Use of *Schistosoma mansoni* calreticulin, a highly immunogenic parasite antigen, in diagnosis of and vaccination against schistosomiasis

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By

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled: **Use of *Schistosoma mansoni* calreticulin, a highly immunogenic parasite antigen, in diagnosis of and vaccination against schistosomiasis**, is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at University of Leicester mainly during the period between October 2006 and May 2010

All of the work recorded in this thesis is original unless otherwise acknowledged in the text or by references

None of the work has been submitted for another degree in this or any other university

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Abstract

Use of *Schistosoma mansoni* calreticulin, a highly immunogenic parasite antigen, in diagnosis of and vaccination against schistosomiasis

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Ten sub-fragments (N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC-domains) of recombinant *S. mansoni* calreticulin (SM-CRT), a highly immunogenic antigen, were expressed and purified. A cDNA library of adult *S. mansoni* was used as a template to amplify the coding sequences of these fragments which were expressed in bacterial expression system using the vector pRSETB.

SM-CRT sub-fragments (except those with N-terminal domain) were shown to bind calcium using "Stains-all" stain or interact with $^{45}Ca^{2}$. With the exception of the P-domain, SM-CRT sub-fragments bound and inhibited C1q-dependent haemolysis *in vitro*.

In *S. mansoni* infected mice, specific antibodies against the carboxy-terminal part of SM-CRT appeared 46 days p.i., while antibodies against the N-terminal part of SM-CRT were detectable at 59 days p.i.. Cercarial Transformation Fluid (CTF) and Solube Egg Antigens (SEA) specific antibodies were detectable at day 12 p.i.. Use for diagnosis of *S. mansoni* in humans, CTF and SEA showed 89.7% sensitivity, while the PC-domain of SM-CRT showed sensitivity of 71.1% (the highest SM-CRT sub-fragment sensitivity). When testing the cross-reactivity with sera drawn from patients with other diseases, SM-CRT N-domain showed the lowest degree of cross-reactivity. Analysing non-endemic controls, a specificity >95% was shown for all of the antigens used. When using endemic controls, SM-CRT N-domain revealed the highest degree of specificity (94.7%), while SEA revealed 26.3% specificity and CTF showed 68.4% specificity.

BALB/c mice immunised with recombinant SM-CRT achieved a 49.9% (P >0.05) reduction in *Schistosoma* adult worm numbers (on exposure to *S. mansoni* cercariae), comparing to the non-immunised mice. The eggs numbers in the liver decreased by 41.8% in the immunised mouse group with a non-significant statistical difference. The immunised mice responded to SM-CRT immunisation by producing specific IgG1 and IgG2a, reflecting a Th1/Th2 with a predominance of Th2 immune response profile.

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Abbreviations

AP	Alkaline phosphatase
BBS	Barbital Buffer Saline
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAA	Circulating Anodic Antigen
CCA	Circulating Cathodic Antigen
cDNA	Complementary DNA
CRT	Calreticulin
CTF	Cercarial Transformation Fluid
dATPs	Deoxyadenosine triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate mixture
EDTA	Ethyl diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ES	Excretory/secretory
Fc	Antibody constant region
FPLC	Fast Protein Liquid Chromatography
HRP	Horseradish peroxidise
IFAT	Immune-fluorescence antibody test
Ig	Immunoglobin
IHA	Indirect haemagglutination test
IL	Interleukin

INF-γ	Interferon gamma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
KLH	keyhole limpet haemocyanin
L.B.	Luria-Bertani
MBL	mannan-binding lectin
mCi	millicurie
NHS	Normal Human Serum
nm	nanometer
O.D.	Optical density
ORF	Open reading frame
p.i.	post infection
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PZ	Praziquantel
RBCs	Red blood cells
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS poly-acrylamide gel electrophoresis
SEA	Soluble Egg Antigens
SLE	Sytemic lupus erythematosus
SM-CRT	*S. mansoni* calreticulin
SRBC	Sheep red blood cells

SWAP	Soluble worm antigen preparations
TAE	Tris-acetate EDTA
TBS	Tris buffred saline
TcCRT	*Trypanosoma cruzi* calreticulin
TEMED	N,N,N',N'-Tetramethyl ethyl enediamine
Th	T helper cell
UV	Ultra-violet
WHO	World Health Organization

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Chapter 1

Introduction

1.1 Schistosomiasis

Schistosomiasis is a tropical parasitic disease caused by digenetic trematodes belonging to the *Schistosomatidae* family. The genus *Schistosoma* includes several species, five of which affect human beings; *mansoni*, *japonicum*, *haemtobium*, *mekongi* and *intercalatum.* The first three species are the most widely distributed (Sturrock 2001, McManus& Loukas 2008).

Schistosomiasis is the second most frequent parasitic disease affecting mankind and ranks immediately after malaria (WHO 1993, Croft *et al.* 2003, King *et al.* 2005). Schistosomiasis is endemic in 74 countries, distributed across the Old World. *S. mansoni* is found in sub-Saharan Africa, Egypt, the Middle East and South American countries especially Brazil. *S. japonicum* is endemic in China and the Philippines. Both *S. mansoni* and *S. japonicum* cause intestinal schistosomiasis, whereas *S. haemtobium* is prevalent in sub-Saharan Africa and causes urinary schistosomiasis (Waine& McManus 1997, Chitsulo *et al.* 2004).

Nearly 200 million people harbour *Schistosoma* infections, of whom about 20 million suffer from severe morbidity, and about 600 million people are at risk of being infected. Sub-Saharan states harbour nearly 85% of the affected persons and two thirds of the at risk individuals (Engels *et al.* 2002, Chitsulo *et al.* 2004). Schistosomiasis is estimated to cause

around 280,000 deaths in sub-Saharan Africa alone each year due to haematemesis and renal failure complicated by the disease (van der Werf *et al.* 2003).

In spite of the efforts of control programmes and the changes in *Schistosoma* distribution over countries, the overall number of infected people has nearly stayed the same for over 50 years. This is attributed to overpopulation, construction of new water projects, immigration and the fact that only a few countries have carried out successful control programmes (Savioli *et al.* 2004a& b, Chitsulo *et al.* 2000). My introduction will mainly focus on the subject organism of my thesis, *S. mansoni*.

1.2 Life cycle of *Schistosoma*

Schistosoma needs two hosts to complete its complex life cycle; an aquatic intermediate snail and a mammalian final host. The cycle is similar among different *Schistosoma* species with species specific differences in intermediate hosts species and the main target organs of the adult parasites inside their mammalian host.

Adult male and female *S. mansoni* and *S. japonicum* live in pairs in the mesenteric venous plexus around the large intestine, while *S. haematobium* adults inhabit the venous plexus surrounding urinary bladder. The male carries its partner female in its gynaecophoric canal. The adult male is shorter and thicker than its partner. Parasite life span is about 3-8 years. The female *Schistosoma* deposits its eggs within the narrowest blood vessels that it can reach. The *S. mansoni* egg has a lateral spine while an *S. hematobium* egg posses a terminal spine. Eggs pass through the walls of these veins, then cross the intestinal wall to be excreted with the stool in the case of *S. mansoni* and *S. japonicum,* whereas *S.*

haematobium eggs pass into the bladder to reach the external environment in the urine (Wilson& Coulson 1986, Wilson 1987).

Upon reaching fresh water, the egg hatches immediately stimulated by the hypotenicity of the water, releasing a free living ciliated miracidium which must find a suitable intermediate snail host within two days, otherwise it will perish. Water snails of the genus *Biomphalaria* are the intermediate host for *S. mansoni*, *Onchomelania* species for *S. Japonicum,* while *S. haematobium* uses *Bulinus* snails as intermediate hosts. Once miracidiae reached their intermediate host, they lose their cilia and multiply asexually, changing into primary sporocysts which then develop into secondary sporocysts. Secondary sporocysts migrate to the snail's digestive gland to transform into cercariae. One miracidium may generate thousands of cercariae in one month. Cercariae are released from the snail host and are able to move independently by undulation of their forked tail. Cercariae have to find their definitive host within 24-48 hours otherwise they will perish. Humans are the definitive host for *S. mansoni* and *S. haematobium,* whereas *S. japonicum* may have also other mammalian definitive hosts in addition to man such as cattle, horse and sheep (Wilson 1987).

The cercaria is a positive phototropic, thermotropic and chemotropic organism responding to free fatty acid in the skin. It penetrates the skin by aid of the enzymes secreted from its acetebuler gland and movement of its tail (Stirewalt 1974, Haas *et al.* 1997, Fishelson *et al,* 1992, Brachs& Haas 2008). Within 1.5 minutes, the cercaria enters the human skin (Haas & Haeberlein 2009).

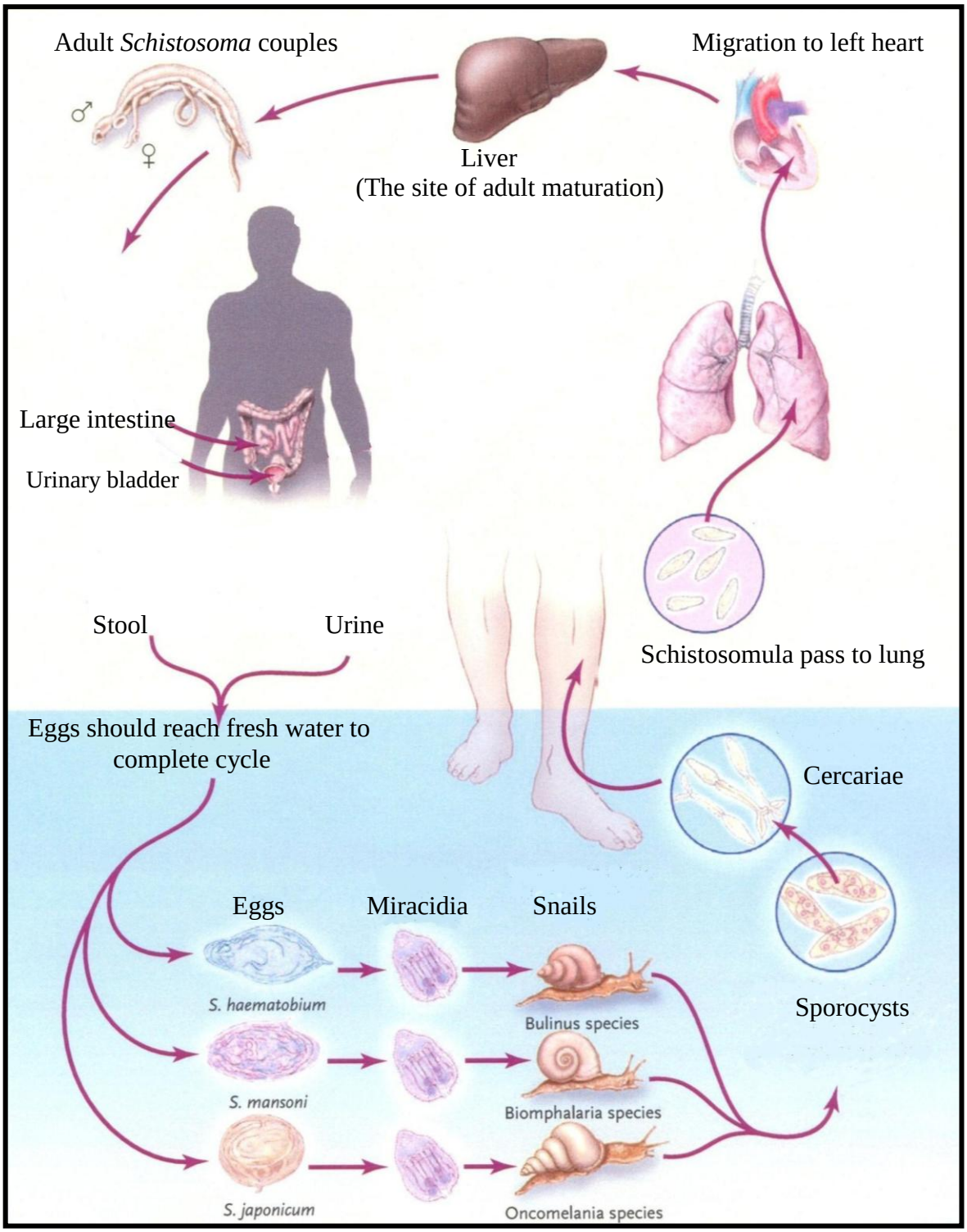


Figure1: Life cycle of *Schistosoma* (This figure is a modified version of a figure published by King 2009).

Upon invasion, cercaria loses its tail and glycocalyx membrane and undergoes several morphological, biochemical and structural changes to transform into a schistosomulum (Samuelson& Caulfield 1982, Samuelson *et al*.1984, Mountford& Trottein, 2004). The schistosomulum leaves the dermis within a time span of few minutes to several hours after the depletion of glycogen stores (McKerrow& Salter 2002).

The schistosomulum passes through local blood or lymphatic vessels to be carried passively by the venous blood flow to the heart. From the heart, it reaches the lung and returns again to the left heart to be carried with systemic circulation to reach the liver sinusoids where it persists throughout its maturation into the adult stage (Wilson *et al*. 1978, Wilson& Lawson 1980).

After reaching maturity, the pairing between male and female *Schistosoma* takes place and both of male and female partners move from the liver through the blood stream to reach their final habitats. The adult female lays several hundred fertilised eggs per day approximately 1.5-3 months post primary infection. The complete life cycle spans a period of about 1.5-3 months depending on temperature of the aquatic environment and the host's immune response (Wilson& Coulson 1986, Wilson 1987). About 50% of the deposited *S. mansoni* eggs succeed in passing to the outer environment with the faeces while the rest remains in the blood stream to be trapped in other organs, especially the liver, where they cause granulomatous inflammatory reactions with its severe sequelae (Cheever 1968).

1.3 Clinical picture of *Schistosoma mansoni*

Many morbidity symptoms and signs caused by *Schistosoma* have socio-economic and public health effects (King *et al*. 2005). The pathogenesis of schistosomiasis occurs in two stages; an acute and a chronic stage.

1.3.1 Acute *Schistosoma* infection

Acute *Schistosomiasis* mainly affects naïve persons who catch the infection for the first time, whereas people living in endemic areas usually do not show such symptoms and signs (Evengard *et al*. 1990, Rabello 1995, Bica *et al*. 2000). Individuals who are living in endemic *Schistosoma* areas might be armed with mechanisms to alleviate their immune response towards the invading cercariae such as synthesis of IgG4 an infection blocking antibody class (Hussain *et al.* 1992) or by release of protective cytokines such as IL-12 (King *et al.* 1998).

The acute stage is associated with dermatitis which occurs in a time span between a few hours to one week after cercariae penetration. Acute infection is often characterised by an immediate hypersensitivity type response due to activation of the dermal mast cells and basophiles triggered by immunoglobulins of the class IgE which specifically respond to the released serine proteases from penetrating cercariae. This leads to itching and to maculopapular rashes (Farid *et al.* 1987, Capron& Capron 1994, Verwaerde *et al.* 1988).

Approximately 3-7 days after infection, schistosomulum reaches the lung causing allergic pneumonitis as part of the immediate hypersensitivity response against serine proteases released by the schistosomulum. There are coughing, leukocyte infiltration of the lung and

eosinophilia. These symptoms usually disappear within 1-2 weeks (Farid *et al*. 1987, Weltman & Senft, 1981, Capron & Capron 1994, Verwaerde *et al*. 1988).

Approximately 1-3 months after infection, when adult worms mature and start to lay eggs, typical Katayama fever symptoms caused by an acute generalized hypersensitivity response are observed in most naïve patients (de Jesus *et al*. 2002, Ross *et al.* 2007). There are non-specific symptoms such as fever, malaise, cough, headache, myalgia, fatigue, diarrhoea and abdominal pain which are more sever in children than in adults (Rabello 1995). Most of the affected people recover spontaneously 2-10 weeks later (Gazzinelli *et al*. 1985, Hiatt *et al*. 1979, Rocha *et al.* 1995). These symptoms could be attributed to formation of circulating immune complex in response to the released *Schistosoma* antigens inducing the release of pro-inflammatory cytokines (Ross *et al.* 2002, Bottieau *et al*. 2006, Visser *et al*. 1995, de Jesus *et al*. 2002). Katayama fever is more common with *S. japonicum* than *S. mansoni* due to higher egg depositions of the former species (Ross *et al*. 2001).

1.3.2 Chronic *Schistosoma* infection and complications

The chronic stage of schistosomiasis occurs several years after primary infection and when effective treatment has not been used. Most of the pathological manifestations occur as a consequence of egg deposition in the tissues, especially the liver and intestine with induction of inflammatory immune response (Caldas *et al*. 2008).

1.3.2.1 Intestinal schistosomiasis

Most of infected individuals living in the endemic areas are more or less asymptomatic during the intestinal schistosomiasis stage (Vennervald *et al.* 1998).

The passage of the deposited eggs through the intestinal wall causes pathological lesions affecting mainly the lower large intestine. There is granulomatous inflammation associated with congestion, ulcers and pseudopolyps (Cheever *et al.* 1978, Farah& Nyindo 1997). Abdominal pain or discomfort, anorexia and bloody or non bloody diarrhoea are the common symptoms (Gryseels 1988, Elliott 1996).

1.3.2.2 Hepatosplenic schistosomiasis

Chronicity of infection is accompanied by continuous deposition of more eggs, which accumulate in the pre-sinusoid space of the liver where they evoke granulomatous inflammation mainly induced by protolytic enzymes and other soluble antigens secreted by the eggs. Granuloma formation is the consequence of a Th2-dependent delayed hypersensitivity reaction. The granuloma is formed by CD4+ cells, eosinophils, plasma cells, multinucleated giant cells and macrophages. After the inflammation subsides, the granuloma induces a periportal fibrosis, called Symmers' clay pipe-stem fibrosis. Around 8-10% of patients suffering from *S. mansoni* and *japonicum* progress to this form of fibrosis within 5-15 years. Periportal fibrosis is the most serious late complication of *Schistosoma* infection and is responsible for portal hypertension with subsequent complications such as oesophageal varices, hepatomegaly, splenomegaly, ascites and fatal gastro-intestinal bleeding (Cheever *et al*. 1978, Boros& Warren 1970).

1.3.2.3 Ectopic schistosomiasis

Schistosoma eggs as well as adult parasites may find their way also to other distant sites and cause granulomatous inflammation and fibrosis, for example in the cerebral or spinal

venous plexus to cause transverse myelitis (Ferrari 2004) or in the lung generating cor-pulmonal and pulmonary hypertension (Lambertucci 1993).

1.4 Schistosomiasis control

Dependent on funds for the treatment, control of schistosomiasis is possible, even though a complete eradication is not easily achieved (Bergquist *et al.* 2002). Successful control programmes need a sustained long-term commitment far in excess of twenty years (Chitsulo *et al*. 2000). Egypt is one of the countries which has a well designed, successful and continued national anti-schistosomiasis control programme (Bergquist 2008).

The main challenges in controlling the parasite are the ubiquitous presence of the intermediate snail hosts, the inefficiency of primary health care measures and the lack of funds in many endemic regions where people have to rely on utilizing infected water to maintain their daily life activities (WHO 2001, Ximenes *et al.* 2000, Gazzinelli *et al*. 2006).

i) Diagnosis

Discovery of infected persons is an essential component of any national control programme which aims to eradicate the infection source (Sturrock 2001). Diagnosis is discussed with detail in the diagnosis section.

ii) Chemotherapy

The introduction of praziquantel (PZ) was a major milestone achievement of the national programmes for control of this disease (Bergquist 2008). It is easily administrated, effective

and posses a long lasting effect on reducing morbidity (WHO 2002, Magnussen 2003 and Savioli *et al.* 2004a&b). The main disadvantage of PZ is that it does not prevent re-infection and thus, it is required that it is to be taken for an unforeseeable period (WHO 2002, Gryseels 1996).

iii) Sanitary measures

The provision of clean water supplies and the establishment of sanitary measures in the endemic areas protect people from using infected water and hence affect the disease distribution and incidence (WHO 1993).

iv) Targeting of the intermediate snail host

Between 1930 and 1985, control of schistosomiasis mainly depended on the use of molluscicides and antimony-based prescriptions for the treatment of patients (Engels *et al.* 2002). The most widely used molluscicides is niclosamide (Andrews *et al.* 1982). Use of predators or competitor snails to substitute molluscicides, has achieved promising results reducing the numbers of intermediate host snails (Giboda *et al*. 1997). Drainage of irrelevant water streams and changes in the irrigation systems to provide unsuitable environmental conditions for the multiplication of intermediates host snail have been effective in limiting *Schistosoma* dissemination (Bergquist 2002). Snail control has, now, a diminished importance in the overall control programme due to the relatively high cost of the molluscicides, their environmental side effects and the availability of PZ (Sturrock 2001, Bergquist 2002).

v) Health education

Health education accompanied with other environmental measures gives good results (Gazzinelli *et al.* 2006). The persons most at risk such as school children and farmers are supplied with general information about the *Schistosoma* life cycle, effects of the disease on their health and precautions to avoid catching the infection and they are encouraged to attend for regular examinations of stool and urine samples (Bergquist 2002). The programmes should be directed to different cultural sectors of the community (Parker *et al.* 2004).

vi) Topical chemotherapy

The use of topical drugs with cercaricidal effects could be a part of the measures which combat *Schistosoma*. N, N-diethyl-m-toluamide (DEET) is a topical lipid therapy which kills cercariae in the skin of animals providing a high level of protection and achieving good results also in humans (Ramaswamy *et al.* 2003). It should be applied before exposure to potentially infected water (Haas& Haeberlein 2009).

1.5 Immunopathology and immune response of schistosomiasis

The degree of severity of *Schistosoma* morbidity is affected by many factors; the host immune response towards *Schistosoma* (Mentink-Kane *et al.* 2004), the multiplicity of infection (Kabatereine *et al*. 2003), the period of exposure (Booth *et al*. 2004), the presence of other infectious agents (Kamal *et al.* 2004) and the genetic background of both the host and the infecting *Schistosoma* (Chevillard *et al.* 2003).

1.5.1 Immune response to migrating schistosomulum

The excretory/secretory (ES) products which can be released within the first 3 hours by infecting cercariae, are less effective in stimulating the proliferation of lymphoid cells than soluble whole schistosomula extracts (Mountford *et al.* 1995). ES products facilitate migration of cerceriae in the skin (Fallon *et al.* 1996). ES products mediate the degranulation of mast cells to release mediators such as histamine and IL-4 *in vitro* (Machado *et al.* 1996). When cercariae migrate through skin, inflammatory cells, including dendertic cells (DC) are attracted to the site of infection which, at a later stage, mature evidenced by the *de novo* expression of CD86 and MHC-II (Angeli *et al.* 2001). Upon activation, DC directs Th2 polarization (Jenkins& Mountford 2005, d'Ostiani *et al.* 2000). DCs are involved in the activation of CD8+ cells (Bennett *et al.* 1998).

1.5.2 Immunopathology of acute schistosomiasis

Acute schistosomiasis is rare in people living in endemic infection zones, because they are exposed to early infection, and a baby born to an infected mother, becomes sensitized to *Schistosoma* antigens *in utero* or by breast milk from infected mothers (King *et al.* 1998, Malhotra *et al.* 1997, Montesano *et al.* 1999). Immunoglobulins specific to antigens present on adult parasite or eggs of the classes; IgG, IgM and IgE are detectable at similar levels during either acute or chronic stages of the infection (Rabello 1995, Kanamura *et al.* 1979).

In the acute stage of infection, peripheral blood mononuclear cells (PBMCs) show significant higher proliferation in response to soluble egg antigen (SEA) and soluble worm antigen preparations (SWAP) than the proliferation seen in chronic patients. Cytokine

profiles are mixed between Th1 and Th2 phenotypes with Th1 predominance with IFN-γ reaching its peak four months after infection. IL-5 responses towards specific parasite antigens are found in acutely infected patients, while IL-10 is only detected at low concentrations (Malaquias *et al*. 1997, Montenegro *et al.* 1999, de Jesus *et al.* 2002).

1.5.3 Immunopathology of chronic schistosomiasis

Most of the morbidity features of chronic schistosomiasis depend on the host's T cell immune response against parasite eggs (Abath *et al.* 2006). During the chronic stage of *Schistosoma* infection, the Th2 cytokine profile generally mounts inflammatory responses which are responsible for most of the pathophysiological manifestations of this stage of the disease (Hoffmann *et al.* 2000). PBMC respond strongly to both SEA and SWAP as the patients move on from intestinal to early of hepatosplenic stage. During late hepatosplenic stage of the disease, patients neither respond to SEA or SWAP (Gazzinelli *et al.* 1985). In the late hepatosplenic stage, cytokine profiles are variable, predominantly of the Th1 phenotype in some cases (Mwatha *et al.* 1998) or of a Th2 profile in other cases (de Jesus *et al.* 2004).

1.5.4 *Schistosoma* granuloma

Granulomas forming around parasite eggs are the most remarkable manifestation of schistosomiasis (Stadecker *et al.* 2004). The granuloma results from a cell-mediated delayed hypersensitivity response occurring around trapped parasitic eggs. It is formed from various types of cells; CD4+, CD8+, eosinophils, macrophages, fibroblast and neutrophils which are held together by a fibrous matrix (Boros& Warren 1970, Grzych *et al.* 1991, Sandor *et al.* 2003). It may cause inflammatory necrosis which can heal leaving

large deposits of extracellular matrix proteins (ECMPs), and then this scar tissue is in turn substituted by normal cells. Fibrosis, which is a pathological condition, results from abnormally heavy and cross-linked depositions of ECMPs in the granulomsa tissue (Kaplan *et al.* 1998, Wynn *et al.* 1997). ECMPs are produced by the myofibroblast satellite cells, which divide and multiply in response to IL-13, IL-4 and TGF-ß (Postlethwaite *et al.* 1988, Tiggelman *et al.* 1995a& b) and are inhibited by INF-γ (Mallat *et al.* 1995, Rockey& Chung 1994). Fibrosis rather than the size of the granuloma determines the severity of human disease (Brunet *et al.* 1998). The granuloma and fibrosis are regulated by independent mechanisms as shown by IL-4 neutralization leading to a decrease of fibrosis without affecting the size of the granuloma (Cheever *et al.* 1992& 1994, Cheever& Yap 1997).

Granuloma formation has multiple effects on both the host and the parasite. From the stand point of the parasite, the granuloma may facilitate the passage of eggs across the intestinal wall to the gut lumen to be secreted and complete the life cycle of the parasite. This is evidenced by low egg output of infected CD4+T cell deficient mice (Doenhoff 1997, Cheever *et al*. 1999) or in immunocompromised patients, for example in individuals suffering from AIDS (Karanja *et al.* 1997). For the host, it forms a physical barrier which hinders penetration of the toxic compounds produced by the parasite eggs into the surrounding tissue (Doenhoff *et al.* 1981& 1986, Dunne& Doenhoff 1983, Fallon& Dunne 1999).

In humans, granuloma formation can be observed around 8 weeks after infection, a period when down-modulation occurs and the infection enters into a chronic stage in most of the

affected individuals. Patients who fail to mount this immuno-modulation progress directly to the hepatosplenomegally stage of the disease (Domingo& Warren 1968, Montesano *et al.* 1990).

1.5.5 *Schistosoma* infection and co-infection

Schistosoma infection increases the severity and susceptibility to other infectious diseases (Secor 2005), the most important example in Egypt is hepatitis B and C, where patients suffering from both, show a significantly higher degree of morbidity than patients with virus hepatitis alone and the Th2 polarization caused by *Schistosoma* infection counteracts the Th1 immune response required to overcome virus infection (Kamal *et al.* 2004, El-Kady *et al.* 2005).

1.6 Human resistant against *Schistosoma* infection

The extend of human resistance against *Schistosoma* infection is difficult to define (Hagan *et al.* 1998) and the mechanisms which are responsible for immune protection against *Schistosoma* infection within a population need to be better understood. Identification of such protective mechanisms may help to understand immune profiles required to design an effective ani-*Schistosoma* vaccine (Hewitson *et al*. 2005). Factors that influence the development human immunity against *Schistosoma* infection include age, severity of the immune response, supportive chemotherapy and genetic factors.

a) Age related immunity in untreated and treated communities

A huge number of studies in endemic communities show that the infection load is higher in younger children than in adults pointing to the role of age in the development of resistance

against parasite infection. The infection peaks at teenage age and then decreases afterwards, but in higher infected areas, it occurs earlier and with higher severity as reported in many studies, for example for *S. haematobium* infection in Gambia (Woolhouse *et al*. 1991) and Zimbabwe (Mutapi *et al*. 1997) and *S. mansoni* in Kenya (Fulford *et al.* 1992).

This difference in susceptibility to infection between the adult and children might be attributed to many factors:

i) Pattern of water exposure

The duration and behaviour in contact with water varies between children and adults and this could partially explain the higher rate of infection in the children sector (Chandiwana 1987, Chandiwana *et al*. 1991, Demeure *et al*. 1993, Fulford *et al*. 1996).

ii) Hormonal effects

During puberty, some of the physiological changes that occur in the human body could alter the host parasite relationship hindering *Schistosoma* metabolism and development (Butterworth *et al*. 1994, Fulford *et al.* 1998, Capron 1992). This was proven in experimental animals, where testosterone was found to affect the development of *Schistosoma* (Nakazawa *et al*. 1997).

iii) Immune response development

During puberty, the innate immune response becomes intensified by increasing thickness of the skin, high depositions of subcutaneous fat and alternations of the skin fatty acids.

All these factors may hinder the cercariae penetration and migration through the skin (Kurtis *et al.* 2006).

Blocking antibodies could explain the higher susceptibility of young children to re-infection than older children and adults (Dunne& Mountford 2001). Younger children develop antibodies against egg carbohydrate antigens which cross-react with schistosomula glycoproteins (Mazza *et al*. 1990, Langley *et al*. 1994). *In vitro* experiments have proven an ability of such blocking immunoglobulins to prevent schistosomulum killing mediated by eosinophils (Khalife *et al*. 1989). Blocking anti-egg carbohydrate antibodies were detected in many groups studied. In Kenya, the younger children show high levels of IgG2 and IgM (Butterworth *et al*. 1985& 1988, Dunne *et al*. 1988) and in Brazil, IgG2 and IgG4 were found in the infected individuals (Demeure *et al.* 1993). Hagan *et al.* (1991) discovered that IgE specific against *S. haematobium* adult and egg antigens is significantly higher in older children and adults who show lower levels of re-infection load. In case of *S. mansoni*, IgE specific against the adult worm was positively correlated with resistance against re-infection in adults and older children, whereas IgM, IgG2 and IgG4 are correlated with higher susceptibility to infection (Dunne *et al.* 1992, Rihet *et al*. 1991& 1992).

b) Length of exposure to *Schistosoma* infection and the development of immunity

The most protective mechanism of defence is accumulated acquired immunity (Dunne& Mountford 2001). This was made clear by Satti *et al*. (1996) in Sudan, who studied two groups of adults who clean the water canals. One group was exposed for a long period and the other was only recently exposed. After one year of successful PZ treatment for both groups, the second group had double the load of infection, despite both groups being exposed under the same circumstances.

Regardless of age factor, people who are exposed to the infection for a long time and are resistant showed higher IgE and IgG1 against the whole worm antigens (Satti *et al*. 1996) or against particular *Schistosoma* antigens for example, *S.mansoni* 28-kD glutathione-S-transferase (Grzych *et al.* 1993) and 22 kDa *Schistosoma* antigen (Dunne *et al.* 1992).

c) Chemotherapy and modulation of the immune response in the direction of resistance

Chemotherapy can modulate the immune reactions against the parasite by abolishing the suppressive effects of *Schistosoma* infection on the immune system. Treatment also kills parasites inside host blood vessels causing the release of novel parasitic antigens to the immune system which senses them for the first time (Viana *et al.* 1995, Grogan *et al*. 1996 a& b, Correa-Oliveira *et al.* 2000). Treatment whether by praziquantel or oxamniquine has many effects on the immune system, for example, after treatment the IgE specificity to worm antigens increases (Dunne *et al.* 1991, Webster *et al.* 1997) and the numbers of eosinophils was 6 fold higher than before the treatment (Kimani *et al*. 1991).

d) Genetic role in human resistance against *Schistosoma* infection

Abel *et al.* (1991) used segregation analysis on 20 Brazilian pedigrees and the results showed that the intensity of *S. mansoni* infection was affected by a codominant major gene with a frequency of 0.2-0.25 for the respective protective allele. This gene, which is called *SM1,* is located on 5q31-q33 chromosome within a cluster of genes that support a protective immune response against *Schistosoma* by controlling many cytokines such as IL-4, IL5& IL-13. This region is linked to another region on the chromosome which is involved in the regulation of eosinophils and IgE (Marquet *et al*. 1996& 1999). A

secondary study in a community in Senegal confirmed this *Schistosoma* resistance gene in clusters (Muller-Myhsok *et al*. 1997).

e) Putative immune & endemic normal

A small group of people in endemic areas are naturally immune to *Schistosoma* infection. Although they are in continuous contact with infected water and do not take any anti-*Schistosoma* chemotherapy, they are *Schistosoma* negative. Generally, they are older individuals and their anti-*Schistosoma* IgE levels are higher than IgG4. The peripheral blood mononuclear cells of the natural resistant individuals secrete higher levels of IFN-γ when exposed to *Schistosoma* antigens (Correa-Oliveira *et al*. 1989, Viana *et al.* 1995).

1.7 Complement

The complement system provides a mechanism which is a part of the innate and adaptive immune response against parasites. It includes a group of plasma proteins which are activated in cascade manner. The terminal activation cascade of complement forms membrane attack complexes which insert in the membranes of the intravascular parasites. Complement activation leads to the generation of numerous activation products which enhance the immune response by opsonising foreign antigens and attracting phagocytes and other leukocytes to the location of infection. There are three complement activation pathways; the classical, the alternative and the lectin. The classical pathway is initiated by immune complexes, the lectin one becomes activated when MBL (mannan-binding lectin) or ficolins bind to carbohydrates found on the surface of the organisms or parasites, whereas the alternative pathway is initiated by direct contact between the activated

complement components and the surface of pathogens. C3 is a key component of the complement system (Bohlson *et al*. 2007, Walport 2001a& b).

1.7.1 *Schistosoma* and complement

a) The classical pathway

Classical pathway activation starts when immunoglobulins of class IgM and nearly all members of class IgG bind to the parasite. Immunoglobulins of different classes were identified on outer surface of *Schistosoma* either by cytochemical or immunocytochemical techniques in addition to Fc regions of Ig receptors (Kemp *et al.* 1977& 1980, Tarleton & Kemp, 1981). The antibodies activate the initial molecule of the classical pathway, C1, which is formed of three glycoproteins; C1r, C1s and C1q as a recognition subcomponent which binds to the fixed antibodies and thereby initiate the classical pathway (Weiss *et al.* 1986, Perkins *et al.* 1986).

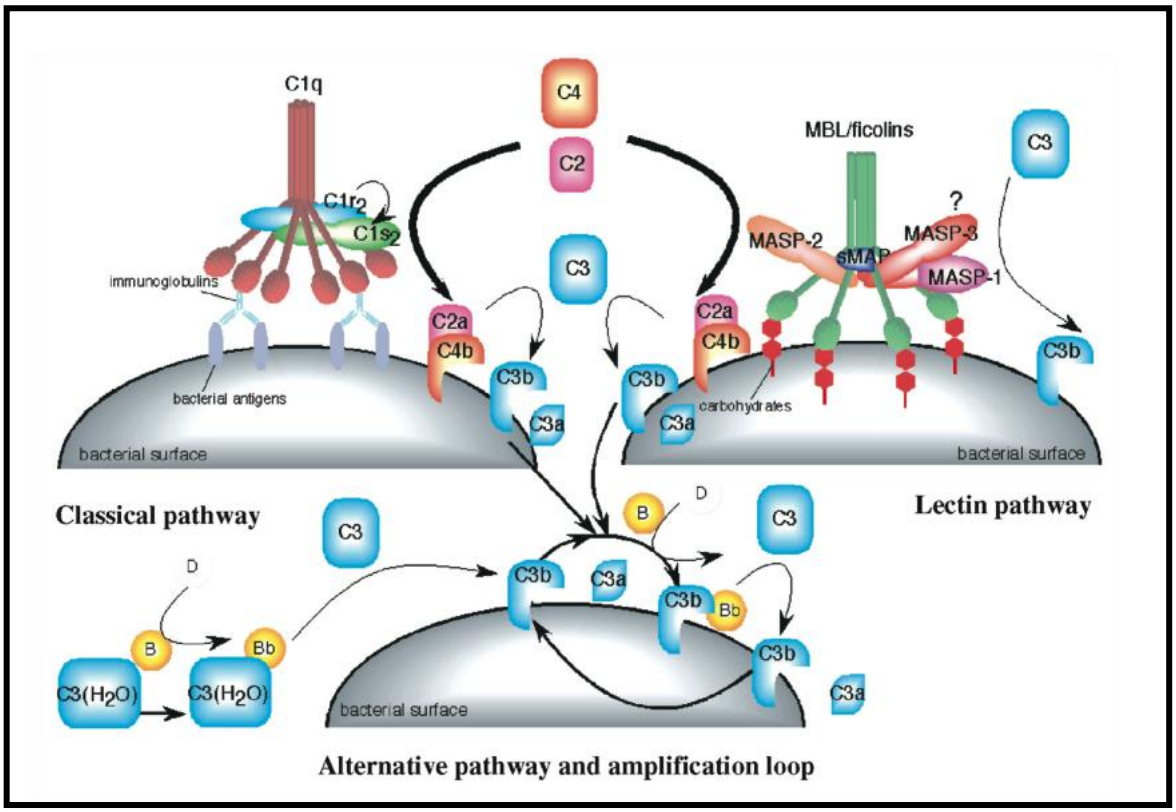


Figure 2: Complement pathways (Fujita 2002)

b) **The lectin pathway**

For the lectin pathway, MBL and L-ficolin, which are plasma components, are the recognition molecules that bind to the carbohydrates in particular to mannose on pathogen surfaces (Schwaeble *et al.* 2002). The surface of cercariae and adult *Schistosoma* expose carbohydrates which bind to host MBL as shown by electron microscopy and fluorescence microscopy. In C1q deficient serum, it was shown that MBL alone is sufficient to fix C4c complement component on the tegument. However, this pathway appears to be not effective in killing *Schistosoma* (Klabunde *et al.* 2000).

1.7.2 Anti-complement *Schistosoma* mechanisms

Adult stage parasites and schistosomulae have developed many mechanisms to avoid the lethal effect of the complement (Fishelson 1995, Capron 1992). *Schistosoma* gets rid of the host immunoglobulin by ingestion through its gut and binding by FcR on the tegument, then these Ig are processed within the excretory system of *Schistosoma* (Thors *et al.* 2006) and this renders the classical complement pathway unable to activate complement. *Schistosoma* may also evade complement attack by breaking down complement components or complement convertases on its surface. The parasite may deplete these components by binding them to the outer membranes and replace those through rapid membrane turnover (Ghendler *et al*. 1996, Brouwers *et al.* 1999).

Schistosoma possess many molecules which inhibit complement activation. CRIT (complement C2 receptor inhibiting trispannin) is firstly discovered in *S. haematobium* then *S. mansoni* and it was detected by its specific antibodies using the electron microscope, in the apical plasma membrane and surface pits of the adults. This protein acts as a receptor for the C2 molecule and can hinder the formation of the C3 convertase, C4b2a (Inal 1999, Inal& Schifferli2002, Inal& Sim 2000). *In vitro* studies showed that the peptides taken from the C-terminal part of this protein inhibited activation of the complement (Inal *et al*. 2003).

Circulating Anodic Antigen (CAA), which is released from the intact *Schistosoma* intestine, acts as a soluble receptor for C1q. It binds to the collagen–like stalks of C1q *in vitro*, so, it could have a role in protecting the parasite intestine against complement activation by immune complexes *in vivo* (Van Dam *et al.* 1993).

A novel 130 kDa protein described on the surface pits and within the multilamellar vesicles of *Schistsosoma*, was shown to be produced by the parasites. This protein is a C3 receptor which binds and inhibits the activation of host C3 thus inactivating all the three pathways at the C3 activation stage (Rasmussen& Kemp 1987, Silva *et al*. 1993).

Another antigen, *Schistosoma* paramyosin, has multiple roles in the protection of *Schistosoma* against host complement. Paramyosin was shown to bind to C1q *in vitro* (Laclette *et al*. 1992) and it also binds to human complement components C8 and C9 preventing the insertion of the membrane attack complex (Deng *et al*. 2003a& b& 2007). *Schistosoma* paramyosin was originally named *Schistosoma* Complement Inhibitory Protein-1(SCIP-1) (Parizade *et al.* 1994). It was later shown that SCIP-1 is an isoform of paramyosin and is present on the surface of the parasite (Deng *et al*. 2003& 2007) Paramyosin was also suggested to act as Fc receptor (Loukas *et al.* 2001).

Delay-accelerating factor (DAF) is a human complement regulatory component which limits the half life of C3 and C5 convertase. This molecule is captured from human plasma by *Schistosoma* protecting the parasite itself from complement attack (Pearce *et al.* 1990, Horta *et al*. 1991).

A 28-kDa cercarial serine protease was shown to cleave many complement components including C9, C3 and iC3b (Fishelson *et al.* 1992, Parizade *et al.* 1994). Another antigen of 56 kDa was found on the *Schistosoma* surface which has serine protease inhibitor activity and interfere with the elastase released from neutrophil (Ghendler *et al.* 1994).

1.8 Diagnosis of schistosomiasis

The diagnosis of schistosomiasis depends on historical evidence of contact with infected water, the clinical picture and laboratory investigations. Passing of blood in urine, haematuria, especially in school children in endemic areas could point to schistosomiasis caused by *S. haematobium*. However, *S. mansoni* has more non-specific symptoms and signs such as bloody diarrhoea which could be a symptom for many other diseases (Savioli *et al.* 1990). The diagnosis is assisted by laboratory tests and radiological investigations.

1.8.1 Parasitological methods

Schistosoma eggs can be identified by microscopy in stool, urine or other tissues through their characteristic shapes. This method is widely used and considered to be the most specific method to diagnosis the infection (Hamilton *et al*. 1998, Doenhoff *et al*. 2004). The detection of parasite egg by microscopy is the gold standard for the diagnosis of schistosomiasis (Goncalves *et al*. 2006). There are many techniques used for the preparation of stool or urine specimens to be examined by microscopy for example, the formol-ether concentration method (Blagg *et al.* 1955, Knight *et al.* 1976) or the Kato-Katz smear (Katz *et al.* 1972) are used for stool specimens, whereas sedimentation and filtration techniques are used for urine samples (Dazo& Biles 1974).

Kato and Miura (1954) described a thick-smear method for stool examination to detect eggs of different helminths including *S. mansoni* and *S. japonicium.* The stool smear is covered with a cellophane strip soaked in glycerol/malachite solution. Katz *et al.* (1972) developed a card with hole in its centre with known dimensions accommodating 50 mg stool and then

the method known as Kato-Katz. Kato-Katz thick smear is mostly recommended for diagnosis of intestinal *Schistosoma* in endemic areas for both national control programmes and epidemiological purposes (WHO 1994, Bowie *et al.* 2004). This technique is quantitative, easily used, of high specificity, rapid and performed at low cost. It requires simple equipments and the persons doing this test can be easily trained (de Vlas& Gryseels 1992, Engels *et al*. 1996, Ebrahim *et al.* 1997). However, a single Kato-Katz smear has low sensitivity and may miss cases excreting fewer than 100 epg (egg per gram of stool), hence may overestimate the cure rate (Barreto *et al.* 1990, Engels *et al.* 1996, Teesdale *et al*. 1985, Ebrahim *et al.* 1997, Eberl *et al.* 2002).

Few eggs in a stool sample may limit the sensitivity of the Kato-Katz test and can be caused by the presence of immature worms that are not yet secreting eggs (Cheever 1968), or by the fact that, the eggs may not be distributed evenly in the stool (Ye *et al.* 1998). There can also be day to day variations in eggs depositions (Engels *et al.* 1996, van Etten *et al.* 1997, Kongs *et al.* 2001). Likewise, severe intestinal fibrosis developed with chronic infection could also hinder eggs secretion (Cheever 1968).

To increase its sensitivity, multiple Kato-Katz smears from the same stool sample or from subsequent stool specimens need to be examined (Rabello, 1997, Engels *et al*. 1997, Gryseels & de Vlas 1996, Kongs *et al.* 2001), however, frequent sampling requires further visits to the clinic, many patients could be of low compliance and sampling two-consecutive-day samples involves higher cost (Berhe *et al*. 2004). Many attempts were made to replace the Kato-Katz test, for example the digestion-sedimentation technique (Borel *et al*. 1999), using negrosin-eosin stain instead of malachite green (Sayed *et al.*

2002), but nearly all of these methods yielded either similar or even lower sensitivity than the routine Kato-Katz test.

Rectal biopsy could be examined microscopically to look for *Schistosoma* eggs, however, it requires a specialised medical doctor to perform this biopsy as this is an invasive procedure, while a two to five stool sampling examination can result in nearly the same level of accuracy as rectal biopsies (Abdel-Hafez& Balbol 1992, Rabello *et al.* 1992 b).

The hatching test is another less popular microscopic test which is designed to see the miracidia coming from the parasite eggs (Fulleborn 1921, Chieffi *et al.* 1978). However, this test produces unsatisfactory sensitivity and is not suitable for field studies (Ross *et al.* 2001, Yu *et al*. 2007).

1.8. 2 Immuno-diagnostic methods

After more than 40 years of using Kato-Katz, it has been realized that the field of diagnosis of *Schistosoma* still needs more sensitive techniques (Bergquist 2008). Serology based tests may be complementary for the routine parasitological methods or even substitute them (Doenhoff *et al.* 2004, Xiang *et al.* 2003).

1.8.1.2.1 Direct methods to detect specific *Schistosoma* circulating antigens

Direct detection of specific antigens shedding from different *Schistosoma* stages as markers of infection have considerable advantages; they indicate ongoing active infection (van

Lieshout *et al.* 1994, de Clercq *et al.* 1997) and some circulating antigens may also be detected in urine samples (van Etten *et al.* 1996). Some of these antigens may be valuable information for follow-up patients having treatment (de Clerq *et al.* 1997). However, these techniques may also have disadvantages, for example low sensitivity in cases harbouring low *Schistosoma* infection (De Jonge *et al.* 1991). The detection of some antigens show often similar sensitivity to that observed in the parasitological examination (Van Lieshout *et al.* 1995), the monoclonal detecting antibodies are of high costs, the procedure may be time consuming and require expensive equipment (Krijger *et al.* 1994, Rabello 1997). In general, all specimens should be pre-treated with trichloroacetic acid or heat-incubation in an alkaline buffer with pH 9.6 (Krijger *et al.* 1994).

a) Adult derived antigens

The most widely studied *Schistosoma* antigens as markers for parasite sero-diagnosis are circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). Both of them are gut-associated proteoglycans and CAA migrates to the anode during electrophoresis, while CCA migrates towards the cathode (Nash *et al.* 1974, Nash& Deelder 1985). The antigens are shed from adult parasites into the host blood (Deelder *et al.* 1994). Both CAA and CCA can be detected in serum and urine, but in urine CCA concentration is higher than CAA (De Jonge *et al.* 1989, Krijger *et al.* 1994).

CCA molecule contains repeating units of Lewis–X trisaccharide epitopes which might cross-react with antigens released by other pathogens sharing these epitopes and thus may produce false positive results (Van Dam *et al*. 1994). In contrast, CAA has unique repetitive carbohydrate units which explains why this parasite antigen give a higher

specificity (Bergwerff *et al.*1994). The serum CAA level was found to correlate with worm burden in many infected subjects (Deelder *et al*. 1994, Agnew *et al.* 1995, van Lieshout e*t al.* 1995), but this may not always be true (Polman *et al.* 2000).

Monoclonal antibodies specific for CAA and CCA are used to capture them in different assays such as sandwich ELISA, indirect haemagglutination, time-resolved immunofluorometric assay, magnetic bead immunoassay and reagents strips (Deelder *et al.* 1989a& b, De Jonge *et al.* 1989, Gunderson *et al.* 1992, Van Etten *et al.* 1994, Van Dam *et al.* 2004, Stothard *et al.* 2009).

b) Larva derived antigens

A circulating polypeptide cercarial antigen of 41 kDa can be detected 3 days after *Schistosoma* infection in experimental mice by a competitive inhibition ELISA using the rabbit antiserum developed against this cercarial antigen. This antigen was prepared by hydrophobic chromatography from cercarial preparation. In a human study, this assay achieved specificity of 85% and 100% sensitivity (Hayunga *et al.* 1986& 1987).

c) Egg derived antigens

Circulating egg antigens were detected in the case of *S. haematobium* in urine by Kahama *et al*. (1998a& b). When using a sandwich ELISA to determine the excreted circulating egg antigens in urine after chemotherapy, it was found that the level of these antigens decrease ,but at a slower rate in comparison to CCA (Nibbeling *et al.* 1998a& b, Nourel Din *et al.* 1994a& b). In elderly patients, their excretion in the urine is significantly lower in

comparison to the egg output and circulating soluble egg antigen levels in serum (Nourel Din *et al.* 1994a).

Recently, *S. mansoni* eggs were found to secrete unique unconjugated oligosaccharides, glycoproteins and glycolipid antigens. It was suggested that mass spectrometric detection of the specific oligosaccharides in urine could be useful in the diagnosis of schistosomiasis (Robijn *et al.* 2007a& b& 2008).

1.8.2.2 Indirect methods for detection of specific *Schistosoma* antibodies

These methods depend on the detection of specific antibodies against certain parasite antigens. Many assays are described for detection of the *Schistosoma* specific antibodies such as dot blot assay (Boctor *et al.* 1987), Dot-dye-immunoassay (Rabello *et al.* 1992a), FAST (Falcon Assay Screening Test)-ELISA (Hancock& Tsang 1986) and TSP (Transferable Solid Phase)-ELISA (Moser *et al.* 1990). The criteria which give superiority of one antigen over others are indicated by the criteria of sensitivity and specificity, in addition to the cost implications of the procedures used (Hamilton *et al.* 1998).

Nowadays, the most widely used system for the detection of the specific antibodies is the enzyme-linked immunosorbent assay (ELISA), which was introduced by Engvall and Perlmann (1971). Voller *et al.* (1974& 1976) first described an ELISA technique which is performed in microtitre plates. Since then it has become the most widely used assay for *S. mansoni* sero-diagnosis. ELISA is easy, reproducible and can accommodate large numbers of samples (Haimlton *et al.* 1998, Doenhoff *et al.* 2004).

a) Adult derived antigens

Materials prepared from adult *Schistosoma* can be obtained easily and in large amounts when compared with other parasite stages (Hamilton *et al.* 1998, Doenhoff *et al.* 2004). Crude adult antigens were applied intra-dermally, or used for ELISA and for blotting assays. The intra-dermal test is of low sensitivity in the children and women and give a high rate of false negative reading (de Noya *et al.* 1992). ELISA coated with soluble worm antigen preparations (SWAP) allows differentiating between acute and chronic patients (Valli *et al.* 1997).

Western blot analyses using crude adult *S. mansoni* antigens achieved higher specificity and sensitivity in comparison to indirect haemagglutination test (IHA) and immune-fluorescence antibody test (IFAT). With blotting, six proteins within the range of 65-120 kDa in size, were shown to be specific for *Schistosoma* infection and these proteins show no cross-reaction with other parasitic diseases (Sulahian *et al.* 2005). Immuno-blotting of some *n*-Butanol extracted antigens from the adult *S. mansoni* membrane is of high sensitivity and specificity (Cesari *et al.* 2005).

Detection of antibodies of the IgM class against *S. mansoni* gut polysaccharide antigens on whole adult parasite sections fixed with paraffin and analysed by immuno-fluorescence microscopy, was used for diagnosis of schistosomiasis. This method is valuable in a low transmission area (Hoshino-Shimizu *et al.* 1992, van Lieshout *et al.* 2000). However, persons who are exposed to *Schistosoma* infection for long periods of times have low IgM levels against *S. mansoni* gut polysaccharides antigens, so IFAT-IgM is not suitable in endemic areas (Sorgho *et al.* 2005). In addition, IFAT-IgM detection is expensive, as it

requires expensive fluorescent microscope and this method is not suitable for examining a large number of samples (van Lieshout *et al.* 2000, Oliveira *et al.* 2005).

Adult worm's vomitus samples were tested by ELISA to diagnosis *S. mansoni* infection in endemic areas in Africa. This method allows identifying as much as 94.6% of the infected individuals (Bahgat *et al.* 2006).

Another parasite antigen termed adult microsomal antigens (MAMA) was characterized by Tsang *et al.* (1983). When using MAMA as an antigen for FAST- ELISA detection of anti-parasite antibody, nearly 100% specificity and sensitivity were seen in the immuno-diagnosis of *S. mansoni* (Hancock& Tsang 1986). The Centre for Disease Control (CDC) (USA) uses MAMA for the initial screening of schistosomiasis in a FAST-ELISA procedure to analysis sera of travellers returning from the endemic areas. A subsequent species-specific confirmation is achieved using the same antigen in an immuno-blotting assay (Tsang& Wilkins 1997).

Cathepsin B, a 31 kDa antigen (Gotz& Klinkert 1993) and asparaginyl endoproteinase, a 32 kDa antigen (Dalton *et al.* 1995) are cysteine proteinases which are proteolytic immunogenic enzymes belonging to the excretory/secretory products of the *S. mansoni* digestive tract. Their specific antibodies appear in sera 4 weeks after the commencement of infection (Ruppel *et al.* 1991& 1987). Approximately 82-88.6% of serum samples of patients recognized these recombinant enzymes in a study carried out in Africa (Klinkert *et al.*1991).

Specific antibodies against recombinant *S. mansoni* Hsp70 were found in patients suffering from chronic infections. They were, however, not detected at the acute stage of infection (Moser *et al.* 1990).

RP26 is a parasitic protein of 26 kDa which was recombinantly expressed using adult *S. mansoni* cDNA library as a template to generate the ORF encoding this protein. The amplified sequence showed 99% identity to the cDNA sequence of the previously described Sm22 antigen. RP26 is useful for the diagnosis of acute infections as 89% of patients with acute *S. mansoni* infections were identified by ELISA. However, only 26% chronic patients were positive. In experimental mouse models of infection, it was shown that these antibodies are detectable up the time point 9 weeks post-infection then diminished (Makarova *et al*. 2003).

b) Larva derived antigens

The Cercarien Hullen Reaction (the formation of a membrane around the cercariae when exposed to infected *Schistosoma* serum) was used for the diagnosis of specific anti-cercariae antibodies (Vogel& Minning 1948). Its efficacy is better than that of IHA and of stool examination, but it is still second to the efficacy of rectal biopsy analysis (Ahmed *et al*. 1993).

There are many individual cercarial antigens which were evaluated for *Schistosoma* diagnosis including, cercarial elastase antigen which showed 87.2% sensitivity (Ramzy *et al*. 1997).

c) Eggs derived antigens

Circumoval precipitin is a very old test developed by Oliver-Gonzales (1954) which was used for the detection of specific antibodies towards *S. mansoni* and S. *haematobium* eggs. This test allows differentiating between active infection and past infection, but it is not suitable for the epidemiological screening as it is very expensive and requires the preparation of live eggs (Spencer *et al.* 1991, Alarcon de Noya *et al*. 1997).

Soluble egg antigens (SEA) are more sensitive and specific than their counterpart adult parasite extracts (Lunde *et al*. 1979& 1980, Mott *et al*. 1987, Khalil *et al*. 1989, Tanabe *et al*. 1990). ELISA-SEA of *S. mansoni* is used in the routine diagnosis of schistosomiasis in parasitology laboratories in U.K for travellers returning from endemic zones (Whitty *et al.* 2000, Van Gool *et al*. 2002). It has been used as a front line diagnostic method for the detection of infected persons in low transmission areas such as Brazil and Venezuela (de Noy *et al*. 1992).

In a comparison between different antigen preparations in endemic areas in Africa, the SEA-ELISA was superior to the SWAP-ELISA in the group of egg passing infected individuals (Sorgho *et al*. 2005). The detection of specific IgA against egg antigens allowed discrimination between acute and chronic *Schistosoma* infection (Liping *et al*. 1996). IgG immunoglobulin specific to SEA were detected not only in human serum, but also in the saliva with nearly the same sensitivity and specificity in the serum when both were tested by ELISA (Garcia *et al*. 1995, Santos *et al*. 2000).

During *Schistosoma* infection, anti-egg antibodies develop against only a limited number of glycan egg antigens (Doenhoff *et al*. 2004). From the six fractions of *S. mansoni* egg homogenates obtained by cationic exchange chromatography, cationic fraction (CEF6) showed the highest specificity and sensitivity (McLaren *et al*. 1981, Turner *et al*. 2004, Doenhoff *et al*. 1985, Dunne *et al*. 1988). CEF6 was used in sero-diagnosis of *S. mansoni* in an endemic area in Saudia Arabia with 90% sensitivity and 50% specificity (Ghandour *et al*. 1997).

It is interesting to note that for *Schistosoma* diagnosis, people have successfully used also a non-derived parasite antigen like keyhole limpet haemocyanin (KLH). KLH (derived from *Megathura crenulata,* marine mollusc) has similar carbohydrate epitopes to those found on the surface of *S. mansoni* schistosomulae (Grzych *et al*. 1987). There is a stringent cross-reactivity between immunogenic elements of KLH and the three major immunogenic egg antigens; omega-1, alpha-1 and kappa-1 (Hamilton *et al*. 1999). Specific IgG and IgM levels are high in acute infection and then decrease, when the infection becomes chronic (Alves-Brito *et al*. 1992, Markl *et al*. 1991, Xue *et al*. 1993). KLH, however, does not allow to differentiate between the acute and chronic infection in non-endemic patients and its sensitivity is relatively low (Verweij *et al.* 1995).

1.8. 3 Detection of *Schistosoma* DNA materials

Hamburger *et al*. (1991) described a highly repetitive 121 bp sequence of *S. mansoni* DNA which represents around 10% of the parasite genome. PCR amplification of this fragment was used in humans with high sensitivity and specificity in comparison to three Kato-Katz

samples (Rabello *et al*. 2002). It was amplified from mouse sera 2 weeks after *Schistosoma* infection without the requirement for special preparation of the serum (Suzuki *et al.* 2006).

Multiplex real time PCR has detected the genome of both *S. mansoni* and *S. haematobium* in human faecal samples by amplifying the species-specific cytochrome C oxidase enzyme gene (ten Hove *et al.* 2008). Detection of parasite DNA is a promising diagnostic tool because of its high sensitivity and specificity, however, it is still expensive and needs complicated laboratory equipment (Abath *et al*. 2006, Sandoval *et al*. 2006).

1.9 Vaccine against *Schistisoma*

Production of vaccine against *Schistosoma* is an important line which could to be included in future *Schistosoma* control programs. Praziquantel, the main anti-*Schistosoma* chemotherapy, has several drawbacks; it is only effective against the adult parasite (Doenhoff *et al*. 1987) and here is also concern about the development of drug resistance (Picquet *et al*. 1998, Ismail *et al.* 1999, Kusel& Hagan 1999), PZ treatment needs to be repeated several times (Doenhoff *et al.* 2000) and it does not affect the transmission rate (Bergquist *et al*. 2002).

1.9.1 Expected role of vaccine in disease control

A complete protection against schistosomiasis through vaccination is presently not achievable. The parasite does not multiply within the human host and therefore a vaccine which even leads to a considerable reduction in the numbers of adults and/ or eggs can

make a significant difference in the severity of the disease (Bergquist 1995, Wilson& Coulson 2006).

1.9.2 Types of the vaccines according to their effects

Vaccines can be categorized according to their mode of action. Vaccines which are designed to prevent infection, vaccines causing female parasite to decrease egg production, anti-fecundity vaccines, and ones which combat the pathology induced by the deposited eggs, anti-pathology vaccine (Bergquist *et al.* 2002)

1.9.3 Ideal immune mechanism of anti-*Schistosoma* vaccine

There is no general agreement amongst parasitologists about the nature of the most effective immune response. It could be cellular (Th1), humoral (Th2) or both but the latter will be very difficult to generate as it requires an optimal synergism between both arms of immune response. An immune response that causes parasite protection against different antigens in experimental animals, might not be as effective in humans (Bergquist *et al*. 2002).

a) Role of Th1 immune response in the protection against *Schistosoma*

Vaccines, that induce a Th1type response protect the host from switching the immune response towards Th2, hence avoiding the occurrence of severe morbidity and act as anti-pathology vaccines (Wynn *et al.* 1994), but this might also lead to early liver disease (Stadecker 1999). In mice, protection against *Schistosoma* challenge occurs under the control of cytokines produced by Th1 cells (Smythies *et al*. 1993). Elimination of the invading cercariae could occur in the lung by the activated macrophages which may be

controlled by IFN-γ (Wilson& Coulson 1998 & Coulson 1997). The pulmonary macrophages generate inducible nitric oxide synthase which mediate nitric oxide (NO) production from L-arginine and NO is proved to kill recently transformed schistosomulum *in vitro* (Wynn *et al*. 1994, Street *et al*. 1999) and mouse which is deficient in iNOS shows lower levels of protection than the wild type (James *et al*. 1998).

In the irradiated attenuated vaccine model, protection is mediated by Th1 through the formation of foci which surround the migrating larva in lung vessels hindering their migration and forcing them to pass into lung alveoli to die there. These aggregates are composed of CD4+ T-cells, CD8+ T-cells and macrophages and their formation depends upon IFN-γ (Kambara& Wilson 1990).

Individuals who did not have anti-*Schistosoma* treatment and are resistant to the infection show Th1 immune response against schitosomulum antigens (Correa-Oliveira *et al*. 2000). The protection against some parasites such as some helminths and many intracellular protozoa such as *Toxoplasma, Trypanosoma* and *Leishmania* is associated with Th1 dependent cytokines responses (Finkelman *et al*. 1991).

b) Role of Th2 immune response in the protection against *Schistosoma*

On the other hand, there are some reports which support Th2 dependent vaccines. In rodents, the protection against *Schistosoma* correlates with predominant Th2 immune response profile (Grzych *et al*. 1991). In humans, the resistance is associated with IgE (Webster *et al*. 1996, Capron and Capron, 1994, Hagan *et al*. 1991). Partial protection in humans is achieved after repeated chemotherapy and is associated with a Th2 type response

(Walter *et al*. 2006). In some parasites, the protection is associated with specific IgE such as *Necator americanus* where patients with a high IgE levels have a lower load of *N. amiricanus* infection in comparison to others with lower IgE levels (Pritchard *et al*. 1999).

1.9.4 Difficulties in the path of anti-*Schistosoma* vaccine development

a) Immune evasion strategies of *Schistosoma*

Schistosoma, in its all developmental stages, use various mechanisms to establish itself in the body, avoiding the immune system (Doenhoff 1997, Davies 1983). The penetrating schistosomulum stays in the skin until it develops strategies to evade the effective immune response (Crabtree& Wilson 1985). It hinders migration of effector Langerhans cells in the skin by induction of prostaglandin D2 formation (Angeli *et al.* 2001). Secreted proteinases such as Sm31, Sm32 and cercarial elastases are involved in the modulation of the immune response against *Schistosoma* (Brindley *et al.* 1997). The removed glycocalyx membrane of the penetrating cercaria is an alternative complement pathway activator consuming this complement pathway in the microenvironment around the parasite (Marikovsky *et al*. 1986& Caulfield *et al.* 1991). The phospholipids of the adult tegument undergo de-acylation and re-acylation using fatty acids from the host (Brouwers *et al*. 1997). Different stages of *Schistosoma* adsorb many host molecules such as blood groups antigens and major histocompatibility products on their surfaces to mimic the host tissues (Sher *et al*. 1978, Goldring *et al*. 1976).

The "Happy Valley Hypothesis", suggested by Wilson *et al.* (1999), speculates that *Schistosoma* has evaded the host immune response by eliciting a mixed Th1 and Th2 cytokines response avoiding polarization to either of them which will be in turn lethal to the parasite.

b) Financial shortage

Funding shortage for vaccine research has slowed vaccine progress. The last international programme targeted towards development of anti-*Schistosoma* is Vaccine Development Programme (SVDP) which closed in 2002 (Bergquist *et al*. 2005, Loukas *et al*. 2007).

1.9.5 Anti-*Schistosoma mansoni* vaccine candidates

a) Antigens which are suggested by World Health Organization (WHO)

WHO had selected six antigens that have been derived from *S. mansoni* and supported independent testing of these antigens in different laboratories, but none of them achieved more than 40% protection in the tested animals (Bergquist 1995, Bergquist& Colley 1998, Bergquist *et al.* 2005).

Glutathione S-transferase (Sm28-GST)

Balloul and his colleagues purified glutathione S-transferase in 1987. Some co-workers did not detect this antigen on the surface of the tegument (Holy *et al*. 1989) while Taylor *et al*. (1988) found it on the tegument. By using mass spectrometry, it was identified in the cytosolic fractions of the tegument but not on outer surface of *Schistosoma* (Braschi *et al*. 2006). *Schistosoma* Sm28-GST has many vital functions for different stages of the parasite, it inhibits migration of the dermal Langerhans cells by formation of prostaglandin D2, thus

protecting schistosomulum from being attacked by dendritic cells (Angeli *et al.* 2001, Herve *et al.* 2003) and it has a role in the productivity functions of the adults by synthesis of prostaglandin D2 (Capron *et al.* 2005).

In vaccine trials, Sm28-GST achieved 40-72% protection in mice and rats exposed to experimental *Schistosoma* infection (Balloul *et al*. 1987). Specific monoclonal antibodies which prevent Sm28-GST enzyme functions caused a significant reduction in the numbers of adult parasite and decreased the viability and numbers of the eggs, whereas the monoclonal antibodies which do not affect its enzymatic function reduced worms numbers only (Xu *et al*. 1993, Capron *et al*. 1995, Grzych *et al*. 1993). Anti-Sm28- GST IgG and IgA antibodies correlate with human resistant to re-infection (Lebens *et al.* 2004). The immune response induced by experimental Sm28 is affected mainly by the type of adjuvants used; with alum or complete Freund's, Th2 cytokine profile predominant but when Sm28 is-GST expressed in recombinant *Salmonella typhimurium,* the response is of Th1 profile. Dose of the protein, route of administration and genetic background of the animal might essentially determine the resultant immune response (Comoy *et al*. 1998).

Paramyosin

Paramyosin was identified by using antibodies generated during immunisation of mice with *S. mansoni* extracts of 97 kDa (Lanar *et al*. 1986). It is a myofibrillar protein forming the main bulk of muscles in invertebrates. It has not been detected on the tegument surface either by immunohistological methods or by Western blotting analysis (Schmidt *et al*. 1996). *Schistosoma* paramyosin has many functions including the binding of C1q and the

Fc portions of the immunoglobulins, hence it has a role in the immune evasion of *Schistosoma* (Loukas *et al*. 2001).

Native and recombinant *S. mansoni* paramyosin preparations have been tested as potential vaccines against *Schistosoma* infection and were shown to achieve protection in the region of up to 30%. Paramyosin stimulates T lymphocytes of vaccinated mice to produce cytokines including IFN-γ which activate macrophages to kill schistosomula, whereas passive immunisation of naïve mice with Sm97 specific antibodies failed to be protective against the infection (Pearce *et al*. 1988). The sera of naturally resistant humans contained Sm 97antibodies (Correa-Oliveira *et al*. 1989) and the immune response showed a typical Th1 cytokine response profile correlating with resistance (Al-Sherbiny *et al*. 2003).

Ir-V5

The sera of mice immunised with irradiated cercariae showed antibodies against a 200 kDa myosin protein. Ir-V5 is found on the surface of newly transformed schistosomula. cDNA encoding a myosin sub-fragment of 62 kDa was cloned and used for immunisation experiments. The recombinant protein achieved a protection of 75% in mice and 97.4% in rats. This protection was not enhanced with adjuvants. The protection action of this antigen appears to be mainly caused by an effective humoral response with a highly specific antibody titre (Soisson *et al.* 1992 & 1993).

Triose phosphate isomerise (TPI)

TPI was discovered by Harn *et al*. (1985) using a monoclonal antibody termed mAb M.1. TPI is detected at different stages of the parasite life cycle mainly in the tegument as well

as in other *Schistosoma* tissues. Native TPI of adult parasites works enzymatically like its mammalian counterpart forming part of the glycolytic pathway as a catalyser for glyceraldehyde 3-phosphate production. *Schistosoma* TPI has 79-87% homology to the mammalian TPI (Reynolds *et al.* 1992).

Monoclonal antibodies against TPI were shown to give partial protection (41- 49%) in mice challenged with cercariae (Reynolds *et al.* 1992). Multiple antigen peptides (MAP), were designed for TPI immunisation, which carry B and T cell epitopes and avoid the highly conserved areas with a high degree of similarity to human TPI. Both of MAP and the full length TPI are highly immunogenic and are strong stimulants for Th1 to produce IL-2 and IFN-γ (Reynolds *et al*. 1994). In 1997, Ferru and his colleagues made a construct using MAP of TPI bearing T and B cell epitopes and 115-131 peptides from *Schistosoma* GST achieving T cell responses against TPI and B cell responses against GST. Peripheral blood mononuclear cells (PBMC) stimulated *in vitro* assay using MAP4 (which is a multiple antigen peptide containing B and T cell epitopes of TPI) produced a typical Th1 cytokine response with a predominant release of IFN-γ (Reis *et al.* 2008).

Sm23

Sm23 was identified using monoclonal antibodies directed against the schistosomulum of *S. mansoni* (Harn *et al*. 1985)**.** Subsequently, the gene was characterized. At all stages, *Schistosoma* expresses Sm23 (Harn *et al.* 1985, Oligino *et al*. 1988). It is the only listed *Schistosoma* antigen on the WHO list that is exposed on the surface of all *Schistosoma* life cycle stages (Harn *et al*. 1985, Braschi & Wilson, 2006). It is a member of the tetraspanin proteins family and the two external domains of Sm23 were found to bear several B cell

specific epitopes and at least four T cell specific domains (Reynolds *et al*. 1992). Sera of infected humans react with recombinant Sm23, especially the C-terminal part of Sm23 (Gaugitsch *et al.* 1991, Koster *et al*. 1993).

In experimental studies using Sm23 DNA as a vaccine, it conferred 21- 44% protection in C57BL/6 mice infected with cercariae, showing an induction of specific IgG2a and IgG1 antibodies, while co-administration of plasmids expressing IL-12 or IL- 4 did not further reduce the worm infectivity and parasitic survival or achieve a significant increase of the antibody response. The protective effect appears to be dependent on the Th1 immune response. The recombinant protein failed to induce protection due to polarizations of the immune response towards a Th2 response with only undetectable IgG2a levels (Da`dara *et al*. 2001, 2002& 2003).

Sm14

Sm14 is a fatty acid binding protein which was first discovered by screening of an *S. mansoni* adult worm cDNA expression library using sera of mice immunised with worm extract suspended in saline (Moser *et al*. 1991). It is found in the dorsal tubercles of adult male (Moser *et al.* 1991), in the basal lamella of the gut and underneath the tegument (Brito *et al*. 2002). Sm14 is implicated in the uptake and transport of host fatty acids through the parasite cytoplasm and the synthesis of the *Schistosoma* membrane (Moser *et al*. 1991). Endemic individuals in Brazil who are resistant to *Schistosoma* infection showed a strong Th1 immune response against recombinant Sm14 responding with a high proliferation rate of their PBMC and a significant release of IFN-γ and TNF following Sm14 exposure (Brito *et al.* 2000).

There are many isoforms of Sm14. Of those, the isoform containing a methionine in amino acid position 20 (Sm14-M20) is the most stable and has a significantly higher fatty acids binding capacity than other isoforms. Outbred Swiss mice vaccinated with Sm14-M20 were 67% protected when challenged with *Schistosoma* cercariae (Ramose *et al*. 2003). Immunisation with the protein alone or as a recombinant fusion protein with tetanus toxin fragment C (TTFC) achieved protection against a cercariae challenge with little differences between the fusion protein and Sm14 alone. The immune response was of a typical Th2 phenotype in this experiment which could be elicited by using Alum as adjuvant (Abreu *et al.* 2004). DNA Sm14 immunisation induced 40.5% reduction of the adult worm which correlated with specific anti-Sm14 IgG antibody and predominance of Th1 type of immune response with high levels of IFN-γ (Fonseca *et al*. 2006).

b) Other promising vaccine antigens

Tetraspanins (Tran *et al*. 2006), Sm29 (Cardoso *et al*. 2006& 2008) and glyceraldehyde 3-phosphate dehydrogenase (El Ridi *et al.* 2001a& b).

1.10 Calreticulin

Calreticulin (CRT) was discovered in 1974, in the rabbit endoplasmic reticulum (ER) originally as a calcium binding protein of 55 kDa weight (Ostwald& MacLennan 1974) and its cDNA sequence was established (Fliegel *et al.* 1989, Smith& Koch 1989). Calreticulin has been identified in every living organism: human, plants, parasites and insects except fungi (Nash *et al*. 1994, Michalak *et al*. 2009).

1.10.1 Calreticulin structure

Most of our knowledge about the structure and function of calreticulin derived from studies on human calreticulin. From the deducted amino acid sequences of calreticulin (Fliegel *et al.* 1989, Smith& Koch 1989), calreticulin is composed of three main domains, called the N-, P- and C-domain. In addition to, another domain called S-domain has recently been identified (Stuard *et al.*1996& 1997). The amino acid sequence of calreticulin starts with a N-terminal hydrophobic signal peptide (the first 15 amino acids) which directs the protein towards ER and ends with a signature of ER retrieval sequence motif KDEL (Fliegel *et al.* 1989& Michalak *et al.* 2009). N- and P-domains are conserved among different calreticulin isolated from different organisms which indicate to the importance of these domains in the fundamental physiological functions of calreticulin (Nakhasi *et al.* 1998& Michalak *et al.* 1999).

N-domain

Residues 1-180 of mature calreticulin form the N-domain which is predicted to be globular in shape, based on the sequnce similarity to calnexin. It consists of 8 anti-parallel ß-strands. Its net charge is neutral. There are two highly conserved sequences motifs found throughout the calreticulin family, which are residues 77-96 and 113-121. However, their functions are still unknown (Michalak *et al.* 2002). The N-domain contains three cysteine residues, which are conserved among all variants of calretculin, two of them form a disulphide bridge which may be responsible for the folding of this domain (Michalak *et al.* 1992, Matsuoka *et al.* 1994).

The N-domain is the site of interaction with alpha-integrins, RNA of rubella virus (host protein for viral replication), DNA-binding domain of glucocorticoid receptor, auto-antibodies and some metals (Rojiani *et al*. 1991, Singh *et al*. 1994, Michalak *et al.* 1999, Burns *et al.* 1994, Eggleton *et al*. 2000). Protein chaperones which are found in the ER such as endoplasmic reticulum protein 57(ERP57) and protein disulfide isomerase (Baksh et *al.* 1995, Corbett *et al.* 1999) bind with calreticulin through the N-domain.

P-domain

The P-domain, a highly charged domain rich in proline, spans amino acid positions 181 to 290. It comprises three antiparallel ß-sheets and contains two amino acid repeats which are found only in calreticulin in each of the antiparallel ß-sheets. The A repeat includes the following amino acids sequences: P-x-x-I-x-D-P-D-A-x-K-P-E-D-W-D-E and B repeat motif is: G-x-W-x-P-P-x-I-N-P-x-Y-x. These repeats are thought to be responsible for its high affinity and low capacity binding to calcium and carbohydrates (Baksh& Michalak, 1991, Vassilakos *et al.* 1998, Ellgaard *et al.* 2001). This domain has a nuclear site signal, which suggests that it is involved in the transport of the glucocortecoid receptor to the nucleus (Holaska *et al.* 2001).

Other chaperone functions assigned to calreticulin, include the binding of glycans of emerging glycoproteins in ER via the lectin–like binding site (Tatu& Helenius, 1997, Vassilakos *et al.* 1998). Calreticulin interacts, through its P-domain, with perforin, one of the components of cytotoxic T cell granules, to protect RBCs from perforin mediated lysis (Fraser *et al.* 2000, Andrin *et al.* 1998).

C-domain

This domain is the least conserved domain of calreticulin. The C-domain starts from residue 291 to the carboxyl terminal end of CRT (Michalak *et al.* 1992). It is highly acidic (negatively charged) with a high capacity but low affinity to bind calcium (Baksh& Michalak, 1991). There is still insufficient knowledge about the structure of C-domain (Gelebart *et al.* 2005).

The C-domain accounts for about 50% of the Ca^{2+} binding capacity of ER proteins (Nakamura *et al*. 2001). An endoplasmic reticulum signal is found in the terminal end of C-domain. This signal is mainly KDEL (Pelham, 1989) (or HDEL in some organisms, such as *S. mansoni*) (Khalife *et al.* 1994). Calcium binding to the C-domain is thought to play a vital role in controlling the chaperone functions of calreticulin (Corbett *et al.* 1999). It binds to the some blood clotting factors (Kuwabara *et al.* 1995).

S-domain

The S-domain of human calreticulin spans over 123 amino acids starting from residues 160- 283 and has structural similarity to a CUB-domain, a motif found in many proteins that bind to C1q, collectins and collagen (Stuart *et al.* 1997, Sim *et al.* 1998). It has two parts; the N-terminal part of S-domain overlaps the N-domain and the C-terminal part is involved in P-domain (Stuart *et al.* 1996, Stuart *et al.* 1997, Sim *et al.* 1998). This domain binds to mannan-binding lectin (MBL) and inhibits the binding of the latter with the MBL Associated Serine Proteases 1, 2 and 3 (MASP-1, MASP-2 and MASP-3) interfering with lectin pathway activation (Stuart *et al.* 1996& 1997).

Under normal physiological conditions *in vivo*, C1q does not bind to calreticulin as the latter is a component of C1 complex, but wherever there is inflammation, activated C1q may bind and interact of calreticulin (Schafer *et al.* 2000, Dietzschold *et al*. 1995). The S-domain of calreticulin inhibits the classical and lectin pathway and impairs the cell functions mediated by C1q receptor at the site of inflammation only (Lynch *et al.* 2002).

1.10.2 Localization of calreticulin

The most common site where calreticulin is found is the endoplasmic reticulum. The calreticulin C-domain contains an ER retention sequence which – depending on the species – can either be KEDL in *Leishmania* (Joshi *et al*. 1996) and *Trypanosoma* (Aguillon *et al.* 2000) or HDEL in *Schistosoma* (Khalife *et al.* 1994, Huggins *et al.* 1995). Calreticulin is also found in other intracellular compartments such as the secretory granules of cytotoxic T-lymphocytes (Dupuis *et al.* 1993), on the cell surface (Gray *et al*. 1995, White *et al.* 1995), within the nucleus (Opas *et al.* 1991), in the cytoplasm (Rojiani *et al*. 1991, Holaska *et al.* 2001), in the saliva of the tick (Jaworski *et al.* 1996) or in serum (Sueyoshi *et al.* 1991).

The presence of calreticulin outside of the ER could easily be explained as many proteins which are synthesized inside ER and accompany calreticulin through the Golgi apparatus and hence to other celluler comportments (Souto-Padron *et al*. 2004). Calreticulin can have various isoforms and is found in many sites inside or outside the cell (Coppolino& Dedhar, 1998, Michalak *et al*. 2009). The ER retrieval peptides are cut by certain ER proteases, allowing protein movement to the Golgi apparatus and then to others sites.

1.10.3 Functions of calreticulin

Most of the identified calreticulin functions were studied mainly in vertebrates but not in lower organisms (Ferreira *et al.* 2004).

1.10.3.1 Functions inside ER

a) Ca^{2+} haemostasis:

Calcium binding and regulation is the most important calreticulin function (Michalak *et al.* 2009). Through the analysis of a CRT gene targeted mouse line which proofed to be lethal as the deficiency of CRT in homozygosity of the CRT disrupted allele leads to an impaired release Ca^{2+} from the ER preventing development of the embryonic heart (Mery *et al.* 1996, Bastianutto *et al.* 1995, Mesaeli *et al.* 1999). With a high level of Ca^{2+} inside the ER, calreticuin binds also to the carbohydrates (Vassilakos *et al.* 1998).

For parasites, the role of calreticulin in regulating calcium levels may be critical for the production of secondary messengers upon contact with the host molecules either soluble molecules or receptors (Nakhasi *et al.* 1998). In the case of *T. cruzi*, the TcCRT calcium binding character could add to the cardiac pathology accompanying Chagas' disease because of its vital role in the calcium hemostasis maintaining heart contractility (Ferreira *et al.* 2004). Calcium per se has an important role in the regulation of many pathways related to metabolism, nuclear DNA cleavage, gene transcription and cell cycle regulation (Nicotera& Rossi 1994).

b) Protein chaperon

Inside the ER, there are many chaperone that interact with nascent unfolded proteins that prevent the exportation of the miss-folded proteins out of the ER (Bedard *et al.* 2005). From those, CRT and calnexin which interact with the glycoproteins through the oligosaccharides present on the glycoprotein surfaces (Ellgaard *et al.* 1999, Trombetta 2003). The glycoproteins are identified by quality-control cycle proteins such as CRT and calnexin. Once the folding process is complete, the terminal glucose is removed by Glucosidase II and the proteins leave the ER. The mis-folded proteins are re-glucosylated by the UDP-glucose: glycoprotein transferase (UGGT) enzyme and remain in the cycle until attaining their proper folding (Hebert& Molinari 2007). The folding unit responsible for the performance of the chaperon duties of calreticulin is formed by both the N- and P-domains (Nakamura *et al*. 2001).

From the prospective of parasites, calreticulin might have a role in folding of some surface proteins such as gp63 of African *trypanosoma, Leishmania* surface antigens and a group of GPI (glycophosphatidyl inositol) anchored proteins which are the main bulk of the surface antigen determinant of the unicellular parasites (Ferguson 1994). Parasite calreticulin is thought to support the correct folding of proteins after being secreted from the parasites such as acid phosphatase and superoxide dismutases which aid in using substrates from the hosts for some of the parasites fundamental biological processes (Nakhasi *et al.* 1998).

1.10.3.2 Extraendoplasmic reticulum calreticulin functions

Also outside the ER, calreticulin has many functions;

a) Apoptosis

Cells which over express calreticulin are more susceptible to apoptosis, in contrast to these cells with low CRT levels. The ectocalreticulin on dead cells attracts other cells involved in systemic removal of dead cells such as phagocytes (Nakamura *et al.* 2000, Chen *et al.* 2005). MBL and C1q bind to apoptotic cells and then ligate to calreticulin expressed on the surface of phagocytes, where calreticulin forms a functional complex with the endocytic receptor protein CD91. On the surface of phagocytes, the CRT/CD91 complex facilitates phagocytosis and triggers cellular activation through ligand binding (Gardai *et al.* 2003, Holmskov *et al.* 2003).

b) Immunity related functions

Cell surface calreticulin associated with CD91 forms a C1q receptor complex (Sim *et al.* 1998), which competes with immune complex to bind C1q (Johnson *et al.* 2001). There are many observations showing to that calreticulin has an important role in the pathogenesis of some autoimmune diseases and it acts as an auto-antigen interacting with other immune complexes (Michalak *et al.* 2009). It is a constituent of the ribonucleoprotein complex RO/SS-A (Smith Surface Antigen), which induces autoantibodies in some autoimmune disease (Franceschini& Cavazzana 2005, Cheng *et al.* 1996, Staikou *et al.* 2003). The peptides of calreticulin are associated with MHC class II molecules on the antigen presenting cells surfaces (Verreck *et al.* 1995). In some patients suffering from autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and celiac disease, there are frequently formed autoantibodies against CRT (Verreck *et al.* 1995, Eggelton *et*

al. 2000, Tuckova *et al.* 1997). Individuals suffering from autoimmune diseases often fail to clear immune complexes, a function largely mediated by C1q. The absence of the C1q mediated scavenger function predisposes for the development of the autoimmune diseases such as systemic lupus erythmatosus (Walport *et al.* 1998, Kovacs *et al.* 1998). Calreticulin is essential for the calcium signalling to modulate immune functions of T cells which is a process involved in the pathophysiology of autoimmune disease (Porcellini *et al.* 2006).

Parasite calreticulin is considered to be responsible for inducing autoimmune pathologies following *Onchocera* and *Trypanosma* infections. In *Onchocera*, parasite CRT does not have an ER retrieval signal suggesting that the protein is secreted from the parasite during infection and triggers the development of antibodies directed against CRT, thus, promoting an autoimmune response against host CRT (Rokeach *et al.* 1994). The degree of similarity in the amino acids sequences between the human and parasite calreticulin, implies that parasitic calreticulin can also bind with the recognition molecules of classical and lectin activation pathway of complement, hindering the activation of the complement during infection and protecting these parasites from the attack by the host complement system (Ferreira *et al*. 2004).

c) Other calreticulin functions

CRT has a role in cell adhesion (Michalak *et al.* 1999, Opas *et al.* 1996) as well as an implicated function in the regulation of steroid gene expression (Burns *et al.* 1994)

1.10.4 Parasites' calreticulin

Parasites with multiple life stages changing from invertebrate to vertebrate hosts, have the ability to adapt quickly to the new environment pH, temperature and the new host immune system, and parasitic calreticulin has been suggested to be a sensor for the changes in different parameters of the environments during the complex life cycle of the parasite and helps the parasite to adapt to the host environment (Nakhasi *et al.* 1998).

There are some reports on parasite calreticulin. It has been isolated and sequenced in various parasites infecting human such as *Onchocerca volvulus, Necator americanus, S. mansoni, Trypanosoma cruzi* and *Leishmania donovani*

Trypanosoma cruzi

The most intensively studied parasite calreticulin, is that of *T. cruzi* (TcCRT). *Trypanosoma* calreticulin was cloned and sequenced by Aguillón *et al.* (1995& 2000). It was found to have a molecular weight of 45 kDa and its C-terminal end contains KEDL, ER retention signal. In *Trypanosoma cruzi,* CRT is secreted from the ER to the flagellum and appears on the parasite surface (Ferreira *et al.* 2004). By using immunoelectron microscopy, CRT was found in the ER, the Golgi apparatus, cytosomic vesicles of the epimastigotes, cytoplasm, chromatin of the nucleus and on the surface of the amastigote forms (Souto-Padron *et al*. 2004). CRT has a 50% homology with the human calreticulin. It is a dimorphic protein and is also known as Tc45 (Marcelain *et al*. 2000). Its immunogenicity is confirmed in the animals (Aguilar *et al.* 2005) and human (Marcelain *et al.* 2000, Aguillón *et al.* 1997). B cells recognize native TcCRT in humans and mice indicating the high immunogeniciy of this protein which is exposed to the immune system

either by shedding by the living organism or after its death (Marcelain *et al.* 2000, Ramos *et al.* 1991).

TcCRT binds to C1q, in particular to its collagenous part, inactivating the classical complement pathway *in vitro* and this could protect the parasite through an immuno-evasion strategy, hence increasing parasite infectivity (Ferreira *et al.* 2004, Norris 1998, Rimoldi *et al.* 1989, Aguilar *et al.* 2005). Binding of C1q and calreticulin could be inhibited *in vitro* using anti-calreticulin F(ab`)2 antibodies and this shed light upon the role of the specific antibodies to achieve a balance between the parasite and human host (Aguilar *et al.* 2005). Native or recombinant TcCRT shows antiangiogenic activity affecting vascularisation *in vivo* on the chicken embryonic chorio-allantoid membrane assay. This could lead partly to an anti-neoplastic effect of this parasite molecule experimentally (Molina *et al.* 2005, Aguilar *et al*. 2005).

Entamoeba histolytica

E. histolytica calreticulin was detected inside *E. histolytica,* which lacks ER. It is a 51 kDa protein and EhCRT specific antibodies were identified in 90% of patients suffering from invasive amoebiasis and decreased by 70 % after 6-12 months, in comparison to only 10% of cyst passers without any symptoms (Gonzalez *et al.* 2000& 2002). Girard-Misguich *et al.* (2008) confirmed its presence on the surfaces of the trophozoite form of the parasite.

Necator americanus

CRT may be secreted by *N. americanus* whether during its feeding (Pritchard *et al.* 1999) or in the secretory pathways of *N. americanus* (Kasper *et al.* 2001). Hookworm CRT was tested as a vaccine to protect against *N. americanus* infection. It was found that, the recombinant hookworm CRT without adjuvant reduced the numbers of adult worms by 43-49% in experimental BALB/c mice which were exposed to the parasite larva (Winter *et al.* 2005).

S. mansoni

S. mansoni CRT was detected when cDNA library of *S. mansoni* adult were screened with anti-serum of rabbit immunised with electroeluted proteins ranging from 60-65-kDa of adult *S. mansoni.* Its full DNA gene length was sequenced and it was expressed in pGEX-2T fusion system. It was detected in different organs of *S. mansoni* by immunohistochemistry methods (Khalife *et al.*1993& 1994) and by recent proteomic studies, it was detected in the *S. mansoni* tegument (Braschi *et al.* 2006). Native S. *mansoni* CRT was proved to be a good B-cell and T-cell immunogen (El Gengehi *et al.* 2000)

Echinococcus granulosus

CRT was cloned from *Echinococcus granulosus* and detected in its hydate cysts (Cabezon *et al.* 2008).

1.10 Aim of the work

My PhD was based on the following aims:

i) The cloning, recombinant expression and purification of *S. mansoni* calreticulin (SM-CRT) sub-fragments representing specific structural domains and combinations thereof.

ii) Analysing the physiological functions of different SM-CRT sub-fragments in terms of calcium binding activity and their interaction with C1q (the recognition subcomponent of the classical pathway of complement).

iii) Evaluation of the diagnostic use of different SM-CRT sub-fragments, CTF (cercarial transformation fluid antigen) in the sero-diagnosis of *S. mansoni* infection using an ELISA-based detection of specific antibodies in serum of experimentally *S. mansoni* infected mice and of infected humans.

iv) Assessment efficacy of the recombinant full length SM-CRT as a vaccine to limit the severity of *S. mansoni* infection in experimental mouse model.

Chapter 2

Materials& methods

2.1 Materials

2.1.1 Reagents

Reagent	Company	Catalogue No.
Acetic acid glacial	Fisher scientific	A/0400/PB15
Acrylamide/bis-Acrylamide, 30%	Sigma-Aldrich	A3699
Agar	Lab M	MC002-A
Agarose	Melford	MB 1200
Alsever`s solution	Sigma-Aldrich	A3551
Ammonium persulfate	Acros Organics	327081000
Ampicillin	Sigma-Aldrich	A9518
***Bam*HI**	New England Biolab	R0136S
Barbital	Fisher scientific	B/0052/50
BCIP/NBT Alkaline Phosphatase Substrate	Sigma-Aldrich	B1911
BL21(DE3)pLysE cells	Invitrogen	V351-20
Bovin serum albumin (BSA)	Sigma-Aldrich	A9647-100G
5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal)	Melford	MB1001
Bromophenol	Sigma-Alrich	114405
Butanol	Sigma-Alrich	B7906
$CaCl_2$	Sigma-Aldrich	C3306
Calmodulin	Sigma-Aldrich	P1431
Chloramphenicol	Sigma-Aldrich	C 0378
Coomassie Blue	Serva	17525
dATPs	Promega	U1201
Developer solution	AGFA	G153A
dNTPs	Promega	U1511
EcoRI restriction enzyme	New England Biolab	R0101S
EDTA	Acros Organics	118432500

Ethidium bromide	Sigma-Aldrich	E-8751
Formamide	Sigma-Aldrich	F7508
Fixer solution	AGFA	G153B
Freund`s adjuvant	Sigma-Aldrich	F5881
GeneRuler ™ DNA Ladder Mix, 100 bp	Fermentas	SM 0331
Glycerol	Sigma-Aldrich	G8773
Glycine	Acros Organics	120070010
Guinea pig serum	Harlan Laboratories	S.R-0006B
HCl	Fisher scientific	11/1100/PB17
Human recombinant CRT	Abcam	ab15729
Human C1q depleted serum	Calbiochem	234401
Human C1q	Sigma-Aldrich	C1740
Imidazole	Sigma-Aldrich	I0125
Industrial methylated spirits (I.M.S)	Fisher scientific	M/4400/17
Isopropanol	Fisher scientific	AC41279
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Melford	MB1008
KCl	Sigma-Aldrich	P9333
K-acetate	Sigma-Aldrich	P5708
L.B. (Luria-Bertani)	Sigma-Aldrich	28713
Methanol	Fisher scientific	A412
$MgCl_2$	Sigma-Aldrich	M8266
2-mercaptoethanol	Sigma-Aldrich	M-6250
$MnCl_2$	Sigma-Aldrich	M8266
Na deoxycholate	Sigma-Aldrich	D5670
Na HCO3	Fisher scientific	S/233
Na2CO3	Fisher scientific	S/2880/S3
NaCl	Fisher scientific	S/3120/63
Na-MOPS	Sigma-Aldrich	M9381
Nitro-cellulose membrane	Bio-Rd	162-0097
Phosphate Buffer Saline (PBS)	Sigma-Aldrich	P4417

Phusion High-Fidelity DNA polymerase	Finnzymes	F-530S
Pefabloc SC (AEBSF)	Roche	429 868 001
***p*-nitrophenyl phosphate substrate**	Sigma-Aldrich	N1891
SeeBlue Plus2 PreStained Standard	Invitrogen	LC925
Sheep red blood cells (SRBC)	Innovative research	IC100-0210
Skimmed milk	Oxoid	LP0031
Sodium Dodecyl Sulfate (SDS)	Fisher Scientific	BP 166-500
Stains-all stain	Sigma-Aldrich	E9379
T4 DNA Ligase enzyme	New England biolab	M0202S
***Taq* DNA Polymerase**	Thermo scientific	AB-1301/A
TEMED	Sigma-Aldrich	T.8133
Tris	Sigma-Aldrich	T 6066
Tris Hcl	Fisher Scientific	BPE 153-1
Tryptone	Lab M	Q29216
Tween-20	Fisher Scientific	BP337
***Xho*I restriction enzyme**	New England Biolab	R0146S
X-ray film	Kodak	Z35, 847-9
Yeast Extract	Sigma-Alrich	Y1625

2.1.2 Antibodies

Anti-human C1q anti-serum (Developed in sheep)	Immune system	SC1Q-80A
Anti-Human IgG (AP conjugated) (Developed in goat)	Sigma-Aldrich	A1543
Anti-mouse IgG (AP conjugated) (Developed in goat)	Sigma-Aldrich	A3688
Anti-Rabbit IgG (AP conjugated) (Developed in goat)	Sigma-Aldrich	A3812
Anti-sheep blood cell stroma fractionated antiserum (hemolysin)(developed in rabbit)	Sigma-Aldrich	S1389
Anti-sheep IgG (AP conjugated) (Developed in donkey)	Sigma-Aldrich	A5187

Monoclonal anti-polyhistidine (HRP conjugated) (Developed in mouse)	Sigma-Aldrich	A7058
Goat Anti-Mouse IgG1, Human ads-AP	Southernbiotech	1070-04
Goat Anti-Mouse IgG2a, Human ads-AP	Southernbiotech	1080-04

2.1.3 Kits

Coomassie Plus – The Better Bradford Assay	Pierce	23236
ECL Western Blotting Substrate System	Pierce	32106
QIAquick Gel Extraction Kit	Qiagen	28704
QIAquick Nucleotide Removal Kit	Qiagen	28304
Wizard® Plus SV Minipreps DNA Purification Systems	Promega	A146

2.1.4 Vectors

pGEM®-T Easy Vector	Promega	A1360
pRSET B vector	Invitrogen	V351-20

2.1.5 Buffers and solutions

2.1.5.1 Protein expression buffers

IPTG, 100 mM	0.24 g IPTG 10 ml dH_2O
TAE (50x), pH 8.5	242 g Tris base 57.1 ml Glacial acidic acid 100 ml EDTA (0.5 M) Completed into 1L H_2O
TAE (1x**),** pH 8.5	200 ml 50x TAE 200 µl Ethidium bromide

Completed to 10 litter with d H_2O

L.B. liquid (1L), pH 7
10 g Tryptone
5 g Yeast Extract
10g NaCl

L.B. agar (1L), pH
10 g Tryptone
5 g Yeast Extract
10g NaCl
15 g Agar

S.O.B. (1L), pH 7
1000 ml deionized H_2O
20 g Tryptone
5 g Yeast Extract
0.5 g NaCl
186 mg KCl

For each autoclaved 1L and before use 10 ml of sterile 1 M Mg Cl2

X-Gal solution
50 mg X-Gal
1 ml N,N´-dimethylformamide
Cover with aluminum foil

2.1.5.2 Competent cells preparations buffer

Tfb1
30 mM K-acetate
50 mM $MnCl_2$
10 mM $CaCl_2$
15 % (w/v) Glycerol

Tfb2
10 mM Na-MOPS
10 mM KCl
75 mM $CaCl_2$
15 % (w/v) Glycerol

2.1.5.3 SDS-page and Western blotting buffers

Resolving gel Tris-glycin SDS-PAGE (15%)	3.4 ml H_2O
(For two mini-gels)	7.5 ml 30% Acrylamide/bis-Acrylamide
	3.8 ml 1.5 M Tris (pH 8.8)
	0.15 ml10 % SDS
	0.15 ml 10% ammonium persulphate
	0.006 mlTEMED (N,N,N',N'-Tetramethylethylenediamine)

Resolving gel Tris-glycin SDS-PAGE (12 %)	4.9 ml H_2O
(For two mini-gels)	6 ml 30% Acrylamide/bis-Acrylamide
	3.8 ml 1.5 M Tris (pH 8.8)
	0.15 ml10 % SDS
	0.15 ml 10% ammonium persulphate
	0.006 mlTEMED (N,N,N',N'-Tetramethylethylenediamine)

*TEMED (was mixed immediately before pouring the gel into the glass plate)

*The mentioned amounts are appropriate to make two gel

Stacking gel Tris-glycin SDS-PAG	4.1 ml d H_2O
(For two mini-gels)	1.0 ml 30% Acrylamide/bis-Acrylamide
	0.75 ml 1.5 M Tris (pH 8.8)
	0.06 ml 10 % SDS
	0.06 ml 10% ammonium persulphate
	0.006 ml TEMED(was mixed immediately before pouring the gel into the glass plate)

SDS-PAGE running buffers (1L), pH 8.3	3.03 g Tris base
	14.4 g Glycine
	1g SDS
	Complete to 1L with dH_2O

Loading buffer, pH 6.8	5.8 ml Tris (1 M) 0.83g SDS 2.5 ml Glycerol 1 mg Bromophenol blue Completed to 10 ml with dH_2O
Coomassie Blue Stain (1L)	600 mg Coomassie blue 260 ml d H2O 240 ml Methanol 100 ml Acetic acid
De-staining Coomassie Blue solution (1 L)	600 ml dH_2O 300 ml Methanol 100 ml Acetic acid
Western blot transfer buffer (1L), pH 8.3	2.9 g Glycine, 5.8 g Tris 0.37 g SDS 200 ml Methanol Completed with d H2O to one litter
5% skimmed milk	5 g Skimmed milk 100 ml PBS
PBS	8 g NaCl 0.2 g KCl 1.44 Na_2HPO_4

0.24 g KH_2PO_4

2.1.5.4 Protein purification buffers

Affinity chromatography buffers

Buffer A, pH 8	20 mM Tris Hcl
	500 mM NaCl
	5 mM Imidazole
Buffer B, pH 8	20 mM Tris Hcl
	500 mM NaCl
	500 mM Imidazole
Desalting Buffer, pH 8	20 mM Tris Hcl
	50 mM NaCl

Ion exchange chromatography

Buffer A, pH 8	20 mM Tris Hcl
	20 mM Na Cl
Buffer B, pH 8	20 mM Tris Hcl
	1 M Na Cl
Dialysing protein buffer, pH 7.4	20 mM Tris Hcl
	145 mM NaCl
2.1.5.5 "Stains-all" stain solution, pH 8.8	30 mM Tris base
	7.5% Formamide
	25% isopropyl alcohol
	0.0025% Stains-all

2.1.5.6 ELISA buffers

TBS, pH 7.4	10 mM Tris

	140 mM NaCl
Blocking ELISA buffer	1g BSA 100 ml TBS
Coating Buffer, pH 9.6	15 mM Na_2CO_3 35 mM Na HCO_3
Washing buffer	1000 ml TBS 0.05% Tween-20 5 mM $CaCl_2$
Washing buffers (for C1q binding assay)	1000ml PBS 0.05% Tween-20
2.1.5.7 *Schistosoma* perfusion buffer	8.6 g sodium chloride 15 g trisodium citrate, 2000 units heparin Complete to 1 litre with H_2O

2.2 Methods

2.2.1 Recombinant *S. mansoni* CRT (SM-CRT) expression and purification

Individual SM-CRT domains within the protein were identified using online programme BLASTP and Signal P v3.0 (www.cbc.dtu.dk/services/). The adult *S. mansoni* cDNA library was amplified with PCR for producing DNA fragments that encode ten SM-CRT sub-fragments : N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC-domains.

2.2.1.1 Amplification of DNA fragments encoding SM-CRT sub-fragments

```
1     acg caa cac ggt ttt gtc tgt cag g

26    ATG CTC TCT ATC TTG TTA ACT TTG TTG CTT TCA AAA TAC GCT CTG GGA CAT
      M   L   S   I   L   L   T   L   L   L S   K   Y   A   L   G   H

77    GAA GTT TGG TTT TCG GAA ACT TTT CCT AAT GAA TCT ATT GAG AAT TGG GTG
      E   V   W   F   S   E   T   F   P   N E   S   I   E   N   W   V

128   CAA TCA ACA TAT AAT GCC GAG AAG CAA GGA GAA TTC AAA GTA GAA GCT GGG
      Q   S   T   Y   N   A   E   K   Q   G   E   F   K   V   E   A   G

179   AAA TCT CCA GTT GAC CCA ATT GAG GAT TTG GGC TTA AAA ACA ACT CAA GAT
      K   S   P   V   D   P   I   E   D   L   G   L   K   T   T   Q   D

230   GCC AGA TTT TAT GGT ATT GCC CGC AAG ATC TCT GAA CCG TTC AGC AAC CGT
      A   R   F   Y   G   I   A   R   K   I   S   E   P   F   S   N   R

281   GGC AAA ACA ATG GTT CTC CAA TTT ACC GTC AAG TTT GAC AAA ACT GTC AGC
      G   K   T   M   V   L   Q   F   T   V   K   F   D   K   T   V   S

332   TGC GGA GGA GCT TAT ATT AAA CTT CTT GGT TCA GAT ATT GAT CCT AAA AAA TTT
      C   G   G   A   Y   I   K   L   L   G   S   D   I   D   P   K   N   F

386   CAC GGC GAA TCA CCT TAC AAG ATC ATG TTT GGT CCT GAT ATT TGC GGT ATG GCC
      H   G   E   S   P   Y   K   I   M   F   G   P   D   I   C   G   M   A
```

441 ACA AAG AAA GTC CAT GTC ATT TTT AAC TAT AAG GGA AAA AAT CAC CTG ATT
T K K V H V I F N Y K G K N H L I

492 AAA AAA GAA ATA CCC TGC AAA GAT GAC CTA AAA ACG CAT CTG TAC ACA TTA
K K E I P C K D D L K T H L Y T L

543 ATC GTG AAT CCA AAC AAC AAA TAC GAA GTT TTA GTT GAT AAC GCG AAA GTG
I V N P N N K Y E V L V D N A K V

594 GAA GAA GGA TCG TTG GAA GAT GAT TGG GAT ATG CTT CCA CCG AAA AAG ATT
E E G S L E D D W D M L P P K K I

645 GAT GAC CCA AAT GAC AAG AAA CCT GAT GAC TGG GTT GAT GAG CAA TTT ATC
D D P N D K K P D D W V D E Q F I

696 GAT GAT CCG GAT GAC AAG AAA CCT GAT AAT TGG GAT CAG CCC AAA ACA ATA
D D P D D K K P D N W D Q P K T I

747 CCT GAT ATG GAT GCC AAA AAG CCA GAT GAT TGG GAT GAT GCT ATG GAT GGT
P D M D A K K P D D W D D A M D G

797 GAG TGG GAA CGT CCT CAA AAA GAC AAT CCG GAA TAT AAA GGC GAA TGG ACA
E W E R P Q K D N P E Y K G E W T

848 CCT AGA CGT ATC GAT AAT CCA AAA TAC AAA GGA GAA TGG AAG CCT GTA CAG
P R R I D N P K Y K G E W K P V Q

899 ATT GAC AAT CCA GAA TAC AAA CAT GAT CCC GAA TTG TAT GTC CTG AAT GAC
I D N P E Y K H D P E L Y V L N D

950 ATC GGT TAT GTC GGC TTT GAT CTG TGG CAA GTC GAT TCA GGG TCA ATC TTT
I G Y V G F D L W Q V D S G S I F

1001 GAC AAC ATT TTA ATT ACT GAC AGT CCG GAT TTC GCT AAG GAA GAG GGC GAG
D N I L I T D S P D F A K E E G E

1052 CGA CTG TGG CGA AAA CGA TAC GAT GCT GAA GTT GCT AAA GAA CAG TCA AGT
R L W R K R Y D A E V A K E Q S S

1103 GCA AAA GAT GAC AAA GAG GAG GCT GAA GAA ACA AAA GAA CGA AAA GAG CTT
A K D D K E E A E E T K E R K E L

1154 CCA GAC GAT GCA AAA GCA TCG GAC GAG CCA TCT GGT GAC CAC GAT GAG CTG
P D D A K A S D E P S G D H D E L

1205 taa aat gtg atg tta gtt gtg gta aga ata cta ttt gct gtt ttt ttt aaa

1256 gtt agg att ctc tgc tcc aaa tct gta cat tta tca ttt tcg tga tta ata

1307 taa att gtt gtg acc

Figure 3: Gene sequence of *S. mansoni* calreticulin, (accession number Q06814**).** The open reading frame of the SM-CRT is in capital letters, blue color is the leader peptide, red color is the N-domain, green color is the P-domain, black color is the C-domain and the S-domain is underlined.

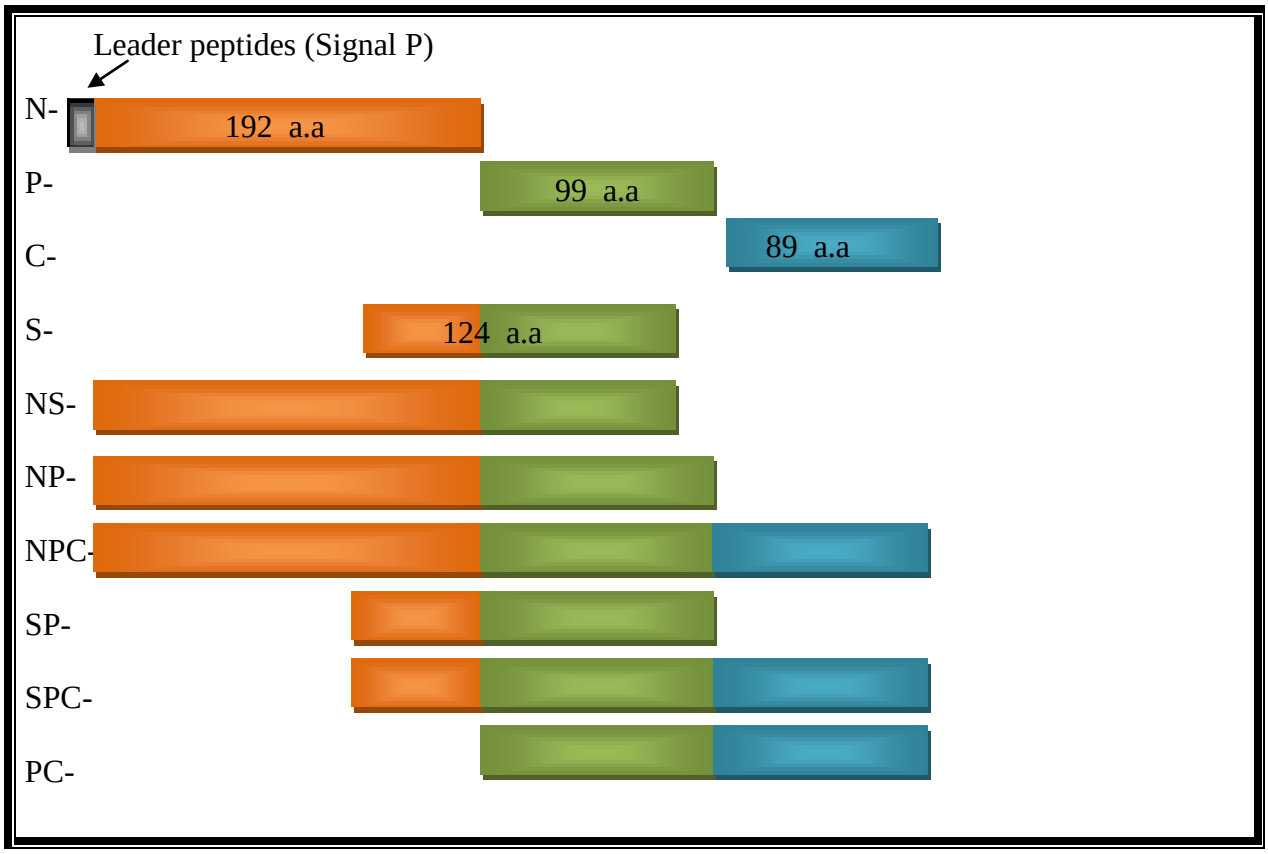


Figure 4: Diagram represents different amplified SM-CRT sub-fragments

2.2.1.1 .1 PCR polymerase chain reaction,

A *S. mansoni* adult cDNA library was kindly supplied by Dr David Johnston (Natural History Museum London). The PCR reaction was prepared in a total volume of 25 µl on ice. Each labelled PCR reaction tube (Fisher scientific**,** AB-0533**)** received 1µl of *S. mansoni* cDNA library and 2.5 µl of each of the corresponding forward and reverse primers that were adjusted at 5 µM. Master mix was prepared in a separate tube to be enough for amplification of 12 samples (extra samples to compensate lost in volume during handling the solutions in-between the tubes). The following amounts were multiplied by 12: 15.8 µl of sterile nanopure water, 2.5 µl x5 Phusion HF reaction buffer containing 1.5 mM $MgCl_2$ in the final reaction, 0.5 µl 10 mM dNTPs (Promga, U1511) and 0.2 µl Phusion High-Fidelity DNA polymerase (Finnzymes, F-530S) which was added at the end to the mixture to avoid degradation of the primers before adding dNTPs. After gentle mixing of the reagents of the master mix, 19 µl of this mixture was added to each the previously labelled prepared tubes.

The PCR reactions were put inside the rack of TGradient Thermocycler (Biometra, 050-801) which was set up with touchdown PCR programme for DNA amplification with the following steps:

- **Initial denaturation** step 95 ºC for 90 seconds

-Cycle 1-15: Denaturation step 95 ºC for 30 seconds

Annealing step 70 ºC for 30 seconds

(decreased by 0.8 ºC per cycle until it became 58 ºC at the fifteenth cycle)

Extension step 72 ºC for 30 seconds.

-**Cycle 16-40** (the next 25 cycles with annealing step at 58 ºC)

Denaturation step	95 ºC for 30 seconds
Annealing step	58 ºC for 30 seconds
Extension step	72 ºC for 30 seconds

-**Final Extension step** 72 ºC for 5 minutes

Domain	The primers
N-	**Forward primer:** 5`-GGA TCC GGG ACA TGA AGT TTG GTT TTC GGA-3` **Reverse primer:** 5`-CTC GAG TTA ATC AAT CTT TTT CGG TGG AAG -3`
P-	**Forward primer:** 5`-GGA TCC GGA CCC AAA TGA CAA GAA ACC TGA-3` **Reverse primer**: 5`-CTC GAG TTA CAG GAC ATA CAA TTC GGG ATC ATG-3`
C-	**Forward primer:** 5`-GGA TCC CCT GAA TGA CAT CGG TTA TGT CG-3` **Reverse primer:** 5`-CTC GAG TTA CAG CTC ATC GTG GTC ACC A-3`
S-	**Forward primer:** 5`-GGA TCC AGA AAT ACC CTG CAA AGA TGA CCT-3` **Reverse primer:** 5`-CTC GAG TTA TGG ATT ATC GAT ACG TCT AGG TGT C-3`
NS-	**Forward primer:** 5`-GGA TCC GGG ACA TGA AGT TTG GTT TTC GGA-3` **Reverse primer:** 5`-CTC GAG TTA TGG ATT ATC GAT ACG TCT AGG TGT C-3`
NP-	**Forward primer:** 5`-GGA TCC GGG ACA TGA AGT TTG GTT TTC GGA-3` **Reverse primer:** 5`-CTC GAG TTA CAG GAC ATA CAA TTC GGG ATC ATG-3`
NPC-	**Forward primer:** 5`-GGA TCC GGG ACA TGA AGT TTG GTT TTC GGA-3` **Reverse primer:** 5`-CTC GAG TTA CAG CTC ATC GTG GTC ACC A-3`
SP-	**Forward primer:** 5`-GGA TCC AGA AAT ACC CTG CAA AGA TGA CCT-3` **Reverse primer:** 5`-CTC GAG TTA CAG GAC ATA CAA TTC GGG ATC ATG-3`
SPC-	**Forward primer:** 5`-GGA TCC AGA AAT ACC CTG CAA AGA TGA CCT-3` **Reverse primer:** 5`-CTC GAG TTA CAG CTC ATC GTG GTC ACC A-3`
PC-	**Forward primer:** 5`-GGA TCC GGA CCC AAA TGA CAA GAA ACC TGA-3` **Reverse primer:** 5`-CTC GAG TTA CAG CTC ATC GTG GTC ACC A-3`

Table 1: Oligonucleotides used in amplifications of different DNA fragments encoding SM-CR sub-fragments. a *Bam*HI restriction site engineered in the forward primer sequence at 5' (under lined bases) and *Xho*I restriction site is included in the reverse primer at 3' (under lined bases).

2.2.1.1.2 Analysing of PCR products by agarose gel electrophoresis

At first, 1.5 g of agarose (Melford, MB 1200) was mixed with 1x TAE buffer [(200 ml 50x TAE, 200 µl ethidium bromide (Sigma, E-8751) and completed to 10 litter with dH_2O) (50x TAE is composed of 242 g Tris base, 57.1 ml glacial acidic acid, 100 ml of 0.5 M EDTA and completed into 1L H_2O pH 8.5)]. The agarose mixture was heated in microwave until the powder completely dissolved, then left to cool in water bath at 58 ºC. The agarose solution was poured into the gel cast tray, containing a sample comb, sealed with autoclavable tape and left at room temperature until solidification. The gel tray, after removing tape and comb, was put into the agarose gel tank full with the running buffer (1X TAE). From each amplified PCR product sample, 5 µl was mixed with 1µl loading dye (5.8 ml of 1 M Tris pH 6.8, 0.83g SDS, 2.5 ml glycerol,1 mg bromophenol blue(Sigma Alrich,114405 **)** completed to 10 ml with d H2O) and loaded into the gel lanes. In the first lane, 6 µl of 100 bp GeneRuler ™ DNA Ladder Mix (Fermentas, SM 0331) was loaded. The electrodes were connected with the cathode in the site of the DNA samples and the anode in the other site. The current was adjusted to 100 V constant and applied until the samples reached near the end of the gel. The gel tray was moved from the tank, seen and photographed under UV light using Olympus digital camera**.**

2.2.1.1.3 Purification of PCR products

Each PCR product encoding individual SM-CRT sub-fragment was purified with QIAquick Nucleotide Removal Kit (QIAGEN, 28304) to get rid of DNA contaminants which could interfere with subsequent sub-cloning process such as salts, enzymes and nucleotides, in

addition to any remaining Phusion DNA Polymerase enzyme in the reaction which will break the terminal-A base again.

QIAquick Nucleotide Removal Kit was used following manufacturer's instructions. Briefly, buffer PN was added and mixed with each PCR product in ratio of 5:1. The sample was loaded into QIAquick column placed into its collection tube and centrifuged for 1 minute at 10,000 xg using bench centrifuge **(**Eppendorf, centrifuge 5417C**)**. After discarding follow-through, 750 µl buffer PE was added and the sample was centrifuged again for another 1 minute. The follow-through was removed completely and the empty column was then centrifuged at high speed of 10,000xg for 1 minute. The QIAquick column was transferred to clean 1.5 ml microcentrifuge tube and 30 µl buffer EB was added into the centre of the membrane of column. The column was centrifuged at maximum speed for 1 minute for the last time to elute the purified DNA. The eluted DNA was analysed by running on 1.5 % agarose gel and DNA was kept at -20 ºC for further use.

2.2.1.1.4 Adding terminal-A to the blunt ended purified PCR products

As proof reading High-Fidelity DNA polymerase enzyme resulted in blunt-ended products, the following steps were used to generate A-tailed products. From each purified PCR product encoding individual SM-CRT sub-fragment sample, 5µl was placed in labelled sterile small microcentrifuge tube. Then, the following reagents were added into each DNA sample; 2 µl buffer *Taq* DNA Polymerase mix 10X, 1 µl of 1mM dATP (Promega, U1201),1µl (5 unit) *Taq* DNA Polymerase (Thermo scientific, AB-1301/A) and autoclaved nanopure water was added to complete the reaction volume to 20 µl. The mixtures were

incubated on heat block (Eppendorff, Thermomixer 5436) at 70 °C for 15 minutes, then the PCR products with terminal-A were stored at -20 °C.

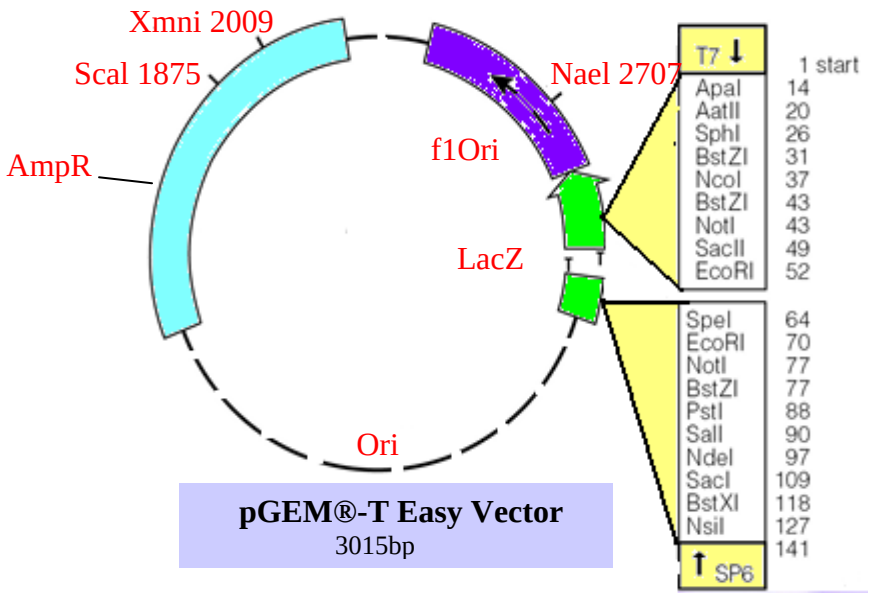
2.2.1.2 Cloning of SM-CRT sub-fragments DNA segments into pGEM®-T Easy Vector

2.2.1.2.1 Preparation of chemically competent cells

This protocol is suitable for many strains of *E. coli* including TOP10F`and BL21 (DE3) pLysS. The bacterial strain was streaked out using a sterile metallic wire loop to spread bacteria on the surface of L.B. agar plate which is enriched with 10 mM Mg Cl2. The plate was left overnight in the incubator at 37 °C. In the next day, the plate was transferred into 4 °C. At the evening prior to competent cell preparation, a single colony was picked up from the corresponding plate with a sterile pipette tip and inoculated into 5 ml sterile L.B. and left overnight in shaker incubator (New Brunswick Scientific (UK) Ltd, Innova 44) at 37 °C with 200 rpm. In the following morning, 1 ml of the overnight culture was transferred into 100 ml of fresh sterile L.B. media and it put back at 37 °C with 200 rpm for 2-3 hours until the OD_{550} reached 0.7-0.8. After that, the cells were spun at 4000 rpm for 10 minutes at 4 °C (Beckman coulter, Allegra® X-22) and the supernatant was discarded. The bacterial pellet was re-suspended with 30 ml of sterile ice cold TfbI (30 mM K-acetate, 50 mM Mn Cl_2, 10 mM $CaCl_2$ and 15 % Glycerol) and placed on ice for 15 minutes for TOP10F` or 30 minutes in case of BL21(DE3)pLysS. The mixture was then centrifuged at 4000 rpm for 10 minutes at 4 °C and the bacterial pellet was re-suspended gently with 4 ml of sterile ice cold TfbII (10 mM Na-MOPS, 10 mM KCl, 75 mM $CaCl_2$ and 15 % Glycerol). The competent cells were aliquoted gently on dry ice in 50 µl fractions into 0.5 ml microcentrifuge tubes and stored at -80 °C.

2.2.1.2.2 Ligation of DNA fragments to pGEM®-T Easy Vector (Promega)

pGEM®-T Easy Vector is a high-copy-number plasmid containing T7 and SP6 RNA polymerase binding site. This vector is linearised and fitted with a 3' terminal T base overhang on either end to facilitate subcloning of an A-tailed PCR product into the multiple cloning site. The ligation reaction was prepared in total reaction of 10 µl on ice. The vector and its accompanying control tubes were centrifuged briefly to allow collection of their contents down the bottom of the containing tubes. A master mix was prepared to be enough for the samples numbers using the following reagents: 1µl 2X rapid Ligation Buffer, 1 µl pGEM®-T Easy Vector, 1 µl T4 DNA Ligase enzyme (New England biolab, M0202S) and 5µl sterile distilled H_2O. Then, 8 µl from the master mix was transferred to each sterile labelled small centrifuge tubes and 2 µl of the corresponding purified PCR encoding a SM-CRT sub-fragment was added and mixed gently. Under the same conditions; a positive control reaction was prepared from 1 µl ligation buffer, 1 µl vector, 2 µl control DNA insert, 1µl T4 DNA ligase and 5 µl sterile distilled H_2O. The negative control was prepared but without any DNA. The reactions were kept at 4°C overnight to maximize the transformants numbers.



T7 RNA polymerase transcription initiation site 1
multiple cloning region 10–128
SP6 RNA polymerase promoter (–17 to +3) 139–158
SP6 RNA polymerase transcription initiation site 141
pUC/M13 Reverse Sequencing Primer binding site 176–197
lacZ start codon 180
lac operator 200–216
β-lactamase coding region 1337–2197
phage f1 region 2380–2835
lac operon sequences 2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site 2949–2972
T7 RNA polymerase promoter (–17 to +3) 2999–3

Figure 5: pGEM®-T Easy Vector map

2.2.1.2.3 Transformation of pGEM®-T Easy constructs into chemically competent TOP10F` *E. coli*

Competent TOP10F` *E. coli* was transformed with chemical transformation method. The cells was thawed on ice, 1 µl of each construct was added and mixed gently with 50 µl cells and incubated on ice for 15 minutes. At the same time, a negative control was done by incubating the competent cells with 1 µl TE buffer. The cells were transferred quickly to

water bath adjusted at 37 °C for 5 minutes (heat shock). After that, the mixture was poured into 950 µl preheated L.B. (at 37 °C) and placed in the shaker incubator for 1 hour at 37 °C with 200 rpm. While incubating the cells, the prepared ampicillin plates were brought from the fridge to be equilibrated at room temperature and 100µl 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Melford, MB1008) plus 20µl of 50mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) (Melford, MB1001) were spread on the plates which were left at 37 °C for half hour before receiving bacterial culture. For each DNA construct, 150 ml from the cell culture was plated on L.B./ ampicillin/ IPTG/ X-gal plate in addition to another plate received 150 µl from the negative control culture cells. All the plates were left overnight at 37 °C. In the next morning, the plats were removed from the incubator and checked for the white/ blue colonies where the white colonies mostly have the recombinant plasmids, whereas the blue ones harbor only vector ligated on itself. The plates showing white colonies were kept in the fridge.

2.2.1.2.4 Small scale production of cloned pGEM®-T Easy Vector, minipreps preparation

For each DNA construct encoding individual SM-CRT sub-fragments, the preparation of plasmids in minipreps scale followed identical procedures. In brief, in the evening, a single white colony was picked up by a sterile pipette tip from its corresponding ampicillin L.B. agar plate. The colony was inoculated into 3-5 ml of L.B. broth supplied with 100 µg/ml ampicillin in sterile 15 ml falcon tubes and incubated overnight at 37 °C in shaker incubator at 200 rpm. In the following morning, bacteria were pelleted by centrifugation of 1-3 ml culture by a tabletop centrifuge for 5 minutes at 10000 xg. The supernatant was discarded and the tubes were inverted and gently tapped against tissue to get rid of any excess

remaining media. Wizard® Plus SV Minipreps DNA Purification Systems (Promega, A1460) was used for purification of DNA plasmid from the bacteria cells following manufacturer's instructions. The pellet was completely re-suspended with 250 µl Cell Resuspension Solution (50 mM Tris-HCl (pH 7.5) and 10 mM EDTA and 100 µg/ml RNase), then lysed with 250 µl Cell Lysis Solution (0.2 M NaOH and 1%SDS), inverted 4 times and left for 1-5 minutes. The endonucleases and other released proteins from the destructed *E. coli* cells were inactivated by adding 10 µl alkaline protease to the sample which inverted 4 times and incubated for 5 minutes. After that, 350 µl Neutralization Solution (4.09 M Guanidine hydrochloride, 0.759 M Potassium acetate and 2.12 M Glacial acetic acid) was added and the tubes were inverted 4 times and the bacterial lysate was centrifuged at 20,000 x g for 15 minutes. The clear supernatant without any white precipitate contaminant was poured into the Spin Column and centrifuged for 1 minute at the maximum speed. The flow-through was removed from the collecting tubes and the Column Wash Solution (162.8 mM Potassium acetate, 22.6 mM Tris-HCl, pH 7.5 and 0.109 mM EDTA pH 8.0), diluted with 95% ethanol, was applied twice, one of 750 µl and after centrifugation for 1 minute and discarding the flow-through, then 250 µl of the washing buffer was added and centrifuged for further 2 minutes. After that, the Spin Columns were transferred to clean 1.5 ml microcentrifuge tubes where DNA was eluted by adding 100 µl of nuclease–free water to centre of the membrane of Spin Column and centrifuged at maximum speed for 1 minute. DNA plasmid was kept at -20 °C.

2.2.1.2.5 Digestion of recombinant pGEM®-T Easy plasmids by restriction enzymes

The aim of digestion was to confirm the success of ligation of DNA fragment with pGEM®-T Easy vector. Digestion reaction was prepared in 20 µl total volume. At first, master mix was prepared (by multiplying number of the samples plus two) using the following reagents: 10 µl sterile water, 2µl NEBuffer EcoRI (10X), 2 µl 10% BSA and 1 µl of EcoRI restriction enzyme (New England Biolab, R0101S). Then 15 µl from the master mix was mixed gently with 5 µl of the DNA construct encoding individual SM-CRT sub-fragment and left in water bath at 37 °C overnight. In the next morning, the whole reaction was loaded into 1.5% agarose gel and after finishing the running, gel was photographed. The recombinant plasmids containing the inserts were stored at -20 °C.

2.2.1.2.6 Sequencing of DNA constructs

From each SM-CRT DNA construct, 25 µl was sent to The Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester for DNA sequencing. The DNA fragment sequences identical with that published in GenBank, was stored at -20°C.

Glycerol stocks were prepared from overnight culture of the transformed TOP10F` cells with pGEM®-T Easy plasmids that were shown to contain the coding SM-CRT DNA sequences identical to the published sequences in the database. From the cell overnight culture, 200 µl was mixed gently with 200 µl of 50 % glycerol in sterile, labelled 1.5 ml microcentrifuge tubes and stored at -80 °C.

2.2.1.3 Sub-cloning of SM-CRT DNA fragments into pRSETB (Invitrogen, V351-20), the protein expression vector

This vector is a pUC-derived expression vector which induces high protein expression. Gene translation is under the control of the T7 promoter. The latter is activated by binding to T7 RNA polymerase which induced by IPTG.

2.2.1.3.1 Propagation of pRSETB vector

The purchased lyophilized vector was centrifuged, re-suspended in 20 µl TE buffer to prepare a stock concentration of 1 µg/µl which then diluted at 1:1000, aliquoted and kept at -20 °C. TOP10F` *E. coli* competent cells were transformed with 2 µl from the diluted pRSETB vector solution following procedures mentioned in the section **2.2.1.2.3**. The selective L.B. media contained 50 µg/ml ampicillin. Minipreps from the vector were prepared using The Wizard® Plus SV Minipreps DNA Purification Systems (see section **2.2.1.2.4**). The amplified vector was then stored at -20°C.

2.2.1.3.2 Digestion of pRSETB vector

The circular closed pRSETB vector was digested by *Bam*HI and *Xho*I restriction enzymes to open the cloning site to be ligated with the DNA insert downstream and in frame with expression of the protein with N-terminal fusion polypeptides. The mixture of digestion was prepared as the following: 25 µl sterile water, 5 µl pRSETB miniprep, 4 µl NEBuffer 2, 4 µl BSA %10 and 1 µl from each of the restriction enzymes *Bam*HI (New England Biolab, R0136S) and *Xho*I (New England Biolab, R0146S). The mixture was vortexed briefly and put in water bath adjusted at 37 °C. In the next day, the digestion mixtures was

purified with the QIAquick Nucleotide Removal Kit. The purified digested pRSETB was visualized by running of 5 µl on 1% agarose gel then labelled and stored at -20°C.

2.2.1.3.3 Purification of DNA inserts from digested pGEM®-T Easy Vectors from agarose gel

The DNA inserts were cut from the cloned pGEM®-T Easy Vectors. For each DNA encoding SM-CRT sub-fragment, the mixture was prepared in 40 µl volume in 1.5 ml centrifuge tube. Master mix was prepared by multiplying samples number plus two by the following reagents volums; 20 µl sterile distilled water, 4 µl NEBuffer 2, 4 µl BSA 10%, 1 µl *Bam*HI and 1 µl *Xho*I, then 30 µl of the mix was distributed to each labelled tube containing 10 µl of the corresponding construct. They were left overnight in a water bath at 37 °C. In the next day, the mixtures were poured into 1.5 % agarose gel. The whole volume of the digestion was loaded into 1.5 % agarose gel and run using 90 volt. When the loading dye reached near the end of the gel, it was removed. Under ultraviolet light, the insert bands were cut using sterile sharp scalpel avoiding excess gel and QIAquick Gel Extraction Kit (QIAGEN, 28704) was used for their purification according to the manufacturer's instructions.

Briefly, the DNA gel fragment was weighed and 3 volumes of buffer QG were added to 1 volume weight of the cut band and put at 50 °C (100 mg equal to 100 µl) for 10 minutes or more with frequent vortex until the gel was completely dissolved. The DNA samples were poured to QIAquick columns placed in its 2 ml collection tube and centrifuged for 1 minute at 13000 rpm (this speed was used in all steps of centrifugation). The flow-through was discarded and 0.5 ml buffer QG was added to the column and centrifuged. After removal of

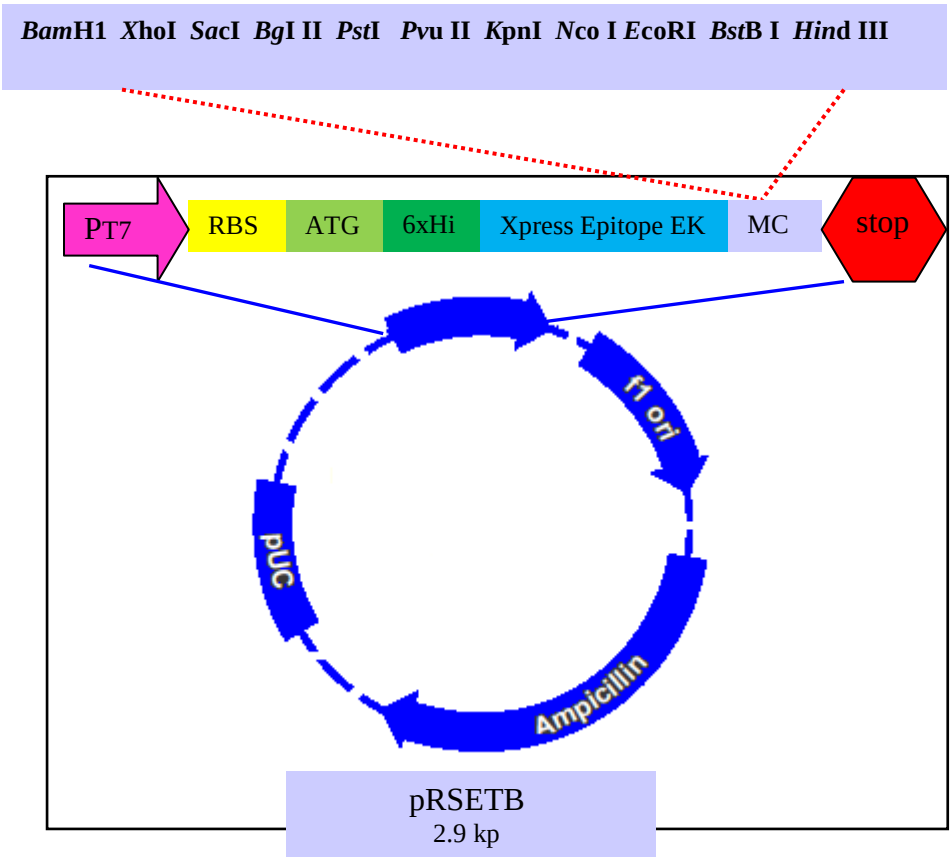
flow-through, 750 µl buffer PE was added to wash QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the column was centrifuged for further 1 minute. Finally, QIAquick column was transferred to clean microcentrifuge tubes and 30 µl buffer EB was added and the column was centrifuged for 1 minute. The eluted DNA was tested by running 5 µl on 1.5 % agarose gel and the remaining amount was kept at -20 °C.

2.2.1.3.4 Ligation of the gel purified DNA fragments to pRSETB vector

Each purified DNA insert encoding a SM-CRT sub-fragment was ligated into pRSETB vector. The ligation procedure was performed as in section **2.2.1.2.2.**

2.2.1.3.5 Transformation of pRSETB constructs

The same procedures in section **2.2.1.2.3** were followed. TOP10F` was transformed and grown overnight on 50 µg/ml ampicillin L.B. agar plate. In the following day, 9-11 colonies were randomly selected from ampicillin /L.B. agar plate.



T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 916-2852

Figure 6: pRSETB vector map

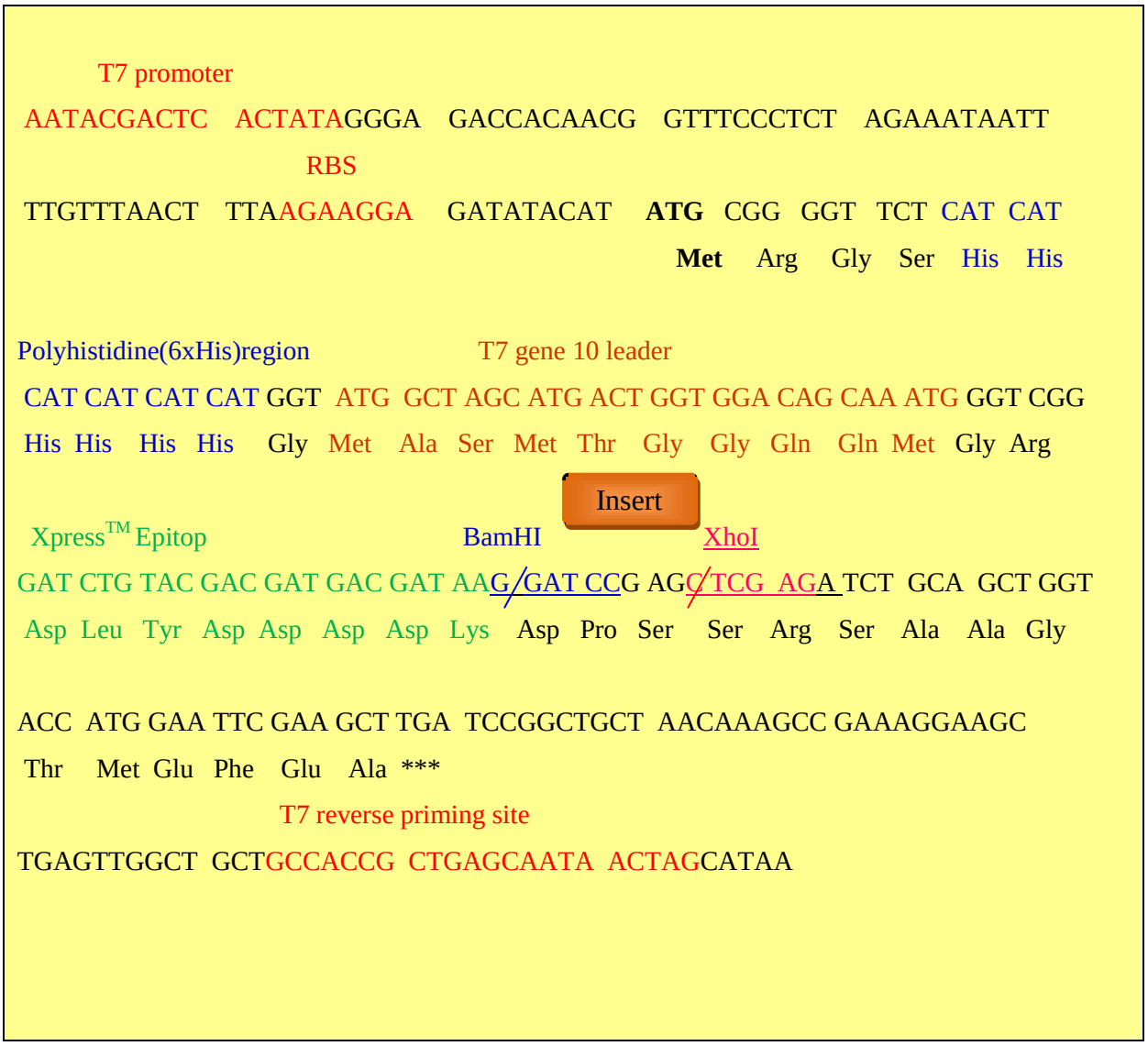


Figure 7: Multiple cloning site of pRSETB vector

2.2.1.3.7 Production of pRSETB constructs minipreps

The randomly selected colonies for each pRSETB construct (9-11) were used to prepare minipreps of the constructs using Wizard® Plus SV Minipreps DNA Purification Systems. (see detail of minipreps preparations in section **2.2.1.2.4**)

2.2.1.3.8 Digestion of pRSETB minipreps

To confirm presence of the DNA inserts ligated to the vector of expression, the 9-11 minipreps prepared from the previous colonies (for each construct) were digested. At first, 5 µl from each DNA miniprep purified from a single colony was put in labelled 1.5 ml microcentrfiuge tube. A master mix was prepared by multiplying the number of the sample plus two by the following volumes: 12.6 µl autoclavable nanopure water, 2 µl NEBuffer 2, 1 µl BSA 10%, 0.2 µl from *Bam*HI and 0.2 µl from *Xho*I. The samples were transferred into water bath at 37 °C and left overnight. In the following day, the digestion products were run on 1.5% agarose gel and one successful ligated pRSETB construct for each SM-CRT sub-fragment was kept in -20 °C.

2.2.1.4 Expression of the recombinant SM-CRT sub-fragments

2.2.1.4.1 Transformation of the chemically competent BL21(DE3)pLysS cells

The competent BL21(DE3)pLysS cells was used for protein expression. These cells contain two plasmid; pLysS plasmid, which is needed for T7 lysozyme, resistant to chloramhpenicol and pRSET B plasmid which is resistant to ampicillin and they produced lower amounts of proteases. From each SM-CRT sub-fragment pRSETB construct, 1µl was incubated with 50 µl of competent BL21(DE3)pLysS, then the rest of transformation steps

(described in section **2.2.1.2.3)** were followed. The transformed cell culture was grown on plate enriched with 50 µg/ml ampicillin and 35 µg /ml chloramphincol

2.2.1.4.2 Pilot protein expression experiment

A pilot experiment was carried out for expression of each SM-CRT sub-fragment separately. A single colony from the plate plated with the transformed Competent BL21(DE3)pLysS cells was incoluted into 2 ml of sterile S.O.B. media (20 g Tryptone, 5 g Yeast Extract, 0.5 g NaCl,186 mg KCl dissolved in 1000 ml distilled water and before use 10 ml of sterile 1 M $MgCl_2$ was added for each 1 L S.O.B. media) supplemented with 50 µg/ml ampicillin and 35 µg /ml chloramphincol and incubated overnight at 37 °C at 200 rpm. In the next day, the media was transferred into 25 ml fresh sterile S.O.B. enriched by the antibiotics and kept in the shaker until OD_{600} reached 0.6-0.8. From that culture, 1 ml was taken and centrifuged at 4 °C for 10 minutes using bench centrifuge (Eppendorf, centrifuge 5415R) and the cell pellet was kept in -20 °C (a zero time sample). IPTG was then added to the culture in concentration of 100 mM and the culture was put again at 37 °C at 200 rpm in the shaker. Every one hour, for four times, 1 ml was removed and centrifuged for preparation of cell pellets.

After that, each pellet was re-suspended in 100 µl of 20 mM phosphate buffer, pH 7.0. The suspension was subjected to 3-4 cycles of freezing and thawing in methanol/dry ice and 42 °C. The samples were centrifuged at 4°C for 10 minutes at the maximum speed. The supernatant was transferred to a new tube and mixed with an equal volume of 2X SDS-PAGE buffer (6.06 g Tris base, 28.8 g Glycine, 2g SDS and complete to 1L with dH2O, pH 8.3), whereas the cell pellet was re-suspended in 100 µl 1X SDS-PAGE buffer (3.03g Tris

base,14.4 g Glycine,1g SDS and complete to 1L with dH2O, pH 8.3). The samples were then analysed with SD-PAGE gel stained with Coomassie blue and with Western blot analysis.

2.2.1.4.3 Analysis of the expressed SM-CRT sub-fragments

This was done by SDS-PAGE Coomassie blue and Western blotting.

2.2.1.4.3.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970)

Mini-PROTEAN Tetra Cell (Bio-Rad, 165-8000) apparatus was used to carry out SDS-PAGE gel electrophoresis. Resolving gel was prepared by using the following reagents (which is enough for 2 mini-gels).

The material	**12% gel**	**15% gel**
H2O	4.9 ml	3.4 ml
30% Acrylamide/bis-Acrylamide	6.0 ml	7.5 ml
1.5 M Tris (pH 8.8)	3.8 ml	3.8 ml
10 % SDS	0.15 ml	0.15 ml
10% ammonium persulphate	0.15 ml	0.15 ml
TEMED	0.006 ml	0.006 ml
(N,N,N',N'-Tetramethylethylenediamine)		

*TEMED (was mixed immediately before pouring the gel into the glass plate).
*The mentioned amounts are appropriate to make two gel.
* 15 % gel was prepared for P- and C-domains.

The resolving gel was loaded until it reached a mark of one centimetre below the level of pre-placed comb teeth. Butanol (Sigma-Alrich, B7906) was added on the top of the gel to prevent air bubble formations**.** The gel was left for 45-60 minutes at room temperature until

completely polymerized. Butanol was washed away with distilled water then dried by the filtered paper. The stacking was prepared from the following amounts which are enough for two mini-gels [(4.1 ml dH_2O, 1 ml 30% Acrylamide/bis-Acrylamide (Sigma-Aldrich, A3699), 0.75 ml 1.5 M Tris (pH 8.8) (Sigma-Aldrich,T6066), 0.06 ml 10 % SDS (Fisher Scientific, BP 166-500), 0.06 ml 10% ammonium persulphate and 0.006 ml TEMED which was mixed immediately before pouring the gel]. The stacking gel was poured on the surface of the resolving gel until reaching the upper border of the short plate.

The clean comb was then placed to construct the wells. The gel was left for 30-45 minutes until occurrence of complete polymerization. The comb was removed and the wells were washed by distilled water. The gel cassettes were placed in the electrode assembly and put inside the tank filled with SDS-PAGE running buffer (SDS-PAGE buffer (10x) is composed of 30.3 g Tris base,144 g Glycine, 10g SDS and complete to 1L with dH_2O, pH 8.3).

To prepare protein sample for loading on SDS-PAGE gel, 15 µl from each protein sample was mixing with 5 µl 5% mercaptoethanol loading buffer (5.8 ml 1 M Tris pH 6.8, 0.83g SDS, 2.5 ml glycerol, 1 mg bromophenol blue and completed to 10 ml with dH_2O) and boiled at 95 °C for 5 minutes. In addition to the proteins samples, 10 µl of protein marker, SeeBlue Plus2 PreStained Standard (Invitrogen, LC925), was loaded in the first well. The power was applied to the cell and the electrophoresis was run using 120 V constant until the front dye reaches the lower end.

2.2.1.4.3.2 Coomassie blue staining of the SDS-PAGE gel

On litre of Coomassie blue solution was prepared (600 mg Coomassie Blue R250 (Serva, 17525), 260 ml water, 240 ml methanol and 100 ml acetic acid) and stored at room temperature for up to six months. After the end of the gel electrophoresis, gels were immersed immediately in the Coomassie Blue solution in a plastic box with a lid and left for 1 hour on a rocking platform at room temperature. The excess stain was removed and a de-staining solution (30% methanol and 10% acetic acid) was poured over the gel. The de-staining solution was changed every ten minutes for three times. Then the gel was kept in 50 % de-staining solution overnight on the rock platform in a tightly sealed box to prevent the gel from drying. The gel was examined for seeing the band of increasing intensity at the expected size range for each protein and then scanned.

2.2.1.4.3.3 Western blotting of the expressed SM-CRT sub-fragments (Towbin *et al.* 1979)

To confirm expression of the proteins, Western blot analysis was performed using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, 170-3930). A piece of nitro-cellulose paper (Bio-rad, 162-0097) was cut slightly larger than the gel and marked by pen on the surface that will face the gel. In a large plate contained transfer buffer (for 1 L volume; 2.9 g Glycine, 5.8 g Tris, 0.37 g SDS, 200 ml Methanol and d H_2O to 1 L, pH 8.3), the Gel Holder Cassette was opened and on its black cathode site, the sandwich was formed in the following order: one Fiber pad, filter paper, the SDS-PAGE gel, nitrocellulose membrane, the other filter paper and finally the other Fiber pad. Then the cassette was closed and transferred into the tank full of the transfer blotting buffer. A frozen Bio-Ice

cooling unit was put alongside the gel holder to cool the system while running. The current was adjusted to 300 milliampire for 45-60 minutes.

After that, the membrane was removed from the cassette and transferred into the blocking buffer (5% skimmed milk in PBS) for 30 minutes at room temperature on a shaking platform. Monoclonal anti-polyhistidine peroxidase conjugate (Sigma, A7058) (1:4000 in the blocking buffer) was incubated with the membrane for 1 hour on the shaking platform. The membrane was washed three times, 10 minutes each using 0.05%Tween-20/PBS. The signal was detected by chemiluminescence technique using ECL Western Blotting Substrate system (Pierce, 32106). The detection reagents 1 (peroxide) and 2 (luminal) were mixed in equal volume and were added to the membrane. After incubation for 1 minute, the excess reagents were removed and the membrane was completely covered with clean plastic sheet. In dark room, the membrane with its previously marked protein side was incubated with X-ray film (Kodak, Z35, 847-9) in the film cassette for 1-5 minutes. The film was put in the developer solution (AGFA, G153A) and shaken until the signal appeared, then it was transferred to the fixer solution (AGFA, G153B). The film was put again on the membrane in the light to mark the bands of the pre-stained standard protein marker on the film. The film was washed with running tap water and left in the room temperature until dried.

2.2.1.4.4 Large scale production of SM-CRT sub-fragments

To produce recombinant SM-CRT sub-fragments in large amounts, the basic steps of protein expression described in the section of preliminary protein expression were followed with increased the volumes of bacterial media. For each SM-CRT sub-fragment, one colony of the transformed competent bacteria was grown in a larger volume of (20 ml)

S.O.B. media supplemented with combined 50µg/ml ampcillin and 35µg/ml chloramphenicol and put in the shaker incubator at 37 °C at 200 rpm overnight. In the next day, the overnight culture was aseptically transferred into 400ml of fresh media, enriched with antibiotics, in autoclaved 2 litre flask (total volume 1 litter for each protein). The Flasks were put in the shaker in the same conditions until OD_{600} reached 0.6-0.7, then sterile IPTG in end concentration of 100 mM was added to the cultures which were put back to the shaker for 2-3 hours. The cultures were centrifuged in large centrifuge Backman 500 ml tubes with 4000 rpm at 4°C (Beckman Coulter, Avanti J-E Centrifuge) for 20 minutes and the cell pellets were stored in -20 °C until used.

2.2.1.5 Purification of the recombinant SM-CRT sub-fragments

2.2.1.5.1 Preparation of protein samples

Each SM-CRT sub-fragment was purified by nearly the same methods. The pellets, derived from 1 litre of the IPTG induced culture, were collectively re-suspended in 25 ml his-tag affinity chromatography buffer A (20 mM Tris HCl, 500 mM NaCl and 5 mM Imidazole, pH 8.0). The proteinase inhibitor, Pefabloc SC (Roche, 11 429 868 001) was added in concentration of 4 mM. The media was subjected to three cycles of freezing in methanol/dry ice, heat shock at 42 °C and three 20 seconds bursts of sonication (Sanyo MSE, London, U.K., Soniprep 150). The destroyed cells were centrifuged at 15000 rpm for 30 minutes at 4 °C. The supernatant was transferred into fresh tubes and were filtered using 0.45 µm filter to get red of the cell debris and cell genome which could block the purifier columns.

2.2.1.5.2 The methods used for SM-CRT sub-fragments the purifications

The buffers were prepared from nanopure water and they were filtered using 0.45 µm filters and they could be used within one week or filtered again. FPLC Amersham with Unicorn 5.01 programme was used in the process of protein purifications.

2.2.1.5.2.1 His-tag affinity chromatography

The proteins were produced as fusion proteins with a Histidine-tag added on their N-terminal end to facilitate purification. The principle of affinity chromatography is that there is a ligand which is bound to the chromatography matrix, and protein bound reversibly and specifically to this ligand. The protein is then eluted by using another competitive ligand, imidazole.

The proteinase inhibitor, Pefabloc SC was added in concentration of 4 mM to the sample before applied to the column. HiTrap Chelating HP column, 5 ml (GE Healthcare, 17-0409-01) was used in performing the process of purification. HiTrap Chelating HP column is pre-packed with 5 ml of Chelating Sepharose™ High Performance which is agarose beads coupled to the chelating iminodiacetic acid. The new column was prepared by application of 5 CV (column volume) distilled water by syringe drop by drop to wash out the 20 % ethanol inside the column. After that, 10 ml 100 mM $NiCl_2$ (Sigma, N 5756) (2 CV) was used to charge the column. The excess unbound Ni was removed by application of 3-5 CV distilled water. The column is now ready to be applied on the machine.

By using FLPC, Amersham set up with Unicorn 5.01 programme, HiTrap Chelating HP column was equilibrated with 5 CV (column volume) of binding buffer, buffer A (20 mM

Tris HCl, 500 mM NaCl and 5 mM Imidazole, pH 8.0) at the flow rat 5 ml /min and the protein was loaded into the column automatically by FLPC machine. After that, the elution buffer, buffer B (20 mM Tris HCl, 500 mM NaCl and 500 mM Imidazole, pH 8.0) was mixed automatically according the programme using linear gradient elution method. The eluted fractions corresponding to the peak representing the purified SM-CRT sub-fragment (on the chromatogram) were collected and analysed. It was found that most of the proteins required further exchange chromatography.

2.2.1.5.2.2 Ion Exchange chromatography

The protein purified with His-tag affinity chromatograph buffer (20-25% buffer b) was desalted by de-salting buffer (20 mM Tris HCl and 50 mM NaCl, pH 8.0**)** using HiPrep™ 26/10 Desalting Column with the FLPC machine. The diluted protein sample was concentrated with Millipore's Amicon® Ultra-15 centrifugal filter devices (Millipore, UFC900308 and UFC901008) to be 25-30 ml. After that, the sample was subjected to anion ion exchange using HiTap Q HP, 5 ml (GE Healthcare, 17-1153-01) column. This column contains Q Sepharose High Performance anion exchanger media and the charged group is $CH_2N+ (CH_3)_3$. The proteins were buffered at few pH units above their iso-electric point, so they become a negatively charged and bound to the anion exchanger.

After washing the preservative from the column with 5 CV of buffer A (20 mM Tris HCl and 20 mM NaCl, pH 8.0), the column was equilibrated with 5 CV of buffer A. The protein sample was loaded into the column in the washing buffer, buffer A at a rate of 5 ml /min automatically by the programme. The elution buffer, buffer B (20 mM Tris HCl and 1M NaCl, pH 8.0) was applied automatically into the column to elute the target protein

according the programme. The elution was carried out with increasing salt concentration (ionic concentration) by using linear gradient method. With increasing salt concentration, it competes with the proteins which are bound to the charged medium in the column launching elution starting with the proteins with weak net charges. The fractions representing the SM-CRT peak on the chromatogram was collected and analysed.

2.2.1.5.2.3 Dialysis and concentration of the purified SM-CRT sub-fragments

HiPrep™ 26/10 Desalting Column was used to dialysis the proteins into a physiological buffer (20 mM Tris HCl and 145 mM NaCl, pH 7.4). The protein sample was concentrated with Millipore's Amicon® Ultra-15 centrifugal filter devices (Millipore, UFC900308 and UFC901008). The filter device with cut off 3 kDa were loaded with SM-CRT N-, P-, C- and S-domains, whereas 10 kDa for other SM-CRT sub-fragments. The centrifugal filter tube was firstly rinsed with the dialysing buffer by centrifugation for few minutes at 4 °C at 4000 xg. After discarding the buffer, dialysed protein sample was added in the filter unit and centrifuged at 4000 xg at 4 °C for 30-45 minutes and proteins was withdrawn from the bottom of the unit.

2.2.1.5.2.4 Measurement of protein concentrations

Coomassie Plus–"The Better Bradford Assay" (Pierce, 23236) was used for the measurement of the proteins concentration**.** The diluted bovin serum albumin (BSA) standards were prepared according to the manufacturer instructions using the diluent's buffer of the protein. The Coomassie Plus Reagent solution bottle was brought from the fridge and inverted several times and the calculated amount was poured into a universal tube which left to be equilibrated in the room temperature. After that, 50 µl from each

previously prepared standard BSA concentrations and the unknown protein sample was added into labelled 5 ml universal tube. Then, 1.5 ml of the Coomassie Plus Reagent solution was added to each tube, mixed and left at room temperature for 10 minutes. After that, 1 ml from each tube was transferred into cuvette tube and water was used to zero the spectrophotometer which was adjusted at 595 nm. The blank (the protein diluent) OD_{595} reading was subtracted from all the readings. The standard curve was constructed between the OD_{595} readings and the corresponding protein concentration of the standard BSA at µg /ml. From the equation generated from that curve, the unknown proteins concentration was calculated.

2.2.1.5.2.5 Storing of the purified SM-CRT sub-fragments

The protein was aliquoted in small clean microcentrifuge tubes labelled by identity, concentration and date. The tubes containing proteins were at first put in the liquid nitrogen briefly, to prevent protein precipitation, and then put at -80 °C.

2.2.2 Characterization of SM-CRT sub-fragments

2.2.2.1 SM-CRT sub-fragment calcium binding assays

The ability of the recombinant SM-CRT sub-fragments to bind calcium was studied by staining with "Stains-all" stain and by interacting with radioactive calcium.

2.2.2.1.1 Staining SDS-PAGE gel by Stains-all stain (Campbell *et al.* 1983)

"Stains-all" is a cationic carbocyanine dye that stain the calcium binding proteins blue. The development of blue colour arise from J complex formation that results from interaction of individual molecules of the dye with the anionic sites on Ca^{2+} binding proteins whether

sialic acid, phosphoryl groups or amino acid residues, while the non-calcium binding proteins accept pink colour.

At first, each SM-CRT sub-fragment was mixed with the loading protein dye (containing 5 % mercaptoethanol) in the ratio 3:1 and heated for 5 minutes on heat block at 95 °C. In one 15% SDS-PAGE gel, 5 µg was loaded from each of the following proteins: N-, P-, C-, NS- and NP-domains in addition to calmodulin (Sgma-Alrich, P114405) (as a positive control), and BSA (Sgma-Alrich, A9647) (as a negative control). Another 15% SDS-PAGE gel was loaded also with 5 µg of SM-CRT NPC-, S-, SP-, SPC- and PC-domains, calmodulin and BSA. In each gel, 10µl protein marker (SeeBlue Plus2 PreStained Standard) was loaded in first lane of each gel. After running the SDS-PAGE electrophoresis, the gels were transferred to 25% isopropyl alcohol overnight for fixation. In the following day, the gels were washed by 25% isopropyl alcohol several times to remove the excess of SDS, then the gel was completely immersed in "Stains-all" solution (30 mM Tris base, 7.5% formamide, 25% isopropyl alcohol and 0.0025% Stains-all, pH 8.8) in a plastic box which wrapped with aluminum foil and left in the dark for 48 hours. Then, gels were examined and scanned.

2.2.2.1.2 Binding with $^{45}Ca^{2+}$ assay

Two mini 15% PAGE gels (without SDS) were prepared. The gels were transferred into Mini-PROTEAN Tetra Cell containing running buffer. 5 µg from each SM-CRT (in addition, calmodulin as a positive control and 5µg BSA as a negative control) was mixed with the non-denature protein loading buffer (without mercaptoethanol) in the ratio 3:1.

The gels were loaded with the prepared proteins samples and protein ladder in the same order mentioned in section **2.2.2.1.1.**

When the front dye reaches the end of the gel, the gels were immediately blotted into nitrocellulose membrane using native Western blot buffer (the Western blot analysis is described in more detail in section 2**.2.1.4.3.3**). After finishing the process of blotting, the membranes were immediately incubated for 1 hour with a solution composed of 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole, pH 6.8. This solution was changed several times during the incubation period. After that, the membranes were immersed for 10 minutes into the same buffer which was supplemented with $^{45}Ca^{2+}$ (1mCi/L). The membranes were then washed with distilled water several times for 10 minutes each to remove excess unbound radioactive calcium and left to dry on filter papers at room temperature for one hour. The membrane was exposed to X-ray film for 48 hours at -80ºC. The signals were developed by exposure to the developer solution then fixed with fixer buffer (see section **2.2.1.4.3.3).**

2.2.2.2 Interaction of the recombinant SM-CRT sub-fragments with C1q, the classical complement pathway recognition molecule

2.2.2.2.1 Assessment SM-CRT sub-fragments binding with C1q using ELISA

One Nunc Maxisorb (Fischer scientific) plate was coated with SM-CRT domains N-, P-, C-, S-, NPC- and (BSA as a negative control), while NS-, NP-, SP-, SPC-, PC-domain and BSA were added to another plate. Each protein was coated in two neighbouring columns in concentration of 5 µg /ml of carbonate/bicarbonate coating buffer (15 mM Na_2CO_3 and 35

mM $NaHCO_3$, pH 9.6) and each well received 100 µl of the prepared protein solution. The plates were warpped in a cling film and left at 4 ºC overnight. The next day, the non specific binding sites were blocked with 250 µl 3% (w/v) BSA /PBS for each well and left 2 hours at room temperature. The plates were washed four times by 250 µl PBS/0.005%T/well. Double serial dilutions of human C1q (Sigma-Aldrich, C1740) in duplicates starting from 0.4 µg (in 100 µl of 1% (w/v) BSA/ 0.005%T/ PBS per well) were incubated with each coated protein and the last row did not receive C1q and left as blank. After incubating the plates for 2 hours at 37 ºC, they were washed and anti-human C1q anti-serum developed in sheep (Immune system, SC1Q-80A) was added in dilution of 1: 200 in PBS/ 0.05%T and left while warpped in cling film at 4 ºC overnight. The following day, the plates were washed four times and then anti-sheep IgG conjugated to alkaline phosphatase (Sigma–Aldrich, A5187**)** in the dilution of 1:5000 in PBS/0.05%T was incubated with plates for 2 hours at 37 ºC. The plates were washed four times for the last time and the reactions were developed by putting 100 µl/well *p*-nitrophenyl phosphate substrate (Sigma, SIGMA*FASTä* p-Nitrophenyl phosphate Tablets, N1891)**.** When the yellow colour developed, the reaction was read at 405 nm wave length using ELISA reader (Bio-Rad, Model 680)**.** The experiment was repeated three times.

2.2.2.2.2 Inhibition of C1q-dependent blood haemolysis by SM-CRT sub-fragments

To test if SM-CRT binding with C1q will affect biological functions of C1q, the C1q dependent blood haemolysis was performed.

2.2.2.2.2.1 Preservation of Sheep red blood cells (SRBCs) in Alsever's medium

On ice, 10 ml fresh sheep red blood cells (SRBC) (Innovative research, IC100-0210) was mixed with equal volume of Alsever's solution (Sigma, A3551). The mixture was centrifuged for 10 minutes with 1200 xg at 4 °C**.** The supernatant was discarded and the SRBC pellet was re-suspended with 10 ml Alsever's solution by inverting the tube gently several times. These cells were aliquoted and kept in the fridge for one month.

2.2.2.2.2.2 Preparation of 5% Sheep red blood cells (SRBCs)= 5 x10^8 cells/ml

In 50 ml Falcon tube containing 40 ml of ice cold BBS (4 mM barbital,145 mM NaCl,1 mM $MgCl_2$ and 2 mM $CaCl_2$, pH 7.4), 5 ml of processed SRBC was mixed gently and centrifuged for 10 minutes with 1200 x g at 4 ºC. The supernatant was discarded and SRBCs pellet was re-suspended with fresh 40 ml of ice cold BBS. The process of centrifugation and re-suspension in 40 ml of ice cold BBS was repeated twice, then the supernatant was discarded and 500 µl of that cell pellet was mixed gently with 9.5 ml of ice cold BBS in a new tube. From this mixture,100 µl was diluted with 1.4 ml water, vortexed and O.D. was measured at 541nm with using a blank of 100 µl of BBS diluted with 1.4 ml water. The checked O.D. should be 0.7 (±5%) and if not, it was adjusted with following formula:

Total Volume (ml) for 5% SRBC= The measured O.D. x total volume (10 ml)/ 0.7

These cells were suitable to be used for two weeks and should be diluted 1:5 before using.

2.2.2.2.2.3 Preparation of 2% Sheep red blood cells (SRBCs)

From the previously prepared (SRBCs) pellet, 250 µl was added to 12.5 ml of BBS. Then 300 µl of this mixture was completed to 2 ml with water then vortexed. The O.D. was

measured at 541 nm with blank composed of 300 µl BBS plus 1.7 water. To prepare 2% SRBCs, O.D. should be 0.5 and the following formula was used to adjust the O.D.:

Total Volume (ml) for 2% SRBC= the measured O.D. x total volume (12.5 ml)/0.5

These cells were kept in the fridge for two weeks.

2.2.2.2.2.4 Titration of hemolysin (rabbit anti-sheep red cell stroma)

Hemolysin (Sigma-Aldrich, S1389) was serially diluted in BBS from 1:100 until 1:12800 and 50 µl from each dilution was mixed with 50 µl of 2% SRBC in 96 well V-bottom plates (Fisher Scientific). This plate was incubated at room temperature for 20 minutes, and then 150 µl of diluted fresh guinea pig serum (Harlan Laboratories U.K. Ltd S.R-0006B) in dilution of 1:10 was mixed with each prepared mixture. The plate was sealed completely with Cling film and put in water bath at 37 °C for 30 minutes, then it was centrifuged at 1200 xg for 10 minutes (Beckman coulter, Allegra X-12). The chosen hemolysin dilution was the one directly before the dilution with visible SRBC sedimentation.

This hemolysin titer was confirmed by mixing 0.5 ml with 0.5 ml of 2% SRBCs and left at room temperature for 10 minutes. Then, 1 ml of fresh guinea pig serum (1: 200) was added and the sample was put in the water bath at 37 °C for 60 minutes. After that, the sample was centrifuged at 1200 x g for 10 minutes and the O.D. was measured at 541 nm using BBS as blank. This reading was compared to a complete lysis made by mixing 0.5 ml of 2% SRBC with 7 ml water with the following formula:-

The measured O.D. x 100/ O.D. of the complete haemolysis.

(70-80% haemolysis was achieved as expected).

2.2.2.2.2.5 Sensitizing of 5% Sheep red blood cells (SRBCs) with hemolysin

The chosen hemolysin dilution was gently mixed with 5% SRBCs by 1:1. This was done by adding the SRBCs drop by drop to the hemolysine while shaking the tube gently. This mixture was incubated at 37 °C for 30 minutes. These sensitized SRBCs were stored at 4 ºC and it could be used for 1 week. The working dilution of SRBCs-hemolysin is 10^8 cells/ ml, therefore, this should be diluted 1:5 of cold BBS before use.

2.2.2.2.2.6 Preparation of sera used in the haemolysis inhibition assay

i) Choosing the dilution of Normal Human serum (NHS) that cause 70-80% haemolysis

NHS was serially diluted starting at 1:20 until 1:1280 and 100 µl from each dilution was transferred into 96 well V-shaped bottom plate. Similar volume (100 µl) of SRBCs-hemolysin (1x 10^8 cells/ ml, sensitized 5% sheep red blood cells (SRBCs) with hemolysin which was diluted 1:5 before use) was added into each dilution. The plate was sealed in cling film and incubated in water bath 37 °C for one hour. Then 50 µl of cold BBS was added to each well and they were centrifuged at 1200 xg for 10 minutes**.** The supernatants were transferred to new flat bottom plate and O.D. of the supernatant which contains hemoglobin was measured at 405 nm. Spontaneous 0% haemolysis was produced by incubation of 100 µl SRBCs-hemolysin without serum, whereas 100 % haemolysis was achieved by incubating 100 µl SRBCs-hemolysin with 100 µl of water, all in the same plate. The haemolytic activity is calculated as a percentage from the total haemolysis after subtraction of the background haemolysis. The NHS dilution which achieved 70-80% hemolysis was selected to perform the assay.

ii) Reconstitution of human C1q deficient serum

Human C1q deficient serum (Calbiochem, 234401) was diluted 1:40 in BBS, then100 µl was added to each well of V-shaped bottom plat (for 12 wells). Human C1q was serially diluted starting from 2µg and each dilution was mixed with the diluted human C1q deficient serum and incubated for 37 °C in water bath for half an hour. Then, 100 µl of sensitized SRBCS (10^8 cells/ml) was added to each well and left for another 30 minutes. The plate was centrifuged at 1200 xg for 10 minutes. The supernatants were transferred into flat bottom 96 plat and the O.D. of the supernatants which were detected at 405 nm using BBS as the blank. The back ground haemolysis was prepared from the sensitized SRBCS cells without any serum whereas the 100 % haemolysis was measured by mixing 100 µl sensitized SRBCS cells with 100µl water and both were put in the same plate during the whole process. The chosen amount of the C1q, is the amount which when re-backed to 1:40 of C1q deficient serum makes the reconstituted serum causing 70-80 % haemolysis .

2.2.2.2.2.7 C1q dependent haemolytic inhibition assay

In the case of NHS, 100 µl of 1:160 NHS was added in V-shaped bottom 96 wells in duplicates. Then, 1, 5, 10, 15 or 20 µM of each SM-CRT sub-fragment were mixed with serum and the plate was sealed and incubated at 37°C in the water bath for 1 hour. After that, 100 µl sensitized 5% SRBCs (1x 10^8 cells/ml) was added for each well and re-incubated at 37 °C for further 1 hour. Negative control was done with 200 µl blood cells alone and the 100 % haemolysis was done by mixing 100 µl of the cells with 100 µl with water, all in the same plate. After the incubation, 50 µl BBS was added to each well and the plate was centrifuged for 10 minute at 1200 xg and 200 µl supernatant from each well was transferred to flat bottom well and O.D. was read at 405 nm to calculate the hemolysis.

In the case of C1q depleted sera, the same process was used but each protein concentration was mixed first with 0.125 µg C1q then the mixture was transferred to 100 µl 1: 40 C1q deficient serum and the rest of procedures were followed as before. For each serum the experiment was done three times in duplicate.

2.2.2.3 Testing immunogenicity of the recombinant SM-CRT sub-fragments

2.2.2.3.1 Anti-SM-CRT rabbit anti-serum production

A Rabbit was immunised at Harlan laboratories, U.K.. The recombinant SM-CRT NPC-domain was adjusted to 1mg/ ml. New Zealand white rabbit was injected S.C. at 4 sites with 0.5 mg of the protein (500µl) plus 500 µl Freund's complete adjuvant in 1 ml total volume. Every two weeks, the rabbit received 4 booster doses but in Freund's incomplete adjuvant. At termination, the blood was collected and left to clot on ice, then serum was obtained and kept at -20 °C.

2.2.2.3.2 Preparation of BL21 (DE3)pLysS cell lysate

From the bacterial BL21 (DE3)pLysS stock, 2-3µl was inoculated into 10 ml L.B., without antibiotic, and incubated at 200 rpm at 37 °C overnight. In the next morning, the bacteria were harvested by centrifugation at 4000 rpm at 4 °C for 20 minutes. The cell pellet was re-suspended in 3 ml PBS and subjected to three 20 seconds bursts of sonication. The bacterial debris were then centrifuged at 1500 rpm at 4 °C for 20 minutes. The supernatant was collected and the protein concentration was measured by Bradford assay and stored at -20 °C.

2.2.2.3.3 SM-CRT sub-fragments probing with anti-SM-CRT rabbit anti-serum by Western blotting analysis

To test the immunoginicity of SM-CRT, the specific anti-SM-CRT rabbit anti-serum was tested against different recombinant SM-CRT proteins. At first, 3ug of each recombinant NPC-domain, BSA and *E.coli* lysates (both were used as negative controls) were loaded into 12% SDS-PAGE gel. The proteins were blotted into nitrocellulose membrane. The membrane was blocked in 5% skimmed milk in TBS for 30 minutes, then the specific anti-SM-CRT rabbit anti-serum in 1: 40 was incubated and left at 4°C overnight at shaker platform. In the next day, the membrane was washed with 0.005%T/ TBS three times, 10 minutes each, and anti-Rabbit IgG (whole molecule)–Alkaline Phosphatase antibody produced in goat (Sigma, A3812) was added in the concentration of 1:10000 and left at room temperature for 90 minutes at shaker platform. The membrane was washed three times, then BCIP/NBT Alkaline Phosphatase Substrate (Sigma-Alderich, B1911-100ml) was added on the membrane that was observed for the colour development, then the reaction was stopped by immersing the membrane in distilled water.

After confirmation of the immunogenicity of the NPC-domain, the anti-SM-CRT rabbit anti-serum was used to probe the different SM-CRT sub-fragments in Western blotting. Two SDS-PAGE gels were prepared and loaded with 3 µg from each protein. One 15% SDS-PAGE gel loaded with N-, P-, C-, NS- and NP-domains, in addition to BSA and *E. coli* lysate (as negative control) and NPC-domain (as positive control), while the other was 12% SDS-PAGE gel which was loaded with S-, SP-, SPC- and PC-domain, in addition to the similar positive and negative controls used in the first gel. Then whole process was repeated as occurred with the NPC-domain in the same section.

2.2.2.3.4 SM-CRT sub-fragments probing with anti-SM-CRT rabbit anti-serum by ELISA

Anti-SM-CRT anti-serum was used for identification of the protein by ELISA. The Nunc Maxisorb plates were coated with 0.5 µg from SM-CRT NPC-domain, in addition to BSA as a negative control in carbonate/bicarbonate buffer and left overnight at 4°C. The following morning, the plates were blocked with 1%BSA/ TBS for 2-3 hours and after washing for four times with TBS/0.05% Tween-20/5 mM $CaCl_2$, the anti-SM-CRT anti-serum was incubated in dilution of 1:1000, 1:10000 and 1:50000 and left at room temperature for 1 hour, then washed four times and they incubated with AP-rabbit antibodies in dilution of 1:10000 for 90 minutes. Plates were washed for the last time and *p*-nitrophenyl phosphate substrate was added at 100µl/well. When the yellow colour developed, the OD_{405} was read.

The ELISA was repeated by using different SM-CRT sub-fragments in two plates coated with 0.5 µg/well from each protein. One plate was coated with N-, P-, C-, NS-, NP-domains and the NPC-domain (+Ve control) and BSA (-Ve control), while the other plate received S-, SP-, SPC-, PC- and NPC-domain (+ Ve control) and BSA (-Ve control). The same ELISA procedures were followed as mentioned in this section. At a dilution of 1:10000, the O.D. readings were compared among different proteins of the same group.

2.2.2.4 Detection of native SM-CRT in *S. mansoni* surface membrane

This was kindly prepared and supplied by Prof. Mike Doenhoff. Briefly, schistosomulum and adult *S. mansoni* were suspended in 2% Na deoxycholate (a detergent that dissolve the parasite tegument) for 30 minutes. After centrifugation at 2000 at 4 °C, the supernatants

were taken and stored at 20°C until use. From the schistosomulum and adult *S. mansoni* supernatants, 1 µl and 2 µl were respectively loaded in 10 % SDS-PAGE, in addition to 3 µg from *E. coli* lysate proteins and BSA as negative controls. After finishing the run, the gels were blotted with Western blot and the nitrocellulose membrane was probed with anti-SM-CRT rabbit anti-serum. The basic steps of Western blot were described in detail in section **2.2.2.3.3.**

2.2.2.5 SM-CRT amino acid sequence identity with CRTs from other species

Amino acids sequences of SM-CRT was aligned with human CRT or some parasitic CRTs using online programme "ClustalW2". Human CRT (accession **N.**P27797)**,** *E. histolytica* (accession N. C4M296), Echinococcus (accession N.Q56JA0), *N. americanus* (accession O76961) and *H.contortus* (accession Q6R5P2) using the online programme **(**http://www.ebi.ac.uk/Tools/clustalw2/index.htmlMuscle).

2.2.3 Using SM-CRT sub-fragments and CTF in the sero-diagnosis of schistosomiasis

2.2.3.1 SEA and CTF preparations

Both soluble egg antigens (SEA) and Cercarial Transformation Fluid (CTF) were prepared and kindly supplied by Prof. Mike Doenhoff

i) SEA preparation

The parasite eggs were harvested from livers and intestine of mice heavily infected with *S. mansoni*. The eggs were homogenized in an ice-cooled glass homogenizer with a rotated mechanical Teflon plunger for 3 minutes. After disruption, the eggs were centrifuged at

20000 x g for 3 hours. The supernatant was collected and the protein concentration was measured. It stored at 80 °C (Dunne *et al*., 1981).

ii) CTF preparation

S. mansoni cercariae were harvested from *Biomphalaria glabrata* snail and allowed to settle by putting them in a universal tube for 2 hours at 4°C. The excess water was removed and 4ml of PBS, pH 7.2 were added to re-suspend a cercarial pellet of 0.75-1 ml volume. The cercarial suspension was mechanically transformed by drawing the suspension 12-15 times through a 2 ml plastic syringe with a 21 gauge needle (Colley and Wikel, 1974). The process of drawing was repeated if all cercariae were not transformed. The schistosomula suspension was left at 37 °C for 45-90 minutes before being centrifuged at 2000 xg for 4 minutes. The supernatant (CTF) was collected and concentrated into to be about 1 mg protein /ml (the protein concentration was measured by BioRad DC protein assay and then aliquoted in 1 ml volumes and stored at -80 °C

2.2.3.2 Experimental mouse

Four groups of *S. mansoni* infected CD1 mice (n= 19 mice and each mouse was infected with 200 cercariae) and one non-infected control group (3 mice), were established. The first infected mouse group (5 mice) was culled at 12 days p.i., the second infected mouse group (5 mice) was culled at 35 days p.i., the third infected mouse group (3 mice) was scarified 46 days after infection, while the mice in the fourth infected mouse group (6 mice) were culled 56-59 days p.i.. The infection of mice and culling were performed kindly by Dr. David Johnston, Museum of Natural History in London. His animal licence unfortunately did not allow frequent blood samplings from the same mouse groups during

the course of infection or allow the infected mice to live beyond the time points of 59 days after infection. The collected mouse sera were aliquoted and stored at -80°C in Lab 231, University of Leicester.

2.2.3.2.1 Detection of specific antibodies against used antigens in mouse sera by ELISA

i) Preliminary ELISA experiment

Preliminary ELISA experiments were performed to test the immunogenicity of different SM-CRT sub-fragments, CTF and SEA in the infected mouse sera by comparing O.D. readings of different titrations of the positive standard serum compared to the negative one reacting with each antigen.

A negative standard mouse serum was constructed by pooling of equal volumes of each non-infected mouse serum, while the positive standard serum was made by mixing equal volumes from the mice sera of 57-59 day group, both sera was aliquted and stored at -80 °C.

The proteins were diluted in carbonate/ bicarbonate coating buffer (15 mM $Na_2 CO_3$ and 35 mM $NaHCO_3$, pH 9.6). Each recombinant SM-CRT sub-fragment and BSA were used at concentration of 5µg/ml, SEA at 1 µg/ ml and CTF antigen was used at 0.4 µg/ml (its optimal concentration was calculated using checkerboard as previously used by Prof. Doenhoff on human sera and I used therefore the same concentration for mouse sera). For coating a Nunc Maxisorb plate,100 µl from each antigen were dispensed per well in duplicate. Each plate was coated with three proteins (four columns for each antigen). The

plate was wrapped in aluminium foil and cling film and left overnight at 4 °C. On the following day, the plate was blocked with 250 µl 1% BSA/TBS/well and left 2 hours at room temperature. The wells were washed four times with 250 µl TBS/0.05% Tween-20/5 mM $CaCl_2$. After that, the negative or positive standard sera were diluted serially starting from 1/20 in the blocking buffer (for each antigen, two neighbouring columns were incubated with the negative serum and other two the positive serum was incubated in the neighboured two columns) and the last row received all reagents except sera. The sera were incubated at the room temperature for 1 hour. After that, the plate was washed four times, the secondary anti-mouse AP-conjugated IgG in dilution of 1: 10,000 (Sigma-Aldrich, A 3688) was added to the wells with 100 µl /well and left at room temperature for 90 minutes. The plate was washed for the last time and *p*-nitrophenyl phosphate substrate was added with 100 µl/well. When the yellow colour developed, plates were read until the O.D. of the higher serum dilution reached 1-1.5 unit at 405 nm wavelength using an ELISA reader.

ii) Detection of antibodies against different proteins in the mice sera, at various points of time

From the previous experiment, the individual mouse serum was used at a dilution of 1:200 (as this value lies in the linear part of the sigmoid curve of the standard positive serum against the used antigens). The goal of this main ELISA experiment was to determine the immunogenicity of the recombinant SM-CRT sub-fragments and CTF as determined by the time of appearance of their specific antibodies in *S. mansoni* infected mouse group. For each protein, the ELISA was repeated three times. Each Nunc Maxisorb plate was coated with one protein with the concentration that used in the preliminary experiment. On each

plate, the first two columns (1& 2) received standard mouse positive serum which is serially diluted (starting from 1: 40 in duplicate) and the last two wells (H1& H2) did not receive any serum but all other reagents. The individual mice sera were incubated at the dilution of 1: 200. The rest of the ELISA steps were performed as previously described in detail in the preliminary experiment. Mean O.D. readings of each *S. mansoni* infected mouse group were compared with the mean O.D. readings of the control mouse group using unpaired Student's *t*-test.

2.2.3.3 Human samples

The human sera were divided into four groups: i) 97 sera collected from *S. mansoni* infected patients resident in Egypt who were diagnosed by microscopic identification of the parasite egg in their stool, ii) 53 sera from patients suffering from other *S. mansoni* unrelated diseases whether parasitic which are: 14 *Ascaris*, 13 *Entamoeba histolytica*, 3 *Fasciola*, 3 *echinococcosis*, 5 *Toxoplasma* (all these sera were kindly collected from an Egyptian patients cohort and sent to me by Dr. Abeer Elhadidi, University of Alexandria, Egypt) or 15 sera taken from autoimmune patients suffering from SLE and rheumatoid arthritis kindly provided by Dr. Michael Browning, Consultant Immunologist at the Royal Infirmary, Leicester, iii) 21 sera collected from healthy U.K. persons who are never exposed to *S. mansoni* infection and iv) 19 sera taken from healthy Egyptian donors. The donors signed consent forms following the ethical protocol put in place. The sera of autoimmune patients were stored in the blood bank of the Royal Infirmary, University of Leicester, U.K.

In the case of the Egyptians patients and healthy persons, a few millimetres of blood were drawn under aseptic condition and left on ice until blood clot has been formed. The samples were centrifuged for 10 minutes at 4000 rpm. Serum was taken and stored in labelled sterile 1.5 ml microcentrifuge tube and stored at -80 ºC until shipping on dry ice to the University of Leicester. Upon arrival, they were aliquoted and stored at -80 ºC in the department of Infection, Immunity and Inflammation, Lab 231.

2.2.3.3.1 Detection of specific antibodies against used antigens in human sera by ELISA

i) **Preliminary ELISA experiment**

A series of preliminary ELISA experiments were performed to select the appropriate amount of SM-CRT sub-fragments antigens, individual serum dilution and secondary anti-human antibody concentration**.** CTF (0.4 µg/ml) and SEA (1 µg/ml) concentrations were measured using checkerboard titration by Prof. Mike Doenhoff.

Nunc Maxisorb plates were coated with 1 µg/ml SEA in carbonate/bicarbonate and left overnight at 4°C. In the following morning, the plates received 250 µl/well of 1% BSA/TBS, the blocking buffer, and left for 2-3 hours at room temperature. After washing with 250 µl TBS/0.05% Tween-20/5 mM $CaCl_2$ per well, the individual *S. mansoni* infected human sera (1:200 in the blocking buffer) were incubated in duplicate on the ELISA plates for 1 hour, in addition to few sera of healthy European were added to each plate as negative control. Two blank wells received the all reagents except the sera and they used to subtract the background. The plates were washed four times and then the AP-conjugated Anti-Human IgG (whole molecule) secondary antibodies (Sigma, A1543) was incubated with

the plates in concentration of 1: 10000 and left for 1 hour at room temperature. The plates were washed and then *p*-nitrophenyl phosphate substrate was added with 100µl/well. When the yellow colour developed, plates were read at $OD_{405,}$ until the highest reading of *S. mansoni* infected serum reached 1-1.5 units.

Equal volumes of 15 sera which gave the highest OD_{405} readings were pooled to prepare the positive reference standard serum. This serum was aliquoted and stored until used. In the same time equal volumes of sera (21) of healthy European persons were pooled to generate the negative control standard reference serum.

To select the individual serum dilution, for SM-CRT sub-fragment in the concentration of 5 µg/ml, 0.4 µg /ml of CTF and SEA in 1 µg/ml were used. The positive and negative standard reference sera were incubated with each protein in duplicates. They were serially diluted from 1: 20 in the blocking buffer in duplicate following the same steps of ELISA mentioned above. From the end point titre, the optimal individual serum was selected from the linear part of the standard curve of the positive standard serum. From that end point titres, 1: 200 was chosen for all antigens except for SM-CRT P-, S-, SP-, C- and PC-domains where 1:100 was used. Concerning the AP-conjugated Anti-Human IgG (whole molecule) secondary antibodies the secondary antibodies different dilutions were tried (1:10000 or 1:5000 or 1:2000) and the latter dilution was found to allow quicker development of the reaction.

ii) ELISA for detection of specific antibodies against used *S. mansoni* antigens in different human sera groups

The same ELISA methodology was followed. The proteins were coated as mentioned above. The sera were diluted in the blocking buffer. In each plate, the first two columns (1& 2) were incubated with serial dilutions of the previously prepared positive standard reference serum starting at 1:20 in duplicate. The last two wells of the first two columns (H1& H2) received all the reagents but not sera. Each plate included the quality control sera which are 5 *S. mansoni* negative sera from the healthy European donors as a negative control and 5 *S. mansoni* +Ve as a positive control. The quality control sera were incubated in the same locations on all plates. The rest of the plate received 30 individual test sera. When the colour developed, O.D. was read until the reading of 1:20 of the standard positive reference serum reached 1-1.5 units.

To correct for variations within the ELISA plates, the O.D. was converted to relative antibody titre. The standard serum was assigned an arbitrary antibody titre of 1.0 and the titre of the samples calculated from the equation of the standard curve.

For assay quality control, both an inter-assay and intra-assay coefficient of variation percent (CV %) was calculated. Within the same plate, CV% was calculated among the relative antibody titres of the 10 quality control sera duplicates by using the following formula= 100x (mean of SD of the relative antibody titres of the duplicates / mean of the duplicates). All the plates achieved intra-assay CV% within range of 3.5-6% which is less than 10%, the acceptable CV%. The inter-assay variations among the plates was also estimated. The inter-assay CV% = 100 X (∑ SD of the relative antibodies titres of the

quality control duplicates among plates / ∑ means of the same samples). The inter-assay variation was <12%.

2.2.3.4 Detection of specific anti-human CRT antibodies in sera of autoimmune patients

Specific antibodies against human CRT were detected in the sera of autoimmune patients by ELISA. The plate was coated with 0.5 µg of recombinant human CRT (Abcam, ab15729). The autoimmune sera which cross-reacted with SM-CRT was pooled together to construct a positive standard serum. The standard serum was serially diluted from 1: 20 and incubate with plate in duplicate. In the same ELISA plate, each individual serum autoimmune sera or the U.K. controls was incubated at 1: 200. The ELISA was performed as in section **2.2.4.2.3.**

2.2.5 Vaccination of BALB/c mouse with SM-CRT NPC-domain

Mice immunisation and portal perfusion of *S. mansoni* infected mice were kindly carried out by Prof. Mike Doenhoff, University of Nottingham where the *S. mansoni* life cycle is established.

2.2.5.1 Testing the immunogenicity of recombinant SM-CRT NPC-domain

A preliminary experiment was performed to test the immunogenicity of the recombinant His-tag fusion SM-CRT NPC-domain in BALB/c mouse strain which will be further infected by *S. mansoni* cercariae.

2.2.5.1.1 BALB/c mouse immunisations with SM-CRT NPC-domain

Two groups of mouse (4 in each group) were used. The immunized mouse group received three doses of SM-CRT intraperitoneally at 2 weeks interval (at 0, 15 and 30 days). The first protein dose was 50 µg and the other two doses were 25 µg (the protein was dissolved in 100 µl PBS/alum). The control mouse group received PBS + alum suspension at the same time points. Blood was drawn from all mice before the first injection (time point 0) and two weeks after each dose. The collected blood was centrifuged at 1000 xg for 10 minutes and stored at -20°C until being shipped on dry ice to me at University of Leicester.

2.2.5.1.2 Measurement of specific SM-CRT antibodies

The profile of specific SM-CRT antibodies response was determined in the sera of immunised mice by ELISA. The ELISA steps were described in detail in section **2.2.4.1.1**. Nunc Maxisorb plate was coated with 0.5µg/well. Each mouse serum collected at the different time points was incubated in duplicate at a dilution of 1:1000 for 1 hour at the room temperature. The plate was washed four times and secondary goat anti-mouse AP-conjugated IgG in dilution of 1: 10000 was added to the wells in 100 µl /well and left for 90 minutes. After washing for the last time, *p*-nitrophenyl phosphate substrate was added and when the yellow colour developed, the OD_{405} was read.

To evaluate the strength of immunogenicity of SM-CRT in BALB/c mouse, SM-CRT specific antibodies were measured by ELISA in the sera of the immunised mice at different serum dilutions of 1:1000, 1:10000 and 1:50000 (from the sera collected after the third dose) in comparison to the control group.

2.2.5.2 BALB/c mice immunisation & challenging with *Schistosoma* infection

2.2.5.2.1 Mice immunisations

After confirmation the immunogenicity of recombinant SM-CRT in BALB/c mouse strain, the actual mouse SM-CRT immunisation and *Schistosoma* infection experiment was performed. Two mouse groups (5 mice in each group) were used. The mice immunisation, blood collection and measurement of specific antibodies were followed as before (see section **2.2.5.1**).

2.2.5.2.2 Preparation of cercariae for infection

The infected snail were transferred from their water tank to be put in beaker (in water bath at 30 °C) containing de-chlorinated de-ionized water under strong illumination for two hours to help the snail to shed cercariae. The water around the snail, which contains shed cercariae, was concentrated and cercariae were counted. Cercariae emerged from the snail were used for mice infection within three hours after emergence from their snail host (Olivier 1966).

2.2.5.2.3 Mice infection with *S. mansoni* cercariae (Doenhoff *et al*. 1978 adapted from Smithers& Terry 1965)

The mice were anaesthetized with Ket/Dom mixture which was given intra peritoneally (0.75 ml Ketamine plus 1 ml Domitor and 8.25 ml sterile isotonic saline, for a 20 g mouse, 0.2 ml from this mixture is used). When the mice lost consciousness, each mouse was placed on its back and its shaved abdomen was moistened with aquarium water. Then a nickel –plated brass ring was taped over its abdomen. The volume of the prepared cercarial suspension (between 0.1ml and 0.9 ml according to cercariae concentration obtained on the

day of infection) which containes 200 cercariae were added to the ring. The mice were left for 20 minutes to allow cercariae penetration. After that, mice were allowed to recover by using Antisedan (0.2 ml Antisedan plus 9.8 ml saline and 0.2 ml from this dilution will reverse a 20 g mouse) in a warm environment.

2.2.5.2.4 Perfusions of *Schistosoma* worms from portal vein (Doenhoff *et al*. 1978 adapted from Smithers& Terry 1965)

After 42 days post-infection, the mice were anaesthetized terminally by injection of 0.2 ml Sagatal containing 25 units of heparin (per mouse) intraperitoneally. The mouse abdominal and thoracic cavities were opened and the ribs were removed. The mouse was attached to a vertical Perspex board by clothes pegs. Then, 25 ml of perfusion fluid (8.6 g sodium chloride, 15 g trisodium citrate, 2000 units heparin and completed to 1 liter with distilled water) was injected into the right ventricle. The perfusion solution coming from incision in the mouse portal vein was collected in a beaker put below the mouse. The collected worms were allowed to settle down, excess solution was removed and the erythrocytes in 1-1.5 ml of fluid were lysed with small volume of 2.5% Saponin. The perfused adult *Schistosoma* worms from each mouse were transferred into Petri dish and the numbers of male, female, total and paired worms were counted under the dissecting microscope.

The level of protection % against *Schistosoma* caused by SM-CRT immunisation is calculated by comparing the numbers of adult worms recovered from the immunised mouse group to the numbers of adult worms recovered from mouse control group using the following formula (where C is the control group and V is the vaccinated mouse group):

$$\frac{\text{mean of worms recovered from (C) group - mean of worms recovered from (V) group}}{\text{mean of worms recovered from (C) group}} \times 100$$

2.2.5.2.5 *Schistosoma* egg counting in the tissues (Doenhoff *et al*. 1978 adapted from Cheever 1968)

Each mouse liver was digested by putting in 15 ml 5% KOH overnight at 37 °C. Three 50 µl aliquots were transferred from the digested liver suspension onto microscopic slides. The eggs were counted under x10 magnification in each aliquot. If any of the three replicate counts deviated more than 10% from the mean count, other slides were prepared. Then, the average of the three slides was taken and from this mean, the total numbers for each mouse was calculated. In each group the numbers was expressed as a group mean. The same formula mentioned above is used to calculate mean % reduction in tissue eggs.

2.2.5.2.6 Measurement of specific SM-CRT IgG1 and IgG2a subclasses by ELISA

A coated ELISA plate with 0.5 µg / well of the recombinant SM-CRT NPC-domain was incubated by each mouse serum (from 2 weeks after the third immunisation) at 1: 200 for 1 hour. After washing, goat anti-mouse IgG1, Human ads-AP (Southernbiotech, 1070-04) or goat anti-Mouse IgG2a, Human ads-AP (Southernbiotech, 1080-04) at 1:2000 dilution was incubated with the mouse sera for 90 minutes. After washing for the last time, *p*-nitrophenyl phosphate substrate was added and when the yellow colour developed, the OD_{405} was read.

Statistical analysis was performed with unpaired Student's *t*-test using computer software package GraphPad Prism 5.02 version to compare control and experimental groups. There were significant statistical difference when the *P*-value, compared to the control group, was lower than 0.05.

Chapter 3

Expression and purification of SM-CRT sub-fragments

3.2 Results

3.1.1 Amplification and cloning of SM-CRT sub-fragments

The SM-CRT was split into 10 different sub-fragments expressed as 6xHis-tagged fusion proteins. The sub-fragments were the N-, the P-, the C-, the NS-, the NP-, the NPC-, the S-,the SP-, the SPC- and the PC- domains.

The SM-CRT cDNA segments encoding such fragments were amplified by PCR from a *S. mansoni* cDNA library. A *Bam*HI restriction enzyme site was engineered into the forward primer sequence at the 5' end and a *Xho*I restriction site was introduced into the sequence of the reverse primer at the 3'end. The coding sequences for different SM-CRT fragments were successfully amplified and each band appeared at the expected molecular size (in bp) **(**see **Figure 8** and **Table 2)**.

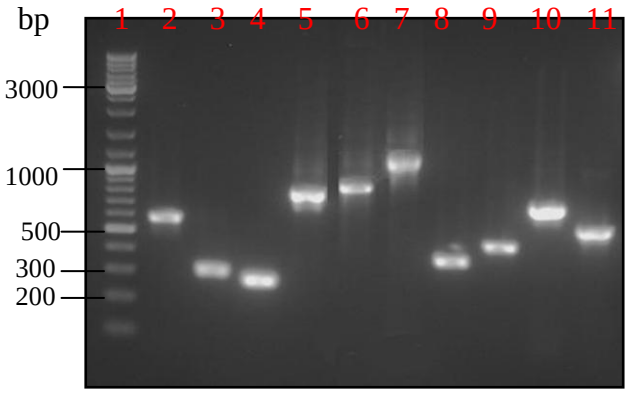


Figure 8: Agarose gel electrophoresis (1.5% agarose) loaded with the amplified PCR products encoding the respective SM-CRT sub-fragments. 5 µl from each PCR reaction was loaded into the gel. Lane 1: standard molecular weight marker (GeneRuler ™ DNA Ladder Mix), lanes 2-11: DNA fragments of the following SM-CRT sub-fragments : N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC- domains (from left to right).

Each PCR product encoding individual SM-CRT sub-fragment was then purified using QIAquick Nucleotide Removal Kit to get rid of contaminants such as salts, enzymes or nucleotides which could interfere with the sub-cloning procedure. After purification, the PCR products were separated and visualized after electrophoretic separation in an agarose gel 1.5% (**Figure 9**).

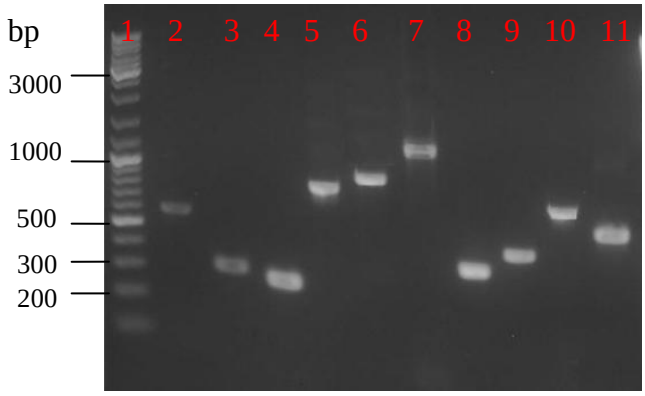


Figure 9: **Agarose gel electrophoresis (1.5% agarose) loaded with the purified PCR products encoding the respective SM-CRT sub-fragments**. 5 µl from each product was loaded into the gel. Lane 1: standard DNA M.W. marker (GeneRuler ™ DNA Ladder Mix), lanes 2-11: DNA fragments of the following SM-CRT domains: N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC- domains (from left to right).

Then, each SM-CRT DNA fragment was ligated into pGEM®-T Easy Vector which has a compatible T overhang to facilitate subcloning using T4 DNA Ligase enzyme. The plasmid was transformed into competent TOP10F` *E. coli*. From transformed colonies, DNA minipreps were prepared using the Wizard® Plus SV Minipreps DNA Purification System. The plasmids were digested by EcoRI restriction digest to test for successful of insertion of the PCR product into the vector and the digested plasmids were separated electrophoretically in a 1.5% agarose gel. In each lane, I observed two bands; one at

approximately 3000 bp (representing the digested pGEM®-T Easy Vector) and the other lower band corresponding to the segments of the amplified DNA **(Figure 10).**

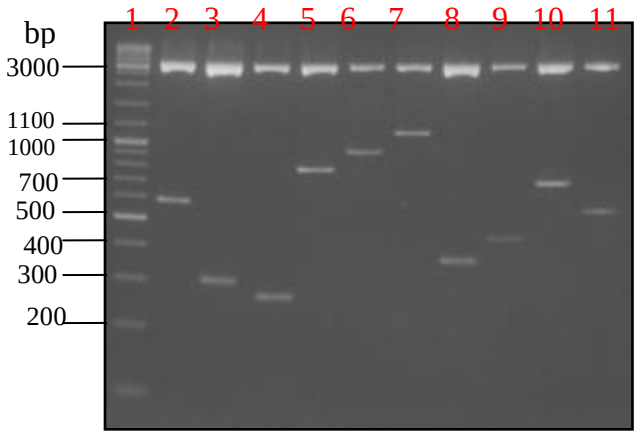


Figure 10: Agarose gel electrophoresis (1.5% agarose) loaded with the digested pGEM®-T Easy constructs with EcoRI restriction enzyme (a collective gel). Lane 1: standard DNA M.W. marker (GeneRuler ™ DNA Ladder Mix), lanes 2-11: the digested DNA fragments representing the following SM-CRT sub-fragments: N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC-domains (from left to right). For each lane, there are two bands, one at 3000 bp representing the cloning vector and the lower band is the DNA fragment encoding a SM-CRT sub-fragment.

3.1.2 Sub-cloning of the amplified SM-CRT sub-fragments DNA into pRSETB, the protein expression vector

Each pGEM®-T Easy construct of the individual SM-CRT sub-fragment was digested with *BamHI* and *XhoI* restriction enzymes overnight to prepare this sub-fragment for the unidirectional subcloning into the expression vector, then it was run on 1.5% agarose gel. Under ultraviolet light, the SM-CRT DNA fragment was cut out and the QIAquick Gel Extraction Kit was used to extract and purify it from the gel. This gel purified SM-CRT DNA segment was sub-cloned into the expression vector pRSETB and the ligation reaction used to transform to TOP10F` *E. coli*. From the ampicillin plate on which the transformed

cells were plated, 9-11 colonies were randomly selected to be grown as liquid cultures overnight to prepare DNA minipreps. These small scaled DNA preparations were digested by *BamHI* and *XhoI* to identify the successfully sub-cloned clone, which was used for the protein expression (see **Figure 11**).

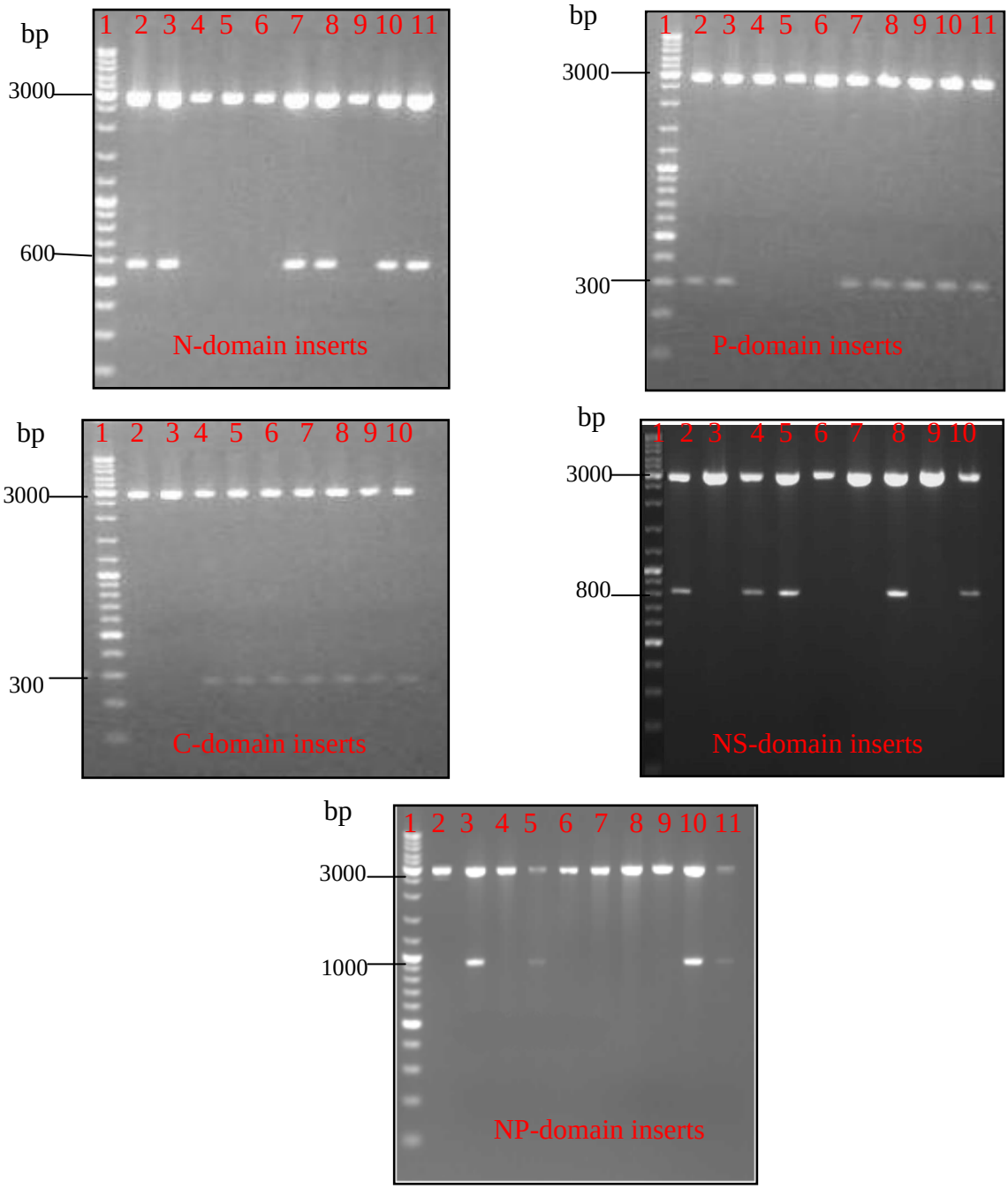


Figure 11: Agarose gel electrophoresis (1.5% agarose) loaded with the restriction enzymes-digested SM-CRT pRSETB constructs. For each construct, 5µl were digested with double digestion using *Bam*HI and *Xho*I. The vector which contains insert appeared in two bands; the vector at 3000 bp and the inserts corresponding to their expected base pair size.

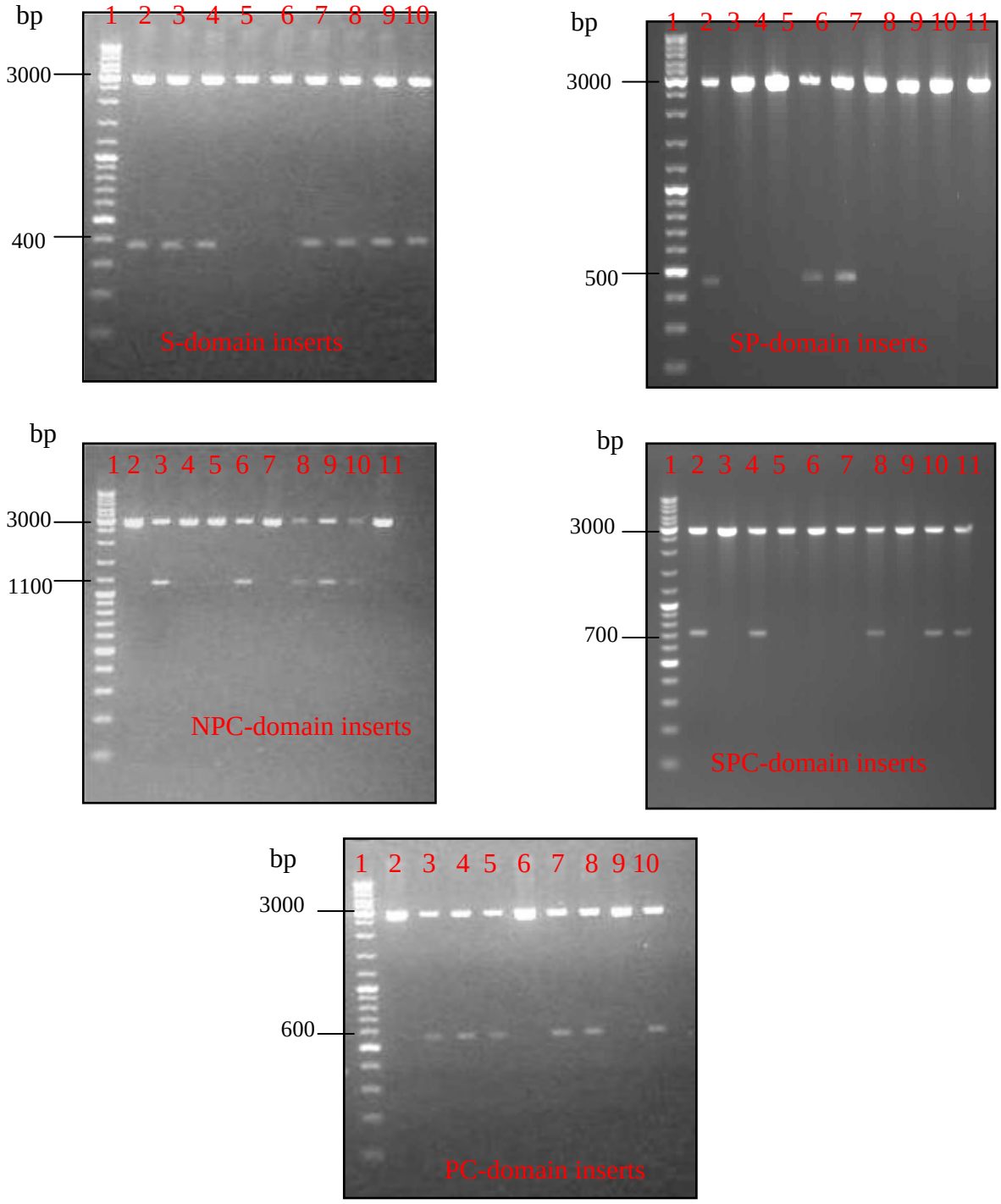


Figure 11: continued….

3.1.3 SM-CRT sub-fragments expression

3.1.3.1 Pilot protein expression experiment

A preliminary protein expression experiment was carried out for each SM-CRT sub-fragment using *E. coli* host BL21 (DE3)pLysS grown in S.O.B. medium. When the OD_{600} of the bacterial culture reached 0.7-0.8, 1 ml was taken and then 100 mM IPTG was added. For four times and every hour, 1 ml was taken from the culture. From the samples collected at these time points, a cell pellet was prepared by centrifugation. Each cell pellet containing the recombinant SM-CRT fragment was re-suspended in phosphate buffer, pH 7.0 and subjected to 3-4 cycles of freezing and thawing in methanol/dry ice and a water bath of 42 °C. The supernatant was mixed with an equal volume of 2X SDS-PAGE buffer, whereas the cell pellet was re-suspended in 100 µl 1X SDS-PAGE buffer.

In the soluble cell lysates, expression of the recombinant SM-CRT fragments was detectable even before IPTG induction, with variable degrees of intensity. After IPTG induction, the amount of the recombinant proteins increased in correlation with the time of induction as shown by the thickness of the protein bands in SDS-PAGE gel stained with Coomassie blue. The SM-CRT P-, C- and PC-domains were not detectable in the cell pellets, whereas the SM-CRT N-domain and other fragments (containing the N-domain or part of it) appeared in the cell pellets but with at very low concentrations in comparison to their corresponding cell lysate (see **Figure 12)**.

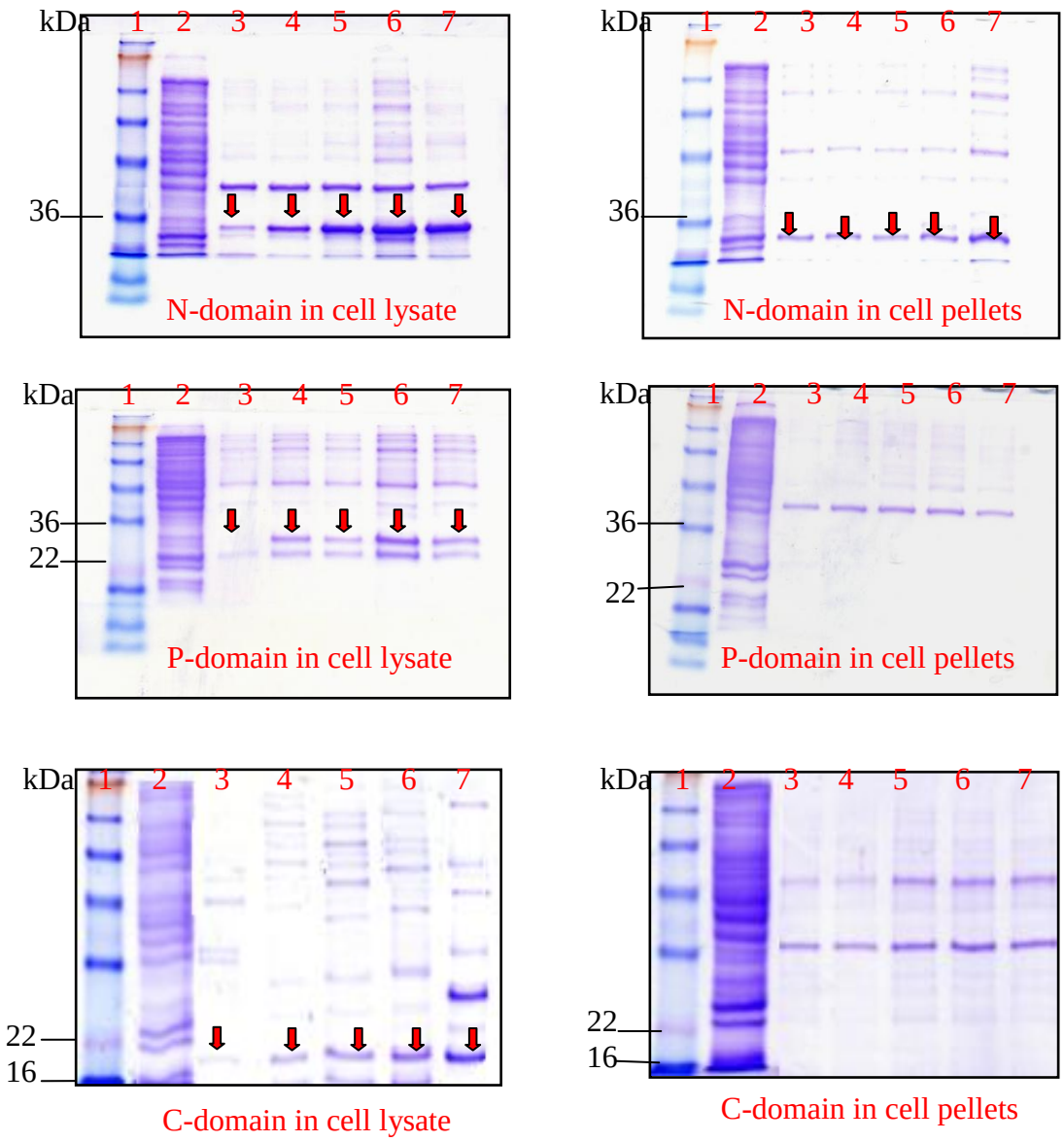


Figure 12: Pilot expression of SM-CRT sub-fragments. Individual fragments were expressed in BL21 cells. For each protein, 15 µl from the corresponding cell lysates or cell pellets at 0, 1, 2, 3 and 4 hours of IPTG induction were loaded onto SDS-PAGE gel. The head of the red arrow points to the recombinant protein band. Lane 1: standard M.W. marker, lane 2: BL21 bacterial lysate without being transformed with expression constructs, lane 3: transformed cells without IPTG induction, lane 4: one hour after IPTG induction, lane 5: 2 hours after IPTG induction, lane 6: 3 hours after IPTG induction and lane 7: 4 hours after IPTG.

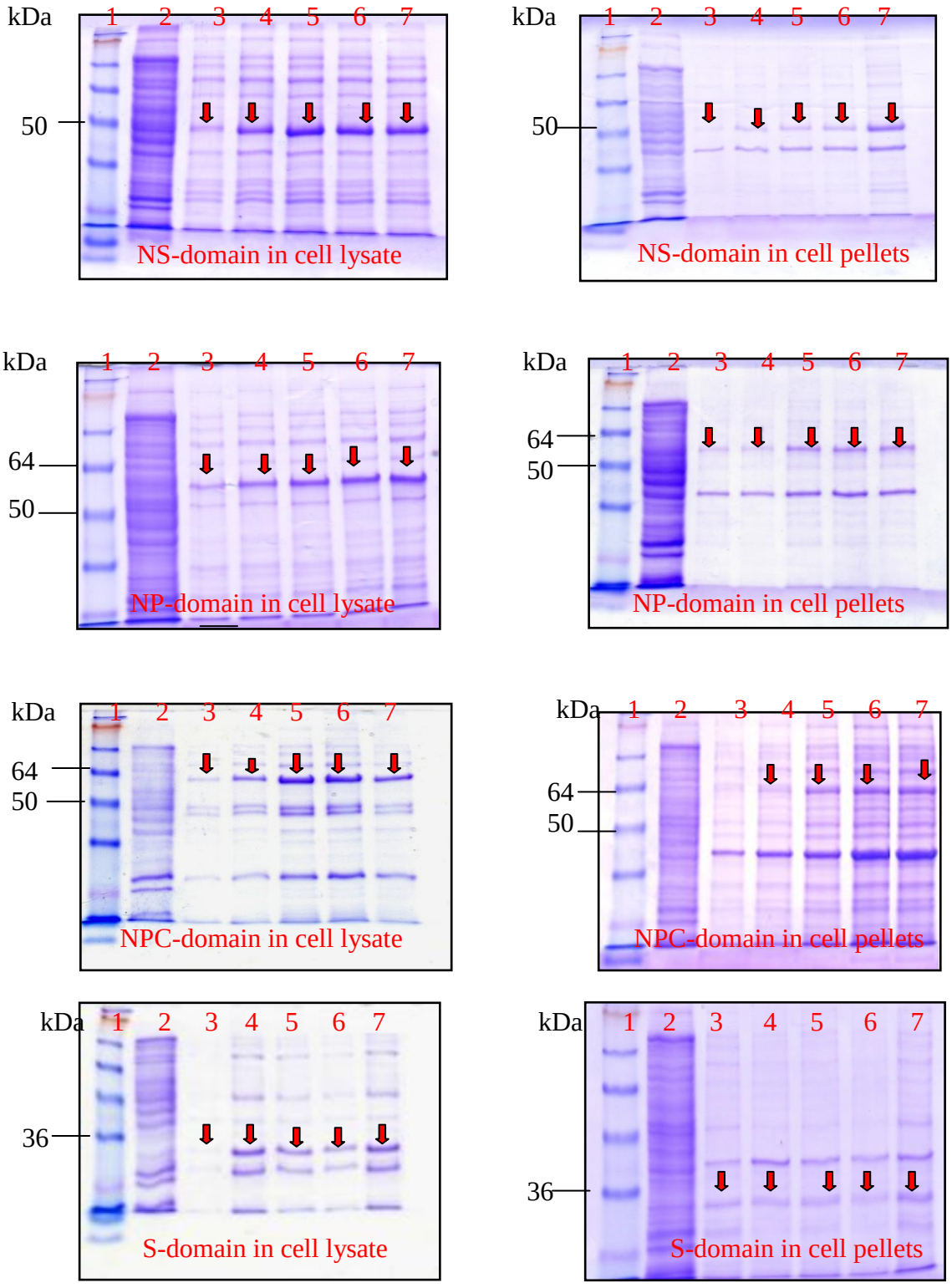


Figure 12: continued ……..

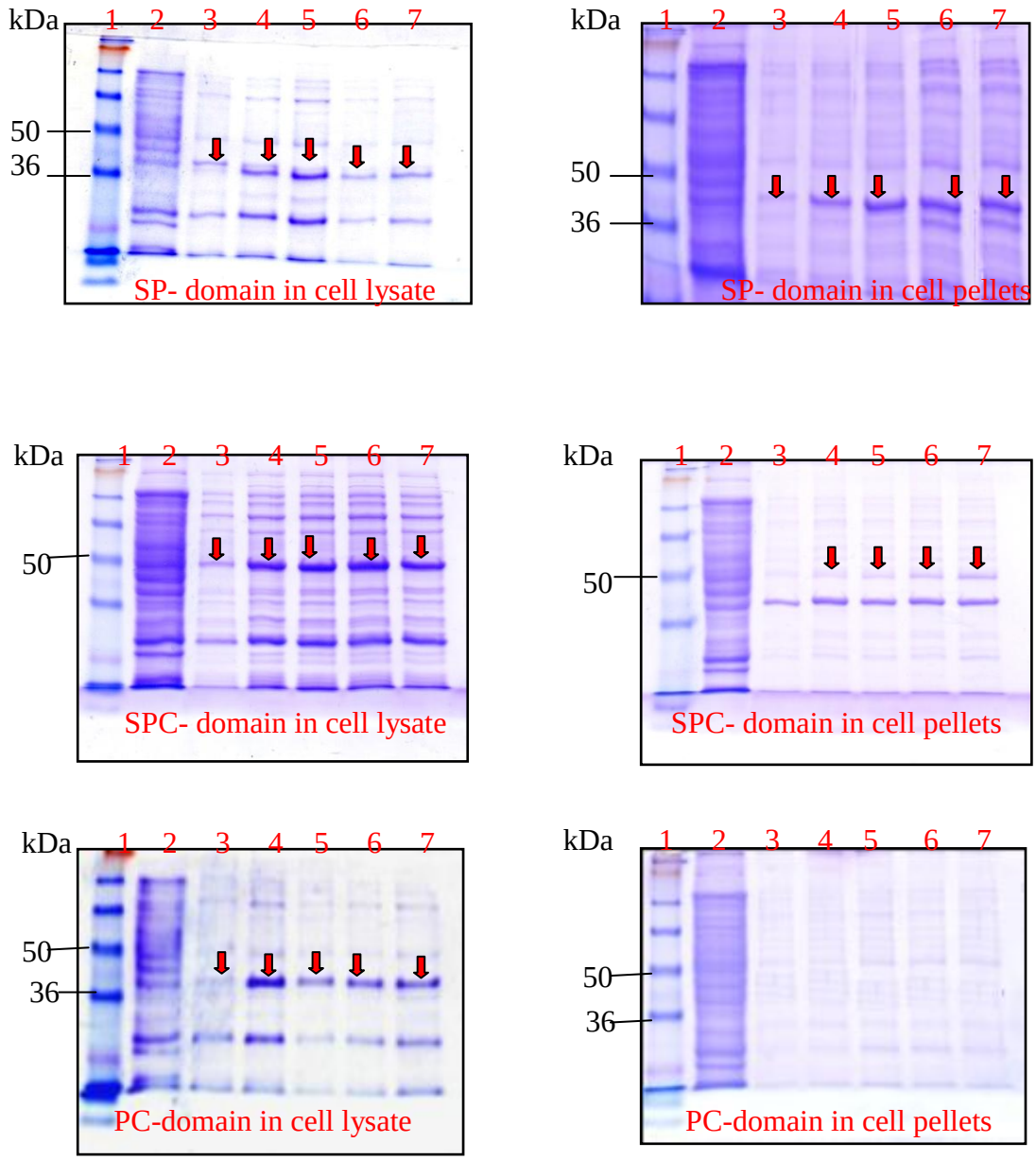


Figure 12: continued ……

To confirm the identity of the expressed SM-CRT proteins in the cell lysates, a Western blot analysis was performed using monoclonal anti-polyhistidine HRP conjugated antibodies. The proteins appeared at their expected M.W. The thickness of the proteins bands increased correlating with the time of IPTG induction. The SM-CRT N-, NS-, NP- and NPC-domains showed protein bands lying below the main protein bands 2 hours after IPTG induction indicating some degradation of recombinant protein. The proteins shown be expressed well 2-3 hours after IPTG induction **(**see **Figure 13).**

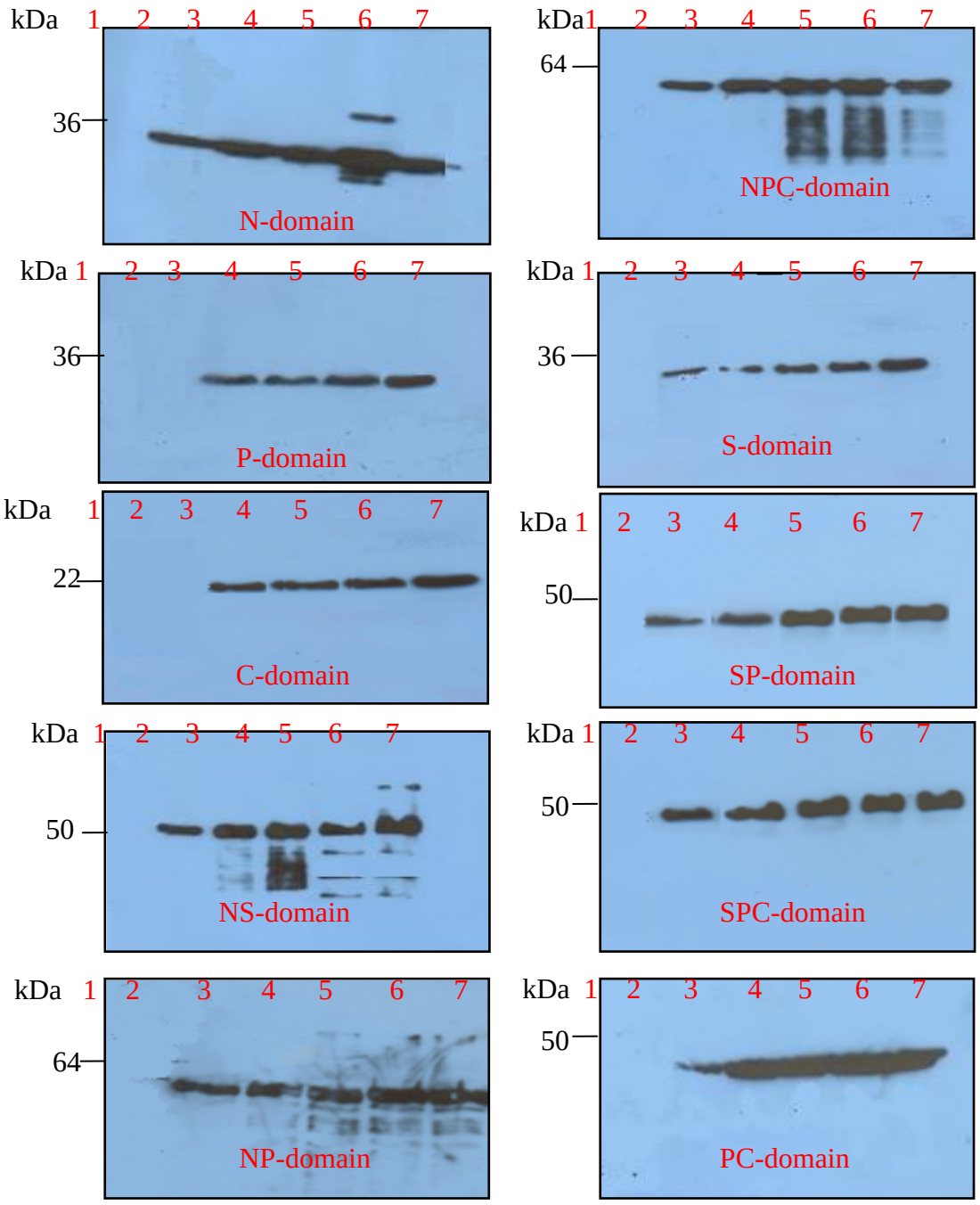


Figure 13: Western blot analysis of SM-CRT sub-fragments expressed in *E. coli* Bl21 using monoclonal anti-polyhistidine HRP conjugate antibodies (in the pilot experiment). The gel was loaded with 15 µl of cell lysate. Lane 1: standard M.W. marker; lane 2: BL21 bacterial lysate without transformed constructs, lane 3: transformed without IPTG induction, lane 4: one hour after IPTG induction, lane 5: 2 hours after IPTG induction, lane 6: 3 hours after IPTG induction and lane7: 4 hours after IPTG.

3.1.3.2 Large scale SM-CRT sub-fragments production

After determining the optimal expression conditions needed for each protein, a large-scaled protein production was performed. The same steps were followed using larger volumes of bacterial media and the transformed bacteria was left for 2-3 hours after IPTG induction.

3.1.4 SM-CRT sub–fragments purification

Each SM-CRT sub-fragment cell lysate was suspended with buffer A (20 mM Tris HCl, 500 mM NaCl and 5 mM imidazole, pH 8.0) and loaded onto HiTrap Chelating HP affinity columns (5 ml) which is charged with 100 mM $NiCl_2$. Then, the elution buffer, buffer B (20 mM Tris HCl, 500 mM NaCl and 500 mM imidazole, pH.8) was injected automatically by the programmed FPLC with linear gradient elution method (from 5-500 mM imidazole). The eluted fractions corresponding to the peak of chromatogram representing the purified SM-CRT were collected and analyzed with Coomassie SDS-PGE gels **(Figure 14).**

The analysis of the purified SM-CRT sub-fragments with affinity chromatography showed that, further ion exchange chromatography is required (**Figure 15**). The protein sample was desalted using desalting buffer (20 mM Tris HCl and 50 mM NaCl, pH 8), then loaded into HiTap Q HP (5 ml) column with washing buffer A (20 mM Tris HCl and 20 mM NaCl, pH 8.0) and elution buffer B **(**20 mM Tris HCl and 1 M NaCl, pH 8). The fractions representing the SM-CRT peak on the chromatogram were collected and analysed.

A **B**

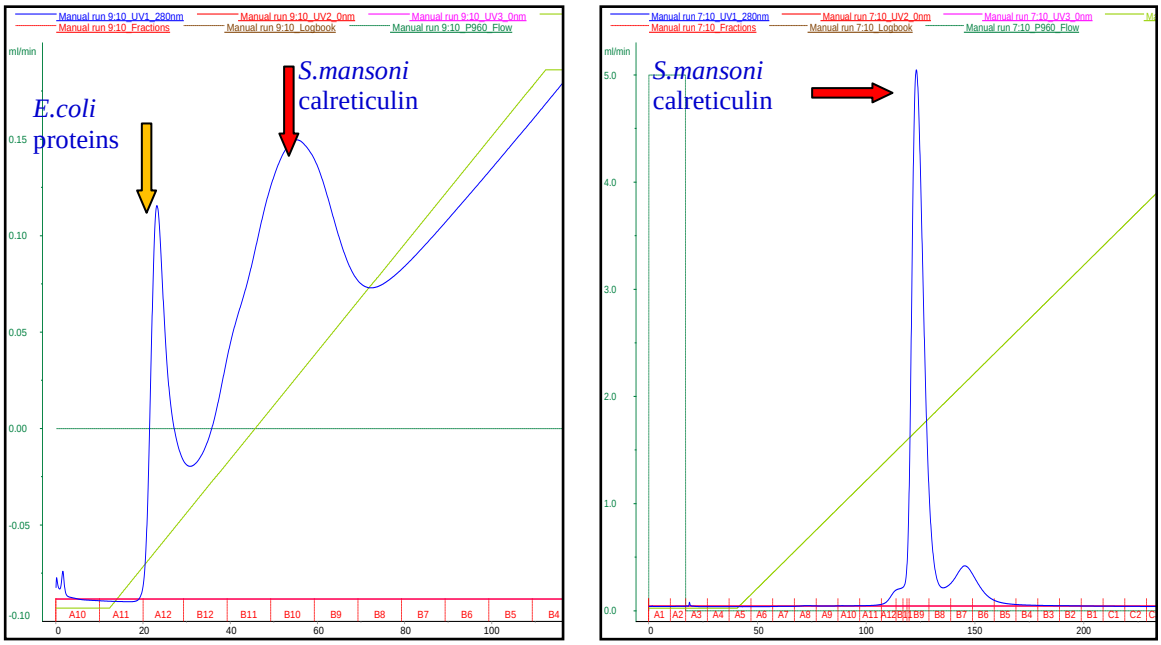


Figure 14: Chromatogram. (A): Affinity chromatography revealed two peaks, the first one representing the eluted *E. coli* proteins, while the second peak represents the eluted SM-CRT protein (at 20-25 % of 500 mM Imidazole, buffer B), as pointed by the corresponding arrows. **(B):** Ion exchange chromatography shows a sharp peak representing SM-CRT sub-fragment (eluted at 20-25% 1 M NaCl, buffer B)**.** The green line represents the linear gradient elution with buffer B and the blue colour is the eluted proteins as detected at 280 nm wavelength (this chromatogram shows the NPC-domain and it is representative of the other SM-CRT sub-fragments chromatograms).

To compare the purity of each SM-CRT sub-fragment, 15 µl were loaded in SDS-PAGE gel stained with Coomassie blue from the following protein preparations: before purification, after affinity chromatography and after ion exchange purification **(Figure 15).**

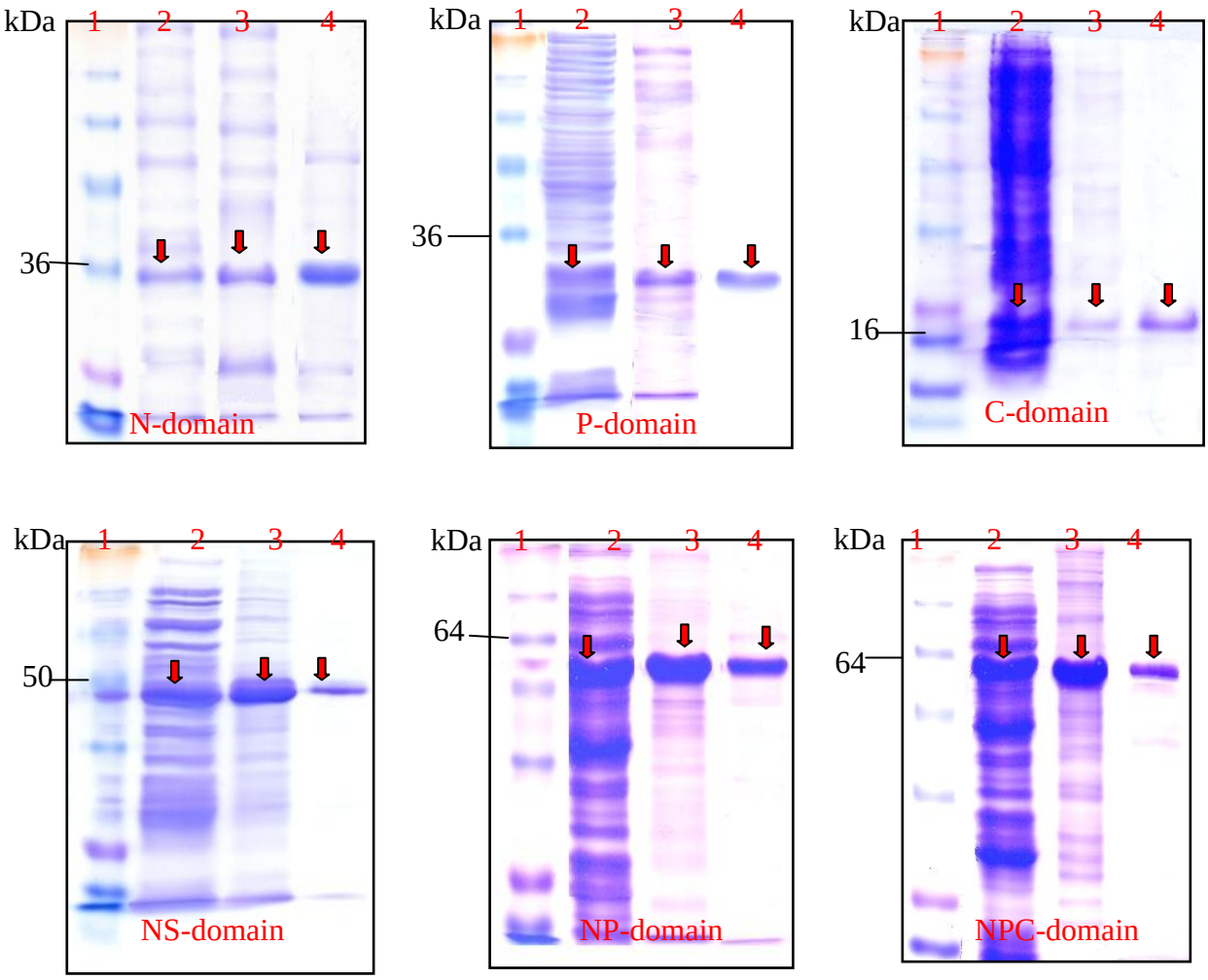


Figure 15: SDS–PAGE gel stained with Coomassie blue loaded with SM-CRT proteins (15 % in the case of P- and C-domains and 12% in other domains). 15 µl from each sample was added. The head of the red arrow point to the protein band. Each graph is labelled with its corresponding protein. Lane 1: standard M.W. (SeeBlue Plus2 PreStained Standard (Invitrogen) marker, lane 2: BL21 bacterial lysate induced with IPTG for 2-3 hours, lane 3: the protein after Ni affinity chromatography and lane 4: after ion exchange chromatography.

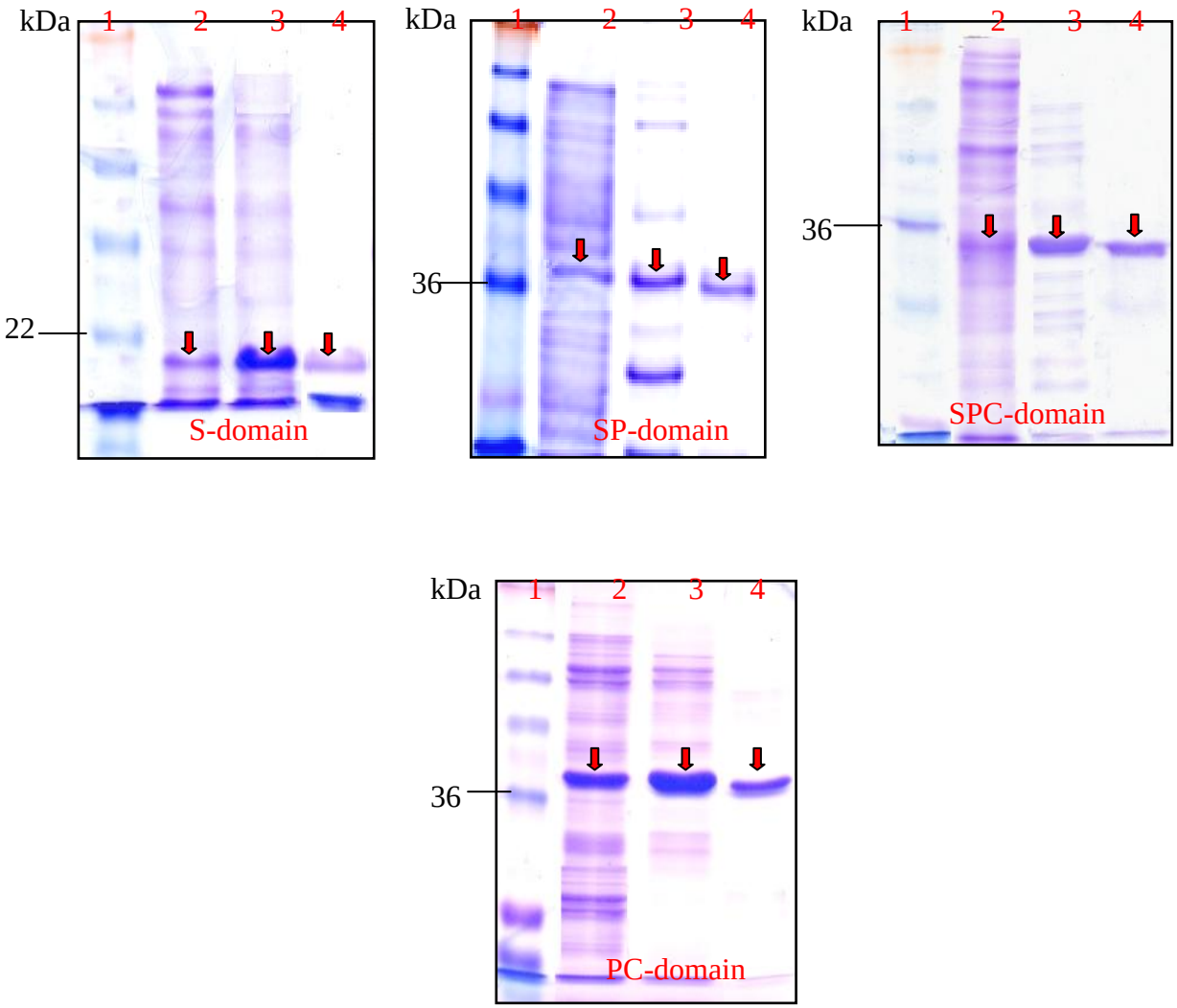


Figure 15: continued …….

3.1.5 Measurement of protein concentration by Coomassie Plus–"The Better Bradford assay"

The standard curve was established using the given BSA concentration coming with the kit (µg/ml correlated with the respective O.D. readings at 595nm). The standard curve was used to calculate concentration of the purified SM-CRT sub-fragments **(Figure 16).**

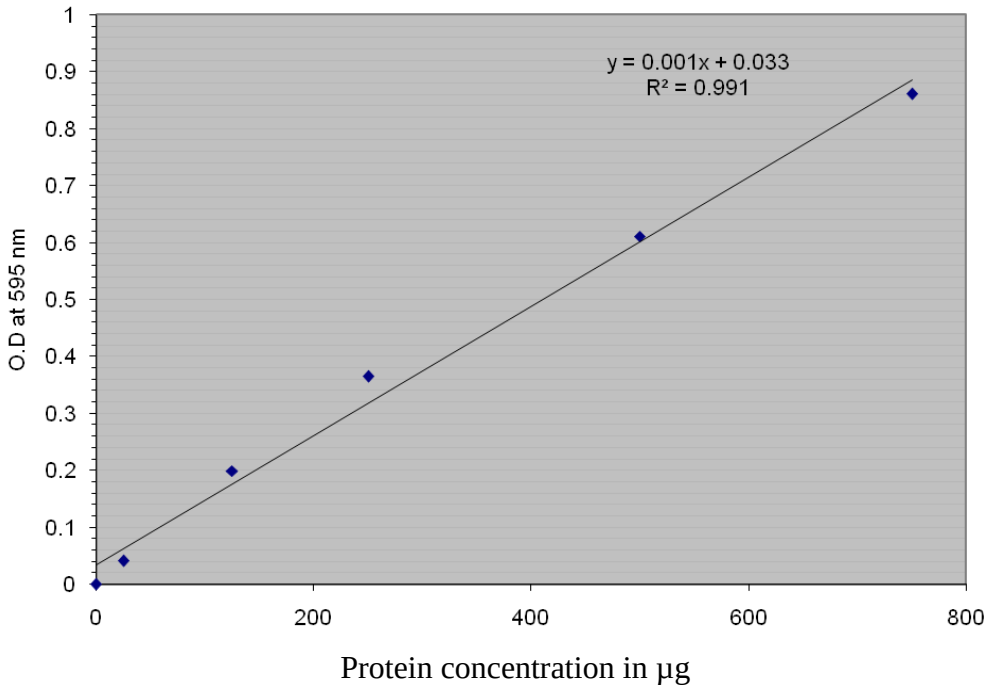


Figure 16: Calculation of proteins concentration using Coomassie Plus–"The Better Bradford Assay". O.D. value measured at 595 nm for each protein was used for calculation of its concentration using the formula; y=0.001x + 0.033, where, x is the protein concentration in µg/ml and y is O.D. measured at 595 nm.

In total, 3 µg of each purified SM-CRT sub-fragment was loaded onto two Coomassie stained SDS-PAGE gels. One 15% SDS-PAGE gel was loaded with N-, P, C, NS and NP-domains and the other 12% SDS-PAGE gel contain the other domains. The gels showed that all SM-CRT fragments were purified to a very high degree of purity **(Figure 17).**

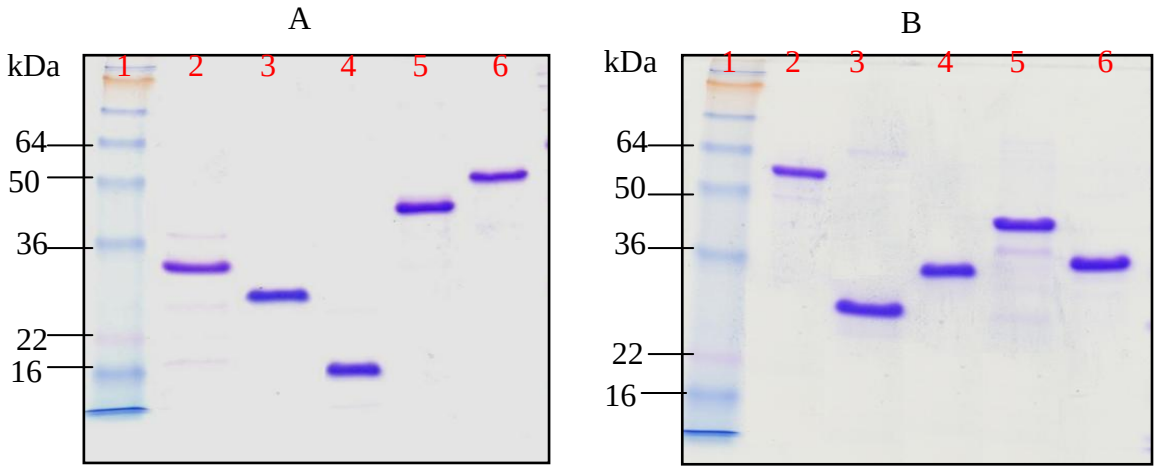


Figure 17: Purified SM-CRT sub-fragments are grouped in two SDS–PAGE gels stained with Coomassie blue (A& B). 3 µg from each protein was loaded in the gel. **(A):** Lane 1: standard M.W. marker (SeeBlue Plus2 PreStained Standard), lane 2: N-domain, lane 3: P-domain, lane 4: C-domain, lane 5: NS-domain and lane 6: NP-domain. **(B)**: Lane 1: standard M.W. marker, lane 2: NPC-domain, lane 3: S-domain, lane 4: SP-domain, lane 5: SPC-domain and lane 6: PC-domain.

3.1.6 Physico-chemical data obtained for the different SM-CRT sub-fragments

The DNA size, expected molecular weight and isoelecteric points (pI) of different SM-CRT sub-fragments were tabulated. These data help to identify the SM-CRT sub-fragments by size of the coding cDNA fragment or the encoded protein and to choose the suitable buffer pH range for the ion exchange step.

Domains	DNA size (bp)	Numbers of residues	Expected MW (kDa)	Isoelectric point (pI)
N	573	205	23.22	6.59
P	294	132	15.7	4.56
C	267	121	13.77	4.51
S	360	157	18.41	4.42
NS	795	299	34.42	4.97
NP	867	324	37.49	5.01
NPC	1134	411	47.41	4.72
SP	444	182	21.49	4.51
SPC	711	269	31.39	4.39
PC	561	219	25.61	4.4

Table 2: Some physico-chemical data SM-CRT proteins using online ExPASY programme (Numbers of residues of each protein include the vector derived ones).

3.2 Discussion

Most of the available knowledge concerning structures and functions of CRT is derived from studies of the vertebrate CRT. From the CRT gene sequences established by Fliegel *et al*. (1989) and Smith and Koch (1989), the protein sequences has been developed and showed to comprise three functional and structural domains; the N-, P- and C-domain. A functional domain termed S-domain was late described with Stuart *et al*. (1996& 1997).

In my work, I split SM-CRT into ten sub-fragments; N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC-domains. The aim of this splitting was to recognize which domain or domain combination is more specific for detection of antibodies against SM-CRT in the sera of *S. mansoni* infected murine and humans. A cDNA library of adult *S. mansoni* was used to amplify the coding cDNA sequences for these segments using PCR. The different coding regions of SM-CRT domains and sub-fragments were cloned (**Figure 8, 9& 10**), expressed using the pRSETB vector in 6 Histidine-tag N-terminal fusion forms (**Figure11, 12& 13**) and purified (**Figure 14 and 15**) successfully.

The amplified SM-CRT DNA sequences were identical to those published by Khalife *et al*. (1993). During PCR amplification, transcription errors were rare for the small segments, such as P-, C- and S-domains, but occurred when amplifying larger fragments like NP-, NS- and NPC-domains which required repeating the amplification attempts.

The pRSETB vector yields the production of recombinant proteins at high concentration and most of the domains were produced as soluble proteins. There are many other microbial protein expression systems that have been used for CRT expression in various

species, for example *S. mansoni* calreticulin was previously expressed using pGEX-2T plasmid expression vector (Khalife *et al.* 1993& 1994), *S. japonicum* CRT was expressed as a fusion protein using pTrcHis plasmid (Huggins *et al*. 1995), *N. americanus* recombinant CRT was expressed by using pQE10 vector (Pritchard *et al*. 1999, Kasper *et al*. 2001, Winter *et al*. 2005), pQE vector expressed *H. contortus* CRT (Suchitra& Joshi 2005), pTrxfus plasmid in thioredoxin fusion form was applied for production of human N-, P-, C- and S-domains (Stuart *et al*. 1997), pMal-c2 expressed different human CRT domains in maltose-binding forms (Kishore *et al*. 1997), Baksh& Michalak (1991) expressed different human CRT domains in GST-fusion form using pGEX-3X plasmid and *L. donovani* CRT was expressed by using pET-22b(+) plasmid (Debrabant *et al*. 2002).

SM-CRT N-domain was expressed mainly in soluble form, but cell pellets still contained some insoluble protein (**Figure 12**) and this denotes the correct folding of the main bulk of the expressed domain. This is somewhat different from what Baksh and Michalak (1991) described. They expressed the human CRT N-domain as a GST fusion protein with only 20% of the total protein was soluble. Suchitra& Joshi (2005) have expressed *H. contortus* calreticulin N-domain yielding an insoluble protein. This variation in the expressed N-domain solubility may be due to the protein species, the expression vector, variation in different conditions of protein expression process such as incubation temperature, or the fact that, the SM-CRT N-domain expressed in pRSETB has twenty extra residues added to the N-terminus (encoded by the vector) which could increase the solubility of the recombinant protein.

SM-CRT NS-, NP-, S-, SP-, SPC- and NPC-domains were produced with varying solubility because they contain the whole or only part of the N-domain, whereas SM-CRT

P-, C- and PC-domains were expressed as completely soluble proteins with no recombinant protein remaining in the cell pellets of the host bacteria (**Figure 12**). This was similar to *H. contortus* CRT P- and C-domains which were produced as soluble forms (Suchitra & Joshi 2005).

Western blot analysis (see **Figure 13**) detected different SM-CRT sub-fragments which confirmed the success of proteins expression in the pilot experiments that helped to optimize the time needed for protein expression and gave an idea about the extent of degradation of the recombinant protein with time. There was a positive correlation between the time of IPTG induction and the yield of the recombinant proteins. Most of SM-CRT sub-fragments expressed well 2-3 hours post-induction. With longer incubation, there was protein degradation with the N-, NS-, NP- and NPC-domains. As these domains all include the N-domain and it seems that this portion of CRT is liable to spontaneous degradation. This is consistent with the observation reported by Ferreira *et al*. (2004) who noticed that the N-terminal part of recombinant *T. cruzi* CRT (TcCRT) is exposed to spontaneous degradation. Other SM-CRT sub-fragments showed increased expression correlating with the incubation time without apparent degradation. It was found that P-, C- and S-domains required a longer period of incubation up to 4-5 hours.

Domains P-, C-, PC-, S-, SP- and SPC- were purified in sufficient quality by His-tag affinity chromatography and with addition of ion exchange chromatography, the degree of purity was excellent. But the segments containing the N-domain needed both the purification steps to attain a sufficient degree of purity and this may be due to the tertiary structure of the expressed SM-CRT sub-fragments that contain the globular N-domain

which could bind non-specifically to BL21 *E. coli* proteins or because of protein dimers formations that occur more frequently with these sub-fragments than with P- or C- domains

The FPLC machine used for protein purifications was run with Unicorn 5.01 software. This purification process was safe, relatively quick, reproducible, the reagents were relatively cheap and the machine was easily managed after training.

Various SM-CRT sub-fragments were analysed on Coomassie blue stained SDS-PAGE gels and shown to be slightly larger than their predicted molecular weight (see **Figure 12, 15& 17 and Table 2**). The expressed NPC-domain, with predicted 47 kDa, ran at a relative size of around 60 kDa in SDS-PAGE gel, and this is in agreement with other expressed calreticulin of different species, such as *Schistosoma* species (Khalife *et al.* 1993, Huggins *et al*. 1995, Scott& McManus 1999), *N. americanus* (Winter *et al.* 2005) and human (McCauliffe *et al*. 1990). The dimorphic phenomenon, that SM-CRT sub-fragments run at a molecular weight larger than the calculated molecular weight, can be explained by the slow run of these proteins in the gel due to their relative high acidity and this acidity may interfere with their binding with SDS (Huggins *et al*. 1995). Also, the occurrence of CRT post-translational modifications may affect the running behaviour.

Chapter 4

Characterization of SM-CRT sub-fragments

4.1 Results

4.1.1 Binding of SM-CRT sub-fragments to calcium

The capability of the different expressed recombinant SM-CRT sub-fragments to bind calcium was evaluated using two methods; staining the proteins with "Stains-all" stain (which identifies Ca^{2+} binding proteins) and by direct calcium binding using $^{45}Ca^{2+}$.

By using "Stains-all" stain, P-, C-, NS-, NP-, NPC-, PC-, S-, SP-, SPC-domains and calmodulin (positive control) all gave a blue colour staining indicating their ability to bind to calcium, whereas the SM-CRT N-domain and the negative control (BSA) stained pink denoting that these protein are not calcium binding proteins (see **Figure 18).**

These results were confirmed by evaluating the binding ability of these proteins with radioactive calcium. This showed that most SM-CRT sub-fragments bound $^{45}Ca^{2+}$. The strength of the signals varied amongst the proteins. The highest capacity to bind calcium was seen with the C-domain. The N-domain did not give any signals, but an unexpected finding was that neither NS- nor NP-domains bound calcium **(Figure 19).**

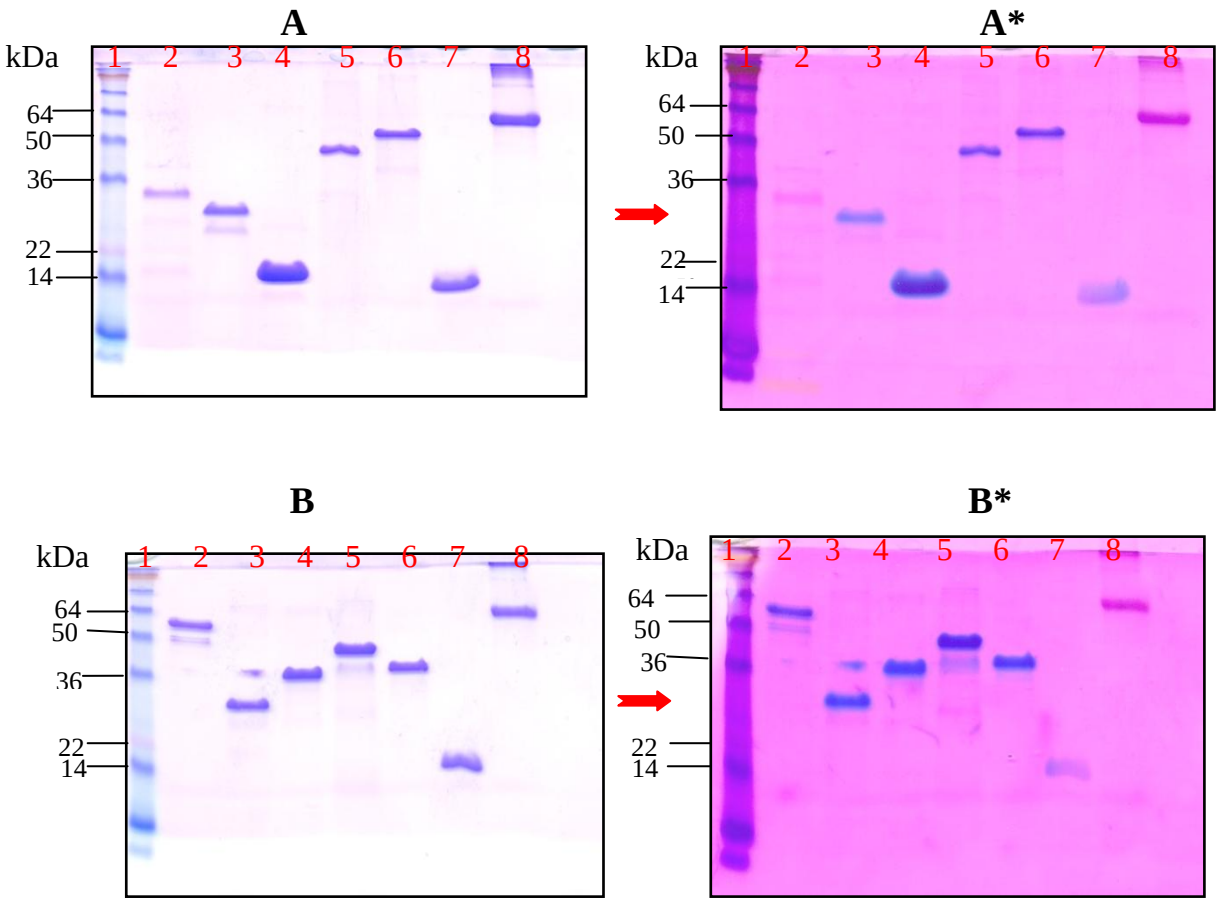


Figure 18: SDS-PAGE gels (15%) stained with Coomassie blue and fixed with 25% isopropyl alcohol (A& B) or stained with "Stains-all" stain (A*&B*) . The gels were loaded with 3 µg from each protein**. (A& A*):** Lane 1: standard molecular weight marker (SeeBlue Plus2 PreStained Standard), lane 2: the N-domain, lane 3: the P-domain, lane 4: the C-domain, lane 5: the NS-domain, lane 6: the NP-domain, lane 7: calmodulin and lane 8: BSA. **(B &B*):** Lane 1: standard molecular weight marker, lane 2: the NPC-domain, lane 3: the S-domain, lane 4: the SP-domain, lane 5: the SPC-domain, lane 6: the PC-domain, lane 7: calmodulin and lane 8: BSA.

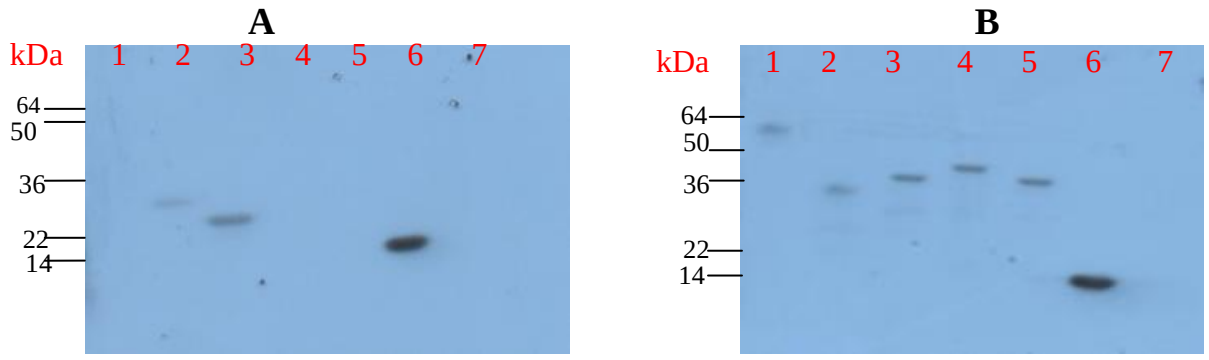


Figure 19: Western blot of recombinant SM-CRT sub-fragments interacting with $^{45}Ca^{2+}$. Nitrocellulose membranes were blotted with 3 µg from each protein and incubated with $^{45}Ca^{2+}$ for 10 minutes and then exposed to X-ray film for 48 hours at -80 °C. (**A)**: lane 1: the N-domain, lane 2: the P-domain, lane 3: the C-domain, lane 4: the NS-domain, lane 5: the NP-domain, lane 6: calmodulin and lane 7: BSA. (**B)**: lane 1: the NPC-domain, lane 2: the S-domain, lane 3: the SP-domain, lane 4: the SPC-domain, lane 5: the PC-domain, lane 6: calmodulin and lane 7: BSA.

4.1.2 SM-CRT sub-fragments interaction with C1q, the recognition molecule of classical pathway

The interaction between C1q and the different SM-CRT sub-fragments was studied by testing the binding between the proteins and C1q, thereafter evaluating the effect of this binding on the inhibition of C1q-dependent haemolysis.

4.1.2.1 SM-CRT binding with C1q

The plates were coated with SM-CRT sub-fragments and BSA and incubated overnight at 4 °C. C1q, diluted in physiological PBS, was added in serial dilution starting with 0.4 µg/well to the coating proteins. The experiments were repeated three times in duplicate.

With the exception of the P-domain, all SM-CRT sub-fragments were able to bind human C1q and this binding was dose dependent (see **Figure 20 A& B**). The P-domain did not show any binding to the serial dilutions of C1q.

The relative C1q binding affinity of different SM-CRT domains was determined from the individual absorption readings using C1q concentration of 0.4 µg/well. It was found that the NPC-domain and S-domain (in the N-, P-, C-, S- and NPC-domains group) (**Figure 20 A***) and NP- and SPC-domains (in the NS-, NP-, SP-, SPC-, PC-domains group) **(Figure 20 B*)** achieved the highest O.D. readings indicating the highest binding interaction.

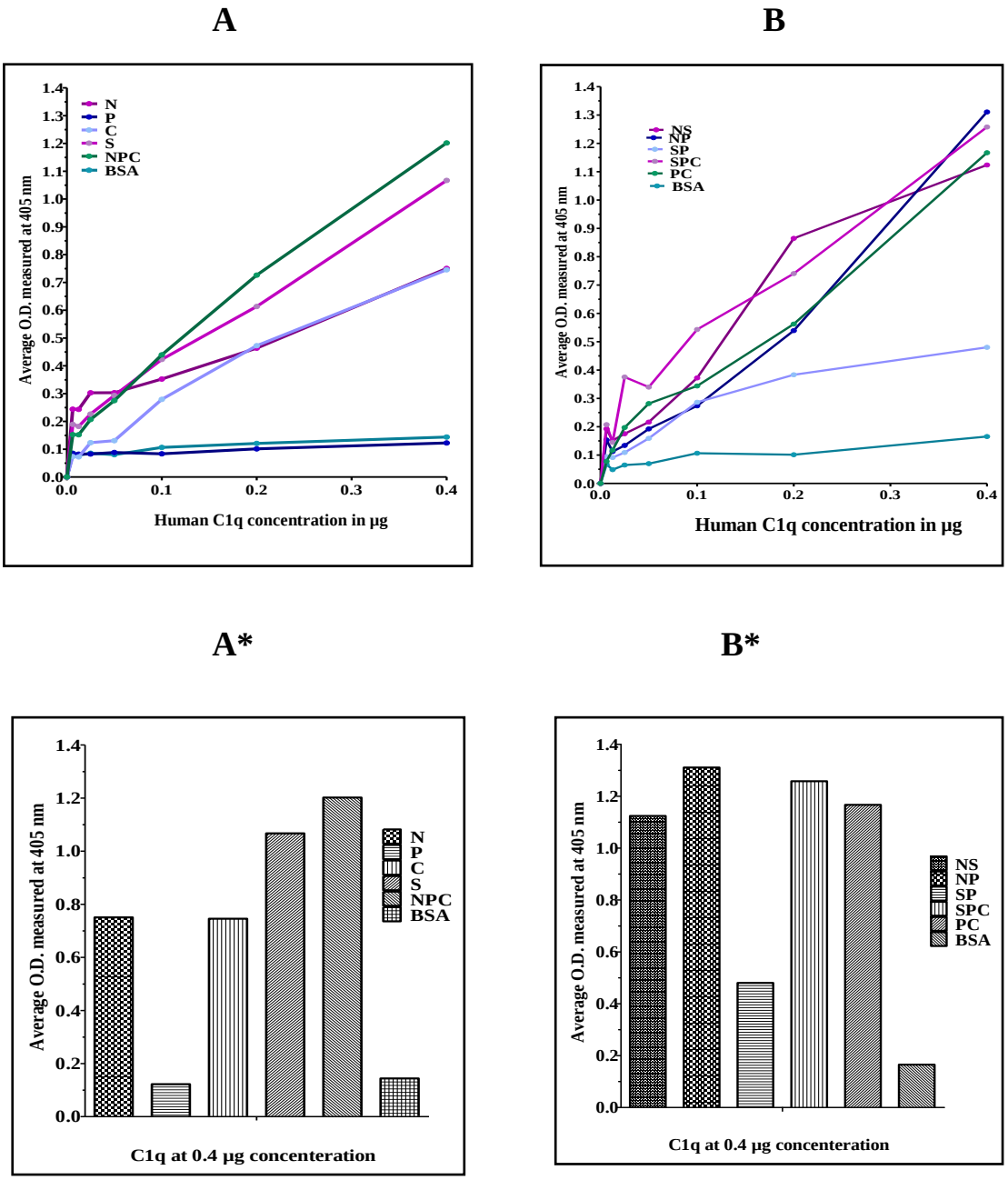


Figure 20: Binding of recombinant SM-CRT sub-fragments to C1q. **(A& B):** Serial dilutions of C1q starting from 0.4 µg was incubated with 0.5 µg of each SM-CRT sub-fragment**. (A*& B*):** with exception of P-domain, SM-CRT sub-fragments bind with C1q at 0.4 µg**.** Each experiment was done three times in duplicate and representative results are shown.

4.1.2.2 Inhibition of C1q-dependent haemolytic assay

To evaluate if SM-CRT sub-fragments can affect the biological functions of C1q, C1q dependent haemolysis inhibition assay was performed using both normal human serum (NHS) and human C1q deficient sera. Prepared sensitized sheep red blood cells (SRBCs) with hemolysin was used to perform the haemolytic assays. At first, series of experiments were performed to optimise assays and choose the appropriate amount of human C1q and sera dilutions. It was found that the 1: 160 NHS titer achieved 70-80% haemolysis and a 1:40 dilution of C1q deficient serum reconstituted with 0.125 μg of human C1q achieved 70-75% haemolysis.

SM-CRT sub-fragments and BSA were prepared in concentrations of 1, 5, 10, 15 or 20 μM. In the case of NHS, the proteins were mixed with 1:160 diluted serum and incubated at 37°C for 1 hour. After that, 5% SRBCs (1x 10^{8} cells/ml) was added for each well and re-incubated at 37 °C for further 1 hour. Then, BBS was added to each well and the plate was centrifuged and 200 μl supernatant from each well was transferred to a flat bottom well and O.D. was read at 405 nm. In case of C1q depleted serum, the same process was used, but the serum was reconstituted with 0.125 μg human C1q and added to 100 μl of 1: 40 C1q deficient serum.

Addition of recombinant SM-CRT P-domain did not affect the haemolysis of the sensitized SRBCS and this underlines the finding that the SM-CRT P-domain does not bind to C1q. All other recombinant SM-CRT domains decreased haemolysis in a dose dependent manner. The inhibitory activity on haemolysis varied among the different domains. SM-CRT sub-fragments effects were similar in both types of sera used, but for the reconstituted

C1q deficient serum, slightly greater amounts of recombinant SM-CRT proteins were required to achieve a similar degree of haemolysis compared to diluted NHS **(Figure 21).**

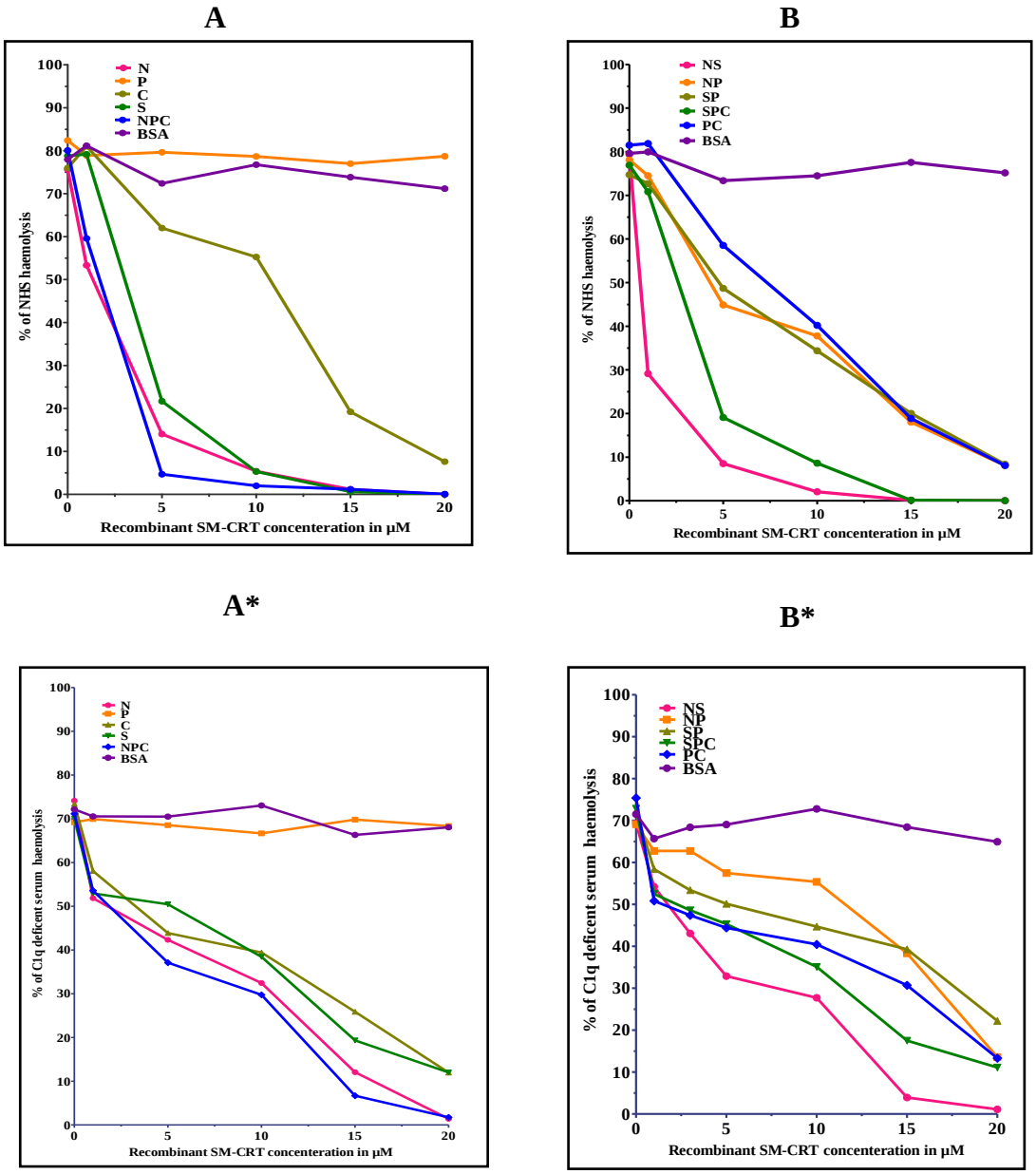


Figure 21: Inhibition of C1q dependent haemolysis assays. NHS **(A& B)** and human C1q deficient **(A*& B*)** sera were tested. Each experiment was done three times in duplicate and representative results are shown.

4.1.3 Evaluating the immunogenicity of SM-CRT sub-fragments

The NPC-domain was used to immunise a rabbit and the anti-serum was used to determine immunogenicity of the SM-CRT NPC-domain. At first, the specific anti-serum recognised the NPC-domain in a Western blot assay of the antigen with BSA and BL21 cell lysate loaded as negative controls. Subsequently, anti-SM-CRT rabbit anti-serum was used to identify all SM-CRT fragments by Western blot.

It was found that anti-SM-CRT rabbit anti-serum reacted with all the SM-CRT fragments in Western blot analysis **(**see **Figure 22)**. On Western blots, there were other bands identified by the anti-serum in addition to the main bands of the recombinant SM-CRT fragments.

ELISA was also applied to test the immunogenicity of SM-CRT. At first, the NPC-domain was coated on a plate and incubated with the pre-bleed and anti-serum using the following dilutions; 1:1000, 1:10000 and 1:50000. The anti-NPC rabbit anti-serum reacted against NPC-domain in different dilutions denoting the good immunogenicity of the SM-CRT NPC antigen **(Figure 23 A)**. Then, different SM-CRT sub-fragments were tested with the same specific anti-serum dilutions. Two plates were coated with the proteins in duplicate and each group received BSA as negative control and the full length NPC-domain as a positive control. The specific anti-SM-CRT anti-serum was added at dilutions of 1:1000, 1:10000 and 1:50000. All SM-CRT sub-fragments gave absorbance with different dilutions of the specific anti-serum with some variations **(Figure 23 B& C)**.

To assess which domain was most immunogenic, O.D.s were compared at 1:10000 dilution. It was found that the C-domain and PC-domains were the most immunogenic achieving the highest O.D. value after the NPC-domain. The fragments with the lowest immunogenicity were the N- and the SP-domains. Other SM-CRT sub-fragments were more or less similar to each other in their degree of immunogenicity (**Figure 23 D& E**).

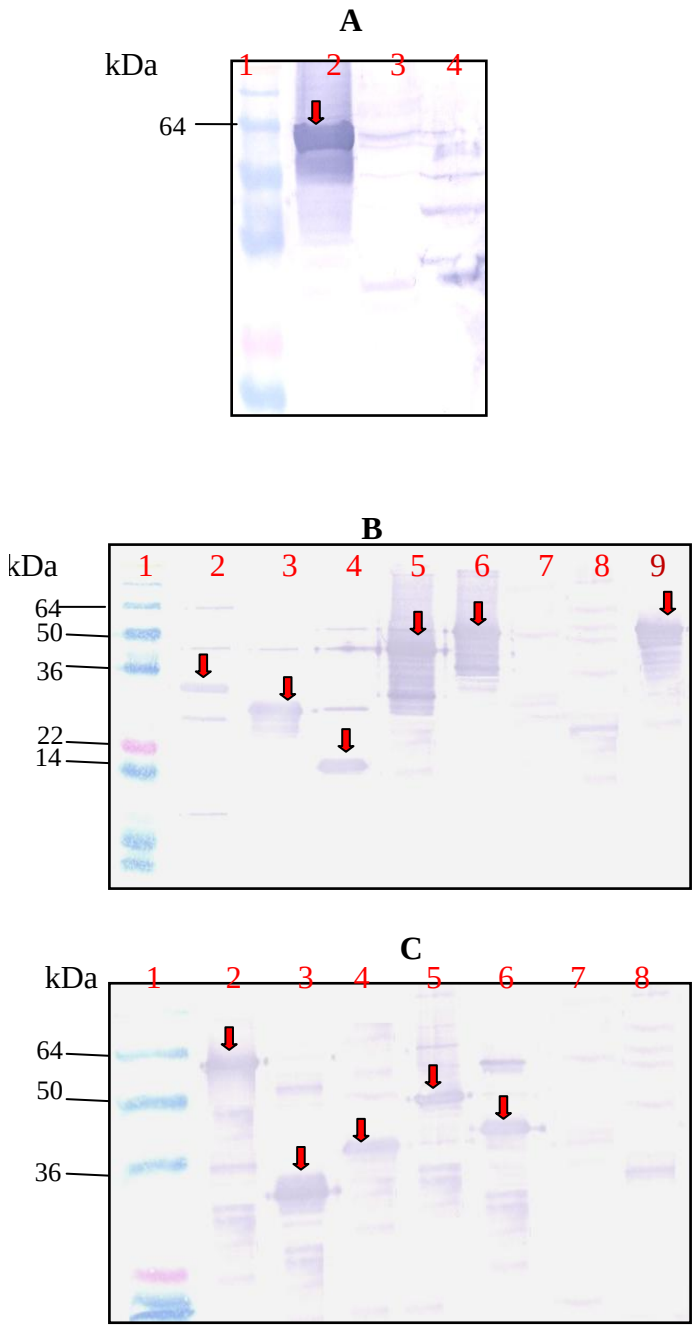


Figure 22: Western blot of recombinant SM-CRT sub-fragments identified by anti-SM-CRT rabbit anti-serum(1:40). SDS-PAGE gels were loaded with 1 µg from each protein. The head of the red arrow points to the protein band. **(A):** lane 1: M.W marker, lane 2: NPC-domain, lane 3: BSA and lane 4: *E.coli* lysate proteins. **(B):** lane 1: M.W marker, lane 2: N-domain, lane 3: P-domain, lane 4: C-domain, lane 5: NS-domain, lane 6: NP-domain, lane 7: BSA, lane 8: *E.coli* lysate and lane 9: NPC-domain. **(C):** lane 1: M.W marker, 2: NPC-domain, lane 3: S-domain, lane 4: SP-domain, lane 5: SPC-domain, lane 6: PC-domain, lane 7: BSA lane and 8: *E.coli* lysate.

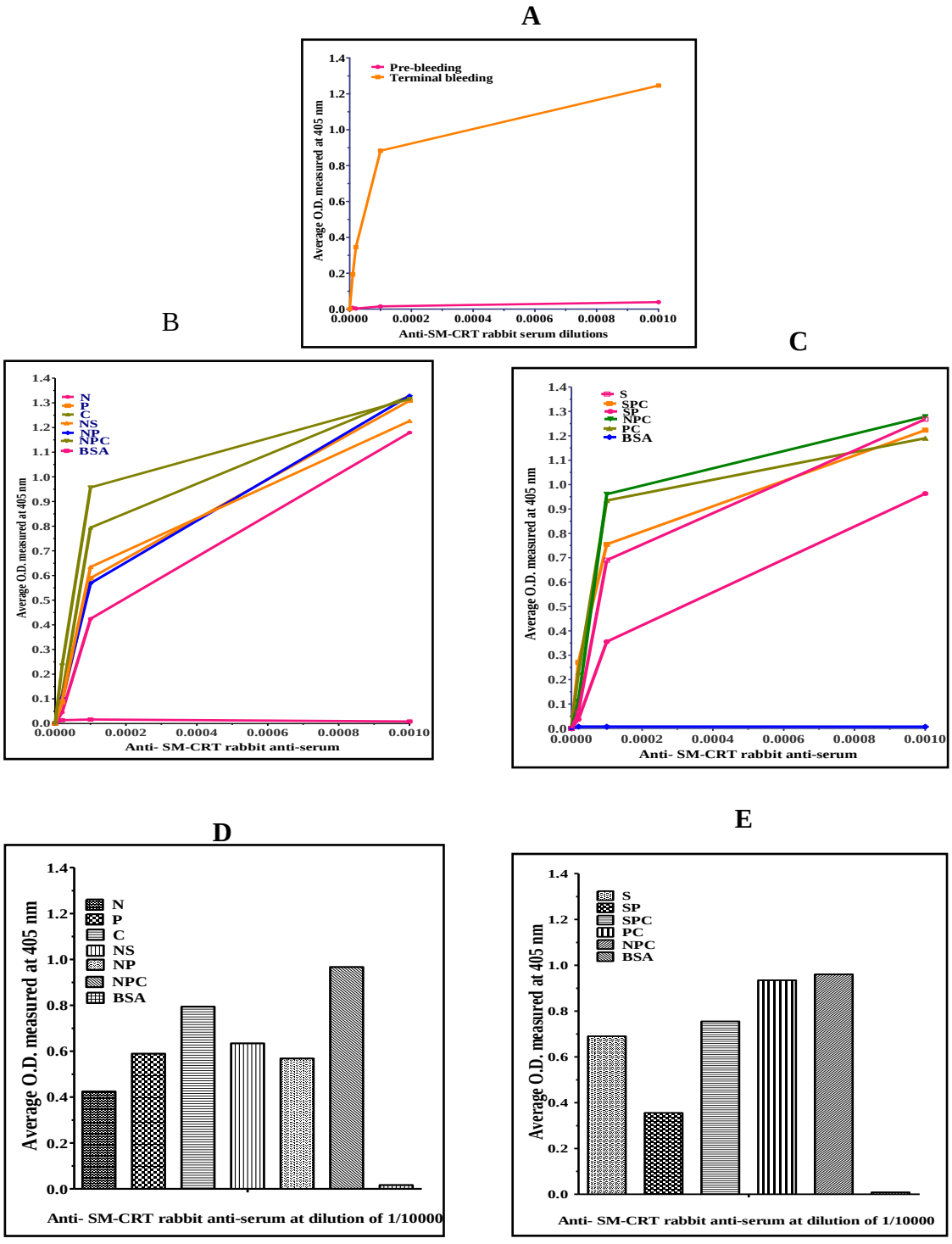


Figure 23: **Anti-SM-CRT rabbit anti-serum identified SM-CRT fragments by ELISA. (A):** the serum was tested at 1:1000, 1:10000 and 1:50000. **(B& C):** it reacted against the NPC-domain and then against different SM-CRT sub-fragments**. (D& E):** from the representative experiment and at 1:10000 dilution, O.D.s were compared among different sub-fragments to compare the immunogenicity of these sub-fragments. The C-domain and PC-domains are the most immunogenic.

4.1.4 Detection of native *S. mansoni* CRT in the tegument of the adult and larva

Anti-SM-CRT rabbit anti-serum identified the native *S. mansoni* CRT (appeared of around 60 kDa in size) in the larval and adult preparations containing the surface membranes antigens using Western blot assay **(Figure 24).**

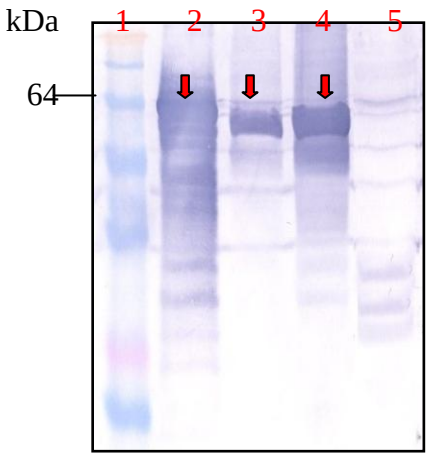


Figure 24: Western blot using anti-SM-CRT rabbit anti-serum to detect native *S. mansoni* CRT in the schistosomulum and adult surface membrane preparation. The head of the red arrow points to the protein. Lane 1: M.W marker, lane 2: recombinant NPC-domain (2µg loaded), lane 3: larval supernatant (1µl loaded), lane 4: adult supernatant (2µl loaded) and lane 5: *E. coli* protein lysate (5µl loaded)**.**

4.1.5 SM-CRT identity with CRT of other species

Amino acids sequences of SM-CRT was aligned with CRT of human**,** *N. americanus, E. histolytica, H. contortus* and Echinococcus using the online programme "ClustalW2". It revealed that the highest degree of amino acids identity between SM-CRT and CRT of other species is located in the P- and the S-domain. Both of them have a degree of identity of more than 60%. SM-CRT P-domain has 66 % identity with P-domain of *E. histolytica,* while S-domain shared 68% identity with human CRT S-domain. The SM-CRT N-domain is of around 50% identity with these CRTs. Whereas, SM-CRT C-domain shows the lowest degree of identity with *E. histolytica,* Echinococcus and human C-domain with 23%, 29% and 38% respectively **(Figure 25** and **Table 3).**

```
S.mansoni         -MLSILLTLLLSKYALGH-EVWFSETFPN--ESIENWVQSTYNAEKQGEFKVEAGKSPVD 56
Human             MLLSVPLLLGLLGLAVAEPAVYFKEQFLDGDGWTSRWIESKHKSD-FGKFVLSSGKFYGD 59
N.americanus      -MRSLVALLPVLCIAVAE--VYFKEEFLD-DSWKERWVQSKHKSD-YGEFVLSAGKYFGD 55
H.contortus       ------------------------------GIRWVQSKHKDD-YGAFKLSAGKYYDD 26
E.granulosus      -------------------------------EGNIDKWQKSKYDESKLGLCEHAKPKGDFD 30
E.histolytica     ------MFTLFLLIALSSAKVYFHETFEN----RDKWIDSTSSGKALGPFKIVSGKWYGD 50
                                                  .* .*. . .  *       *   *

S.mansoni         PIEDLGLKTTQDARFYGIARKISEPFSNRGKTMVLQFTVKFDKTVSCGGAYIKLLG-SDI 115
Human             EEKDKGLQTSQDARFYALSASF-EPFSNKGQTLVVQFTVKHEQNIDCGGGYVKLFP-NSL 117
N.americanus      ATRDQGMKTSQDAKFYSRAAKFPKAFSNKGKTVVIQFTVKHEQGIDCGGGYVKVMS-SDV 114
H.contortus       AKRDQGLRTSQDAKFYSLAAKFPKKFTNKGKTVVIQYTVKHEQGIDCGGGYVKVMS-SDV 85
E.granulosus      DREDGGIRTTQDARFYRYSAPFTKPLSSKGKTVCVQFTVKHEQNIDCGGGYVKLLG-ESF 89
E.histolytica     AN-NKGLQTSEDNKFYIAAAKLDEEFSNKDKNLIVQYNLKFEQGIDCGGGYIKLLPKKSI 109
                     : *::*::* :**  :  : : ::.:.:.: :*:.:*.:: :.***.*:*::  ...

S.mansoni         DPKN-FHGESPYKIMFGPDICGMATKKVHVIFNYKGKNHLIKKEIPCKDDLKTHLYTLIV 174
Human             DQTD-MHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIV 176
N.americanus      DLSD-FHGETPYNVMFGPDICGP-TKKVHDIFSYKGKNHLIKKDIRCKDDELTHLYTLIL 172
H.contortus       DLKD-FHGETPYNVMFGPDICGP-TKKVHVIFSYKGKNHLIKKDIRCKDDELTHLYTLIL 143
E.granulosus      KPED-FHGESPYEIMFGPDICGYDKKIVHVIFSYKGKNHLVKKDIPCKSDTLTHLYTLII 148
E.histolytica     ESEEKFTPESEYNIMFGPDVCGG-SKRTHVIMNYKGKNNLIRKEIKCESDDISHLYTLII 168
                  .  : :  :: *::*****:**  .* .* *:.***** *:.*:* *:.*  :******:

S.mansoni         NPNNKYEVLVDNAKVEEGSLEDDWDMLPPKKIDDPNDKKPDDWVDEQFIDDPDDKKPDNW 234
Human             RPDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDW 236
N.americanus      NPDNTYEVQIDGEKVESGELESDWDLLPPKKIKDPDAKKPEDWDEREYIDDADDKKPEDW 232
H.contortus       NPDNTYEVKIDGEKVESGELEADWDMLPPKKIKDPDAKKPEDWDEREYIDDADDKKPEDW 203
E.granulosus      RPDNTFEVLIDNKTSETGSLVADFDMIPSKTIDDPDAEKPEDWVDVAEIPDPDDRKPDDW 208
E.histolytica     RPNNTYVVKIDGVEKQEGKFDEDWDMLAPKEIDDPNVSKPADWVDEKEIDDPNDKKPEGW 228
                  .*:*.: * :*.   : *.:  *:*::..* *.**: .** ** :   * *. * **:.*

S.mansoni         D-QPKTIPDMDAKKPDDWDDAMDGEWERPQKDNPEYKGEWTPRRIDNPKYKGEWKPVQID 293
Human             D-KPEHIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGTWIHPEID 295
N.americanus      D-KPEHIPDPDAKKPDDWDDEMDGEWEPPMIDNPEYKGEWKPKQIKNPAYKGKWIHPEID 291
H.contortus       D-KPEHIPDPDAKKPDDWDDEMDGEWEPPMIDNPEYKGEWKPKQIKNPDYKGKWIHPEID 262
E.granulosus      D-QPKTIVDTNAKQPEDWNEETDGEWTAPIIDNPDYKGEWSPRRIPNPAYKGQWKPPQIP 267
E.histolytica     DDIPKTIVDPNAKKPEEWNDEDDGEWEAPTIENPEYKGEWKPKRIPNPAYKGEWVHPQIA 288
                  *  *: * * :**:*::*::  ****  *  :**:*****.*::* ** *** *   :*

S.mansoni         NPEYKHDPELYVLNDIGYVGFDLWQVDSGSIFDNILITDSPDFAKEEGERLWRKRYDAEV 353
Human             NPEYSPDPSIYAYDNFGVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKAAEK 355
N.americanus      NPEYTPDDELYLYKDWGAIGFDLWQVKSGTIFDNVLVSDSVDEAKAHAAETFEKLKPVEK 351
H.contortus       NPEYTPDDELYLYKDWGAIGFDLWQVKSGTIFDNILVTDSVDDAKAHAAETFEKLKAVEK 322
E.granulosus      NPDYFEDDELYAR-TFAYIGLDLWQVKSGTIFDNFIVSDDVSECQAH-AEYWQKRFTFEE 325
E.histolytica     NPDYVYDPELYKYDSFAYIGIDVWQVKAGTIYDDILITDDIEEAEKEAKVILERN-AAEK 347
                  **:*  * .:*     . :*:*:***.:*:*:*:.::::.   .:             *

S.mansoni         A-KEQSSAKDDKEEAEETKERKELP-----DDAKASDEPSGD----------------HD 391
Human             QMKDKQDEEQRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKD 415
N.americanus      ELKEKADEENRKKMEEEAKKQEEEE--KKKKEKEEKEEKEDEDEEKADE--------GHE 401
H.contortus       EKKDKADEEERKKIEEEAKKREEED--KKKKEAKEKEEKEDEDEDKEEE--------AHD 372
E.granulosus      EQEKKGFEEKEKESSTIESLPEESG-----DEEVDLEEAGDAS--------------PKD 366
E.histolytica     KMRDEIKEAEKQKEEEAKKEAEKQK------EEETKEEIKKEE--------------NKE 387
                    ..:    .  :     .  ::        :    :*                    ::

S.mansoni         EL 393
Human             EL 417
N.americanus      EL 403
H.contortus       EL 374
E.granulosus      EL 368
E.histolytica     EL 389
                  **
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Figure 25: Alignment of amino acid sequences among various CRTs using the online programme"ClustalW2" (http://www.ebi.ac.uk/Tools/clustalw2/index.htmlMuscle). *S. mansoni* CRT (accession N.Q06814)**,** human CRT (accession N.P27797)**,** *N. americanus* (accession O76961), *H. contortus* (accession Q6R5P2), *E. histolytica* (accession N. C4M296) and Echinococcus (accession N.Q56JA0) (Asterisks indicated identities across all six CRTs, colons indicates one or more conservative substitutions and points denote non-conservative changes).

	S. mansoni				
	Full length	N-domain	P-domain	C-domain	S-domain
Human	49	53	62	38	68
Echinococcus	51	54	64	29	63
E. histolytica	47	48	66	23	61

Table 3: The amino acids identity (%) of SM-CRT N-, P-, C- and S-domains with other CRTs species.

4.2 Discussion

The regulation of *S. mansoni* calcium may be vital for many central functions at different developmental stages of the parasite such as muscle contraction and cercariae skin penetration (Davies 1983). Many calcium binding proteins were described in different stages of *Schistosoma,* for example Sm20 protein which is found in *Schistosoma* muscle layer (Havercroft *et al*. 1991), a calmodulin–like protein was recognized within adult parasite tegument (Siddiqui *et al*. 1991), in addition to other proteins ranging from 15-205 kDa in size (Fuhrman 1990).

The calcium binding ability of different recombinant SM-CRT sub-fragments was evaluated by two methods; staining SM-CRT sub-fragments loaded on SDS-PAGE gels with "Stains-all" stain and testing the binding of the recombinant proteins separated on acrylamide gel to radioactive calcium.

Most SM-CRT sub-fragments have the ability to bind to calcium as they stained blue with "Stains-all" stain, while the N-domain band stained pink indicating that this domain does not bind calcium. This point to the conclusion that the SM-CRT P- and C-domain are the domains responsible for calcium binding (**Figure 18**). These results were in agreement with those of Baksh& Michalak (1991) who tested calcium binding ability of different human CRT forms; native, recombinant GST fusion, non-fusion CRT or expressed C-, P-, NP- and PC-domains. The authors found that all these segments gave blue colour bands when stained with the stain, while the N-domain stained pink. Also, *H. contortus* CRT proved to bind calcium using this stain (Suchitra& Joshi 2005).

"Stains-all" stain was used under reducing conditions and the dye interacted with the protein after being run and denatured with SDS. It is an easy, simple technique which requires only few reagents.

To confirm that SM-CRT sub-fragments bind to calcium, they were incubated with $^{45}Ca^{2+}$. Radioactive calcium was diluted in buffer of pH 6.8, which is near the physiological condition (Maruyama *et al.*1984) and avoids low pH that would decrease the negativity of the protein fragments, leading to less calcium binding as it is well known that calcium ion is a positively charged molecule and calreticulin especially its C-terminal part is acidic negatively charged molecule.

Most SM-CRT sub-fragments interacted with $^{45}Ca^{2+}$ and showed signals corresponding to their band, but N-, NS- and NP-domains did not show such signals (see **Figure 19**). Similar results were observed by other researchers who tested CRT-calcium binding of other species. Calreticulin from many parasitic species bound $^{45}Ca^{2+}$ such as *S. mansoni* in a previous report (Khalife *et al*. 1994), *S. japonicum* (Huggins *et al*. 1995, Scott& McManus 1999) and *L. donovani* (Joshi *et al*. 1996). Recombinant full length *H. contortus* CRT and its P- and C-domains interact with calcium, but its N-domain does not bind to the calcium (Suchitra& Joshi 2005). Recombinant *N. americanus* CRT failed to bind calcium *in vitro* using band shift electrophoresis and this may be due to an improper folding of the protein (Kasper *et al.* 2001). Different forms of human CRT bind to $^{45}Ca^{2+}$ (Baksh& Michalak 1991), but in addition to my results where SM-CRT NP-domains did not show any $^{45}Ca^{2+}$ binding in a Western blot analysis, these authors found that human NP-domain bind calcium.

The SM-CRT C-domain is rich in aspartic acid (D) and glutamic acid (E) residues especially at the last 50 residues (it contains 18 aspartic acid or glutamic acid amino acids). This part is also remarkably similar to the human counterpart (Bakash& Michalak 1991). This might account for the high capacity of this region to bind calcium.

The failure of SM-CRT NS- and NP-domains to bind to $^{45}Ca^{2+}$, may be caused by that the P-domain (which binds to calcium in high capacity and low affinity) being masked by the globular N-domain during expression.

The radioactive overlay technique has many advantages: the nitrocellulose membrane is easily handled, the reagents and chemicals (except the radioactive calcium) are relatively cheap and stable, the time required for incubation with the radioactive calcium is relatively short and it is an efficient procedure to test specific protein binding to calcium.

The successful demonstration of calcium binding to SM-CRT confirms that the recombinant protein retains its function after being produced in bacterial expression system. The proven calcium binding activity of SM-CRT might suggest that native *S. mansoni* CRT can bind tcalcium in the micro-environment around the parasite in blood and consume calcium which is required for blood coagulation, hence CRT may help prevent blood coagulation around the parasite.

Schistosoma secretes and/ or excretes some proteins which were shown to interact and inactivate some complement components in order to protect the parasite from lethal complement activities. CRIT acts as a receptor for C2 and hinders production of the C3 convertase, C4b2a (Inal 1999, Inal& Schifferli 2002, Inal& Sim 2000), CAA is as a

soluble receptor for C1q (Van Dam *et al.* 1993) and *Schistosoma* paramyosin binds C1q (Laclette *et al.*1992), C8 and C9 (Deng *et al*. 2003& 2007).

This is the first report addressing the interaction of *S. mansoni* calreticulin with the complement system. I have shown that most SM-CRT sub-fragments bound to C1q using physiological PBS, while the P-domain did not. The binding was dependent on the protein concentrations and occurred under physiological conditions. My experiments show a direct binding of SM-CRT (except P-domain) to C1q and an inhibition of C1q-dependent haemolysis. The proteins were added to two plates, as each plate could only accommodate six samples. The C1q binding affinity of different SM-CRT sub-fragments were compared to each other (in the presence of BSA) using C1q concentration of 0.4 µg/well as this concentration was expected to give the highest signal of binding allowing easy comparison.

Within the protein group comprising N-, P-, C-, S- and NPC-domains, the NPC-domain bound to C1q slightly stronger than the S-domain, followed by both N- and C-domains, which were nearly equal in binding affinity, but the P-domain did not show any binding signal even with this high C1q concentration (**Figure 20 A***).

In the other group (see **Figure 20 B***), NP- and SPC-domains bound with similar affinity to C1q, followed by NS- and PC-domains, whereas the SP-domain bound C1q with the lowest affinity, despite the fact that it contains the whole S-domain. There are two explanations for this observation. Firstly, SP-domain contains only one confirmed SM-CRT C1q binding motif in its N-terminal part which is KEIPCKDD (this will be discussed in detail below).

Secondly, the P-domain, which is linear in shape, may mask the S-domain during expression not allowing the S-domain to expose its C1q binding site and interact with C1q.

To evaluate if this binding would affect the biological function of C1q, a classical pathway dependent haemolysis assay was performed using NHS (normal human serum) and C1q deficient serum reconstituted with purified human C1q to re-gain haemolytic activity of about 70-75%. As expected, all SM-CRT domains, except the P-domain, decreased the reactive percentage of haemolysis with some degree of variation. In one protein group (N-, P-, C-, S-, NPC-domains and BSA which was tested using NHS), 10µM from each NPC-, N- and S-domains decreased haemolysis from 75-80% to under 10% when using NHS diluted at 1:160. The C-domain required a final concentration of 20µM to reduce haemolysis to 10 %, while the P-domain did not have any effect on haemolysis (see **Figure 21A**). On the other plate (NS-, NP-, SP-, SPC-, PC- domains and BSA using NHS), 10 µM of NS- and SPC-domains decreased the haemolysis from 75-80% to less than 10% whereas a final concentration of 20 µM of domains NP, SP and PC was required to reduce haemolysis to approximately 10% (**Figure 21B**).

When using the reconstituted human C1q deficient serum, similar effects were seen, but larger quantities of recombinant SM-CRT sub-fragments were required (**Figure 21 A* & B***). This is probably due to relatively higher amounts of calreticulin required to bind to the excess free C1q molecules added to reconstitute the C1q deficient serum. Domains N, NPC and NS needed to be added at a final concentration of 20 µM to drop down the haemolysis from 70-75% to less than 10%. Likewise, S-, C-, SPC-, PC- and NP-domains required to be added at a final concentration of 20 µM to lower the haemolysis to less than only 20%. The

SP-domain was the least potent inhibitor of C1q-dependent haemolysis inhibition. The addition of a final concentration of 20 µM of the SP-domain only decreased haemolysis to approximately 30 %.

It was noted that N- and S-domains are the individual domains which achieved the strongest binding and haemolytic inhibition of haemolysis followed by the C-domain. Inhibition of classical pathway dependent haemolysis in NHS has shown that SM-CRT can bind to C1q in the C1 complex and this event is likely to happen *in vivo* during *S. mansoni* infection.

Most of the previous studies with CRT were done using human CRT, while only few reports analysed the effects of parasite CRT. The SM-CRT P-domain did not bind to C1q which is in agreement with Kishore *et al.* (1997) who found that under physiological salt concentration, C1q bound to human CRT N-domain, while human CRT P- and C-domains did not bind C1q. Contrasting with my results, the human CRT C-domain did not bind to C1q, whereas P-domain did (Stuart *et al.* 1996). Subsequently, when the same group repeated the experiment and included the S-domain, the human CRT S-domain achieved a higher percentage of relative binding activity than the human CRT P-domain, while the least binding affinity was seen with the N-domain. Again, the C-domain did not bind C1q (Stuart *et al*. 1997).

Native and recombinant TcCRT and its S-domain and R-domain (which is the S-domain plus 20 a.a of the N-domain) were all found to bind to C1q *in vitro* under a physiological salt concentration and this interaction was found to be a specific, saturable and dose

dependent (Ferreira *et al.,* 2004). In another study, its S-domain bound to C1q in an ELISA assay in a concentration dependent manner using physiological PBS (Aguilar *et al.* 2005).

The *H. contortus* CRT, immobilized to CNBr-activated Sepharose, showed binding affinity to C1q. When the authors tested the ability of the N-, P-, C-domains and the full length protein to inhibit C1q-dependent haemolysis of sensitized SRBCs, *H. contortus* CRT N-domain inhibited the destruction of sensitized sheep RBCs in a dose dependent manner, but the P-domain had no inhibitory effect despite having two putative C1q binding sites. These results are in agreement with my results, however, the SM-CRT C-domain binds to C1q and inhibits haemolysis, whereas, *H. contortus* CRT C-domain did not inhibit haemolysis (Suchitra& Joshi 2005).

Recombinant *N. americanus* CRT does not bind calcium, but it bound to C1q immobilized on microplates under physiological conditions in a dose dependent manner. The same binding occurred when *N. americanus* CRT was coated on a plate. Likewise, *N. americanus* CRT inhibits C1q dependent haemolysis (Kasper *et al.* 2001). This is in full agreement with what I observed using the SM-CRT NPC-domain.

To explain why SM-CRT P-domain did not bind to C1q, while the SM-CRT C-domain not only bound C1q, but also it inhibited its activity in the haemolytic assay, *S. mansoni* CRT C1q binding sites sequences were compared with that of other parasite CRT sequences. Human CRT N-domain has four C1q binding sites of which, IESKHKSDF which has weak C1q binding activity, DEEKDKG, KDIRCKDD which has the most potent C1q binding activity and FNYKGKN shows only partial binding to C1q and does not affect

haemolysis. The human CRT P-domain has only two C1q binding sites which are WDERAKIDD and GEWKPRQ (Kovacs *et al.*1998). *N. amercanius* CRT has three C1q binding sites which are KDIRCKDD, FSYKGKN and VQSKHKSDY in its N-domain, while the P-domain contains WDEREYIDD and GEWKPRQ (Kasper *et al*. 2001). *H. contortus* N-domain has KDIRCKDD, FSYKGKN and VQSKHKDDY, while its P-domain contains WDEREYIDD and GEWKPKQ (Suchitra& Joshi 2005). Naresha *et al*. (2009) reported further two unique binding sites in the *H. contortus* N-domain.

S. mansoni SM-CRT protein sequencing was compared with that of human CRT to identify the potential C1q binding sites in the *S. mansoni* protein. It was found that SM-CRT N-domain contains 4 potential C1q binding sites which correspond to the human C1q binding sites and are as follows: KEIPCKDD (75% identity with its human counterpart), FNYKGKN (100% identity with its human counterpart), VQSTYNAEK (11% identity with its human counterpart) and DPIEDLG (42% identity with its human counterpart)**.** Its P-domain has two motifs WVDEQFIDD (44.4% identity with its human counterpart) and GEWTPRR (71% identity with its human counterpart) (the underlining a.a shows identity to the human counterpart).

SM-CRT P-domain binding sites did not bind or affect C1q dependent haemolytic activity and this is generally in agreement with Suchitra& Joshi (2005) who found that *H. contortus* P-domain did not affect the biological activity of C1q in a C1q dependant haemolytic assay. This could be a consequence of minor amino acids substitutions that inactivate these motifs.

On the other hand, SM-CRT C-domain bound and inhibited the classical pathway dependent haemolysis in contrast to most other reports. This may be due to the involvement of C1q binding sites. When trying to identify similar motifs, a motif RKELPDD was identified showing a degree of similarity to KEIPCKDD which is found in the SM-CRT N-domain. This suspected SM-CRT C-domain new motif is in the same location as the KEEEEAE motif in the human sequence. This corresponding human sequence motif was shown to be without C1q binding activity. The human CRT motif KDIRCKDD was shown to be the most potent motif inhibiting of C1q dependent haemolysis (Kovacs *et al*., 1998) and my SM-CRT C1q binding motif RKELPDD could be a repeated sequence located in a new site within SM-CRT C-domain causing this domain to bind C1q. In addition to this newly identified sequence motif, there might be other unidentified binding sites in SM-CRT C-domain causing binding to C1q.

For parasites, including *Schistosoma*, CRT binding to C1q may allow the parasites to escape from host complement attack. For TcCRT which breaks down the innate immune protection of C1q, at least in the microenvironment around the extracellular parasite stages (Ferreira *et al.* 2004), and in the case of *Trypanosoma*, this binding might be a factor that facilitates the process of parasite invasion into the host cells (Ogden *et al.* 200, Rimoldi *et al.* 1989). I hypothesise that SM-CRT may protect the parasite from complement activation via the classical pathway in its surrounding environment and in the parasitic gut by binding C1q and inactivation of the classical pathway of complement away from the parasite. This is similar to what has been reported for the *S. mansoni* CAA antigen which is found to be excreted to the serum and is suggested to bind to C1q in the lumen of its gut away from its vulnerable epithelia (van Dam *et al*. 1993). Also, *Schistosoma* is a blood feeding parasite

and it could swallow C1q while taking its blood meal and parasite CRT could interact with the ingested C1q protecting its gut from classical pathway activation as previously suggested in for *H. Contortus* CRT (Naresha *et al.* 2009). *Schistosoma* could benefit from the presence of multiple C1q binding sites where this could allow the protein to interact with C1q directly at multipoint positions hence inactivate the classical pathway completely as suggested for *H. contortus* CRT (Naresha *et al.* 2009).

The rabbit anti-SMCRT anti-serum, which was prepared by immunisation with the recombinant SM-CRT NPC-domain, reacted not only with SM-CRT NPC-domains, but also different fragments thereof in both Western blot analysis and ELISA assays. This confirms the immunogenicity of my recombinantly expressed SM-CRT and this is in agreement with others who reported that CRT is highly immunogenic in different species such as rabbit (Aguilar *et al.* 2005) and human (Marcelain *et al.* 2000, Aguillon *et al.* 1997).

Using rabbit anti-SM-CRT anti-serum in Western blots (see **Figure 22**), specific reactive protein bands were detected. The faint bands which appear above and below SM-CRT NP-, NS-, NPC-, SPC-, PC-domains might be due to the formation of proteins dimers, they could also be degradation products or these faint bands are proteins derived from the host BL21 *E. coli*. In ELISA and at 1:10000 titre (**Figure 23**), the reactivity with the anti-serum was still detectable and it gave strong signals with the C-, PC- and SPC-domains indicating that these domains may be more immunogenic than SP- and N-domains.

Native *S. mansoni* CRT was identified as one of the surface antigens of schistosomulum and the adult parasite preparations containing the membrane antigens using anti-SM-CRT specific rabbit anti-serum. This suggests that CRT is one of the excretory/secretory products which may perform various functions for the parasite. SM-CRT was detected in the excretory/secretory products of *S. mansoni* primary sporocysts (Guillou *et al.* 2007). *S. mansoni* CRT was found in the tegument (Khalife *et al.* 1994, van Balkom *et al.* 2005). It has also been detected using a proteomic analysis inside cisternae of tegument cell bodies organelles and it was suggested that it reaches the tegument through secretory vesicles (Braschi *et al.* 2006). Parasite CRT was detected in the excretory/secretory products of *H. contortus* (Suchitra& Joshi 2005), it is proved to be found on the surface of *E. histolytica* and could have role in the pathogenesis of amoebic liver abscess (Girard-Misguih *et al.* 2008) and *N. americanus* CRT was detected in parasite homogenates (Kasper *et al.* 2001).

Chapter 5

Use of recombinant SM-CRT sub-fragments and CTF in the sero-diagnosis of *S. mansoni*

5. 1Results

5.1.1 Murine antibody response against SM-CRT, CTF and SEA

5.1.1.1 Mouse groups

Five groups of CD1 mice were used in this study: Non-infected control mice and four mouse groups infected with *S. mansoni* (each mouse was subjected to 200 *S. mansoni* cercariae) for periods of either 12 days, 35 days, 46 days or 57-59 days (**Figure 26**). The members of each mouse group were culled after the indicated times post-infection. The blood was collected by cardiac puncture and the worms were recovered. From the blood, the sera was prepared , aliquoted and stored at -80 °C until sent to me at the University of Leicester.

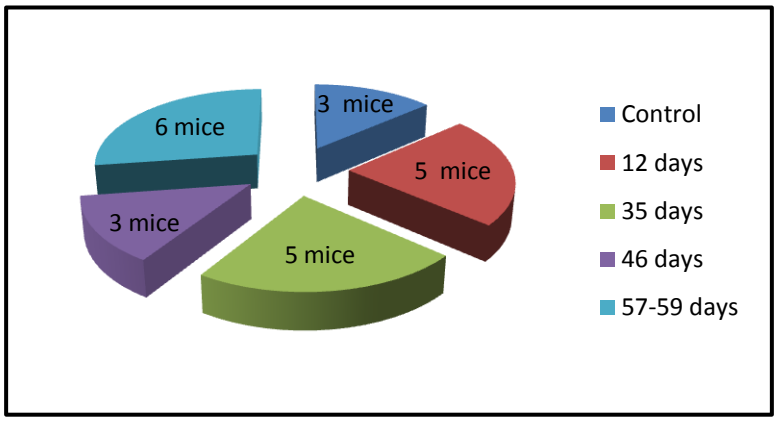


Figure 26: Mouse groups used to test the efficacy of different antigens in sero-diagnosis of *S. mansoni.*

All the infected mice showed after 35 days post-infection, that the *S. mansoni* life cycle was fully established. There were tiny adult *S. mansoni* worms in the 35 day p.i. mouse group. In mice 46 and 57-59 days p.i., the paired worms were easily counted and varied in their numbers amongst individual mice. Mice 46 and 57-59 days p.i. suffered from severe hepatic pathology. Complete pathology results are shown below in **table 4**.

Mice group	**Mouse Numbers**	**Mouse code**	**Adults *S. mansoni* worms numbers**	**Liver pathology**
First group:	3	Mouse.1		
Control group		Mouse. 2		
		Mouse. 3		
Second group	5	Mouse. 4		
12 days p.i.		Mouse. 5		
		Mouse. 7		
		Mouse. 8		
		Mouse. 9		
Third group	5	Mouse .10	in all members of	
35 days p.i.		Mouse.11	this group , there	
		Mouse .12	were very small	
		Mouse .13	paired worms	
		Mouse .14		
Fourth group		Mouse. 15	90 pairs	major liver pathology
46 days p.i.	3	Mouse. 16	40 pairs	was detected in all
		Mouse. 17	80 pairs	group members
Fifth group		Mouse. 18	few worms	major liver pathology
57-59 days p.i.	6	Mouse. 19	38 pairs	was detected in all
		Mouse. 20	108 pairs	group members
		Mouse. 21	25 pairs	
		Mouse. 22	37 pairs	
		Mouse. 23	74 pairs	

Table 4: Mouse groups used in *S. mansoni* sero-diagnosis against different antigens. The number of each mice group, numbers of adult *Schistosoma* worm pairs for each mouse and the liver condition are mentioned.

5.1.1.2 Detection of specific antibodies against *S. mansoni* antigens used in mouse sera using ELISA

Because CRT is a highly conserved protein, there is a significant risk to that SM-CRT will be identified by antibodies directed against CRT from other parasites or by host auto-antibodies. To recognize the most specific fragment, SM-CRT domains were expressed individually or in combination.

Each ELISA plate was coated with antigen. The standard positive reference serum was prepared by pooling equal volumes of sera from the 57-59 day mouse group. One plate was tested for antigen binding using the serially diluted standard positive serum starting with a 1:40 dilution in duplicates in two neighbouring columns. The individual mouse sera were incubated with the antigen at a dilution of 1:200 in duplicates. The ELISA plates were read until the OD_{405} of the 1:40 standard dilution reached 1-1.5 units. For each antigen, the specific antibody response was evaluated by comparing O.D. readings of each mouse group separately with the O.D. readings of the non-infected mouse control group and the statistical significant difference was assessed using Student's unpaired *t*-test.

SM-CRT P-($P<0.001$), C- ($P<0.05$), PC- ($P<0.05$ at sera taken 46 days after infection, then $P<0.001$ with sera taken 57-59 days after infection) and SPC-($P<0.05$) domains were significantly more reactive than other SM-CRT sub-fragments and their specific antibodies started to appear in the sera 46 days post-infection. Likewise, SM-CRT N-($P<0.01$), NS-($P<0.001$), NP-($P<0.05$), S-($P<0.001$), SP-($P<0.01$) and NPC-($P<0.01$) domains were identified later by the sera of mice 57-59 days after infection. On the other hand, the CTF and SEA antigens gave a positive reading in sera as early as 12 days after infection

(*P*<0.05) and the level of significance increased in mouse sera taken at later time points after infection (*P* became <0.001) (**Figure 27**).

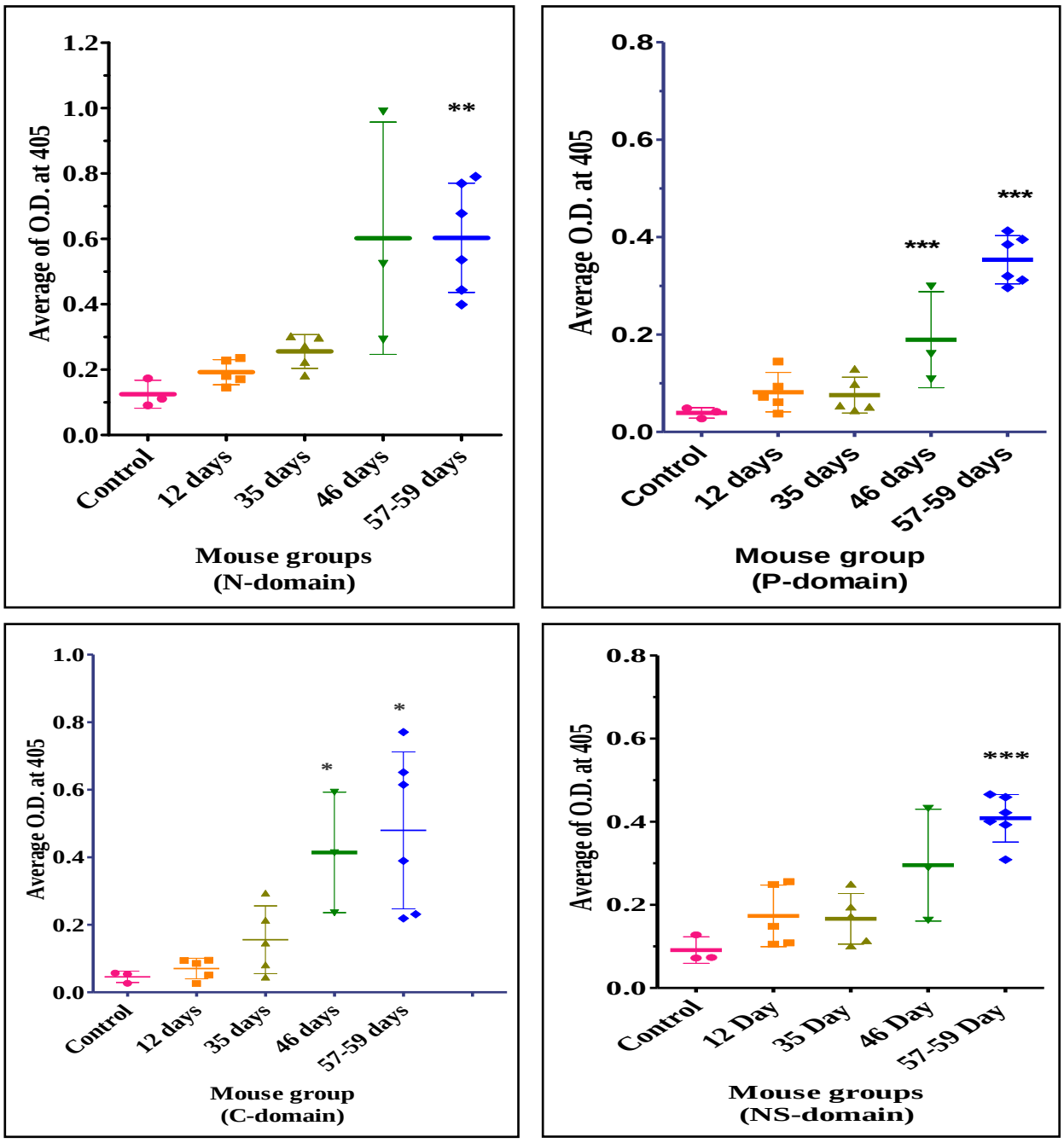


Figure 27: **Specific antibody responses against different antigens in mice infected with *S. mansoni*.** Each point represents the mean O.D. values of duplicates taken from serum dilutions of one mouse. An unpaired Student's *t*-test was used to compare the significance of antibody response at different post-infection times in relation to the control group. The results are presented as mean ±SD. The probability values were *$P < 0.05$, **$P < 0.01$ and *** $P < 0.001$. A representative experiment for each antigen is shown.

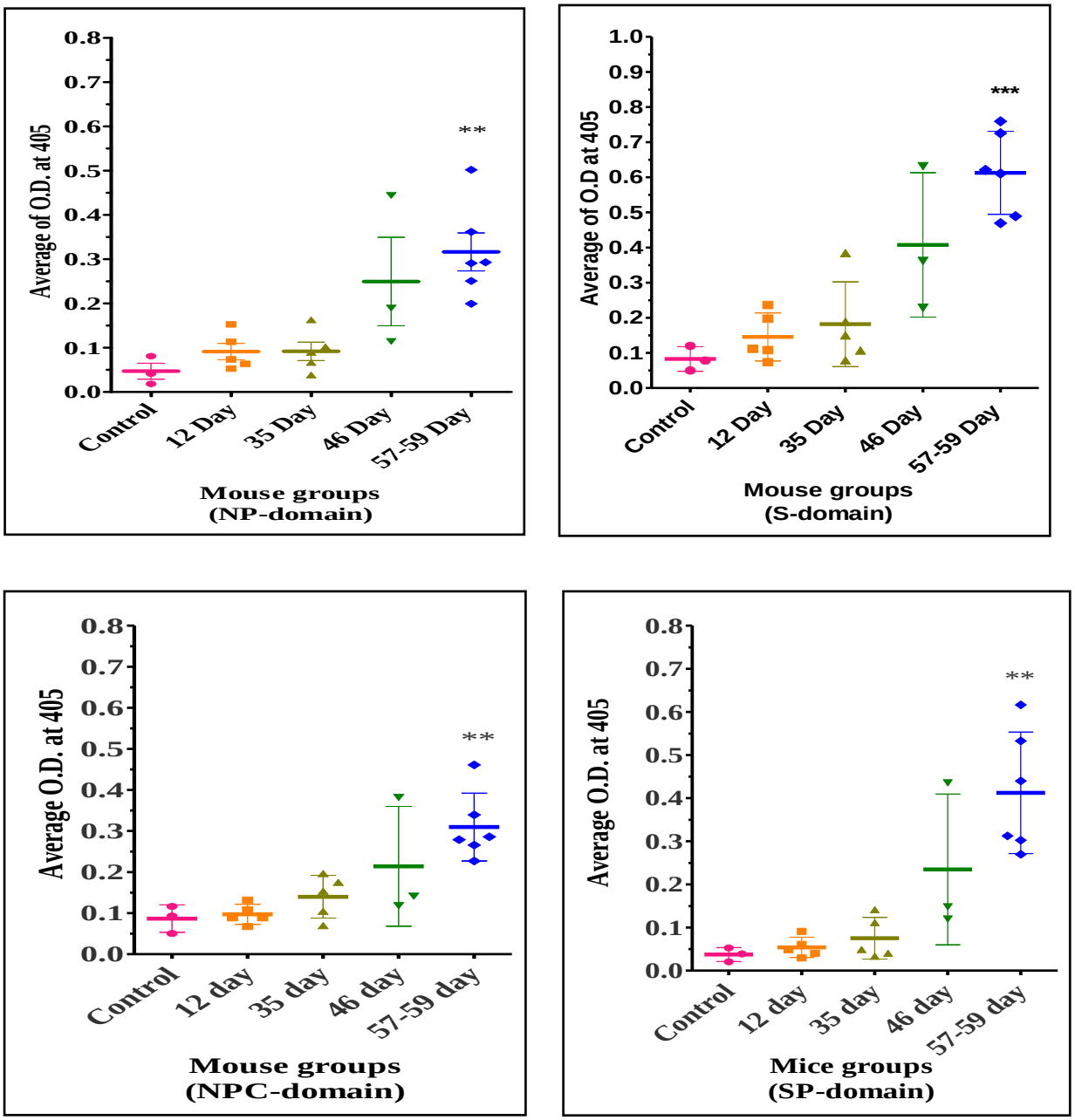


Figure 27: continued……

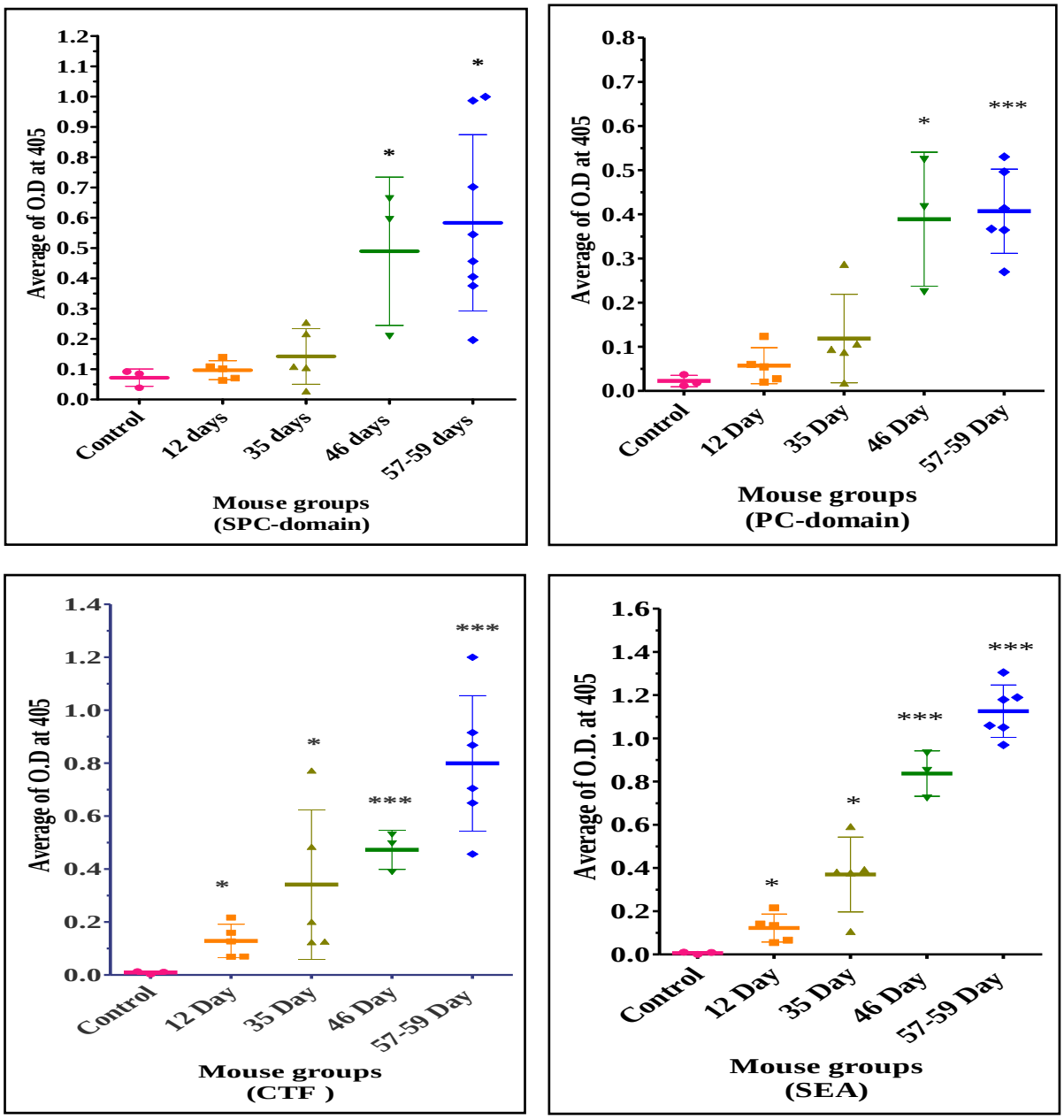


Figure 27: continued……

5.1.2 Sero-diagnosis of Schistosomiasis in humans

5.1.2.1 Human samples

My work then progressed to evaluate these antigens as diagnostic markers of infection in human sera in an attempt to generate a simple, easy and reliable serological test. The human samples included four groups: (i) 97 sera from *S. mansoni* infected patients that were positive for the parasite eggs (kindly provided by Dr. Abeer Elhadidi, University of Alexandria, Egypt), (ii) 21 sera from healthy U.K. volunteers, none of them had visited endemic *Schistosoma* endemic areas (iii) 53 human sera from patients suffering from various diseases in absence of *S. mansoni* infection collected from different localities and (iv) 19 sera collected from healthy Egyptian volunteers in which *S. mansoni* infection was excluded by microscopic examination (**Figure 28).**

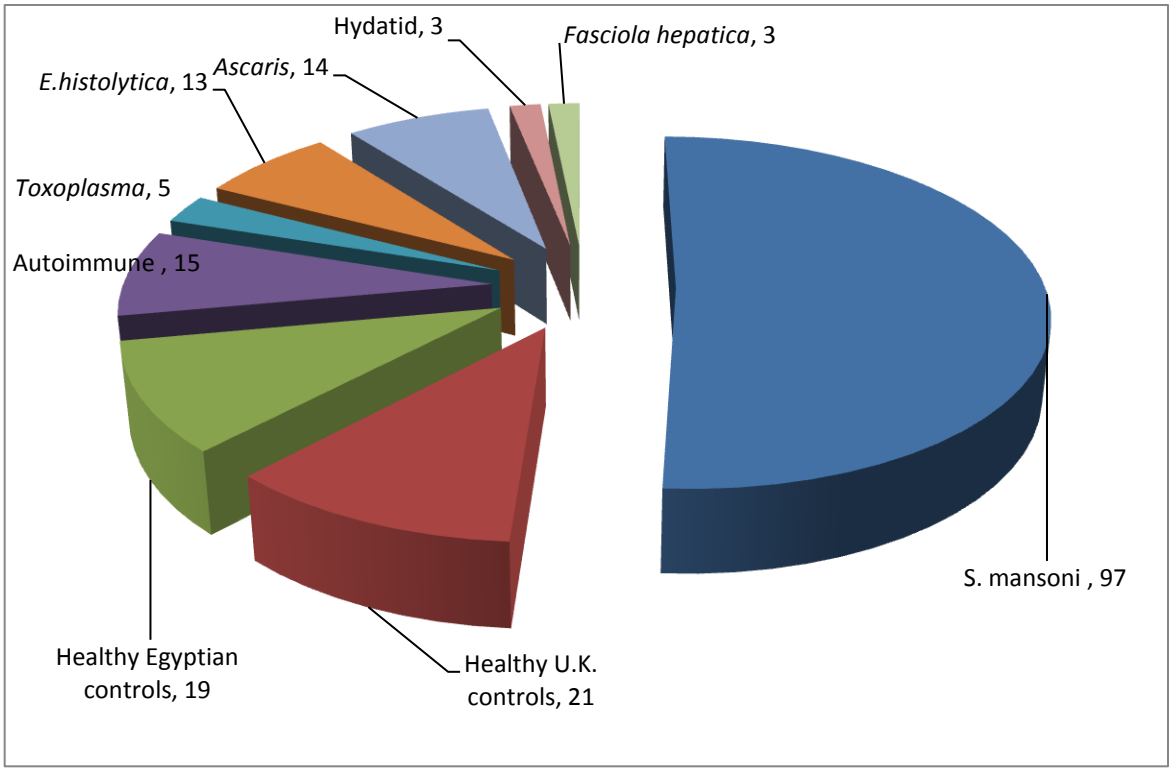


Figure 28: **Human samples used in the study.**

Series titration ELISA experiments were carried out to optimize the antigens concentrations and the individual human serum dilutions. The following antigens concentration was used: 5µg/ml in case of SM-CRT fragments, and 1µg/ml for SEA and 0.4 µg/ml for the CTF antigen. The standard reference positive control sera were prepared by mixing equal volumes of 15 *S. mansoni* +Ve sera which gave the highest responses to the SEA antigen. The first two columns (1& 2) of each coated plate were incubated with serial dilutions of the prepared standard reference positive sera (1:20-1:1280) in duplicates to generate a standard curve. The last two wells of the first two columns (H1& H2) received all the reagents except sera as negative controls. Each plate also contained quality control sera: 5 *S. mansoni* -Ve sera from the healthy U.K. donors as a negative control and 5 *S. mansoni* +Ve as a positive control. The quality control sera were incubated in the same locations on all plates. The rest of the plate received 30 individual test sera. The reading was taken when the O.D. of the standard reference serum at 1: 20 dilution reached 1-1.5 units. To correct for variations within the ELISA plates, the O.D. was converted to relative antibody titre. The standard serum was assigned an arbitrary antibody titre of 1.0 and the titre of the samples calculated from the equation of the standard curve.

5.1.2.2 The cut-off values of the antigens

The cut-off for each antigen was equal to mean + 2SD of the relative antibody titres of the sera collected from the healthy U.K. donors (21 sera). SM-CRT sub-fragments have relatively high cut-off values. The SM-CRT N-domain cut-off was the highest with 0.817, while the cut-off for the SM-CRT P-domain was the lowest amongst SM-CRT with 0.399. CTF antigen cut-off was 0.119, which was higher than that of the SEA (0.091) (see **Table 5**).

Antigen	N	P	C	NS	NP	NPC	S	SP	SPC	PC	CTF	SEA
Cut off	0.817	0.399	0.565	0.595	0.654	0.651	0.815	0.542	0.736	0.577	0.119	0.091

Table 5: Cut-off values, calculated in relative antibody titre, of the different used antigens.

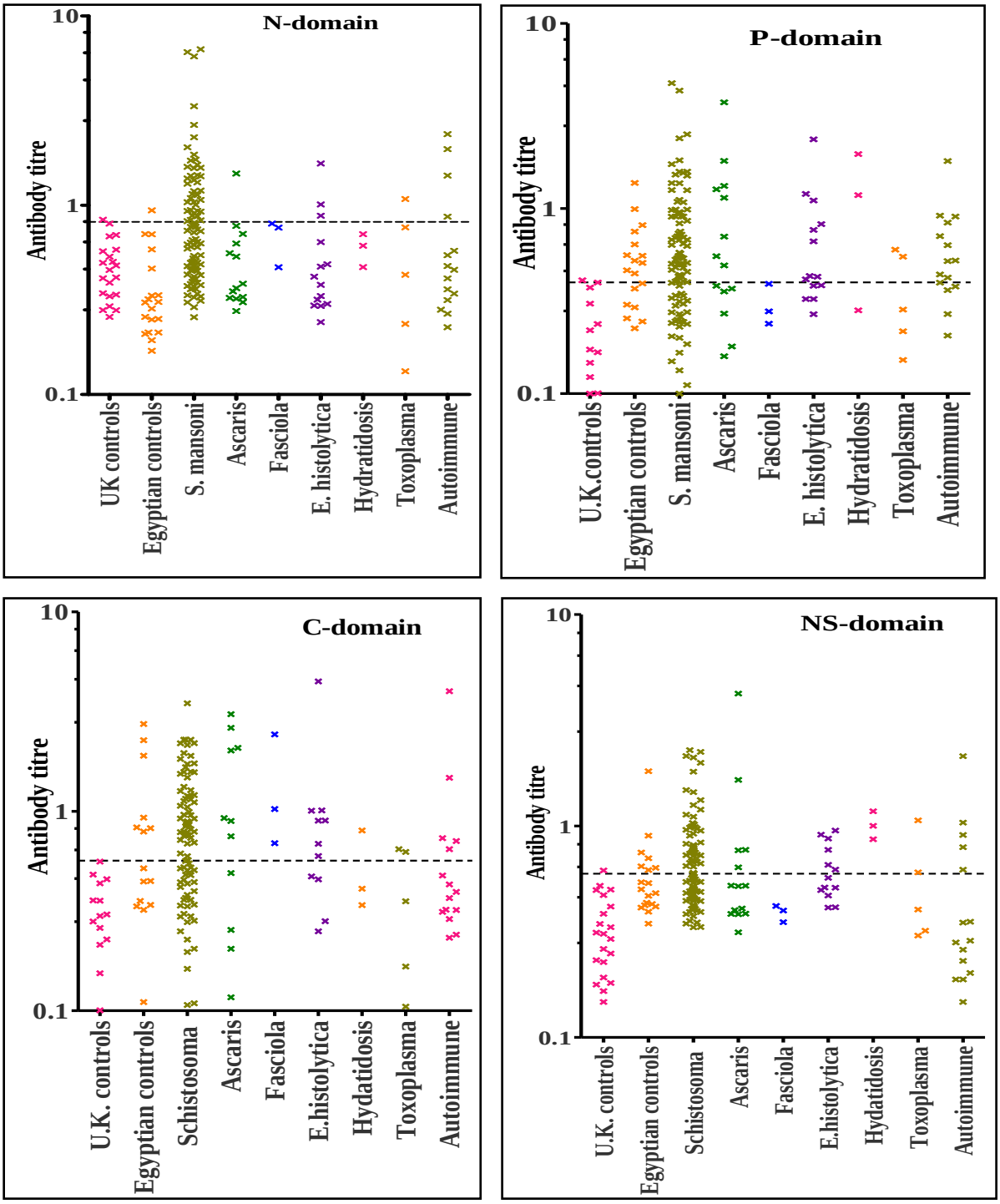
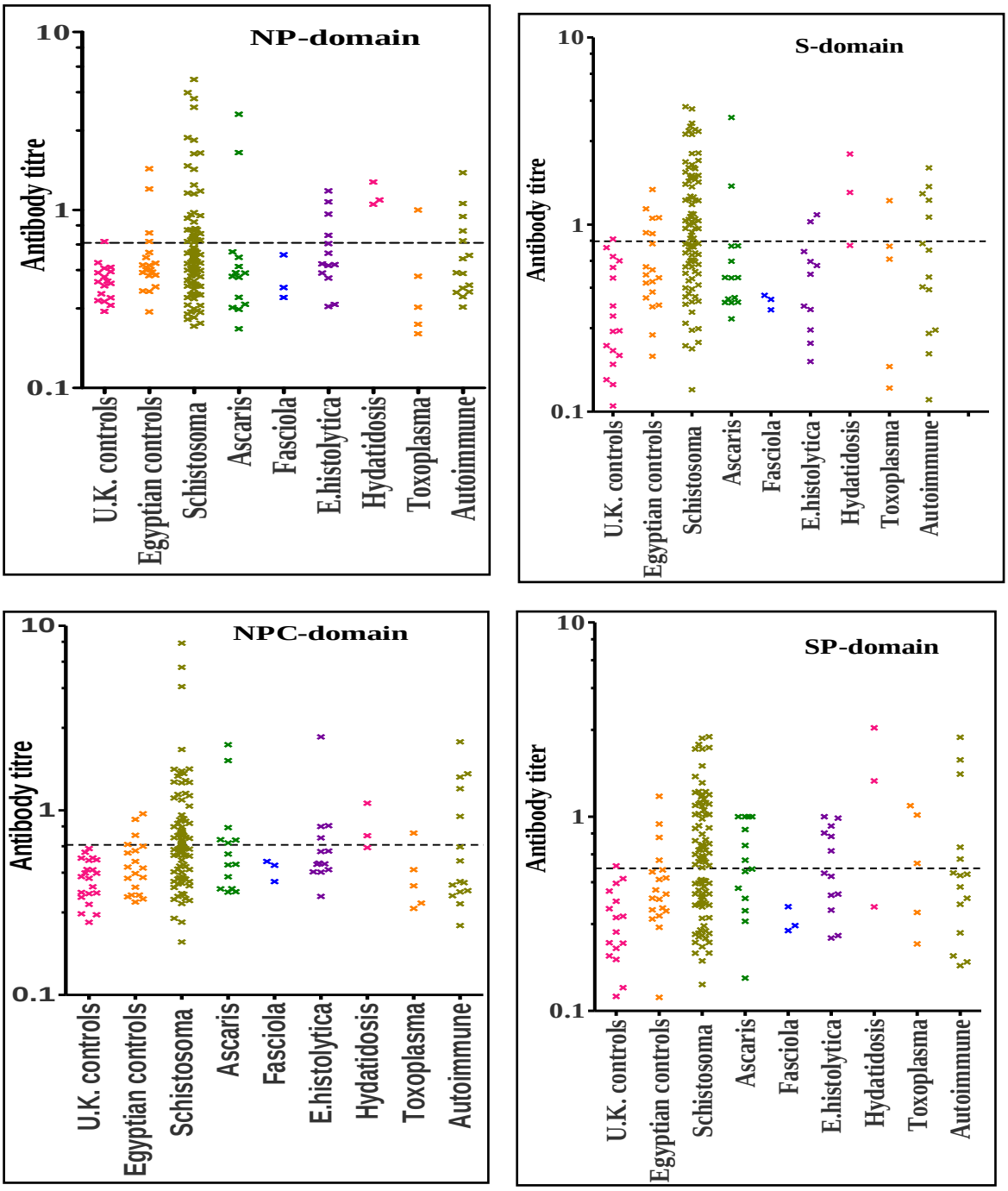


Figure 29: Sero-diagnosis of *S. mansoni* infection in Egyptian patients and controls. Antibody titres were determined using 12 different antibody capture ELISAs, as indicated. Horizontal lines indicate the lower cut-off limits for positive calls (mean ±SD of healthy U.K. controls)



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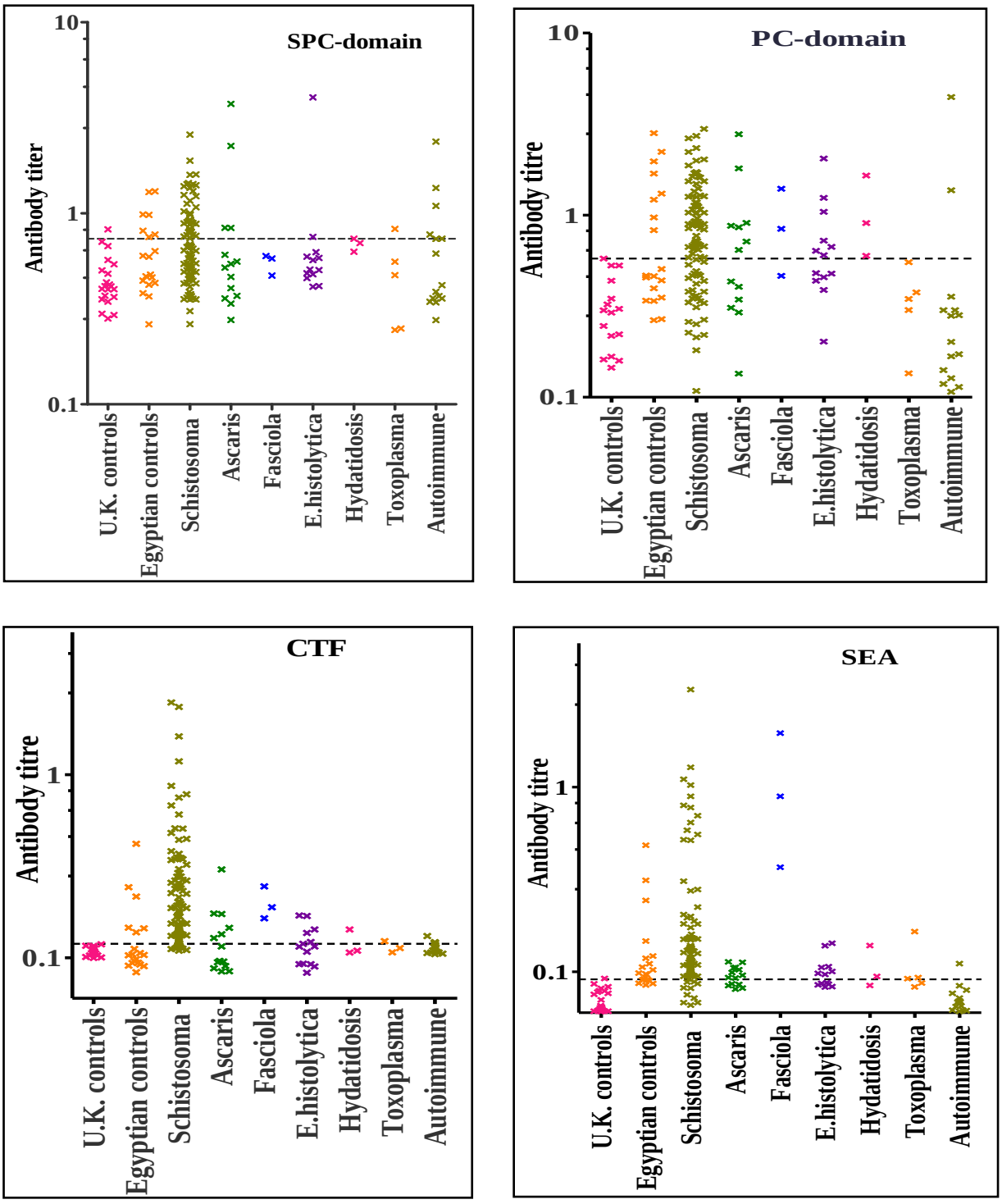

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5.1.2.3 Sensitivity of SM-CRT sub-fragments, CTF and SEA

The sensitivity (%) = $\frac{\textbf{100 X True positives}}{\textbf{True positives + False negatives}}$

True positive serum is a serum collected from an *S. mansoni* infected patient and this serum showed relative antibody titres **above** the cut off of an antigen, while **false negative serum** is a serum collected from an *S. mansoni* infected patient (who has *S. mansoni* eggs proven microscopically) and that serum showed relative antibody titres **below** the cut off of this antigen.

When using the stool examination as the gold standard test for *S. mansoni* diagnosis, CTF ELISA antigen and SEA ELISA gave positive reactions with 87 sera out of 97 sera collected from *S. mansoni* infected patients who showed to have parasite eggs in their stool by microscopic examination. Both antigens showed the highest degree of sensitivity (both achieved 89.7% sensitivity). SM-CRT PC-domain achieved the highest degree of sensitivity amongst SM-CRT sub-fragments with 71.1%, followed by the P-domain (66 % sensitivity) and C-domain (60.8% sensitivity). While, SM-CRT NP-domain showed the lowest degree of sensitivity achieving 35.1% where it identified 34 sera from these 97 sera (**Table 7**).

In comparison to SEA sensitivity (which was used as serological gold standard antigen), CTF showed the best sensitivity amongst all the used antigens, where it identified 81 sera from 87 sera which showed a positive reaction in the SEA ELISA revealing 93.1%. Each antigen (SEA or CTF) recognized 6 *S. mansoni* infected patient sera which were not

identified by the other, while both SEA ELISA and CTF ELISA gave false negative reactions with 4 *S. mansoni* infected patient sera **(Table 6 and Figure 30).**

When comparing to SEA ELISA sensitivity, SM-CRT PC-domain achieved the highest degree of sensitivity amongst all of the SM-CRT sub-fragments followed by the P- then the C-domains showing 72.4%, 69% and 61% sensitivity respectively. The SM-CRT NP-domain recognized the least number of *S. mansoni* infected sera which gave positive reactions with SEA ELISA. It reacted with only 33 sera out of the cohort of 87 sera identified with SEA ELISA with a sensitivity of 37.9%. The results are summarized in **Table 6& Figure 30.**

	SEA +Ve	SEA -Ve	Total
N-domain +Ve	**37(43%)**	3	**40**
N-domain -Ve	50	7	57
Total	87	10	97
P-domain +Ve	**60 (69%)**	4	**64**
P-domain -Ve	27	6	33
Total	87	10	97
C-domain +Ve	**53 (61%)**	6	**59**
C-domain -Ve	34	4	38
Total	87	10	97
NS-domain +Ve	**46 (52.8%)**	6	**52**
NS-domain –Ve	41	4	45
Total	87	10	97
NP-domain +Ve	**33 (37.9%)**	1	**34**
NP-domain -Ve	54	9	63
Total	87	10	97
NPC-domain +Ve	**44 (50.5%)**	5	**49**
NPC-domain -Ve	43	5	48
Total	87	10	97
S-domain +Ve	**44 (50.5%)**	7	**51**
S-domain –Ve	43	3	46
Total	87	10	97
SP-domain +Ve	**49 (56.3%)**	5	**54**
SP-domain -Ve	38	5	43
Total	87	10	97
SPC-domain +Ve	**37(42.5%)**	4	**41**
SPC-domain –Ve	50	6	56
Total	87	10	97
PC-domain +Ve	**63 (72.4%)**	6	**69**
PC-domain –Ve	24	4	28
Total	87	10	97
CTF +Ve	**81 (93.1%)**	6	**87**
CTF –Ve	6	4	10
Total	87	10	97

Table 6: Comparison of CTF and SM-CRT sub-fragments to SEA.

	N	P	C	NS	NP	NPC	S	SP	SPC	PC	CTF	SEA
S. mansoni												
True +Ve	**40**	**64**	**59**	**52**	**34**	**49**	**51**	**54**	**41**	**69**	**87**	**87**
False –Ve	**57**	**33**	**38**	**45**	**63**	**48**	**46**	**43**	**56**	**28**	**10**	**10**
Sensitivity (Using eggs +Ve as gold standard**)**	**41.2 %**	**66 %**	**60.2 %**	**53.6 %**	**35.1 %**	**50.5 %**	**52.5 %**	**55.7 %**	**42.3 %**	**71.1 %**	**89.7 %**	**89.7 %**
Sensitivity (Using SEA +Ve as gold standard**)**	**42.5 %**	**68.9 %**	**60.9 %**	**52.9 %**	**37.9 %**	**50.5 %**	**50.5 %**	**56.3 %**	**42.5 %**	**72.4 %**	**93.1 %**	**-**

Table 7: Sensitivity (%) of the antigens. Sensitivity was calculated twice, by using *S. mansoni* eggs +Ve as the gold standard (total 97 patients) and using SEA ELISA as the serological gold standard (87 patients).

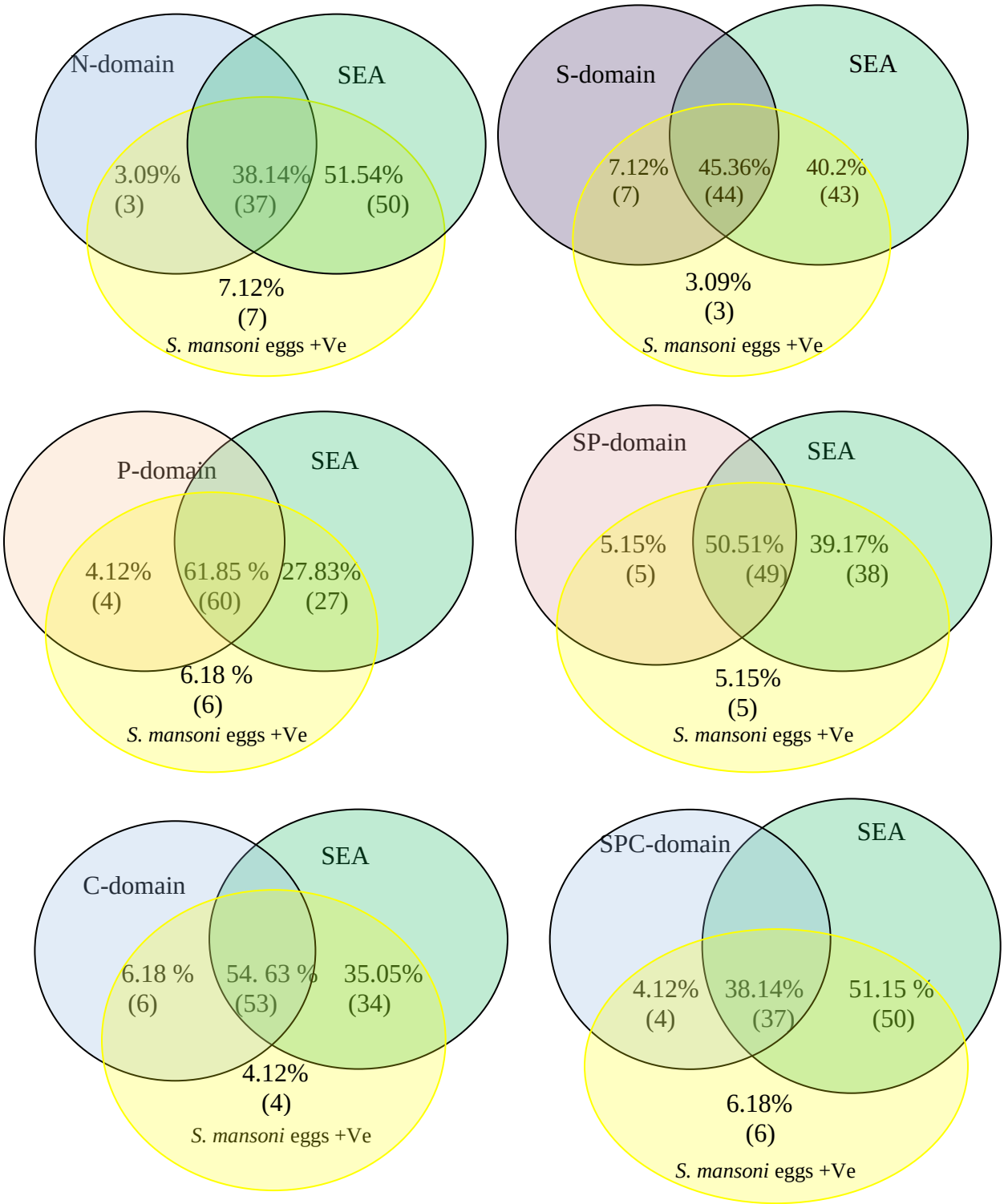

Figure 30: A comparison between *S. mansoni* eggs +Ve, SEA as the serological gold standard antigen and each of the other antigens tested.

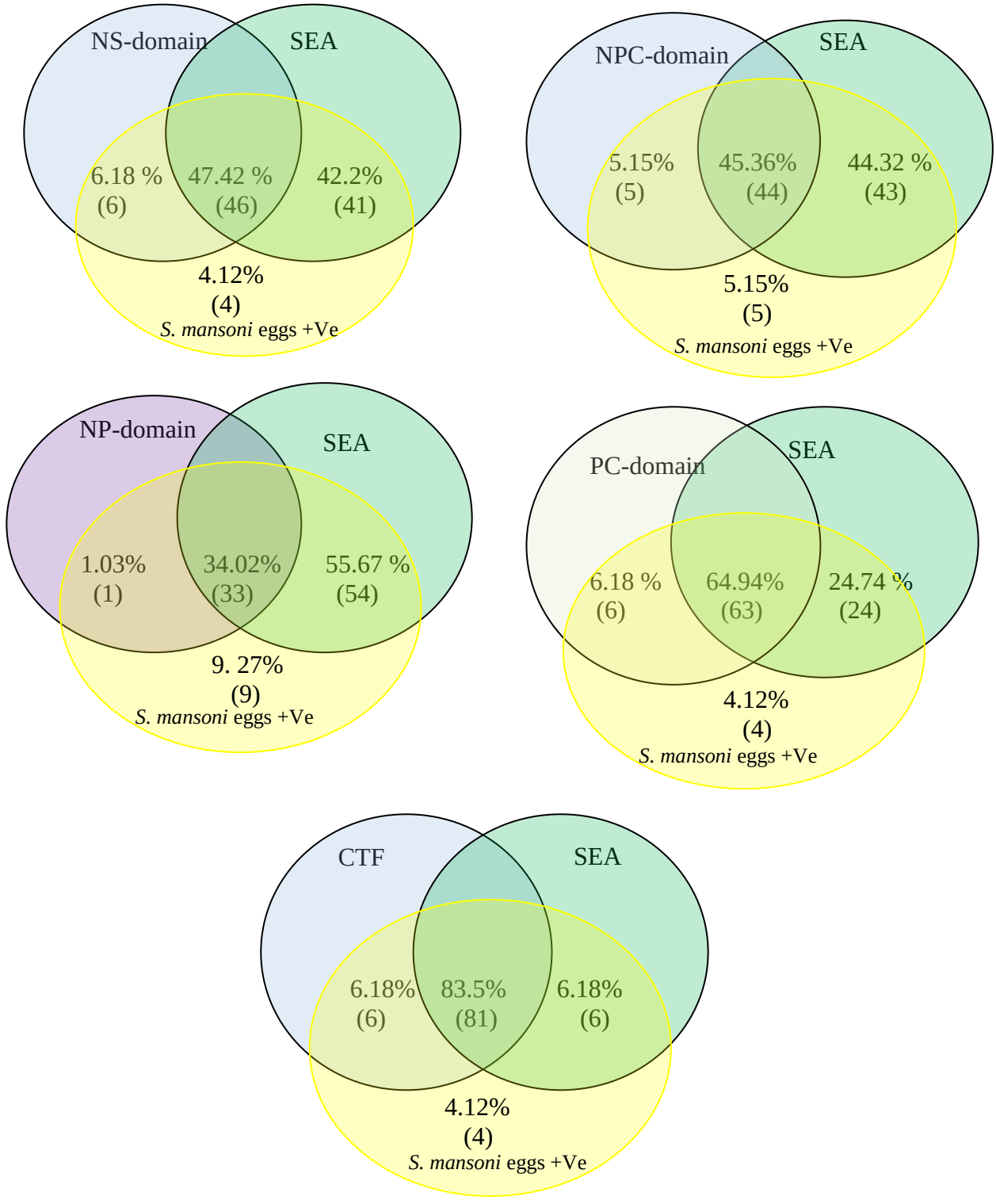

Figure 30: Continue.....

5.1.2.4 Correlation of relative antibody titers between CTF and SEA

There was correlation between anti-CTF and anti-SEA antibody titers in blood of *S. mansoni* infected individuals who had the infection confirmed by the detection of specific eggs in their stool and the *S. mansoni* negative control groups (cohort of sera collected from healthy U.K. persons and that withdrawn from healthy Egyptian controls) **(Figure 31).**

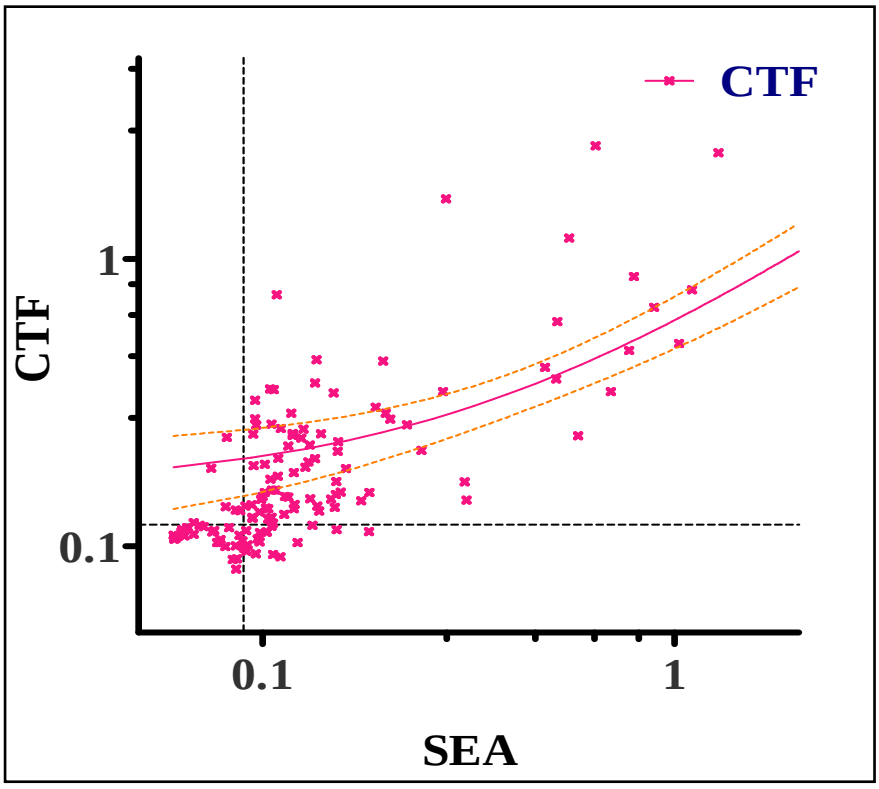

Figure 31: Correlation between anti-CTF and anti-SEA antibody titres. Values seen in S. *mansoni* patients, as well as in the U.K. and Egyptian controls. Solid and the two dashed lines indicate linear regression and 95% confidence intervals. Vertical and horizontal lines indicate the cut-off. r^2= 0.216, 95% confidence interval=0.307- 0.592.

5.1.2.5 Cross-reactions with other diseases

The cross-reactivity of the SM-CRT sub-fragments and CTF with other non-relevant *Schistosoma* disease was evaluated by testing their reactivity with 53 sera collected from patients suffering from some parasitic and autoimmune diseases, all are *S. mansoni* egg -ve. The SM-CRT N-domain showed the least degree of cross-reactivity, where it cross-reacted with only 9 sera from the cohort of these 53 sera. The highest degree of cross-reactivity amongst the SM-CRT sub-fragments were shown by SM-CRT C- and SP-domains which cross-reacted with 22 sera, for each, out from the total 53 sera collected from patients suffering from different parasitic diseases plus autoimmune patients. CTF showed a lower degree of cross-reactivity than SEA. CTF false positively recognized 19 patients sera, while SEA cross-reacted with 25 sera.

All *Fasciola* infected patient sera cross-react with CTF, SEA and SM-CRT C-domain. SM-CRT N- and SPC-domains did not cross-react with any hydatid infected patient sera, while SM-CRT NS-, NP- and PC-domains cross-reacted with these sera. Likewise, *Toxoplasma* patient sera did not cross-react with CTF or the SM-CRT PC-domain, while 1 *Toxoplasma* patient serum cross-reacted with the SM-CRT N-, NP-, NPC-, S- and SPC-domains. In contrast, SEA cross-reacted with 3 of the 5 tested *Toxoplasma* infected patient sera.

When testing autoimmune patients sera on an ELISA plate coated with recombinant human CRT, four out of 15 sera from patients with autoimmune disease were positive for auto-antibodies against human CRT (see **Figure 32)**.

The highest degree of cross-reactivity with autoimmune sera was shown with the N-terminal part (including full length protein) of SM-CRT where NS-, NP-, NPC-domains identified falsely 5 autoimmune patient sera. However, SM-CRT PC- and C-domains showed the least degree of cross-reactivity with the autoimmune sera, where they cross-reacted with only two sera (the sera 5 and 14 which shown auto-antibodies). The SM-CRT P-domain did not cross-react with any of the autoimmune patient sera. The SEA antigen showed a lower cross-reactivity with autoimmune patient sera (cross-reactivity with 1 serum) than CTF (which was positive in 4 autoimmune patient sera) **(Table 8)**. There were 7 sera out of these 15 autoimmune sera (46.7%) which did not cross-react with any of the SM-CRT sub-fragments. Two out of 4 autoimmune sera (that showed auto-antibodies against human CRT) showed cross-reactivity with all of SM-CRT sub-fragments with the exception of the P-domain.

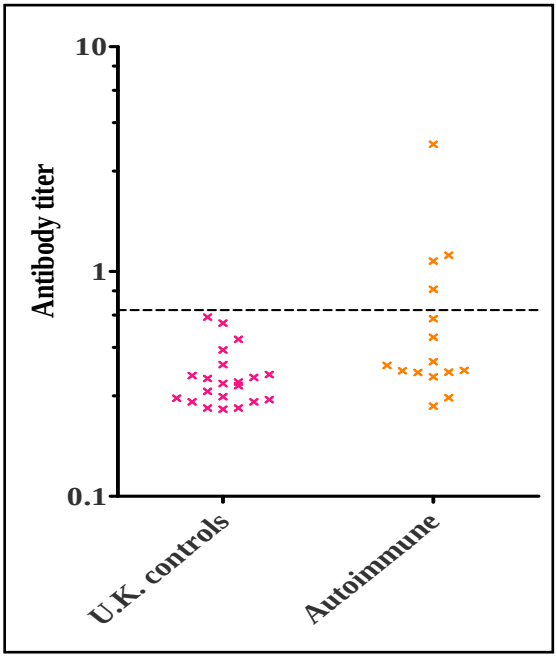

Figure 32: The relative antibody titres against human CRT in healthy U.K. donors sera and autoimmune sera. Horizontal line indicates the lower cut-off limits for positive cases (mean±SD of healthy U.K.controls).

	N	P	C	NS	NP	NPC	S	SP	SPC	PC	CTF	SEA
i) Parasites (38)												
***Fasciola* (3)**												
-Ve	**3**	**3**	**0**	**3**	**3**	**3**	**3**	**3**	**3**	**1**	**0**	**0**
+Ve	**0**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**3**	**3**
Hydatid (3)												
-Ve	**3**	**1**	**2**	**0**	**0**	**1**	**1**	**1**	**3**	**0**	**2**	**1**
+Ve	**0**	**2**	**1**	**3**	**3**	**2**	**2**	**2**	**0**	**3**	**1**	**2**
***Ascaris* (14)**												
-Ve	**13**	**6**	**7**	**9**	**12**	**8**	**12**	**7**	**10**	**7**	**8**	**5**
+Ve	**1**	**8**	**7**	**5**	**2**	**6**	**2**	**7**	**4**	**7**	**6**	**9**
***E. histolytica* (13)**												
-Ve	**10**	**5**	**6**	**7**	**9**	**9**	**11**	**7**	**11**	**6**	**8**	**6**
+Ve	**3**	**8**	**7**	**6**	**4**	**4**	**2**	**6**	**2**	**7**	**5**	**7**
***Toxoplasma* (5)**												
-Ve	**4**	**3**	**3**	**3**	**4**	**4**	**4**	**3**	**4**	**5**	**5**	**2**
+Ve	**1**	**2**	**2**	**2**	**1**	**1**	**1**	**2**	**1**	**0**	**0**	**3**
ii) Autoimmune (15)												
-Ve	**11**	**15**	**13**	**10**	**10**	**10**	**10**	**10**	**11**	**13**	**11**	**14**
+Ve	**4**	**0**	**2**	**5**	**5**	**5**	**5**	**5**	**4**	**2**	**4**	**1**
Total–Ve	**44**	**33**	**31**	**32**	**38**	**35**	**41**	**31**	**42**	**32**	**34**	**28**
Total + Ve	**9**	**20**	**22**	**21**	**15**	**18**	**12**	**22**	**11**	**21**	**19**	**25**

Table 8: Patients suffering from non-*Schistosoma* diseases and cross-reacted with the different antigens used.

5.1.2.6 Specificity of the antigens

$$\textbf{Specificity (\%)} = \frac{\textbf{100 X True negatives}}{\textbf{True negatives + False positives}}$$

True negative serum is a serum taken from healthy U.K. individuals or healthy Egyptian controls, all *S. mansoni* egg –Ve and that serum shows relative antibody titres **below** the cut-off for an antigen. **False positive serum** is a serum taken from healthy U.K. individuals or healthy Egyptian controls, all *S. mansoni* egg –Ve and that serum showed relative antibody titres **above** the cut off for that antigen.

The specificity (%) for each used antigen in the study was calculated twice. In one method, cohort of sera from healthy U.K. blood donors (21) was considered "*S. mansoni* negative group" or a cohort of sera collected from healthy Egyptians (19) which is considered the "*S. mansoni* negative group", all are *S. mansoni* egg –Ve.

With using cohort of healthy U.K. sera, the specificity (%) was 100% with the CTF antigen and SM-CRT C-, PC- and NPC-domains, whereas SEA and other SM-CRT sub-fragments showed a specificity of 95.2%. When using the cohort of sera drawn from the healthy Egyptian controls (19), the specificity of all used antigens decreased. The SM-CRT N-domain achieved the highest specificity with 94.7%, while the SM-CRT P-domain achieved the lowest specificity (42.1%) amongst all SM-CRT sub-fragments. CTF specificity was higher than SEA, the former achieved 68.4% and the latter revealed 26.3% **(Table 7).**

Antigen	N	P	C	NS	NP	NPC
Egyptian controls (19)	**94.7%**	**42.1%**	**63.2%**	**63.2%**	**78.9%**	**78.9%**
U.K. controls (21)	**95.4%**	**95.4%**	**100%**	**95.4%**	**95.4%**	**100%**
Antigen	**S**	**SP**	**SPC**	**PC**	**CTF**	**SEA**
Egyptian controls (19)	**68.4%**	**78.9%**	**63.2%**	**57.9%**	**68.4%**	**26.3%**
U.K. controls (21)	**95.4%**	**95.4%**	**95.4%**	**100%**	**100%**	**95.4%**

Table 9: Specificity (%) of the antigens using sera of healthy U.K. or healthy Egyptian persons.

The specificity was also calculated as a function of the sensitivity for CTF, SEA, SM-CRT PC-domains (the antigens achieved the highest sensitivity). When using a cohort the healthy U.K. sera as the *S. mansoni* negative group, SEA and CTF ELISAs showed > 95 specificity when their cut-off were set to detect 90 % of the positive *Schistosoma* sera. The specificity of the PC-domain goes down when its cut-off was set to achieve 70% sensitivity. For healthy Egyptian control sera, CTF retained >65% specificity when its cut off was set to achieve 95% sensitivity. SEA and PC-domain showed around 60% specificity when their cut-off was set to show 60% sensitivity **(Figure 33).**

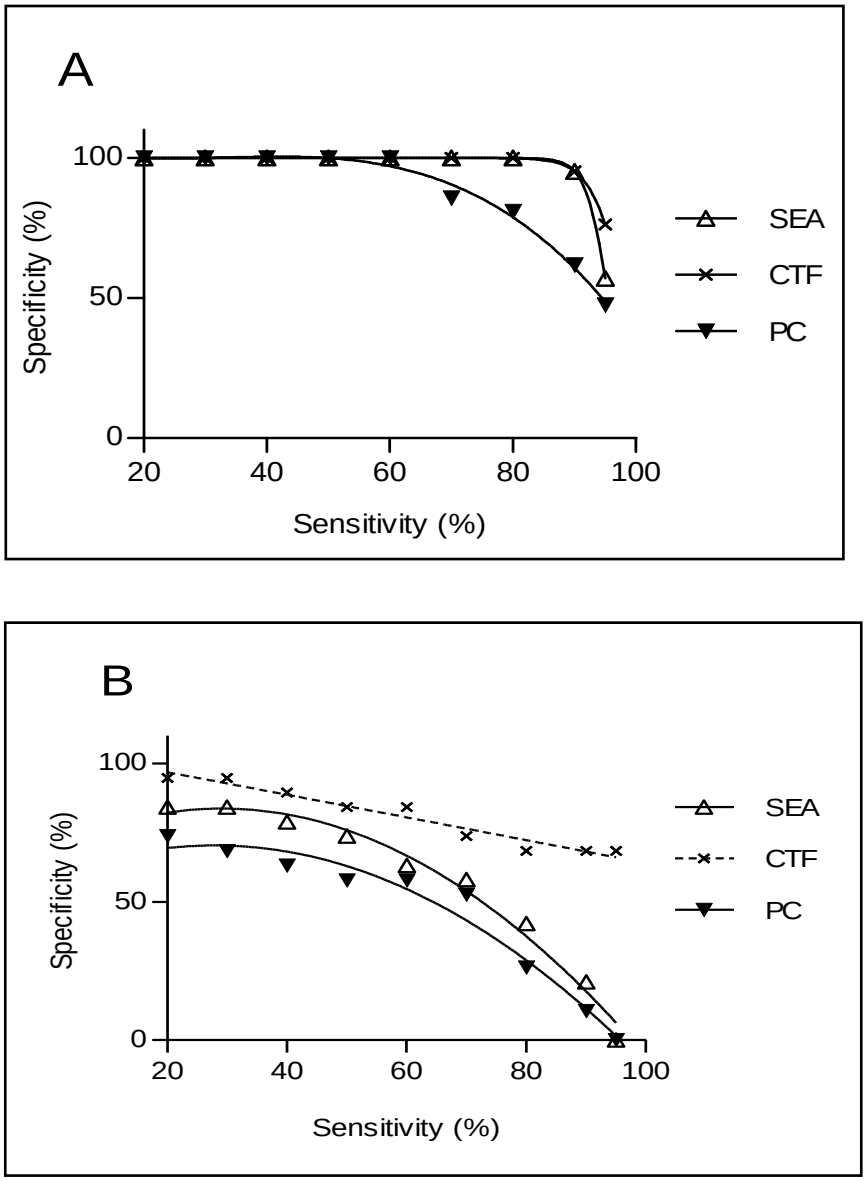

Figure 33: Specificity as a function of sensitivity for SEA, CTF and PC-domain ELISAs. Cut-off limits for each ELISA were re-calculated to produce the sensitivity shown and plotted against specificity. **A.** Using U.K. controls, **B.** Egyptian controls

5.2 Discussion

The aim of this part of my study was to evaluate the ability of different SM-CRT sub-fragments and CTF (Cercarial Transformation Fluid) to diagnose *S. mansoni* infection using ELISA through detection of their specific antibodies in experimentally infected mice and infected patients from endemic areas in Egypt.

It is the first time that recombinant SM-CRT sub-fragments have been tested in *Schistosoma* sero-diagnosis. The main reasons for choosing *S. mansoni* CRT as a potential diagnostic antigen is that it is well established that parasite CRT has a high degree of immunogenicity and the detection of specific anti-CRT antibodies in patients suffering from other parasitic diseases, such as *O. volvulus* (Lux *et al.* 1992), *T. cruzi* (Marcelain *et al.* 2000) and *E. histolytica* (Gonzalez *et al.* 2000& 2002). *T. cruzi* CRT and *T. brucei CRT* have been used with promising results for the diagnosis of Chagas' disease and sleeping sickness through detecting of CRT specific antibodies with ELISA assay in South American and sub-Sahara African patients (Marcelain *et al.* 2000, Schwaeble& Lynch 2007, Prof. Schwaeble, personal communication).

CTF is schistosomula supernatant which contains the soluble materials which are released by the ceracariae after being mechanically transformed to schistosomula *in vitro* (Colley& Wikel 1974). This preparation has not far been widely used for sero-diagnosis of schistosomisis. To my knowledge, the work presented in this thesis shows for the first time an evaluation of the CTF antigen in an endemic Egyptian cohort of sera. The efficiency of CTF and SM-CRT sub-fragments were compared to that of SEA. The latter was chosen

because it is well known to be more sensitive and specific than SWAP (McLaren *et al*. 1978, Mott& Dixon 1982, Sorgho *et al*. 2005).

At first, the reactivity of the antigens were tested in the mice which were experimentally infected with *S. mansoni* and blood samples taken and analysed at different time points after infection. Specific antibodies against the N-terminal part of SM-CRT (NS-, NP-, S-, SP-domains and full length protein, NPC-domain) appeared later where the antibody titre elevated significantly in the sera of mice 57-59 days p.i. **(**see **Figure 27)**. The late appearance of specific antibodies against these SM-CRT sub-fragments may be due to the low immunogenicity of SM-CRT N-domain or due to the low amount of native SM-CRT available to the immune system during early *Schistosoma* infection. The finding of a low immunogenicity of the N-domain is supported by my previous finding that rabbit anti-SM-CRT anti-serum was less reactive to the N-domain **(Figure 23)** and this is in agreement with Rokeach *et al.* (1994) who studied the immunogenicity of different recombinant *O. volvulus* CRT segments in patients suffering from the oncocercosis. These authors reported that, the protein segment A (spans from 1-99 a.a position) and segment B (spans from 100-167 a.a position) (which correspond to the SM-CRT N-domain) reacted weakly with these patients sera.

However, the specific antibodies against the C-terminal domains of SM-CRT (P-,C- and PC-) and SPC-domain start to appear in the sera of infected mice at 46 days after infection starting with the onset of adult development **(Figure 27)**. This could be attributed to several reasons. First, the P-, C- and PC-domains do not include the less antigenic N-domain, but the SPC-domain (which includes a considerable fragment of the N-domain) is

still identified earlier because its main bulk is formed of P-domain plus C-domain. Second, it seems that P- and C-domains were highly immunogenic to a degree that the amount of the native CRT shed from the parasite was sufficient to induce significant levels of specific antibodies in the mice during *S. mansoni* infection. Third, the C-domain potentially involves a site for N-linked glycosylation (Michalak *et al*. 1999) hence; it could be a glycoprotein antigen that induce relative early production of specific antibodies. Despite the possibility that the glycosylated C-domain of the parasite CRT induces a good response against the carbohydrate components of that glycoprotein, but these anti-glycostructure specific antibodies do not bind to the recombinant non-glycosylated SM-CRT C-domain used for my antibody detection assay. It is well established that there are good anti-*Schistosoma* antibody response, especially after the egg production, mainly towards the parasitic glycan antigens (Doenhoff *et al.* 2004).

When human CRT N- and C-domains interact with calcium or zinc, they become more susceptible to proteolysis (Corbett *et al.* 2000) and this may also be the case for SM-CRT, where the degradation of extra-cellular SM-CRT C-domain in the *in vivo* environment might be accelerated by the binding of ions and this makes the C-domain accessible at an early stage to antigen presenting cells, hence elicits antibodies at earlier stage compared to N-domain which appears to be less immunogenic.

This behaviour of SM-CRT was similar to that previously described for recombinant *O. volvolus* CRT (Rokeach *et al.* 1994). It was found that specific antibodies were directed primarily against the central (C segment, which spans from 167-248 a.a. position and nearly corresponds to the SM-CRT P-domain) and its terminal carboxyl (D segment which

spans from 248-374 a.a. position and corresponds to the SM-CRT C-domain). The day 12 p.i. and day 35 p.i. mouse groups were not sero-reactive to any SM-CRT sub-fragment. This might be because the protein is highly expressed at the adult stage and in eggs when compared to relative lower levels of expression in the newly transformed schistosomula (Khalife *et al.* 1994). However, CTF was recognized by sera from infected mice at day 12 p.i. onwards. The unexpected early antibody response to CTF implies the early mounting of specific antibody response against cercariae.

SEA behaved like CTF. An explanation for detection of anti-SEA in mice sera as early as 12 days p.i. is offered by a recent proteomic study which shows that SEA includes antigens which are expressed at every stage of *Schistosoma* life cycle. Notably SM-CRT is a minor constituent of SEA (Mathieson& Wilson 2010). However, this was in contrast with many reports which studied the time points from which a significant anti-SEA antibody response was seen in experimental mice ranging from 3-13 weeks post infection. Anti-SEA antibodies were detectable in mouse sera as early as 3 weeks after infection with 60 (+/-10) *S. mansoni* cercariae (Helmy 2007), highly specific antibodies were recognized from 4 weeks post-infection onwards in BALB/c mice infected with 25 cercariae only (Makarova *et al.* 2003). In another study, anti-SEA antibodies were detectable in sera of *S. mansoni* infected ICR mice (with 200 cercariae) by ELISA assay six weeks p.i. (Suzuki *et al*. 2006). Anti-SEA specific antibodies were detected in sera of inbred CBA/H mice infected with *S. mansoni* at 35 days post-infection by the ELISA, while in the same study using immune-electrophoresis, the anti-SEA antibodies were only detectable at 42 days p.i. with showing strong precipitation at the very late time point of 13 weeks after infection (Dunne *et al.*1984).

S. mansoni infected human sera were collected from Northern Nile delta Egypt, where *S. mansoni* is prevalent. I used sera from healthy U.K. donors which have never been exposed to *Schitosoma* to calculate the cut-off for detection of each antigen, as it is otherwise difficult to identify individuals who are truly *Schistosoma* negative in endemic regions (DeVlas& Grysels 1992, Polman *et al.* 2000).

Cut-off values, which were expressed in relative antibody titre, were relatively high for different SM-CRT sub-fragments **(Table 5)** reaching its highest level for the N-domain with 0.817 and the lowest was for the P-domain with 0.399. The general increase of the cut-off of SM-CRT sub-fragments could due to the presence of cross reacting anti-human CRT antibodies found in the normal individuals. The difference in the cut-off of different fragments could be due to the difference in the antigenicity of the different domains.

The C-terminal part (P-,C- and PC-domain) of SM-CRT gave the best sensitivity among different SM-CRT sub-fragments **(Table 7).** The PC-domain was the most informative SM-CRT-sub-fragment and recognized 69 of 97 sera of *S. mansoni* patients (71.1%), P-domain reacted with 64 of 97 sera (66%) and the C-domain reacted with 59 of 97 positive patient sera (60.8%).

Amongst the calreticulin domains, its C-terminal part is the least conserved domain (Michalak *et al*. 1999) with the SM-CRT C-domain showing only 38% identity with its human counterpart **(Table 3).** This might explain its relative high sensitivity, which was also observed for the PC-domain. In addition, the sensitivity of these domains may also be related to their relatively high immunogenicity. This explanation is also supported by the

results of my immunogenicity study in mice. This hypothesis is also supported by a previous study analysing *O. volvulus* CRT which showed high reactivity of sera with central and carboxyl parts of recombinant *O. volvulus* CRT. In my study, however, the SM-CRT PC-domain which showed stronger reactivity than C- and P-domains on their own, while the recombinant CD fragment (central and carboxyl) of *O. Volvulus* CRT was less reactive than either sub-fragments separately (Rokeach *et al.* 1994).

SM-CRT sub-fragments containing the N-domain shown lower sensitivity with the worst sensitivity was for the NP-domain detecting only 35.1% of infected sera (34 of 97 sera tested) **(Table 6)**. The lower sensitivity of the SM-CRT NP-domain in comparison to the SM-CRT P-domain might be due to the fact that the P-domain is somewhat hidden in the NP-domain fragment, as a result of recombinant expression in a prokaryotic system.

In my hands, the recombinant SM-CRT NPC-domain achieved a sensitivity of 50.5% (49 out of 97 tested sera), that was lower than that reported for the complete purified SM-CRT antigen used by Khalife *et al.* (1993) (87.5% sensitivity). They analysed recombinant SM-CRT in presence or absence of the fusion partner GST for reactivity only with few children sera (8 total) who were infected with *S. mansoni*, from an endemic area in Kenya, Africa. Reactivity was analysed on Western blots and by ELISA. The higher sensitivity in their study might be explained by the fact that the children are serologically more reactive to some *Schistosoma* antigens such as SEA (Tanabe *et al*. 1990, Naus *et al.* 2003).

SM-CRT revealed a lower sensitivity than TcCRT for the detection of *T. cruzi* infection which produced excellent results for the sero-diagnosis of Chagas' disease (Prof. Schwaeble, personal communications) and this may be attributed to several factors. SM-CRT may not be immunogenic enough to induce sufficient specific antibodies in humans infected by *Schistosoma,* whereas *Trypanosoma* does, SM-CRT may be not become available to immune system in sufficient quantities during the course of *Schistosoma* infection and SM-CRT specific antibodies levels may decrease during chronic infection.

The SEA ELISA, in my hands, achieved a sensitivity of up to 89.7% which is slightly better than that shown in some other reports. The sensitivity was 86 % in a non-endemic area in Venezuela, South America (de Noya *et al.,*1992), or 72% sensitivity in patients passing *Schistosoma* eggs, examined in another U.K. laboratory analysing sera from immigrants and Europeans returning from Africa during the period of 1993-1997 (Whitty *et al.* 2000). The SEA-ELISA sensitivity was 79% in infected sera taken from a community with low transmission in Venezuela (Spencer *et al.* 1991). On the other hand, the sensitivity of SEA ELISA reported in this study is slightly lower than previous reports. Tanabe *et al.* (1990) reported that SEA ELISA sensitivity was up to 98% in an endemic area. The SEA ELISA yielded a sensitivity of 94% for a large population in an endemic *S. mansoni* area in Kenya (Doenhoff *et al.* 1993). In Venezuela, SEA ELISA sensitivity was compared and reached the same sensitivity of stool sample and approached 98% (Alarcon de Noya *et al*. 1997). In a study analysing patients from an endemic area in Brazil, there were 98.8% of stool egg positive patients showing anti-SEA specific antibodies (Santos *et al.* 2000). In an endemic area in Africa, the SEA ELISA achieved 95.8% sensitivity in cohort of sera collected from patients of different age categories (Sorgho *et al.* 2005).

The difference in the sensitivity of SEA ELISA among various studies can be attributed to many factors. i) Whether the infected persons were living in endemic *S. mansoni* areas or were just tourists, as the latter patient category usually reveal better sensitivity of the serological *Schistosoma* tests (van Gool *et al*. 2002). ii) The prevalence rate of *Schistosoma* infection in the locality and the severity of *Schistosoma* infection in individuals (Hamilton *et al*. 1998, Doenhoff *et al*. 2004). iii) Age of the patients as the immune response against *Schistosoma* antigens changes with the age, for example, the immune response against SEA decreases with increasing the age of patients (Naus *et al.* 2003, Van Dam *et al.* 1996, Webster *et al*. 1997). iv) PZ administration also affects the anti-*Schistosoma* immune response by exposing new *Schistosoma* antigens to the host immune system after the drug kills adult worms (Viana *et al.* 1995, Correa-Oliveira *et al.* 2000, Mutapi 2001) and by eliminating the immune hypo-responsiveness caused by the adults (Feldmeier *et al*. 1988). v) The variations in the assays procedures include the use of different types of negative control sera and whether or not the infected persons are living in or outside *Schistosoma* endemic areas. vi) Finally, the performance of the same serological diagnostic test might significantly differ amongst sera of infected blood donors in different areas (van Lieshout *et al.* 2000).

CTF antigen gave the highest sensitivity in this study (89.7%) which was similar to that revealed with SEA ELISA. The CTF ELISA reacted with 87 sera from the 97 sera collected from *S. mansoni* infected patients. There were 81 out of 97 patient sera which were positive for both SEA and CTF antigens and 93.1% of SEA positive sera were positive for CTF (which produces a sensitivity of 93.1% in these sera). Nearly similar results were reported by Chand *et al.* (2010), the only previous report on this antigen, who found that the CFT

ELISA achieved 99.2% sensitivity in SEA ELISA positive sera. The slight difference in sensitivity between my study and Chand *et al.* (2010) might be due to the type of the patients, who were in my study persons living in endemic area, while in the other study, they analysed sera from infected European tourists.

There was correlation between the antibody response to both CTF and SEA. This is in agreement with Chand *et al.* (2010), who reported such correlation between O.D.s of both antigens. This correlation in antibody titres of both antigens could be due to the fact that, most of the produced antibodies are directed against glycoproteins which are shared between cercariae and eggs (Wuhrer *et al*. 1999).

There were 4 *S. mansoni* infected patient sera which were not positive with either SEA or CTF ELISAs. It cannot be excluded that these *S. mansoni* infected patient sera were improperly handled for example, by keeping the sera for longer periods exposed to room temperature before freezing.

A group of 53 sera from patients (*S. mansoni* negative) with either other parasitic infection (14 *Ascaris,* 13 *Entamoeba histolytica,* 3 *Fasciola,* 3 *echinococcosis* and 5 *Toxoplasma*) or established autoimmune diseases (15 sera) were used to test for cross-reactivity against other diseases. The SM-CRT N-domain was shown to give the highest degree of specificity for schistosomiasis by cross-reacting with only 9 sera out of 53 *S. mansoni* negative sera, while SM-CRT SP- and C-domains showed the highest degree of reactions showing reactivity in 22 of these 53 *S. mansoni* negative sera. CTF showed less cross-reactivity

than SEA in this cohort of patient sera. CTF cross-reacted with 19 of 53 patient sera, whereas SEA cross-reacted with 25 of these 53 patient sera **(Table 8).**

All the *Fasciola* sera of infected patients (3) were falsely positive with CTF, SEA and the SM-CRT C-domain antigens. The other SM-CRT sub-fragments did not react with any of these sera. The *Fasciola* patients reacted more strongly with SEA than with CTF as shown by the relative antibody titre. It was observed that the N-terminal part of SM-CRT did not cross react with *Fasciola* positive sera. Unfortunately, *Fasciola* CRT sequences has not yet been characterized to compare amino acids identity. In the cases of CTF and SEA, there may be cross-reactive antigens in *Schistosoma* and *Fasciola.* The cross-reactions between other parasitic antigens for *Schistosoma* and *Fasciola* were reported in many previous reports (Rodriguez-perez& Hillyer 1995, Hillyer 1981&1985, Aronstein *et al.* 1985a& b, Alarcon de Noya *et al*. 1996).

In the cases of hydatid patients (3), the recombinant N-domain antigen did not cross react with any patients sera, but a high cross-reactivity between hydatid antigens was seen with recombinant SM-CRT NS-, NP- and PC-domains (containing P-domain). The SMCRT N-domain alone seems to have a high degree of specificity with low cross reactivity with hydatid associated antigens. CTF showed cross reactivity with one of the three hydatid sera, while SEA was positive with two of the 3 sera tested. The highest degree of amino acid identity between SM-CRT and CRT of Echinococcus is located in the P-domain with 64%, while SM-CRT N-domain has 54% identity with N-domain of Echinococcus (**Table 3)** . Cross-reactivity between hydatide and *Schistosoma* was reported previously (Lacllette *et al.* 199, Sulahian *et al.* 2005).

The SM-CRT N-domain showed cross-reactivity with only 1 out of 14 sera from patient suffering from *Ascaris*. CTF cross-reacted with 6 out of the 14 *Ascaris* sera. The highest degree of cross-reactivity was seen with SEA which was positive in 9 out of these 14 sera. SM-CRT N-domain showed a higher degree of specificity in identifying *Schistosoma* infection in *Ascaris* patient sera than CTF or SEA. I speculate that *Ascaris* CRT carboxyl part is highly immunogenic and elicit antibodies in the sera of affected patients which cross-react with the counterpart SM-CRT. Also, a high degree of amino acid identity between SM-CRT and CRT of *Ascaris* could be located in the P-domain. At present, there is unfortunately no published sequence information available for *Ascaris* CRT. Cross-reactivity of other *Ascaris* and *Schistosoma* antigens has been reported previously (Sulahian *et al*. 2005, Correa-Oliveira *et al.* 1988, Pritchard *et al.* 1991).

In sera of patients infected with *E. histolytica* (13 sera), the S- and the SPC-domains cross-reacted with only 2 sera out of 13, the N-domain recognized 3 sera of this cohort and SM-CRT P-domain showed the highest degree of cross-reactivity with 8 out of 13 sera being positive. CTF reacted with 8 out of 13 *E. histolytica* infected sera while SEA reacted with 9 out of these 13 sera. The carboxyl portion of SM-CRT showed the highest degree of reactivity with *E. histolytica* antigens and this might be due to a high degree of identity in sequences between both parasite CRTs which would explain their high cross reactivity. The degree of amino acids identity between SM-CRT P-domain and CRT P-domain of *E. histolytica* is 66%. I speculate that *E. histolytica* CRT carboxyl part is highly immunogenic and elicit antibodies in the sera of affected patients which can cross-react with the counterpart SM-CRT. The specific anti-*E. histolytica* CRT antibodies were generated during the course of invasive amoebiasis. Gonzalez *et al.* (2000& 2002) reported that in

91% of patients with invasive amoebiasis, *E. histolytica* CRT specific antibodies are present in their serum and saliva and the antibody level decreased by about 70% 6-12 months after treatment. These antibodies did cross-react with SM-CRT.

Most of the antigens used in this assay showed low cross-reactivity with toxoplasmosis (5 sera). The SM-CRT PC-domain and CTF did not cross-react with any of the 5 *Toxoplasma* patients sera. SM-CRT P-, NS-, SP- and C-domains cross-reacted with 2 of the 5 *toxoplasma* patients sera. Generally speaking, the cross-reactivity of the different SM-CRT sub-fragments with *Toxoplasma* antigens was low and a good specificity could be achieved. This may be attributed to a lower degree of homology between both *S. mansoni* and *Toxoplasma* CRT (although at present, no sequence information for *Toxoplasma* CRT is available). *Toxoplasma* CRT appears to be less immunogenic as only insufficient quantities of specific antibodies against *Toxoplasma* CRT were present to cross-react with highly homology *S. mansoni* CRT. It could also be that the *Toxoplasma* CRT does not shed from this parasite in sufficient quantities to elicit an effective immune response. CTF shows a better degree of specificity with none of these 5 sera being positive in the CTF ELISA, while 3 out of the 5 *Toxoplasma* patient sera were positive in the SEA ELISA.

There were 4 sera from the 15 autoimmune patients sera which gave positive readings with recombinant human CRT on ELISA. When testing the cross-reactivity of sera collected from autoimmune patients against SM-CRT sub-fragments on ELISA, it was observed that 7 auto-immune sera out of these 15 (46.7%) did not cross-react with any SM-CRT sub-fragment. The carboxy-terminal part of SM-CRT (PC- and C-domains) showed the least degree of cross-reactivity with the autoimmune sera, where none of these 15 sera

cross-reacted with the SM-CRT P-domain and the SM-CRT PC- and C-domains cross-reacted with only two sera (which shown to have auto-antibodies against human CRT).

It was interesting to find that the SM-CRT P-domain did not cross-react with any of the autoimmune sera, despite the fact that it has 62% identity with its human counterpart and the human P-domain has auto-antigenic epitopes which are able to induce auto-antibodies in some autoimmune diseases (Boehm *et al.* 1994, Routsias *et al*. 1993, Kishore *et al.* 1997, Sontheimer *et al.* 1991). The lower degree of cross-reactivity of PC- and C-domains with autoimmune sera might attributed to that the SM-CRT C-domain has only 38 % identity in amino acids with the human C-domain.

The highest degree of cross-reactivity with autoimmune sera was revealed for the N-terminal part of SM-CRT (N-, S-, NS-, NP-, SP-, NPC-domains, containing N-domain or part of it) where all of these recombinant antigens gave 5 positive readings. This could be attributed to the fact that i) the N-terminal part of human CRT includes auto-antigenic epitopes which can induce auto-antibodies in patients suffering from autoimmune diseases such as rheumatoid arthritis and SLE (Boehm *et al.*, 1994, Kishore *et al.*, 1997, Sontheimer *et al.*, 1991, Routsias *et al.*, 1993). This may in part explain the cross- reactivity with the autoimmune sera which were shown to have auto antibodies against human CRT, ii) the amino acid identity between the N-domain of SM-CRT and human CRT is 53%, and iii) that human CRT is a major component of cytolytic T-cells (Dupuis *et al.* 1993) and during parasitic infections, through the activation of the cytolytic T-cells, larger amounts of human CRT are released and may account for a higher concentrations of auto-antibodies which in turn may cross react with SM-CRT.

The SM-CRT NPC-domain cross-reacted with 5 (three of them have auto-antibodies against human CRT) out of the 15 autoimmune sera (33.3%). This degree of cross-reactivity was lower than that shown by a previous report in which 50% of European SLE patients sera cross-reacted with *S. mansoni* CRT being tested by Western blot or by the ELISA assay (Khalife *et al.* 1993).

SEA cross-reacted with only 1 autoimmune serum, while CTF showed cross-reactivity with 4 patients sera out of 15 suffering from autoimmune diseases. However, none of these cross-reacting autoimmune sera, with SEA or CTF, showed auto-antibodies against human CRT by ELISA.

Collectively, the SM-CRT N-domain achieved the least cross-reactivity degree (cross-reacted with 5 from the 38 sera collected from patients suffering from parasitic disease) than the SM-CRT C-terminal parts (P-, PC- and C-domain which cross-reacted with 20, 19 and 20 sera respectively). Three main explanations were addressed to explain this finding. First, the identity between SM-CRT and these parasites CRTs may be mainly in the P-domain of the CRT. Second, the CRT P- and C-domain are more immunogenic than the N-domain, therefore both P- and C-domains can generate a higher antibody response which during the parasitic infection, hence these generated parasites anti-CRT antibodies may cross -react with the recombinant SM-CRT sub-fragments expressed from its central and carboxyl parts. Third, the cut-off limit for SM-CRT N-domain ELISA was very high, largely due to the presence of a significant amounts of anti-SM-CRT N-domain in the control sera, in other words, the specificity for N-domain was high and its sensitivity was

low because the controls and patients alike had relatively high anti-SM-CRT N-domain antibody.

In total, The SM-CRT N-domain showed the least degree of cross reactivity with patients sera infected by the parasitic disease or suffering from autoimmune disease where it reacted with 9 sera out of these 53 (16.9%) patients. The CTF antigen cross-reacted with 19 sera out of 53 sera of patients suffering from parasitic or autoimmune disease (30.2%) and it achieved a lower degree of cross-reactivity than did SEA which falsely recognized 25 sera out of these 53 sera (47.2%) suggesting that CTF lacks epitopes responsible for cross-reactivity in SEA. This was slightly different about what reported by Chand *et al*. (2010) who found that CTF identified 16 sera out of 74 patients sera suffering from different parasitic disease and hepatitis B virus (21.6%) while 10 sera out of these 74 sera (13.5%) were reacted with SEA.

With analysis of the 21 sera from healthy U.K. donors as the *"S. mansoni* negative control group", the specificity was 100 % with the SM-CRT NPC-, C- and PC-domains, while a specificity of 95.2% was seen for other SM-CRT sub-fragments. Likewise, CTF revealed 100% specificity and compared to SEA (which achieved a specificity of 95.2%) appeared more reliable. When the cohort including the 19 sera from healthy Egyptian individuals was included in the specificity study, I observed an increased the number for sera which gave cross-reactions with the different *S. mansoni* antigens used. For this cohort, the SM-CRT N-domain revealed by far the highest degree of specificity with 94.7%, whereas the SM-CRT P-domain achieved the lowest degree of specificity with 42.1% amongst SM-

CRT sub-fragments. SEA revealed the lowest degree of specificity in this study with 26.3%, but CTF showed a higher specificity with 68.4% (see **Table 9**).

In my study, the SEA ELISA specificity was 95.2% for the "*S. mansoni* negative group" of sera collected from healthy U.K. donors. This is in agreement with previously published studies, the SEA ELISA achieved variable specificity results such as 100% in a non-endemic area (Tanabe *et al.* 1990), while another study achieved a specificity of 98.2% for the SEA ELISA. This study analysed sera of *S. mansoni* infected Europeans which contracted infection whilst being abroad and the negative control group included various cohorts of sera from patients suffering from bacterial, viral, parasitic or autoimmune diseases (van Gool *et al.* 2002).

Likewise, the specificity of the SEA ELISA was only 26.3 % when the *"S. mansoni* negative group" was formed out of 19 sera of healthy Egyptian donors. This is in agreement with other studies performed in endemic areas. The SEA ELISA specificity was lower than 20% in an endemic area in Africa (Doenhoff *et al.* 1993). In another recent study in an endemic *Schistosoma* area in Africa, the specificity of the SEA ELISA was lower than 30% (Sorgho *et al.* 2005).

There is straightforward trade-off between sensitivity and specificity (Doenhoff *et al*. 1993 and results presented here). This illustrates a general problem with the use of antibody detection assay in endemic areas, whether the antigen is SEA, SM-CRT and CTF or the previously popular *S. mansoni* adult worm antigen (SWAP).

There are many factors that could explain why the individuals previously diagnosed as negative for *S. mansoni* infection using the stool examination method gave false positive reactions with SM-CRT, CTF and SEA in the ELISA. i) Low sensitivity of stool examination (Doenhoff *et al.* 2004), so some patient sera that were collected from Egypt, might be from individuals that have harboured *S. mansoni* infection with a very low parasites load, where the eggs cannot be seen using microscopic analysis of stool. ii) The development of mainly adult male (unisexual infection) *Schistosoma* where no egg deposition can be found in stool (Oliveira *et al*. 2005, Doenhoff *et al*. 2004). iii) Anti-*Schistosoma* specific antibodies may persist for months or even years, despite a successful elimination of the infection (Doenhoff *et al.* 1989). iv) *Schistosoma* infection might not have been completely eliminated by chemotherapy and the infection may still continue with only few eggs being secreted which are not detectable microscopically (Doenhoff 1998). v) The presence of cross-reacting antibodies, which are generated against certain antigen epitopes found in other parasites such as *Fasciola* and *Ascaris* could be responsible for the false positive readings with some of the *Schistosoma* antigens (Correa-Oliveira *et al.* 1988& 2002, Alarcon de Noya *et al.*1996, Aronstein *et al.*1986, Hanna &Hillyer 1984 and Sulahian *et al.* 2005). For example, Sm14 and tropomyosin are cross-reacting antigens with a high degree of homology between *Schistosoma* and *Fasciola* (Hillyer 1995). Some SM-CRT sub-fragments could be incorrectly folded and expose new epitopes that may be identified with cross reacting antibodies.

In conclusion, the ideal test required for the sero-diagnosis of *Schistosoma* infection should cover the following certain criteria. It should be simple, the reagents should be cheap and stable, the test should be reproducible, interpretation of the results should be easy and it

should be of satisfactory sensitivity and specificity (Hoshino-Shimizu *et al.*1992, Rabello *et al.* 1997,Turner *et al.* 2004). Most of these criteria were covered more or less by CTF and the SM-CRT antigens PC-, P and C-domains. Some SM-CRT sub-fragments gave relatively good results, especially the PC-domain (71.13% sensitivity), the P-domain (65.97% sensitivity) and the C-domain (60.82% sensitivity). Using SM-CRT in the sero-diagnosis of *Schistosoma* has certain advantages. The use of recombinant *Schistosoma* antigens in the serological assays avoids cross-reactivity to a large extent. In addition to achieving a sensitivity equal to that of SEA (89.7%), CTF antigen is ideal as it is a quick and cheap to produce. Large amounts could be prepared from the schistosomulum and only few mice are needed to generate considerable amounts of the antigen.

Chapter 6

Use of recombinant SM-CRT in vaccination of mice against *S. mansoni* infection

6.1 Results

6.1.1 Testing immunogenicity of the recombinant full length His-tagged SM-CRT in BALB/c mouse

A preliminary experiment was done to evaluate the immunogenicity of the recombinant full length SM-CRT in BALB/c mouse strain given prior to them being infected with *S. mansoni* cercariae. Two mouse groups (4 mice in each group) were established. Each mouse in the immunised group received intra-peritoneally three doses of recombinant SM-CRT protein in two weekly intervals; the first dose was 50 µg SM-CRT and other two doses were 25 µg, all doses were mixed with equal volumes of alum adjuvant (total volume was 100 µl /dose). The control mouse group received PBS/alum at the same points of time parallel to that of the immunised group. In addition to the pre-bleed (time point 0), serum of each control or immunised mouse was collected 2, 4 and 6 weeks after the first immunisation.

The SM-CRT specific antibodies (whole IgG fraction) were measured in the sera collected from both mouse groups at 0, 2, 4 and 6 weeks after the first immunisation by ELISA assays. It was found that SM-CRT specific antibodies were detectable in the sera of the immunised mouse group as early as 2 weeks after the first dose. The intensity of the immune response in the immunised mice against SM-CRT increased in correlation with subsequent doses. SM-CRT specific antibodies were detectable up to a titre of 1:50000 in

the sera of immunised mice group, two weeks after the third immunisation dose **(Figure 34**).

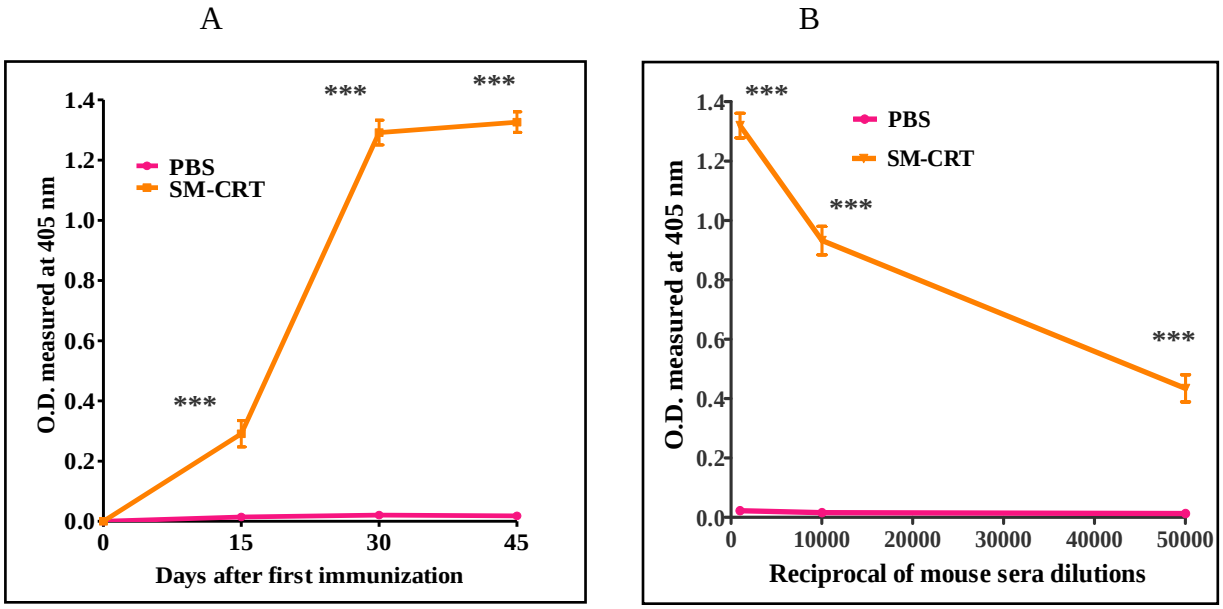

Figure 34: Anti-SM-CRT specific IgG responses in the immunised mice. (A): The sera of the immunised mice were tested on plates coated with recombinant SM-CRT by ELISA. Antibodies against SM-CRT were detectable 2 weeks after immunisation and the antibody titre increased significantly in sera taken at later time points (results were expressed as mean± SD of each group at different points of time)**. (B):** The antibody titre in SM-CRT immunisation mice and PBS control mice in sera taken after the third round of immunisation (results were expressed as mean± SD of each group at mouse sera dilutions of 1:1000, 1:100000 or 1:50000). The difference was statistically significant compared to the control group as denoted with three asterisks (P<0.001).

6.1.2 Immunisation and challenging of BALB/c mouse

6.1.2.1 Detection of specific anti-SM-CRT antibody response in the immunised mice

After confirmation the immunogenicity of the recombinant SM-CRT in BALB/c mouse strain, the main vaccination experiment was launched. Two mouse groups; a control and an immunised group (5 mice in each group) were established. The same regimen of immunisation and sera collection was repeated as in the first experiment. The specific anti-SM-CRT antibody response showed a profile similar to that of the pervious experiment (**Figure 35**).

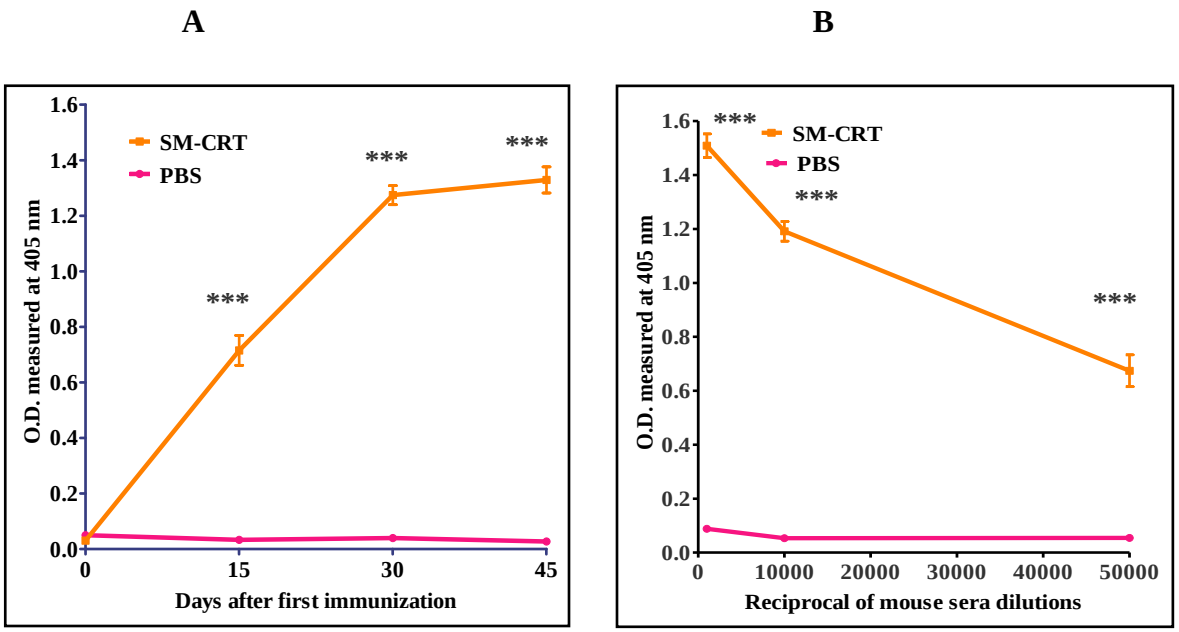

Figure 35: Anti-SM-CRT specific IgG responses in the immunised mice (second experiment). (A): The sera of the immunised mice were tested on plates coated with recombinant SM-CRT by ELISA. Antibodies against SM-CRT were detectable 2 weeks after immunisation and the antibody titre increased significantly in sera taken at later time points (results were expressed as mean± SD of each group at different points of time). **(B):** The antibody titre in SM-CRT immunisation mice and PBS control mice in sera taken after the third round of immunisation (results were expressed as mean± SD of each group at mouse sera dilutions of 1:1000, 1:100000 or 1:50000). The difference was statistically significant compared to the control group as denoted with three asterisks (P<0.001).

6.1.2.2 Worms, eggs in liver and eggs/worm pair counting

Both mouse groups were then challenged with 200 cercariae per mouse. 42 days post infection, the mice were culled. The severity of infection was assessed using the following criteria; the recovered adult worms, eggs in liver and eggs/ worm pair numbers in each mouse. The mean of these measures was calculated for each mouse group (one mouse died in the control group during the course of the infection).

The mean % protection in the vaccinated (V) mouse group was compared to the mean of the control (C) group with following formula:

$$\frac{\text{mean of worms recovered from (C) group - mean of worms recovered from (V) group}}{\text{mean of worms recovered from (C) group}} \times 100$$

The mouse group immunised with recombinant His-tagged SM-CRT showed a mean reduction of 49.9% in the number of adults worms which gives a statistically significant difference (P=0.03) to the non-immunised control group. However, when looking at the numbers of eggs in liver tissue (41.8% lower than the control group), no statistical significance could be detectable between immunised and non-immunised mice. It was found that recombinant SM-CRT achieved mean reduction of 10% in fecundity (eggs/ worm pair) in comparison to the mouse group given PBS/alum and this difference is not statistically difference. It seems that SM-CRT immunisation did not cause any further anti-fecundity effect (**Table10 and Figure 36**).

	PBS (control) **(4 mice)**	**SM-CRT(immunised)** **(5 mice)**
Total worms counts (Mean± SD)	95 ± 25.4	47.6 ± 26
Mean % reduction		49.9%
Two-tailed *p* value		**P*=0.03(significant)
Eggs counts in the liver (Mean± SD)	44300±17556.5	25780 ± 22393.5
Mean % reduction		41.8%
Two-tailed *p* value		*P*=0.2(insignificant)
Eggs/ worm pair counts (Mean± SD)	1047.8± 140.2	942.6± 379.4
Mean % reduction		10%
Two-tailed *p* value		*P*=0.96(insignificant)

Table 10: Worm burden, tissue eggs and eggs/worm pair numbers in immunised and control groups (mean ± SD)**.** The mean reduction % was calculated in relation to the control mouse group. The two mouse groups were compared with unpaired Student's *t*-test using computer software package GraphPad Prism 5.02 version. There were significant statistical difference when *P*-value, compared to the control group, was lower than 0.05 (**P* < 0.05).

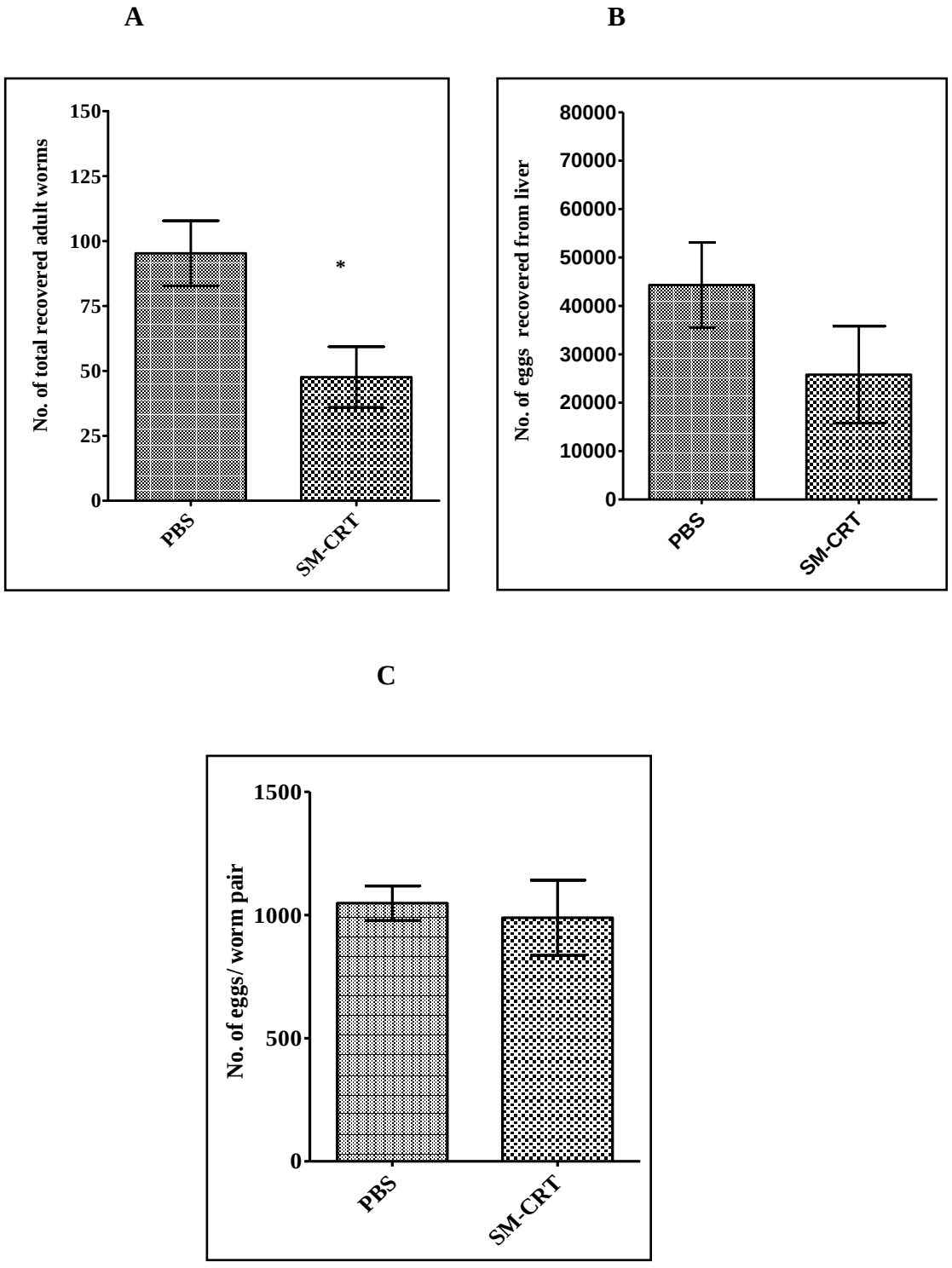

Figure 36: Scattergram of the worm burden (A), eggs in the liver (B) and eggs/worm pair in both mouse groups. In the case of worms, the immunised mouse group showed a significant statistical difference (*$P < 0.05$) in comparison to control mouse group. Error bars represents the standard deviations.

6.1.2.3 Anti-SM-CRT specific IgG subclasses

The vaccinated mouse group with recombinant SM-CRT showed a significant reduction in experimental *Schistosoma* infection, therefore I decided to analyse the immune response in the immunised mouse group. The type of the immune response was determined by measurement of subclasses IgG1 and IgG2a in the sera of both groups two weeks after the third SM-CRT immunisation. The immunised mouse group sera showed high both IgG2a and IgG1 subclasses levels with a statistically significant difference in comparison to the control mouse group ($P < 0.001$). The immunised mouse group showed that, the mean of IgG1 O.D. readings was higher than the mean of O.D. readings of IgG2a with a high statistical significant difference between both IgG subclasses ($P < 0.001$) (showing a mixed Th1/Th2 with a predominant Th2 immune response profile) (see **Figure 37**). The mean of IgG1 O.D. readings/ mean of IgG2a O.D. readings ratio was 2.4

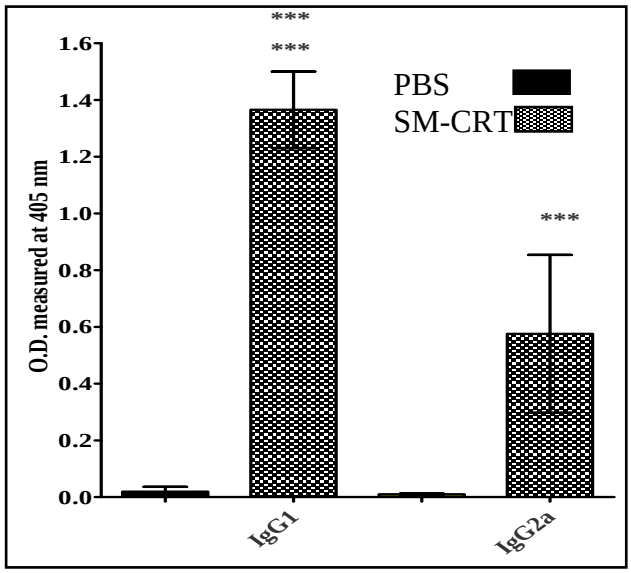

Figure 37: Levels of specific SM-CRT IgG1 and IgG2a subclasses. On one ELISA plate coated with SM-CRT, the individual mouse sera were probed (collected two weeks after the third immunisation) with IgG1or IgG2a. The results were expressed as mean ±S.D. in both mouse groups. IgG1 column showed 2 sets of 3 asterisk, one indicates a significant difference compared to the control group, the other indicates a statistically significant difference in comparison to IgG2a of the same immunized group. IgG2a column showed 3 asterisk denoting a statistically significant difference when compared to the control group (***$p < 0.001$).

6.2 Discussion

Anti-*Schistosoma* vaccine development is an achievable goal as suggested by many previous studies for example, those vaccinating with the irradiated *S. mansoni* cercariae that resulted in a degree of protection up to 70-80% (Hewitson *et al*. 2005, Smythies *et al.* 1996, Ganley-Leal *et al*. 2005). Some individuals in endemic *Schistosoma* areas manifest various degrees of natural resistance against the parasitic infection (Butterworth *et al*. 1985& 1988& 1994, Hagan *et al.* 1991) and some anti-parasitic vaccines have been successfully developed such as *Echinococcus granulosus* and *Taenia ovis* (Dalton& Mulcahy 2001).

SM-CRT appears to provide a potentially effective anti-*Schistosoma* vaccine antigen due to a number of reasons: i) SM-CRT is present in the tegument of larva and adult *Schistosoma* (van Balkom *et al.* 2005, Braschi *et al.* 2006, Khalife *et al*. 1994 and this study), so that it is present at the parasite/ host interface. ii) In this study my recombinant SM-CRT binds and inhibits the biological function of the C1q molecule *in vitro,* therefore SM-CRT neutralization *in vivo* may expose *Schistosoma* to complement attack. iii) SM-CRT is identified by T-cell and B-cell of the *Schistosoma* infected mice and rabbits (El Gengehi *et al*. 2000).

Until now, there is no published report that assessed the efficacy of recombinant or native SM-CRT in the protection against experimental *S. mansoni* infection in animals. My work is the first study to evaluate the protective efficacy of recombinant SM-CRT against *Schistosoma* infection in experimental animal models of infection.

The preliminary experiment which was performed to evaluate immunogenicity of the recombinant full length His-tag SM-CRT in BALB/c mic, showed that the immunised mouse group produced specific SM-CTRT antibodies as early as 2 weeks after immunisation with recombinant SM-CRT. The antibody levels increased after further boosting doses (as assessed by ELISA) (see **Figure 34 A**). The immunised mouse sera **(**at 6 weeks after first immunisation) reacted with the antigen at a titre of 1:50000 **(Figure 34B).** This implies that SM-CRT produced strong immune response in BALB/c mouse strain. Subsequently, a challenging experiment was established. When analysing sera two weeks after each immunisation, the sera of the immunised mouse group showed antibody response profiles similar to those seen in the preliminary experiment **(Figure 35 A& B**).

The detection of specific SM-CRT antibodies in both immunisation experiments indicates the immunogenicity of the recombinant SM-CRT in BALB/c mouse strain and this is in agreement with the previously shown immunogenicity of my expressed recombinant SM-CRT in rabbit (see **Figure 22& 23**). It is interesting to observe that despite the fact that CRT is a highly conserved protein among different organisms, SM-CRT was able to induce a remarkable antibody response in the mice immunised with the protein. This is in line with similar observation for other CRT species like TcCRT (Aguilar *et al.* 2005).

Recombinant SM-CRT achieved a 49.9% reduction in *Schistosoma* adult worm numbers with a statistically significant difference (*P*=0.03) when compared to the control group **(Table 10 &Figure 36).** This percentage of protection is good when compared to other *Schistosoma* antigens which were tested in experimental mouse model of *Schistosoma* infection. For example, recombinant caplin caused 29-39% protection (Hota-Mitchell *et al.*1997), Sm23-pcDNA reduced the worm burden up to 36-44% (Da'dara *et al*. 2003), rSm14 showed protection of 36.9-49.5% (Ribeiro *et al*. 2002) and recombinant *Schistosoma* 14-3-3 protein achieved protection of 25-43% (Schechtman *et al*. 2001).

By achieving the 49.9% reduction in adult worm numbers, SM-CRT fulfils an important anti-*Schistosoma* vaccine criterion suggested by WHO. WHO indicates that the antigen, which cause 40% reduction in the adult worms in the mouse model, is a promising vaccine antigen. To reduce morbidity and mortality in humans, it is most important that such a vaccine gives a relative reduction of the parasitic load, as this determines the severity of pathology. So, even a vaccine that gives not 100% of protection, but reduces the severity of parasitic disease by reducing the numbers of parasites following infection is highly valuable as it reduces the morbidity and mortality of *Schistosoma* (Bergquist& Colley 1998).

Concerning liver eggs counting, the immunised mouse group showed a mean reduction of 41.8% in comparison to the control group, but the difference between both groups was statistically insignificant (*p* value = 0.22) and this is could be due to the wide SD of the immunised groups (eggs number in the immunised group was 25780 ± 22393.5) (**Table 10),** as it shown that**,** there was a varied individual protection from 14.7-80.4% compared to

the mean of the worm adult of the control mouse group. However, SM-CRT did not show further anti-fecundity effect **(Table 10),** where the count of eggs per worm pair of the immunised group was reduced with only 10% with insignificant difference in comparison to the control mouse group.

To explain the protective effect of recombinant SM-CRT, I measured IgG sub-classes in the sera of both mouse groups (control and immunised group) two weeks after the third injections. The measurement of IgG1 and IgG2a are indicators for Th2 and Th1 immune profiles type responses (Martin& Lew 1998). It was found that, in comparison to the control mouse group, the immunised mouse group sera had significantly higher antibody levels of both IgG1 and IgG2a antibodies (**Figure 37**). However, the immunised mouse group sera showed that IgG1 levels was significantly higher than IgG2a (p value= 0.0004). The IgG1:IgG2a ratio in the sera of the immunised mouse group was 2.4 (measured by dividing the mean of O.D. readings of IgG1 by O.D. readings of IgG2a). This shows that the protective immune response generated with recombinant SM-CRT was a mixed Th1/Th2 response with a predominant Th2 immune response profile. The alum adjuvant combined with recombinant SM-CRT may be partly responsible for this immune response profile. My SM-CRT study is therefore in the line with other mouse vaccination trails where aluminium hydroxide was used as adjuvant and shifted the immune response predominantly towards Th2 immune response profile (Yip *et al*. 1999, Grun *et al*. 1989).

I concluded that the SM-CRT specific antibodies generated during vaccination helped to neutralise or kill the invading *S. mansoni* cercariae in immunised mice. These SM-CRT specific antibodies are likely to bind to the surface of cercariae where SM-CRT is exposed

and may alter calcium binding capacity of parasitic CRT which in turn may affect muscle contractions and the signalling functions of calcium and thereby hinder the migration of cercariae. The specific SM-CRT antibodies may inhibit C1q binding function of SM-CRT and this would let the larvae exposed to the lethal effects of complement activation via the classical pathway. In addition to that, immunisation with recombinant SM-CRT may trigger Th1 cells to produce proinflammatory cytokines such as IFN-γ which in turn will activate local macrophages (in lung and skin) to kill invading larvae (Reynolds *et al*. 1990, Mounteford *et al*. 1992, Smythies *et al*. 1992). IFN-γ may also help with the elimination of parasites from the lung through the induction of adhesion molecule on epithelial cells and hinder schistosomulum migration (Wilson *et al*. 1996).

Previous works describe protective immune responses against *Schistosoma* of the Th1 type (Smythies *et al*. 1993, Zhang *et al*. 2001, Farias *et al*. 2010) and others of the Th2 (Grzych *et al.* 1991, Pearce *et al.* 1991). However, a mixed Th1/Th2 appears to provide maximal protection against experimental *Schistosoma* infection in mice involving both cellular and humoral immune responses (Jankovic *et al.* 1999, Hoffmann *et al.* 2000, Caulada-Benedetti *et al*. 1991, Wynn& Hoffmann 2000).

Two previous reports tested the protective phenotype of *S. japonicium* CRT immunisation using a nucleic acid based vaccination approach. In one report, *S. japonicium* CRT cDNA cloned into the pCDNA (the expression vector) failed to induce specific antibodies response in the immunised mice (Waine *et al*. 1997). The authors did not test the cloned *S. japonicium* CRT construct's ability to express protein *in vitro* and the cause of failure in generation of specific antibodies upon mouse injection may be attributed to a technical

failure of the cloning vector. In another study, *S. japonicum* CRT-DNA did not show any protection against experimental *S. japonicum* infection, however, there was damage to external membranes of the adult *S. japonicium* tegument, (which were obtained from the immunised mouse group) shown by electron microscope examination (Scott 1998). The failure to induce protection may have been due to the failure of the CRT-DNA expression construct to generate sufficient recombinant *S. japonicum* CRT which in turn failed to induce high specific antibody titres to cause protection against the challenging cercariae. On the other hand, CRT from *S. japonicium* may be not very immunogenic.

Winter *et al*. (2005) tested recombinant hookworm CRT in a BALB/c mouse model using the recombinant hookworm CRT dissolved in PBS or encapsulated in poly-lactide-coglycolide microparticles (PLG). It was found that the immunisation with parasite CRT (dissolved in PBS) reduced the number of adult hookworms in the immunised mouse group by 43-49% when compared to the control group, even in the absence of any adjuvant. Administration of recombinant hookworm CRT without using PLG achieved protection, despite the fact that no specific IgE could be found and the degree of eosinophilia and total IgE was lower than in the other immunised mouse group where PLG have been used (Winter *et al*. 2005).

In conclusion, my SM-CRT immunisation experiment showed that recombinant SM-CRT is an effective and promising vaccine antigen. It should be further tested in future vaccine studies using larger numbers of mice and possibly different types of adjuvants. The protective immune response induced by recombinant SM-CRT deserve to be investigated in more details.

Chapter 7

Summary

In my thesis I fully achieved the goals of my project, which assessed the use of recombinant *S. mansoni* calreticulin, a highly immunogenic parasitic antigen in the diagnosis of and vaccination against *S. mansoni*. i) I expressed 10 SM-CRT sub-fragments representing individual domains or combinations, thereof. ii) I characterised the physiological function of SM-CRT sub-fragments in terms of calcium binding activity and their interaction with C1q (the recognition subcomponent of the classical pathway of complement). iii) I evaluated the use of different SM-CRT sub-fragments and another parasite antigen called CTF (cercarial transformation fluid antigen) in the sero-diagnosis of *S. mansoni* infection in murine and human sera using an ELISA-based detection of specific antibodies, iv) and finally, I assessed the efficacy of the recombinant full length SM-CRT in the protection against experimental *S. mansoni* infection in an animal model.

I split SM-CRT into ten sub-fragments which were N-, P-, C-, NS-, NP-, NPC-, S-, SP-, SPC- and PC-domains. A cDNA library of adult *S. mansoni* was used to amplify the coding cDNA sequences for these segments using PCR. The different coding regions for these sub-fragments were cloned and expressed using the pRSETB vector transformed to *E. coli* BL21 (DE3) pLysS host.

SM-CRT N-domain was expressed mainly in soluble form, but *E. coli* host pellets contained some insoluble protein. The SM-CRT NS-, NP-, S-, SP-, SPC- and NPC-domains were produced with varying solubility because they contain the whole or a part of the N-

domain, whereas SM-CRT P-, C- and PC-domains were expressed as completely soluble proteins.

The different fragments were expressed with 6 His-tag N-terminal fusion to facilitate the process of protein purification. The proteins were purified using affinity chromatography with HiTrap Ni Chelating HP columns (using linear gradient elution using buffer containing 5-500 mM imidazole), then the proteins were further purified by ion exchange using HiTap Q HP column (using linear gradient elution with buffer containing 20 mM-1 M NaCl).

Calcium binding capacity of the recombinant SM-CRT sub-fragments was evaluated by two methods; "Stains-all" staining of the protein loaded onto SDS-PAGE gel or by the interaction with the radioactive calcium binding to the recombinant proteins separated on a non-denaturing PAGE and blotted to nitrocellulose membrane Western blot assay. With the exception of the N-domain, all SM-CRT sub-fragments showed the ability to bind calcium when stained blue with "Stains-all" stain. On incubation with $^{45}Ca^{2+}$, most SM-CRT sub-fragments bound $^{45}Ca^{2+}$, however N-, NS- and NP- domains did not. The failure of SM-CRT NS- and NP-domains to bind $^{45}Ca^{2+}$, may be because the P-domain (which binds to calcium in high capacity and low affinity) is masked by the globular N-domain in NS- and NP-domains.

The proven calcium binding activity of SM-CRT might suggest that native *S. mansoni* CRT can bind to calcium in the micro-environment around the parasite in blood and

consume calcium which is required for blood coagulation, hence CRT may help to limit blood agglutination around the parasite.

I tested the interaction of SM-CRT sub-fragments with C1q *in vitro*. It was shown that, all SM-CRT sub-fragments (except the SM-CRT P-domain) bound to C1q using physiological PBS and that binding was dose dependent. It was found that, all SM-CRT sub-fragments (except P-domain) inhibited the C1q dependent haemolysis not only using normal human serum but also using the reconstituted C1q deficient serum. I observed that SM-CRT N- and S-domains are the individual domains which achieved the strongest binding with C1q and inhibition of C1q dependent haemolysis.

In trying to explain why SM-CRT P-domain did not bind to C1q, while the SM-CRT C-domain not only bound C1q, but also inhibited its activity in the haemolytic assay (which was in contrast to most of previous reports on CRT), *S. mansoni* CRT C1q binding sites sequences were compared with that of human and other parasite CRT sequences. I observed that the SM-CRT C-domain has a suspected C1q motif binding site, in addition to the presence of other unidentified C1q motifs. On the other site, the SM-CRT P-domain C1q binding sites seem to be functionless. I hypothesise that SM-CRT may protect *Schistosoma in vivo* from complement activation via the classical pathway in its surrounding environment and in the parasitic gut by binding C1q and inactivating the classical pathway of complement.

My recombinant full length SM-CRT protein (NPC-domain) showed high immunogenicity when immunising a rabbit. The rabbit anti-SM-CRT anti-serum reacted with different

recombinant SM-CRT sub-fragments in both Western blot analysis and ELISA assays. This specific anti-SM-CRT identified the native *S. mansoni* CRT in the surface preparations of schistosomulum and the adult parasite using.

The third goal of my project was to evaluate the use of different SM-CRT sub-fragments and CTF (cercarial transformation fluid) antigen in the sero-diagnosis of *S. mansoni* infection in experimental mice and humans using an ELISA-based detection of specific antibodies. The work presented in this thesis shows for the first time an evaluation of both antigens in an endemic Egyptian cohort of sera. The efficiency of CTF and SM-CRT sub-fragments were compared to that of SEA.

Specific antibodies against the carboxy-terminal part of SM-CRT (P- and C-domain) appeared at 46 p.i. days in *S. mansoni* infected mouse sera, while the specific antibodies against the N-terminal part of SM-CRT became detectable only 57-59 days after infection. This suggests the high immunogenicity of the carboxy-terminal part of the protein. CTF and SEA were recognised by sera of *S. mansoni* infected mice as early as 12 day p.i.. The unexpectedly early antibody response to CTF implies the early mounting of specific antibody response against cercariae.

S. mansoni infected sera (n=97) were recruited from the Northern Nile Delta, Egypt, while I used sera from healthy U.K. donors (which have never been exposed to *Schistosoma)* to calculate the cut-off limit for the detection of each antigen. The carboxy-terminal part of SM-CRT gave the best sensitivity amongst different SM-CRT sub-fragments. The PC-domain was the most informative SM-CRT-sub-fragment showing a sensitivity of 71.1%,

the P-domain revealed 66% and the C-domain produced a sensitivity of 60.8%. It seems that SM-CRT (which does not contain the N-domain) reacts strongly with specific antibodies and the antibodies generated against SM-CRT during the course of schistosomiasis are directed mainly against its central and terminal parts.

For the other recombinant SM-CRT sub-fragments, I observed an unsatisfactory low degree of sensitivity with very low sensitivity of detection for the NP-domain detecting only 35.1% of *S. mansoni* infected sera. However, CTF showed 89.7% sensitivity which was equal to sensitivity of the SEA ELISA. CTF achieved a sensitivity of 93.1% in the *S. mansoni* infected sera which gave positive readings with SEA ELISA. I found a correlation between the antibody response to CTF and SEA.

I tested the cross-reactivity of the antigens against a cohort of 53 sera drawn from patients suffering from other diseases including either other parasitic infection (14 *Ascaris,* 13 *Entamoeba histolytica*, 3 *Fasciola*, 3 *echinococcosis* and 5 *Toxoplasma*) or established autoimmune diseases (15 sera). All of these control sera are *Schistosoma* egg -Ve. The SM-CRT N-domain showed the highest degree of specificity for schistosomiais by cross-reacting with only 9 sera out of 53 *S. mansoni* negative sera, while SM-CRT SP- and C-domains showed the highest degree of cross-reactions showing reactivity with 22 sera of these 53 *S. mansoni* negative sera. CTF showed less cross-reactivity than SEA in this cohort of patients sera. CTF cross-reacted with 19 sera, whereas SEA cross-reacted with 25 of these 53 patients sera. It was interesting to find that SM-CRT P-domain did not cross-react with any of the tested autoimmune sera, despite the fact that the P-domain of SM-CRT has 62% identity with its human counterpart.

When using the 21 sera from healthy U.K. donors as the *"S. mansoni* negative control group", CTF and SM-CRT NPC-, C- and PC-domains achieved 100% specificity, while a specificity of 95.2% was seen for SEA with similar specificity seen for the other SM-CRT sub-fragments. When the cohort including the 19 sera collected from healthy Egyptian individuals was used for the specificity study, the SM-CRT N-domain revealed by far the highest degree of specificity with 94.7%, whereas the SM-CRT P-domain achieved the lowest degree of specificity with 42.1% amongst the SM-CRT sub-fragments. SEA revealed the lowest degree of specificity in this study with only 26.3%, while CTF showed a specificity of 68.4%.

My fourth goal was to test the protective effect of recombinant SM-CRT against experimental *Schistosoma* infection. My work is the first study to evaluate the protective efficacy of recombinant SM-CRT against *Schistosoma* infection in experimental animal models of infection. In my study, the recombinant SM-CRT produced strong immune response in the immunised BALB/c mouse strain. It is interesting to observe that despite the fact that CRT is a highly conserved protein among different organisms, SM-CRT was able to induce a remarkable antibody response in the mice immunised with this protein.

In my study, recombinant SM-CRT achieved a 49.9% reduction in *Schistosoma* adult worm numbers with a statistically significant difference (P=0.03) when compared to the non-immunised control mouse group. By achieving this percentage of reduction in adult worm numbers, SM-CRT fulfils an important anti-*Schistosoma* vaccine criterion set out by the WHO. The WHO states that an antigen, which can cause a 40 % reduction in the adult worms numbers in a mouse model of infection is a promising vaccine antigens as it will

cause a valuable reduction of morbidity and mortality caused by *Schistosoma* infection (Bergquist and Colley, 1998).

In correlation with the reduction of adult *Schistosoma* numbers, the eggs numbers in the liver tissues decreased by 41.8% in the immunised mice when compared to the control mouse group, however, the difference was not statistically significant due the wide SD within the immunised groups (egg numbers was 25780 ± 22393.5). This was attributed to the variation in individual mouse protection which ranged from 14.7 to 80.4% compared to the mean of the adult worm numbers of the control mouse group. However, SM-CRT did not show further anti-fecundity effect.

On dissecting the immune response in the immunised mouse group, I found that the immunised mice responded against SM-CRT by producing specific IgG1 and IgG2a subclasses with ratio of 2.4. The IgG subclasses reflected that the immunised mice showed Th1/Th2 with a predominate Th2 immune response profile and this profile appeared to cause the maximal protection against experimental *Schistosoma* infection in mice as suggested in other reports (Jankovic *et al.* 1999, Hoffmann *et al.* 2000, Caulada-Benedetti *et al.* 1991, Wynn& Hoffmann 2000).

I speculated that the SM-CRT specific antibodies generated during vaccination may kill the invading *S. mansoni* cercariae in SM-CRT immunised mice. These SM-CRT specific antibodies are likely to bind the exposed SM-CRT on surface of cercariae and may alter the calcium binding capacity of the parasitic CRT which in turn may affect muscle contractions and the signalling functions of calcium. All these consequences could hinder the migration

of cercariae. In addition to that, the specific SM-CRT antibodies may inhibit C1q binding function of SM-CRT and this would allow the larvae to be exposed to the lethal effects of complement activation via the classical pathway. Also, in response to SM-CRT immunisation a prevalent Th1 response profile may be triggered to secrete IFN-γ cytokine which have important roles in killing the invading larvae.

In conclusion, i) the SM-CRT P- and C-domain are the domains responsible for calcium binding activity of SM-CRT, ii) all SM-CRT sub-fragments (except the SM-CRT P-domain) bound to C1q and inhibited the C1q dependent haemolysis, iii) CTF shows better performance in sero-diagnosis of *Schistosoma* infection in endemic areas than SEA and gives similar results in non endemic areas. This suggests that the CTF antigen (which is a quick and cheap to produce) can replace the expensive SEA. Also, SM-CRT PC-domain achieved a sensitivity of 71.13% with specificities of 57.9% in Egyptian controls and iv) the immunisation experiment using recombinant full length SM-CRT showed that this antigen is an effective and promising vaccine candidate as it caused 49.9% of reduction in *Schistosoma* adult worm numbers.

Chapter 8

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Appendix

This manuscript was accepted for publication in the Journal Immunobiology

The title of manuscript is

" Use of recombinant calreticuln and cercarial transformation fluid (CTF) in the serodiagnosis of Schistosoma mansoni"