Identification and molecular characterization of membrane antigen gene(s) of *Schistosoma haematobium*

By

Mohamed Aden Ali (B. Sc)

This thesis is submitted in partial fulfilment for the award of the degree in Master of Science in applied parasitology of Kenyatta University.

Ali, Mohamed Aden
Identification and molecular
99/255223

October, 1998

KENYATTA UNIVERSITY LIBRARY

DECLARATION

I, *Mohamed Aden Ali*, declare that the contents of this thesis entitled " Identification and molecular characterization of membrane antigen gene(s) of *Schistosoma haematobium*" is all my own work, except where acknowledgement has been made in the text. I, further declare that no part of this thesis has been submitted to any other university for the award of a degree.

Signed ....Mohamed A. Ali......... Date ...Oct 19, 1998...

Supervisors' approvals

This thesis is submitted with the approval of supervisors:-

1. Dr. Ephantus W. Kabiru ....
   Department of Zoology
   Kenyatta university
   P. O. Box. 43844
   Nairobi Date. 19/10/98.

OFFICE OF THE
DIRECTOR OF RESEARCH
AND SCIENTIFIC AFFAIRS
NATIONAL MUSEUMS OF KENYA

2. Dr. Rashid A. Aman ....
   Director Research & Scientific
   Affairs. National Museums of Kenya
   P O Box 40658
   Niarobi Date. October 12, 1998

ACKNOWLEDGEMENT

The completion of this project would not have been possible in the absence of either direct or indirect contribution of several people and institutions. The list is long, but only a few will be mentioned here. First thanks to all those under whom I have had the previlege to study as my supervisors Dr. Ephantus W. Kabiru and Dr. Rashid A. Aman provided valuable support, encouragement and keen supervision throughout the entire course of my study. Both provided much needed guidance especially during some of the critical times of my research work. Special thanks to Dr. Aman for providing me the facilities to conduct the research and write up this thesis. This project also afforded me the opportunity to work with the staff of the Department of Molecular Genetics, National Museums of Kenya and everybody I met at the National Museums of Kenya went out of their way to help me and their company and conversation were helpful. I would also like to express my sincere gratitude to Githiu E. Kem, Mohamed A. Jama, and Alex Osanya for their hospitality and valuable contribution to this work and my appreciation to them is beyond the print. I acknowledge my parents, brothers, sisters, endured endless grumbling with a smile and nothing but encouraging words while they needed my help due to man-made tragedy in our country (Somalia). And they have been doing this for a long time, but during the past few years, I have learned to appreciate it. I am grateful to the German Academic Exchange Services (DAAD) for awarding me a scholarship and support for the M. Sc study and all those who encouraged me throughout this course of study, especially Mrs Osiniski (DAAD programme officer), my lecturers and members of the Somali Student Association (SOSA).

DEDICATION

This thesis is dedicated to my parents and my beloved wife, for without them this would not have been possible.

TABLE OF CONTENTS

Page

Declaration..II

Acknowledgement.. III

Dedication.. IV

Table of contents.. V

Abstract X

Glossary of abbreviations.. XI

List of figures.. XIII

List of plates... .... X1V

List of tables.. XV

CHAPTER ONE

1.0 Introduction and literature review 1

1.1 Introduction...... 1

1.1.1 Impact and epidemiology of human schistosomiasis.. 1

1.2 Literaterature review 5

1.2.1 Morphology and general life cycle... 5

1.2.2. Diagnosis of the schistosomiasis.. 9

1.2.3. Immune response to schistosomes 10

1.2.4. Surface membranes of schistosomiasis. 12

1..2.5. Biochemistry and physiology of schistosomiasis. 15

1.2.6. Molecular biology of schistosome................. 16

1.2.7. Management of schistosomiasis 18

1.2.8. Vaccine development for schistosomiasis.. 20

1.2.8.1 Native and recombinant protein vaccines... 22

1.2.8.2.Peptides based vaccines.. 23

1.2.8.3. Nucleic acid vaccines.. 24

1.3. The *rationale* for the study.. 26

1.3.1 Hypothesis in the study 27

1.3.2. The objectives of the study 28

1.3.2.1 General objective of the study... 28

1.3.2.2 Specific objectives 28

CHAPTER TWO

2.0. Materials and methods.. 29

2.1 Culture of *S. mansoni* 29

2.2. Isolation of membrane proteins.. 30

2.2.1. Extraction of peripheral proteins 30

2.2.2. Extraction of tegumental proteins 30

2.2.3. Determination of extract proteins. 31

2.3. Electrophoresis of membrane proteins... 31

2.3.1 SDS polyacrylamide electrophoresis... 31

2.4. Immunization of experimental rabbits.. 32

2.5. Immuno blotting(Western blot)... 33

2.6. Screening of adult *S.haematobium* cDNA library 35

2.6.1 Titration of cDNA library 35

2.6.2 Plating of the lambda gt11 library 36

2.6.3 Induction of the expressed proteins onto 36

2.6.4 Lifting, washing and blocking of the filter papers 37

2.6.5 Antibody probe onto the filter 37

2.6.5.1 Preparation of acetone powder... 37

2.6.5.2 Treatment of the antisera with acetone powder 38

2.6.6 Primary and secondary antibody probes 38

2.6.7 Picking and storage of positive lambda clones 39

2.7 Characterization of the positive clones 39

2.7.1 Preparation of templates for PCR reactions.................................... 39

2.7.1.1 Rapid preparation of lambda DNA 40

2.7.2 Amplification of bacteriophage library inserts using PCR 41

2.7.2.1 Polymerase chain reaction 41

2.7.3 Agarose gel eletrophoresis 43

2.7.3.1 Gel purification 43

2.7.4 Direct cloning of the PCR products...45

2.7.4.1 Ligation of PCR products into pMOSBlue DNA 47

2.7.4.2 Preparation of *E. coli* competent cells 47

2.7.4.3 Transformation into *E. coli* cells 48

2.7.5 Screening of the transformants 48

2.7.5.1 Plasmid DNA extraction 48

2.7.5.2 Rapid direct colony screening 49

2.7.5.3 Restriction enzyme analysis 49

2.7.6 Phenol-chloroform extraction 50

2.7.6.1 Ethanol precipitation of the DNA 50

2.8 cDNA clone sequencing 50

2.8.1 Single stranded pMOS template sequencing 50

2.8.2 Preparation of sequencing gel 51

2.8.3 Gel loading and running 52

2.8.4 Drying and auto-radiography of the gels 52

CHAPTER THREE

3.0 Results 54

3.1 Membrane protein extraction and characterization 54

3.1.1 Bovine serum albumin standard curve 57

3.2. Screening of adult *S.haematobium* library 62

3.3. Characteriaztion of positive clones 66

3.7.1. Polymerase chain reaction 66

3.7.4. Sub cloning of the cDNA inserts into vectors 71

3.7.4.1 Preparation inserts for cloning 71

3.8 Partial cDNA clone sequencing 77

CHAPTER FOUR

4.0 Discussion 86

4.1 The importance of the tegument in the adult parasite 86

4.2 Characteriazation of adult worm proteins 88

4.3. Molecular cloning and charateriazation 90

CHAPTER FIVE

5.0 Conclussions and sugestions for future work.. 97

5.1 Summarry of the conclusions.. 97

5.2 Sugestions for the future works.. 99

References 100

Appendices.. 113

ABSTRACT

In the last decade substantial resources have been invested to identify, characterize and purify various schistosome antigens for the purpose of designing and testing potential vaccines. The investigation of the schistosome tegumental antigen gene(s) was based upon the fact that the unusual structure of the tegument in the adult schistosomes is an adaptation to survival in the blood stream of the human host. The hypothesis put forth in this study was that the tegument proteins of the adult *S. mansoni* could be potential vaccine immunogens. The membrane proteins from the *S. mansoni* were extracted and characterised on SDS-polyacrylamide gel electrophoresis. Rabbit anti sera were then raised against the membrane extract. Western blot analysis revealed conspicuous immuno-bands of 84, 26 kDa. Thereafter an adult *S. haematobium* cDNA expression library was screened using rabbit anti-*S.mansoni* membrane sera. Several immunoreactive clones were isolated by the screening exercise and the presence, size of the cDNA inserts determined by the polymerase chain reaction. Inserts ranged in size from 0.5 to 1.3 Kb. Two of the clones (*Sh* 3-4, *Sh* 4-1) with insert sizes of 0.5 and 0.9 Kb were sub-cloned into the pMOS-plasmid vector (Amersham, UK) and sequenced partially using Sanger's dideoxy chain termination method. Sequence homology search through the Gene bank data base showed that both clones contained membrane encoding genes. One clone had 94% nucleotide identity to *S. mansoni* myosin encoding gene. The other one scored 98% homology to dynein light chain of *S. mansoni* and 77% nucleotide identity to T–cell stimulating antigen of *S. mansoni* . Therefore, these antigen genes are likely candidates that could contribute to the development of a sub-unit vaccine against schistosomiasis.

GLOSSARY OF ABBREVIATIONS

Aps :	Ammonium per sulfate
ATP:	Adenosine triphosphate
Bp(s):	Base pair
BSA:	Bovine serum albumin
cDNA:	Complementary DNA
dCTP :	Deoxy cytosine triphosphate
DNA:	Deoxyribonucleic acid
Ds:	Double stranded
EDTA:	Ethylenediaminetetra-acetate
ETOH:	Ethyl alcohol
dGTP:	Deoxy guanosine triphosphate
Hr(s):	hours
IPTG:	Isopropyl –1-thio B-D-galactoside
Kb:	Kilo base
mg:	Milligram
min:	Minutes
ml:	Milli litre
mM:	Milli molar
nM:	nano molar
OD:	optical density
Oligo:	Oligonucleotide

ORF:	open reading frame
PEG:	Polyethylene glycol
PBS:	phosphate buffered saline
RF:	Replicative form (of M13 DNA)
RNA:	Ribonucleic acid
rpm:	revolutions per minute
SDS:	sodium dodecyl sulfate
SM:	Phage storage buffer
Ss:	Single stranded
dTTP:	Deoxy thymidine tri-phosphate
μ :	Micro
μ g:	Microgram
μ l:	Microlitre
μ M:	Micromolar
X-gal:	5-bromo -4-chloro-indoly B-D-galagtoside

LIST OF FIGURES

Figure number Page

1 Current world-wide distribution of schistosomiasis… ……………………………..3

2 Schematic diagram of adult *S.mansoni* in blood …… ..…………………………... 6

3 Snail vectors of schistosomes of med-importance……...………………………….. 7

4 Cercariae showing its penetration glands………………. …………………….. 8

5 *S .mansoni* surface membrane… …..…………………...……………….… 13

6 BSA standard curve………..…………………………..…………………….... 46

7 pMOS Blue vector……… ……………………………. ……..……………… 57

8 Nucleotide sequence of the 3-4 clone…..…...…………………………………… 78

9 Nucleotide sequence of the 4-1 clone…..…………………...……………………. 79

10 DNA sequence of the 3-4 clone and the deduced amino acid
using the single-lettered code………………………………………………….80

11 DNA sequence of the 4-1 clone and the deduced amino acid
using the single-lettered code……..……………………………………… 81

12 Alignment of the 3-4 clone sequence with the *S. mansoni*
dynein light chain sequence……………………………………………………… 84

13 Alignment of clone 4-1 sequence wit *S.mansoni* myosin and human genes……… 85

LIST OF PLATES

Plate number page

1 Bio-Rad Mini-Protean II gel... 34

2 Protein transfer (Hoeffer instrument) 34

3 PCR-machines..44

4 Bio-Rad agarose mini-gel 44

5 Electrophoresis analysis of the worm proteins...,.. . 59

6 Immunoblot analysis..........................,.. .. 60

7 Dot blot analysis 61

8 Isolation of the *S.haematobium* cDNAs 64

9 PCR analysis and extraction of λDNA..... 67

10 PCR on Pure lambda DNA... .68

11. Analysis of EcoRI digests of λDNA... . 69

12 Screen lambda DNA on PCR..70

13 Recovery of DNAs from agarose gel and PCR products................................ 72

14 Verification of recombinant transformants.. 75

15 Dual digestion of restriction enzyme of the recombinant DNA......................... 76

LIST OF TABLES

Table number		Page
1	A typical PCR reactions..	42
2	Sequencial extraction of proteins from *S.mansoni*................................	56
3	Concentration of soluble proteins at 37^0 C for 30 minutes.....................	58
4	Plaque forming units count and titre...	63
5	Isolation of the specific Sh cDNA encoding tegument proteins	65
6	Transformation results of *E.coli*...	74
7	List of proteins of 3-4 clone after homology search with Blast Programme.........	82
8	List of myosin proteins of clone 4-1 after homology search......................	83

CHAPTER ONE

1.0: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Impact and epidemiology of human schistosomiasis

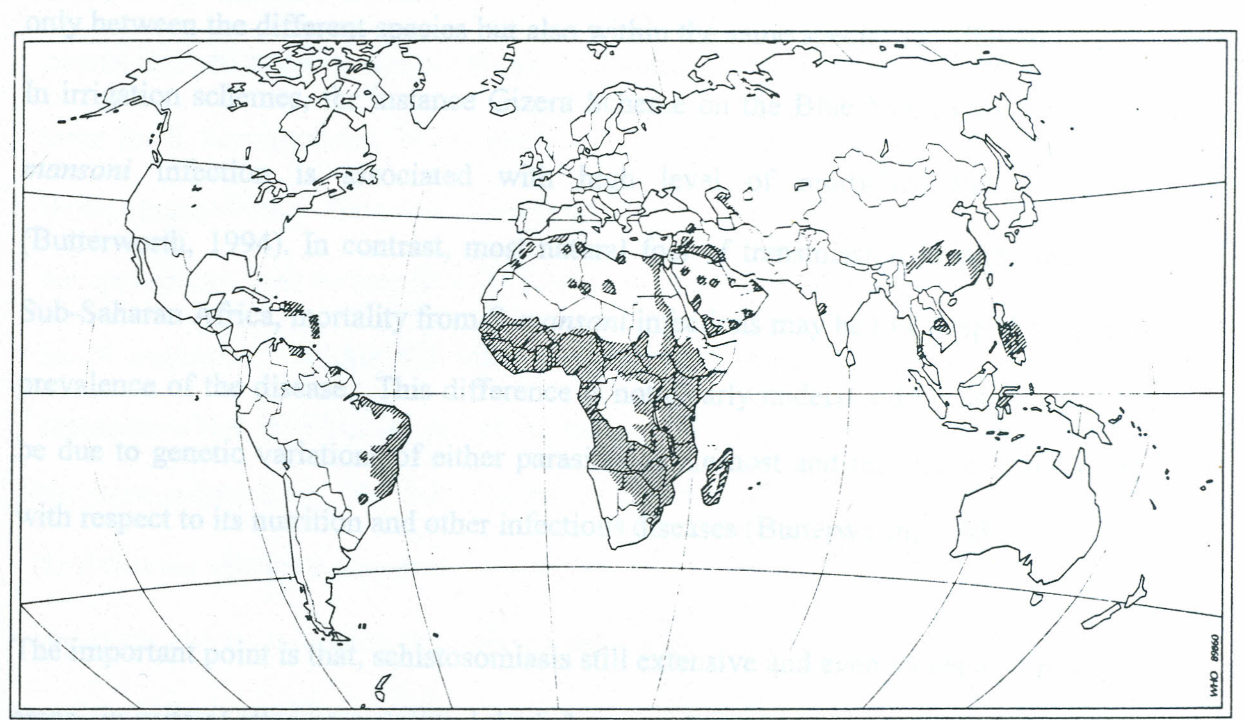
Schistosomiasis, also known as Bilharziasis, is one of the most significant parasitic disease of humans caused by the flatworm of the genus *Schistosoma,* which lives in the blood vessels of the human hosts (Lucey and Maguir, 1993). The World Health Organisation (WHO) estimates that over 200 million people are currently infected world -wide mainly in rural agricultural and pre-urban areas (WHO, 1996). Of these 20 million suffer severe consequences from the disease which claims 800,000 lives annually (Capron and Dessiant, 1992). It is the second most prevalent tropical disease occurring in Africa, Asia, and South America, where as many as 600 million people are at risk (WHO, 1993). However in many endemic areas schistosomiasis is not yet recognised as an important public heath problem and receives a low priority for control.

There are at least nineteen recognized species of the genus *Schistosoma* of which only five species affect humans. *Schistosoma mansoni*, *S. haematobium*, and *S. japonium* are the most important and widespread, human parasites while the other two, *S. intercalatum* and *S. mekongi* have a more localised distribution (Johnson *et al.*, 1993). These species differ not only biologically from one another, but also in their geographical distribution and in the type of symptoms they produce in the infected hosts.

Acute schistosomiasis is characterised by chills, weakness, weight loss, cough, arthralgias, marked eosinophilia and often culminating in abdominal pain and bloody diarrhoea (Fallon *et al.,* 1994; Gryseels *et al.,* 1994). Depending on the type of schistosomiasis caused by the parasite the clinical manifestations involve the liver, intestinal and urinary complications resulting from reactions to Schistosome eggs lodged in the tissues of the infected hosts. The chronic effects of the disease are very high in endemic areas and associated with agricultural and economic water development projects, which make the disease a great public health concern (Fig 1). Approximately 90% of all schistosomiasis cases are found in Africa, and it is of major socio-economic importance in Brazil, China and Egypt (Butterworth, 1988). In many endemic regions, three out of four children (75%) may be infected (WHO, 1995).

However, a rough approximation might be that in areas of high transmission, 5-10% of heavily infected people will eventually die as a result of infection while many others suffer more or less severe, chronic ill-health, specially during childhood or adolescence. However, this is only an estimate, but the disease can also cause death, through the rupture of enlarged collateral blood vessels. However, the actual number of deaths is largely unknown. Available information on the social and economic importance of schistosomasis is controversial. Studies of the effect on the productive capacity and economic output have produced inconsistent results, partly because they have been undertaken in communities of different endemicity.

Figure 1: Current world-wide distribution of schistosomiasis (Adopted from WHO 1993). The figure shows the key areas affected by schistosomiasis.



For example, in rural Nigeria, abortion and reproductive tract infections were reported among adolescent girls (Feldmeir, 1995) and in Egypt vesical schistosomiasis has been considered a common cause of malignancy of the bladder in male agricultural workers (Abdel-rahman, 1994). Preliminary anthropological studies indicate that, the disease may have significant social impact such as stigma associated with haematuria in women (WHO, 1993). There is marked difference in the severity of the disease, not only between the different species but also within the same species in different areas. In irrigation schemes, for instance Gizera Scheme on the Blue Nile in the Sudan *S. mansoni* infection is associated with high level of morbidity and mortality (Butterworth, 1994). In contrast, most natural foci of transmission in rural areas of Sub-Saharan Africa, mortality from *S. mansoni* infections may be low despite the high prevalence of the disease. This difference is not clearly understood but is thought to be due to genetic variations of either parasites or the host and the status of the host with respect to its nutrition and other infectious diseases (Butterworth, 1988).

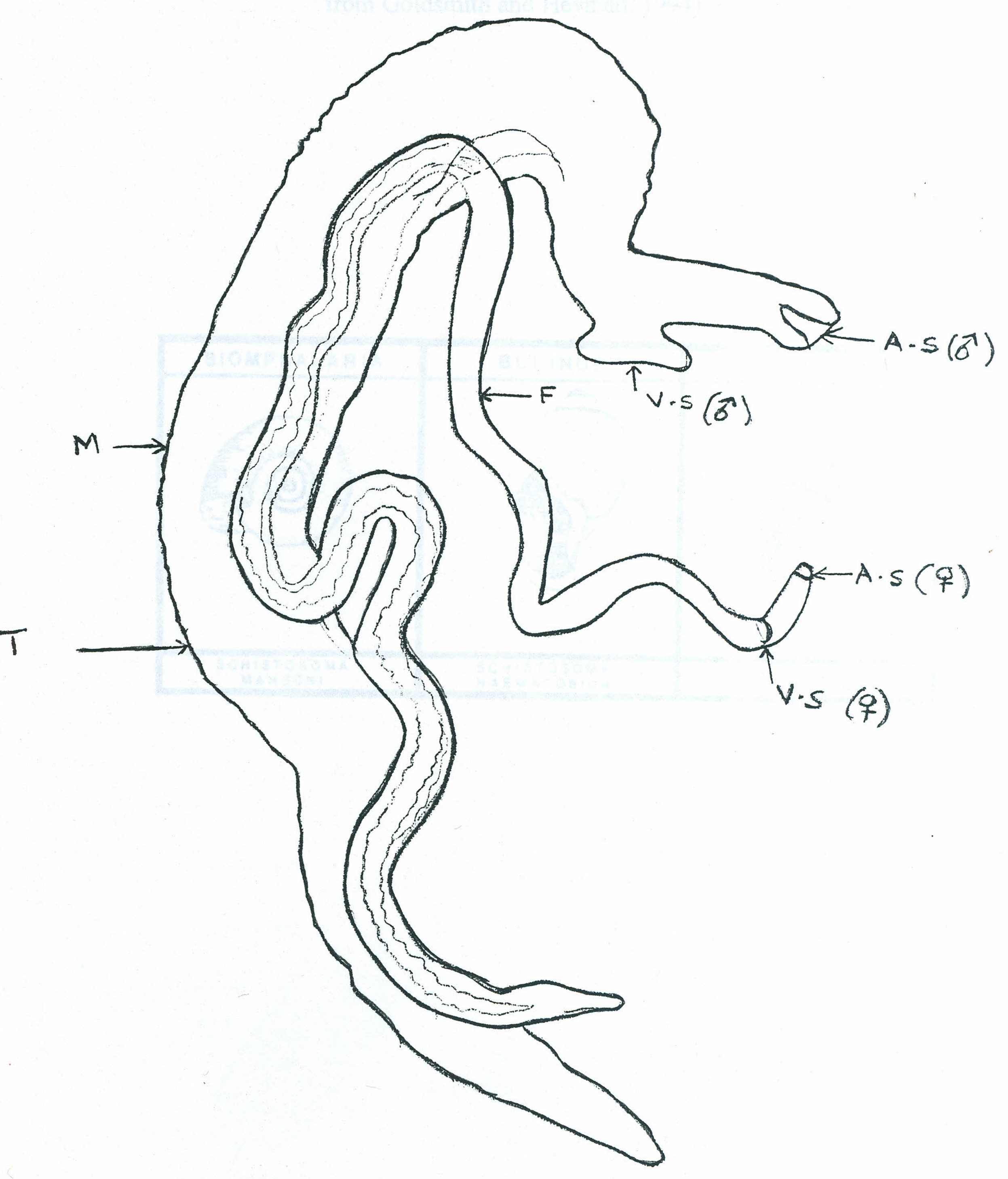
The important point is that, schistosomiasis still extensive and even increasing in some areas, in spite of attempts to control the infections. This is largely due to increasing development of both large and small scale irrigation schemes as rapid population growth and extensive drought puts increasing pressure on more effective land and water usage (WHO, 1995).

1.2: Literature Review

1.2.1: Morphology and general life cycle

Schistosomes are elongated (up to 20 mm in length) and well adapted to life in the blood vessels of their hosts (Fig. 2). The sexes are separate and they stay permanently in copula, the larger male holds the thinner female in a groove on its ventral side called the gynaecophoric canal (Neva and Brown, 1994). Adult females produce eggs constantly, which are passed out either in faeces for S. *mansoni* and *S.japonicum* or in urine for S. *haematobium.* When they encounter fresh water, they hatch into ciliated larvae (miracidium.). The miracidium is an active free-swimming stage and penetrates the appropriate snail vectors (Fig. 3), (*Bulinus,* for *S. haematobium*, and *Biomphalaria* for *S. mansoni*). Within the snail, it develops to mother sporocycts and finally cercariae, which then burrow out of the snail into fresh water completing the cycle in the intermediate host. The initial phase of infection of susceptible mammals by the *Schistosoma* species involves the penetration of the skin by the cercarial stage of the organism and its migration through the dermis (Chavez-olortegui *et al.*, 1992). This process is facilitated by proteolytic enzymes present in the cercarial penetration glands (Dalton, 1997) (Fig. 4). The tail is cast off and the body enters the skin of the definitive host. Several other major changes accompany transformation into a new form called the schistosomulum (Waine and McManus, 1997). The young flukes penetrate the wall of a nearby vein, and then are carried via the host's blood and eventually reach the liver where they grow and attain maturity. The mature male and female worm pair migrate to their dwelling sites. Many eggs pass through the intestinal or bladder wall and are excreted in the faeces or urine, others remain trapped in the liver.

Figure 2 : Schematic diagram of adult *S.mansoni* in the blood. The schematic presentation was adopted from Paniker, (1991)

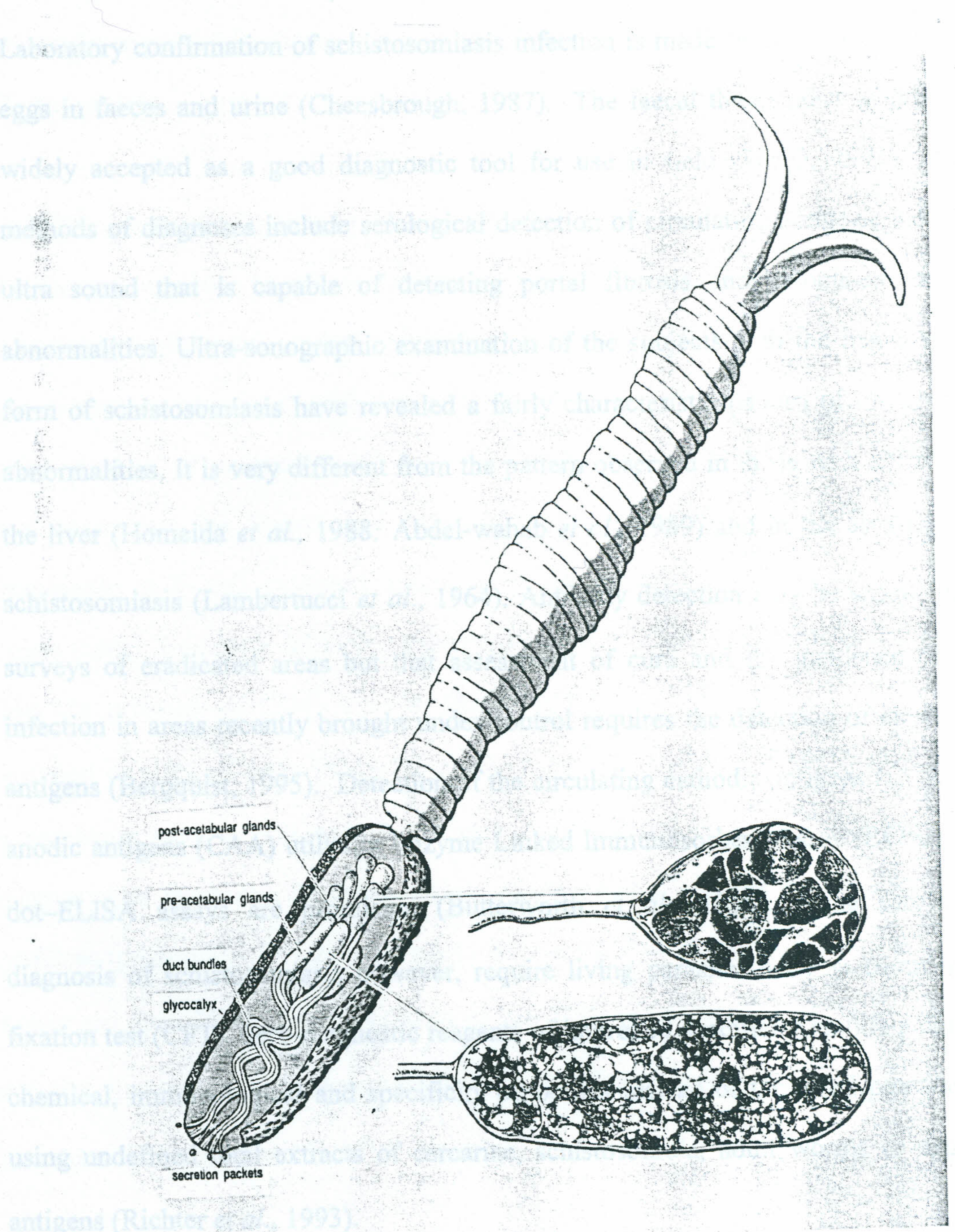


A.S(♂), Anterior sucker (male); V.S(♂), Ventral sucker (male); F, Female; M, male; T, tegument; A.S(♀), Anterior sucker (female); V.S(♀), Ventral sucker (female)

Figure 3: Snail intermediate hosts of Schistosomes of medical importance (adopted from Goldsmith and Heyman, 1994)

BIOMPHALARIA	BULINUS	ONCOMELANIA
SCHISTOSOMA MANSONI	SCHISTOSOMA HAEMATOBIUM	SCHISTOSOMA JAPONICUM

Figure 4: Diagram of cercariae illustrating the location the acetabular glands and the penetration ultrastructure (adopted from Marikovsky, 1990)



1.2.2: Diagnosis of shistosomiasis.

Schistosome infections continue to be diagnosed by well standardised direct parasitological techniques, but their relative insensitivity and the variability of egg excretion is a drawback, particularly in low transmission areas (WHO, 1995). Laboratory confirmation of schistosomiasis infection is made by finding schistosome eggs in faeces and urine (Cheesbrough, 1987). The faecal thick-smear technique is widely accepted as a good diagnostic tool for use in field (WHO, 1993). Other methods of diagnoses include serological detection of circulating antigens and using ultra sound that is capable of detecting portal fibrosis and associated vascular abnormalities. Ultra-sonographic examination of the subjects with the hepatosplenic form of schistosomiasis have revealed a fairly characteristic pattern of intra-hepatic abnormalities. It is very different from the pattern observed in those with cirrhosis of the liver (Homeida *et al.,* 1988; Abdel-wahab *et al.,* 1989) and in the case of acute schistosomiasis (Lambertucci *et al*., 1964). Antibody detection may be sufficient for surveys of eradicated areas but that assessment of cure and the incidence of new infection in areas recently brought under control requires the detection of circulating antigens (Bergquist, 1995). Detection of the circulating cathodic antigens (CCA) and anodic antigens (CAA) utilizing Enzyme Linked Immunosorbent Assay (ELISA) and dot–ELISA assays are promising (Butterworth *et al.,* 1985). Some serological diagnosis of schistosomiasis however, require living parasites such as complement fixation test (CFT). Sero-diagnostic reagents of improved specificity require a defined chemical, immunological and specificity properties instead of the usual practices of using undefined total extracts of cercariae, schistosomula, adult worms or eggs as antigens (Richter *et al*., 1993).

World Health Organisation is concerned about the development of diagnostics not only because of their need in the field but also because future vaccine trials can't be properly evaluated without a reliable way of determining worm burdens. A number of pure antigens have been described (Simpson & Smithers, 1985) but until now genetically engineered antigens have not yet been made available for the diagnosis of schistosomiasis.

1.2.3: The immune response to schistosomiasis

The immune response to schistosomiasis in both animal models and humans is vast and has been extensively studied (Mitchel, 1991; Pearce, 1995) focusing primarily on aspects that are associated with the development of protective immunity. The two major models of animals used for studies of immune response and effector mechanisms are the mouse and the rat. Studies in mice have established the prominence of T- cell mediated immunity in the acquisition of resistance. In this mouse model, immunity following vaccination with irradiated cercariae is dependent upon the presence of $CD4^+$ T cells, (Kelly & Kelly, 1988) and is unaffected by depletion of $CD8^+$ T cells (Vignalli, *et al.*, 1989; Thomson, S. A.1995). Activated macrophages play a role as effector cells in protection against schistosomiasis, together with cytokines, showing that a vaccine that preferentially induces Th 1 cytokines (particularly IFN.gamma) might be beneficial in controlling schistosomiasis (James and sher, 1990). Treatment with an anti-IFN-γ monoclonal antibody is able to abrogate immunity to *S. mansoni* in mice vaccinated with attenuated cercariae (Smythies *et al.*, 1992).

Furthermore, IL-12 promotes Th 1 responses and suppresses Th 2 response. Both Th1 and Th2 responses enhanced attenuated cercarial induced immunity in terms of reduced worm burdens (Wynn *et al.,* 1995) and dramatically reduced the tissue fibrosis induced by natural infection (Wynn, 1995; Sher, 1995). While activated macrophages associated with Th 1 responses appear to be the major effector cells in mice protectively vaccinated once with attenuated cercariae. Other experiments have demonstrated that repeated vaccination, while still protective is associated with Th 2 cell response (Caulada-Benedetti *et al.,* 1991) suggesting that both Th-1 and Th-2 responses may be protective. In primate, human and rat schistosomiasis, the immune response is characterized by the production of anaphylatic antibodies of immunogloblolin G 2a and Immunogobulin E dependent cell mediated cytotoxicity (ADCC). Mechanisms directed against schistosomula with effector cells such as macrophages, eosinophils and platelets (Harrison *et al.*, 1992; Capron & Capron, 1986) demonstrated that adoptive transfer of eosinophils or platelets bearing cytophillic Immunoglobulin E could induce resistance to infection (Capron & Dessiants, 1985). Monoclonal anti-schistosome antibodies of the rat Ig E and IgG 2a isotypes could also passively transfer protection (Verwaerde, 1987, Kigoni *et al.,* 1986). In humans resistance to schistosome infection is age dependent and studies have demonstrated that this is not attributable to differences in exposure after chemotherapy. Such acquired immunity has been demonstrated for *S. mansoni* in Kenya (Butterworth *et al.,* 1985) and Brazil (Dessein *et al.*, 1988) and for *S. haematobium* in the Gambia (Wilkins *et al.,* 1987). The observation that humans can acquire immunity to schistosomes through natural infection suggests that development of a vaccine against human schistosomiasis is feasible.

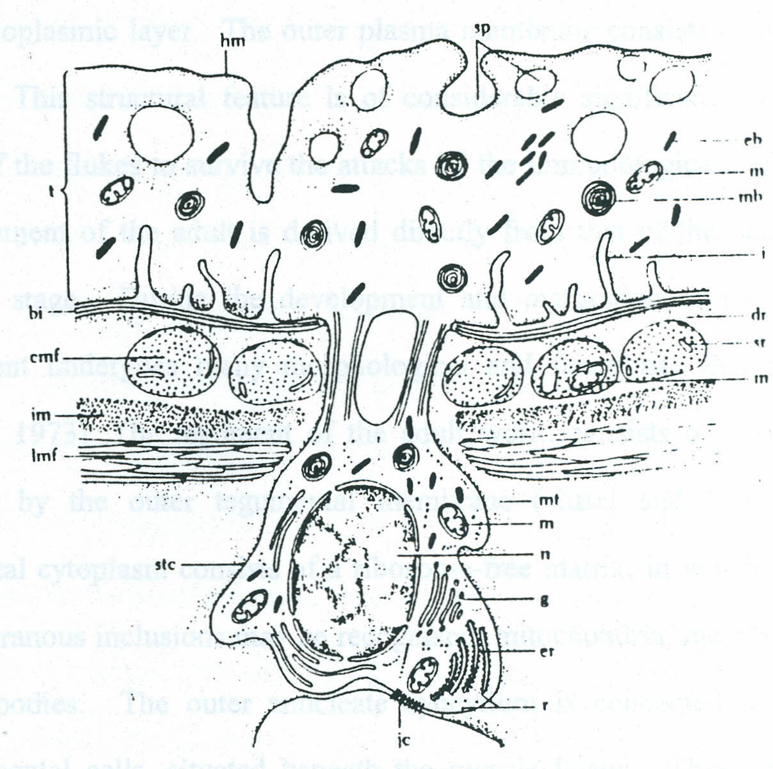
A number of immuno-epidemiological studies have established correlation between specific immune responses and resistance (acquired immunity) or susceptibility to infection. In particular, a build up of 1mmunoglobulin E antibodies has been correlated with the development of resistance to re-infection for both *S. haematobium* (Hagan *et al.,* 1991) and *S. mansoni* (Demeure *et al.,* 1993). In contrast, 1mmunoglobulin G4 and 1mmunogolubulin G2 were found to correlate with susceptibility (Demeure *et al.,* 1993). Antibodies recognising specific parasite antigens have also been associated with resistance to re-infection in humans. The qualitative presence of 1g E antibodies to a 22 kDa antigen was shown to be associated with the expression of resistance in *S.mansoni* patients in Kenya (Dunne *et al.,* 1992). Immunoglobulin G antibodies against a 37 kDa parasites antigen have been associated with resistance to re-infection in Brazil (Dessein *et al.,* 1988; Goundot-Crozel *et al.,* 1989) and Immunoglobulin A antibodies against 28 kDa antigen (Glutathione-S-transferace) have been associated with resistance in *S. mansoni* patients in Kenya (Buterworth, 1994).Homologues of the 22 kDa antigen of *S. japonium* (Waine *et al.*, 1993; Waine *et al.,* 1994), as well as a panel of other *S. japonium* antigens (Becker *et al.,* 1995) are currently being studied with the aim of correlating immune responses to these antigens with resistance to infection with *S.japonium* in China and Phillipines (Yang *et al.*, 1995;Yang *et al.*, 1994).

1.2.4: Surface membranes of schistosome

Schistosomes have a major interface that exists between itself and its external environment called the tegument. This consists of an outer cytoplasmic layer containing mitochondria, secretory bodies, spines and sense organs (Fig. 5).

Figure 5: Representation of adult schistosome tegument and underlaying structure

(Adopted from Davis, 1993)



cmf, circulatory arranged muscle fibres; dr, peripheral dense region of muscle fibre; el elongate body; g, golgi region; hm, heptalaminate outer membrane; I, invagination of bas: membrane; im, interstetial material; jc, junctional complex with paranchymal cell; Lm longitudinal muscle fibre; m, mitochondrion; mb, membraneous body; mt, microtubule; nucleus; r, ribosomes; sp, surfacepits; sr, sarcoplasmic reticulum; stc, sub tegumental cell; t, tegumental.

These rest on a fibrous basal layer and are connected by slender microtubules and lined cytoplasmic processes to cytons and lie below the muscle layers which contain the nucleus, GER, golgi complexes and mitochondria. These cytons actively synthesize the secretory bodies, which move along the cytoplasmic connections to the outer cytoplasmic layer. The outer plasma membrane consists of two layers of lipid bilayer. This structural feature is of considerable significance with regard to the ability of the flukes to survive the attacks by the immunological defences of the host. The tegument of the adult is derived directly from that of the penetrating infective cercarial stage. During the development and maturation of the schistosome, the integument undergoes many morphological and functional changes (Hockley and Mclaren, 1973) .The tegument of the adult worm consists of a synctial cytoplasm enclosed by the outer tegumental membrane (Kusel and Gordon 1989). The tegumental cytoplasm consists of a ribosome-free matrix, in which three major types of membranous inclusions may be recognised; mitochondria, membranous bodies and discoid bodies. The outer anucleate syncytium is connected to sunken nucleate subtegumental cells, situated beneath the muscle layers. These cells are typically secreting cells responsible for the synthesis of the tegumental inclusion bodies. The membranous inclusion bodies with the tegument probably have a role in the formation and replacement of the double outer membrane. The tegument contains cytoplasmic as well as membrane molecules and the double outer membrane is not the only membranous structure of it (tegumental antigen). The cyto-skeletal proteins, actin and paramyosin play a major role in the organisation of the tegument structure (Matsumoto *et al.*, 1988). The surface is composed of arrays of actin filaments. This structure takes essential role of the parasite's survival in the vertebrate host.

1.2.5: Biochemistry and physiology of parasitic schistosomes

Metabolic studies in parasitic schistosomes have been confined almost exclusively to parasitic stages in the final vertebrate hosts and to cercaria (a free-living stage preceding it). Whereas the former mainly have anaerobic energy metabolism and the latter generate energy by aerobic mechanisms (Tielens *et al.*, 1992). It is known that adult *S. mansoni* degrades glucose mainly to lactate but also relies on Krebs cycle activity for energy generation (Bueding, 1950). Cercariae on the other hand have aerobic energy metabolism which degrade their endogenous glycogen reserve mainly to carbon dioxide (Oordt *et al.*, 1989). During the transformation of cercaria to schistosomula, the energy metabolism changes towards a more anaerobic one. It was shown that this biochemical transition occurs only in the head of cercaria and is induced by rise in external glucose concentration that accompanies the *in vivo* transformation as well as the commonly used *in vitro* transformation (Horemans *et al.*, 1991 and 1992). *S. mansoni* cercariae have been shown to express both cathepsin L and β cysteine proteinase present in the post-acetabular glands (Modha and Doenhorf, 1994). The activity of these two enzymes may be involved in the process of skin penetration together with the serine proteinases. Newly transformed schistosomulae do not express serine proteinases but do express cysteine proteinases activity. This cathepsin-like cysteine proteinases may be involved in the degradation of the heamoglobin, which is the probable function of some of these enzymes in adult worms. The nascent digestive track of the schistosomes becomes active within hours following transformation of cercaria to schistosomula and is well developed within seven days.

In vitro studies show that erythrocytes are ingested by schistosomula within days of transformation (Basch, 1981). The expression of cathepsin L and β in schistosomula suggest that the parasite have the digestive machinery in place in the preparation for

feeding once the skin is penetrated (Dalton *et al.*, 1997). Glucose uptake by the schistosomes across the surface membrane appears to be important but the schistosomes gut has nutritional functions (Becker *et al.*, 1995). Female adult worms process an estimated 400,000 erythrocytes per hour, ten times more than the males. Gut associated proteinases are considered to play a vital role in the digestion of haemoglobin to produce amino acids (Fallon and Hagan, 1996). Adult worms are metabolically active and release macromolecules from the epithelial surfaces, such as the tegument and gut as part of their normal activities (Wilson and Barnes, 1974). This propensity means that the mature schistosomes represent a rich source of potential immunogens (Lewis and Strand, 1991).

1.2.6: Molecular biology of schistosome

The genetic constitution of the schistosomes reflects a complex morphology and life-cycle (Simpson & Mcutchan,1982). The genome size of the schistosomes of medical importance is 2.7×10^8 base pairs and is composed of approximately 60% highly and moderately repetitive DNA and 30% single copy sequences. Thus, in comparison to their hosts, the genome size is approximately tenth that of man and exhibits a similar complexity to that of molluscs (Rollinston *et al., 1997).* All members of the genus *Schistosoma* so far examined are normally diploid and have 8 pairs of chromosomes, comprising 7 pairs of autosomes and a pair of sex chromosomes. The female is a heterogametic sex (ZW) whilst the male is homogametic (ZZ). The advent of new techniques in molecular biology has created opportunities for examining genetic variations of schistosomes (Johnson *et al., 1993)* and unlike *plasmodium* and trypanosomes, polymorphism in schistosome genes and chromosome structures are limited which facilitate phylogenetic analysis (Dalton *et al*, 1997). A short tandemly repeated DNA sequence which is highly represented in the genomes of both male &

female *S.mansoni* has been cloned, characterizated (Simpson and Pena, 1991) and used as a probe for detecting parasites and identifying their taxon (Mc Cutchan *et al.*, 1982; Simpson *et al.*, 1982). The advent of new techniques in molecular biology has been used to access the relationships of the major species and to examine intraspecific diversity of schistosome. There is a special need to understand the genetic diversity, both within and between schistosome populations, and this may relate to differences in transmission, disease pathology and immunological response. To date, only 1.5% of the schistosome genome (270Mb) is cloned and expressed and number of genes expressed in schistosomes is probably in the order of 20,000 (Franco *et al.*, 1995) before the schistosome genome project commenced. The need for the sequencing of the whole schistosome genome exist and this could help the understanding of infectivity and the pathogenicity of the parasite (Tanaka *et al.*, 1995). However there is a long way to go before a complete sequence and physical map of the parasite is obtained. The announcement on the success in the Yeast Artificial Chromosome (YAC) project is a boost to those working on the schistosome genome (Tanaka *et al.*, 1995). Despite the analysis of the parasite genomic libraries, recent investigations have been focussed on the cloning of complementary DNA (cDNA) copied from mRNA purified from the parasite. These cDNAs of the parasite could be cloned on either an efficient plasmid or bacteriophage lambda derivatives, within the inducible gene (βeta-galactosidase). Then the expressed fusion proteins can be screened with anti-sera, where many schistosome cDNAs have been isolated and their function characterized.

1.2.7: Management of schistosomiasis

The management of schistosomiasis has been the focus of the investigations world-wide for many decades. It requires multiple inputs and all methods should be utilized

simultaneously, but in reality this is rarely feasible or affordable (WHO, 1997). Appropriate management measures must be selected for each endemic population based on local characteristics of transmission, available resources, and other health priorities. Chemotherapy plays a leading role in the control of schistosomiasis, as all other helminthic diseases (Butterworth, 1988). Reduction of morbidity after treatment has now been validated with three safe and effective drugs, Paraziquantel (PZQ), Oxamniquine (OX) and metrifonate (Andrews *et al.,* 1983). Praziquantel (Biltricide) is effective against all forms of schistosomiasis with few and only transient side effects (Waine & McManus, 1997). Both paraziquantel (PZQ) and oxamniquine (OX) have been used to treat schistosomiasis in population based studies in Africa (Rihet *et al.,* 1991). Althougth they have different modes of schistosomicidal action for both drugs, a prelude to ultimate schistosome death and degeneration is damage to the worm tegument exposing the host to a range of parasite antigens (Fallon *et al.,* 1996*).* Treatment of adult worm with PZQ induces a characteristic instantaneous muscular contraction and pronounced tegumental damage and disruption (Day, 1992). Differences in membrane structures between juvenile and adult, male and female have been observed (Sabah *et al.,*1986; Modha *et al.*, 1990). These differences suggest membrane composition may be an important factor in susceptibility PZQ-induced damage (Lima *et al.,* 1994). PZQ induces the immune system to act preferentially against new exposed surface antigens by PZQ (Fallon *et al.*, 1994) and two of these are 27 KDa antigen with esterolytic activity and an alkaline phosphatase (Doenhoff *et al., 1988*; Brindley *et al.,* 1989). The synergistic activity of PZQ plus antibody is attributed to the antibody's binding to specific exposed epitodes and thereby immunologically enhancing worm death (Fallon *et al.,* 1992*;* Doenhoff *et al.,* 1988). One of the PZQ-exposed *S. mansoni* antigens is tubercle glycoprotein (200 kDa), attached to the membrane via glycosly phosphatidyl Inositol (GPI) anchors (Sauma

and Strand, 1990) has potential as a chemotherapeutic target antigen (Hall, 1995).

The chemotherapy of many helminthic infections is complicated by the occurrence of drug resistance to certain anti-helminthics. Drug resistance in schistosomes began essentially to compounds of the Hycanthone /Oxamniquine family when the worm lost drug activating enzymes present in the sensitive parasites (Cioli *et al.,* 1993). Resistance to a number of schistosomicidal drugs has developed in the laboratory and in the field often shortly after their introduction (Cioli *et al*., 1993; Brindley, 1994). Occurrence of PZQ resistance in schistosomiasis patients has been reported in Senegal and Egypt (Fallon *et al*., 1996). Drug therapy alone is not effective in lowering the incidence of the disease (WHO, 1993). Furthermore the efficient use of PZQ is limited by the high re-infection rates in endemic areas, even after mass chemotherapy. Repeated treatments are therefore necessary and the cost of the drug is prohibitively high (Waine and McManus, 1997). This calls for research for alternative strategies for the management of the disease. In vector management, although there are alternatives, but the most wide spread approach to snail control is by the use of molluscicides, which can be applied in several ways. Molluscicides have always had a role in integrated control programmes (Mc Cullough, 1980). The most extensively used agent is Niclosamide (WHO, 1993). The main advantage of the use of mollucicides in general is that it is independent both of community support and population movement. The disadvantages, however, are that it is extremely expensive and requires extensive and skilled pre-control studies and virtually indefinite continuation of control. Molluscicide-resistant strains may also eventually be selected. Another method of snail control is biological control by predators and competitors although this has not been routinely used.

Health education now ranks as the highest priority in control programmes (WHO,

1985) as community participation is vital in schistosomiasis management. But Haaland (WHO, 1995) stated that this normally fails to work because it is usually badly done. Telling people about *Schistosoma* life cycle will not make them change their behaviour and attitudes. The potential of environmental control, which can provide a sustainable control of transmission, has not always been fully exploited, due to social, economic and even legal implications (Hagan *et al.*, 1997).

Vaccination is believed to be the one shot that can break the gordian knot binding humans to schistosome infections. The possibility of an effective vaccine is based on the fact, that as the people get older egg counts fall, and that animals and humans develop partial immunity to new schistosome infection leaving established worms unaffected (Capron *et al.*, 1987). Vaccines that have been tried against schistosome infections include, live attenuated cercaria, recombinant proteins like Glutothione-S-transferase, multiple antigenic peptides and recombinant DNA (Marguerite *et al.*, 1992; Sher *et al.*, 1998). To date, no effective vaccine is available for this disease, but the possibility of developing of a novel vaccine in the near future is growing in order to protect those at risk against the infection.

1.2.8: Vaccine development for schistosomiasis

The possibility that a schistosome vaccine might eventually be developed began to emerge during the 1960s, when it became clear that a variety of experimental animals, in particular the mouse and the rhesus monkey, acquired the capacity to resist re-infection following the primary infection. This observation led to the concept of concomitant immunity (Smithers and Terry, 1969). The feasibility of a vaccine for schistosomiasis is provided by successful vaccination/challenge experiments using attenuated cercariae in animals. Research using laboratory mice has demonstrated that it is possible to protect mice against challenge infection with schistosomes prior to

immunisation/exposure of the mice to the cercariae previously attenuated either using ultra-violet (u v) or gamma- radiation (Bickle *et al.,* 1985). Successful UV-attenuated *S. japonium* vaccines have been tested in pigs (Shi *et al.,* 1993) and buffaloes under field conditions in China (Shi *et al.;* 1990). However, due to the difficulty of producing large scale, quality controlled, reproducible batches of these vaccines, and the associated safety considerations, they are not suitable for use in humans. Much attention has therefore focused on investigating the protective mechanisms of the immunized response, and subsequently on identifying particular parasite antigens that may be involved in inducing protective immune responses, with a view of developing a recombinant derived, synthetic peptide based or DNA vaccine. In addition to the antigens described, a number of parasite antigens have also been shown to confer protection in experimental animal models. Such have been tested in a number of different forms. These include purified native antigens, recombinant antigens encoding partial or full length proteins, vaccines based on peptide sequences such as multiple antigenic synthetic peptides (MAPS) and more recently, nucleic acid vaccines, where the DNA encoding parasite antigen is delivered, rather than the protein itself. Various defined antigens have been tested for their protective efficacy against schistosome re-infection. These include glutathione-S-transferase (GST) (Smith *et al.,* 1994; Taylor *et al.,* 1988; Mitchell *et al.*, 1991; Xu *et al.,* 1993), immunogenic glycoproteins from adult worms for example GP62, GP38, and GP16 in reference to their molecular weights in kDa (Simpson and Smithers, 1985; Taylor *et al.*, 1994). In the schistosomula stage, a host of schistosomula surface antigens including a 37 kDa, 38 kDa and two different 22 kDa molecules, one of which induces a specific 1mmunoglobulin E response have been described (WHO, 1993). Neutral glycolipids from *S. mansoni* adult worms, cercarial and eggs are also antigenic and immunogenic (Alving *et al.*, 1974; Weiss *et al.*, 1986; Baumeister *et al.*, 1992). The

potential of attenuated parasites has been explored in mice and primate models. In baboons for instance, vaccination with radiation attenuated cercaria can provide a high level of protection (up to 8O%) against subsequent challenge by infection with *S. haematobium* (Harrison *et al.,* 199O). In cattle, an irradiated larval vaccine was quite effective against infection of *S. bovis* and *S. Matthei* (James, 1987).

1.2.8.1: Native and recombinant protein vaccines

Paramyosin, a myofibrillar protein found exclusively in invertebrates, is one such protective antigen. Both the native molecules and recombinant expression products encoding half of the full length protein of *S. mansoni* have been shown to confer significant levels of protection in mice (Pearce *et al.*, 1988; Flanigan *et al.,* 1989). Higher levels of protection have been obtained for the Philippine strain of *S. japonicum* using purified paramyosin (Ramirez *et al.*, 1996). Furthermore, paramyosin of the Japanese strains of *S. japonicum* was identified as the target of a partially protective murine 1g E monoclonal antibody (Nara *et al.,* 1994). The absence of homologous antigen in vertebrates may ensure that cross-reactivity with self does not occur. Another molecule that has been shown to confer protection in animal models is glutathione-s- transferase (GST) and it has been shown to exist as 26 kDa and 28 kDa molecules, each consisting of several isoforms in *S. mansoni.* Immunization with either the purified native 28 kDa GST or the recombinant 28 kDa GST can confer protection against experimental *S. mansoni* infection in a range of animal models, including rat (Balloul *et al.,* 1987). However, the recombinant 26 kDa GST of *S. japonicum* has been shown to induce a prominent anti-fecundity effect in experimental *S. japonium* infection, as well as a low but significant level of protection in terms of reduced worm burden (Shuxian *et al.,* 1995). A recombinant 14 kDa fatty acid-

binding protein of *S. mansoni* has been shown to protect outbreed swiss mice by up to 67% against challenge with *S. mansoni* vaccination of mice with a 62 kDa recombinant antigen designated irrV-5, of *schistosoma mansoni* has shown to induce protection against *S . mansoni* challenge infection in baboons (Soisson *et al*., 1993).

1.2.8.2 Peptide based vaccines.

Peptide based vaccines are alternative to vaccines based on the entire native or recombinant proteins. They are more clearly defined and have advantage of being easier to produce, as they do not require expression in and purification from a foreign host. Further, it may be possible to construct synthetic peptide based vaccines that retain immuno-protective responses, while removing sequences with undesirable sequalae such as cross-reactivity with host tissue. A disadvantage with peptide vaccine, however, is that unlike native and recombinant proteins they can present only linear, but not conformational epitopes. One antigen that has been investigated with respect to constructing peptide vaccines in the 28 kDa glutathione-S-transterase of *S. mansoni*. A peptide comprising amino acids 115-131, and known to incorporate both T and B cells recognition sites in a variety of experimental models, was used to synthesize an octameric `octapus' construct. Rats immunized with the construct were partially protected against a challenge infection with *S. mansoni* (Wolowezuk *et al.*, 1991). Another approach of peptide based vaccines is the construction of MAPS. Such an approach has been used with some success for *S. mansoni* using MAPS based on two *S. mansoni* antigens Sm 23 and triose-phosphate isomerase (TPI). Vaccination trials in mice using either of these construct was reported to confer partial protection (Harn and Reynolds.,1993).

1.2.8.3 Nucleic acid vaccines

One of the most exciting new fields in vaccinology is the development of nucleic acid vaccines, touted as the third generation of vaccines (Waine & McManus, 1995). In these type of vaccines nucleic acids are used for immunization rather than proteins and peptides. Usually a cDNA encoding a protective antigen is cloned into a plasmid containing a eukaryotic promoter. This plasmid construct is then delivered to the host to be immunized, usually by injection into muscle, or by delivery to the skin using a device called `gene gun'. The plasmid DNA is then taken up and expressed by the host cells themselves, subsequently generating a specific immune response. Nucleic acid vaccine has several potential advantages over other types of vaccine. A major concern is, live vaccines for instance, such as the attenuated cercarial vaccines is the risk of some organisms failing to attenuate or the possibility of attenuated organisms reverting to a pathogenic form. Nucleic acid vaccines do not carry this risk, yet are still able to induce all the same immune responses including cytotoxic-T lymphocytes (CTLs), T - helper (Th) cell and humoral (antibody) immunity. This gives them an advantage over recombinant proteins and peptides, which are generally unable to induce specific CTL responses. Other advantages include the simplicity of producing plasmid DNA compared to purifying native or recombinant proteins, eukaryotic expression (by the vaccinated host), and the potential to generate long term immunity resulting from continued expression in the host. DNA vaccines are also stable at room temperature, thus eliminating a need for a `cold chain' and reducing the hosts and logistics associated with delivering vaccines to remote areas. Nucleic acid vaccines have been shown to confer potential protection against a diverse range of infectious agents including parasites (Sedegah *et al.*, 1994). Their effectiveness against parasites as well as viruses and bacteria (Kriesel *et al.*, 1996) suggests that nucleic acid vaccines may hold promise for the development of a vaccine for

schistosomiasis. Recently a panel of *S. japonicum* cDNA has been cloned into eukaryotic expression vectors and tested for their efficacy in mice (Yang *et al.*, 1995, Waine *et al.*, 1997). A nucleic acid vaccine for *S. mansoni* reported to have high levels of protection against *S. mansoni* challenge in mice vaccinated with a DNA construct encoding an integral membrane protein Sm 23 of *S. mansoni* (Harn, 1997). While still in its infancy, nucleic acid vaccination is a rapidly expanding area and two areas immunogenetics, oral vaccination are gaining an impact on schistosomiasis. In summary, a wide range of approaches are being taken towards the development of an effective vaccines for schistosomiasis and vaccination trials utilizing experimental animals ranging from mice to water buffaloes showed that a vaccine for the disease is substantial and it can be anticipated that these efforts will result in a successful out-come. Preliminary results (WHO, 1996) have however highlighted some problems that might delay vaccine development. One of these is that the results have demonstrated that the stated goal of consistence induction of 40% protection or better has not been reached with any of the antigens formulations tested in the trials. Continued research in the development of putative vaccine candidates is therefore imperative and remains a highly potential field that needs to be addressed. The use of recombinant DNA techniques to elucidate and characterize potential vaccine candidates is a useful strategy for development of vaccines.

1.3: The *rationale* for this study

Currently, diagnosis followed by appropriate chemotherapy remains the cornerstone of control strategy against schistosomiasis. At the moment three effective and safe drugs are available for treating schistosomiasis: Oxamniquine, metrifonate and praziquantel (WHO, 1993). For a number of reasons, including commercial viability, Oxamniquine and Metrofonate may soon cease to be readily available, leaving paraziquental as the sole generally available drug. Paraziquental is the drug of choice for case-management in schistosomiasis and it is the main tool of a country level schistosmiasis control programmes. It has thus been administered to tens of millions infected individuals in endemic areas for morbidity control (WHO, 1993). Moreover the effective and efficient use of PZQ is limited by highly infection rates in endemic areas even after mass chemotherapy and the need for repeat treatment making the cost of chemotherapeutic regimens prohibitively high and largely unaffordable. This scenario, however, has recently been tainted by reports of low cure rates in Senegal. The isolation of potential praziquantel–resistant schistosome in the laboratory and field, though these report need further confirmation. In the mean time, there is a need for increased vigilance in both monitoring and reporting of any emerging praziquantel tolerance/resistance, which would obviously have major implications for control strategies in the future. There is also a strong need to promote research and development of additional anti-schistosomal drugs and/or vaccines if human cases of schistosomiasis have to be reduced significantly. Immunological intervention in the form of a schistosome vaccine would complement the success of our present control efforts by adding to existing control strategies. Vaccines for schistosomiasis has been targeted as a priority by WHO. This is based on the ability of the humans to acquire natural immunity to schistosome infections, together with the successful use of attenuated vaccines in animals both under laboratory and field conditions, suggesting that development of vaccines is feasible. Several candidate immunogens have already

been developed but few elicited significant level of protective responses compared to those observed in experimentation using attenuated larva in non–human primate models of the disease. Continued research in the development of putative vaccine candidates is therefore necessary and remains a highly potential field that needs to be addressed. The use of recombinant DNA techniques to elucidate and characterize potential vaccine candidate is a powerful strategy for development of vaccines. The aim of this study was to extract and characterize tegumental proteins from *S. mansoni* worms and immunoscreen *S. haematobium* adult worms lambda gt11 expression cDNA library and isolate, characterize clones encoding for tegumental antigens with a view of being a potential vaccine antigen and/or as diagnostic antigen.

1.3.1: Hypothesis in the study

The null hypothesis put forward of this study was that adult *S. mansoni* worms have tegumental proteins that can be extracted and characterized. The tegumental extracts are potential vaccine and diagnostic antigens. There was also an assumption that a *S. haematobium* cDNA library can be screened with rabbit anti-sera and isolate Sh cDNAs encoding for tegumental antigens. These cDNAs could then be characterized by cloning and sequencing its composition.

1.3.2: O B J E C T I VE S OF THE STUDY

1.3.2.1:GENERAL OBJECTIVE.

The general objective of the study was to study tegumental extracts of *Schistosoma mansoni* adult worms with the aim of isolating and identifying *S. haematobium* antigen gene(s) with potential as vaccine/ diagnostic agents.

1.3.2.2: SPECIFIC OBJECTIVES:-

The specific objectives were to:

I) extract and characterize tegumental proteins form adult *S. mansoni* worms.

II) screen adult *S. haematobium* cDNA expression library with rabbit anti *S. mansoni* sera (raised from rabbits challenged with tegumental extract) in order to identify and isolate cDNAs encoding for tegumental antigens.

III) sub-clone, characterize and express select clones for production of recombinant proteins and evaluate their efficacy as potential vaccine or diagnostic antigens.

CHAPTER TWO

2.0: MATERIALS AND METHODS

2.1: Culture of *Schistosoma mansoni*

Schistosoma mansoni eggs were recovered from faeces of infected school children in Kisumu and experimental animals (baboons) at the Institute of Primate Research (IPR) Nairobi, Kenya. The eggs were hatched to miracidia at 35° C under powerful electric lamp and within two hours, they were exposed to laboratory raised F1 progeny of *Biomphalaria* snails of 3.4 mm in size, at 27° C for 5 hours under an electric lamp. Six weeks post exposure, snails were kept in darkness for a period of 16 hours prior to inducing them to shed. The snails were then placed individually in glass vials (20 x 30 mm) without water for a period of 30 min under powerful electric lamp at room temperature of about 25° C. After this period, 5 ml of water was added to each vial and placed under 15-M white fluorescent light (18 inches above vials) for two hours. With the help of dissecting microscope, the vials were examined for the presence of cercariae. Newly shed cercaria were counted and put into petri dish. Ten drops of water were poured into the petri dishes to cover the bottom. Balb/c mice belly was inoculated using Pouch method. Eight weeks post inoculation, the mice were anaesthetized and perfused as described by (Sturrock *et al.,* 1976) to recover mature worms. The recovered mature worms were immediately transported on dry ice from Institute of Primate Research, Nairobi, to Department of Molecular Genetics, National Museums of Kenya for further processing and use in experiments.

2.2: Isolation of membrane proteins

Fresh eluted adult *S. mansoni* worms were washed several times with phosphate buffered saline (PBS) pH 7.4 to remove any host molecules.

2.2.1: Extraction of peripheral protein

Peripheral proteins, which are loosely bound surface membrane proteins, were detached by soaking the worms in a highly concentrated salt solution (0.75 mM NaCl in phosphate buffered saline containing 12 mM hypotonic sucrose solution). These were gently mixed and allowed to stand for 20 min. at a room temperature, then 10 min on ice. The suspension with the parasite was centrifuged at low speed (500g) for 5 min in order to recover the worms stripped of their peripheral proteins and the supernatant preserved. The worm pellets were washed in PBS twice before subsequently treated with detergent.

2.2.2: Extraction of tegumental proteins

After removing the extrinsic proteins, the worm pellets were incubated in a detergent solution to solubilize integral (intrinsic) proteins, submerged in the hydrophobic lipid bi-layer. The parasites were left in the detergent (0.1% Triton x-100 in PBS) for 20 min in ice with occasional gentle mixing, which then pelleted and the supernatant preserved. This extract was named as tegument (a). The worm pellet was then washed with PBS and this was named as wash proteins. A separate method of tegument extraction was used (Vasconcelos *et al.,* 1993). Adult worms were incubated for 10 min at 37^0 C in phosphate buffered saline containing 2.7 mM KCl/ 0.9 mM $CaCl_2$ /0.5 mM $MgCl_2$. The material was vortexed for 10 sec and after sedimentation of the worms, the tegument fraction was recovered from the supernatant by centrifugation. This kind of extract was termed as tegument (b). The pellet was homogenized in 5 mM Tris-buffer pH 7.4, containing 8%(w/v) sucrose. To prevent protein degradation, extracts were stored with addition of 2 drops of cocktail protease inhibitors (10 mM

PMSF, 0.5M EDTA, 0.5μg/ml Leupeptin), and samples were placed at –20 ^{0} C for later use

2.2.3: Determination of protein concentration

Determination of protein concentration was performed using PIERCE BicinChoninic Acid (BCA) protein assay (Brown *et al.* 1989). A series of protein standards of known concentration were prepared by diluting the stock 2 mg/ml BSA standard and test samples also were prepared in 100 μl of the same reagent. 100μl of the known (standard) and test samples was then pipetted into the appropriately labelled test tubes, and one blank 0.1ml of diluent was used. Then 2.0 ml of the working reagent was added into each tube and mixed well. All tubes were then incubated at 37^{0}C for 30 min. The protein absorbance at 562 nm of each tube versus water reference was then measured. A standard curve was then prepared and the protein concentration of each unknown protein sample was determined.

2.3: Electrophoresis of schistosome proteins

Extracted proteins from the surface of adult *S. mansoni* were characterised on polyacrylamide gel electrophoresis in the presence of Sodium Dodecyl Sulfate (SDS).

2.3.1: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method of fractionating proteins on polyacrylamide gels adopted in this study was that originally described by Laemmli (1970), using a Mini-protein II dual slab cell (Bio-Rad San fransisco CA, USA) apparatus (Plate 1). PAGE gels contained SDS, with a discontinous buffer system in which there was 4% of stacking-gel (0.375M Tris-base pH 8.8) or sharpening of individual protein bands and 10% of separating gel (0.125M Tris base pH 6.8) were used. Extracted protein samples were treated in a way that allows complete denaturation and reduction of disulfide bonds of proteins before electrophoresis. This was achieved by mixing the protein sample with sample

buffer (Appendix III) into an eppendorf tube. The mixtures were then heated for 5 min at 95- 100° C on heat block and then chilled on the bench at room temperature before using. The prepared samples were loaded into wells and run in Electrode buffer (Appendix III) at 100 volts for 90 min. They were then stained with 0.0125 mM Coomassie Blue R250 (appendix III) overnight. To visualize the separated proteins, the gel was washed with de-staining solution I (Appendix III) and then de-staining solution II for two hrs each, with gentle agitation. Then separated proteins were detected by photography. The protein marker comprised a mixture of proteins, such as α-macroglobulin (mol.wt, 180,KDa), β-glactosidase (116,KDa), Fructose-6-phosphate kinase (84,KDa), pyruvate kinase (58,KDa), lactatedehydrogenase (36,KDa) and triose phosphate isomerase (26KDa). The band sizes of the unknown proteins were compared with a standard protein marker.

2.4: Immunization of experimental rabbits

Two New Zealand white rabbits, at Institute of Primate Research (Ngong forest) were used to raise sera against *S. mansoni* tegumental immunogens. Twenty millilitre of blood was collected from both experimental rabbits prior to immunization. For the initial inoculum (100μg) of crude tegumental extract emulsified in Freund's Complete Adjuvant (FCA) was inoculated sub-cutaneously at several points between the shoulders and back. Three weeks later the rabbits were given the first boost or injection (50μg tegumental antigens constituted in Freund's Incomplete Adjuvant (FIA)). At the same time 10-15 ml of blood was collected. The second and final boosting was done two weeks after the first boost using 50μg of the immunogens in FIA. Twenty mls of the blood were again collected. Three weeks after the second boost later, the animals were exsanguinated. After collection, blood was allowed to clot for 60 min at 37° C. The clot was then separated from the sides of the collection vessel, using a pasteur-pipete and placed at 4 °C over night. Serum was separated by

centerifugation at 10,000g for 10 min at 4° C. All sera was stored at -20° C .

2.5 : Immuno-blotting (Western blot)

Electrophoretically separated proteins were transferred from gel to nitrocellulose membrane obtained from Schleicher and Schull. Western blotting was carried out following the procedure described previously (Towbin *et al.*; 1979; Burnette, 1981). The electrophoresis apparatus TE70 semiphor™ semi dry transfer unit (Hoeffer CA. USA) was used (Plate 2). Electroblotting was carried out in a transfer buffer (Appendex II) at a current of 0.65 mA/sq.cm for a period of 60 min. The NC filters was washed with 1x TBS and blocked with 5% of non-fat skimmed milk (BLOTTO) at room temperature on shaker for 30 min. Primary sera (1:50) in 1x TBS was incubated with the blot overnight at 4° C, with constant agitation. The membrane was rinsed briefly with TBS, then washed three times, for 5 min each, and blocked with the same blocking solution. Goat anti-rabbit Immunoglubulin G conjugated with Horse-radish peroxidase 1:1000 in TBS was incubated with the blot at room temperature shaking for 2 hrs. The membrane were removed, rinsed three times in TBS, for 5 min each, and developed in 4 chloro-1-naphthol solution to detect the immuno-reactive bands. The immunoblot was photographed as soon as possible before bands faded away. Dot blot analysis of extracted worm proteins was also carried out on the nitrocellulose filter paper.

PLATE 1: SDS- PAGE equipment
BIO- RAD mini- protean^{R} II
2000 Alfred Nobel Drive
Hercules, CA 94547 U.S.A,

PLATE 2: Semiphor Transfer Unit
TE70 `91-1017
Hoeffer Scientific Instruments
San Fransisco, CA 94107
U.S.A,

2.6: Screening of adult *S. haematobium* cDNA expression library

An aliquot of 100 µl adult *S. haematobium* cDNA library constructed in lambda gt11 expression vector (Young and Davis, 1983) (New England Bio-lab, USA) was provided by Dr. Rashid A. Aman at the Department of Molecular Genetics, National Museums of Kenyan (NMK) Nairobi. *Escherichia coli* strain Y1090hsdR (Young and Davis, 1983) which is commonly used as the host for immunological screening libraries constructed in Lambda gt11 was recovered from a frozen glycerol stock (–80 ^{0}C). The bacterial cells were streaked onto the surface of Luria Bertani (LB) agar plate containing appropriate ampicillin (50µg/ml) and incubated at 37 ^{0} C overnight.

2.6.1: Titration of the cDNA library

A single colony of *E. coli* Y1090 strain (Appendix 1) picked from the original LB agar plate was inoculated into 10 ml of LB medium (10g Bacto-tryptone,5g NaCl, 5g Bacto-yeast exstract, pH 7.0, into one litre) containing 50 µg/ml ampicillin and supplemented with maltose final concentration of 0.2%. The culture was grown overnight in a 37° C incubator with shaking at 220 rpm. The bacteria culture was pelleted by centrifugation at 3500g for 10 min at 4^{0}C and re-suspended in 0.4 volume of ice-cold 10 mM $MgSO_4$ on ice. 400µl of this bacterial suspension was used as lawn cells in the subsequent plating of the lambda gt11 library on 150 mm petridishes. 10µl of the stock cDNA library was serially diluted in SM buffer starting with hundred fold dilution (that is 10µl of the stock library into 990 µl of SM buffer). That was followed by another 100 fold dilution of the second and third, continued up to $10.^{-8}$ For plating 100µl of the 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, dilutions were mixed with 400µl of the plating cells in a pre labelled 1.5 ml sterile microfuge tubes and incubated at 37 ^{0}C for 10 min with out shaking. Seven ml molten top agar at 45^0 C was added to each tube and mixed. The mixture was plated on LB agar plates immediately. The plates were left

for 15 min on the bench to harden the soft top agar and incubated at 37°C in an inverted position for at least 5 hrs. To determine the titre of plaque forming units per microliter, the plaques obtained from each dilution were counted. This number was divided by 100 and multiplied by the respective dilution factor. The average of the two middle dilutions was then used as an estimate of the titre of the library.

2.6.2: Plating of the lambda gt11 library

For the primary screening of the cDNA library, five large plates (150 mm) each at a density of 50,000 plaque forming units (pfu) were plated. For secondary screening, small plates (90 mm) were used and plated with 50µl of a 10^{-5}dilution of the primary positive plaques and 100 µl of the Y1090 plating cells in 3 ml top agar. All positive plaques picked up in the secondary screening were subjected to tertiary screen using small plates, in a way that they could give single well isolated plaques. The adult *S. mansoni* cDNA library was diluted to 500 pfu/µl in SM buffer .100µl of these diluted phage was mixed with 200µl of plating cells and incubated at 37°C for 30 min and then 7ml of molten top agar were added. The whole mixture was then poured onto the surface of the 150 mm-LB agar plate, with gentle tipping of the plate from side to side, so that the top agar spread evenly over the entire surface of the plate. The plates were left on the bench for the top agar to solidify, then the plates were incubated at 42^0 C inverted for phage induction. There after, nitrocelullose filters were overlaid for three hours at 37 ^{0} C.

2.6.3: Induction of expressed proteins onto

Nitrocelullose filter papers (Schleicher & Schuel, 0.45µ pore size) were placed on petri dish containing 50 ml of 10mM Isopropyl thio-β-D-galactoside (IPTG) solution and each filter was allowed to wet. After 20 min the filters were taken out, placed on

paper towel and allowed to dry thoroughly. The dried filters were carefully overlaid on the plates by holding opposite sides with forceps and lowering the centre of the filter onto the centre of the plate and allowing the filter to wet slowly from the centre to the edges. Care was taken not to form air bubbles. The nitrocellulose filter papers and the LB agar plates were marked for orientation by puncturing them with a needle in three slabs asymmetrical and then labelled. The filters remained on the plates incubated for 4 hours at 42° C and after the plaque appearance, the plates were transferred to 37° C incubator for another 4 hours.

2.6.4: Lifting, washing and blocking of the filter papers

After the incubation, the plates were kept at 4° C for overnight to ease peeling the filter papers off the agar plates without difficulties. Sometimes the top agar could stick and complicate the process of lifting the paper. Thus, clean forceps were used to remove the NC filters in a way to avoid a retouch of the filter paper on the plate once removed. The filters were then washed in PBS-Tween three times for 5 min each by a shaker at room temperature. Then the filters were blocked with 3% bovine serum albumin (BSA) for one hour shaking at room temperature.

2.6.5: Antibody probe onto the filters

The primary antibody was raised in two experimental rabbits challenged with *S. mansoni* tegumental immunogens. Most rabbit anti-sera contain IgG components that bind to *E. coli* proteins, which will cause extensive non-specific reactions. To avoid the cross-reaction of specific with non-specific components, it was absolutely necessary to adsorb out any *E. coli* antibodies.

2.6.5.1: Preparation of acetone powder

A single colony of *E. coli* strainY1090 was inoculated into 200 ml of 2x YT broth supplemented with an appropriate amount of ampicillin at 37°C with shaking at 200

rpm. The culture was pelleted in a pre-weighed 50 ml falcon tube and re-suspended using 1 ml of 0.9% NaCl per gram of the pellet. The suspension was transferred to the tubes for five minutes. After that 8 ml of ice-cold acetone (-20^0 C) were added to each 2 ml of cell suspension, vortexed and placed on ice for 30 min with intermittent vortexing. The precipitate was collected by centrifugation at 700g for 10 min and the supernatant discarded. The pellet was re-suspended with fresh acetone (-20 ^{0}C), vortexed then kept on ice for 10 min. Thereafter, the suspension was pelleted by centrifugation at 700g for 10min, the supernatant was decanted and the pellet left on the bench to dry at room temperature. Afterwards, the pellet was broken down into fine particles.

2.6.5.2: Treatment of anti-sera with acetone powder

The amount of powder needed to prevent background staining depended on the concentration of the sera, higher concentration of the anti-sera required the use of more powder to adsorb the bacterial antibodies out. The acetone powder was added into the primary sera to a final concentration of 1%. The primary anti-sera were then incubated for 60 min shaking at 4^0 C then spun at 10,000g for 10 min. The supernatant was kept –20 °C and used as the pre-adsorbed primary antibody.

2.6.6: Primary and secondary antibody probe

The pre adsorbed sera was diluted 1:50 PBS incubated with the filters for 16 hrs, at 4°C with gentle shaking. Then the primary antibody was saved and reused several times. The filters were washed with PBS-Tween and blocked with 0.3% BSA in 1xPBS for 1hr, shaking at room temperature. Goat anti- rabbit (IgG) conjugated with Horse-radish peroxidase (New England Bio-lab. USA) was used to detect primary antibodies (IgG) bound to the immunogens. Secondary antibody was diluted in a ratio of 1:1000 in PBS, and incubated with the blot for two hours at room temperature with shaking. Protein-A conjugated with Horse-radish peroxidase (NEB BIO-LAB) was

also used as secondary antibody on occasion. 4- chloro-1-naphtol substrate (0.03 g in 10 ml of absolute ethanol to 40 ml of 0.7% Tris-base pH 8.0.) and H_2O_2 (30 µl of 30%) were used as developing substrate.

2.6.7: Picking and storage of positive lambda gt11 clones

After the band development, the clones showing immuno-reactivity with the primary antibodies against tegumental immunogens were traced. The agar plates were removed from 4 °C and the filters were placed in plastic bag with the help of light box. The filters were matched to the needle mark of the agar plates, which contained three asymmetrical slabs. The positive recombinant lambda plaques were picked from the agar plate using micro-pipette tips, and placed in sterile 1.5ml microfuge tubes containing 0.5ml of SM buffer (5.8g NaCl, 2g MgSO4.7H2O, 50ml of 1M Tris-HCl, pH 7.5, 5ml of 2% gelatin solution, made to 1 liter with water). A drop of chloroform was added and the lambda clones stored at 4 °C. Secondary and tertiary screenings were plated in a way that can give isolated plaques that could be picked without difficultly.

2.7: Characterisation of positive clones

The positive clones selected by immuno-screening were further characterised by cloning into plasmid vectors. First of all, the recombinant plaques were subjected to polymerase chain reaction (PCR) to isolate the cloned inserts from the lambda gt11vector and establish their sizes.

2.7.1: Preparation of templates for PCR reactions

About 20 µl of phage lysate was pipetted into a sterile 0.5ml microfuge tubes and boiled in a heat block at 100° C. This caused the lysing of the phage particles to release its DNA. Immediately the tubes containing the lysed particles were chilled on ice for 5 min and centrifuged at 10,000g for 2min. 5 µl of the supernatant was used as

DNA sample for 50µl PCR.

2.7.1.1: Rapid preparation of lambda gt11 DNA

The positive lambda clones isolated from the screenings were appropriately diluted to give confluent growth with *E. coli* Y1090 on LB agarose. After 6 hrs of incubation at 37^0 C the plaques appeared on the media. Then agarose plates were transferred to 4° C and 5 ml of SM buffer were poured on the plates and kept overnight. The plates with the SM buffer were shaken at room temperature for 1 hour. The buffer was harvested into sterile 50 ml Falcon tubes and centrifuged at 5000 rpm at 4° C for 10 min. To the supernata was added DNase, RNase and both at a final concentration of 1µg/ml and the nucleic acid digestion allowed to occur for 30 min at room temperature. Proteinase K was then added to a final concentration of 50µg/ml. The suspension was incubated at 50° C for 1 hour and then cooled for 15 min room temperature. An equal volume of 2M NaCl, 20% PEG (w/v) in SM buffer was then added into mixing by inversion and the suspension left for 1 hour on ice. λ Phage precipitates were collected at 10,000 rpm at 4° C for 1 hour. The supernatant was poured off and the pellet free from any PEG trace solution was resuspended with 0.5 ml of TE on shaker mixer for 10 min. Then SDS was then added to a final of 1% and incubated for 15 min at 73° C. Then 0.5M NaCl was added. Thereafter, Phenol-Chloroform DNA extraction was applied and the recovered phage DNA was precipitated with 1 ml of ice cold Isopropyl alcohol and placed at -70° C for 30 min. This was then centrifuged at 12000g for 10 min to recover the phage DNA. The DNA was washed with 70% ethyl alcohol and spun 12000g for 10 min. The alcohol was poured off and the remaining pellet was dried, re- suspended with 50 µl TE RNase and dissolved at 37° C for 15 min. To ensure presence of DNA recovered, 5 µl of this solution was analyzed on 0.8% agarose gel.

2.7.2: Amplification of bacteriophage library inserts using polymerase chain reaction

The cDNA of adult *S . haematobium* were cloned into the EcoRI site of the lambda gt11 expression vector. Isolation of the cloned foreign DNA from the vector polymerase chain reaction was carried out using lambda foward primer (5'-ATTGGTGGCGACGACTCCTGGAG-3') and reverse primer (5'-CAGACCAACTGGTAATGGTAGCG – 3') that flank the cloning site of the vector.

2.7.2.1: POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) was set up as follows:- 10x reaction buffer for Taq 1 polymerase (strategene, la Jolla, CA, USA), 10 mM Tris-Cl pH 8.3, 50 mM Kcl, 1.5mM MgCl2, 0.02% [w/v] gelatin, 0.2 mM of each deoxynucleotide, 1μm of each primer(forward and reverse), 2.0 Units of Thermus aquatus (Taq) DNA polymerase and 1.0 μl of lambda genomic DNA and total volume of the reaction was made up to 50 μl with double distilled water (ddH2O). The reaction material was spun briefly and overlaid with 50μl of mineral oil (Sigma). The reaction was run in either Hybaid thermol reactor or Coy thermocycler (Plate 3) under the following conditions; initial denaturation at 94° C for 3 min and the followed by 30 cycles of: Strand separation at 94° C for 30 sec, primer annealing at 55°C for 1 min and extension at 72° C for 2 min. A final extension at 72° C for 5 min was often included. The volumes and final concentrations are shown in table 1 below.

Table 1: A typical 50 µl volume of PCR reactions.

Reagents	**50µl volume**	**Final concentration**
10 x Taq buffer	5µl	10mM Tris-Cl pH 8.3, 50mMKCl, 1.5mM$MgCl_2$, 0.02%(w/v) gelatin.
100mM dNTP	0.5µl	0.2mM each dNTP
λgt11 forward primer	5µl	1µM
λgt11 reverse primer	5µl	1µM
Taq polymerase	0.5µl	1 Unit
Sterile water	32.7µl	To make up 50 µl
DNA	1µl	

2.7.3 Agarose gel electrophoresis

Standard method was used to separate, identify, and purify amplified DNA fragments through 0.8% and/or 1.0% agarose gel electrophoresis. This technique was mostly performed using Mini-agarose gel unit (model HE-99) of Hoefer Scientific Instruments (San Fransisco) (Plate 4). Ethidium bromide (0.5 μg/ml) was included in the gel matrix. 5μl of PCR product was pipetted out, mixed with a gel loading dye and loaded carefully into the sample wells. BRL's 1Kb ladder was used as marker. Gels were run at 80 Volts for 60 min. The gel was photographed under short wavelength UV light using Polaroid type 667 film on a Polaroid mp-4camera fitted with an orange filter in the dark room.

2.7.3.1: Gel purification

PCR poducts were screened on either low or high melting temperature agarose in Tris acetate buffer (TAE). After electrophoresis, bands were located using a long wavelength (300-360 nm) UV amp to minimise the damage to gene fragment. The bands of interest were cut out as closely as possible using a sharp blade and placed in 1.5 ml tubes. Promega's Wizard purification system was used to recover from the excited bands.

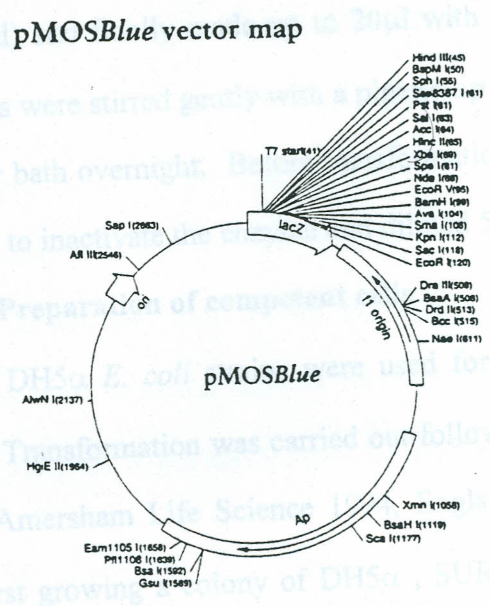
PLATE 3:The Hybaid Thermal Reactor
Hybaid ltd III-113 Waldegrave Road,
Teddigton, Middlesex,TW 99 8LL.
England and COY Thermocycler,
Model 50 Tempcycler Version 'r-1.05'
COY Laboratory Produts INC.

PLATE 4: Miniphor Electrophoresis Unit
Hoeffer Scientific Instruments
San Fransisco, CA 94107
U.S.A.

2.7.4: Direct cloning PCR product

The following PCR products 3-4 and 4-1 were cloned into pMOS-T plasmid vector (Amersham UK)(Fig 6), using T4- ligase enzyme to create recombinant molecules. this cloning vector (pMOS) is a T-plasmid vector which exploits the template-independent activity of thermostable polymerases which preferentially add a single adenosine nucleotide to the 3'end of double stranded DNA. This plasmid is thymidine tailed vector and it has been specifically constructed for direct cloning of the PCR products.

Figure 6: pMOS Blue vector map, It is adapted from pMOS Blue manual book (1997).



T7 promoter primer →

Sal I
Sse8387 I *Hinc* II

lacZ start → T7 promoter *Hind* III *Sph* I *Pst* I *Acc* I *Xba* I *Spe* I

ATGACCATGATTACGCCAAGCTCTAATACGACTCACTATAGGGAAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCTACTAGT

Sma I
Ava I *Ban* II

Nde I *EcoR* V *BamH* I *Kpn* I *Sac* I *EcoR* I

CATATGGATATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCT

↓ T-vector cloning ← U-19mer primer

...tatggatT atcggat...
...ataccta Ttagccta...

pMOS*Blue* Multiple Cloning Region

2.7.4.1: Ligation of the PCR products into pMOS-Blue T-vector.

For each PCR product to be cloned the following ligation reaction were set up, using a ratio of 1:2 (vector:insert respectively). 10μl of DNA to be ligated with vector, 5μl of the vector DNA, 2 μl of 10x ligation buffer (Appendix III) 1μl of T4-DNA ligase enzyme (20 U/ul) and finally made up to 20μl with nuclease free water. Then the ligation reactions were stirred gently with a pipette tip and the tubes were incubated at 16° C in a water bath overnight. Before transformation the ligated mix was heated at 75 ^{0}C for 5 min to inactivate the enzyme and diluted 5- fold with de-ionized water.

2.7.4.2: Preparation of competent cells

SURE ^{TM,} and DH5α *E. coli* strains were used for preparation of competent cells (Appendix 1). Transformation was carried out following the protocol of pMOS-Blue T-vector Kit (Amersham Life Science 1994, England). The competent cells were prepared by first growing a colony of DH5α , SURE ^{TM} or JM109 from LB agar plate, in 5 ml LB broth (with no antibiotics) at 37 ^{o} C overnight with shaking 200 rpm. 500 ml of LB-broth was inoculated with the O/N culture grown at 37^{0} C vigorously shaking at 225 rpm. When the optical density at 600 nm (O. D_{600}) reached 0.5, the cells were chilled on ice for 20 min and transferred to sterile, pre-chilled 50 ml falcon tubes which were then spun for 5 min at 5000 rpm (4°C) in a Hereaus centrifuge. The supernatant was discarded and to the pellet was added one tenth of the volume of (original cultured) of ice-cold solution I (Appendix III) for competent cells. The suspension was incubated on ice for 30 min, with occasional swirling and mixing, using a 1ml pipette. The cells were centrifuged again at 5000rpm at 4° C for 5min and the pellet was re-suspended gently in 5 ml (one twentieth of the original volume) of ice-cold solution II for competent cells. The cell suspension was aliquated into 200μl fractions into sterile prechilled 0.5 ml Eppendorf tubes and stored frozen at -70 ^{o} C until use.

2.7.4.3: Transformation into *E. coli*

Frozen *E. coli* competent cells were thawed on ice and mixed to evenly suspend the cells . 5µl of ligated mix was added directly to 200 µl competent cells by gentle mixing. The mixture was allowed to stand on ice for 30 min, after which the cells were heat shocked by placing the tubes in at 42° C water bath for 90 sec and then placing on ice for 60 sec. 200µl of SOC medium (Appendix III) was then added and the cells incubated at 37° C shaking with 200 rpm, for 1 hr to allow the cells to recover. To concentrate the cells before plating them, the culture was micro-centrifuged for 30 sec and most of the supernatant poured, leaving 150 µl. After resuspension, the transformation mixture was plated out on LB-agar plates containing 50 µg/ml amp, 50µg/ml X-gal and 0.5mM IPTG. Plates were incubated at 37 ^{0} C for 16 hrs.

2.7.5: Screening of the recombinants

The transformants were examined after 16 hrs of incubation and positive and negative transformants were observed in different colours (White and Blue). These colonies were screened for their presence of insert in the true positive transformants by direct colony PCR and by standard plasmid mini-preparation followed by restriction enzymes analysis.

2.7.5.1 Plasmid DNA extraction

Three millilitre of LB broth containing 100 µg/ml ampicillin was inoculated with one white bacterial colony from the plate and the tube was incubated at the 37 ^{o}C shaker overnight. Culture cells were poured into Eppendorf tubes and microfuged for 1min. The supernatant was poured off and pellet re-suspended with 100µl of lysis buffer (4mg/ml lysozyme in 50 mM glucose, 10mM EDTA, 25mM Tris-Cl). The tubes were

thoroughly vortexed and left for 5 min at room temperature. After that 200µl freshly prepared solution II (0.2 N NaOH/1% SDS) was added at room temperature, inverted gently to mix, and incubated 30 minutes on ice. 200µl of 3M potassium acetate pH 4.8 was then added and mixed by inversion before placing on ice for 30 min. The suspension was microfuged for 5min and the supernatant recovered in a fresh Eppendorf tubes. Samples were then extracted, once with Phenol- Chloroform and the DNA precipitated with absolute ethyl alcohol. The dry DNA pellets were re-suspended in 50µl 1xTE (10mM Tris-HCl, pH. 8.0, 1mM EDTA) and to soften the pellet they were incubated at 37 °C for 15min and vortexed.

2.7.5.2: Rapid direct colony screening

A recombinant pMOS-plasmid colony was picked from a plate containing the transformants using a sterile tooth pick and transferred to a tube containing 50 µl of sterile water. After vortexing, the tube was placed in boiling water for 5 min, to lyse the cells and denature DNAses. The suspension was then centrifuged at 12,000 g for 1 min to remove cell debris. 10 µl of the supernatant was used as PCR template.

2.7.5.3: Restriction enzyme analysis

To cut the cloned inserts out of the cloning site. **Pst I** and **Sac I** restriction enzymes were digested with the pMOS DNA. 20µl of digestion reaction was set up containing 1Unit of each of the enzymes (New Eng. Bio-Lab), 1X of the reaction buffers (NEB 3), and the final volume was made up to 20µl with water. The samples were incubated at 37^0 C for 12 hours. Then, the digestion enzymes were inactivated by incubating at 70° C for 5 minutes.

2.7.6: Phenol chloroform extraction

An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to sample DNA, which was then vortexed vigorously and microfuged for 5min to separate phases. Most of the aqueous phase (top layer) was pipetted into a new Eppendorf tube,(the interface was avoided)and an equal volume of Chloroform was added . After mix, the tube was microfuged for 2min. The top aqueous layer was placed into a new tube, and residual chloroform was allowed to evaporate off at 37 ° C incubator for 15 min.

2.7.6.1: Ethanol precipitation of the DNA

After estimating the volume of the DNA solution, twice its volume of ice- cold ethanol was added, mixed well and chilled at -70° C for 30 min. DNA was recovered by centrifugation at 14,000g for 10 min. Supernatant was carefully removed and the pellet was completely dried. The pellet was then washed with 70% ethanol for 2 min at 14,000. The DNA pellet was dissolved in the desired volume of Tris-EDTA buffer and incubated to 37^0 C for 5 min to assist in dissolving the pellets.

2.8: cDNA Clone sequencing.

Two pMOS-T clones were partially sequenced according to the Dideoxy nucleotide chain termination method of Sanger *et al.,(* 1977). Plasmid DNA was prepared as discussed in section (2.7.5.1). About 3 µl of this DNA was then used as the template in the sequencing reactions. The sequenase™ kit (USB) was used for sequencing single stranded DNA after denaturing the extracted recombinant pMOS DNA at 94° C.

2.8.1: Single stranded pMOS template sequencing.

To 7 µl of ssDNA in a 0.5 ml microfuge tube, 2µl of 5x sequenase buffer (200 mM

Tri-HCl, pH 7.5, 100mM Mgcl$_2$, 25O mM NaCl) and 1 μl of M13 - 40 primer [Appedix D (0.5 pmole)] was added. Annealing of the primer was done by heating the 10 μl reaction for 2 minutes at 65° C, then cooled slowly to below 35° C and placed on ice. While the annealing reaction was incubating, the extension/termination reaction was prepared. The labelling reaction reagents were added into the annealed DNA while on ice, 1 μl of 0.1m DTT, 2μl of diluted labelling mix, (1.5 μm dGTP, 1.5μm dCTP, 1.5 μm dTTP), O.5μl of [^{35}s] dATP (1Oμ Ci/μl, 1398 Ci/mmol, Dupont) and 2μl (3.2 units) of 1:8 diluted sequenase (T7 polymerase) (diluted in 10mM Tri-HCL, pH 7.5, 5 mM DTT, 0.5 mg/ml BSA, to make about 1.6 units /μl of sequenase). The labelling reaction was left a room temperature for 5 minutes. Then four termination reactions tubes labelled G, A, T, and C with 2.5μl of the ddGTP termination mix added into the tube labelled G and similarly 2.5μl of ddATP, ddCTP, ddTTP was added to the other respective tubes and placed for a minute at 37° C. There after 3.5μl of the primer/labelling mix were added to each of the tubes with ddNTPs and incubated at 37° C for 5 minutes. Some 4μl of the stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol ff) was added to the termination reactions and mixed thoroughly. The sequenase reactions were stored at - 20° C.

2.8.2 Preparing of sequencing gel:

The sequencing gel was cast in either IBI or Bio-Rad sequencing apparatus. The glass plates were through cleaned in warm water and rinsed then with distilled water. The surface was cleaned with 95% ETOH and dried with lint-free towels. The gel mold was assembled using the two 35x43 cm glass plates with 0.4 mm spacers A liquid-tight seal was essential by taping the plates together and clipping. 6% gels were made form 40% acrylamide stock (acrylamide: bis acrylamide ratio 19:1) by pipeting 15 ml

into a 25Oml beaker. To the beaker, 50g of urea (to make 8M) and 10ml of 10x TBE were added. After dissolution of the urea, the volume was made up to 100ml with distilled deionized water. The acrylamide was then filtered through a Whatman 3MM paper. 350µl of 10% ammonium per sulphate and 30 µl of TEMED was then added to the acrylamide, which was poured into the space between the glass plates held at an angle of 45° C. The sharks tooth comb was then inserted with the teeth facing outwards. The combs and plates were then clamped with several clips, and the gel left to polymerise in horizontal position overnight (16 hours).

2.8.3: Gel loading and running.

After polymerization, the comb and the bottom spacer were removed and the gel sandwich assembled on the sequencing apparatus. Both upper and lower chambers were filled with 1x TBE and the wells flushed using a syringe and needle to wash away, any unpolymerized acrylamide, urea, which had diffused out into wells. Electrophoresis was carried out at 70W using an EC 600™ power pack. The gel was pre run for 45 minutes until the temperature was 55 °C before loading 3 µl of the sequencing reactions, which had just been denatured at 72°C for 2 minutes. All the wells were loaded as quickly as possible to prevent possible renaturation and diffusion of samples.

2.8.4 Drying and autoradiography of the gels

At the end of electrophoresis, one glass plate was carefully lifted off. A 3MM Whatman paper was then placed on the gel, and the gel peeled from the second glass plate. The gel on the Whatman paper was covered with saran wrap and dried at 80° C under vacuum for 1 hour using the Savant ᵗᵐ gel drier. The saran wrap was then peeled off the gel and then dried gel placed in the metal cassette with an x-ray

(Kodak) film in contact with it. The cassette was then kept at room temperature in the dark, the X-ray film was developed after 24 hours.

CHAPTER THREE

3.0: RESULTS

3.1: Membrane protein extraction and characterization

Schistosoma mansoni adult worms were recovered by perfusion of Balb/c mice at the Institute of primate research (IPR) Nairobi. Then membrane proteins were isolated adopting two different methods of. *S mansoni* surface protein extraction. The use of high concentrated salt (0.75 mM NaCl in PBS pH 4.5) provided a convenient method of extraction of loosely-bound surface membrane proteins. Use of Triton-X 100 detergent after this initial stripping off of peripheral proteins by salt results in extraction of integral membrane proteins in the membrane lipid bi-layer. A modified method by Vasconcelos *et al*., (1993) was used in extracting the membrane proteins. The remaining worm pellet was also homogenized (Table 2). Extracted *S. mansoni* membrane proteins were separated according to their molecular sizes by SDS-PAGE as described by Laemmli, (1970). Protein bands ranging in size from 84 KDa to 26KDa were observed. The peripheral proteins had more bands in contrast to tegument samples, but Triton-X 100 extracts showed less bands compared to the extracts obtained by Vasconcelos method. All tegument samples had not apparent protein bands smaller than 26KDa (Plate 5). The band sizes of the unknown proteins were compared with a standard protein marker. Rabbit anti-membrane sera (RAMS) was raised in two rabbits and pooled from both animals. Then sera was pre-adsorbed with *E.coli* lysate and its immuno-reactivity was evaluated by Western blot analysis using the various extracts as antigens. Electro-blotting on to nitrocellulose filter paper (Scheiler&Schull 0.5μm pore size) was carried out using Hoefer (San Fransisco CA, USA,) semidry blotter according to the instructions of the supplier (50 mA for 60 min). The gel showed complete transfer after staining with Commasie Blue stain.

Immuno-bands greater than 26 kDa were present in all samples. Most extracted fractions showed one or two conspicuous bands. The tegument fractions had major bands of Mr approximately 84 kDa and 26 kDa. These were not conspicuous in the protein washes of the worms stripped of their peripheral proteins. The peripheral protein extract had prominent antigens of approximately Mr 26, 36, 58, 84, 116 and 130 k Da. Unlike teguments, peripheral proteins showed conspicuous complex bands like Mr 20-26 kDa and 58-84 kDa and antigens less than 20 kDa were not conspicuous (Plate 6). A quick analysis of the sera was done of blotting crude extracts directly on to the nitrocellulose papers (Plate 7).

Table 2: Sequential extraction of proteins from *S. mansoni*

Extractant	*No. of worms*	*Volume of extractant*	*Volume of extract*	*Protein [conc] extracts (mg/ml)*
High salt	1000	4 ml	3 ml	0.09
Triton-X 100	1000	3 ml	2 ml	0.16
Vasconcelo's method	1000	5 ml	4 ml	0.22
Homogenate	1000	4 ml	4 ml	0.49

The first column of the Table shows different methods used in the extraction of worms. For the first batch of worms, high salt and Triton-X 100 was used sequentially. The next batch of worms was extracted using the vasconcelos method. Stripped, extracted worms were then homogenized.

3.1.1: Bovine serum albumin standard curve

A bovine serum albumin (BSA) standard curve (Fig 7) was used to determine the protein concentration of the extracted material. Protein concentrations of unknown samples were read from this standard curve (The linear part), are shown in table 3.

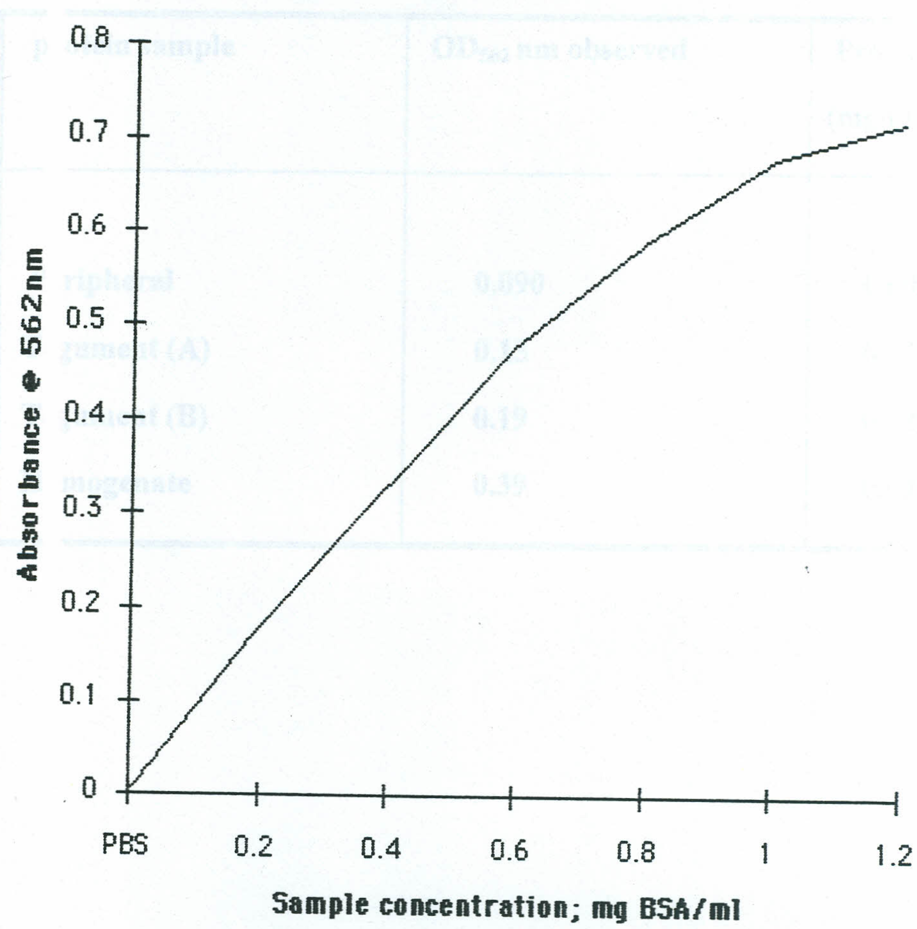
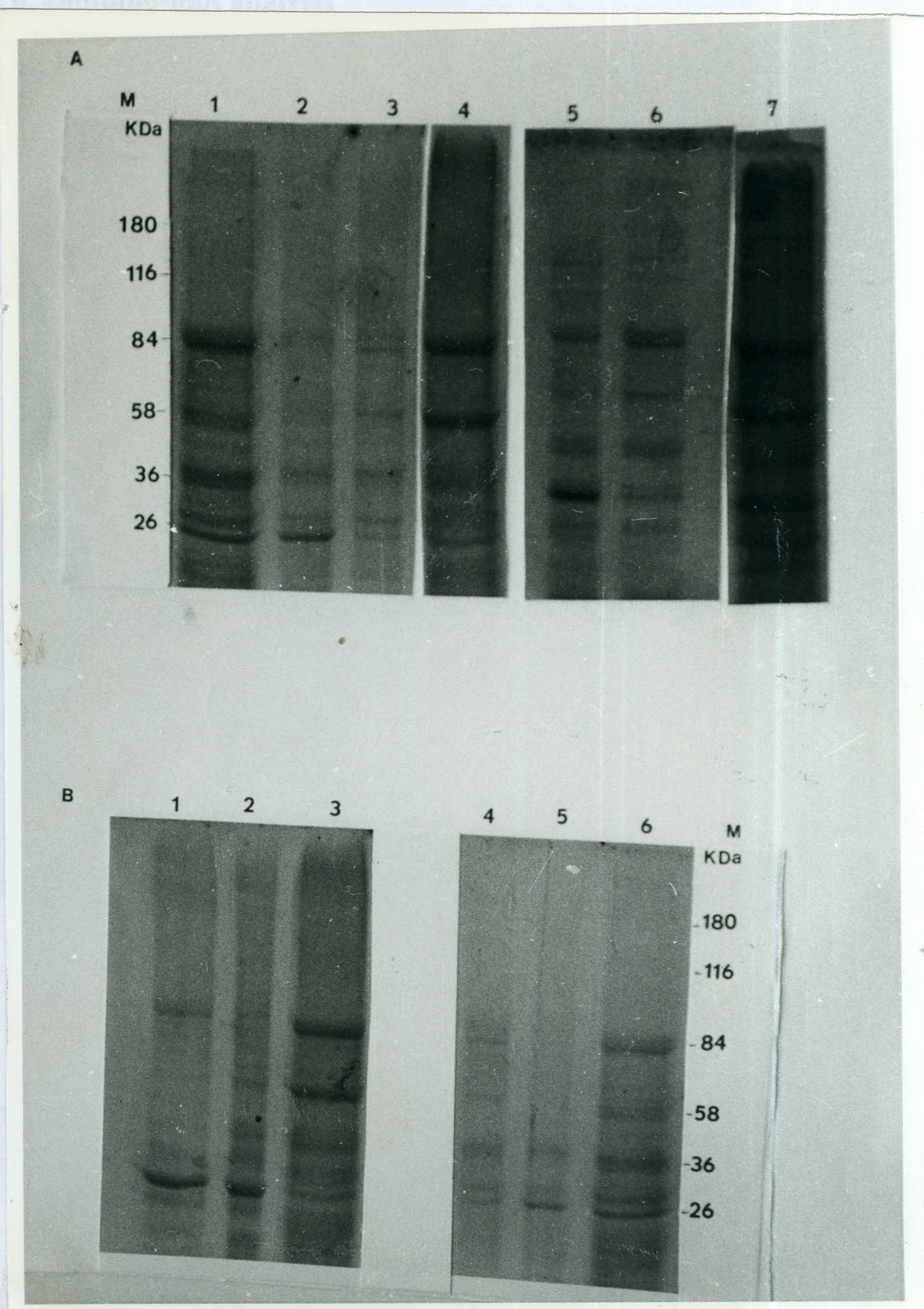


Figure 7: Bovine serum albumin (BSA) standard curve.

Table 3: Concentration of soluble proteins at 37^0 C for 30 min

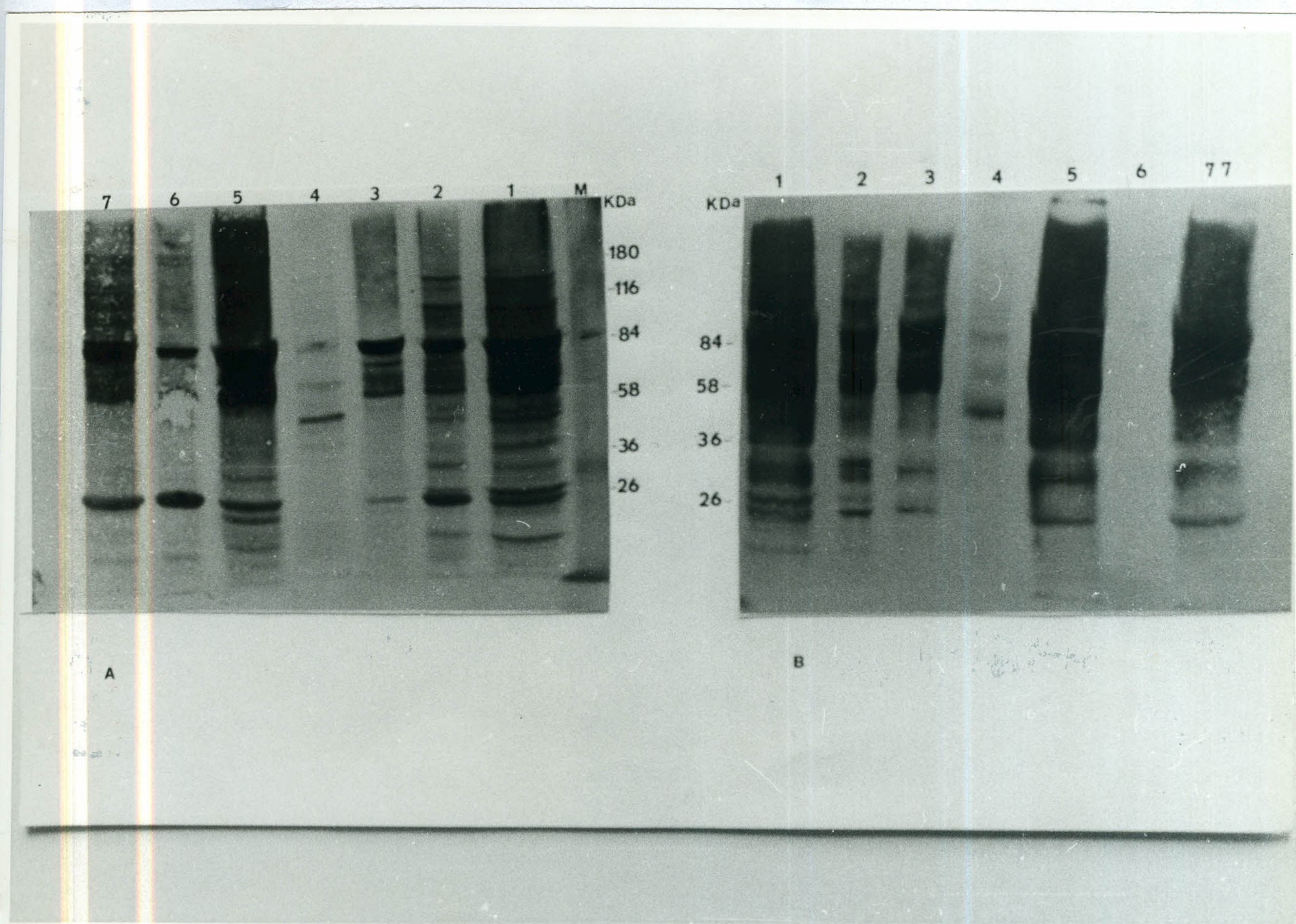
protein sample	OD_{562} nm observed	Protein concentration (mg/ml)
Peripheral	0.090	0.09
Tegument (A)	0.15	0.16
Tegument (B)	0.19	0.22
Homogenate	0.39	0.49

Plate 5: Electrophoretic analysis of the worm proteins



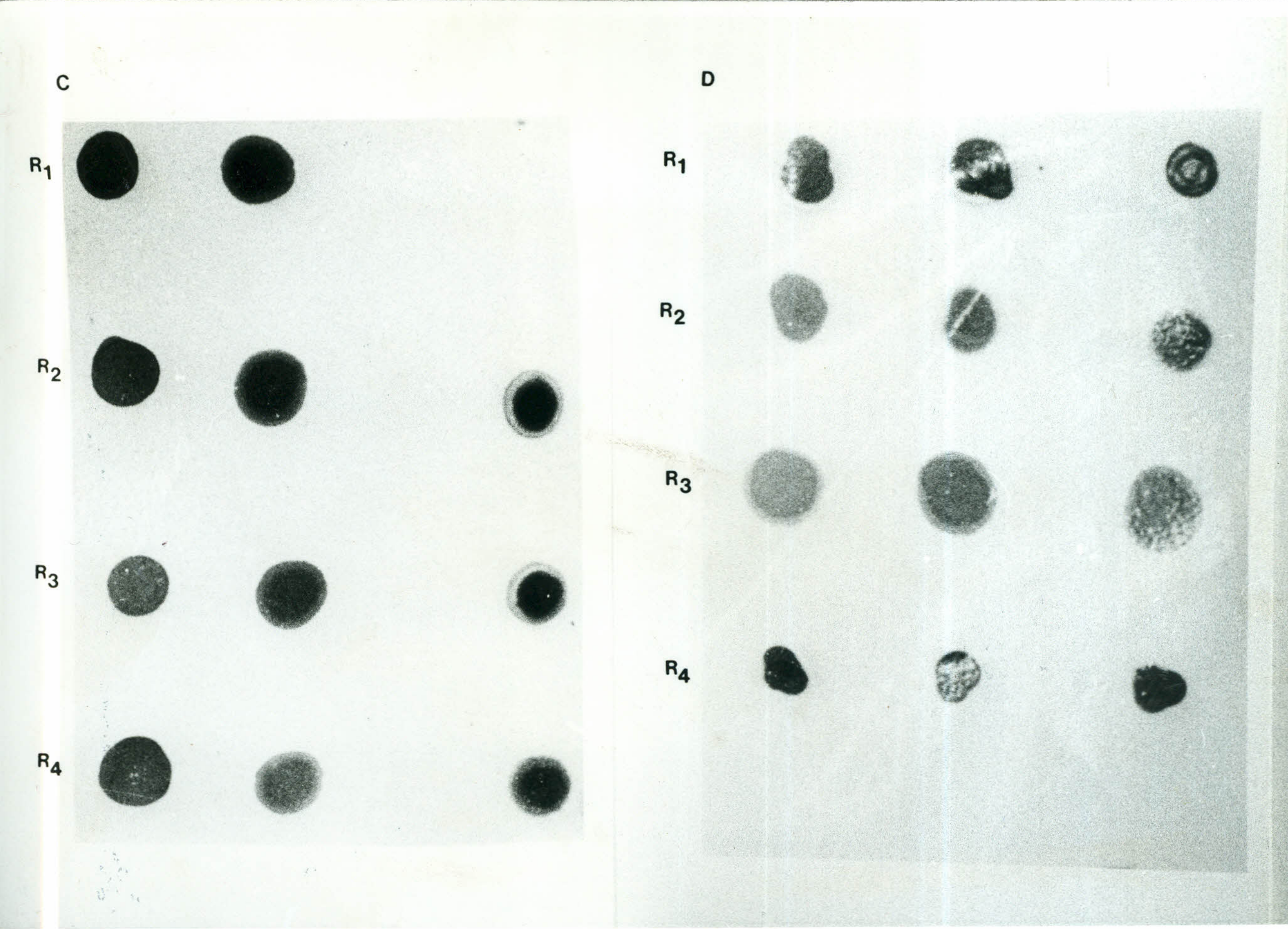
Each lane was loaded with 35µl of protein sample containing 1x sample buffer. **A:** M; protein marker, Lane1; peripheral proteins, Lane 2; tegument (a), Lane 3; tegument (b), Lane4; homogenate of the worm, lane5; tegument (a), lane6; tegument (b) and lane 7; Bacteria lysate. **B:** lane1; tegument (a), lane 2; tegument (b), lane 3; homogenate, lane4; tegument (b), lane5; tegument (a), lane 6; peripheral, M; protein marker.

Plate 6: Immuno-blot analysis



A: M; Protein marker, Lane1; peripheral, lane2; tegument (a), lane3; tegument (b), lane4; washes, lane5; homogenate, Lane 6 tegument (a)., lane7; tegument (b) ; **B:** M; Protein marker, lane1; peripheral proteins, Lane 2; tegument (b), lane3; tegument (a), lane4; washes, lane5; homogenate, lane 6; *E.coli* lysate lane7; homogenate

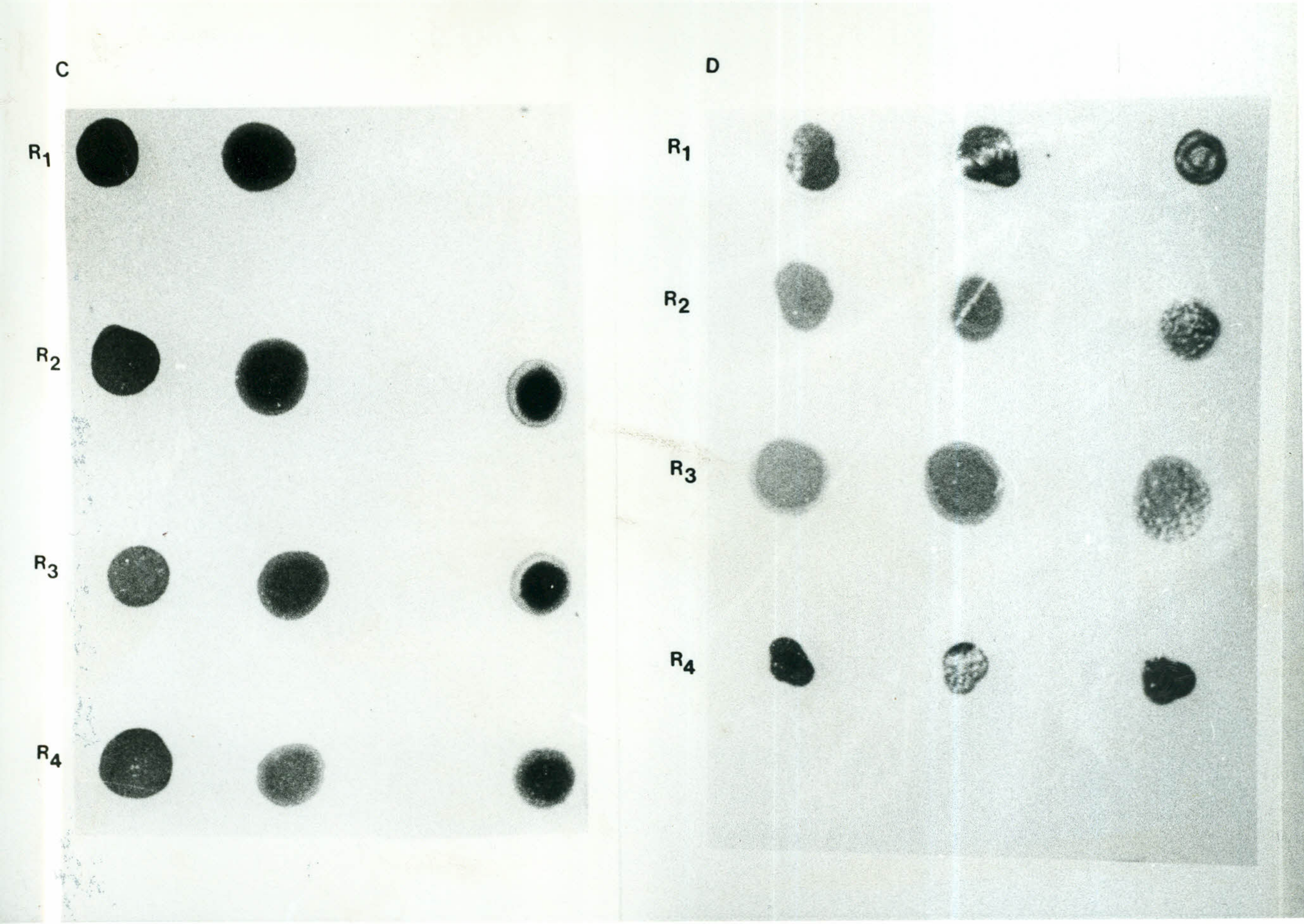
Plate 7: Dot blot analysis



This plate shows that a quick test of that rabbit anti membrane sera reacts against *S. mansoni* extracts spotted on two separate nitrocellulose filter papers. **C** ; Row one (R_1) contained 1 µl of (1:2) peripheral proteins with TBS buffer, R_2 up-to R_4 ; contain 1 µl dots of same dilution tegument extracts.

D; Row one (R_1) contained 1 µl of (1:5) peripheral proteins with TBS buffer, R_2 up-to R_4 ; contain (1:5) dots of tegument extracts.

Plate 7: Dot blot analysis



This plate shows that a quick test of that rabbit anti membrane sera reacts against *S. mansoni* extracts spotted on two separate nitrocellulose filter papers. **C** ; Row one (R_1) contained 1µl of (1:2) peripheral proteins with TBS buffer, R_2 up-to R_4 ; contain 1µl dots of same dilution tegument extracts.

D; Row one (R_1) contained 1µl of (1:5) peripheral proteins with TBS buffer, R_2 up-to R_4 ; contain (1:5) dots of tegument extracts.

3.2: Screening of adult *S.haematobium* cDNA library

To determine the titre of plaque forming units (pfu)/μl, the number of plaques obtained from each plate were counted and this number was divided by the volume of the phage plated (in μl) and multiplied by the respective dilution factor. The average titre of the cDNA library was 4.6×10^6 pfu/μl and that calculation was deduced, after pfu count per plate as shown on the Table 4. The average of the plate 2 and 3 was calculated and used as the titre of the library. $1.2 \times 10^6 + 8 \times 10^6 /2$ and $9.2 \times 10^6 /2 = 4.6 \times 10^6$. In the primary immunoscreening, five plates were used designated 1, 2, 3, 4 and 5. Thus the plaques picked from plates were given a series numbers for example, Sh 1-1 stands for *Schistosoma haematobium (Sh)* clone one from plate one. The following were picked from the primary screening: *S h* 1-1, *S h* 1-2, *S h* 2-1, *S h* 2-2, *S h* 3-1, *S h* 3-2, *S h* 3-3, *S h* 3-4, *S h* 4-1, *S h* 4-2, *S h* 4-3, *S h* 5-1, *S h* 5-2, *S h* 5-3, and *S h* 5-4. All 15 primary clones were rescreened (2^0 screening) using 15 small (90mm) petridishes. Clones *S h* 3-1, *S h* 3-2, *S h* 4-2 and *S h*5-2 which gave no signal but the remaining eleven were positive. The third round of screening eliminated three more clones. 27 clones were picked from the tertiary screening and stored in SM buffer under chloroform (Table 5).

Table 4: Plaque forming units (pfu) count and titre

Plate	Dilution factor	Pfu count/plate	Calculated pfu/μl
1	10^{-4}	Confluent	Not counted
2	10^{-5}	1200	1.2×10^{6}
3	10^{-6}	800	8×10^{6}
4	10^{-7}	200	0.2×10^{6}
5	10^{-8}	60	0.6×10^{7}

Plate 8: Isolation of the *S.haematobium* specific cDNAs.

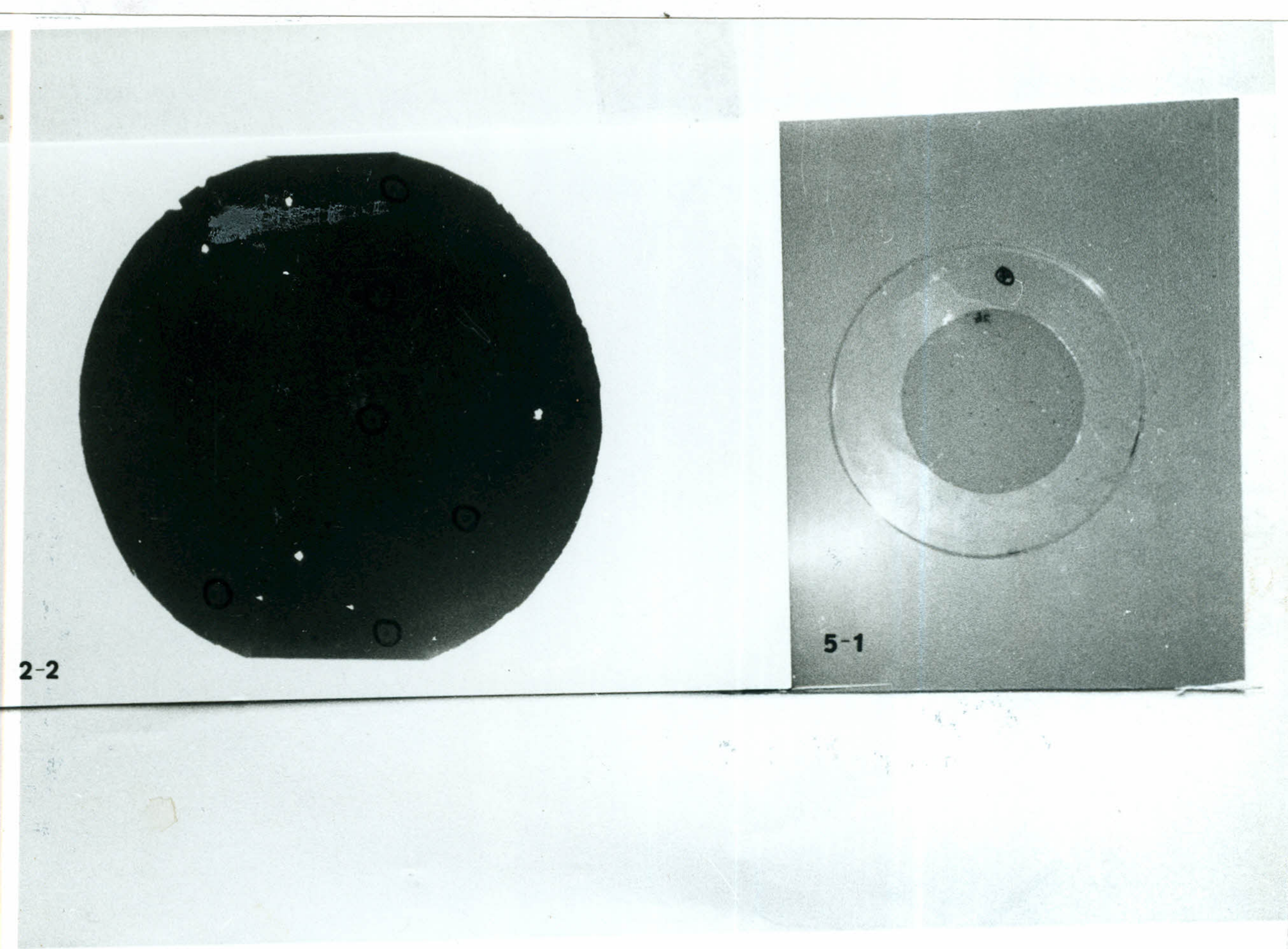


Plate 3.4 show secondary and tertiary screens of the primary clones plated on 90-mm size to produce single well-isolated plaques. Several clones were picked on each. **Clone 2-1**: This primary clone was replated and screened in a secondary screening. The plaques were clearly separated. The circled clones were picked for tertiary screening. The white dot marks show the points for orientation of the nitro-cellulose filters with respect to the agar plates. **Clone 5-1:** A tertiary round was screened this clone from 2^0 plate of 5-1.Well separated tertiary clones were picked and stored in phage storage buffer under chloroform.

Table 5: Isolation of specific Sh cDNAs encoding tegument antigens after three rounds of screening.

Plate	Primary clones	Secondary clone	Tertiary clones
1	Sh 1-1, Sh 1-2	Sh 1-1, Sh 1-2	Sh 1-1, Sh 1-2
2	Sh 2-1, Sh 2-2	Sh 2-1, Sh 2-2	Sh 2-2
3	Sh3-1, Sh3-2, Sh3-3, Sh 3-4	Sh 3-3, Sh 3-4	Sh 3-4
4	Sh4-1, Sh4-2, Sh4-3	Sh 4-1, Sh 4-3	Sh 4-1, Sh 4-3
5	Sh5-1, Sh5-2, Sh5-3, Sh 5-4	Sh 5-1, Sh 5-3, Sh 5-4	Sh 5-1, Sh 5-3

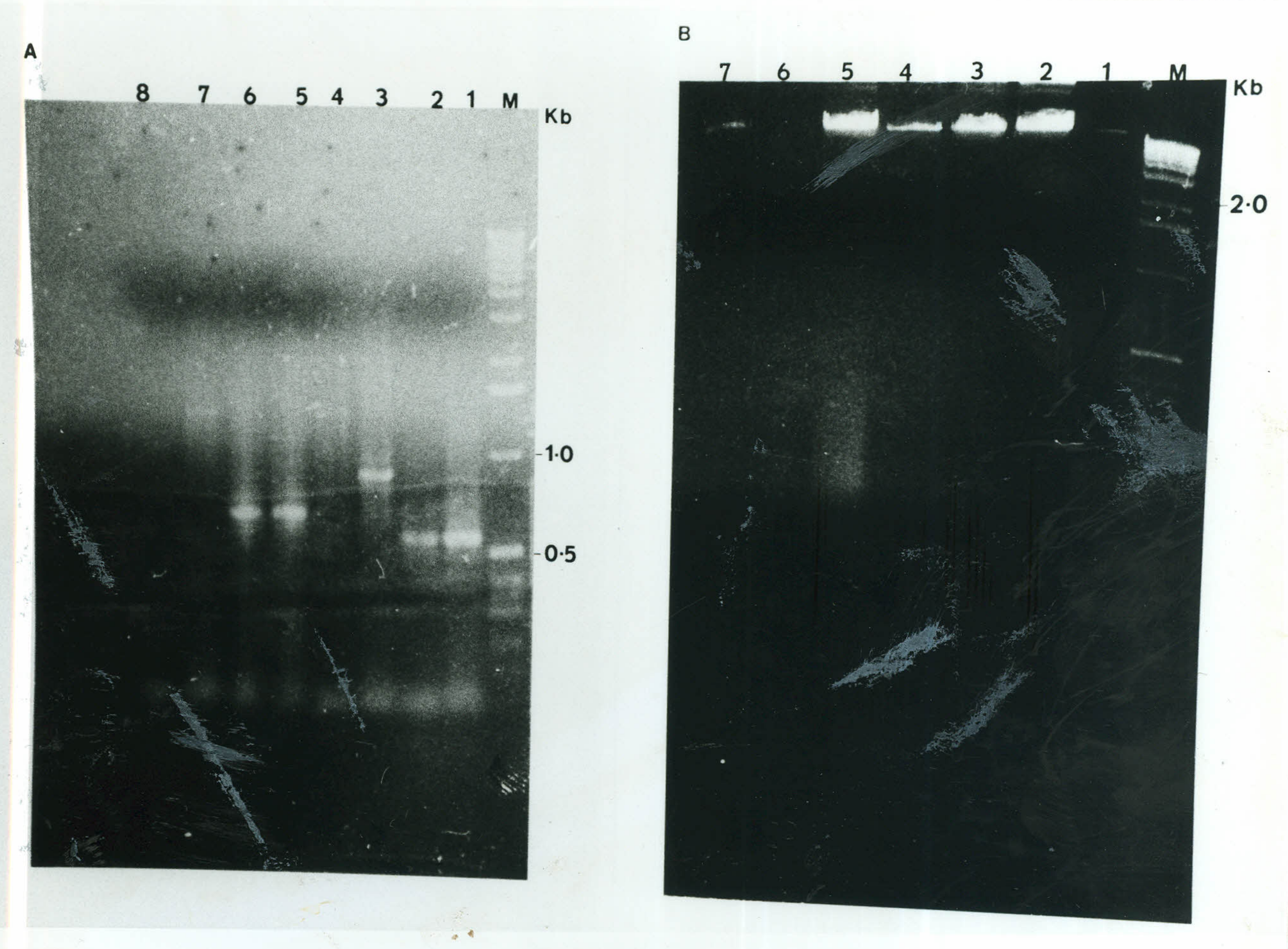
The results of immunoscreening are summarised in table 5.

3.3: CHARACTERISATION OF POSITIVE CLONES

3.3.1: Polymerase chain reaction

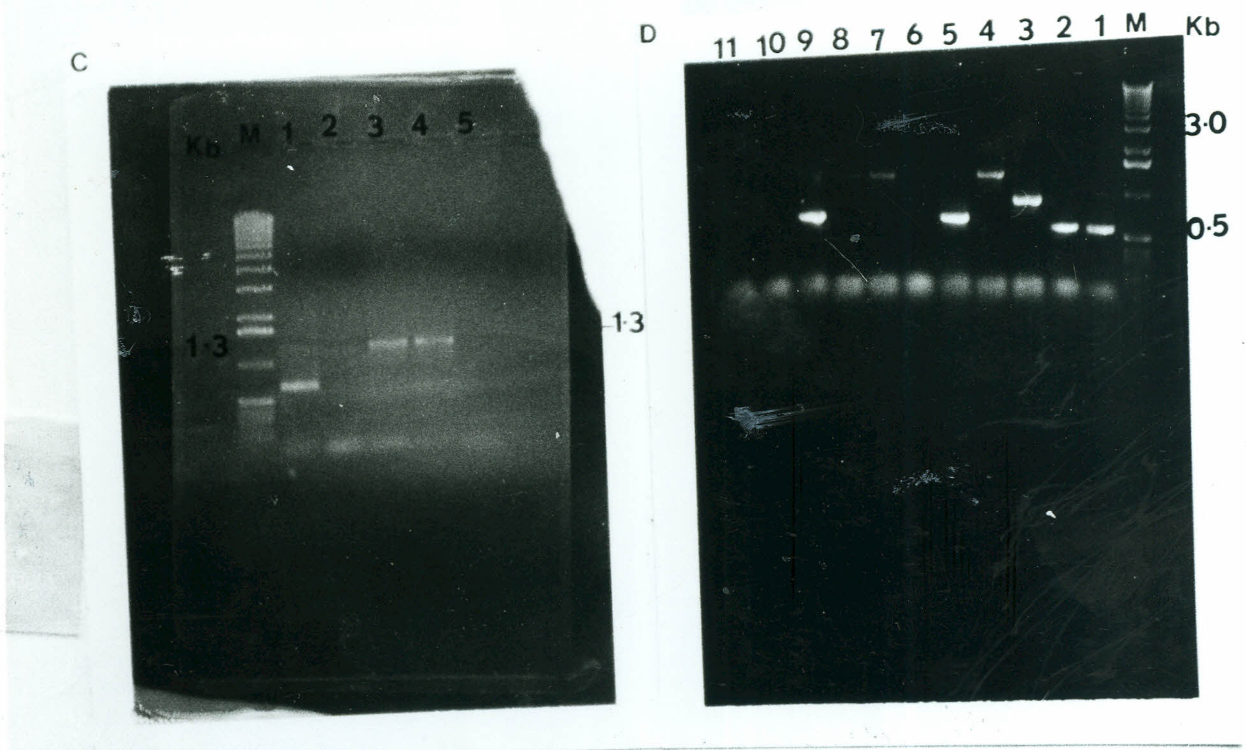
The purified positive clones from the immuno-screening were checked for the presence of an insert by the polymerase chain reaction using either phage lysate or pure extracted lambda DNA (Plate 9). The initial PCR reactions on the lysate produced no amplification. The reaction was optimised and several PCR Products of different sizes were generated with Phage lysates and Pure DNA extracts. The PCR products range from 400bp-1.3kb. All the lambda gt11 clones contained foreign DNA fragments. None of the isolated clones was found to be false positive and they were grouped into five classes according to their sizes. When three lambda DNAs from same plate i.e. 2-2a, 2-2b, 2-2c subjected to polymerase chain reaction (PCR), two of the clones (2-2b and 2-2c) produced 1.3Kb insert sizes, while the other one (2-2a) did not give any amplification. These two (1.3 Kb) fragments were the first two inserts observed (Plate 10). Eighteen lambda samples selected from 27 tertiary clones were screened with PCR. On analysis of the PCR products on a 0.8% agarose gel it was seen that most clones amplified (plate 12). In addition, all cDNA inserts generated fall between 0.5Kb-1.3Kb in size. Rapid lambda DNA extraction was carried out on phage lysates grown on LB-agarose plates containing lawn of *E. coli* cells of strain Y1090 (Plate 9b). The lambda DNA of the clones Sh 1-1, Sh 2-2, Sh 3-4, Sh 4-1, Sh 5-3, Sh 5-4, Sh 5-2, Sh 5-1 were extracted. Lambda genomic DNA was digested with EcoRI enzyme to release the insert. No insert was seen after electrophoresis of the digest on 1% agarose gel (Plate 11). However, lane1 of Plate 11b, had faint band on the gel.

Plate 9: PCR on the phage lysate & lambda DNA extraction



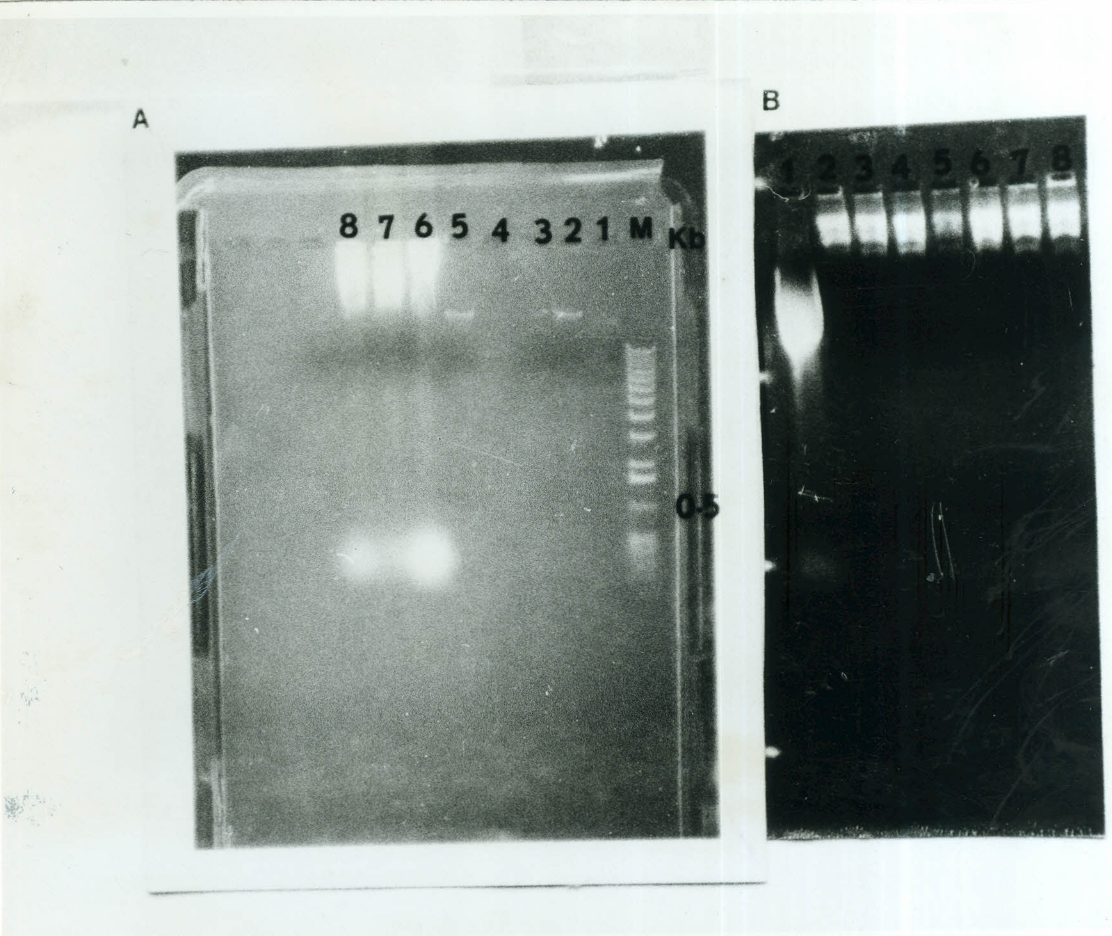
A: PCR was carried out using 5µl of phage lysate. Then 5µl of the PCR products were analysed on agarose gel. M; 1Kb DNA molecular markers, lane 1; Clone 3-4, lane 2; Clone4–3, lane 3; Clone4–1, lane 4; Clone2 –2, lane 5; Clone1-1, lane 6; Clone1–2, lane 7; Clone 5–1, lane 8; negative control (no template added). **B:** Lambda gt11 DNAs isolated from the clones were examined on 1% agarose gel. M; 1Kb DNA molecular marker, lane 1; Clone1-1, lane 2; Clone1–2, lane 3; Clone2–2, lane 4; Clone3–4, lane 5; Clone4–1, lane 6; Clone4– 3, lane 7; Clone5– 1.

Plate 10: PCR of the lambda DNA



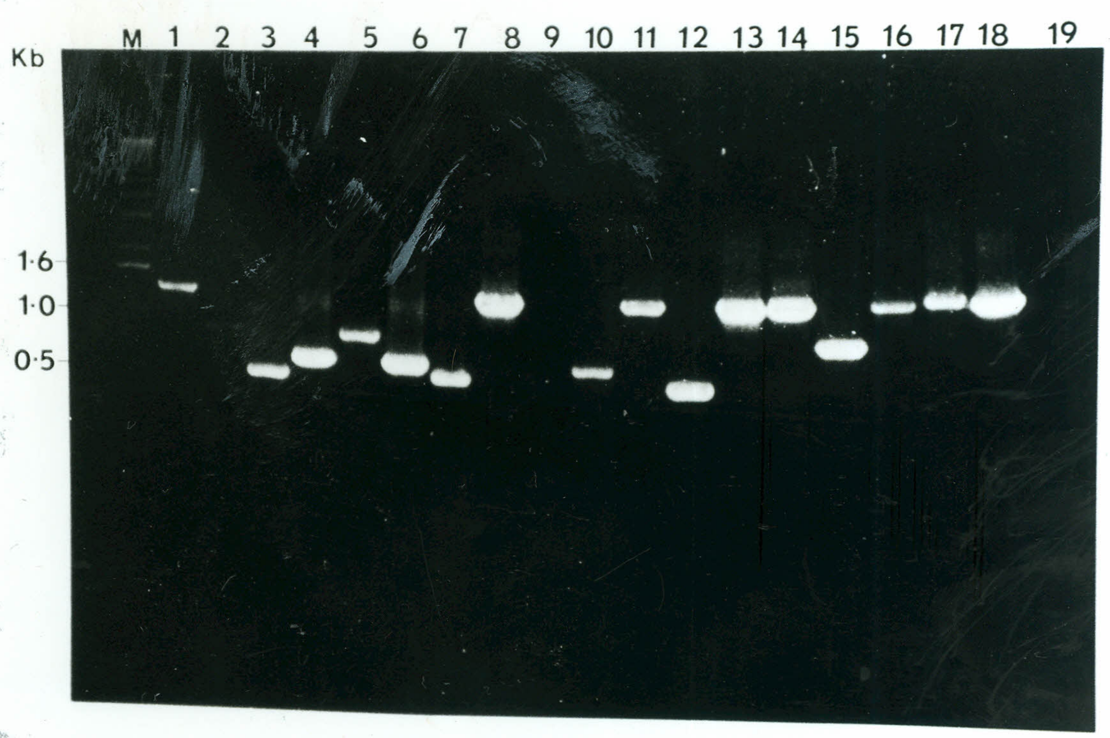
C: Analogous lambda DNAs from plate two i.e. Clone2-2a, Clone2-2b, Clone2-2c were subjected to polymerase chain reaction (PCR) and 5µl of the product was then analysed. M; 1Kb DNAmarker, Lane 1; Known lambda gt11 (clone carrying 700bp insert) for positive control. lane2; Clone2-2a , lane 3; Clone2-2b, lane 4; Clone2-2c , lane 5; negative control (no template added). In **D:** Ten amplified lambda DNAs were analysed on agarose gel. M; 1Kb DNA molecular marker, lane1; Clone3-4a, lane2; Clone4-3, lane3; Clone4-1, lane4; clone2-2a, lane5; Clone1-1, lane6; Clone3-4b, lane7; Clone5-1, lane8; Clone5-3, lane9; Clone1-2, lane10; Clone2-2b, lane11; negative control (no template added).

Plate 11: Analysis of EcoR1 digests of the lambda clones



To release inserts, eight lambda DNAs digested with EcoR1 were analysed in agarose gel. **A:** 10 µl of DNA was digested and the entire digests was analysed. M; 1Kb DNA molecular marker, lane1; Clone1-1, lane2; Clone1-2, lane3; Clone2-2a, lane4; Clone2-2b, lane5; Clone3-4, lane6; clone4-1, lane7; Clone4-3, lane8; Clone5-3. In **B:** 20µl of the DNA was digested and complete of the digests was examined. M; 1Kb DNA molecular markers, lane1; clone1-1, lane2; Clone1-2, lane3; Clone2-2a, lane4; Clone2-2b, lane5; Clone3-4, lane6; Clone4-1, lane7; Clone4-3, lane8; Clone5-3.

Plate 12: Screen lambda DNAs with PCR



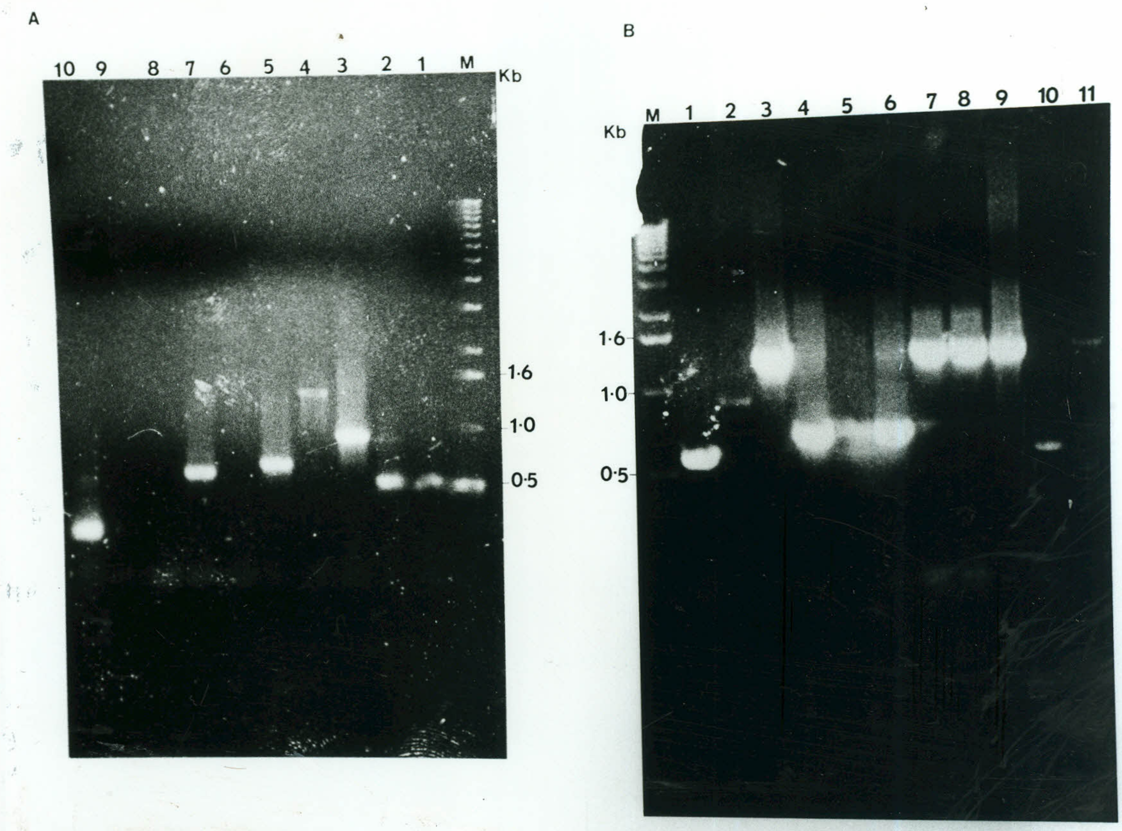
This plate shows a number of lambda DNAs were prepared from the clones and screened with PCR for their presence of inserts. M; 1Kb DNA molecular markers, lane1; Clone 2-2a, lane2; Clone 2-2b, lane3; clone 3-4a, lane 4; Clone 1-1a, lane 5; Clone 4-1a, lane6; Clone 1-2a, lane 7; Clone 3-4b, lane 8; Clone 4-3a; lane 9; Clone 4-3b, lane 10; Clone 1-2b, lane 11; Clone 2-2c, lane 12; Clone 1-1b, lane 13; Clone 2-2d, lane 14; Clone 2-2e, lane 15; Clone 4 -1b, lane 16; clone 5-1a, lane 17; Clone 5-1b, lane 18; Clone 5-3a, lane 19; negative control (no template added).

3.3.2: Sub cloning of the cDNA inserts into vectors

3.3.2.1: Preparation inserts for cloning.

Lambda phage DNA did not release good insert bands that could be excised and purified but that opportunity was provided by PCR bands recovered from the agarose gel and PCR products purified of (Plate 12). These DNA fragments recovered from agarose gel (Plate 13b) were used for cloning. Phenol-chloroform extractions were observed as a time consuming method and typically resulted in some loss of DNA at the extraction and subsequent ethanol precipitation steps. Attempts to clone inserts prepared by EcoRI digestion of lambda DNA and PCR products into EcoRI cut M13 mp18/19 bacteriophage or PUC18/19 plasmid vectors failed. Numerous white plaques/colonies were observed on the agar plates. Particularly M13 plates. When replicative form (RF) of M13 bacteriophage DNA was analysed for the cloned inserts, it gave false positives. Several attempts to clone these inserts into the EcoR1 site of pRSET A, B, C, expression plasmid vectors (Invitrogen, USA) were also made following. Transformation of the resultant recombinant vectors into *E. coli* JM109, XL–1 blue competent cells was performed. The positive (white) transformants could not produce single expected bands on PCR using T7 and T3 primers as well as EcoRI restriction enzyme analysis. It has not been explained the multiple bands observed (not shown). It was possible that there could have been non-spacific or mismatched priming of regions of the pRSET vectors.

Plate 13 : Recovery of DNAs from PCR products and agarose gel



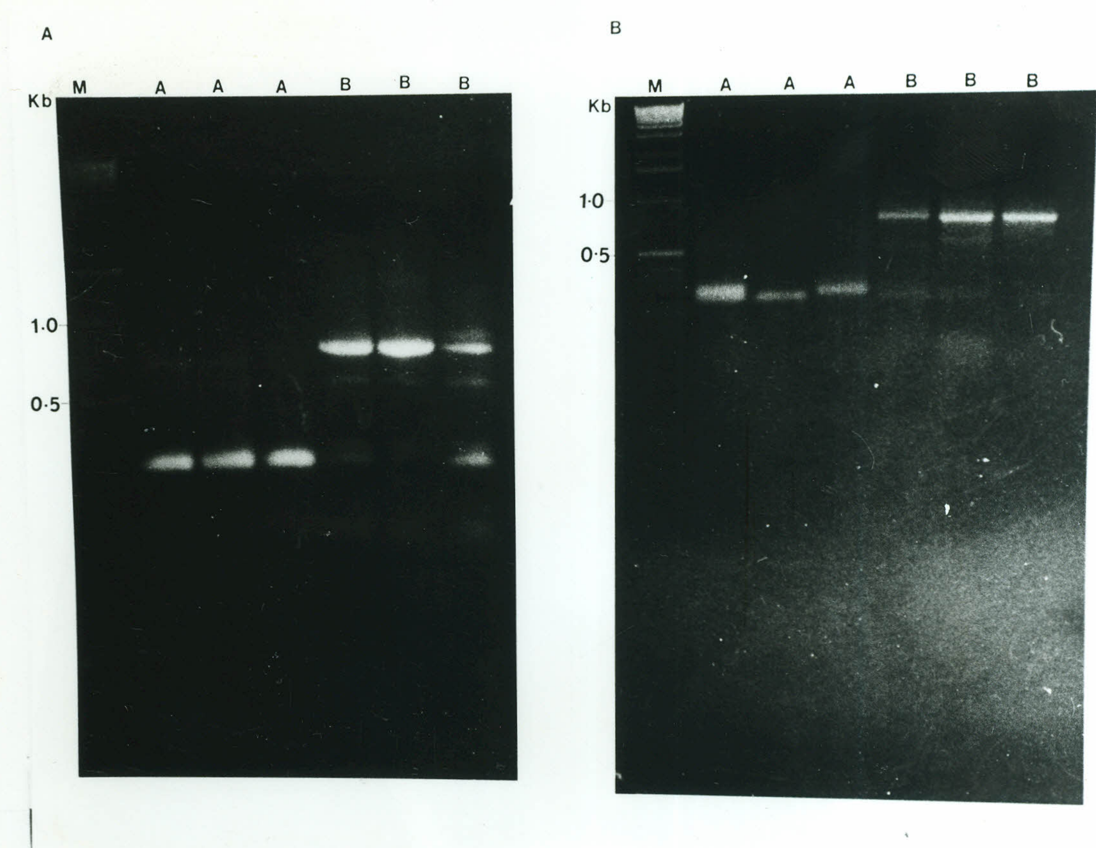
A. The remaining PCR products were purified from the impurities of the PCR reagents using Promega Wizard purification. 5µl of the DNA was loaded on the gel. M; 1Kb DNA molecular markers, lane1; Clone 3-4a, lane2; Clone 4-3, lane3; Clone 4-1, lane4; Clone 2-2a, lane5; Clone 1-1, lane6; Clone 2-2b, lane7; clone 1-2, lane8; Clone 5-1,lane9; Clone 5-3, lane10; clone 3-4b. **B:** Ten gene bands from the figure3.8 were cleaned using Promega Wizard purification system. M; 1Kb DNA molecular markers; lane1; Clone 3-4a, lane; clone 4-1, lane3; Clone 2-2, lane4; Clone 1-1a, lane5; Clone 1-1b, lane6; clone 1-2, lane7; Clone 5-1a, lane8; Clone 5-1b, lane9; clone 5-3, lane10; Clone3-4b.

Finally, two inserts, Sh 3-4, sh 4-1 were cloned directly into pMOS-plasmid vector. The recombinant pMOS-vector DNAs were transformed into *E. coli* SURE^{TM} competent cells. All six plates used produced colonies (Table 6). *E. coli* colonies contained a recombinant pMOS-plasmid were selected by plating on Amp, X-gal, IPTG containig plates. Only five and three white transformants were observed on the first and third plates against a background of 30 and 20 blue colonies respectively. Not a single white colony was observed when ligase enzyme was absent in the cloning process and numerous blue colonies grew on the agar. The last two plates, positive and negative control DNAs were transformed. A negative control of DNA cut with EcoRI enzyme did not produce a single positive colony. Positive control was ligation of a 214Kb fragment provided with the Plasmid kit. It produced a large number of white colonies. The presence of cloned inserts on the pMOS vector was verified by PCR (Plate14). The restriction enzyme analysis could not give clear insert bands, though there was a noticeable 700 bp on the lane (b) in (Plate 15c).

Table 6: Results of transformations of *E.coli*

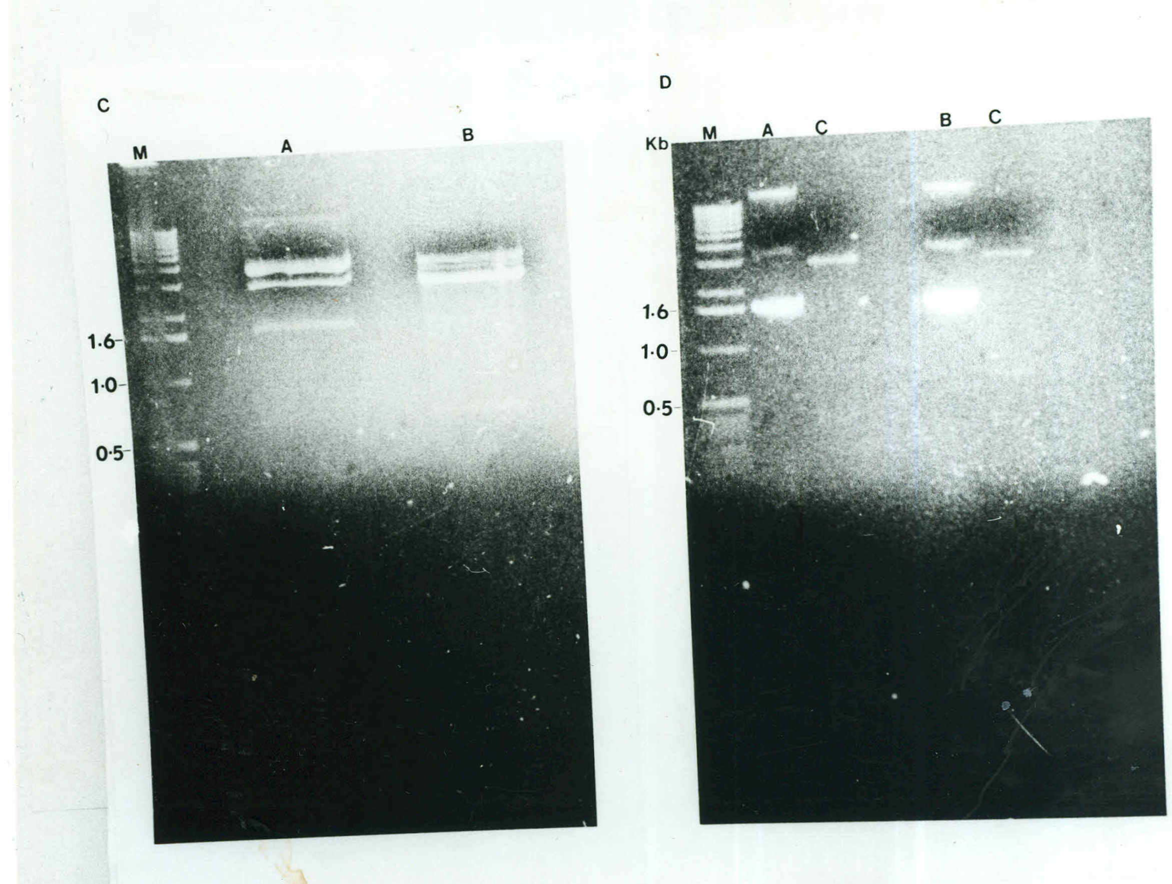
Plate	ligation	Number of colonies		
		Blue	*ligth-blue*	*White*
1	Vector + 0.5Kb + ligase	30	4	5
2	Vector + 0.9 Kb + ligase	40	5	3
3	Vector + 0.5Kb (No ligase)	20	1	0
4	Vector + 0.9 Kb (No ligase)	15	2	0
5	Vector +Positive control insert + ligase	9	50	60
6	Vector + EcoRI cut insert + ligase	20	5	0

Plate 14: Verification of positive transformants



This plate shows PCR on white positive colonies of *E. coli.* The primers used were m13 forward and reverse primers. **A:** Source of the PCR template was bacteria lysate. 5μl of the PCR product was then analysed. M; 1Kb molecular markers; Lanes A: Clone 3-4 ; lanes B; clone 4-1 **B:** Plasmid DNA was extracted (Maniatis *et al.*, 1982) and this DNA was used as a template in 20μ l PCR reaction at the annealing temperature of 60^0 C for one minute. M; 1Kb DNA molecular markers, lanes A: Clone 3-4 clone, lines B: Clone4-1.

Plate 15: Dual restriction enzyme analysis on the recombinant plasmid



C: Entire 20μl of the digested reactions was loaded. M; 1Kb DNA molecular marker, Lane (A) digest of the vector plasmid DNA carrying 3-4 clone , lane (B) digest of the vector plasmid DNA carrying 4-1 clone. In **(D):** There is a comparison between digested plasmid DNA and undigested DNAs. M; 1Kb DNA molecular marker, A; undigested pMOS DNA, C; digested 4-1plasmid, A; undigested pMOS, C; digested 3-4 clone.

3.3.3: Partial cDNA clone sequencing.

3-4, 4-3 clone sequences were analysed by the GeneWorks program 2.3 (Fig 8, 9) and their protein sequence were deduced (Fig 10,11). 400 bp sequence of each clone was submitted to the Gene Bank at USA, for homology search. Clone3-4 showed 98% nucleotide identity with dyenien light chain (DLC) of *S. mansoni* and 77% identity with T-Cell stimulating antigen of the same parasite. The 3-4 sequence shared homology to DLC of other organisms as well (Table 7). The second clone showed 94% similarity with Myosin heavy chain of the *S.mansoni* and close identity to *Brugia malayi* myosin heavy chain gene (62%). The Blast analysis for the 4-1 clone also revealed similarities with myosin gene of 50 organisms as highlighted in (Table 8). The nucleotide sequence generated from clone 3-4 was aligned with a DLC sequence. It showed only three bases altered along the 300 base sequences aligned (Fig 12). On the other hand, clone 4-1 was compared with human and parasite myosin genes on the Gene Bank (Fig 13 a, b, c, d). It shared 100% identity with mRNA for myosin two heavy chain of *S. mansoni* and a complete sequence myosin gene of *S. mansoni* (7Kb) (Fig 13 a, b). But when aligned with human heavy myosin chain (MHC) for fetal-myosin heavy chain and *Homo sapiens* mRNA for myosin had only 53% identities to the 4-1 clone sequence (Fig 13 c, d).

Figure 8: Nucleotide sequence analysis of the 3-4 clone on Gene Works^{TM} Sofware programme 2.3. It contains 480 bases, each line comprises 60 bases and on the right side shows the nucleotide numbers.

```
            10          20          30          40          50          60
123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890
GCC CGT CAG TAT GGC GGA TTC GCG GCC GCG TAA AGC AGT TAT TAA AAA TGC TGA TAT GAG    60

TGA AGA AAT GCA AGA GGA TGC AAT ACA TAT AGC TGC TGG TGC TCT AGA TAA ACA TGA TTT   120

AGA AAA AGA TAT AGC TGC TAA TAT AAA AAA AGA TTT TGA TCG AAA ATA TCA TCC TAC ATG   180

GCA TTG TAT AGT TGG ACG ACA TTT TGG AAG CTA CGT AAC ACA TGA AAC CCA TAA TTT TAT   240

ATA TTT CTA TTT GGA TGA TCG AGC ATT CCT TCT TTT TAA ATC AGG ATG AAA GGA TGA TTT   300

TTT TTA CAA ATG AAC TTA TGA AGA CAA TTA TTA TGT AAC ATT GCA AAT TTT ATG TAT CTA   360

TTT AAT GTA TTT TGC TTT TCT TCA TTT ATG TCT TCA TTT ACT GGA TTA CAT TAT GTT AGT   420

AAA AAG AAA GGG AAA CTA ATT GAT TTA AAC AGA ATA TAC CAT TAT TGT TAA ATA AAA AAA   480
```

Figure 9: Nucleotide sequence analysis of the 4-1 clone at Gene works^{TM} software programme 2.3. This clone has been sequenced 421 bases, each line comprises 50 bases and the right side shows the nucleotide numbers.

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
GAATTCAAAC AACGTTACTC GATTTGGCAC CAAATGTTAT ACCTGATGGA    50

TTTTGTTGAT GGTCGTAAGT TACAGAAAAA ATATTGGAAG CAACACAATT   100

AGATAAAAAT CTTTATCAAT GCGGTAATAC AAAAGTATTT TTCAAAGCTG   150

GCACCCTAGC TCATTTAGAA GATTTACGTG ATGATAAATT AAATGGTATT   200

ATTAGTTTAT TCCAAGCAGA AATTCGTGGT TATTTAATGA GAAAACAATA   250

TAAAAAGCTA CAAGATCAAC GTGTTGCACT TACATTAATG CAACGTAATA   300

TACGTAAATA TTTAGTATTA CGTAATTGGC CATGGTGGAA GATTATATAC   350

AAAAGTGAAA CCAATGTTAA ATATAGCACG TCCAAGAAGA AGAAATGAAA   400

AAAGCCGCTG AAGAATTAGC C                                  421
```

Figure 10: DNA sequence of the 3-4 clone and the deduced amino acid sequence using the single-letter code. The translation starts on +2 ORF. The analysis was done at GeneWorks software programme 2.3. The upper row is nucleotide sequence of the 3-4 clone and the second row is the translated amino acids from the clone.

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
CCCGTCAGTA TGGCGGATTC GCGGCCGCGT AAAGCAGTTA TTAAAAATGC     50
P V S M  A D S  A P A  K A V I  K N A

TGATATGAGT GAAGAAATGC AAGAGGATGC AATACATATA GCTGCTGGTG    100
 D M S  E E M Q  E D A  I H I  A A G A

CTCTAGATAA ACATGATTTA GAAAAAGATA TAGCTGCTAA TATAAAAAAA    150
 L D K  H D L  E K D I  A A N  I K K

GATTTTGATC GAAAATATCA TCCTACATGG CATTGTATAG TTGGACGACA    200
D F D R  K Y H  P T W  H C I V  G A H

TTTTGGAAGC TACGTAACAC ATGAAACCCA TAATTTTATA TATTTCTATT    250
 F G S  Y V T H  E T H  N F I  Y F Y L

TGGATGATCG AGCATTCCTT CTTTTTAAAT CAGGA                    285
 D D A  A F L  L F K S  G
```

Figure 11: DNA sequence of the 4-1clone and the deduced amino acid sequence using the single-letter code. Translation starts at +1 ORF. The analysis was done at GeneWorks software programme 2.3. The upper row is nucleotide sequence of the 4-1 clone and the second row is the translated amino acids from the clone.

```
         10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
GAATTCAAAC AACGTTACTC GATTTGGCAC CAAATGTTAT ACCTGATGGA     50
E  F  K  Q  R  Y  S  I  W  H  Q  M  L  Y  L  M  D

TTTTGTTGAT GGTCGTAAGT TACAGAAAAA ATATTGGAAG CAACACAATT    100
 F  V  D  G  R  K  L  Q  K  K  Y  W  K  Q  H  N  X

AGATAAAAAT CTTTATCAAT GCGGTAATAC AAAAGTATTT TTCAAAGCTG    150
  I  K  I  F  I  N  A  V  I  Q  K  Y  F  S  K  L

GCACCCTAGC TCATTTAGAA GATTTACGTG ATGATAAATT AAATGGTATT    200
A  P  X  L  I  X  K  I  Y  V  M  I  N  X  M  V  L

ATTAGTTTAT TCCAAGCAGA AATTCGTGGT TATTTAATGA GAAAACAATA    250
 L  V  Y  S  K  Q  K  F  V  V  I  X  X  E  N  N  I

TAAAAAGCTA CAAGATCAAC GTGTTGCACT TACATTAATG CAACGTAATA    300
  K  S  Y  K  I  N  V  L  H  L  H  X  C  N  V  I

TACGTAAATA TTTAGTATTA CGTAATTGGC CATGGTGGAA GATTATATAC    350
Y  V  N  I  X  Y  Y  V  I  G  H  G  G  R  L  Y  T

AAAAGTGAAA CCAATGTTAA ATATAGCACG TCCAAGAAGA AGAAATGAAA    400
 K  V  K  P  M  L  N  I  A  R  P  R  R  R  N  E  K

AAAGCCGCTG AAGAATTAGC C                                   421
  S  R  X  R  I  S
```

Table 7: List of similar proteins to 3-4 clone after homology search. The Table shows the various dynein light chain genes, which scored highly with frame +2 of the 3-4 clone. The genes with the lowest probability scores are considered to have the highest homology to the clone. Key: ORF: open reading frame of the clone, P(N): Probability.

Organism	Title/Definition	Frame	Score	P(N)
S.mansoni	Dynein ligth chain	2	464	6.6e -59
S.mansoni	T-cell stimulating antigens	2	376	1.0e -46
Drosophila melanogaster	Cytoplamic dynein ligth chain	2	350	4.0e -43
Homo sapiens	Cytoplasmic dynein ligth chain	2	350	4.0e -43
Caenorhabditis elegens	T26A5.9	2	344	2.7 e-42
Chlamydomonas reinhardtii	Outer arm dynein ligth chain	2	339	1.3e -41
Anthocidaris crassispina	Outer arm dynein lc6	2	337	2.5e -41
Arabidopsis thaliana	Strong similarity toDLC	2	225	7.7e -30
Saccharomyoces cerevisiae	Dynein ligth chain 1	2	252	1.5e -29

Table 8: List of similar proteins to 4-1 clone after homology search. The Table shows the various Myosin heavy chain genes, which scored highly with frame +1 of the 4-1 clone. The genes with the lowest probability scores are considered to have the highest homology to the clone. Key: P(N): Probability.

Organism	Title/definition	Score	P(N)	N
S.mansoni	S.masoni myosin heavy chain	1700	5.1 e - 179	6
S.mansoni	S..mansoni mRNA for myosin II heavy chain	1700	2.7 e - 177	5
S.mansoni	S.mansoni myosin heavy chain	570	4.4 e - 91	5
Homo sapiens	Homo sapiens myosin heavy chain mRNA	276	2.2 e - 12	1
Human	Human MHC mRNA for fetal-myosin heavy	276	2.3 e - 12	1
Homo sapiens	H. sapiens mRNA for myosin	276	2.3 e - 12	1
Human	Human perinatal myosin heavy chain mRNA	276	2.3 e - 12	1
Brugia malayi	Brugia malayi myosin heavy chain gene	268	1.1 e - 11	1
Mouse alpha	Mouse alpha cardiac myosin heavy chain	238	3.5 e - 09	1
Plasmodium	Plasmodium falciparum (strain FCR3) varia	151	2.2 e - 05	4
Plasmodium	P. yoelii major merozoite surface antigen	177	6.7 e - 05	2
Rat	Rat polymeric immunoglobulin receptor mRNA	180	0.00018	10
Human	Human cosmid g1346a045; HTGS phase 3	175	0.00065	7
Human	Human cosmid g1346a 094; HGTS phase 3	175	0.00065	7

Figure 12: Alignment of the 3-4 sequence with the *S. mansoni* dynein light chain (Sm DLC) sequence revealing differences shown in bold letters pionted by arrows at bases 105, 213, 314 of the 3-4 clone. 3-4 stands our clone of study where SmDLC is for S. *mansoni* dynein light chain.

3-4clone29 CGTAAAGCAGTTATTAAAAATGCTGATATGAGTGAAGAAATGCAAGAGGA 79
SmDLC 1 CGTAAAGCAGTTATTAAAAATGCTGATATGAGTGAAGAAATGCAAGAGGA 65

↓
3-4clone80TGCAATACATATAGCTGCTGGTGCT**C**TAGATAAACATGATTTAGAAAAAG 129
SmDLC66 TGCAATACATATAGCTGCTGGTGCT**A**TAGATAAACATGATTTAGAAAAAG 115

3-4 clone130 ATATAGCTGCTAATATAAAAAAAGATTTTGATCGAAAATATCATCCTACA 180
SmDLC 116 ATATAGCTGCTAATATAAAAAAAGATTTTGATCGAAAATATCATCCTACA 166

↓
3-4 clone 181 TGGCATTGTATAGTTGTACGACATTTTGGAAG**C**TACGTAACACATGAACC 229
SmDLC 167 TGGCATTGTATAGTTGTACGACATTTTGGAAG**T**TACGTAACACATGAACC 215

Sample 230 CATAATTTTATATTTCTATTTGGATGATCGAGCATTCCTTCTTTTAAAT 279
SmDLC216 CATAATTTTATATTTCTATTTGGATGATCGAGCATTCCTTCTTTTAAAT 266

↓
Sample 280 CAGGATGAAAGGATGATTTTTTTACAAATGAA**C**TTATGAAGACAATTAT 329
SmDLC267 CAGGATGAAAGGATGATTTTTTTACAAATGAA**A**TTATGAAGACAATTAT 317

Figure 13: Alignment of 4-1 clone sequence with *Schistosoma mansoni* myosin genes and human myosin genes.

(A) Alignment of the 4-1 clone sequence with *Schistosoma mansoni* myosin heavy chain mRNA (SCMYH). *Sample*: stands for the 4-1 clone sequence and the first 340 of the study sequence falls nucleotide sequence from 2230 to 2569 bases of the complete sequence of *Schistosoma mansoni* myosin heavy chain.

(B) Alignment of the 4-1 clone sequence with mRNA for myosin heavy chain of *Schistosoma mansoni* (SMMIIHC). Both clone sequences have 100% similarities from the beginning up to the end of the sequence.

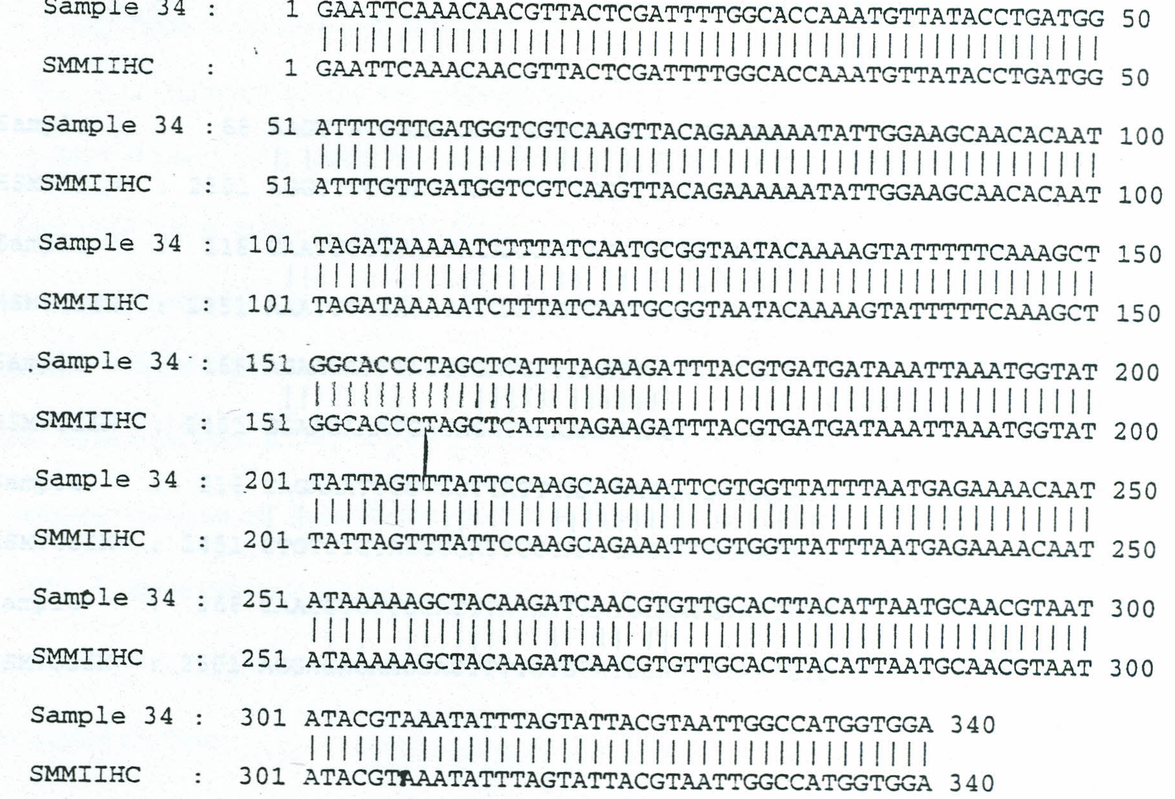
(C) Alignment of the 4-1 clone sequence with Homo sapiens myosin heavy chain mRNA (HUMMYHPN). There are many deference points between these two sequences.

(D) Alignment of the 4-1 clone sequence with *Homo sapiens* mRNA for myosin (HSMYOSIN). There are clearly seen many deference between these two sequences.

A

```
Sample   :    1 GAATTCAAACAACGTTACTCGATTTTGGCACCAAATGTTATACCTGATGG 50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2230 GAATTCAAACAACGTTACTCGATTTTGGCACCAAATGTTATACCTGATGG 2279

Sample   :   51 ATTTGTTGATGGTCGTCAAGTTACAGAAAAAATATTGGAAGCAACACAAT 100
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2280 ATTTGTTGATGGTCGTCAAGTTACAGAAAAAATATTGGAAGCAACACAAT 2329

Sample   :  101 TAGATAAAAATCTTTATCAATGCGGTAATACAAAAGTATTTTTCAAAGCT 150
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2330 TAGATAAAAATCTTTATCAATGCGGTAATACAAAAGTATTTTTCAAAGCT 2379

Sample   :  151 GGCACCCTAGCTCATTTAGAAGATTTACGTGATGATAAATTAAATGGTAT 200
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2380 GGCACCCTAGCTCATTTAGAAGATTTACGTGATGATAAATTAAATGGTAT 2429

Sample   :  201 TATTAGTTTATTCCAAGCAGAAATTCGTGGTTATTTAATGAGAAAACAAT 250
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2430 TATTAGTTTATTCCAAGCAGAAATTCGTGGTTATTTAATGAGAAAACAAT 2479

Sample   :  251 ATAAAAAGCTACAAGATCAACGTGTTGCACTTACATTAATGCAACGTAAT 300
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2480 ATAAAAAGCTACAAGATCAACGTGTTGCACTTACATTAATGCAACGTAAT 2529

Sample   :  301 ATACGTAAATATTTAGTATTACGTAATTGGCCATGGTGGA 340
                ||||||||||||||||||||||||||||||||||||||||
SCMMYH   : 2530 ATACGTAAATATTTAGTATTACGTAATTGGCCATGGTGGA 2569
```

B



```
Sample 34 :   1 GAATTCAAACAACGTTACTCGATTTTGGCACCAAATGTTATACCTGATGG 50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SMMIIHC   :   1 GAATTCAAACAACGTTACTCGATTTTGGCACCAAATGTTATACCTGATGG 50

Sample 34 :  51 ATTTGTTGATGGTCGTCAAGTTACAGAAAAAATATTGGAAGCAACACAAT 100
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SMMIIHC   :  51 ATTTGTTGATGGTCGTCAAGTTACAGAAAAAATATTGGAAGCAACACAAT 100

Sample 34 : 101 TAGATAAAAATCTTTATCAATGCGGTAATACAAAAGTATTTTTCAAAGCT 150
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SMMIIHC   : 101 TAGATAAAAATCTTTATCAATGCGGTAATACAAAAGTATTTTTCAAAGCT 150

Sample 34 : 151 GGCACCCTAGCTCATTTAGAAGATTTACGTGATGATAAATTAAATGGTAT 200
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SMMIIHC   : 151 GGCACCCTAGCTCATTTAGAAGATTTACGTGATGATAAATTAAATGGTAT 200

Sample 34 : 201 TATTAGTTTATTCCAAGCAGAAATTCGTGGTTATTTAATGAGAAAACAAT 250
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SMMIIHC   : 201 TATTAGTTTATTCCAAGCAGAAATTCGTGGTTATTTAATGAGAAAACAAT 250

Sample 34 : 251 ATAAAAAGCTACAAGATCAACGTGTTGCACTTACATTAATGCAACGTAAT 300
                ||||||||||||||||||||||||||||||||||||||||||||||||||
SMMIIHC   : 251 ATAAAAAGCTACAAGATCAACGTGTTGCACTTACATTAATGCAACGTAAT 300

Sample 34 : 301 ATACGTAAATATTTAGTATTACGTAATTGGCCATGGTGGA 340
                ||||||||||||||||||||||||||||||||||||||||
SMMIIHC   : 301 ATACGTAAATATTTAGTATTACGTAATTGGCCATGGTGGA 340
```

C

```
Sample   :   68 AAGTTACAGAAAAAATATTGGAAGCAACACAATTAGATAAAAATCTTTAT 117
HUMMYHPN : 2301 AGGCTTCTGAGAAACTTCTTGCATCTATTGATATTGATCATACTCAATAT 2350

Sample   :  118 CAATGCGGTAATACAAAAGTATTTTTCAAAGCTGGCACCCTAGCTCATTT 167
HUMMYHPN : 2351 AAATTTGGACATACCAAGGTTTTCTTCAAAGCTGGACTTCTGGGTCTTCT 2400

Sample   :  168 AGAAGATTTACGTGATGATAAATTAAATGGTATTATTAGTTTATTCCAAG 217
HUMMYHPN : 2401 GGAAGAAATGAGAGATGAAAAATTAGCCCAAATTATAACAAGAACACAAG 2450

Sample   :  218 CAGAAATTCGTGGTTATTTAATGAGAAAACAATATAAAAAGCTACAAGAT 267
HUMMYHPN : 2451 CTGTCTGTAGGGGATTCCTAATGAGGGTAGAATATCAGAAGATGTTGCAA 2500

Sample   :  268 CAACGTGTTGCACTTACATTAATGCAACGTAATATACGTAAATATTTAGT 317
HUMMYHPN : 2501 AGGAGAGAAGCACTTTTCTGCATCCAGTATAATGTCCGTGCCTTCATGAA 2550

Sample   :  318 ATTACGTAATTGGCCATGGTGGAAGATTATATACAAAAGTGAAACCAATG 367
HUMMYHPN : 2551 CGTCAAGCACTGGCCCTGGATGAAACTCTTTTTCAAGATTAAGCCCCTCC 2600
```

D

```
Sample   :   68 AAGTTACAGAAAAAATATTGGAAGCAACACAATTAGATAAAAATCTTTAT 117
HSMYOSIN : 2301 AGGCTTCTGAGAAACTTCTTGCATCTATTGATATTGATCATACTCAATAT 2350

Sample   :  118 CAATGCGGTAATACAAAAGTATTTTTCAAAGCTGGCACCCTAGCTCATTT 167
HSMYOSIN : 2351 AAATTTGGACATACCAAGGTTTTCTTCAAAGCTGGACTTCTGGGTCTTCT 2400

Sample   :  168 AGAAGATTTACGTGATGATAAATTAAATGGTATTATTAGTTTATTCCAAG 217
HSMYOSIN : 2401 GGAAGAAATGAGAGATGAAAAATTAGCCCAAATTATAACAAGAACACAAG 2450

Sample   :  218 CAGAAATTCGTGGTTATTTAATGAGAAAACAATATAAAAAGCTACAAGAT 267
HSMYOSIN : 2451 CTGTCTGTAGGGGATTCCTAATGAGGGTAGAATATCAGAAGATGTTGCAA 2500

Sample   :  268 CAACGTGTTGCACTTACATTAATGCAACGTAATATACGTAAATATTTAGT 317
HSMYOSIN : 2501 AGGAGAGAAGCACTTTTCTGCATCCAGTATAATGTCCGTGCCTTCATGAA 2550
```

CHAPTER FOUR

4.0: Discussion

4.1: The importance of the tegument in the adult parasite

Proteins occur in all component parts of living organisms and several major targets of immunity of schistosomes are proteins that are highly conserved in evolution. These polypeptides will no doubt share a lot of sequence homology with those in the host and therefore, the immune response to these antigens is probably directed to a very low number of epitopes. The adult schistosomes stay in permanent copula for several years in the mesenteric vasculature of the host and this is responsible for the pathology and transmission of the disease. The adult worm is covered by a specialized syncytial epithelial, capable of unidirectional transport of solutes and water. The structure of the syncytial surface of the blood-dwelling schistosomes consists of fairly inert membranous layer overlaying the apical plasma membrane, hence a double bilayer (McDiarmid *et al.*, 1983). It has been suggested that this extra-membrane layer in the schistosomes may serve the same function as the envelope in the Gram-negative bacteria by resisting immunological attack particularly by the complement system which act by insertion into and formation of a pore in the membrane (Chendler *et al.*, 1994). The evasion mechanisms employed by the adult worm are still poorly understood, and their elucidation will be significant for a better understanding of host-parasite relationship. Several surface proteins shown to play a critical role in the *S. mansoni* infection may be anti-parasitic vaccines or chemotherapy targets such as serine proteases (Marikovsky *et al.*, 1990). A serine protease inhibitor was identified in the tegumental detergent extracts from adult worms (Blanton *et al*; 1994; Chendler *et al.*, 1994) which can block activity of neutrophil elastase.

Previous studies were targeted to the discovery of new set of physiologically active biomolecules that are expressed by growing parasite to make it adapted to the human host. Female pairing with males are considered to be a pre-requisite for the completion of female growth and reproductive morphogenesis (Popiel, 1986). It has been shown that egg-laying female schistosomes, separated from their male partners and surgically implanted into naive animals, stop laying eggs and begin to regress physically and reproductively to the immature state (Clough, 1981). But, if such regressed females are allowed to pair again with males, normal reproductive activity resumes even after months of regression. The mating system in schistosomes is generally assumed to be monogamous (Tchuem *et al.*, 1996) and there is physical stimulation and chemical transfer in the process (Skuce, 1990). The nature of the developmental stimuli provided by the adult male parasite is unknown. A glycoprotein antigen of *S. mansoni* expressed on the gynecophoral canal of the male is shown to be widely distributed on the surface of adult female worms. But in males surface expression is limited to the gynocophoric canal, the site of direct interaction between the mating pair. The reproductive antigens of the adult schistosomes have been identified as potential targets for therapeutic interventions because of the egg's primary importance in morbidity and mortality. Drug therapy such as PZQ acts within the tegument and muscle cells of the schistosome. Adult male which face the drug action have a highly complex membrane structure and this may complicate the susceptibility of the PZQ-induced damage. However, PZQ-sensitive sites must be considered. This drug is more effective against females than against male worms. Further studies on the composition of the male tegument proteins would provide molecules of interest such as drug induced antigens which play a fundamental role in the destruction of the parasite. The possible targets of the drug design are membrane-associated proteins, 200 kDa, Tubercle glycoprotein. The strains of the parasite resistant to the PZQ action lost a drug-activating enzyme that is present in the sensitive sites on

the schistosome membrane. Further study of this enzyme may yield valuable clues for drug design and for a basic understanding of the parasite metabolism (Cioli *et al.*, 1993).

4.2: Characterisation of adult worm proteins

In order to obtain membrane associated proteins, fresh adult worms were recovered from infected Balb/c mice perfusion. Schistosome membrane contains both extrinsic (peripheral) and intrinsic (integral) proteins. Extrinsic proteins are loosely attached to the surface of the worm membrane by electrostatic bonds. They are easily extracted by mild procedures which do not disrupt the membrane. In this case, they were removed in soluble form using high salt concentration at acidic pH 4.5. On the other hand, the intrinsic proteins are integrated into the structure of the worm membrane. They are partially submerged through the phospholipid bilayer of the membrane. So they can't be removed from the membrane without the use of conditions which disrupt the membrane. In this study, to solubilize these integral proteins out of the hydrophobic lipid bilayer utilising non-ionic Triton-x 100 detergent was aimed and this is widely used to solubilize such proteins. During solubilization, the non-ionic detergent replaces most lipid molecules in contact with the hydrophobic domains of the integral membrane protein and forms a soluble protein-detergent mixed micelles. In a separate attempt tegument proteins were also isolated from adult worm by using a mixture of salt solutions (Ca^{2+}, K^{+}, Mg^{2+}). Both protocols (McDiarmid *et al*, 1983; Vasconcelos *et al.*, 1993) adopted in this study for tegument extraction was accorded, although both were studying protein removals and specific protein gene(s) on the worm surface. But here proteins were required which could be used to challenge experimental animals and raise antisera. *In vivo,* worm membrane proteins are stable. However, *in vitro* the proteins are unstable since they are exposed to the external (experimental) conditions, which would lead to degradation. These conditions include pH, temperature and enzymatic

degradation by proteases. To avoid this from happening, a cocktail of protease inhibitors was used. The concentration of the extract proteins was dilute and any efforts to concentrate was futile. Protein assay of Brown *et al.,*(1989) provided a good approximation of the sample concentration. It was found that the temperature plays a big role in the determining the concentration of proteins i.e. there was a difference in concentration when the protein samples were incubated at different temperatures; room temperature and 37° C with time difference. Electrophoretic separation of the tegumental proteins under denaturing conditions facilitated separation of a very minute extracts into their polypeptide composition. To test the hypothesis of this study, rabbits were immunized using tegument extracts, thus eliminating the possibility of contamination with other parasite proteins, which could have occurred. Numerous studies on schistosomes have been conducted in experimental models, in order to demonstrate molecules from the parasite allowing a better understanding of its immunogenic and biological functions. In this study, rabbits were chosen because they are the ideal animal model for raising antisera. In order to analyse the molecular basis of the host immunity to *S. mansoni,* it was essential to test the immunoreactivity of the sera. Western blot analysis by Towbin *et al.,* (1979) was adopted. The analysis of the reactivities of antibodies from rabbit inoculated with tegument proteins revealed two molecules approximately 84 and 26 KDa bands. However, results obtained in this study showed that a number of immuno-dominant conserved proteins including Mr 58, 116 and 130 kDa were specific for peripheral proteins. More recently, the work of Smithers *et al.,*(1989) revealed that mice can be protectively vaccinated using a purified surface membrane preparation from adult *S. mansoni* . Adult worms tegumental proteins 25, 30, kDa size were the most recognised bands by immunoblotting (Smithers *et al*., 1990). Accumulated data affirm that Sm25 may be responsible for the protective immunity obtained when the mice were inoculated with isolated tegumental surface.

4.3: Molecular cloning and characterization of worm protein gene(s)

A major focus of research on schistosomiasis continues to be the development of a usable vaccine. The progress towards the development of a vaccine against schistosomiasis has been made both via the understanding of the mammalian immune response, characterisation and molecular cloning of target antigens. Most of the recombinant vaccines being studied today were originally identified as important antigens in animals protectively vaccinated with ill-defined crude antigen inocula or radiation attenuated infections. Many defined vaccines have been developed and probably they are the most promising present available. Interestingly, they were initially developed through quite different routes. Present study is focused on screening of *Schistosoma haematobium* cDNA expression library utilising rabbit anti *S. mansoni* sera as a tool for identification. Generally several situations may call for, screening strategies using antibodies as hybridization probes. One strategy, which has been taken advantage of, was probing of recombinant proteins on NC papers using antibodies raised in animals against the expressed proteins. This capitalizes on the fact that antibodies hybridize to the immunogens, which induced their production. However, successful screening for antigen production generally depends on the availability of high tittered polyclonal antibodies. The pre-adsorbed rabbit anti-membrane sera had identified only antigen-positive clones. Sometimes antigens do not elicit high titre antibody responses making the screening of libraries difficult and unreliable as the signal on antigen–positive clones becomes weak. This limitation of the technique has thus hampered the identification of clones expressing many proteins of biological relevance. Screening with rabbit anti membrane sera proved to be efficient which resulted in the isolation of very many clones. The number of the primary clones was adequate, in view of the 25 X 10^4 pfu screened. While the other rounds of screening polished the primary cDNA clones. The clones, which were followed to tertiary screening had homology to *S. mansoni* membrane protein genes. To verify the positivity of the isolated clones a

Polymerase Chain Reaction (PCR) technique was applied. It is a widely used method that has greatly simplified cloning and modification of nucleic acids or the identification of specific sequences from individual organisms. Recently a direct PCR protocol was presented for intact multicellular organisms such as adult males of *S. mansoni* (Grevelding *et al.,* 1996). But, in this study the ordinary protocol was performed (Mullis *et al*., 1986) on each isolated plaque using complementary lambda gt11 forward and reverse primers. It was found that phage lysate was not ideal as a PCR template. Possibly phage storage buffer(SM) might could have had Magnesium ions which hindered PCR reactions. Pure lambda DNA extract deciphered this puzzle and resulted in the identification of four cDNA insert classes. PCR technique established the presence of inserts in all clones. To subclone some of the inserts, the PCR products were purified and attempts made to clone into a variety of cloning vectors. The restriction enzymes might have not released inserts due to factors inhibiting their functions. The ligation enzymes might have not joined vector–insert DNAs. It could also have be a genetical mutation of the vector DNAs. Efforts were also made to clone "end-filled and kinassed" PCR products into Sma I or EcoRV (blunt sites) of cloning vectors of M13, PUC18, pRSET A, B, C plasmid vectors. All such attempts failed. Cloning was however successful when a plasmid T-vector, pMOS-T was used. Promega purification system was also utilized and found better in DNA yield than organic purification system. These purified cDNA fragments eased the ligation process to pMOS-T vector. Two clones, Sh 3-4, Sh 4-1 inserts were selected for further study. T-plasmid vector was used because taq polymerase has a inherent activity of adding a single deoxyadenosine (A) to the 3' ends of PCR products. Linearized T-plasmid vectors such as pMOS-T have a single overhanging 3' deoxythymidine (T) residues at the cloning site. This allowed PCR inserts to ligate efficiently with the vector. Screening of *S. haematobium* cDNA libraries with antibody probes identified a promising glycoprotein protein at Department of Molecular genetics NMK, Kenya (Un published). Most of the schistosome vaccine

candidates were identified in this screening process using either anti-soluble worm antigen preparation (SWAP) or tegument sera. In this study, *S.haematobium* library was screened with sera raised against tegument membrane of *S. mansoni* adult worm.

SURE^{TM} competent cells were found good in transformation and most white colonies had inserts. Few failed to show the inserts, that could be the single 3' T- overhangs on the vector may have degradated. To determine the presence and orientation of the inserts, at least 10 white colonies were picked and mini plasmid extraction was done (Sambrook, 1989). PstI and SacI restriction enzyme were used to release the cloned inserts on to the plamid DNA. Digest analysis on agarose gel did not show very clear insert bands. PCR verified the presence of the cloned inserts in the plasmid vectors. The results of the current study indicate that *S. mansoni* tegument extracts induce immune response to cytoplasmic dynein light chain, T cell antigen and myosin heavy chain of the *S.haematobium* worm. Thus, the cloned *S.haematobium* cDNA of the worm appears to express protein epitopes responsible for the reactivity of the antisera. Further characterization of the two cDNA fragments showed that both clones had sequence homology to *S. mansoni* genes. The first, Sh 3-4 clone had an open reading frame +2 encode cytoplasmic dynein light chain (DLC) from *S. mansoni* (Hoffman and Strand, 1996). Dynein light chain are the components of dynein, an enzyme complex involved in various aspects of tubule-based motility. The identification of that gene within the *S. mansoni* tegument (a layer that does not contain flagella or cilia) is debatable. In the present study, it was shown that rabbit-sera inoculated subcutaneously with a tegument extracts accompanied by FCA adjuvant identified a gene encoding SmDLC. In contrast in Hoffman and Strand (1996) they generated monoclonal antibody (mAb709A2/2) against antigens associated with isolated tegumental membranes of *S. mansoni*. Then, the antigen recognized by this mAb was characterized and found to be highly similar to

cytoplasmic DLC of *S. mansoni.* Until recently, cytoplasmic dyneins were not believed to contain light chains, but a report by King and Patel-King (1995) raised the possibility that dynein light chains exist in the organinisms that do not contain cilia and flagella. They characterized two axonemal dynein light chains from *Chlamydomonas reinhartii* and verified that these molecules have sequence homology in organisms that don't contain either flagella nor cilia. Additionally, this report presents novel characteristic that may uniquely be associated with schistosomes or may be applicable to other proteins in this growing family. But the role Sm DLC plays in the tegument as well as the identification of the associated proteins is the focus of the future work of schistosome molecular immunologists. Elucidation of the function of SmDLC may help us to have better understanding in the protective role of the tegument as well as provide an insight in the mechanism of organelles trafficking in *S. mansoni* that may be applicable to other systems.

Vaccine-induced immunity against *s*chistomes depends on the specific priming of certain T-cell subsets and on the recall of this response by natural infection after vaccination. Previous studies have shown that the protective immunity induced in schistosomes is to be T-cells dependent. The activation of the appropriate T-cell subset is critical for the recruitment and activation of efficient immune defences against schistosome infections. It has been shown that S.h3-4 clone also has 77% similarity to T-cell stimulating antigen of *S. mansoni* Sybille *et a*l., (1997). Parasite antigens that are strong T-cell immunogens represent potential candidates for vaccines against pathogens susceptible to T-cell mediated immunity. Due to the time and cost this clone could not have been expressed and tested its protectivity against cercarial infection. In variance, Sybille *et a*l.,(1997) identified T-cell clone from schistosomula cDNA expression library using mouse anti schistosomula extracts sera. Sybille *et a*l.,(1997) cloned, expressed

poypeptides stimulated T-cells and yielded tryptic peptides whose sequence matched the sequence of the cloned molecule. Another study searched for a schistosome antigen capable of strong T-cell stimulation in mice vaccinated with adult worm extracts constitute Bacillus of Calmette and Guerin (BCG) adjuvant. It detected schistosome paramyosin (90-kDa) as being a major T-cell stimulating antigen in these immunized animals (Lanar, 1986). T-cell antigen illustrated in this investigation suggests that, it is present in the tegument preparation of adult worms in addition to schistosomula. This study postulated that if the recombinant protein of this clone could have been examined for the Tcell responses of subjects living in endemic areas, then its efficacy would have been observed. It is also possible to use this recombinant antigen alone or as a cocktail with antigens like paramyosin. The second clone sequence of this study revealed that it encodes for myosin protein of *S. mansoni.* Many *S. mansoni*-derived contractile proteins such as myosin heavy chain, paramyosin and tropomyosin play important role, in the immune responses of infected hosts (Grossman *et al.,* 1990). This protein is localised in muscle tissue and elicits immunodominant antibody responses in infected mice and rats. The finding that myosin gene in the tegument extracts leads to examinations of the localization of this gene. It is assumed that it could be attached to the schistosome surface. Alternatively, it seems that during protein extraction longitudinal muscle fibres of the worm were included. Thus, anti-myosin antibodies reacted with the fusion-myosin protein of the expression vector. In early vaccine studies immunization of murine hosts a strong immune response with a major component being the production of antibodies against a 97 kDa antigen was realized (Pearce *et al.,* 1988). A more recent study by Ramirez *et al*, (1996) has again demonstrated the effectiveness of paramyosin as a vaccine target antigen in *Schistosoma japonicum* infection. This antigen was subsequently studied and a native paramyosin without adjuvant, induced 62–86 % resistance against cercarial challenge in four separate experiments. However, injection

of myosin did not induce similar protection (Pearce *et al.,* 1988) although both molecules share similarities in amino acid sequence. The production and purification of the recombinant proteins of these genes (myosin and paramyosin) would be a potential immuno–diagnostic tool for the disease (Xu *et al*., 1991). Alignment of the clone 4-1 sequence with parasite and human myosin protein genes showed that it has very little human nucleotide similarity compared to parasite sequences. That would verify that this gene is more likely to be found in the muscle tissue of the parasite than any other organisms. It has long been obvious that antigen exposed on the surface of the parasitic schistosomes are primary targets of protective immune responses. It is becoming apparent that not all protective schistome antigen are surface antigens and that not all surface antigens are necessarily targets of protective immunity because neither paramyosin nor myosin exists on the surface of the schistosome tegument. Recombinant polypeptide rIrv-5, that is encoded by a cDNA exhibits a high degree of homology with the cDNA of myosin two heavy chain from several species. It induces high levels of protection in rodents (Soisson *et al*., 1992) and baboon (Soisson *et al.,* 1993). One of the leading antigen for vaccine candidates in schistosomes (Sm28 kDa) were co-purified with T-cell antigen and has shown to induce protective immunity in animals (Grezel *et al.,* 1993) and to reduce schistosome fecundity (Xu *et al.,* 1993). Both soluble protein associated with the parasite surface and integral proteins are important for protective immunity. Some of them having the potential to be vaccine candidates. Other studies have shown that Sm 97 schistosome paramyosin and triose–phosphate isomerase (TPI) (Shoemaker *et al;* 1992 Reynolds *et al* 1994) induces T-cell responses in animals. Paramyosin is one of the vaccine antigens for the prevention of the *S. mansoni* infection with humans and thus 4-1 clone could have similar function to paramyosin because of their homologues amino acids sequences. Currently WHO assessed the protective potential of six promising antigens. These include Paramyosin 97 kDa, Sm 23, and

Sm14 membrane associated antigens. The accumulated results demonstrated that the stated goal of consistant induction of 40% protection or better was not reached with any of the antigens formulations tested in the trials. Although their protection was not good enough, it does not necessarily imply that they are not candidates anymore. But it is clear that, they are worthy of further studies in this regard. It is possible that a cocktail vaccine consisting a paramyosin and 28 kDa (GST) molecule could be developed. These are two immunogens thought to work through quite different mechanisms and may induce levels of protection high enough to allow consideration for human use. Further characterisation of the membrane protein encoding genes discovered in this study is vital. The techniques of the genetic engineering can produce recombinant proteins, but to carry on this characterisation was beyond the scope of this study. It is hoped that this will provide sufficient information that might be utilised for the development of vaccine and/or immunodiagnostic agents against schistosomiasis.

CHAPTER FIVE

5.0: Conclusions and suggestions for future work

The identification and characterization of membrane-associated proteins within the tegument of *Schistosoma haematobium* is relevant to an improved understanding of its role and importance in the adult worm's survival in the host. This would create avenues to take advantage of the existing interface between the parasite and the host, with a view of developing a vaccine, diagnostic antigens or a chemotherapeutic molecule, hence facilitating effective control of the schistosomiasis menace. Disruption of the membrane structure would hinder the existence of the parasite in the host and would hence provide effective control of the disease. The study performed presents its conclusions and suggestions for future work.

5.1: Summary of the conclusions

I) A culture of *S. mansoni* was established from which the isolation of membrane proteins was performed.

II) Characterization of membrane proteins on polyacrylamide gel and the raising of anti *S. mansoni* membrane sera in experimental rabbits were possible.

III) Antigenic bands of 84 kDa and 26 kDa were found to be major tegument proteins in western blotting. The various extracts of worms were not found to contain immunogenic protein smaller than 25 kDa.

IV) *Schistosoma. heamatobium* and *Schistosoma mansoni* share membrane protein genes, which were identified by screening of membrane protein clones using antisera raised against either of the species.

V) Polymerase chain reaction (PCR) gave the best results in determining cloned inserts and their sizes providing five insert classes range from 0.5Kb-1.3Kb.

VI) Polymerase chain reaction products, Sh 3-4, and Sh 4-1 were cloned into pMOS-Blue vector. The presence of the inserts was checked with PCR. Dual restriction enzyme digestion did not release the cloned inserts out of the cloning site.

VII) One of the clones of this study was found to be coding cytoplasmic dynein light chain of the *S .mansoni* (SmDLC). It also has high similarity to T cell stimulating antigen gene of *S. mansoni.*

VIII) Clone Sh 4-1 was found to be similar to myosin gene of *S mansoni* . There was significant difference between Sh 4-1 sequence and human myosin genes. But there was great similarity with *S. mansoni* myosin genes and the study clone (Sh 4-1)

5.2: Suggestions of future work

I) The result revealed in this study offer grounds for considerable optimism about the rapid development of a schistosome vaccine. It is recommendable to continue studying the schistosomes surface proteins. Further studies on the composition of the tegument proteins would provide molecules that will play a fundamental role in the destruction of the parasite. Furthermore, 84 kDa and 26 kDa tegument bands are to be electro-eluted and its functions further studied.

II) There is a need to clone and sequence the remaining cDNA inserts. Each clone may then be tested for its protective effects. Further characterization remains to be done on these clones.

III) Clone Sh 3-4 could be subcloned into an expression vector and have its efficacy ascertained. This could be done by immunizing in animal models followed by cercaria challenge. Then assaying for protective immune responses and carrying out histopathological studies. In addition to that, there is a need to search for the role SmDLC plays in the tegument as well as the identification of the associated proteins.

IV) It is also necessary to decide whether a single antigen will be sufficient or whether a cocktail vaccine would be preferable. A combination of T cell antigen and myosin, each eliciting different effector responses may be more effective than a single product.

V) The Sh 4-1 was observed to be identical of *S. mansoni* myosin gene. The recombinant protein of this gene would be or part of diagnostic molecule of this disease.

REFERENCES

Abdel-rahman, M. M., (1994). Cytophotometric estimation of hepatocytes DNA in chronic diseases including schistosomiasis for detection of early preneoplasmic changes. *J. Egypt. Soc. Parasitol.* **24** 633-641

Abdel-wahab, M. F., Esmat, G., Milad, M., Abdel-rezell, S., & Strickland, G. T., (1989). Characteristic sonographic schistosomal hepatic fibrosis. *American Journal of Trop. Med And Hyg* . **40,** 72-76

Alving, C, R., Joseph, K, C., Lindsley, H. B., and Shoenbechler, M. S., (1974). Immune damage to liposome containing lipids from *Schistosoma mansoni* worms (38125). *Proceedings of the society for experimental biology and medicine* **146,** 458-61

Amersham Life Science (1994). A manual book for pMOS Blue vector, *Amersham international plc , Lincoln place , Green End Aylesbury Bucks HP20 2TP, England U.K*

Andrews, P., Thomas, H., Pohlke, R., and Seubert, J., (1983). Praziquentel. *Med. Res. Rev.* 3, 147-200

Balloul, J. M., Sondermeyer, P., Dreyer, D., Capron, M., Grzych, J. M., Pierce, R. J., Carvallo, D., Lecocq, J. P., and Capron A., (1987). Molecular cloning of a protective antigen of schistosomes. *Nature* London. **326,** 149 - 153

Basch, P. F., (1981). Cultivation of *Schistosoma mansoni in vitro*. Establishment of cultures from cercariae and development until pairing. *Journal of Parasitol.* **67,** 179-85

Baumester, S., Dennis, R. D., Kunz, J., Wegant, H., & Geyer, E., (1992). Comparative serological reactivity of *Taenia crassicepts, Taenia solium* and *Taenia saginata* metacestode neutral glycolipids to infection serum from *Taenia crassiceps* infected mice. *Molecular and Biochem. Parasitol.* **53,**53 – 62

Becker, M. M., Kalina B. H., Yang, W., Harrop, S. A., Scott, J. C., Waine, G. J., Kurtis, J. D., & McManus, D. P., (1995). Gene cloning and complete nucleotide sequence of Philippine *Schistosoma japonicum* paramyosin. *Acta. Tropica.* **59,** 143-147

Bergquist, N. R (1995). Controlling schistosomiasis by vaccination; A realistic option? *Parasitology Today* **11,** 191-194

Bickle, Q. D., Andrews, B. J., Doenhoff, M. J., Ford, M. J., and Taylor, M. G., (1985). Resistance against *Schistosoma mansoni* induced by highly irradiated infections: studies on species specificity of immunization and attempts to transfer resistance. *Parasitology.* **90,** 301 - 312

Blanton, R. E., Licate L. S., Aman, R. A., (1994a). Characterization of a native and recombinant *schistosoma haematobium* serine protease inhibitor gene product. *Molecular. Biochem. Parasitology,* **63,** 1-11

Brindley, P. J. (1994). Drug resistance to schistosomicides and other anthelminthcs of medical importance. *Acta. Tropica.* **56**, 213 -231

Brindley, P. J., Strand, M., Norden, A. P., and Sher, A., (1989). Role of the host antibody in the chemotherpeutic action of praziquentel against *Shistosoma mansoni*: Identification of target antigens. *Molecular. Biochem. Parasitol.* **34**, 99-108

Brown, R., Jarvis, K., and Hyland, K., (1989). Protein measurement using bicinchoninic acid: Elimination of interfering substances. *Anal. Biochem.* **180**, 136-139

Bueding, E., (1950). Carbohydrate metabolism of *Schistosoma mansoni*. *Journal of General. Physiology.* **33,** 473-95

Burnette, W. N., (1981) `Western Blotting': Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. *Analytical Biochemistery* **112**,195-203

Butterworth A. E. (1988). Control of shistosomiasis in man. The biology of parasitism (*ed.P.T.Englund and A.sher*), pp. 43-59. New york.

Butterworth, A.E (1994). Immunity and morbidity in human schistosomiasis mansoni. *Trop. Geog. Med.* **46**, 197-208

Butterworth, A. E., Capron, M., Cordingley, J. S., (1985). Immunity after treatment of human schistosomiasis mansoni. Identification of resistant individuals and analysis of their immune responses. *Trans. R. Soc. Tro. Med. Hyg.* **79,** 393 - 408

Capron, A. and Dessaint, J. P. (1985). Effector and regulatory mechanisms in immunity to schistosomes: a heuristic view. *Ann. Rev. Immunol.* **3,** 455 - 476

Capron, M and Capron, A (1986). Rats, mice and men models for immune effector mechanisms against schistosomiasis. *Parasitol. Today* **2**, *69-75*

Capron, A., Dessaint, J. P., Capron, M., Ouma, J. H., and Butterworth A. E., (1987). Immunity to schistosomes: Progress towards vaccine. *Science* **238**, 1065-69

Capron, A and Dessiant J. P (1992). Vaccine strategies against schistosomiasis. *Immunol. Biology.* **184**, 282 -294

Caulada–Benedetti, Z., Al-Zamel, F., Sher, A and James, S., (1991). Comparison of Th1 and Th2–associated immune reactivities stimulated by a single versus multiple vaccination of mice with irradiated *Schistosoma mansoni* cercarial. *J. Immunnol.* **146,** 1655 – 1660

Chavez-olortegui, C; Resend, M., and Tavares, C. A., P.(1992). Purification and characterization of a 47 kDa protease from S*chistosoma mansoni* cercarial secretion. *Parasitology.* **105,** 211 218

Cheesbrough, M., (1987). Medical Laboratory Manual for Tropical countries. vol.1 Cambridgeshire Heineman ltd Oxford England ESBS pp 326 - 327.

Clough, E, R.,(1981). Morphology of reproductive organ and oogenesis in bisexual and unisexual transplants of mature *Schitosoma mansoni* females. *J. Parasitol* **67**, 535-539

Chendler, Y., Arnon, R., and Fishelson, Z., (1994). *Schistosoma mansoni*: Isolation and charactrization of smpi 56, a novel serine protease inhibitors. *Exper.Parasitol.* **78**, 121-131

Cioli, D., Pica-Mattoccia, L., and Archer, S., (1993). Drug resistance in schistosomes. *Parasitol. Today* **9**, 162-166

Colley, D. G. and Colley M. D. (1989). Protective immunity and vaccine to schistosomiasis. *Parasitiology. Today.* **5**, 351 -55

Dalton, J. P., Day, S. R., Drew, A. C., and Brindley, P. J. (1997). A method for the isolation of *schistosome* eggs and miracidia free of contaminating host tissues. *Parasitology,* **115**, 29 - 32

Davis A, Jordan, J., webbe, G., and Sturrock, R. F., (1993). In human schistosomiasis, eds. pp 367-404, *CAB International,*Wallingforg, UK

Day, T. A., (1992). Praziquantel: the enigmatic antiparasitic. *Parasitology. Today.* **8**, 342 -344.

Demeure, C. E., Rihet, P. L., Ouattara, M., Bourgois, A. and Dessein, A. J., (1993). Resistance to *Schistosoma mansoni* in humans: influence of IgE/IgE4 balance and IgE2 immunity to reinfection after chemotherapy. *J. Infect. Dis.* **168**, 1000-1008

Dessein, A. J., Begley, M., Demeure, C., Caillol, d., Feuri, J., Dos Reis, M. G., Andrade, Z. A., Prata, A., and Pina, J. C. (1988). Human resistance to schistosomiasis is associated with 1gG reactivity to a 37– kDa larval surface antigen. *J. Immunol.* **140**, 2727–2736

Doenhoff, M. J., Modha J., & Lambertucci J.R., (1988a). Anti-schistosome chemotherapy enhanced by antibodies specific for a parasite esterase. *Immunol.* **65**, 507-510

Dunne, D. W.; Butterworrth, A. E.; Fulford A. J. C.; Kariuki, L. J. G.; Ouma, J. H.; Capron, A.; Pierce R. J.; and Sturrock, R. F.; (1992). Immunity after treatment of human *schistosomiasis.* Association between IgE antibodies to adult worm antigens and resistant to reinfection. *Eur. J. Immunol.* **22**, 1483 - 1494

Fallon, P. G., Redman, C. A., Robertson, A., Modha, J., Kusel, J. R., Doenhoff, M. J., and Marhn, R. J., (1996). Praziquantel: an urgent and exciting challenge. *Parasitology. Today* **12:** 14-20

Fallon, P.G., Fookes, R. E., & Wharton, G.,(1996). Temporal differences in Praziquantel and Oxamniquine-induced tegumental damage to adult *S. mansoni:* implications for grug-antibody synergy. *Parasitology* **112**, 47-58

Fallon P.G., and Hagan, P.,(1996). Blood and guts of Bangor. *Parasitol Today* **12,** 460-462

Fallon, P. G., Cooper, R. O., Probert, A. J., & Doenhoff, M. J. (1992). Immune-dependent chemotherapy of schistosomiasis. P*arasitol.* **105**, 541-548

Fallon, P. G., Smith, P., Nicholls, T., Modha J., & Doenhoff, M. J., (1994). Praziquentel-induced exposure of *Schistosoma mansoni* alkaline phosphatase: drug-antibody synergy which acts preferentially against female worms. *Parasite immunol.***16** 529-35

Feldmeir, H., (1995). Female genital schistosomiasis as a risk-factor for the transmission of HIV. *Int. J. STD AIDS*, **5**, 368-72

Flanigan, T., King, C., Left, R., Nanduri, J., and Mahmoud, A., (1989). Induction of resistance to *Schistosoma mansoni* infection by mice by purified parasite paramyosin. *J. Clin. invest.* **83,** 1010-1014

Franco, G. R., Adams, M. D., Soares, M. B., Simpson, A. J. G., Venter, J. C., and Pena, S. D. J., (1995). Identification of new *Schistosoma mansoni* genes by the EST strategy using a directional cDNA library. *Gene* **152.** 141-147

Goldsmith, R and Heynman, D (1992). Tropical medicine and parasitology, Prentice-Hall International Editions pp766, (U.K) limited, London

Goudot-crozel, V., Caillo, D., Djabali and Dessein, A. J. (1989). The major parasite surface antigen associated with human resistance to schistosomiasisis a 37 kDa glyceraldehyde-3P-dehydrogenase. *J. Exp. Med.* **170,** 2065-2080

Grezel, D., Capron, M., Grzych, J. M., Fontaine, J., Lecocq, J. P., and capron, A (1993). Protective immunity induced in rat schistosomiasis by a single dose of the Sm28GST recombinant antigen: effoctor mechanisms involving IgE and IgA antibodies. *Eur. J. Immunol.* **23**, 454-460

Grevelding, G. G., Kampkotter, A., Hollmann, M., Schafer, U., and Kunz, W., (1996). Direct PCR on fruit-flies and blood flukes without prior DNA isolation. *Nucleic Acids Research, in press.*

Grossman, Z., Ram, D., Markovics, A., Tarrab-Hazdac, R., Lantner, F., Ziv, E., and Schechler, I., (1990). *Schistosoma mansoni*: stage-specific expression of muscle-specific genes. *Exp. Parasitol.* **70**, 62-67

Gryseels, B., Stelma, F. F., Talla, I., VanDam, G. J., Polyman, K., Sow, S., Diaw, M., Sturrock, R. F., Doenhring, S. E., Kardorff, R., Decam, C., Niang, M., and deelder, A. M., (1994). Epidemiology, immunology, and chemotherapy of *Schistosoma mansoni* infections in a recently exposed community in Senegal. *Trop. Geogr. Med.* **46,** 209 – 219

Gyrch, J. M., (1993). IgA antibodies to protective antigen in human schistosomiasis mansoni. *J. Immmunol.* **150,** 527 -535

Hagan, P., Mbye, A., and Horstamann R., (1997). Schistosomiasis in Senegal. *Parasitiology Today,* **13,** 449 – 453

Hagan, P., Blumenthal, U. J., Dann, D., Simpson, A. J. G., and Wilkins, H. A., (1991) human IgG4 and resistance to reinfection with *Schistosoma haematobium. Nature* **249,** 243-245

Hall, M., (1995). *Schistosoma mansoni*: molecular cloning and sequencing of the 200 kDa chemotherapeutic target antigen. *Exper. Parasitol.* **80**, 242-249

Harn, D. A., and Reynolds, S. R., (1993). Immunoreactivity and vaccine trials with schistosome candidate antigen triose phosphate isomerase and Sm23. Symbosium on Schistosomiasis, 8–12, November, 1993. *Research Institute of Tropical Medicine, Manilla.*

Harn, D., (1997). Sm23 DNA vaccine for *Schistosoma mansoni . vaccine* (in press)

Harrison, R. A., Bickle, Q. D., Kiarie, S., James, E. R., Andrews, B. J., Sturrock, R. F., Rihet, P., Demeure C. E., Dessein, A. J., and Bourgois, A.,(1992). Strong serum inhibition of specific IgE correlated to competing IgG4, revealed by a new methodology in subjects from a *S. mansoni* endemic area. *Eur. J. Immunol.* **22,** 2063-2070

Harrison, R. A., Bickle, Q. D., Kiare, S., James, E. R., Andrews, B. J., Sturrock, R. F., Taylor, M. G., and Webbe, G., (1990). Baboons with attenuated schistosomula of *Schistosoma heamatobium*: Levels of protection induced by immunization with larvae irradiated with 20 and 60 Krad. *Trans, Roy, Soc, Trop, Med and Hyg.* **84,** 89-99

Hockey, D. J., and Mclaren, D. J., (1973). *Schistosoma mansoni* changes in the outer membrane of the tegumental during development from cercaria to adult worm. *International Journal of Parasitiology.* **3,** 13 - 25

Hoffman, K. F., and Strand, Mette., (1996). Molecular identification of a *Schistosoma mansoni* tegumental proteins with similarities to Cytoplasmic Dynein Light chains. *The Journal of Biological chemistry.* **271,** *26117-26123*

Homeida, M., Abdel-gadir, A. F., Cheever, A. W., Bennet, J. L., Arbab. B. O., Hibrarium, S. Z., Abdelsalam, I. M. Dafall. A. A. and Nash, T. E., (1988a). Diagnosis of pathogically confirmed symmerrs fibrosis by ultrasonography. a prospective blinded study *American Journal Tropical Medicine and Hygiene* **38,** 86 - 91

Horemans, A. M. C., Tielens, A. G. M., and Vanden bergh, S. G., (1991). The transition from an aerobic to anaerobic energy metabolism in transforming *Schistosoma mansoni* cercarial occurs exclusively in the head. *Parasitiology.* **102,** 259 - 265

Horemans, A. M. C., Tielens, A. G. M., Van den bergh, S.G., (1992). The reversible effect of glucose on the energy metabolism of *Schistosoma mansoni* cercarial and *schistosomula. Mol. Biochem. Pasitology.* **51**, 70 - 80

James S. L. (1987). A review *schistosma spp.* progress towards a defined vaccine. *Exp. Parasitology,* **63**, 247-252

James S. L., Salzman, C., Pearce, E. J., (1988). Induction of protective immunity against *Schistosoma mansoni* by a non-living vaccine. VI Antigen recognition by non-responder mouse strains. *Parasite immunology* **10**, 71.

James S. L., and Sher, A., (1990). Cell mediated immune response to schistosomiasis. *Curr. Top. Microbio. Immuno.* **155,** 21 -41

Johnson, D. A., Dias N. E., Simpson A. J. G., and Rolliston D.O.(1993). Opening the can of worms Molecular analysis of schistosome populations. *Parasitology. Today* **9**, 286 – 291

Kelly, E. A., and Colley D. G., (1988). *In vivo* effects of monoclonal anti -L3T4 antibody on immune responsiveness of mice infected with *S. mansoni*. Reduction of irradiated cercariae-induced resistance. *J. Immunol.* **140,** 2737 -2741

Kigoni, E. P., Lenzi, H. L., and Dessein, A. J., (1986). IgE antibody and resistance to infection. Effects of IgE suppression on the early and late skin reactions and resistance of rats to *Schistosoma mansoni* infection. *Eur.J. immunol.* **16,** 589 –595 **King, M. Stephan., and Patel-King, S. Ramila., (1995).** The Mr = 8000 and 11,000 outer dynein light chains from Chlamydomonas flagela have cytoplasmic homoloues. *The Journal. Biol.. Chem.* **270**, 11445-11452

Kriesel, J. D., Spruance, S. L., Daynes, R. A., and Areneo, B. A., (1996). Nucleic vaccine encoding gD2 protects mice from herpes simplex virus type 2 disease. *J. Infect. Ids*. **173,** 536-541

Kusel J. R., & Gordon J. F., (1989). Point of view. Biophysical studies of the schistosome surface and their relevance to its properties under immune and drug attack. *Parasite Immunology* **11,** 431-451

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature;* **227**, 680 - 685

Lambertucci, J. R., Pinto dassilva, R. A., Gerspecher-lara, R., and Barata, C. H., (1964). Acute mansoni schistosomiasis: sonographic features. *Trans. Royal. Trop. Med & Hygiene.* **88,** 76 – 77

Lanar, D., Pearce, E., James, S., and Shar, A., (1986). Identification of paramyosin as schistosome antigen recognized by intradermally vaccinated mice. *Science* **234**, 593–596

Lewis, S. A., and Strand, M., (1991). Characterization of proteins and immuogens released by adult *Schistosoma mansoni.. J .Parasitol.* **77**, 263-271

Lima, S. F., Vierra, R. Q., Hader, A., & Kusel, J. R. (1994). Effects of culture and paraziquantel on membrane fluidity parameters of adult *Scistosoma mansoni. Parasitiology.* **109**, 54 – 64.

Lucey, D. R., and Marguir, J. H. (1993). Schistosomiasis. *Parasit. Dis. 7*, 635 -645

Maniatis, T., Frisch, E. F., and Samrook, J., (1986). Molecular cloning. Laboratory manual, Cold Spring Habour, N.Y.

Marguerite, M., Bossus, M., mazigue, C., Gas-Masse, H., Tartar, A., Capron, A., Auriault, C., (1992). Analysis of antigenicity and immunogenicity of live different chemically defined constructs of a peptide. *Molecular. Immunology.* **20,** 793–800

Marikovsky, M., Parizade, M., Arnon, R., and Fishelson, Z., (1990). Complement regulation on the surface of schistosomula and adult worm of *Schistosoma mansoni. Eur. J. Immunol* **20,** 221-227

Matsumoto, Y., Perry, G., Levine, R. J .C., Blanton, R., Mahmoud, A. F., and Aikowa, N., (1988). Paramyosin and actin in schistosoma teguments. *Nature* **333,** 76–78.

Mc Cutchan, T. E., Simpson, A. J. G., Mullins, J. A., Sher, A., Nash, T.E., Lewis, F., and Richards, C., (1984). Diffrentiation of Schistosomes by species, strain and sex by using cloned DNA markers. *Prot. Natl. Acad. Sci. USA*, 889-893

McDiarmid, S. S., Dean, L. L., and Podesta, R. B., (1983). Sequential removal of apical plasma membrane from surface epithelial syncytium of *S. mansoni. Molecular Biochemical.* 7,141 - 57

McCulough, F.S., (1980). Molluscicides in schistosomiasis control. *Bull. WHO.* **58,** 68 - 69.

Mitchel, G. F., (1991*).* Infection characteristics of *Schistosoma japonicum* in mice and relevance to the assessment of the schistosome vaccines. *Adva. Parasit.* **30**, 167 - 200

Modha, J., and Doenhoff, M. J., (1994). *Schistosoma mansoni* host-parasite relationship: Interaction of contrapsin with adult worms. *Parasitiology.* **109**, 487-495.

Modha, J., Lambertucci J. R., Doenhoff, M. J., McLaren, L., (1990) Immune dependence of schistosomal chemotherapy: an ultrastructural study of *Schistosoma mansoni* adult worms exposed to praziquentel and immune serum *in vivo. Parasitol. Immunol.* **12**, 321-324

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology* **1**, 263-273

Nara T., Matsumoto N., Janecharut T., (1994). Demonstration of the target molecule of a protective IgE antibody in secretory glands of *Schistosoma japonicum* larvae. *International Journal of Immunology.* **6,** 963-71

Neva, F. A. & Brown, H. W. (1994). Basic clinical parasitology sixth edition pp 245-262. Prentice - Hall International Inc,(U.K) limited, London

Oordt, B. E. P., Tielens, A. G. M., and Van den Berg. S. G., (1989). Aerobic to anaerrobic transition in the carbohydrate metabolism of *Schistosoma mansoni* cercarial during transformation in vitro. *Parasitiology.* **98,** 409 – 415

Paniker, C.K.J (1991). Textbook of medical parasitology, *Jaypee brothers medical publishers, New Delhi, India*

Pearce, E., (1995). The immunology of schistosomiasis. In molecular approaches to parasitology (*ed. J.C boothroyd and R. Komuniecki)*, pp 497 - 510. *Wiley - Liss, incl.*

Pearce, E. J., James, S. L., Lanar, D. E., and Sher, A., (1988). Induction of protective immunity against *Schistosoma mansoni* by vaccination with schistosome paramyosin (Sm97). A non-surface parasite antigen. *Proc. Natl. Acad. Sci.* USA. **85,** 5678 - 5682

Popiel, I., (1986). Male-Stimulated female maturation in schistosoma; a review. *J. Chem.Ecol.* **12,** 1745-1754

Ramirez, B. L., Kurtis J. D., Wiest, P. M., Arias, P., Aliqui, F., Acosta L., Peters, P., & Olds G. R., (1996). Paramyosin: A candidate vaccine antigen against *Schistosoma japonicum. Parasit. Immunol.* **18,** 49-52

Reynolds, R., Dahl, C. E., and Harn, D. A., (1994). T and B epitope determination and analysis of multiple antigenic peptides for the *Schistosoma mansoni* experimental vaccine triosephosphate isomerase. *J. Immunol* **152,** 193-199

Rihet, P., Demeure, C. E., Bourgois, A., Prata, A., and Dessein, A. J., (1991). Evidence for an association between human resistance to *Schistosoma mansoni* and high anti-larval IgE levels. *Eur. J. Immunol.* **21,** 2679-2686

Ritcher, D., Reynolds, S. R., and Harn, D. A., (1993). Candidate Immunogen that stimulates the cellular immune response of mice vaccinated with irradiated cercaria of *S. mansoni. J. Immunology,* **151,** 256 - 265

Rodriguez-perez, Rodriguez-medina, J. R., Gacia-Bianco, M. A. and Hilyer, G.V. (1992). *Fasciola Hepatica:* molecular cloning. nucleotide sequence and expression of a gene encoding polypeptide homologous to *Schistosoma mansoni* fatty-acid binding protein. *Exp. parasitol.* **74,** 400-407

Rollinson, D., Kaukas, A., Johnson, D. A., Simpson, A. J. G., and Tanaka, M., (1997). Some molecular insights into schistosome evolution. *International Journal for Parasitology* **27,** 11-28

Sabah, A. A., Fletcher, C., Webbe, G., and Doenhoff, M. J., (1986). *Schistosoma mansoni*: Chemotherapy of infections of different ages. *Exp. Parasitol.* **61,** 294-303

Sambrook, J., Fritsch, E., and Maniatis, T., (1989). Molecular cloning, A laboratory manual, *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R., (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences* USA **74**, 5463-67

Sauma, S., and Strand, M., (1990). Identification and characterization of glycosyl-phosphatidyl-inositol linked *Schistosoma mansoni* adult worms immunogens. *Mol. Biochem. Parasitol.* **38**,199-210

Sedegah, M., Hedstrom, R. P., and Hoffman, S. L., (1994). Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl Acd. Sci. USA.* **91**, 9866-9870

Sher, A., (1995). An IL-12 based vaccination method for prevention fibrosis induced by schistosome infection. *Nature.* **376**, 594-6

Sher, A., James, S. L., Olivera, R. C., and Pierce, E., (1998). Schistome vaccine current progress and future prospects. *Parasitology.* **98**, 561-568

Shi, Y. E., Jiang, C. F., Han, J. J., and Ruppel, A., (1993). Immunization of pigs against infection with *Schistosoma japonicum* using utra-violet attenuated cercariae. *Parasitology.* **106**, 459- 462

Shi, Y., Jiang, C., Han, J., Li, Y., Ruppel, A., (1990). *Schistosoma japonicum*: An utraviolet-attenuated cercariae vaccine applicable in the field to water buffaloes. *Exp. Parasiot.* **71**, 100-106

Shoemaker, C., Gross, A., Gebremichael, A., and Harn, D. A., (1992). cDNA cloning and functional expression of the *Schistosoma mansoni* protective antigen triose phosphate isomerase(TPI). *Proceedings of the National Academy of Sciences, USA* **89**, 1842-1846

Shuxian, L., Guangchen, S., Yuxian, X., Yang, W., and McManus, D. P., (1995). Immunization of mice with recombinant Sj26 GST induces a pranced anti-fecundity effect after experimental infection with Chinese *Schistosoma japonicum. Vaccine.* **13.** 603 - 607

Simpson, A. J. G and Pena, S. D. J (1991). Host-related DNA sequences in the schistosome genome. *Parasitology Today.* 7, 266

Simpson, A. J. G., Sher, A. and McCutchan, T. f.(1982). The genome of *Schistosoma mansoni,* Isolation of DNA, its size, bases and repititive sequences. *Biochem Parasitol.* **6**, 125-137

Simpson, A. J. G., and Smithers, S. R., (1985). Schistosomes surface, egg and circulating antibodies. *Current Topics in Microbiology and Immunology* **120,** 205 - 39

Skuce, P. J., (1990). Immunoreactivity to the pancreatic polypeptide family in the nervous system of the adult human blood fluke *Shistosoma mansoni. Cell. Tissue. Res.* **261**, 573-581

Smith, A. M., Dalton, J. P., Clough, K. A., Kilbane, C.L., Harrop, S. A., Hole, N., and Brindley, P. J (1994). Adult *Schistosoma mansoni* express cathepsin L proteinase activity. *Mol.Bioch.* **67,** 11-19

Smithers, S. R., Hacket, F., Omer, Ali., Simpson, A. J. G., (1989). Protective immunization of mice against *S. mansoni* with purified adult worm surface membrane. *Parasite Immunology.* **11,** 301-315

Smithers, S. R., Hackett, V. B., and Simpson, A. J. G., (1990). Immunoblotting identifies additional antigens recognized by mice protectively vaccinated with adult *Shistosoma mansoni* tegumental membranes. *Parasitol. Research.* **76,** 454-456

Smithers, S. R., and Terry, R. J., (1969) Resistance to experimental infection *with Schistosoma mansoni* in rhesus monkey induced by the transfer of adult worms. *Trans. R. Soc. Trop. Med. Hyg.* **61,** 517-533

Smythies, E. L., Coulson, P., and Wilson, R. A. (1992). Monoclonal antibody to Interferons (IFN) modifies pulmonary inflammatory responses and abrogates immunity to *Schistosoma mansoni* mice vaccinated with attenuated cercariae. *J. Immunol.* **149,** 3654 -3658

Soisson, L. M. A., Masterson, C. P., Tom, T., McNally, M.T., Lowell, G. H., and Strand, M., (1992). Induction of protective immunity in mice using a 62-kDa recombinant fragment of a schistomansoni surface antigen. *The Journal of immunology* **149,**3612- 20

Soisson, L. A., Reid, G. D. F., Farah, I. O., Nyindo, M., and Strand, M., (1993). Protective immuninity in baboons vaccinated with a recombinant antigen or radiation-attenuated cercariae of *Schistosoma* mansoni in antibody-dependent. *J. Immunol.* **151,** 4782-4789

Sturrock, R. F., Butterworth, A. E., and Houma, V., (1976). *Schistosoma mansoni* in Kenya baboons (Papio anubis). Parasitological response of Kenya Baboons to different exposures of local parasite strain. *Parasitology. Today,* **73,** 293-252

Sybille, K., Patricia, C., Alain, B., Bernadette, B., Karen, P., Hanno, K.,and Alain J. D., (1997). Characterization of schistosome T-cell stimulating antigen (Sm10) associated with protective immunity in humans. *Mol. Bioch. Parastiol.* **84,** 155–165

Tanaka, M., Hirai, H., Loverde, P. T., Nagatachi, S., Franco, G. R., Simpson, A. J. G., & Pena, S. D. J., (1995). Yeast artificial chromasome (YAC) based genome mapping of *Schistosoma mansoni. Mol. Bioch. Parasitol.* **69,** 41-51

Tanaka, T. M., Joseph, G. T., & Strand, M., (1995). *Schistosoma mansoni* molecular cloning and sequencing of the 200 KDa chemotherapeutic target antigen. *Exper. Parasitiol.* **80,** 242–249

Taylor, J. B., Vidal, A., Torpier, G., Meyer, D. J., Roitsch, C., Balloul, J. M., Southern, C., Sondermeyer, P., Pempe, S., Capron, A., and Ketterer, B., (1988). The glutathione S- transferase activity and tissue distribution of cloned Mr 28 KDa protective antigen of *Schistosoma mansoni. EMBO Journal.* **7,** 465–472.

Taylor, M. G., (1994). Schistosomiasis vaccines: Farewell to the god of plague? *Journal of Tropical Medicine and Hygiene.* **97,** 207 -68

Tedler, M. (1996). A *Schistosoma mansoni* fatty acid-binding protein. Sm14 is the potential basis of a dual-purpose anti-helminth vaccine. *Proc. Natl. Acad. Sci.* **93**, 269-273

Tchuem Tchuente, L. A., Southgate, V. R., Combes, C., and Jourdane, J., (1996). Mating behaviour in Schistosome; are paired worms always faithfull? *Parasitol. Today.* **12,** 231-235

Tielens, A. G. M., Horemans, A. M. C., and Van Den Bergh, S. G., (1992). The reversible effect of glucose on the energy metabolism of *Schistosoma mansoni* cercerial and schistosomula. *Mol. Bioch. Parasitol.* **51**, 78–80.

Thomson, S. A., (1995). Minimal epitopes expressed in a recombinant polyepitope protein are processed and presented to CD8+ cytotoxic T cells: Implication of vaccine design. *Pro. Natl Acd. Sci. USA*, **92,** 5845-5849

Towbin, H., Staehelin, T., and Gordon, J., (1979). Electrophoresis transfer of proteins from polyacryamide gels to nitrocelullose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences U.S.A.* **76**, 4350–4354

Vasconcelos E. G., Paul, S. N., Nazareth, M. L., Sergio, V. A., and Sergio T. F., (1993). Characterization and localization of an ATP-diphosphorylase on the external surface of the tegument of *Schistosoma mansoni. Mol. Bioch. Parasitiol.* **58,** 205–214

Verwaerde, C., Joseph, M., Capron, M., Pierce, P. J., Damonelville, M., Velge, F., Auriault, C., and capron, A., (1987). Functional properties of a rat monoclonal IgE antibody specific for *Schistosoma mansoni*. *J. Immunol.* **138,** 4441-4446

Vignali, D. A., Crocker, P., Bickle, Q. D., Cobbold, S., Waldmann, H., and Taylor, M. G., (1989). A role for CD 4+ but not CD 8 + Tcells in immunity to *Schistosoma mansoni* induced by 20 Krad-irriadiated and R0 11-3128 terminated infections. *Immunology.* **67**, 466-472

Waine, J, G., and McManus, P. D., (1997) Schistosomiasis vaccine development- the current picture. *Bio. Essays.* **19**, pp 435- 443 I CSV press 1997.

Waine, G. J., Becker, M., Scott, J., Kalinna, B., Yang, W. and McManus, D. P. (1994). Purification of recombinant *Schistosoma japonicum* antigen homologous to the 22 kDa membrane associated antigen of *S. mansoni,* a putative vaccine candidate against schistosomiasis. *Gene.* **142,** 259-263

Waine, G. J., Yang, W., Scott, J. C., McManus, D. P., and Kalinna B. H., (1997). DNA-based vaccination using *Schistosoma japonicum* (Asian blood fluke) genes. *Vaccine* (in press)

Waine, G. J., and McManus, D. P., (1995). Nucleic acids: Vaccines of the future *Parasitology Today.* **11,** 133-116

Waine G, J., Becker M., Yang, W., Kalinna, B., and McManus D. P., (1993). Cloning, molecular characterization and functional activity of *Schistosoma japonicum* gelyceraldehyde *3-P*-dehydrogenase, a putative vaccine candidate against schistosomiasis japonica. *Infection and immunity.* **61**, 4716-4723

Weiss, J. B., Magnani, J. L., and Strand, M., (1986). Identification of *Schistosoma mansoni* glycolipids that share immunogenic carbohyrate epitopes with glycoproteins. *Journal of Immunol.* **136,** 4275-82

Wilkins, H. A., Blumenthal, U. J., Hayes, R. J. and Tulloch, S. (1987). Resisance to reinfection after treatment of urinary schistosomiasis. *Trans.Roy. Soc.Trop. Med.& Hyg.* **81,** 29-35

Wilson, R. A., and Barnes, P. E., (1977). The formation and turnovers of the membranocalyx on the tegument of *Schistosoma mansoni. Parasitology.* **74**, 61-71

Wolowczuk, I., Aurault, C., Bossus, M., Boulanger, D, Gass-Masse, H, Mazingue, C, Pierce, R . J., Grezel, D., Reid, G. D., and Tartra., (1991). Antigenicity and Immunogenicity of a multiple peptiditic construction of the *Schistosoma mansoni* Sm - 28 GTS antigen in rat, mouse, and monkey. partial protection of Fischer rat after active immunization. *J. Immunol.* **146,** 987-95

World Health Organization (1985). The control of schistosomiasis. Report of a WHO Expert Committee (Technical report series 728), WHO, Geneva, Switerzarland

World Health Organization/TDR (1993). Progress 1991-1992. Eleventh programme report, Geneva, Switzerland

World Health Organization (1993). The control of schistosomiasis. WHO technical report 830, second report of the WHO expert committee. WHO, Geneva, Switzerland

World Health Organization/TDR., (1995). Progress report 1975-1995. 20th anniversary. Geneva

World Health Organization (1996). WHO facts sheet on schistosomiasis. May 1996

World Health Organization (1997). Tropical disease research thirteenth programme report, WHO Geneva, Switzerland

Wynn, T. A., Jankovic, D., Hieny, S., Cheever., and Sher, A., (1995). IL-12 enhances vaccine-induced immunity to *Schistosoma mansoni* in mice and decreases T helper 2 cytokine expression, 1gE production, and tissue eosinophilic. *J. Immunol.* **154,** 4701-4709

Wynn, T. A., (1995). An IL-12 based vaccination method for preventing fibriosis induced by schistosome infection. *Nature.* **276,** 594-496

XU, C. B., Verwaerde, C., Grych, J. M., Fontaine, J., and Capron, A., (1991). A monoclonal antibody blocking the *Schistosoma mansoni* 28 kDa glutathione-S-transferase activity reduces female worm fecundity an egg viability. *Eur. Journal of Immunol.* **21,** 1801-*1807*

Xu, C. B., Verwarde, C.,and Grass–Masse, H., (1993). *Schistosoma mansoni* 28 kDa glutathione-S-transferase and immunity against parasite fecundity and egg viability. *J. Immunol.* **150,** 940-949

Xu, H., Rekosh, D., Andrews, W., Higashi, G., Nicholson, L., and Loverde, P., (1990) *Schistosoma mansoni* tropomyosin production and purification of the recombinant protein and studies on its immuno-diagnosis potential. *Am. J. Trop. Med. Hyg.* **45,** 121-131

Yang, W., Waine, G. J., Sculley, D. G., Lui, X., and McManus, D. P., (1992). Cloning and partial nucleotide sequence of Schistosoma japonicum paramyosin: a potential vaccine candidate against schistosomiasis. *International Journal for Parasitology.* **22,** 1187

Yang, W., Wane, G. J., Becker, M. M., Lulls, S. X., and McManus, D. P., (1995). The 26 kDa glutathione-s-transferases from Chinese and Philippine *Schistosoma japonicum* are identical. *Acta. Tropica.* **57,** 345-349

Yang, W., Waine, G. J., and McManus, D. P., and Kalinna, B. H., (1994). Antibodies to *Schistosoma japonicum* (Asian bloodfluke) paramysoin induced by nucleic acid vaccination. *Biochem. Biophys. Res. Commun.* **121,** 1029-1039

Young, R. A., and Davis, R. W., (1983). Efficient islolation of genes using antibody probes. *Proceedings National Academy of Science of U.S.A.* **80,** 1194-98

APPENDICES

Appendix I: Bacterial Host Strains and primer sequences

SURE: el4 (mcrA), (mcrCB-hsd SMR - mrr) 171, end A1 SUPE 44, thi-1, gyrA96, relA1,lac, reCB, reCJ, sbcC, umuC: Tn5 (kan), uvrC [F pro AB, lacl_M15,Tn10 (Fet)].

Y1090: FD (lac u 169). pro A, A, clon) araD 13a, str A, supF, [trq c 22:: In10 (tet)], (pmc9), hsdR (r, mk).

JM109: rec A1, SUPE 44, end A1 hsdR17 gyrA 96

XL-1 Blue: SUPE 44 hsdR 17 rec A1 gyr A46 thi rel A lac F[pro AB+ lac I lacZ m15 Tn10 (tet)]

M13, FORWARD, - 40: 5' - GTTTTCCCAGTCACGAC -3'

M13, Forward, - 20: 5' - GTAAAAGGACGGCCAGT -3'

M13, Reverse : 5' - CAGGAAACAGCTATGAC -3'

Lambda gt 11, forward 5'- ATTGGTGGCGACGACTCCTGGAG -3'

Lambda gt11 Reverse : 5'- CAGACCAACTGGTAATGGTAGCG – 3'

Appendix II: Bacterial media and plates:

Luria-Bertani (LB) medium per litre: 10g Bacto-tryptone, 5g Bacto- yeast extract, 10g NaCl, Adjust pH to 7.0 with NaOH and autoclave where indicated, add ampicillin to 50 µg/ml, after autoclaved solution have cooled to 50°C

LB Agar/agarose for plates: Add 15 g agar or agarose to 1 litre of LB medium.

2YT-broth (per liter): 16.0 Bacto-typtone, 10 yeast extract, 5.0g NaCl and distilled water added to make 1 litre after pH adjustment to 7.0. This solution was sterilized by autoclaving for 20 minutes. It was stored at 4°C then antibiotic was added just before growing.

Top (soft) agar/agarose (250 ml): 2 g bacto-tryptone, 1.25 g bacto-yeast extract, 1.25 g NaCl, 1.75 g bacto- agar or agarose. This solution was sterilized by autoclaving for 20 minutes. It was stored at room temperature. It was melted in the microwae oven and cooled to 42° c before use.

SOB Medium : 2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto - yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM mg Cl, 10 mM Mg SO_4

SOC media: SOB media containing glucose (0.2% w/v)

APPENDIX III : Buffers and chemical solutions

Solutions for competant cells.

Solution I:- 10 mM NaOAc pH 7.0, 70 mM MnCl, 5mM NaCl.

Solution II:-10 mM NaOAc pH 7.0, 70 mM CaCl, 5% glycerol, 50mM MnCl, this solution is filter sterilised and stored at 4°C.

Plasmid extraction solutions:

Solution I: 20% glucose (50 mM), 0.5 M EDTA (10 mM),Tris-HCL pH 8.0 (25 mM) plus 4mg/ml of Lysozyme enzyme.

Solution II: 10 M Na OH, 10% SDS plus ddH_2 0

Solution III: 5 M Potassium acetate 60 ml, and 11.5 ml glacial acetate acid, 28.5ml of distilled water, The resultant solution with is 3 M respect to potassium and 5 M with respect to acetate.

Solutions for protein work

Monomer solution (30% T, 2.7% Bis). 192 g acrylamide 8.0 g Bis 1.0 litre deionized water Filter though whatman filter paper, store at 4° c at in a light protected container.

Separating gel buffer. 181.6 tris-base (1.5 M), 1.0 litre ddH_20 water adjust pH 8.8 with HCl, store at store at 4°C after filtering

Stacking gel buffer:_60.6 g tris-base (0.5M), 1.0 litre ddH_20, adjust pH to 6.8 with HCl, fliter and sotre at 4°C.

2 x protein loading dye: 2.5 ml stacking gel buffer (0.125 m in Tris), 4.0 ml 10% SDS (4%SDS), 2.0 glycerol (20% u/v), 1.0 ml ß-mecapto-ethanol (10% v/v), 0.25 ml 0.5% bromophenol blue, aliquot 1.0 ml and store at - 20°C insoluble proteins add urea in 2 M concentration

Electrode buffer (5x) stock .6.0 g Tris-base (0.05M), 28.8g glycine (0.384M),1.0g SDS (0.1%) Adjust pH to 8.3, do not use with NAOH because SDS precipitates out.

1% Coomase Blue stain stock.1.0 g of coomase blue R-250, 100 ml double distilled water stir and filter(**Working stain solution):** 500 ml methanol 50% , 100 ml acetic acid 10%, 1 litre deionised water, 62.5 g of 1% stain stock, i.e 0.125% in commasie blue R-250 and gels stained for 8 -12 hrs.

Destaining solution 1. 500 ml absolute ethanol 50%, 100 mls acetic acid 10%, 1.0 litre dH_20 destain gel for 2 hrs.

Destain solution II. 70 ml acetic acid 7%, 50 ml methanol 5%, 1.0 litre dH_2O destain for 2 hrs.

10 x Taq polymerase buffer (magnesium free) 500 ml KCl, 100 mm Tris-HCl pH 9.0, 1 mg/ml gelatine, 1% triton x - 100.

10 x Tris-buffered saline (TBS).Nacl - 43.75 g 150 mM Tris - base - 12.1 g 10 mM Add 500 ddH2O **10 x phosphate buffered saline** (PBS): 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO4, 0.24 g KH2PO4

make up to 1 litre with ddH2O, Adjust pH 7.4 ≤ HCL, store at room temperature.

10 x ligation buffer: 500 mM Tris-Cl pH 7.8, 100 mM $MgCl_2$, 200 mM dithio-threital, 10 mM ATP, and 500 µg/ml bovine serum albumin.

6 x DNA loading buffer:- 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose water)(or 30% glycerol in water).

50 x TAE/L: 242 G Tris base and 57.1ml (0.5 M ` EDTA pH 8.0) make up to 1 litre to double distilled water.

5 x TBE/L: 54 g tris base, 27.5 g boric acid and20ml (0.5 M EDTA pH 8.0) make up 1 litre of double distilled water.

TE buffer:- 10 mM Tris-HCL,pH 8.0,1 mM EDTA,

Blocking solution: (5% Blotto) 5g of powdered non fat milk into 100 ml of 1 x TBS. (3% BSA) 3g of Bovine serum albumin into either TBS or PBS.

Sequencing gel-loading buffer:- 98% deionized formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue.

Transfer buffer /liter : 2.9g of glycine(39 mM),5.8gtris-base(48 mM),0.37g of SDS(0.037%)a 200 ml absolute methanol(20%) pH 8.3.