. falciparum* antigens	
	S. haematobium	*S. mansoni*	*S. japonicum*	Circum-sporozoite antigen	Crude antigen extract[a]
Sh3/34.10	+	+	+		+
Sh3/38.2	+	+	+	-	
Sh4/14.3	+	+	+	-	
Sh5/32.30	+	+	+	-	-
Sh5/34.10	+	+	+	-	+

[a] Crude antigen extract of different *P. falciparum* life cycle stages obtained from culture.

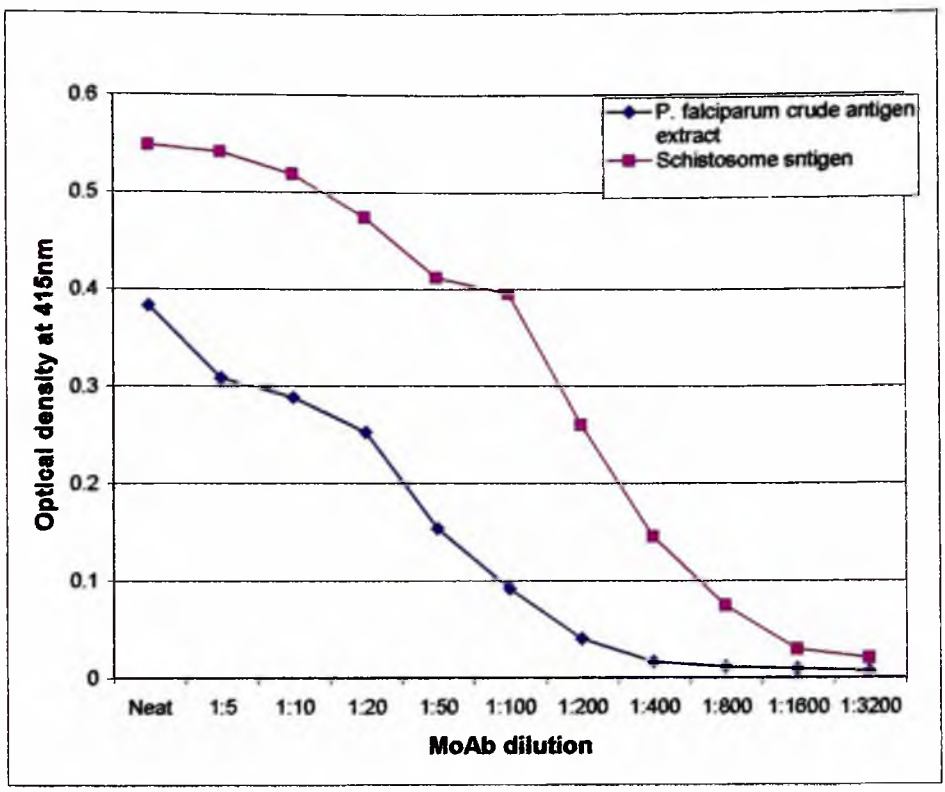

Figure 1. **Reactivity of Sh3/34.10 with schistosome antigen and *P. falciparum* crude antigen extract at different MoAb dilutions**

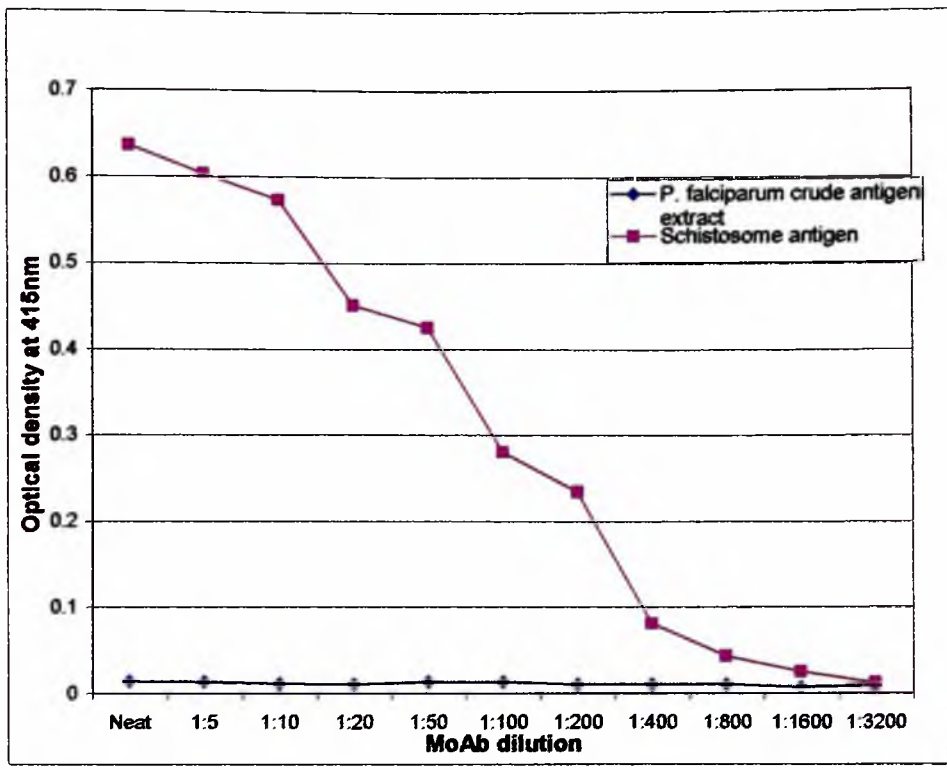

Figure 2. **Reactivity of Sh3/38.2 with schistosome antigen and *P. falciparum* crude antigen extract at different MoAb dilutions**

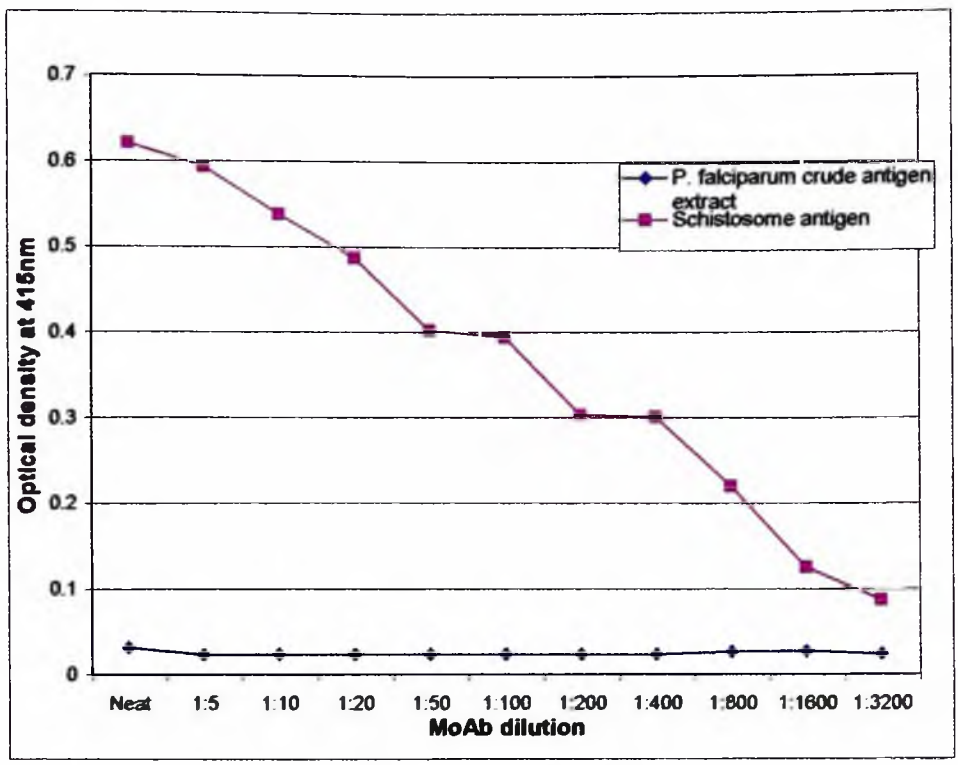

Figure 3. **Reactivity of Sh4/14.3 with schistosome antigen and *P. falciparum* crude antigen extract at different MoAb dilutions**

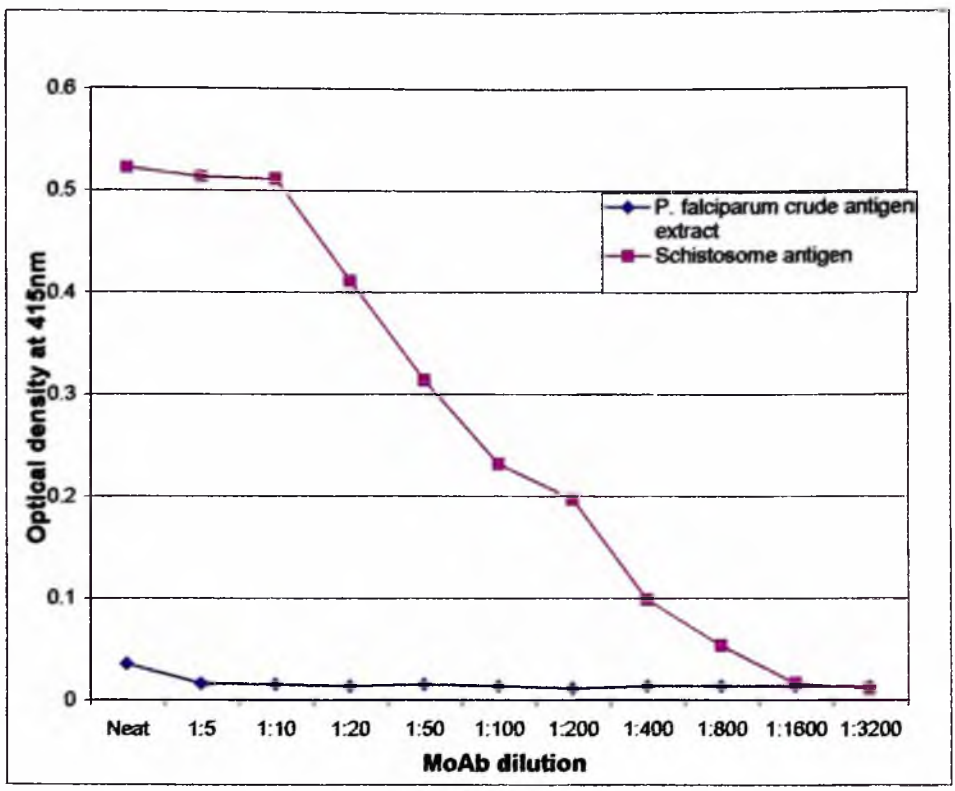

Figure 4. **Reactivity of Sh5/32.30 with schistosome antigen *and P. falciparum* crude antigen extract at different MoAb dilutions**

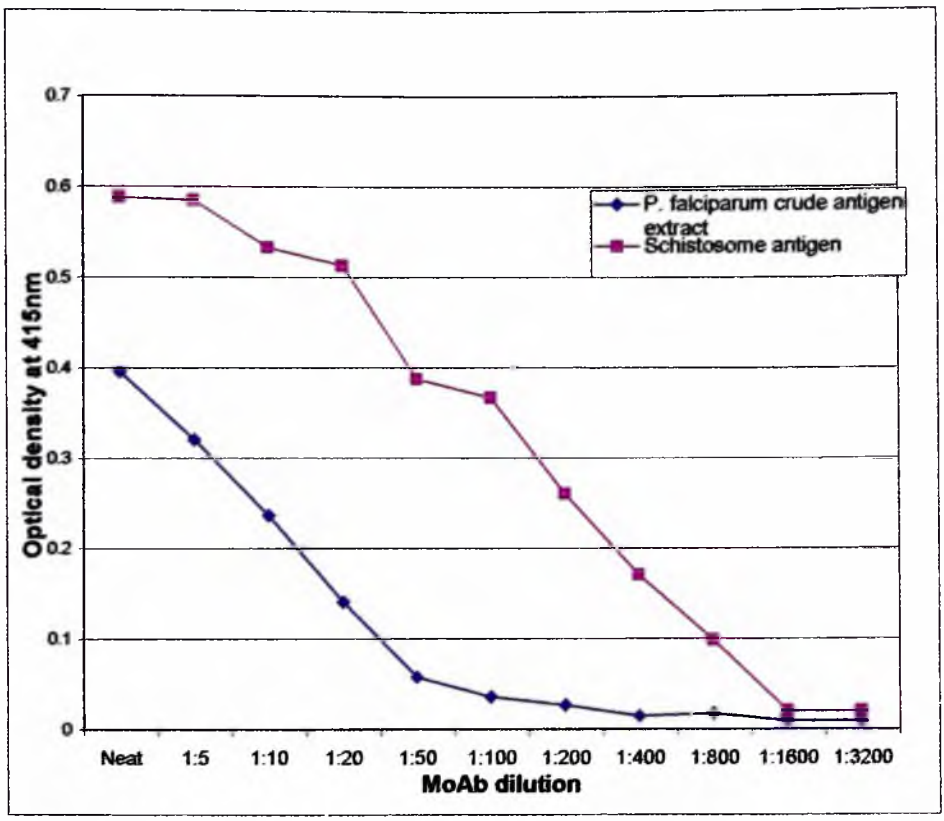

Figure 5. **Reactivity of Sh5/34.10 with schistosome antigen and *P. falciparum* crude antigen extract at different MoAb dilution**

Development of assays

Detection of *Schistosoma* antigens in human urine using the selected monoclonal antibodies

Table 3 summarises results of microscopic identification of parasite ova in human urine and the ability of the selected monoclonal antibodies to detect *Schistosoma* antigens when evaluated using the standard dipstick-ELISA protocol. The result obtained with Sh2/15.F, the monoclonal antibody utilized in the standard urinary schistosomiasis dipstick-ELISA, was used as the basis to assess the other monoclonal antibodies. Out of 74 urine samples examined, Sh2/15.F detected antigen in 65 (87.8%) (Table 4), and microscopy revealed *S. haematobium* ova in 60/65 (92.3%) of them (Table 3). The five *Schistosoma* genus-specific monoclonal antibodies (Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10) detected antigen in different numbers of the infections identified by the standard Sh2/15.F dipstick-ELISA (Table 3). As shown, they recorded comparatively lower relative sensitivities (ranging from 60.0 to 71.7%) when compared with microscopy as the gold standard test (Table 3). There were no significant differences ($p<0.05$) in relative sensitivities of the assays utilizing the different Schistosoma genus specific MoAbs in detecting schistosome antigens. However they were significantly different from the performance of the standard Sh2/15.F dipstick-ELISA, which gave a relative sensitivity of 100%. Interestingly, each of the *Schistosoma* genus-specific MoAbs detected schistosome antigens in only urine samples, which were microscopically positive, thereby indicating 100% specificity. On the other hand the standard Sh2/15.F dipstick-ELISA recorded a specificity of 64.3% (9/14). As shown in Table 5, the *S. haematobium* egg positive samples detected by the dipstick assays utilizing the selected MoAbs had intensities ranging from 2-3000 eggs/10ml urine (median = 295, mean = 498), however the egg positive samples not correctly detected by the different assays had overall egg count ranging from 50-2800 eggs/10ml urine (median = 274, mean = 492). Each of the dipstick assays failed to detect some infections within egg count ranges that were revealed in other

subjects. Of the different dipstick assays, Sh3/34.10, Sh5/32.30 andSh5/34.10 detected antigens in the widest range of egg counts. Assays utilizing MoAb Sh4/14.3 detected infections involving the minimum number of egg counts.

Using urine samples of individuals who were infected with *S. mansoni,* the ability of the selected monoclonal antibodies to detect *S. mansoni* antigen was evaluated by dipstick-ELISA. Microscopic analysis revealed that 44 out of 55 (80.0%) individuals examined were *S. mansoni* egg positive, and 40/44 (90.9%) had mixed infections with *S. haematobium* (Table 3). This gave a prevalence of 72.7% for mixed infections involving *S. mansoni* and *S. haematobium.* With the exception of *S. mansoni*, no other human intestinal parasite ova were detected. The relative sensitivity of the different *Schistosoma* genus-specific MoAbs in detecting *S. mansoni* infections, using microscopy as gold standard test ranged from 61.3% to 63.6% (Table 3). However, the differences in sensitivity of the various assays were not significant ($p>0.05$). On the other hand, the relative sensitivity of the Sh2/15.F based-dipstick recorded a higher sensitivity of 95.5% (42/44). As shown in Table 3, 40 out of the 42 Sh2/15.F-dipstick positives were also confirmed to be infected with *S. haematobium* by demonstration of the parasite's eggs. Interestingly, Sh2/15.F dipstick-ELISA detected antigens in 2/4 (50%) of single infections involving *S. mansoni* as determined by microscopy. None of the selected genus-specific MoAbs could detect antigens in single infections involving *S. mansoni.*

Table 3 **Prevalence of urinary and intestinal schistosomiasis as determined by microscopy and dipstick-ELISA using different MoAbs**

MoAb	Detection of *S. haematobium*					Detection of *S. mansoni*				
	No.	No. positive		Prevalence (%)		No.	No. positive		Prevalence (%)	
	tested	Microscopy	Dipstick[a]	Microscopy	Dipstick[a]	tested	Microscopy	Dipstick[a]	Microscopy	Dipstick[a]
Sh2/15.F	74	60	65	81.1	87.8	55	44	42 (40)[b]	80	76.4
Sh3/34.10	74	60	36	81.1	48.6	55	44	27	80	49.1
Sh3/38.2	74	60	38	81.1	51.3	55	44	28	80	50.1
Sh4/14.3	74	60	36	81.1	48.6	55	44	27	80	49.1
Sh5/32.30	74	60	37	81.1	50.0	55	44	27	80	49.1
Sh5/34.10	74	60	43	81.1	58.1	55	44	28	80	50.1

[a] Number of urine samples testing positive for schistosome antigens in dipstick utilising the different MoAbs.
[b] Number of samples that were also positive for *S. haematobium* by microscopy

Table 4 **Diagnosis of schistosomiasis by microscopy and dipstick-ELISA using the selected MoAbs**

MoAb	Detection of *S. haematobium*					Detection of *S. mansoni*				
	No. tested	Microscopy positive	Dipstick positive[a]	Specificity (%)	Relative sensitivity[b] (%)	No. tested	Microscopy positive	Dipstick positive[a]	Specificity (%)	Relative sensitivity[b] (%)
Sh2/15.F	74	60	65	64.3	100.0	55	44	42 (40)[c]	100.0	95.5
Sh3/34.10	74	60	36	100.0	60.0	55	44	27	100.0	61.4
Sh3/38.2	74	60	38	100.0	63.3	55	44	28	100.0	63.6
Sh4/14.3	74	60	36	100.0	60.0	55	44	27	100.0	61.4
Sh5/32.30	74	60	37	100.0	61.7	55	44	27	100.0	61.4
Sh5/34.10	74	60	43	100.0	71.7	55	44	28	100.0	63.6

[a] Number of urine samples testing positive for schistosome antigens in dipstick utilising the different MoAbs.
[b] Sensitivity of dipstick assays utilising the different MoAbs as compared to microscopical detection of schistosome egg as gold standard test.
[c] Number of samples that were also positive for *S. haematobium* by microscopy

Table 5. Comparison of egg counts of *S. haematobium* egg positive urine samples detected by the genus-specific MoAb dipstick with those not detected by the dipstick assay

MoAb Utilized in Dipstick	Microscopy positive	Number of *S. haematobium* egg positive not detected by dipstick and their egg				Number of *S. haematobium* egg positive not detected by dipstick and their egg count			
			Egg count/10ml urine				Egg count/10ml urine		
		Dipstick positive	Mean	Median	Range	Dipstick negative	Mean	Median	Range
Sh3/34.10	60	36	550	316	1-3000	24	530	261	50-2800
Sh3/38.2	60	38	441	301	1-2800	22	501	300	50-2800
Sh4/14.3	60	36	520	288	1-2800	24	447	253	2-2800
Sh5/32.30	60	37	514	279	1-3000	23	492	264	50-2800
Sh5/34.10	60	43	467	293	1-3000	17	488	292	50-2700
Total	60		498	295			492	274	

Ability of the selected MoAbs to coat onto different micro-titre plates and membranes

Table 6 shows the ability of the selected MoAbs to bind onto different micro-titre plates [sero-wel (Cat No. 612F96), sero-well (Cat. No. 611U96), sumilon (Ms-8496F)] and membranes [PVDF (Cat No. 1PUH20200) and nitrocellulose (Cat No. 13H0457)]. MoAb coated plates were probed using goat anti-mouse immunoglobulin HRPO conjugates and revealed with ABTS substrate. As shown in table 6 all the MoAbs (Sh3/34.10, Sh3/38.2, Sh4/14.3, Sh5/32.30 and Sh5/34.10) could coat onto the three different micro-titre plates tested. However, the assessment of binding based on colour intensity showed that the MoAbs were better retained by the sero-wel (Cat No. 612F96) plate, followed by Sumilon before sero-well (Cat. No. 611U96). Sero-well (Cat No. 612F96), which exhibited the highest binding of MoAbs, was therefore selected for further experiments to detect schistosome antigens in human urine.

Investigation into the binding of the MoAbs to different membranes in dot-ELISA also revealed that the antibodies could bind to both membranes (Table 6). Bound antibodies remained detectable in both membranes after extensive washing in PBS buffer. Both membranes showed higher MoAb binding at the highest, pH 7.4 compared to pH 7.0 and pH 7.2 that were evaluated. The intensity of colour development was however higher on PVDF (Table 6). The PVDF membrane was therefore used in the subsequent assays.

Table 6 Binding studies of the selected MoAbs onto different micro titre plates and membranes

MoAb	Micro titre plate			Dot-ELISA reactivity on different membrane and pH					
				PVDF			Nitrocellulose		
	Sero-wel	Sero-wel U-bottomed	Sumilon	7.0	7.2	7.4	7.0	7.2	7.4
Sh3/34.10	+++	±	+	++	+ +	+++	+	<++	++
Sh3/38.2	+++	±	+	++	++	+++	+	<++	++
Sh4/14.3	+	±	±	++	+	+++	+	<++	++
Sh5/32.30	++	±	+	++	++	+++	+	<++	++
Sh5/34.10	++	±	+	++	++	+++	+	<++	++

Detection of schistosome antigens in human urine using the selected monoclonal antibodies in sandwich ELISA

Following the successful detection of schistosome antigens in infected human urine using the selected MoAbs in dipstick ELISA, experiments were conducted to capture the antigens in sandwich ELISA utilizing micro-titre plates and membranes coated with the MoAbs. Interestingly, none of the selected MoAbs could demonstrate captured schistosome antigen when used along side its own enzyme-conjugate in a homologous ELISA.

Prevalence of *Schistosoma* infection in cattle as determined by microscopy

In order to identify cattle infected with schistosomes, faecal specimens from 32 animals were screened by microscopy. As shown in Table 7, ova of *Schistosoma bovis* were identified in 17 (53.1%) of the faecal specimen examined. Also, one specimen (3.1%) was positive for *S. indicum* eggs. Typical *S. bovis* eggs found are shown in Plate 2. In addition to the schistosome infections, other cattle parasites were identified (Table 7). The prevalence of the other infections ranged from 3.1% to 21.9% (Table 7). Interestingly, each of the 32 faecal samples examined showed the presence of at least one parasite infection.

Table 7 **Detection of parasite ova in stool samples of cattle (short horn variety) by microscopy from Agbekpotsepko**

Parasite	Number of stool samples examined	Number of cattle infected	Prevalence %
Schistosoma bovis	32	17	53.1
Schistosoma indicum	32	1	3.1
Mecistocirrus digitatus	32	5	16.0
Eimeria auburnensis	32	5	15.6
Eimeria subspherica	32	7	21.9
Syngamus laryngeus	32	1	3.1
Eimeria zurnii	32	5	15.6
Oesophagostomum radiatum	32	5	15.6
Trichostrongylus axei	32	7	21.9
Ascaris vitulorum	32	1	3.1
Bonustomum phlebotomum	32	1	3.1
Cooperia sp	32	1	3.1
Fasciola	32	4	12.5

Plate 2: **Eggs of *S. bovis* identified in faecal specimen of cattle at X20 magnification.**

Schistosoma infection rates in cattle as determined by IFAT and micro-plate ELISA

Schistosome infections in cattle were also diagnosed by detection of anti-*schistosoma* antibodies using IFAT, or antigen using micro-plate ELISA. Out of 32 cattle sera screened, 16 tested positive for schistosome antibodies, giving a seroprevalence of 50%. The sero-positives included 16 of the 17 microscopically confirmed schistosome infections in the cattle (94.1%).

The ability of the selected MoAbs to detect schistosome antigens in cattle sera was assessed using micro-plate ELISA protocol. The different MoAbs detected antigens in sera of cattle confirmed to be infected with *S. bovis* by microscopy. They recorded different relative sensitivities (ranging from 46.8 to 50.0%) when compared with microscopy as the gold standard test (Table 8). However, there were no significant differences ($p>0.05$) in relative sensitivities of the assays. All the antigen positive sera were also positive for anti-*Schistosoma* antibodies as determined by IFAT.

Table 8 **Diagnosis of schistosomiasis in cattle by microscopy and micro-plate ELISA using the selected MoAbs**

MoAb	No of sera examined	Microscopy positve	Plate-ELISA positive[a]	Relative sensitivity[b] (%)	Prevalence (%)
Sh3/34.10	32	17	15	88.2	46.9
Sh3/38.2	32	17	16	94.1	50.0
Sh4/14.3	32	17	16	94.1	50.0
Sh5/32.30	32	17	15	88.2	46.9
Sh5/34.10	32	17	15	88.2	46.9

[a] Number of sera samples testing positive for schistosome antigens in micro-plate ELISA utilising the different MoAbs.

[b] Sensitivity of Plate-ELISA assays utilising the different MoAbs as compared to microscopical detection of schistosome egg as gold standard.

CHAPTER FOUR

DISCUSSION AND CONCLUTION

The objective of the series of experiments described in this thesis was to evaluate the diagnostic potential of selected anti-*Schistosoma* genus-specific MoAbs (Sh3/34.10, Sh3/38.3, Sh4/14.3, Sh5/32.30 and Sh5/34.10) in detection of *S. mansoni* in humans and *Schistosoma* species in animals. The strategy was to purify the MoAbs, determine their reactivity and utilize them in diagnosis of schistosomiasis, whilst comparing with the established urinary schistosomiasis dipstick (Bosompem *et al.*, 1998).

Analysis of culture supernatants from the selected hybridoma cell lines in this study revealed the presence of MoAbs of IgG and IgM isotypes which were also confirmed to react with schistosome antigens. In a previous study Yeboah (1996), reported the lack of cross reactivity of the MoAbs (Sh3/34.10, Sh3/38.3, Sh4/14.3, Sh5/32.30 and Sh5/34.10) with antigens of *Necator americanus* a common endemic parasite in Ghana. In this study, the specificity of three of the selected MoAbs (Sh3/38.3, Sh4/14.3 and Sh5/32.30) with schistosome antigens was further demonstrated by the lack of cross reactivity with antigens of the highly endemic malaria parasite *P. falciparum.* It is therefore unlikely that the presence of hookworm and/or *P. falciparum* infections would influence the results of any assays developed with these MoAbs. However two of the MoAbs, Sh3/34.10 and Sh5/34.10 reacted with crude antigen extract of *P. falciparum* at high MoAbs concentrations, suggesting that these MoAbs could only be utilized in diagnosis at high antibody titres where cross-reactivity did not occur; even though the sensitivity of the assays developed could be compromised.

The diagnostic potential of the selected *Schistosoma* genus-specific MoAbs was first assessed using *S. haematobium* infected human urine samples. This approach was thought feasible because of two reasons. Firstly, as a species of the *Schistosoma* genus, *S.*

haematobium antigens were detected by each of the MoAbs. Secondly, the more sensitive urinary schistosomiasis dipstick assay (Bosompem *et al.*, 1996a, 1997, 1998) was available to be used alongside microscopy to facilitate the assessment. Interestingly, the urinary schistosomiasis prevalence estimated by the established Sh2/15.F MoAb-based urinary schistosomiasis dipstick (87.8%) was not statistically different ($p>0.05$) from that of microscopy (81.1%). However, the assays utilizing the selected MoAbs gave significantly lower prevalence estimates (48.6-58.1%) than either microscopy or the urinary schistosomiasis dipstick, indicating that the developed assays were less sensitive. Furthermore, the higher relative sensitivity (100%) recorded by the urinary schistosomiasis dipstick compared to that of the assays (60.0-71.1%) utilizing the selected *Schistosoma* genus-specific MoAbs suggested that the established dipstick was more sensitive in detecting *S. haematobium* infections in humans. However, this may not be true when the same MoAbs are utilized in assays based on principles other than that of the urinary schistosomiasis dipstick. This is because the urinary schistosomiasis dipstick is based on random binding of antigens in urine onto a membrane and then probed with specific antibodies. This could be less sensitive than assays based on selective binding of antigen by membrane coated antibodies. This notwithstanding, the specificity of the assays developed with the selected MoAbs was each 100% compared with the urinary schistosomiasis dipstick, which gave a specificity of 64.3%. In earlier studies Bosompem *et al*, 1997 observed that the high sensitivity of the Sh2/15.F MoAb-based urinary schistosomiasis dipstick gave a corresponding lower specificity when compared with microscopy as the gold standard test.

The inability of the developed genus-specific dipstick assays to detect some microscopically confirmed infections did not appear to be associated with a particular intensity of infection (low or high) as determined by egg count analysis. The mean egg count of microscopically confirmed infections detected by the dipstick assays was 498 egg/10ml urine. This was not significantly different from the mean egg count (492

egg/10ml) of microscopically confirmed infections that were not detected by the MoAb dipstick assays. In a previous study Bosompem *et al*, 1998 reported that the failure of urinary schistosomiasis dipstick-ELISA to detect known *S. haematobium* infections was likely to be due to peculiar immunological reactions. They emphasised that schistosome antigens occur in infected human urine mainly as immune complexes together with human immunoglobulins and complement, possibly leading to masking of diagnostic epitopes during the course of infection. There is the likelihood that the same phenomenon underlies the observation in the present study. Investigations into the pre-treatment of urine samples to dissociate immune complexes in order to improve sensitivity should therefore be pursued.

The suitability of the genus-specific MoAbs to detect *Schistosoma* antigens was further assessed using *S. mansoni* infected human urine specimens. Similar to the observation made in the diagnosis of *S. haematobium* infection using the *Schistosoma* genus-specific MoAb-based dipstick assays, the estimated prevalences (49.1-50.1%) of *S. mansoni* infections were statistically lower ($p<0.05$) than that by microscopy (80.0%). Furthermore, the developed assays recorded significantly lower relative sensitivities (61.3-63.6%) compared with microscopy as the gold standard test. The dipstick assay could detect antigens in single infections involving *S. haematobium* and mixed infections involving *S. haematobium* and *S. mansoni*. The failure of the dipstick assays to detect infections involving only *S. mansoni* may suggest that the genus-specific antibodies are incapable of detecting *S. mansoni* infections. However, the small number (4/44) of *S. mansoni* single infections involved makes it difficult to draw conclusions, and the ability of the antibodies to detect *S. mansoni* antigens *in vitro* suggests that this may not be the case.

Bosompem *et al*. (1997; 1998) emphasized the need for repeated urine examination to identify low intensity *S. haematobium* infections by microscopy. The observation that urine samples from two *S. mansoni* infected individuals in this study were positive for

antigen by the urinary schistosomiasis dipstick assay was therefore not surprising. This is because concomitant *S. haematobium* infection could be missed by microscopy without repeated testing. Furthermore, Amanor *et al.* (1996), observed that the MoAb (Sh2/15.F) utilized in the urinary schistosomiasis dipstick did not cross-react with *S. mansoni* antigens.

In this study, successful coating of each of the selected MoAbs onto solid support provided an opportunity for the antibodies to be utilized in sandwich ELISA. Studies have shown that the sandwich ELISA is one of the most sensitive and specific assay systems in immunodiagnosis (Chi Fu and Cater, 1990; Barsoum *et al.*, 1991). However, none of the MoAbs investigated could reveal antigens in both the plate- and membrane-based sandwich ELISA. One possible explanation for the inability of the MoAbs to detect antigens in sandwich ELISA even though they detected antigens in direct ELISA is that the antigens possess single diagnostic epitopes. This may be because the sandwich ELISA requires that target antigens to have multiple epitopes that make it possible to use similar antibodies to capture and reveal the antigens. Hence the exploration of the selected MoAbs to diagnose schistosomiasis in humans using sandwich ELISA technique was discontinued.

Development of alternative diagnostic techniques for animal schistosomiasis was investigated using cattle. In a previous study, Boulanger *et al.* (1999) reported that *S. bovis* is one of the main schistosome species affecting domestic ruminants in Africa of which cattle is among the potential reservoirs. In highly endemic areas of human schistosomiasis, the possibility of identifying some species of human schistosomes in animals such as cattle cannot be underestimated. For example, cattle and dogs could be naturally infected with *S. mansoni* and they are sources of continual transmission (Mayaudontarbes and Power, 1969; 1970; Nelson *et al.*, 1962; Mango, 1971). The choice of cattle in this study to investigate the importance of animal schistosomiasis was therefore justifiable.

Analysis of cattle faeces for parasites by microscopy showed a high prevalence of *S. bovis*. Rollinson and Southgate (1987) reported that this parasite is a serious veterinary problem. In Sudan a prevalence of nearly 90% in 18-month-old cattle was demonstrated by Majid *et al.* (1980). *S. bovis* is capable of utilizing a wide variety of *Bulinus* species as molluscan host, often developing naturally in snails of *B. truncates/tropicus, B. africanus* and *B. forskalii* group (Southgate and Knowles, 1975), which may also be the intermediate hosts for the human schistosomes. It is not surprising therefore that *S. bovis* is reported to incidentally infect man (Rollinson and Southgate, 1987). The relatively high prevalence (53.1%) of *S. bovis* found in this study may not rule out the possibility of incidental infections in the local population. Interestingly, the prevalence of *S. bovis* by microscopy was not significantly ($p>0.05$) higher than the 50.0% prevalence obtained using the newly developed IFAT. Likewise, the newly developed *Schistosoma* genus-specific MoAb-based plate-ELISAs estimated prevalences (46.9-50.0%) that were not statistically lower than that of microscopy. The high sensitivity (94.1%) and specificity (100%) of the IFAT clearly indicated its potential as an alternative diagnostic tool for detecting schistosomes in cattle. Also, the MoAb-based plate-ELISAs utilizing (Sh3/38.2 and Sh4/14.3) had high attributes, 100% specificity and 94.1% relative sensitivity in detecting *S. bovis* in cattle. This work has therefore established two possible assays (IFAT and plate-ELISA) as potential alternatives to microscopic diagnosis of schistosomiasis in cattle. There is an urgent need to further evaluate these tests in order to adopt one for routine diagnosis of animal schistosomiasis, preferably in the field. The use of ELISA as a screening test is justified, considering its advantages over other methods of diagnosis. It is highly sensitive, specific, non-hazardous to technicians, can be semi-automated and quantitative (Waltman *et al.*, 1984; Hirvela-Koski, 1990). However it has been explained that IFAT utilises whole cells of parasites, while the antigen for ELISA consists of soluble proteins. IFAT is therefore able to detect antibodies which appear at the early stage of infection against antigenic components of the intact parasites (Karim and Ludlam, 1975), whereas ELISA is

better suited for detecting antigens that appear later in infection. The choice between IFAT and ELISA therefore could be influenced by the kind of diagnostic tool required. Thus, for example, a screening assay capable of detecting early infections will be preferred in disease surveillance so as to enable early intervention. On the other hand, the ELISA is extremely economical in the use of reagents (Riott *et al.*, 1993) and large numbers of tests can be performed in a relatively short time. It is therefore recommended that ELISA is developed for field diagnosis of animal schistosomiasis whilst IFAT is used for confirmatory purposes in the laboratory.

The study also demonstrated 3.1% prevalence of *S. indicum* in mixed infections with *S. bovis*. In India, *S. indicum* is known to infect a variety of domesticated animals including sheep, camel, goat buffalo and cattle (Rollinson and Southgate, 1987). It is known to be among the veterinary schistosomes which are of less medical importance because its range is free of human schistosomes (Chandler, 1926). However, it is known that cercariae of many non-human schistosomes penetrate man but die in the skin. Such infections are not usually significant but can cause a cercarial dermatitis referred to as swimmers itch and repeated exposure may lead to a severe allergic reaction (Macfarlane, 1949; Olivier, 1949). The observation of bovine schistosomiasis in parts of Ghana where human schistosomiasis is known to be endemic is therefore important. It is expected that the information would be useful as a basis to launch a thorough investigation into animal schistosomiasis, which has previously received little attention in Ghana.

Even though eleven other cattle parasites were identified by microscopy, none of them showed cross-reactivity with the MoAbs used. This is because none of the sera from the *Schistosoma* negative animals was positive for antigen by the MoAb-based micro-plate ELISA, indicating a 100% specificity of the assays.

In conclusion, alternative assays (micro-plate ELISA and IFAT) have been developed and shown to be highly sensitive and specific in detecting *Schistosoma* infections in cattle. The assays are likely to contribute towards better understanding of the

distribution and interactions of the animal schistosomiases in Ghana, and thereby create opportunities to explore zoonotic schistosomiasis in the country.

In the case of human schistosomiasis, the established *S. haematobium* species-specific dipstick (Bosompem et al., 1998) proved to be sensitive in detecting urinary schistosome antigens. On the other hand, the lower sensitivities recorded by the newly developed *Schistosoma* genus-specific dipstick assays even though the specificities were very high, underscore the need to explore the MoAbs in different assay systems.

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