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A portion of this work has been presented in a poster format at the South African Parasitology Association conference in 1993.

ABSTRACT

The study was performed and is reported here in two parts. The first part examined the cross-reactivity between three species of African schistosomes, *Schistosoma mansoni*, *S.haematobium*, and *S.leiperi*. Using sera from patients infected with *S.haematobium* or *S.mansoni* and control sera, it was found that the three species of schistosomes are antigenically closely related. In addition, a common antigen of approximately 68 kD was consistently recognised by both the seropositive groups in all three antigen preparations.

The second part describes the use of alternative, non-schistosome antigens for the serological diagnosis of bilharzia. The presence of cross-reactivity between *F.gigantica* adult worm crude extracts and schistosome-infected persons was established. The cross-reactive regions were limited mainly to high molecular weight proteins, and in particular, a 60-75 kD complex. The partially purified 60-75 kD complex had a slightly higher specificity than the crude *F.gigantica* preparation when utilized in RIA, and thus showed immunodiagnostic potential for schistosomiasis.

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of Wiwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Cheryl Dawn Brand

27th day of MARCH 1997

WESTERN BLOT ANALYSIS OF BILHARZIAL INFECTIONS

Cheryl Dawn Brant

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science.

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of the intestine, particularly the rectosigmoidal region and the appendix, and liver granulomas (but not pipe-stem fibrosis). However these granulomas are rarely of any significance. The major lesions form in the urinary system. Lesions of the bladder may be classified into three groups:

- early egg patches;
- sandy patches; and
- fibrous patches.

All three lesion types cause the bladder or ureter to become less pliable. The lesions in the first and second categories may sometimes be reversed by chemotherapy, but fibrous lesions are unresponsive.

The clinical symptoms of urinary schistosomiasis include cystitis, haematuria, proteinuria, bacturia and pain on urination. The more serious signs of urinary schistosomiasis are those indicative of kidney involvement. Severe cases result in kidney failure. An added complication of urinary schistosomiasis is the correlation between bladder carcinoma and *S.haematobium*

formation and ulceration. With few exceptions, the intestinal lesions contribute very little to the pathophysiology.

Many of the eggs also become lodged in the hepatic capillary bed of the liver. The disorder commonly associated with hepatosplenic schistosomiasis is Symmers' pipe-stem fibrosis. The characteristic lesion of pipe-stem fibrosis has wide bands of fibrosis around portal veins, with the parenchyma between fibrotic areas being well preserved, correlating with the nearly normal liver function.

The clinical symptoms of the disease include abdominal pain and diarrhoea with blood, mucus and pus being present in the faeces. Splenomegaly may also occur due to the lodged eggs and chronic congestion of the liver. Some eggs may pass through the liver and become trapped in secondary sites such as the lungs, nervous system and other organs.

Urinary schistosomiasis may also cause mucosal lesions

excretion levels. However, in intestinal schistosomiasis, the relationship between morbidity and infection intensity remains unclear, since patients with low egg output may still exhibit severe disease manifestations, such as periportal pipe-stem fibrosis (Doehring-Schwerdtfeger *et al.*, 1990). Thus, the worm load is not the only factor responsible for the development of disease. Another element that may define the severity of the disease is whether the patient has had prior exposure to schistosomiasis (Evans *et al.*, 1991).

Although the adult worms do stimulate an immune response from the host, the focal lesions that may be formed lack clinical significance when compared to the lesions caused by the eggs.

In chronic intestinal schistosomiasis, the eggs become lodged in the venules and submucosa of the intestine. The eggs elicit an inflammatory response with leucocytic and then fibroblastic infiltration. These develop into pseudotubercles, leading to abscess

Stage III

At this stage, there are lesions caused by the deposition of eggs in the tissues and subsequent granuloma formation and fibrosis. The final stage of the disease may be classed into two groups:

- subclinical chronic schistosomiasis; and
- morbid chronic schistosomiasis.

Most infected patients only have minor, non-specific symptoms or none at all, and may continue in apparent good health indefinitely, despite the continual accumulation of ova in the tissues. Eventually, several years into the infection, predisposed individuals begin to show characteristic symptoms specific for their infecting schistosome species.

In general, morbidity and mortality may be correlated with the intensity of infection, which may be crudely reflected by the number of eggs excreted. In *S.haematobium* studies, there has been shown to be a close correspondence between pathological features of the upper and lower urinary tract and the urinary egg

known as swamp itch or Kabure Syndrome. It is characterised by a papular rash and pruritus.

Such a disease may also result from massive penetration by human schistosome species, or from repeated exposure to, for example, bird or rodent schistosome cercariae.

Stage II

Acute or toxaemic schistosomiasis is associated with the migration of the schistosomulae to the lungs and liver and the onset of oviposition. This is a self-terminating phase which begins one or two months after the first exposure to cercariae.

The symptoms of toxaemic schistosomiasis are the same for infections caused by all the schistosome species: intermittent fever, abdominal pain, bronchitis, enlargement of the liver and spleen and diarrhoea. Most children only develop minor symptoms, or none at all.

1.2 DISEASE SPECTRUM

Since the adult worms engage in permanent copula and lay eggs for many years, schistosomiasis is a disease of long chronicity which ranges from first water contact during childhood to well into adult life, albeit with decreasing prevalence after the second decade. In one study (Hornstein et al., 1990), it was found that the lifespan of *S.mansoni* worms in an isolated population can exceed 38 years.

The symptoms of the disease differ according to the species of fluke involved because of the preferred venous sites. In general, however, the progression of the disease in all three species may be divided into three basic stages:

Stage I

Swimmer's itch or cercarial dermatitis results from cercarial penetration of the exposed skin. Cercariae, particularly those that die in the skin, may be responsible for the development of this condition, also

- *S.japonicum* is more concentrated in the veins of the small intestine.

The adult worms reside in pairs, the female worm lying in the gynaecophoric groove of the male. The eggs are passed daily in the venules. The number of eggs excreted daily (evaluated as the number of eggs per worm pair) differs greatly between species (Loker, 1983). The period between successful cercarial penetration and the first appearance of eggs in the urine or stools of the definitive host may vary between 30 to 40 days, depending on the species.

Developing in the eggs is the larval form of the parasite, the miracidium, which releases lytic enzymes that rupture the venule wall, liberating the eggs into perivascular tissues of the intestine or urinary bladder. If the eggs do not become trapped in the tissue, they effect passage into the lumen of these organs and are evacuated in the faeces or urine. On contact with fresh water, the eggs hatch and the miracidia are released.

Some of the schistosomulae reach the hepatic portal system and are trapped in the liver. The remainder traverse the systemic capillary beds to the venous system and travel back to the lungs. They make several circuits of the vasculature before either being trapped in the liver or lodging in an environment that is unfavourable for development.

Active growth occurs in the portal vessels of the liver. The trapped flukes grow rapidly while feeding on the nutrient rich hepatic portal blood. Pairing of the worms occurs in the liver. Approximately three weeks after exposure, the adolescent worms migrate against the portal blood flow into the mesenteric, vesical and pelvic venules.

The three major human schistosome species have distinct preferences:

- *S.haematobium* is found principally in the veins of the urinary system;
- *S.mansoni* prefers the portal veins draining the large intestine; and

The free-swimming cercariae are infective to the vertebrate host.

The cercariae have only a brief period in which to locate and penetrate the definitive host. The usual portal of entry is the skin. The cercariae penetrate the skin and burrow down to the peripheral capillary bed. The cercariae, now referred to as the schistosomulae, are transported via the afferent blood vessels to the heart and lungs. The lung phase is a period of adaptation to the intravascular environment.

The route of migration of the schistosomulae from the lungs to the hepatic portal system has been the subject of controversy for many years. Of the proposed theories, the intravascular route of migration seems to be the most plausible (Miller *et al.*, 1989; Wilson and Coulson, 1989; Wilson *et al.*, 1990). The parasites leave the lungs via the pulmonary vein and pass via the left side of the heart to be distributed around the body.

over a succession of stages: egg, miracidium, first stage sporocyst, second stage sporocyst, cercaria, schistosomulum and adult worm. The basic life cycle has an alternation of generations. Sexual reproduction by adult worms takes place in the definitive vertebrate host, and asexual multiplication in the molluscan host.

The eggs are expelled from the vertebrate host by way of the urine or faeces. The hatching of the eggs in the outside environment only occurs under certain environmental conditions, thus optimizing the chances of the parasite meeting with a suitable snail host. A relatively short lived, free swimming stage, the miracidium, hatches from the egg and is infective to the snail intermediate host.

The asexual reproductive phase of the life cycle occurs in the snail host. In the appropriate snail host, the miracidium becomes a first stage sporocyst; this gives rise to second stage sporocysts which produce numerous cercariae. The cercariae leave the snail following a specific daily rhythm for each species of schistosome.

estimated 200 million people infected, and a further 600 million people at risk of infection (WHO Report, 1985). Schistosomiasis is also a disease of domestic stock and wild animals. It is principally a disease of rural areas, and is acquired from surface water during contact for domestic, occupational and recreational purposes. The development of man-made lakes and irrigation projects has increased the risk of infection for large populations.

There are currently 18 species of *Schistosoma* recognised, of which 5 species are considered to be important parasites in man. *S. haematobium*, *S.japonicum* and *S.mansoni* are the most important and widespread, while the other two, namely *S.mekongi* and *S.intercalatum*, are more localized in distribution. Further species such as *S.mattheei* are known to occasionally infect man (Fripp and Joubert, 1991).

1.1 BIOLOGY

The schistosomes have similar life cycles and develop

CHAPTER ONE

GENERAL INTRODUCTION

The aetiological agent of bilharzia was first described by Theodore Bilharz in 1851. The parasite was named *Distomum haematobium*. Seven years later, two researchers independently noted that only one of the suckers of the adult *D.haematobium* worm had an oral cavity. Weinland changed the generic name to *Schistosoma*, and soon afterwards Cobbold changed the name to *Bilharzia*, after its discoverer (Rollinson and Simpson, 1987). In terms of the International Code of Zoological Nomenclature, the generic name *Schistosoma* has been upheld. However, both schistosomiasis and bilharzia (or bilharziasis) are used to describe the various diseases caused by the numerous species of the genus in man and animals.

Schistosomiasis is one of the most widespread of all parasitic diseases in Africa, parts of the Middle and Far East, South America and the Caribbean. Schistosomiasis is endemic in 76 countries, with an

Ten *S.haematobium* and ten *S.mansoni* serum samples were used, along with five negative controls. The *S.mansoni* sera used in this study were obtained from the Caprivi Strip area. *Schistosoma* infections were confirmed by the presence of eggs in the patient's urine or faeces. The negative controls were from healthy individuals with no history of prior contact with schistosomiasis.

2.3.3 Nitrocellulose dot blots

1µl of antigen sample was dotted onto nitrocellulose discs 3mm in diameter and allowed to air dry for ten minutes. Following this, the discs were blocked in a 0,5% blocking solution (Appendix) for half an hour. After three, five-minute washes with TBS, the discs were incubated overnight at room temperature in a 1:50 primary antibody solution (human sera).

The three, five-minute TBS washes were repeated prior to the addition of the secondary antibody (sheep anti-human Ig, peroxidase linked; Amersham) at a dilution of 1:300. The discs were incubated for a period of 2

2.3 MATERIALS AND METHODS

2.3.1 Parasite antigens

The parasites were kindly donated by Drs C.D. Dettman and S.B. Higgins-Opitz (both of the South African Medical Research Council).

The antigen extracts of the adult worms of *S.mansoni*, *S.haematobium* and *S.leiperi* were all prepared in the same manner. Briefly, several worm pairs were homogenized in 100µl of Tris Buffered Saline (TBS; Appendix) and 50µl of double lysis buffer (Appendix). After mixing, the homogenate was boiled for five minutes and then spun down in an Eppendorf centrifuge for 5-6 minutes. The parasite extract was made up freshly, as required.

2.3.2 Human sera

Human sera were kindly donated by Dr C.H.J. Schutte (South African Medical Research Council).

2.2 RATIONALE

The levels of cross-reactivity between the three major human schistosome species and thus their antigenic relationships, have been well documented. In contrast, the antigenic relationships between human and non-human schistosomes are not that well defined.

In this investigation, three species of African schistosomes, namely two human species (*S.mansoni* and *S.haematobium*) and a species that infects ruminants, *S.leiperi*, were to be studied.

The aim of the research was to determine the extent of antigenic cross-reactivity between these three species of schistosomes using the Western blot technique and radioimmunoassay (RIA).

polypeptides common to the schistosomulae of *S.mansoni* and *S.haematobium*. The adult worms of *S.haematobium* and *S.mansoni* also showed marked differences in the major polypeptides associated with the adult tegumental membrane. However, since not all surface polypeptides can be readily radiolabelled, the presence of cross-reactive surface polypeptides cannot be totally ruled out.

The profile of antigens presented to the host's immune system gives an indication of the antigenic complexity of the schistosome species, as well as the relationship between the different schistosome groups. It thus appears that despite numerous morphological and physiological differences, the three major schistosome species infecting man, namely *S.haematobium*, *S.mansoni* and *S.japonicum*, are antigenically related. The two African schistosome species are more closely related antigenically to one another than to the Oriental schistosome species.

cell free translation products, they did not contain detectable amounts of antibody that recognised and bound to the major low molecular weight *S.mansoni* polypeptide surface antigens.

In contrast to this, the major high molecular weight antigens that did not enter the resolving gels were precipitated by most of the *S.haematobium* sera. This confirms the findings of Norden and Strand (1984a, 1984b), who found that the low molecular weight antigens appear to be species-specific, while the high molecular weight antigens are cross-reactive. It would thus seem that the polypeptide surface antigens are not strain specific, but are species-specific.

Roberts *et al.* (1988) proposed a rationale for the species-specific recognition described by Simpson *et al.* (1986), whereby the lack of interspecific cross-reactivity is a major feature of the schistosome surface in contrast to the bulk of somatic antigens. On the basis of electrophoretic mobility, there appear to be no surface antigens, and few if any surface

S.japonicum sera. These results are similar to those of Barral-Netto *et al.* (1983) and confirms the antigenic relationship between the two African schistosomes and the Oriental schistosome.

The cross-reactivity observed is not limited to the adult worm glycoprotein preparations, but also extends to the glycoprotein extracts of cercariae and eggs. On the basis of immunoreactivity, it was impossible to distinguish between *S.mansoni* and *S.haematobium* infections even though the soluble egg extracts of the two species have differert 2-D electrophoretic patterns. Thus, although there are several genus and species-specific egg antigens, the majority of egg antigens are cross-reactive (Norden and Strand, 1984b).

Simpson *et al.* (1986) established the identity of polypeptide surface antigens of the schistosomulae recognised by human sera taken from patients infected with either *S.mansoni* or *S.haematobium*. Although the *S.haematobium* sera contained antibodies that bound to the *S.mansoni* schistosomulum surface and precipitated

of schistosome, and minor cross-reactivity with the Oriental schistosome. Both *S.mansoni* and *S.haematobium* patient sera immunoprecipitated the same major *S.mansoni* adult glycoproteins. In contrast, *S.japonicum* human sera precipitated less that half the major *S.mansoni* glycoproteins.

The major glycoproteins were further separated by two dimensional (2-D) analysis to determine the extent of species-specificity. The precipitation pattern of *S.mansoni* glycoproteins with either *S.haematobium* or *S.mansoni* patient sera was indistinguishable, while *S.japonicum* sera precipitated only nineteen out of thirty *S.mansoni* glycoproteins.

This pattern of cross-reactivity is also evident when *S.haematobium* glycoproteins are precipitated with human sera exposed to either *S.mansoni*, *S.haematobium* or *S.japonicum*. Sera of patients infected with *S.haematobium* or *S.mansoni* precipitated the same major glycoproteins of *S.haematobium*, while less than half of the major glycoproteins were precipitated by

cycle stages, 10 components shared by the adult worm and egg and 10 components shared by the adult and cercaria.

The presence of common antigens is not exclusive to *S.mansoni* life cycle stages, but is also evident in other schistosome species. Viyanant *et al.* (1991) identified common epitopes in the tegument of adult worms and schistosomulae of *S.japonicum* (Chinese strain), using monoclonal antibodies.

2.1.2 Inter-species cross-reactivity

The serological cross-reactivity exists not only between the different life cycle stages, but also between the different schistosome species. Sera from patients infected with heterologous schistosome species are cross-reactive irrespective of the schistosome developmental stage used in the diagnostic assay.

Norden and Strand (1984a, 1984b) demonstrated extensive cross-reactivity between the two main African species

Although both the egg and adult worm antigens cross-react extensively with those of the schistosomulum surface, there is a minimal level of cross-reaction between the adult tegument membrane and the egg antigens. These observations have been supported by Dunne *et al.* (1988) in a study using sera from Kenyan school children. It was shown that epitopes shared by schistosomulae and adult worms are different from those epitopes shared by the schistosomulae and eggs.

Analysis of silver-stained gels has demonstrated that extracts of schistosomulae and cercariae contained identical polypeptides. Despite this, there were differences in the staining intensities of some polypeptides in the extracts. Of the ninety-four polypeptides identified in the schistosomulum extract, sixty-one (approximately 60%) were present in the adult tegument membrane (Miller *et al.*, 1989).

Using the technique of crossed immunoelectrophoresis, Akhiani *et al.* (1991) showed that there are at least 7 components shared by the egg, cercaria and adult life

extensive cross-reactivity occurs not only between different schistosome species, but also between the different schistosome life cycle stages and between schistosomes and parasite species not related to schistosomes.

2.1.1 Intra-species cross-reactivity

Omer-Ali *et al.* (1989) demonstrated that antibodies from Egyptian patients infected both acutely and chronically with *S.mansoni* bind to the three-hour-old schistosomulum surface and immunoprecipitate surface antigens. Individual serum samples from both chronic and acute patients consistently immunoprecipitate antigens of M_r >200, 38, and 32 kilodaltons (kD). The 38 and 32 kD antigens on the three-hour schistosomulum surface express species-specific polypeptide epitopes that cross-react with the adult worm, but not the egg. In contrast, the epitopes of the M_r >200 kD antigen recognised by the sera of infected patients were found to be carbohydrate moieties, which are cross-reactive with the schistosome egg (Simpson, 1990).

CHAPTER TWO

A STUDY OF THE CROSS-REACTIVITY BETWEEN THREE SPECIES OF AFRICAN SCHISTOSOMES

2.1 HISTORICAL PERSPECTIVE

In the course of a normal infection, the host's immune system must respond to a complex profile of schistosome antigens. Various experimental approaches have been adopted by investigators to identify the antigenic polypeptides present during an infection. These techniques include the isolation and purification of tegument membranes (Roberts *et al.*, 1988), characterization of the labelled surface antigens recognised by human sera (Kelly *et al.*, 1985, 1987; Simpson *et al.*, 1985), the extraction and purification of soluble schistosome proteins (Abbas *et al.*, 1989; Harn *et al.*, 1989) and the cell free translation of schistosome mRNA (Kelly *et al.*, 1987).

From these studies, it has become evident that

infection.

1.3 OBJECTIVES OF THE STUDY

From the above, it can be seen that schistosomiasis is a debilitating disease which infects millions of people worldwide. There are, however, several problems with the routine diagnostic assays currently employed. These include cost, ease of use and as far as serological assays are concerned, the lack of sensitivity and specificity.

The study was performed and is reported here in two parts. The first part deals with the immunological cross-reactivity between three schistosome species. This aspect is covered in Chapter Two. The second part describes the use of alternative, non-schistosome antigens for the serological diagnosis of bilharzia (Chapter Three). The current diagnostic methods for detecting bilharzia are also be discussed in some detail in this chapter.

The controls (sera from uninfected persons) were significantly different (0,05 significance level) from the infected homologous patients' serum reactions in both the *S.mansoni* (p[control vs. *S.mansoni*-positive sera]=3,83; p[control vs. *S.haematobium*-positive sera]=2,19) and *S.haematobium* (p[control vs. *S.mansoni*-positive sera]= 2,24; p[control vs. *S.haematobium*-positive sera]=2,46) antigen groups, and in the heterologous *S.leiperi* system (p[control vs. *S.mansoni*-positive sera]=2,38; p[control vs. *S.haematobium*-positive sera]=2,41).

serum dilution as the optimal serum dilution instead of the 1:50 serum dilution.

2.4.5 Specificity of test antigens

The reactivity of the *S.mansoni* and *S.haematobium* patients' sera and the normal controls was each compared at the 1:800 dilution using three test antigens: the *S.haematobium*, *S.mansoni* and *S.leiperi* adult worm antigens referred to in section 2.3.1.

The results are summarized in Figures 6 to 8.

None of the antigen preparations were able to distinguish between *S.mansoni* and *S.haematobium* patients' sera, i.e. there was no significant difference between the binding of the *S.mansoni* or the *S.haematobium* patients' sera (cpm), irrespective of the antigen type used (0,05 significance level). However, although not at a significant level, the homologous systems did produce higher cpm values than the heterologous systems.

group are summarised in Figure 5.

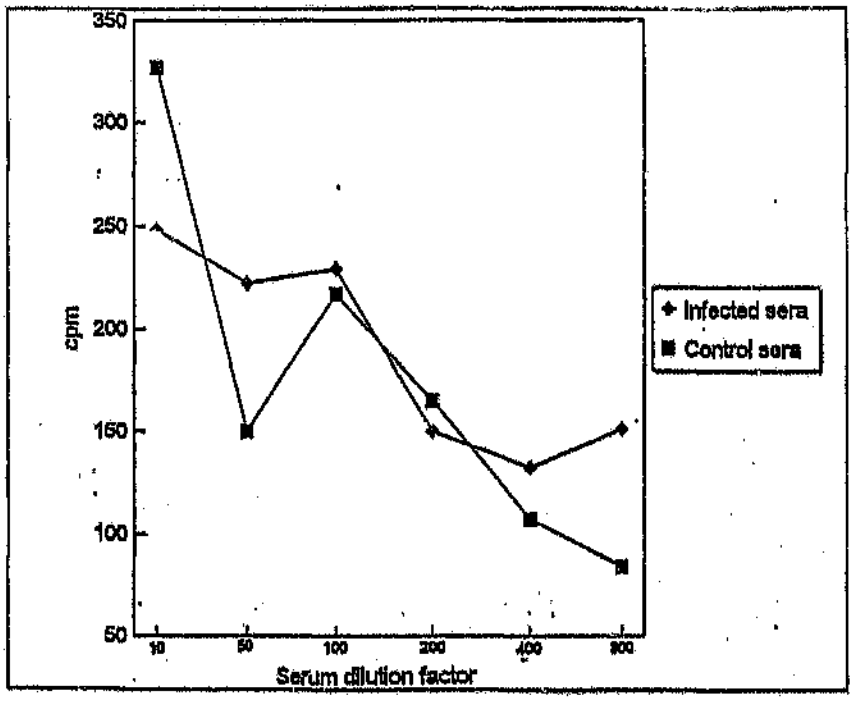

Figure 5: Titration curve of sera from infected persons and normal controls, for determining the optimal serum dilution for RIA.

Overall, there was poor discrimination between the control sera and the infected patients' serum groups, with none of the dilutions producing significant differences between the control and infected sera cpm. Although both the 1:50 and the 1:800 serum dilutions produced suffiently different biological results, because of the poor discrimination in the 1:10 and 1:100 to 1:400 range, it was decided to use the 1:800

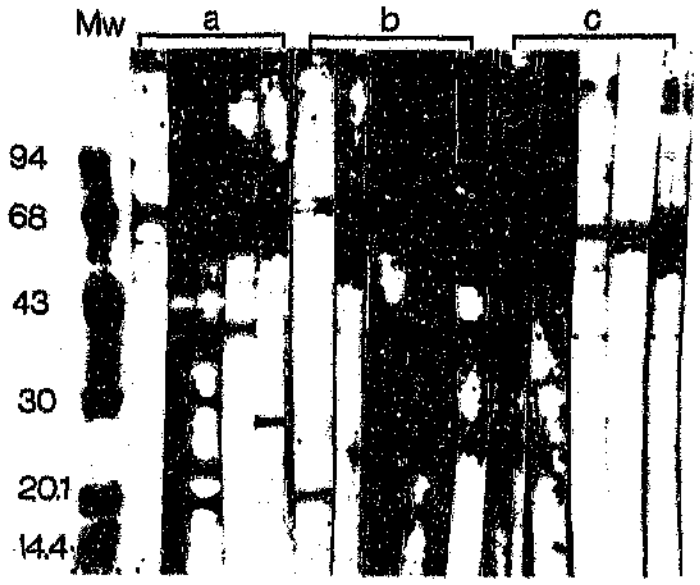

Figure 4: Immunoblot reactions of *S.haematobium*-infected sera against a: *S.lelperi* b: *S.haematobium* and c: *S.mansoni* adult worm antigen preparations.

The control sera consistently produced negative results.

2.4.4 Optimal serum dilution for RIA

Six serum samples, namely three control and three infected patients' sera were selected at random to determine the optimal serum dilution to run the RIA. Adult *S.mansoni* protein extract was used in the assay. The serum samples were compared at 1:10, 1:50, 1:100, 1:200, 1:400 and 1:800 dilutions. The means of each

antigens recognised by the individual serum samples was reproducible.

A common antigen of approximately 68 kD was recognised by both schistosome serum groups in all three antigen preparations Apart from this common band, the antigen patterns produced were highly variable between the individual patients' sera. This variation of antigen patterns was evident in both the heterologous and homologous systems.

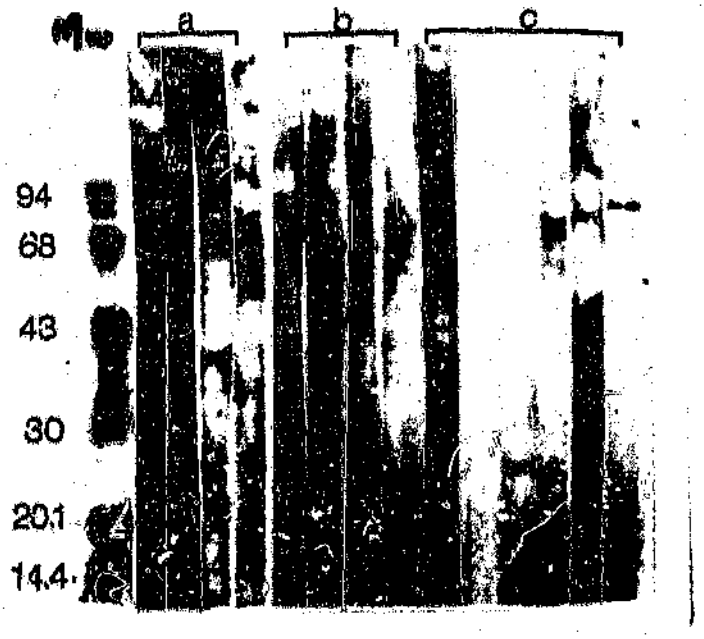

Figure 3: Immunoblot reactions of *S.mansoni*-infected sera against a: *S.leiperi*, b: *S.haematobium* and c: *S.mansoni* adult worm antigen preparations.

There was no difference in the intensity of the colour reaction either between the three antigen preparations or between the two infection-positive serum groups (Figure 2).

2.4.3 Analysis of Western Blots

Crude extracts of *S.mansoni*, *S.haematobium* and *S.leiperi* adult worms were separated on a 12,5% SDS polyacrylamide gel, blotted onto nitrocellulose paper (400 mA for 75 minutes) and incubated overnight with patients' sera (1:100). The anti-human antibody was used at a dilution of 1:400.

Three groups of patients' sera were used: *S.mansoni* (10 samples), *S.haematobium* (10 samples) and normal, non-infected patients' sera (5 samples) to probe the blots. The representative data are shown in Figures 3 and 4.

The schistosome-infected serum groups were limited to a relatively small range of proteins, mainly in the high molecular weight (<43 kD) range. The pattern of

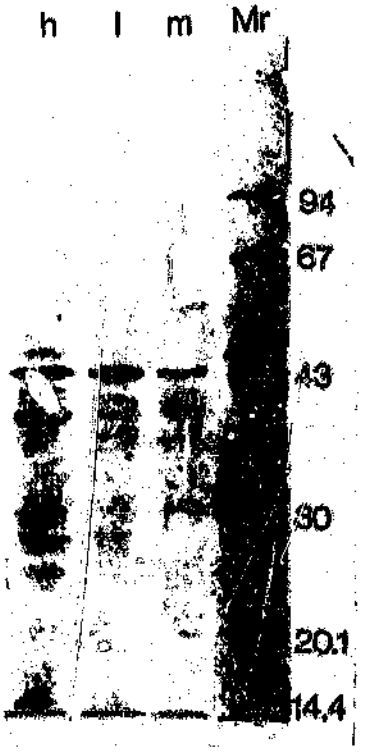

Figure 1: Protein profiles of the crude whole worm extracts of h: S.haematobium, l: *S.leiperi* and m: *S.mansoni*. Mr = Molecular weight markers.

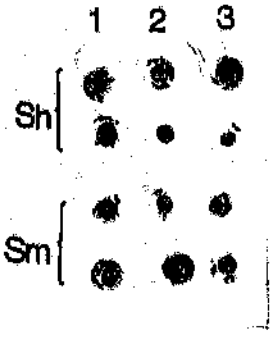

Figure 2: Nitrocellulose dot blot reaction of *S.haematobium* (Sh) and *S.mansoni* (Sm) sera groups against 1: *S.leiperi*, 2: *S.haematobium* and 3: *S.mansoni* antigen preparations.

2.4 RESULTS

2.4.1 Protein profiles of antigen preparations

The protein extracts of the three preparations were run on a 12,5% SDS polyacrylamide gel and then stained with PAGE blue stain.

The protein staining reactions are shown in Figure 1. It can be seen that on the basis of the whole worm protein staining pattern, it is not possible to distinguish between the three worm species. There are minor differences in staining intensities of certain bands but overall, the patterns are indistinguishable.

2.4.2 Nitrocellulose dot blots

1µl of crude extract was dotted onto nitrocellulose paper and reacted with serum from either infected or non-infected persons (1:100). Both the schistosome serum groups produced colour reactions, while the control serum group was negative (no colour reaction).

After washing with PBS as above, the discs were incubated for one hour at room temperature with test sera diluted with PBS. Following further PBS washes, the discs were incubated with anti-human ^{125}I (Amersham) at a concentration of 0.1μCi/ml for one hour. The final washing stage consisted of three, ten-minute washes to remove all excess labelled antibody. The discs were then air dried and counted in a Packard Autogamma counter.

2.3.7 Statistical analysis

After unmasking the sera, the RIA readings were grouped into *S.mansoni*, *S.haematobium* and negative control groups. The mean counts per minute (cpm) for each group was calculated and statistically analysed for significance using Student's t-test analysis.

additional blocking stage of TBS Tween 20 was added, followed by two, five-minute TBS washes.

The anti-human antibody (Amersham) was used at a 1:400 dilution and incubated with the strips for two hours at room temperature. The washing and enzyme substrate reactions were the same as for the dot blot procedure, utilizing 4-chloronapthol as a substrate.

2.3.6 Radioimmunoassay (RIA)

All the radioimmunoassay (RIA) experiments were performed in triplicate. The RIA was run blind so as to preclude any experimental bias.

Briefly, nitrocellulose discs were incubated overnight at 4°C in a test antigen diluted to 1mg/ml. After three, five-minute washes with phosphate buffered saline (PBS), the discs were incubated in a 5% blocking solution for one hour to prevent non-specific absorption of the reagents added subsequently.

(1979).

The proteins were blotted onto nitrocellulose paper (Hybond C; Amersham) at 400 mA for 75 minutes. The blot was stored in a sealed bag containing TBS at 4°C until required.

The complete pattern of proteins transferred to the nitrocellulose was revealed by staining single strips with Fast Green stain (Appendix).

2.3.5 Analysis of Western Blots

The procedure for the Western blot reactions was similar to that of the dot blot protocol. In short, the nitrocellulose strips were placed in a multi-well chamber and blocked with a 0,5% blocking solution for 15 minutes. After three, five-minute TBS washing periods, the strips were incubated with human sera (dilution 1:100) overnight at room temperature.

Between the primary and secondary antibody stages, an

hours at room temperature.

The TBS washes were repeated once again to rinse off any unbound anti-human serum. After the washing step, the discs were placed in enzyme substrate solution containing 4-chloronapthol (Appendix).

The presence of a purple dot on the nitrocellulose indicates a positive reaction.

All the antibody dilutions and blocking solutions were made up in TBS, pH 7.5.

2.3.4 Electrophoresis and immunoblotting

150µl of sample was loaded into one large well of 8cm on a 12,5% SDS polyacrylamide slab gel (Appendix). The gel was run at constant current (28 mA) for 1-2 hours until the bromophenol blue front was approximately 1cm from the bottom of the gel. After the electrophoretic run had been completed, the gel was cut to size and blotted using the method devised by Towbin *et al.*

stage of infection and infection intensities. In the chronic stage of infection, few or no eggs may be passed, resulting in low egg counts which can mask the infection. Similarly, low infection intensities may result in low egg counts, therefore increasing the risk of false negative diagnoses.

Another factor that may influence the egg count is the urine flow rate and the faecal consistency (McCullough and Bradley, 1973). Problems in day-to-day variations in individual egg counts, as well as variations over seasons also occur (Wilkens and Scott, 1978; Schutte et al., 1994). By replicating the observations, the probability of correctly classifying individuals into specific categories of egg output, increases (Warren et al., 1978; Rashed et al., 1990). However, in large population groups and in epidemiological studies, these methods of repeated stool and urine examination are neither practical nor economical.

Other parasitological techniques such as hepatic or rectal biopsies generally reveal a higher prevalence

CHAPTER THREE

THE USE OF ALTERNATIVE ANTIGENS FOR DIAGNOSING SCHISTOSOMIASIS

3.1 HISTORICAL PERSPECTIVE

The diagnostic methods for schistosomiasis can be grouped into two broad categories:

- parasitology
- serology

3.1.1 Parasitology

The parasitological diagnosis of bilharzia involves the demonstration of the schistosome eggs in either the urine or the faeces, depending on the worm species infecting the patient. The presence of eggs in the excreta or urine results in the classification of patients as infected or non-infected. However, this method of diagnosis has disadvantages.

Firstly, egg excretion levels can be influenced by the

three species of African schistosomes are antigenically related; and

- a common antigen of approximately 68 kD was consistently recognised by both the *S.haematobium* and *S.mansoni* serum groups in all three antigen preparations.

The Sm31/32 complex has been expressed in a Baculovirus vector (Felleisen et al., 1990). Fusion proteins have been shown to be a feasible option for detecting circulating antibodies (Klinkert *et al.*, 1991). It is believed that Sm32 may be a digestive enzyme, possibly participating in the degradation of haemoglobin (Chappel *et al.*, 1989), while Sm31 has been identified as Cathepsin B (Klinkert *et al.*, 1989).

Other antigens that have been identified include the heat shock protein (HSP) 70 (Newport *et al.*, 1988; Moser *et al.*, 1990). The 68 kD protein identified in this study may be a HSP70 homologue. *S.mansoni* HSP70 has been found to share epitopes with *S.haematobium* HSP70, but is antigenically distinct from the *S.japonicum* HSP (Moser *et al.*, 1990). The results described here show that there is no divergence between the three species of African schistosome in respect of the 68 kD protein identified.

Based on the above, the following can be concluded:

- with respect to the antigenic reactivity, the

S.mansoni sera were obtained from patients in the Caprivi Strip, while the origin of the *S.mansoni* worms is unknown. Hackett *et al.* (1987) compared a Puerto Rican laboratory isolate of *S.mansoni* with an isolate from Egypt. It was found that the major surface antigens of the two isolates were conserved. It therefore seems unlikely that strain variation is the reason for the low reactivity of the *S.mansoni* antigens, although the possibility cannot be ruled out.

The low reactivity of the *S.mansoni* antigens and the lack of detectable immunoreactive, low molecular weight antigens (<43 kD) has important implications. Most important is the fact that we are not getting a true reflection of the antigen recognition pattern by the various serum groups. In particular, a band identified as the 31/32 kD region (Ruppel *et al.*, 1985a, 1985b) which shows immunodiagnostic potential, was not located. This Sm31/32 protein complex has been shown to react with *S.japonicum* (Ruppel *et al.*, 1987, 1991), *S.mansoni* (Ruppel *et al.*, 1985b, 1990) and *S.haematobium* (Idris and Ruppel, 1988) sera.

and/or they are being masked by other (non-antigenic) polypeptides.

In addition to not being of low molecular weight, the *S.mansoni* antigens also appeared to have a low sensitivity in the Western blot assay compared to the other two antigen preparations (Figures 3c and 4c). This was the case for both the *S.haematobium* and *S.mansoni* sera groups. The reason for this low sensitivity is also unclear, considering that no difference was evident between the *S.mansoni* antigen and the *S.leiperi* or *S.haematobium* antigen preparations in the RIA tests.

Since the worms used in the antigen preparations were laboratory strains, it is possible that due to inbreeding, the immunogenicity of the proteins may have decreased. However, no such documented cases have been published in the literature.

Another factor to consider is differences between geographic isolates, i.e. strain variation. The

the human host.

The cross-reactive proteins are mainly of high molecular weight (>43 kD). This finding is in agreement with previous studies (Norden and Strand, 1984a, 1984b; Simpson et al., 1986), which revealed that the low molecular weight antigens are apparently species-specific.

In the present study, however, very few low molecular weight antigens (<43 kD) were identified, either in the heterologous or homologous antigen systems. The reason for this is unclear. The presence of low molecular weight proteins (<43kD) was confirmed by Fast Green staining of the Western blot strips after the transferring procedure. It is possible that the antigenicity of the proteins may have been modified at some stage of the experimental procedure. However, if this was the case, one would expect the whole range of proteins to be affected, not just one fraction. It is also possible that the low molecular weight antigenic polypeptides are present at low concentrations

study confirms the antigenic relationship between the two African human schistosome species, and extends the finding to a non-human schistosome species.

Based on taxonomic studies, *S.leiperi* is placed in the same broad group as *S.haematobium*. The high level of cross-reactivity observed in the present investigation between these two species would seem to further confirm their relationship. A more accurate means of defining their phylogenetic affinities would be to perform isoenzyme and DNA profiling studies.

Similar levels of cross-reactivity were noted between *S.mansoni* and *S.leiperi*. This may be because *S.mansoni* and *S.leiperi* are antigenically closely related, like *S.mansoni* and *S.haematobium*. Alternatively, exposure of patients to *S.leiperi* may have occurred. The *S.mansoni* sera used in this study were obtained from infected humans in the Caprivi Strip area. *S.leiperi* is endemic in ruminants in the same area (Pitchford, 1970). Although this schistosome will not mature in man, large scale exposure to cercariae would sensitise

2.5 DISCUSSION AND CONCLUSION

The purpose of this study was to investigate the common antigenicity between three species of African schistosomes: the two human schistosome species *S.mansoni* and *S.haematobium*, and a species that infects ruminants, *S.leiperi*.

The results showed cross-reactivity between all three species of schistosome. There were no proteins that were exclusive to any of the species, with both serum groups (*S.mansoni* and *S.haematobium*) identifying a range of reactive proteins. A common antigen of approximately 68 kD was recognised by both serum groups in all three antigen preparations.

Previous work (Norden and Strand, 1984a, 1984b) has shown extensive cross-reactivity between the two species which appear to have originated in Africa, *S.mansoni* and *S.haematobium*, and minor cross-reactivity with the Oriental species, *S.japonicum*. This present

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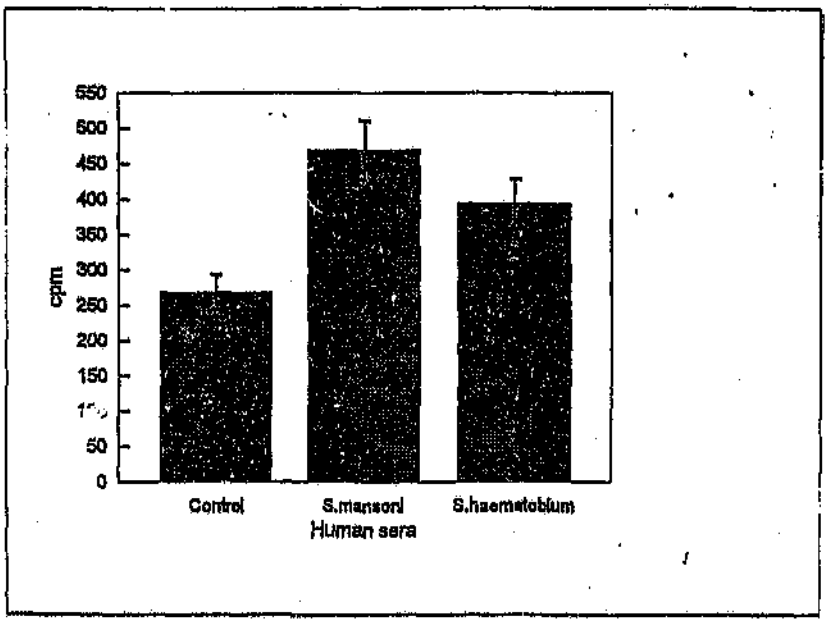

Figure 8: RIA specificity of *S.mansoni* adult worm antigen when reacted with control, *S.mansoni*-positive and *S.haematobium*-positive sera.

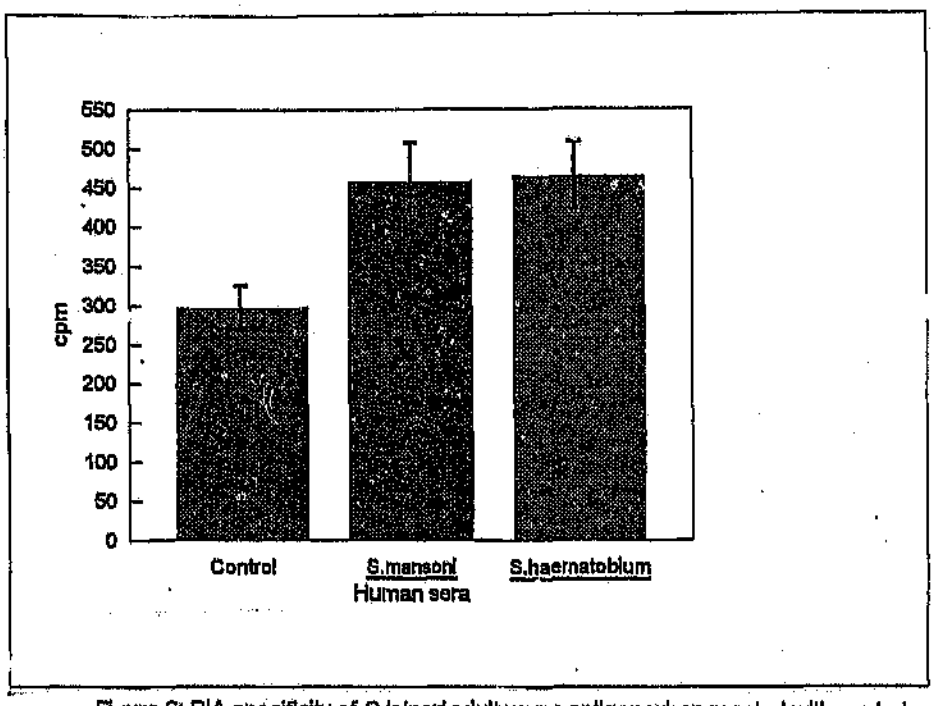

Figure 6: RIA specificity of *S.leiperi* adult worm antigen when reacted with control, *S.mansoni*-positive and *S.haematobium*-positive sera.

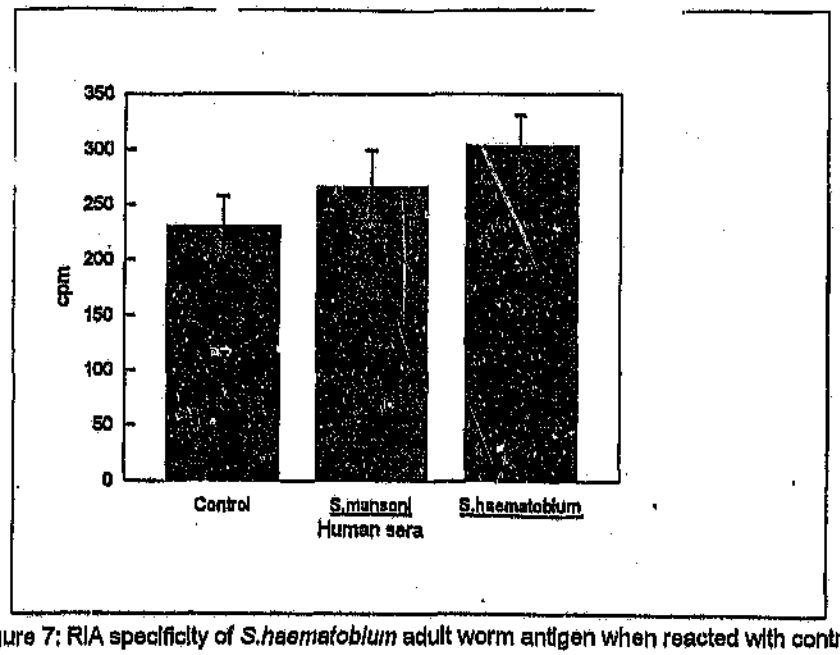

Figure 7: RIA specificity of *S.haematobium* adult worm antigen when reacted with control, *S.mansoni*-positive and *S.haematobium*-positive sera.

3.3 MATERIALS AND METHODS

3.3.1 Parasite antigens

Fasciola antigen preparation:

Adult *Fasciola* worms were obtained from bovine livers condemned at the City Deep Abattoir, Johannesburg.

The worms were washed repeatedly in 0.8% saline (Appendix), followed by extended washes with distilled water. This was to ensure that all contaminants - such as the host's bile fluids and worm exudates - were removed. The worms were then stored in a small volume of Tris buffered saline (TBS, Appendix), pH 7.5, at -70°C until required.

Individual worms were shell frozen in liquid nitrogen and lyophilised as needed. After lyophilisation the worms were homogenized in TBS pH 7.5 using approximately 1ml TBS per 20mg worm dry weight. The sample was left overnight in the refrigerator at 4°C,

3.2 RATIONALE

From the above, it is clear that because of extensive serological cross-reactivity, it is not feasible to utilise the serological assays currently in use as confirmatory tests or in large-scale epidemiological studies. The monitoring of egg excretion is still the best marker of active infection.

The aim of this study was two-fold:

- Since it is difficult to maintain schistosomes in the laboratory which, in turn, affects the production of antigens (SEA and adult worm antigens), the use of alternative cross-reacting (non-schistosome) antigens for serodiagnosis was investigated.
- Using techniques such as radioimmunoassay and Western blotting, the potential immunodiagnostic agent was compared with existing antigens (SEA) to determine the feasibility of replacing currently used antigens with others which are more readily available.

The presence of common antigens shared between *Schistosoma* species and *Fasciola* has long been known (Hillyer *et al.*, 1976a, 1978; Pelley and Hillyer, 1978; Reddington *et al.*, 1984) and has been the subject of numerous studies in regard to vaccine development (Hillyer *et al.*, 1976a; Hillyer and Capron 1976b; Hillyer and Serrano, 1982b; Ford *et al.*, 1987). This cross-reactivity has also been noted in the diagnostic assays (Table 1) utilizing *Fasciola* antigens.

water may be infective.

Human infection with *F.hepatica* is rare in most countries, but it is considered a serious public health problem in the Caribbean and North Africa (Bacq et al., 1991; Pulpeiro et al., 1991; Verappon et al., 1991).

Table 1: *Fasciola and Schistosoma: common antigenicity.*

Antigen Extract	Patients' Sera		Reference
	Fascioliasis	Schistosomiasis	
F.hepatica: adult antigens partially purified	+	+	Santiago and Hillyer, 1986
F.gigantica: crude adult worm extract	+	+	Youssef and Mansour, 1991
F.gigantica: partial purified adult worm extract	+	-	Youssef and Mansour, 1991
F.hepatica: E/S antigens	+	+	Hillyer and Soler de Galanes, 1991

investigate a member of the family Fasciolidae as a possible source of antigen.

F.hepatica is the best known species of the family Fasciolidae and is a parasite of, mainly, herbivorous mammals. *F.hepatica* is a common liver fluke of domestic animals worldwide, causing severe economic loss due to mortality, liver condemnation, reduction of milk and meat production and secondary bacterial infections. Because of its economic importance, this fluke has been the focus of intense scientific research. Infected wild rabbits and hares serve as natural reservoirs in spreading and maintaining the populations of the parasite in grazed and ungrazed lands. Other *Fasciola* species include *F.gigantica* (a parasite of cattle and that used in the present study) and *F.jacksoni* (a parasite of Asian elephants).

Man is an accidental host in the life cycle of the *F.hepatica*. Human infections often occur as a result of the ingestion of encysted metacercariae, although other aquatic horticultural plant species and even

also been found between an epitope of virion infectivity factor (vif) encoded by the HIV-1 genome and a surface antigen of *S.mansoni* (Brand and Tsang, 1989).

The presence of this heterologous cross-reactivity is a hinderence in diagnostic testing, resulting in a decrease in the specificity and sensitivity of the test. However, by exploiting the presence of this cross-reactivity, perhaps a serological assay can be developed using antigens that are more easily accessible.

The ideal antigen candidate should have the following properties:

- large biomass for maximum protein harvest;
- cheap production or easy access; and
- sufficient levels of cross-reactivity so that normal from infected patients' sera can be distinguished.

Keeping the above criteria in mind, it was decided to

1985a, 1985b, 1987, 1990; Idris and Ruppel, 1988; Newport et al., 1988; Moser et al., 1990). The main limitation to the further development of recombinant antigens for immunodiagnosis is the difficulty of large scale production of uniform, partially purified antigens at low cost. Another important factor is that some of the major immunogenic components are proteoglycans and glycoproteins, and cannot, therefore, be cloned.

Another approach which has received very little attention involves the exploitation of the occurrence of cross-reactivity between schistosomes and unrelated organisms (Brand and Tsang, 1989; Correa-Oliveira et al., 1988). A number of cross-reactive serological responses against schistosome antigens have been observed in patients infected with other parasite species (Hillyer et al., 1979b; Aronstein et al., 1986; Mott et al., 1987). Such false positive results have occurred with sera from patients infected with fascioliasis, trichinosis, cysticercosis, *Ascaris* and echinococcosis. Immunological cross-reactivity has

only a small percentage of the parasites in the inoculum used for infecting the definitive host, survive (McLaren and Smithers, 1987). Also, *S.haematobium* is widely believed to hybridize naturally with *S.mattheei* which makes it difficult to be sure that one has a pure source.

An additional factor related to the availability of research data on the schistosome species was their geographic location with respect to the United States, where the bulk of the research in this field was taking place. Sources of *S.mansoni* (for example, Pueto Rico and Brazil) were closer to the United States than Africa. Similarly, the paucity of work on *S.haematobium* was partly because it was not available close to the United States.

This problem of antigen production may be overcome by using molecular techniques. The rapid development of recombinant DNA technology has facilitated the identification and purification of defined antigens for vaccine development and immunodiagnosis (Ruppel et al.,

of the adult worms, eggs and schistosomulae, large volumes of the respective parasite stages are required to produce the necessary amounts of antigen for the various serological assays. To harvest these antigens, a continuous life cycle must be maintained in the laboratory. This is an expensive and technically complex procedure involving the maintenance of both the reservoir hosts (snails) and the definitive hosts.

Most of the laboratory-based research which has been performed has focused on *S.mansoni*, not because it is the most important species, but because it is easier to maintain in the laboratory. Although *S.japonicum* has a similar, but not identical host specificity, it is not as easy to maintain since the cercarial harvest from the intermediate host is very low. Furthermore, the intermediate host is semi-aquatic. Consequently, it is more difficult to culture. *S.haematobium* has received the least amount of attention because of its more restricted host specificity and the fact that parasites take longer to mature (11 weeks) in the definitive host as compared to the other two species. Coupled to this,

both the adult worm and the eggs. Investigators have adopted various experimental approaches to identify the antigenic polypeptides present during an infection. These techniques include the isolation and purification of tegument membrane proteins (Roberts *et al.*, 1988), the characterization of labelled surface antigens recognised by human sera (Kelly *et al.*, 1985, 1987; Simpson *et al.*, 1985), the extraction and purification of soluble antigens (Abbas *et al.*, 1989; Harn *et al.*, 1989) and the cell-free translation of schistosome mRNA (Kelly *et al.*, 1987).

It has become evident from these studies that extensive cross-reactivity occurs not only between different schistosome species (Norden and Strand, 1984a, 1984b; Simpson *et al.*, 1986; Roberts *et al.*, 1988), but also between the different stages of the schistosome life cycle (Miller *et al.*, 1989; Omer-Ali *et al.*, 1989) and between parasite species not related to schistosomes.

Further problems with the available serological tests are of a more practical nature. Due to the small size

However, as with the egg detection methods, numerous disadvantages have been noted:

- Serological assays produce limited information regarding infection intensities. Collaborative studies have been carried out to compare the sensitivity and specificity of various serological assays and the relationship between the infection intensities and the serological assays (Mott and Dixon, 1982) Thus far, it has not been possible to obtain serological correlates with either morbidity or infection intensity with either crude or partially purified antigen preparations (Hillyer et al., 1979b; Long et al., 1981; Yasawy et al., 1989).
- Immunological cross-reactivity has been demonstrated not only between different species of schistosome, but also between schistosomes and unrelated helminth species.

During a normal infection, the schistosome presents a complex array of antigens to its host's immune system, with major immunogenic stimulus believed to come from

rate and are valuable for confirming diagnosis in individual cases (Yasawy et al., 1989; Mohamed et al., 1990). However this technique requires specialized training and advanced facilities and is therefore not feasible for epidemiological studies and large population groups.

3.1.2 Serology

Because of the difficulties encountered with the parasitological techniques, researchers have been focusing on the development of serological techniques for diagnosing schistosomiasis. These immunodiagnostic techniques are becoming increasingly diverse and refined. Numerous tests have been developed using various antigen preparations, eg. the circumoval precipitin test (COPT) (Hillyer and Rivera-Marrero, 1982a), indirect fluorescent antibody test (IFAT), radioimmunoassays (RIA) (Pelley et al., 1977) and enzyme-linked immunosorbent assays (ELISA) (Hillyer and Gomez de Rios, 1979a; Hillyer et al., 1979b; Lunde et al., 1979).

experimental conditions were then used in the Western blot system. However, the Western blot system failed to produce any results. Various changes were made regarding the blocking solutions and times and the concentrations of the primary and secondary antibody, but with no improvement. It was then decided to investigate the antigen preparation.

It was found that SDS and the presence of ß-mercaptoethanol inhibited the binding of the primary and secondary antibodies to the antigen (Figure 9). Hillyer and Ramos (1980) also found that a high concentration of SDS (>0,05%) inhibited the antigen-antibody reactions.

Because of this, it was decided to run the SDS gels using antigen prepared without the double lysis buffer. This preparation produced gels with protein bands that were not very sharp, but the antigen preparation was immunoreactive.

Dot blots are prepared for two reasons. Firstly, the procedure is a means of determining whether immunogenic components are present in the extract being tested. Since the dot blots were positive for both the infected and non-infected sera, the presence of antigenic components in the extract was established. To identify the immunogenic components in the preparation, the extract was separated according to molecular weight on an SDS-gel and blotted onto nitrocellulose paper.

A second function of the dot blot procedure is to optimize the experimental system. By changing selected variables in the system, the ideal conditions for the experiment may be determined. In this way, various blocking agents, blocking times and substrate reagents were tested. It was found that blocking with 0,5% fat-free milk powder for twenty minutes provided sufficient blocking when the primary and secondary antibodies were diluted 1:50 and 1:400, respectively. The ideal substrate was found to be 4-chloronapthol.

Since the dot blot system was working, the defined

3.4 RESULTS

3.4.1 Identification of parasites

After the final saline wash and prior to freezing, ten worms were set aside for identification purposes. On the basis of size and shape, it was determined that the infecting species was *F.gigantica*. There were no *F.hepatica* harvested from the liver. *F.gigantica* is a large parasite measuring 25 to 75mm by 3 to 12mm. *F.hepatica* is shorter and broader than *F.gigantica*, reaching a length of 30mm and a width of 13mm (Schmidt and Roberts, 1985).

3.4.2 Nitrocellulose dot blots

1µl of crude extract was dotted onto nitrocellulose paper and reacted with either infected or non-infected human sera (1:100). Both the normal and the infected sera produced positive results, with the intensity of the reaction varying.

required.

3.3.9 Statistical analysis

The RIA readings were grouped into *S.haematobium*, *S.mansoni* and negative control groups. The mean counts per minute (cpm) were calculated for each group and statistically analyzed for significance using the Student's t-test.

3.3.8 Protein elution

Crude *F.gigantica* adult worm extract was separated on a 10% polyacrylamide gel and eluted as follows:

After running the gel, a portion of the gel was sliced off and stained in PAGE blue for an hour and then destained with 10% acetic acid and 10% alcohol solution. This was done to locate the region of interest, that is, the 60-75 kD protein complex. The gel was then divided into three portions according to molecular weight, and placed in a sterile test tube. The gel was incubated 37°C in an elution buffer overnight.

After the incubation period, the elution buffer was removed and the proteins precipitated using the acetone precipitation technique. The protein concentration was then determined using the Bradford method.

The eluted proteins were stored at -20°C until

3.3.7 Radioimmunoassay (RIA)

All the radioimmunoassay experiments were performed in triplicate. Nitrocellulose paper discs (Hybond C, Amersham), 3mm in diameter, were soaked overnight in a 1mg/ml solution of antigen extract. The protein concentrations of the various samples were determined by the Bradford Method (Segal, 1976; Appendix). Any remaining background was masked by a one-hour treatment with a 5% blocking solution. After three, five-minute washing periods, the discs were incubated in 1:50 dilution of human sera for one hour. This serum dilution was determined by incubating the discs in serial dilutions of human sera in order to identify the optimum antibody titre for the assay.

After four, ten-minute washes with PBS, the discs were incubated with ^{125}I anti-human antibody (Amersham) at a concentration of 1μCi/ml for one hour. The discs were again washed in PBS as above, air dried and counted on a Packard Autogamma counter.

sera used in the assay.

Immunoplots of simple frequencies and frequency differences were constructed using the frequency values calculated as above. A simple frequency immunoplot is a plot of the frequency with which each individual band in the Western blot reacts with infected patients' sera, compared with the frequency of occurrence of that same band when reacted with the normal, non-infected sera.

For the frequency difference plot, the frequency of each band reacting with uninfected human sera is subtracted from the frequency of reaction of that band with infected patients' sera. By subtracting the control sera frequencies, the excess background reaction is effectively being reduced. This plot allows for the comparison of the frequency differences between two separate diseases under the same experimental conditions.

temperature. The washing and enzyme substrate reaction were the same as for the dot blot procedure.

3.3.6 Immunoplotting

The molecular weights of the various proteins were calculated using a standard curve of Rf values plotted against log molecular weights of the molecular weight standards. The Rf value is calculated by dividing the distance travelled by a particular band by the total distance travelled by the front.

The frequency value graphs were constructed according to Larralde et al. (1989). The frequency values for each band were calculated by first constructing a Western blot master pattern of all the bands appearing at least once in the blots when reacted with the patients' sera. The bands were sorted by molecular weight and then scored for their presence or absence in the individual serum samples. The frequency value for each band was calculated by dividing the sum of all sera that reacted with that band by the total number of

blot was stored in a sealed bag containing TBS at 4°C until required.

3.3.5 Analysis of Western Blots

The nitrocellulose sheets were cut into strips approximately 0,5cm wide and placed into a multichamber incubation plate.

3ml of 0,5% blocking solution was added to each well and left there for 20 minutes, followed by three, five minute TBS washes.

The antibody was diluted to 1:50 in TBS and left to incubate overnight at room temperature. Between the primary and secondary antibody stages, an additional blocking stage was added. One, five-minute TBS-Tween 20 wash was followed by two, five-minute TBS washes.

The anti-human antibody was used at a 1:400 dilution and incubated with the strips for two hours at room

any unbound anti-human sera. After the washing step, the discs were placed in enzyme substrate solution containing 4-chloronapthol (Appendix).

The presence of a purple dot on the nitrocellulose represents a positive reaction.

TBS, pH 7.5, was used for all the antibody dilutions.

3.3.4 Gel electrophoresis and Western Blotting

The samples were run on a 12,5% SDS polyacrylamide gel, with a 5% stacking gel overlay (Appendix). The gels were run at a constant current of 28 mAmps.

For the Western blot procedure, the sample was loaded into one large well of 8cm. After the gel had completed its run, it was cut to size and blotted using the method devised by Towbin et al. (1979).

The proteins were blotted onto nitrocellulose paper (Hybond C, Amersham) at 400 mA for 75 minutes. The

Schistosoma infections were confirmed by the presence of eggs in the patient's urine or faeces. The negative controls were from healthy individuals with no history of prior contact with schistosomiasis.

3.3.3 Nitrocellulose dot blots

1µl of antigen sample was dotted onto 0,5 cm^2 nitrocellulose discs and allowed to air dry for ten minutes. Following this, the discs were blocked in a 0,5% blocking solution (Appendix) for half an hour. After three, five-minute washes with TBS, the discs were incubated overnight at room temperature in a 1:50 primary antibody solution (human sera).

The three, five-minute TBS washes were repeated prior to the addition of the secondary antibody (sheep anti-human Ig, peroxidase linked; Amersham) at a dilution of 1:300. The discs were incubated for a period of 2 hours at room temperature.

The TBS washes were repeated once again to rinse off

then spun down in an Eppendorf centrifuge for 5 minutes.

The resulting supernatant was then aliquoted into smaller volumes and stored at -20°C.

S.mansoni soluble egg antigen preparation:

Prior to the antigen preparation, the eggs were washed three times in phosphate buffered saline (PBS). A small quantity of eggs was diluted in PBS and homogenised on ice. The homogenate was then centrifuged at 4°C for 60 minutes at 30 000 rpm. After centrifugation, the supernatant was stored at -20°C until required.

3.3.2 Human sera

Human sera were kindly donated by Dr C.H.J. Schutte.

Thirty *S.haematobium* and twenty-six *S.mansoni* serum samples were used, along with five negative controls.

antigenic components, that is, they are cross-reactive (Mansour et al., 1983).

In terms of the criteria stated in the Introduction, *F.gigantica* appeared to be the ideal candidate for investigation. The three criteria are discussed below.

The first criterion deals with maximum protein harvest. The adult worms of *F.gigantica* are large, leaf-shaped flukes that can be easily seen with the naked eye. They measure 75mm by 12mm. The large biomass is also associated with a high protein content, measured in this case by the Bradford method.

The second of the criteria involves the cheap production of or easy access to the test antigen. The adult worms used in this study were obtained from an infected ox liver supplied by the City Deep Abattoir, Johannesburg. Approximately 80 worms were harvested from a single infected liver, without any dissection procedure being necessary.

3.5 DISCUSSION AND CONCLUSION

The cross-reactivity between *Fasciola* and *S.mansoni* was first documented in 1968 (Reddington *et al.*, 1984), and has since been well described (Hillyer *et al.*, 1976a, 1978; Pelley and Hillyer, 1978; Khalil *et al.*, 1990). Because of the existence of this common antigenicity, it was decided to investigate the nature of the cross-reactivity between adult *Fasciola* antigens and *Schistosoma*-infected patients' sera, and the possible consequences for the diagnosis of schistosome infections.

Because of its great economic importance, *F.hepatica* has been the subject of intense investigation. This is usually the *Fasciola* species described in textbooks. By comparison, relatively little is known about *F.gigantica*, although more information is now becoming available. The life cycle, morphology and pathogenicity of the two species are similar, but they have different snail hosts (Schmidt and Roberts, 1985). Serological studies have shown a similarity in their

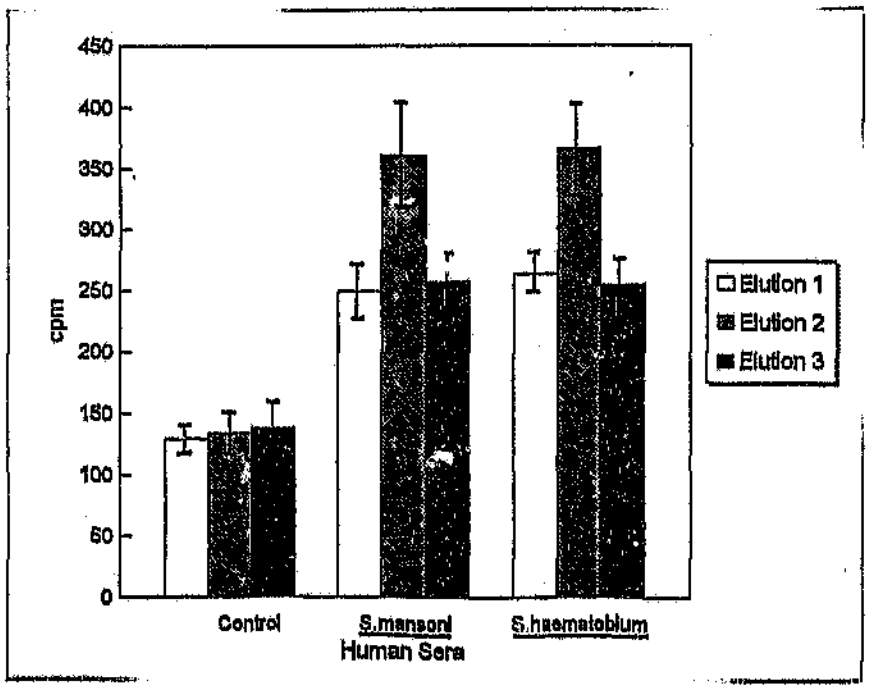

Figure 16: RIA specificity of *F.gigantica* eluted antigen profiles using *S.mansoni*, *S.haematobium* and normal patients' sera.

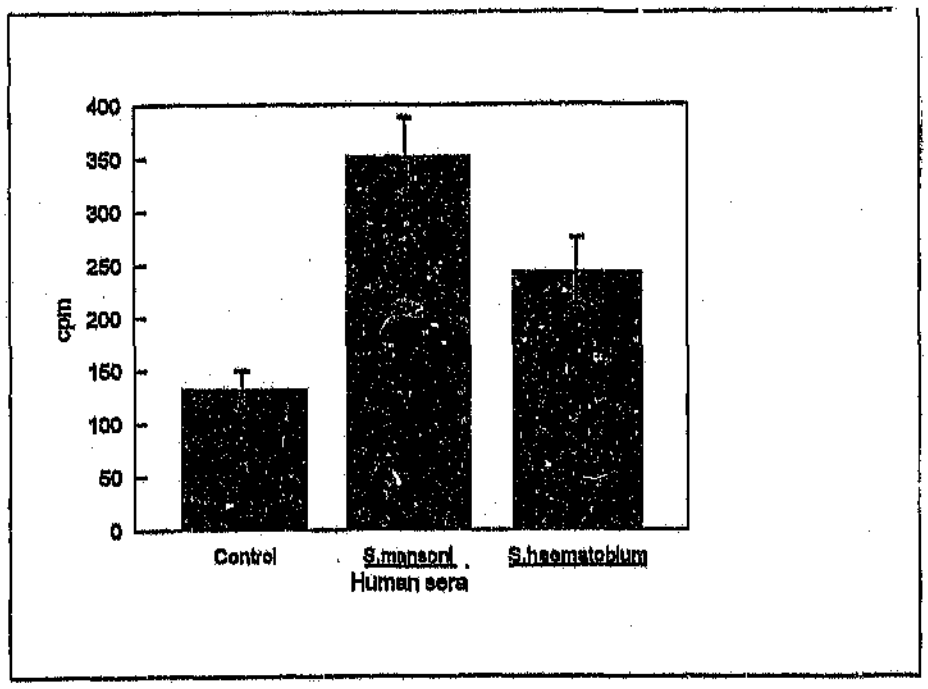

Figure 17: RIA specificity of *S.mansoni* SEA using *S.manconi*, *S.haematobium* and normal patients' sera.

extract.

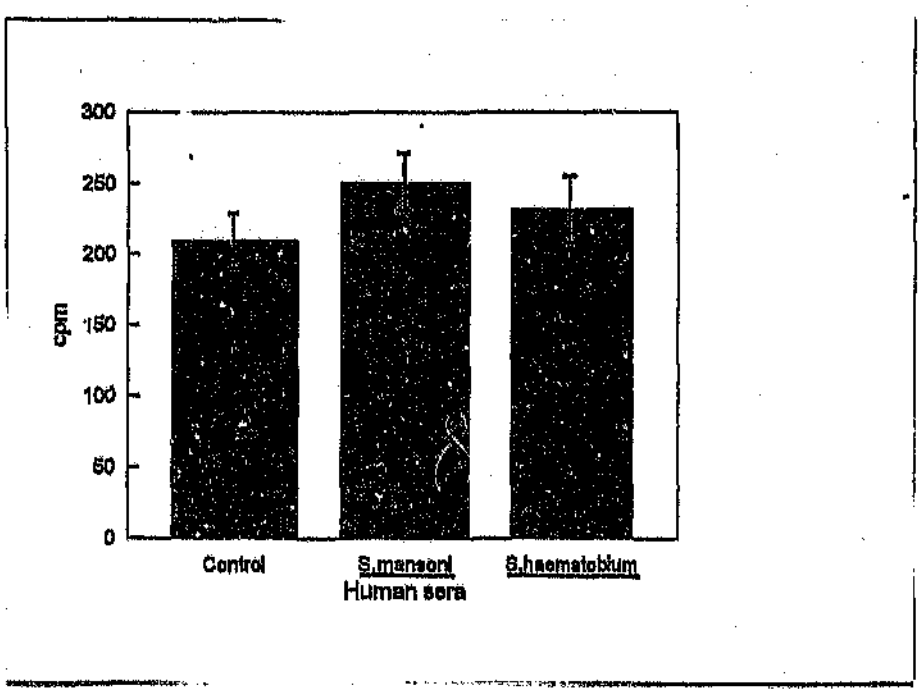

Figure 15: RIA specificity of *F.gigantica* crude protein extracts using *S.mansoni*, *S.haematobium* and normal patients' sera.

The eluted antigen profile consisted of three antigen groups: elution 2 contained the 60-75 kD antigen group identified in the Western blots, while elutions 1 and 3 contained regions above and below the 60-75 kD cluster, respectively. From the RIA, it can be seen that there is a significant difference between the amount of primary antibody binding to elution 2 when compared to the binding of the sera to either elution 1 (p[control vs *S.mansoni*-positive sera]=2,23; p[control vs *S.haematobium*-positive sera]=2,35) or elution 3 (p[control vs *S.mansoni*-positive sera]=2,56; p[control vs *S.haematobium*-positive sera]=2,37). This was the case for both the *S.haematobium* and *S.mansoni* patient groups (Figure 16).

Comparison of the average cpm values obtained with the various antigen preparations shows that SEA (Figure 17) and elution 2 (Figure 16) are not significantly different (0,05 significance level) for *S.mansoni* human sera (p=1,89). However, for *S.haematobium* human sera, there is a significant difference (p=2,48), due to the low reactivity of the sera with the *S.mansoni* SEA

3.4.5 Specificity of test antigens

The reactivity of the normal controls, *S.mansoni* and *S.haematobium* patients' sera were each compared at a 1:50 dilution using three test antigens: crude *F.gigantica* adult antigen, eluted *F.gigantica* antigen and *S.mansoni* soluble egg antigen (SEA).

The results are summarized in Figures 15 to 17. With the crude *F.gigantica* preparation, there were no significant differences (0,05 significance level) between the two schistosome infected patient groups and the control group (p[control vs *S.mansoni*-positive sera]=1,69; p[control vs *S.haematobium*-positive sera]=1,23). However, this was not the case with either the SEA (p[control vs *S.mansoni*-positive sera]=4,32; p[control vs *S.haematobium*-positive sera]=3,16) or the *F.gigantica* eluted antigens , where there was a significant difference between control and experimental groups. In all of the test groups, there was no significant difference between the *S.haematobium* or *S.mansoni* serum groups.

3.4.4 Optimal serum dilutions for RIA

Six serum samples were selected at random to determine the optimal serum dilution to run the RIA using the adult *F.gigantica* crude protein extract. The serum samples were compared at 1:10, 1:50, 1:100, 1:200, 1:400 and 1:800 dilutions. The means of each group are summarised in Figure 14.

As there was no significant difference between the various antibody dilutions, it was decided to run the RIA at a 1:50 dilution since this was the dilution used in the Western blot assays.

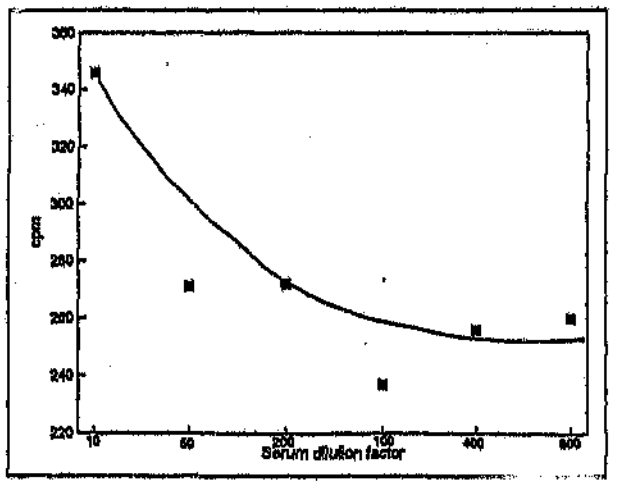

Figure 14: Titration curve of human serum to determine the optimal serum dilution to be used in the RIA.

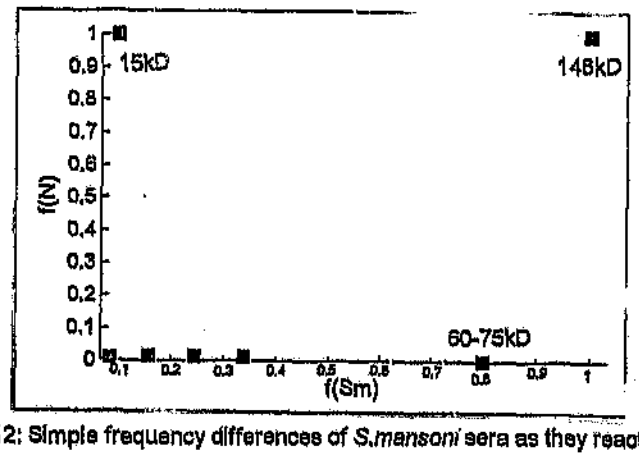

Figure 12: Simple frequency differences of *S.mansoni* sera as they reacted with *F.gigantica* crude protein extract.

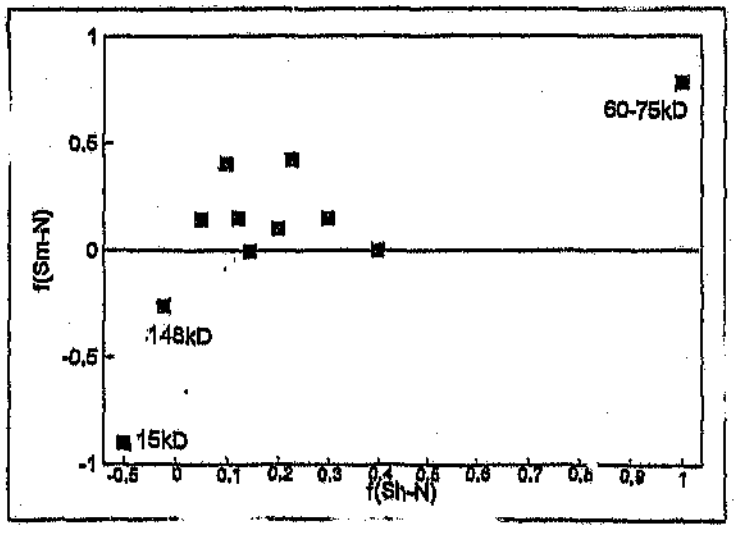

Figure 13: Frequency difference immunoplots of *S.mansoni* and *S.haematobium* patients' sera as they reacted with crude *F.gigantica* protein extracts.

The plots of simple frequencies and frequency differences are useful for the identification of potential diagnostic antigens and cross-reactive antigens in a given system. However, the graphs do not give any information regarding the intensity of the colour reaction for a particular band.

The band intensities for all the blots ranged from very clear and dark to extremely faint (Figure 10). However, overall the *S.mansoni* patients' sera appeared to have consistently weaker reactions and thus fainter bands than the *S.haematobium* patients' sera.

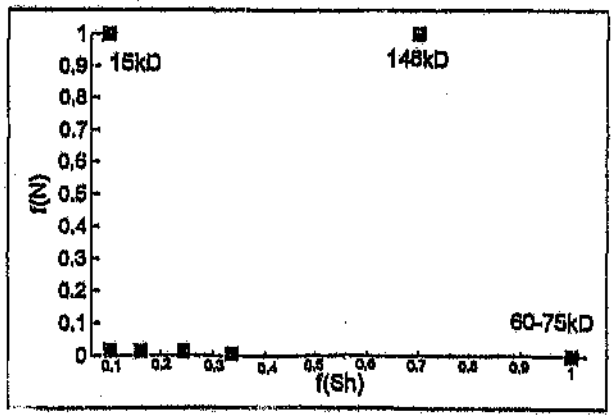

Figure 11: Simple frequency differences of *S.haematobium* patients' sera as they reacted with *F.gigantica* crude protein extract.

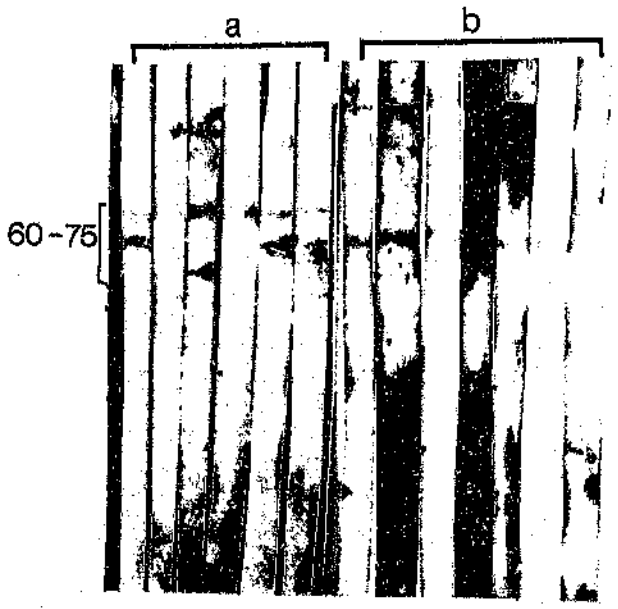

Figure 10: Immunoblot analysis of adult *F.gigantica* worm proteins assayed with sera from patients infected with a: *S.haematobium* and b: *S.mansoni*.

Numerous bands were found to be common to both the *S.mansoni* and *S.haematobium* serum groups: Mw 148 kD, 100 kD, 60-75 kD cluster, 50 kD, 42 kD to 33 kD range and a low molecular weight 15 kD band. Of these bands, the two high molecular weight bands (148 kD and 60-75 kD) have the highest frequency of occurrence (Figures 11 and 12).

None of the bands that are unique to the different serum groups are of sufficiently high frequency to be of any use diagnostically (Figure 13).

serum groups (Table 2, Figure 10). The frequency of occurrence was calculated by dividing the number of positive serum samples for that band by the total number of serum samples.

Table 2: Listing, by molecular weight ranking, of all the different antigen bands in a crude extract of adult *F.gigantica* worm that are recognised by *S.mansoni*, *S.haematobium* and normal patients' sera.

Band Mw (kD)	*S.haematobium* sera (n=30)	*S.mansoni* sera (n=26)	Normal sera (n=5)
148	1	1	1
100	2	2	
85	3		
60-75	4	3	
55	5		
50	6	4	
45		5	
42	7	6	
38	8	7	
33	9	8	
28		9	
15	10	10	2

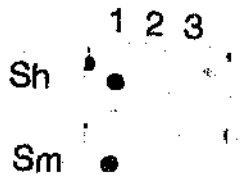

Figure 9: Dot blot assay of *F.gigantica* crude extracts utilizing *S.haematobium* (Sh) and *S.mansoni* (Sm) infected human sera. Three groups of antigen preparations were tested: 1: *F.gigantica* in TBS, 2: *F.gigantica* in double lysis buffer containing no ß-mercaptoethanol and 3: *F.gigantica* in double lysis buffer with ß-mercaptoethanol.

3.4.3 Analysis of Western Blots and immunoplotting

The crude extract of *F.gigantica* adult worms was separated on a 12,5% SDS polyacrylamide gel, blotted onto nitrocellulose paper (400 mA for 75 minutes) and incubated overnight with patients' sera (1:50). The anti-human antibody was used at a dilution of 1:400.

Three groups of patients' sera were used: *S.mansoni* (26 samples), *S.haematobium* (30 samples) and normal, non-infected patients sera (5 samples).

The molecular weight (Mw) bands reactive with the patients' sera ranged from 148 kD to 15 kD in all the

schistosomes, namely two human species (*S.mansoni* and *S.haematobium*) and a species that infects ruminants, *S.leiperi*, were be studied.

The aim of the research was to determine the extent of antigenic cross-reactivity between these three species of schistosomes using the Western blot and radioimmunoassay (RIA) techniques. It was found that with respect to the antigenic reactivity, the three species of African schistosomes are closely related, and that a common antigen of approximately 68 kD was consistently recognised by both the *S.haematobium* and *S.mansoni* infected patient serum groups in all three antigen preparations.

Since there are problems with the maintenance of schistosomes in the laboratory and with the production of antigens (SEA and adult worm antigens) for serological assays, the use of alternative cross-reacting (non-schistosome) antigens for serodiagnosis was also investigated. This is reported in the second part of the study.

Chapter Four

Summary and Conclusion

Schistosomiasis is a debilitating disease which infects millions of people worldwide. Current diagnostic methods for schistosomiasis include serological and parasitological assays. Because of the extensive serological cross-reactivity both between the various schistosome species and between schistosome species and other organisms, it is not feasible to utilise the serological assays currently in use as confirmatory tests or in large-scale epidemiological studies. The monitoring of egg excretion is still the best marker of active infection.

The levels of cross-reactivity between the three major human schistosome species, and thus their antigenic relationships, have been well documented. In contrast, the antigenic relationships between human and non-human schistosomes are not that well defined.

In this investigation, three species of African

Thus in this assay system, specificity was increased by purifying the crude extract.

- The 60-75 kD *F.gigantica* complex shows immunodiagnostic potential when compared to existing assays, such as that employed in this study, namely RIA using *S.mansoni* SEA as the test antigen. However, future research involving larger population groups and more sophisticated purification techniques should be undertaken to determine the exact status of the potential diagnostic agent.

appears to be the ideal candidate. Future research should focus on the further purification, location and identification of the complex. At this stage, it is not possible to distinguish between *S.haematobium* and *S.mansoni* infections utilizing the 60-75 kD complex. Perhaps by carrying out further purification steps and two dimensional gel analysis, it may become possible to separate the two diseases on the basis of their immunoreactivity.

From the above results, therefore, the following conclusions may be drawn:

- The presence of cross-reactivity between *F.gigantica* adult worm crude extracts and schistosome-infected persons was established. The cross-reactive regions were limited mainly to high molecular weight proteins, with the 60-75 kD complex being targeted as potentially useful for the immunodiagnosis of schistosomiasis.
- The partially purified the 60-75 kD complex had a slightly higher specificity than the crude *F.gigantica* preparation when utilized in RIA.

Another factor that influenced the choice of *F.gigantica* as a potential test antigen was the very low frequency of *Fasciola* infections in human populations in South Africa. Man is merely an accidental host in the life cycle of the parasite. The usual route of human infection is through the inge. ion of watercress contaminated with metacercarial cysts. Human cases of fascioliasis caused by *F.hepatica* are rare in most countries, but isolated cases have been reported in Europe, the Caribbean and North Africa (Pulpeiro *et al.*, 1991; Verrapon *et al.*, 1991; Bacq *et al.*, 1991). The incidence and prevalence of *F.gigantica* infection are unknown, as no cases have yet to be reported in the literature. Therefore, it is unlikely that the cross-reactivity between the sera from the schistosome-infected patients and the *F.gigantica* adult worm antigens is the result of undiagnosed *F.gigantica* infections.

The initial results are promising with respect to the presence of potential immunodiagnostic proteins in the crude *F.gigantica* preparation. The 60-75 kD complex

the SEA; the difference between the average cpms of the two groups was not significant. Similarly, the 60-75 kD complex could also be used to distinguish between the uninfected and infected serum groups. On this basis, the initial findings show promise regarding the use of this alternative antigen for diagnosing schistosome infections.

The technique used to elute the alternative antigen was a crude method, and future studies should address this point. Large quantities of the antigen preparation were required to produce a relatively small amount of the eluted antigen, and so the process is not that cost-effective.

In addition, the sample size in this study was relatively small, and did not take into account the age of the patients or the infection intensities. Future research should, therefore, concentrate on refining the testing procedure and extending the study to larger population groups.

sensitivity when used in various assays (Mott and Dixon, 1982; Doenhoff *et al.*, 1993; El-Tahawy *et al.*, 1993). Similarly, RIA has been found to be a sensitive technique for diagnostic assays (Long *et al.*, 1981; Hillyer and Capron, 1976).

It can be seen from the RIA results that there was good discrimination between the control sera and sera from persons with schistosomiasis (both *S.haematobium* and *S.mansoni*). However, between the two schistosome sera groups, even though the *S.haematobium* group had a lower average cpm value than the *S.mansoni* group, there was no significant difference overall as regards their cpm values. This shows that the crude egg antigen preparation is not specific with respect to the infecting schistosome species, but can differentiate between sera from infected and uninfected persons.

The RIA values for the SEA were then compared to those of the crude and fractionated *F.gigantica* antigen preparations. The elution 2 profile containing the 60-75 kD complex produced results comparable to those of

with *S.mansoni* patients sera. *F.hepatica* ES antigens have been shown to cross-react with *S.mansoni* egg antigens (Hillyer and Soler de Galanes, 1991).

Studies using sera from animals vaccinated with schistosome worm or egg subfractions have revealed an interesting association. *F.hepatica* tegumental worm preparations cross-react with *S.mansoni* egg antigens, while *F.hepatica* parenchymal preparations cross-react with adult *S.mansoni* worms (Hanna and Hillyer, 1984). Similarly, monoclonal antibodies raised against surface glycoproteins of *S.mansoni* adult worms cross-reacted with parenchymal tissue (Aronstein *et al.*, 1985a) and the gut and vitelline glands (Aronstein *et al.*, 1985b) of mature *F.hepatica* flukes.

The second part of the study involved comparing the proposed test antigen with existing schistosome serological assays. For this purpose, we employed SEA derived from *S.mansoni* eggs in a radioimmunoassay. Previous studies have shown that schistosome egg antigen derivatives have a high specificity and

In similar studies, Hillyer and Santiago de Weil (1981) found that the cross-reactivity between *F.hepatica* and anti-*S.mansoni* adult worm (SmWWE) and anti-*S.mansoni* egg (SmEE) serum was limited to pI and pII of Sephacryl S200 purified worm extracts. These two peaks correspond to the high molecular weight proteins of the crude extract. Low molecular weight *Fasciola* proteins appear to be specific and immunogenic to experimental and fascioliasis patients' sera (Hillyer and Santiago de Weil, 1981; Santiago de Weil et al., 1984; Santiago and Hillyer, 1986).

Comparison of the elution profiles of *F.gigantica* and *F.hepatica* reveal a similar pattern. Furthermore, serological studies using partially purified *F.gigantica* antigens suggests that *F.gigantica* contains antigens common to both *F.hepatica* and *F.gigantica*.

The location of the 60-75 kD protein complex is also of interest. Hillyer and Soler de Galanes (1988) identified a 63 kD protein in a *F.hepatica* excretory/secretory (ES) preparation that cross-reacts

with normal human control sera or *Fasciola* sera, but did react with *Schistosoma* patients' sera. This pI extract contains proteins in the 70 kD range. Partially purified *F.gigantica* antigens of pII and pIII are specific to *Fasciola* sera, exhibiting no cross-reactivity with protozoal or other helminth infections by CIEP.

With the ELISA technique, however, cross-reactivity was noted. After further fractionating pII and pIII, the cross-reactivity was eliminated. Those extracts that were specific to *Fasciola* sera lacked proteins in the 37, 54, 60 and 66 kD range (Youssef and Mansour, 1991).

In the present study, reactions of the *Schistosoma* patients' sera with the 60-75 kD protein complex would be analogous to the cross-reactivity with the high molecular proteins of pI in the above studies. Similarly, there was limited cross-reactivity with the low molecular weight *F.gigantica* proteins, confirming the low specificity of these proteins with heterologous sera.

extracts of *F.gigantica* showed that the 60-75 kD complex was recognised by *S.mansoni* and *S.haematobium* sera, but had limited specificity with control sera. These results correlate with the Western blots, where the control sera failed to produce bands in the 60-75 kD range.

Previous studies have shown that specificity and sensitivity of assays increase with increasing purification of crude extracts (Hillyer and Capron, 1976b) This was also found to be the case in the present study, where no significant difference was found between the infected patients' sera and the control sera binding to crude *F.gigantica* antigen in RIA assays. Following partial purification of the crude antigen, the cross-reactivity with the control sera was eliminated (Figure 16).

In a study by Mansour *et* al. (1983), F.gigantica adult worm antigens cross-reacted with sera from with schistosomiasis and amoebiasis patients. After partial purification, eluted antigens from pI did not react

The final criterion relates to the levels of cross-reactivity between the test antigen and the schistosome patients' sera and control sera. The cross-reactivity between the *F.gigantica* antigen preparation and the normal control sera was limited to a low molecular weight, 15 kD protein and a high molecular weight, 148 kD protein. However, both *S.mansoni* and *S.haematobium* patients' sera identified a range of bands that are cross-reactive. Of the bands identified, only the 60-75 kD protein complex was found to be consistently recognised by both the *S.haematobium* and *S.mansoni* patients' sera groups. None of the bands were found to be consistently unique to either one of the serum groups.

On the basis of Western blot reactivity, therefore, it was possible to distinguish between normal human sera and infected patients sera, but it was not possible to distinguish between the *S.mansoni* or *S.haematobium* patient groups.

The RIA studies utilizing the crude and purified

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Substrate Reaction

2.5ml of 0.3% 4-chloronapthol solution mixed with

22.5ml of TBS

2-3 drops of Hydrogen peroxide

The solution must be made up fresh.

Tris Buffered Saline (TBS) 5x

146,1g of NaCl

12,1g of Tris made up in 1l of distilled water.

The pH is adjusted to 7.5 with HCl.

25μl TEMED

12.5% Separating Gel:
1.86g Acrylamide
3.75ml Separating buffer
0.6ml Bis solution (25mg/ml)
0.6ml SDS solution (50mg/ml)
Make up to 15ml with distilled water, then add
0.3ml 1.5% Ammonium persulphate
32μl TEMED

8% Separating Gel:
1.2g Acrylamide
3.75ml Separating buffer
0.6ml Bis solution (25mg/ml)
0.6ml SDS solution (50mg/ml)
Make up to 15ml with distilled water, then add
0.3 ml 1.5% Ammonium persulphate
32ul TEMED

Separating Buffer pH 8.8
9,08g of Tris made up in
50ml distilled water.
The pH is adjusted to 8.8 with HCl.

Stacking Buffer pH 6.8
3,01g of Tris made up in
50ml distilled water.
The pH is adjusted to 6.8 with HCl.

water.

Running Buffer

2.42g Tris

0.8g Sodium Dodecyl Sulphate

11.56g Glycine

Make up with 800ml distilled water.

0.8% Saline

8g Sodium chloride made up in

1l distilled water

Sealing agar

0.8g of agar dissolved in

40ml water

SDS Solution (50mg/ml)

0.5g of Sodium dodecyl sulphate in

10ml distilled water

Sodium Dodecyl Sulphate Polyacrylamide (SDS) Electrophoresis

5% Stacking gel:

0.5g Acrylamide

2.5ml Stacking buffer

0.48ml Bis solution (25mg/ml)

0.4ml SDS solution (50mg/ml)

Make up to 10ml with distilled water, then add

0.25ml 1.5% Ammonium persulphate

The solution may be stored at 4°C for up to 6 months.

Destain

10% acetic acid and
10% alcohol made up to 1l with distilled water.

Elution Buffer

The excised gel pieces were incubated overnight at 37°C in a 1x solution of Tris buffered saline.

Fast Green Stain

0,1% Fast green
45% methanol
10% acetic acid
Make up to 100 ml with distilled water.

Lysis Buffer (2x)

3,028g 0,0625M Tris-HCl (pH6.8)
8g SDS
20% glycerol
10% ß-mercaptoethanol
Make up to a final volume of 200ml with distilled water.

PAGE Blue Stain

1,3g PAGE blue made up in
250ml methanol and
50ml acetic acid
The final volume of 500ml is made up with distilled

0.5mg/ml Bovine Serum Albumin (BSA)

5mg BSA dissolved in
10ml distilled water
The solution must be kept at 4°C.

Bradford Protein Assay

In duplicate, add 5µl, 10µl, 15µl and 20µl of 0,5mg/ml Bovine serum albumin (BSA) into Eppendorf tubes.
Make up the volume in the tubes to 100µl using distilled water.
Add 1ml of Coomasie brilliant blue solution and vortex.
Leave for 2 minutes.
Read absorbence using a spectrophotometer set at visible spectrum A_{595}.
Construct a graph of Absorbence (A_{595}) versus protein concentration. Use this graph to read off protein concentration for the unknown samples.

0.3% 4-Chloronapthol

0.15g 4-chloronapthol dissolved in
50ml Methanol
The solution is kept at 4°C.

Coomasie Brilliant Blue

0.1g Coomasie brilliant blue G-250 dissolved in
50ml 95% ethanol and
100ml phosphoric acid

Add distilled water to a final volume of 200ml.
The dye solution must be filtered before use.

APPENDIX

Acetone Precipitation

One volume of sample was mixed slowly with nine volumes of ice cold acetone.
The solution was left at -20°C for one hour.
The tubes were then spun at 2000 rpm for 10 minutes to form a protein pellet.
The pellet was resuspended in 1ml TBS and then precipitated, and spun down again.
The pellet was dried off to remove traces of acetone and then resuspended in an appropriate volume of lysis buffer or TBS.

Bis Solution (25mg/ml)

0.25g of Bis in
10ml distilled water

Blocking Solution (0.5%)

0.5g Fat Free Milk Powder made up in 100ml TBS.
The solution must be kept at 4°C, and if possible made up fresh as required.

Blot Buffer

500ml Methanol
37.5g Glycine
7.5g Tris
Make up to 2.5l with distilled water.

preparation when utilized in RIA. Thus in this assay system, specificity was increased by purifying the crude extract. The 60-75 kD *F.gigantica* complex also shows immunodiagnostic potential when compared to existing assays, such as that employed in this study, namely RIA using *S.mansoni* SEA as the test antigen.

The method used for partially purifying the *Fasciola* antigens was an extremely crude method. Future research should concentrate on refining the purification technique to ensure more accurate results. In addition, for both parts to this study, the sample populations were very small. There was also limited information available regarding infection intensities, age analyses and gender. Future research projects should also take these factors into consideration.

Using techniques such as radioimmunoassay and Western blotting, the potential immunodiagnostic agent was compared with existing antigens (SEA) to determine the feasibility of replacing currently used antigens with others which are more readily available.

Fasciola gigantica was identified as a potential immunodiagnostic agent for several reasons - it has a relatively large biomass and it is readily available. In addition, the number of human fascioliasis infections is considered to be very low in South Africa.

The presence of cross-reactivity between *F.gigantica* adult worm crude extracts and schistosome-infected persons was established. The cross-reactive regions were limited mainly to high molecular weight proteins, with the 60-75 kD complex being targeted as potentially useful for the immunodiagnosis of schistosomiasis.

The partially purified 60-75 kD complex had a slightly higher specificity than the crude *F.gigantica*

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