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Use of recombinant calreticulin and cercarial transformation fluid (CTF) in the serodiagnosis of *Schistosoma mansoni*

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ABSTRACT

Schistosomiasis is traditionally diagnosed by microscopic detection of ova in stool samples, but this method is labour intensive and its sensitivity is limited by low and variable egg secretion in many patients. An alternative is an ELISA using *Schistosoma mansoni* soluble egg antigen (SEA) to detect anti-schistosome antibody in patient samples. SEA is a good diagnostic marker in non-endemic regions but is of limited value in endemic regions, mainly because of its high cost and limited specificity. Here we assess seven novel antigens for the detection of *S. mansoni* antibody in an endemic region (the Northern Nile Delta). Using recombinant *S. mansoni* calreticulin (CRT) and fragments thereof, anti-CRT antibodies were detected in the majority of 97 patients sera. The diagnostic value of some of these antigens was, however, limited by the presence of cross-reacting antibody in the healthy controls, even those recruited in non-endemic areas. Cercarial transformation fluid (CTF), a supernatant that contains soluble material released by the cercariae upon transformation to the schistosomula, is cheaper and easier to produce than SEA. An ELISA using CTF as the detection antigen had a sensitivity of 89.7% and an estimated specificity of 100% when used in non-endemic regions, matching the performance of the established SEA ELISA. CTF was substantially more specific than SEA for diagnosis in the endemic region, and less susceptible than SEA to cross-reacting antibody in the sera of controls with other protozoan and metazoan infections.

Introduction

Schistosomiasis is one of the commonest parasitic diseases in humans, ranking second only to malaria (Croft et al., 2003; King et al., 2005). It is endemic in 74 countries, affecting about 200 million people, nearly 20 million of who suffer severe morbidity. A further 600 million are at risk of infection (Engels et al., 2002; Savioli et al., 2004; Chitsulo et al., 2004).

Schistosomiasis is a water-borne disease; the infectious agents are the cercariae, which develop in the intermediate host, aquatic snails of the genus *Biomphalaria*. The cercariae attach to and rapidly penetrate human skin, where they transform into an endoplasmic larval stage, the schistosomule. From there, the schistosomule enters the circulation and migrates via the lungs and the left side of the heart to the hepatoportal vessels, where it pairs and develops into the sexually mature adult worm. Worm pairs then move to the mesenteric blood vessels surrounding the large intestine and begin egg production. In the case of *S. mansoni*, eggs are secreted in the faeces, from where they can re-infect the intermediate host. Deposition of eggs in host tissue, chiefly the liver, is the primary pathogenic mechanism in human schistosomiasis.

An efficient and reliable method of diagnosis is essential for effective disease control (Hamilton et al., 1998; Doenhoff et al., 2004), but current methods are inadequate: in particular microscopic examination of faeces, which remains a frequently-used method, has low sensitivity, the detection limit being >100 eggs/g of stool (Barreto et al., 1990; Engels et al., 1996; Teesdale et al., 1985; Ebrahim et al., 1997; Doenhoff, 1998). Serological tests are available, the most widely used being a commercially-available indirect haemagglutination test (IHA) using erythrocytes coated with *S. mansoni* adult worm antigens and in-house ELISAs to detect antibody against *S. mansoni* egg antigens (SEA) in patient blood, but their use is generally restricted to rich Westernised non-endemic regions (Van Gool et al., 2002). The IHA has a reported sensitivity of 71–94% and a specificity ranging from 80% to 100%, with

Abbreviations: CRT, calreticulin; CTF, cercarial transformation fluid; IHA, indirect haemagglutination test; SEA, *Schistosoma mansoni* soluble egg antigen; SWAP, *S. mansoni* adult worm antigen.

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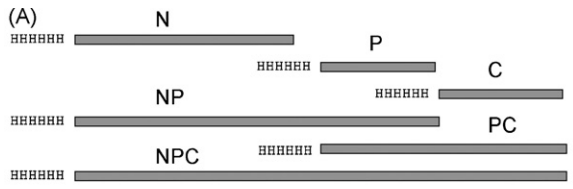
some trade-off between specificity and sensitivity. The SEA ELISA is marginally better, combining 94% sensitivity with 98% specificity. A combination of these two serodiagnostic tests gave 98% sensitivity with 97% specificity (Van Gool et al., 2002).

In this study we investigate two promising alternatives to SEA as capture antigens for the detection of anti-*S. mansoni* antibodies; *S. mansoni* calreticulin (Sm CRT) and 'cercarial transformation fluid' (CTF), an antigen prepared from *S. mansoni* cercariae.

CRT is a multifunctional protein found in all eukaryotic cells except fungi and erythrocytes. Mammalian CRT it is largely restricted to the endoplasmic reticulum, where is acts as a calcium-binding and chaperone protein. In contrast, protozoan and metazoan CRT appear to be secreted proteins. Several protozoan and metazoan parasites, including *Trypanosoma brucei* and *T. cruzi* (the causative agents of sleeping sickness and Chagas' disease, respectively), *Necator americanus* (hookworm) and pathogenic species of schistosome have been shown to secrete CRT and/or express CRT on their surface (Ferreira et al., 2004a; Pritchard et al., 1999; Jones et al., 2006). Parasite CRT inhibits the host complement system by binding to C1q, the first component of the classical complement cascade (Ferreira et al., 2004b; Kasper et al., 2001). Inhibition of the complement system reduces the effectiveness of the host antibody response, and these mechanisms appear to be an integral part of the parasite's strategy to evade the host immune response. It has been shown that trypanosome CRT is potentially useful as a capture antigen in the serodiagnosis of African and South American trypanosomiasis (sleeping sickness and Chagas' disease, respectively) and onchocerciasis (Schwaeble and Lynch, 2007; Marcelain et al., 2000; Rokeach et al., 1994).

CTF is a supernatant that contains soluble material released by schistosome cercariae after they have been mechanically-induced to transform to schistosomula *in vitro* (Colley and Wikel, 1974). This type of preparation has not so far been widely used for detecting anti-schistosome antibodies, but it may have some advantages compared with egg and worm antigens, particularly a requirement for fewer laboratory animals to produce it when compared with the number required for schistosome egg and worm production.



(B)

Peptide	Primers	Base pairs	Residues	MW (kDa)
N	N_BamH1& N_Xho1	573	204	23.16
P	P_BamH1& P_Xho1	294	130	15.5
C	C_BamH1& C_Xho1	267	121	13.77
NP	N_BamH1& P_Xho1	867	324	37.49
PC	P_BamH1& C_Xho1	561	219	25.61
NPC	N_BamH1& C_Xho1	1134	411	47.41

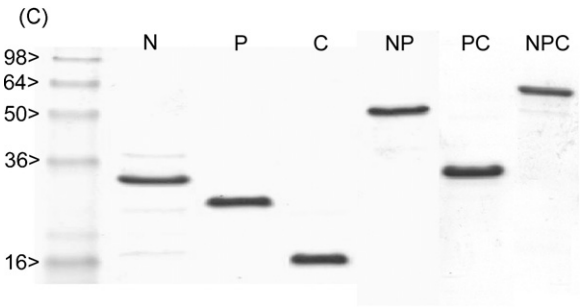


Fig. 1. Expression of recombinant *S. mansoni* CRT antigens. Six 6× histidine-tagged polypeptides representing all three domains, combinations thereof and the full-length protein (NPC) were expressed in *E. coli* using pRSETBand purified by metal affinity and ion-exchange chromatography. (A) Schematic drawing of the CRT fragments. (B) Biochemical data for the antigens (including the 6× his-tag and vector-derived residues). (C) Coomassie stained SDS-PAGE gel showing the purified antigens.

Materials and methods

Materials

Unless otherwise stated, all chemical reagents were obtained from Sigma–Aldrich. The *S. mansoni* adult worm cDNA library was kindly supplied by Dr. David Johnston (Natural History Museum, London).

Murine sera

Mice were purchased, housed, maintained and infected using procedures agreed with the local ethics committee and licensed by the UKHO. CD1 mice were infected with 200 cercariae, culled by cervical dislocation at 0, 12, 35, 46 and 57–59 days post-infection, and blood collected by cardiac puncture. Animals were perfused after sacrifice and adult worms counted. Serum was prepared from the blood, aliquoted and stored at −80 °C.

Human sera

Sera were collected in the Northern Nile Delta from Egyptian patients with *S. mansoni* (97), ascariasis (14), *Fasciolia hepatica* (3), echinococcosis (3), *Entamoeba histolytica* (13) and *Toxoplasma* (5) infection. 19 healthy controls were recruited from the same region. *S. mansoni* infection was confirmed in the patients and excluded in the controls by microscopic examination of stool samples. Sera from 15 Caucasians suffering from rheumatoid arthritis and SLE, and 21 control sera were obtained in the UK, none of whom had previously visited endemic areas. All patients and controls gave informed consent and the study was approved by the appropriate institutional ethical review board.

Preparation of S. mansoni antigens

S. mansoni CRT, and sub-fragments thereof, were expressed as 6× his-tagged fusion proteins in *Escherichia coli* using pRSETB (Invitrogen). Individual CRT domains and the signal peptide were identified using BLASTP and Signal P v3.0 (www.cbs.dtu.dk/services/), respectively. PCR products encoding the CRT fragments illustrated in Fig. 1 were amplified from a *S. mansoni* adult worm cDNA library using primers modified to incorporate a BamHI site at the 5′ end of the fragment and anXhoI site at the 3′ end (Table 1). Phusion high fidelity DNA polymerase (New England Biolabs) was used for amplification, following the manufacturer's recommendations. The products were purified with silica spin columns (Qiagen), tailed with deoxyadenosine using Taq polymerase (Abgene), cloned into pGEM-Teasy (Promega) and sequenced. The fragments were subsequently excised from pGEM-Teasy with BamHI and XhoI, sub-cloned into pRSETB and transformed into *E. coli* BL21(DE3)pLysS.