ANEXO

PUBLICACIONES

Veterinary Parasitology 173 (2010) 76–84

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Cloning and characterization of a plasminogen-binding surface-associated enolase from *Schistosoma bovis*

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ARTICLE INFO

Article history:
Received 16 April 2010
Received in revised form 2 June 2010
Accepted 7 June 2010

Keywords:
Schistosoma bovis
Enolase
Plasminogen
Fibrinolysis
Haemostasis

ABSTRACT

Schistosoma bovis is a ruminant parasite able to survive prolonged periods in the vasculature of its host without either being cleared by the host defensive systems or inducing thrombotic or coagulation disturbances. This suggests that the parasite modulates both the immune and haemostatic host responses. Previous studies have shown that host plasminogen binds to the surface of *S. bovis* adult worms, and that a tegument extract from *S. bovis* fixes and activates host plasminogen, generating plasmin, which in turn could both inhibit blood clotting and dissolve clots. Enolase has been identified among the tegumental proteins that bind plasminogen. The aim of the present study is to determine the physiological role of the enolase found in the tegument of *S. bovis* adult worms as regards plasminogen-binding and activation, and to confirm its surface exposure on the parasite. The study included the cloning and sequencing of *S. bovis* enolase cDNA, collection of the corresponding recombinant protein and evaluation of its plasminogen-binding and activation activity, and an exploration of the expression and localization of native enolase in adult worms and lung schistosomulae. Here we show that *S. bovis* male adult worms express enolase on their tegumental surface and that this protein binds host plasminogen and increases its activation in the presence of host tissue plasminogen activator (t-PA). This suggests that the surface-associated enolase found here is a physiological receptor of plasminogen that plays a role in the activation of the host fibrinolytic system, most probably to avoid blood clot formation on the worm's surface.

1. Introduction

Schistosomiasis is a parasite disease affecting man and several domestic and wild animals worldwide, and it represents a significant health and veterinary problem. Human beings are mostly infected by *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*, while animals can be infected by ten different species. Of these, *S. mattheei* and *S. bovis* have attracted the most attention, owing to their veterinary importance (De Bont and Vercruysse, 1998; Vercruysse and Gabriel, 2005).

Like all schistosome species, *S. bovis* migrates to and lives in the host's blood vessels, without being cleared by the immune or haemostatic systems. The immune evasion mechanisms of schistosomes have been studied in detail (rev. in Abath and Werkhauser, 1996). In contrast, studies of the interaction of schistosomes with host haemostatic mechanisms are scarce, and have been limited to the reporting of ATP-diphosphohydrolase activity in the tegument of *S. mansoni*, which could be related to the inhibition of platelet activation (DeMarco et al., 2003), and to the demonstration of thrombocytopenia in infected individuals (Stanley et al., 2003), which could partially explain the lack of blood clot formation on adult worms (Ruppel et al., 2002). Recently, it has also been shown that the tegument

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doi:10.1016/j.vetpar.2010.06.011

antigen Sm22.6 inhibits thrombin activity (Lin and He, 2006).

The interaction between host haemostatic mechanisms and blood parasites such as schistosomes should result in regulatory mechanisms affecting the host-parasite relationship and its outcome. In a previous approach, we began a study of the interaction between *S. bovis* adult worms and the host fibrinolytic system (Ramajo-Hernández et al., 2007). The fibrinolytic system, in which plasminogen plays a key role, has two main functions: namely, blood clot dissolution and extracellular matrix disintegration (Plow et al., 1995). The activation of fibrinolysis could be used by the parasite to avoid clot deposition or to dissolve clots on its surface. Thus, we showed that the tegument extract from adult worms binds and activates plasminogen, resulting in either the presence or absence of tissue plasminogen activator (t-PA) in the generation of plasmin, which in turn degrades fibrin (Ramajo-Hernández et al., 2007). We also showed that plasminogen binds to the tegumental surface of male, but not female, worms, and that this binding is inhibited by aminocaproic acid (εACA), a lysine analogue. The subsequent experiments allowed us to identify 10 plasminogen-binding proteins in *S. bovis*, among them several isoforms of enolase, GAPDH, and actin (Ramajo-Hernández et al., 2007).

Enolase is a multifunctional glycolytic enzyme included in a new group of proteins, called moonlighting proteins, that are present on the surface of several pathogens, although they lack a signal peptide to be secreted or a transmembrane region to be anchored to the surface of cells (Pancholi, 2001; Jefferry, 2009). Enolase has been characterized in detail as a plasminogen receptor in different pathogens – bacteria (Bergmann et al., 2001; Jones and Holt, 2007), fungi (Jong et al., 2003) and protozoa (Vanegas et al., 2007; Mundodi et al., 2008) – and it has been found in *Onchocerca volvulus* tissues (Jolodar et al., 2003), and in *Fasciola hepatica* and *Echinostoma caproni* secretions (Bernal et al., 2004; Marcilla et al., 2007). We have successfully identified eight plasminogen-binding isoforms of enolase in the tegument of *S. bovis*, four of them being immunogenic for *S. bovis*-infected sheep (Pérez-Sánchez et al., 2006; Ramajo-Hernández et al., 2007).

The aim of the present work was to study the physiological role of the enolase found in the tegument of *S. bovis* adult worms as regards plasminogen-binding and activation, and to confirm its presence on the surface of the parasite, including the following objectives: (i) isolation, cloning and sequencing of the *S. bovis* enolase cDNA, (ii) production of the corresponding recombinant protein, (iii) evaluation of the plasminogen-binding and activation activity of the above recombinant enolase, and (iv) study of the expression and localization of native enolase in adult worms and lung schistosomulae, with a view to better understanding the disease and developing control strategies against schistosomiasis.

We show that the *S. bovis* enolase binds plasminogen through lysine residues and enhances plasminogen-activation and plasmin generation induced by t-PA. Additionally, we demonstrate that enolase is expressed on the tegumental surface of adult worms, and hence is in contact with the host's blood.

2. Materials and methods

2.1. Parasite material

A strain of *S. bovis* from Salamanca (Spain) is routinely maintained in our laboratory in *Planorbarius metidjensis* snails, sheep, and golden hamsters. Adult worms and lung schistosomulae were recovered, respectively, from infected sheep and hamsters as explained below.

In order to obtain cercariae, each snail was infected with five miracidia from eggs collected from experimentally infected sheep faeces. The infected snails were maintained under controlled conditions until the emission of cercariae. Sheep were infected percutaneously with 2000 *S. bovis* cercariae by submerging a fore-limb in a suspension of the above-mentioned cercariae for 30 min. At 4 months post-infection, the sheep were sedated with ketamine (10 mg/kg) and then sacrificed by bleeding through the jugular vein. Sera from these animals were kept at −20 °C for further use. Adult *S. bovis* worms were recovered by dissection of the mesenteric vessels from the entire gut. The viability and integrity of the adult worms were assessed microscopically and only intact parasites were processed for further experiments. Tegument and excretory-secretory extracts from adult worms were obtained as described by Pérez-Sánchez et al. (2006).

Lung schistosomulae were isolated using the modified technique of Gui et al. (1995). The hamsters were infected percutaneously by bathing each animal for 1 h with 2000 cercariae per hamster. Six days after the initial infection, the lungs were removed, minced, and incubated in RPMI medium at 37 °C for 2 h on a rocker-shaker. The suspension was sieved and mobile schistosomulae were carefully collected using a fine-tipped glass pipette. The schistosomulae were immediately fixed and stored a 4 °C for immunolocalization studies.

2.2. RNA isolation, RT-PCR, and cloning of SbEno cDNA

Total RNA from adult parasites was extracted using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturers' instructions. First-strand cDNA was synthesized from *S. bovis* adults worms RNA using the first-strand cDNA synthesis kit (Roche) as recommended by the manufacturer. The cDNA sequence of the *S. bovis* enolase (SbEno) was amplified using two degenerate primers: EnoFw (5′-ATGKCMATTWTARCGATYCACG) and EnoRev (5′-TYAKAYTTKRGGATGGCGG). These primers were designed on the consensus sequence resulting after the alignment of enolase cDNA sequences from *S. mansoni* and *S. japonicum* (GenBank accession numbers AAC46886 and AAA29874, respectively). PCR was performed in 35 cycles at 94 °C for 40 s, 42 °C for 40 s, and 72 °C for 1 min 30 s for the 5 first cycles, and 94 °C for 40 s, 48 °C for 40 s, and 72 °C for 1 min 30 s for the remaining 30 cycles.

The PCR product was electrophoresed in an agarose gel and the band was purified from the gel using the StrataPrep DNA Gel Extraction kit (Stratagene) as recommended by the manufacturers. The enolase PCR product was cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene) following the

manufacturer's instructions, and the sequencing of both strands was performed at the Salamanca University DNA sequencing service. BLAST analysis of the sequence was performed at http://blast.ncbi.nlm.nih.gov/. The motifs characterizing enolase were positioned in the corresponding sequence by the server http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (Marchler-Bauer et al., 2009). In addition, the servers http://www.cbs.dtu.dk/services/SignalP (Bendtsen et al., 2004), http://www.cbs.dtu.dk/services/TMHMM-2.0/ and http://gpi.unibe.ch/ were used to search for signal peptides, transmembrane helices or glycosyl-phosphatidyl anchors in the sequence.

2.3. Expression and purification of the rSbEno protein

A PCR fragment containing the whole SbEno coding sequence was obtained using the construction in pSC-A as a template and two primers: SbEnoFw (5′-GGGGATCCATGTCCATTATAGCGATCCACG) and SbEnoRev (5′-GGGGATCCTTATTTTTTAGGATGGCGG). These primers contained the restriction site for *Bam*HI (underlined) to assist subsequent cloning into the expression vector pQE30, which fuses the protein to a hexahistidine peptide in the N-terminal region (Qiagen). PCR was performed in 35 cycles of 94 °C for 15 s, 64 °C for 30 s, and 72 °C for 40 s.

The PCR product was digested with *Bam*HI, and cloned into the pQE30 vector. The recombinant plasmid was transformed into the *Escherichia coli* M15 strain (Qiagen), and expression of the recombinant protein was induced by adding isopropyl β-d-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37 °C for 4 h. The induced cells were harvested and sonicated in a buffer containing 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris-Cl, pH 7.9. After a 20 min centrifugation step at 10,000 g, the supernatant was applied to a His-Bind resin (Novagen) for affinity purification of the histidine-tagged rSbEno, according to the manufacturer's instructions. Urea was eliminated by washing the column with wash buffer (100 mM NaH_2PO_4, and 10 mM Tris-Cl pH 6.3) containing decreasing concentrations of urea (from 6 M to 0 M). Then, the recombinant protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 7.9). The eluted rSbEno was dialysed in PBS for 24 h at 4 °C and stored at −80 °C until use.

2.4. Collection of an anti-rSbEno hyperimmune serum

An antiserum against *S. bovis* enolase was prepared by subcutaneously immunizing two New Zealand female rabbits with three doses of 50 μg rSbEno plus 200 μg of saponin per dose, administered at 15 days intervals. Rabbits were bled 15 days after the last boost. Antibody titres were measured by ELISA following a standard protocol (Astigarraga et al., 1995). The ELISA plates were coated with 0.5 μg/well of rSbEno and rabbit sera were analysed in serial dilutions from 1/100 to 1/3200.

The reactivity and specificity of the sera were also checked by Western blot on rSbEno or on tegument (TG) and excretory-secretory (ES) extracts from adult worms containing the corresponding native protein, as described previously (Pérez-Sánchez et al., 2006). Briefly, samples of TG, ES (10 μg each) and rSbEno (2 μg) were subjected to electrophoresis in a 12% acrylamide gel. After running, the gels were electrotransferred onto nitrocellulose membranes that were then cut into 4 mm strips, blocked with 2% BSA, and incubated with the rabbit anti-rSbENO serum diluted 1/6400 or anti-*S. bovis* serum from infected sheep diluted 1/50. After washing, the strips were incubated with 1/600 diluted peroxidase-conjugated anti-rabbit or anti-sheep antibodies. Finally, the reactive bands were developed using 4-chloro-1-naphthol as chromogen.

2.5. Plasminogen-binding assays

To determine whether rSbEno binds plasminogen, an ELISA was performed. Multiwell plates (Sigma) were coated with 0.5 μg of rSbEno per well diluted in carbonate buffer, pH 9.6, overnight at 4 °C. Non-specific binding sites on the plates were blocked by incubation with 1% BSA in PBS (PBS-BSA). After washing with PBS containing 0.05% Tween 20 (PBST), different wells were incubated with increasing amounts (from 0 μg to 3 μg) of human plasminogen (Acris Antibodies) diluted in PBS-BSA. The wells were then washed and incubated with 0.1 μg/well of a peroxidase-conjugated goat anti-human plasminogen IgG (Cedarlane Laboratories). All incubations were performed for 1 h at 37 °C. Ortho-phenylene-diamine was used as a chromogen substrate for peroxidase. In parallel, competition experiments were carried out by including 40 mM of the lysine analogue εACA during the plasminogen-binding reaction. Positive binding controls consisted of wells coated with 1 μg of TG extract, and negative controls consisted of BSA-coated wells.

To verify the ability of rSbEno to bind plasminogen, rSbEno and human plasminogen (Acris Antibodies) as a positive control were electrotransferred from 12% polyacrylamide gels to nitrocellulose membranes. The nitrocellulose membranes were blocked with 3% BSA in PBS for 1 h at 37 °C, rinsed 3 times with PBST, and incubated overnight at 4 °C with 35 μg/ml of human plasminogen diluted in PBST-1% BSA. After four washes, the blots were incubated for 1 h at 37 °C with 0.5 μg/ml of the above-mentioned peroxidase-conjugated goat anti-human plasminogen IgG diluted in PBST-1% BSA. The blots were revealed with 4-chloro-1-naphthol. Negative controls were also included in which the rSbEno or the plasminogen were omitted.

2.6. Plasminogen-activation assays

Plasminogen-activation was evaluated by measuring the amidolytic activity of the plasmin generated, as described by Mundodi et al. (2008). The assay was carried out in 96-well plates. The reaction mixture in each well contained 2 μg of human plasminogen, 1 μg of rSbEno, 3 μg of plasmin substrate (D-Valyl-L-leucyl-L-lysine 4-nitroanilide dihydrochloride; Sigma), 15 ng tissue-type plasminogen activator (t-PA) (Acris Antibodies), and PBS at a final volume of 150 μl. In parallel, plasmin generation was also measured in the absence of t-PA and in the presence or absence of 40 mM εACA. Plates were incubated at

37 °C for 2 h and the hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm every 30 min. Each sample was analysed in quadruplicate.

2.7. Immunolocalization of enolase in S. bovis adult worms and lung-stage schistosomulae

Confocal microscopy studies were carried out on adult worm sections and on whole adult and schistosomulae mounts. Parasites were fixed in 4% saline formalin for 24 h (adults) or 5 h (schistosomulae).

For assays on parasite sections, the worms were dehydrated and embedded in paraffin. Microtome-cut 5 μm sections were placed on microscope slides, deparaffinised in xylene (2 × 8 min each), and rehydrated. Sections were then blocked with 1% BSA in PBST for 1 h a 37 °C, after which they were incubated with the anti-rSbEno rabbit serum diluted 1:50 in blocking buffer for 1 h at 37 °C. Samples were washed three times with PBST and incubated at 4 °C overnight with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Molecular Probes) diluted 1:400 in blocking buffer containing phalloidin-Alexa Fluor 488 (Molecular Probes) diluted 1:200, which binds to actin microfilaments. The samples were then washed four times and mounted in an antifade reagent (Prolong Gold, Molecular Probes).

A similar protocol was followed for whole-mount assays. The reactions were performed in 1.5-ml test tubes containing 100 schistosomulae or 5 adult pairs each. The parasites were blocked for 2 h at 4 °C, incubated with the anti-rSbEno serum for 3 h at room temperature, and then with the above-mentioned Alexa Fluor reagents at 4 °C overnight. Whole parasites were washed five times and mounted in 90% glycerol in PBS pH 9.0.

In each assay, serum from a non-immunized rabbit was used as a negative control.

3. Results

3.1. Amplification, cloning, sequencing, and expression of S. bovis enolase

Amplification of *S. bovis* enolase cDNA by RT-PCR resulted in a PCR product of around 1300 bp, as expected. This was cloned into the pSC-A vector and fully sequenced. BLAST analysis of the sequence demonstrated its identity as enolase. The new sequence, in which the degenerate positions from the corresponding primers were excluded since they were not validated, was deposited in the GenBank under accession number EU595759.1. The full cDNA contained 1305 nucleotides and encoded a protein of 434 amino acids, with a theoretical molecular weight and pI of 46,796 Da and 6.77, respectively. The protein sequence did not show a signal peptide, transmembrane helices or glycosyl-phosphatidyl inositol anchors. Owing to the potential plasminogen-binding activity of lysine its positions were checked and two carboxy-terminal lysines were found at positions 433 and 434.

A comparison of the amino acid sequence from the *S. bovis* enolase with ten enolase sequences from other *Schistosoma* species, two trematodes, two cestodes and four nematodes is shown in Fig. 1. This comparison shows that enolase is a highly conserved protein in the above-mentioned organisms, with slight changes in size (from 431 to 437 aa) and with percentages of identity with the enolase of *S. bovis* ranging from 93% to 69%, taking into account that degenerate positions defining ambiguous amino acids (lower case letters) were excluded from this calculation. Fig. 1 also shows the conservation in all sequences of the following motifs: (i) the four amino acids binding Mg^{2+} (S^{37}, D^{245}, E^{249} and D^{321}), (ii) the seven amino acids for substrate (2-phosphoglycerate) binding (H^{158}, E^{210}, K^{346}, H^{374}, R^{375}, S^{376} and K^{397}), and (iii) the fourteen amino acids constituting the so-called enolase signature (LLLKVNQIGSLTES, at positions 341–354).

The *S. bovis* enolase cDNA was cloned into the expression vector pQE30. After induction of expression in *E. coli*, the hexahistidine-tagged recombinant protein was purified under denaturing conditions using nickel affinity chromatography. The purified recombinant protein rSbEno had a molecular weight of 48.2 kDa in polyacrylamide gel.

3.2. Plasminogen-binding assays

The ELISA test revealed that rSbEno bound plasminogen and that this binding increased when the plasminogen concentration was increased in the reaction mixture (Fig. 2 A). The TG extract – the positive control – also bound plasminogen, and the negative control – BSA – did not show any non-specific plasminogen or anti-plasminogen antibody binding, as expected. When 40 mM εACA was added to the reaction, the binding of plasminogen to the rSbEno was inhibited by 70% (Fig. 2A).

The ligand blot assay confirmed the ability of rSbEno to bind plasminogen. As shown in Fig. 2B, plasminogen bound to electrotransferred rSbEno, while no non-specific binding of the anti-plasminogen antibody was observed in the control lane, to which neither rSbEno nor plasminogen were added.

3.3. Plasminogen-activation assays

The objective of these assays was to investigate the ability of the rSbEno to influence the plasminogen-activation induced by a physiological activator (t-PA), and also its ability to activate plasminogen by itself. We also included a competition assay with a lysine analogue – εACA – to determine whether this potential activation was mediated by lysine residues in the rSbEno. Fig. 3 shows that the generation of plasmin by t-PA was enhanced by rSbEno (reaction mixture 4), but rSbEno was unable to generate plasmin without t-PA (reaction mixtures 5 and 6). When εACA was included in the reaction together with rSbEno and t-PA, plasmin generation was inhibited by 50% (reaction mixture 3), indicating that the enhancement by rSbEno of t-PA-induced plasminogen-activation relied on lysine residues. The negative control (substitution of rSbEno by BSA) resulted in a lack of effects on plasminogen-activation.

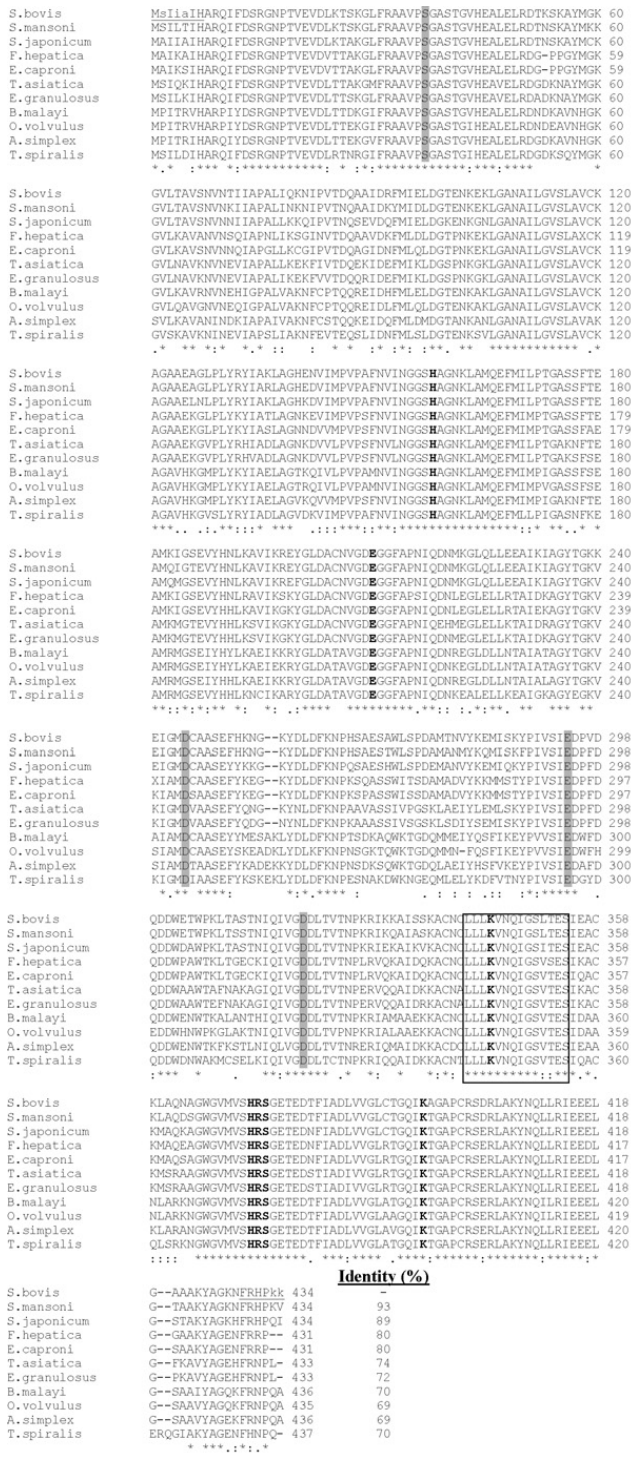

Fig. 1. Alignment of the *S. bovis* enolase sequence (B2LXU1) with enolase from *S. mansoni* (Q27877), *S. japonicum* (P33676), *Fasciola hepatica* (Q27655), *Echinostoma caproni* (CAK47551), *Taenia asiatica* (C9V487), *Echinococcus granulosus* (D0VLV3), *Brugia malayi* (A8PFE3), *Onchocerca volvulus* (Q7YZX3), *Anisakis simplex* (Q8MU59) and *Trichinella spiralis* (Q967U0). The percentage of sequence identity between the *S. bovis* sequence and the others is indicated. The amino acids conserved in all the sequences are labelled with asterisks, and conservative and semiconservative substitutions are labelled with two and one point, respectively. The Mg^{2+} binding motifs are shaded in grey, the enolase signature is boxed, and the motifs for substrate binding are indicated in bold (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The underlined amino acids in the *S. bovis* sequence are those defined by the degenerate primers used for its amplification, and specific amino acids affected by the degenerate positions in the above-mentioned primers are indicated in lower case letters.

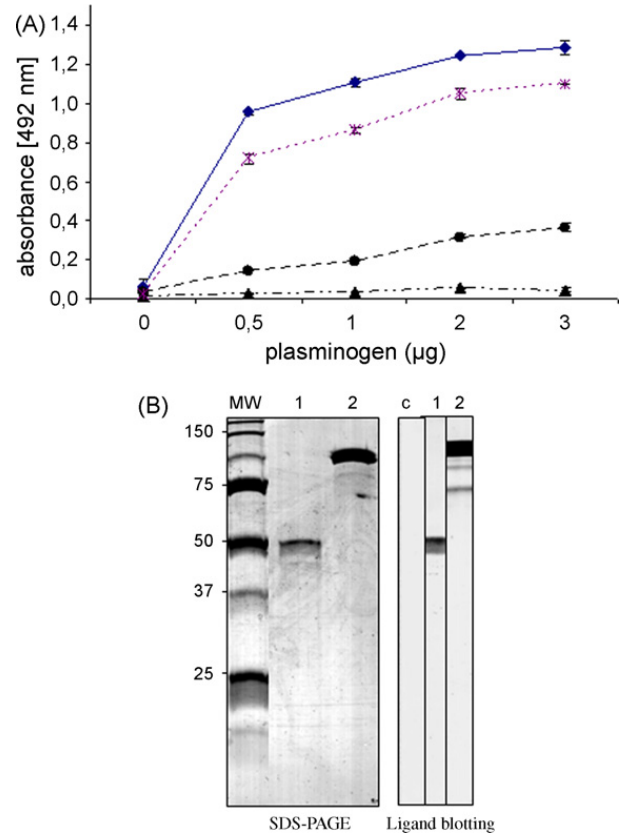

Fig. 2. (A) Plasminogen-binding to 0.5 μg of rSbEno using a microtitre plate method. (♦) Incubation with increasing amounts of plasminogen, 0–3 μg. (●) Competition assay: 40 mM εACA was included during the incubation with plasminogen. (X) Positive control using 1 μg of tegument extract instead of rSbEno. (▲) Negative control without rSbEno or TG, coated only with BSA. Each point is the mean of three replicates ± standard deviation. (B) SDS-PAGE stained with Coomassie blue with 1 μg rSbENO (lane 1) or plasminogen (lane 2). Ligand blotting containing no protein (c, negative control) or the rSbEno (1) or plasminogen (2 positive control), incubated with plasminogen and developed with an anti-plasminogen antibody.

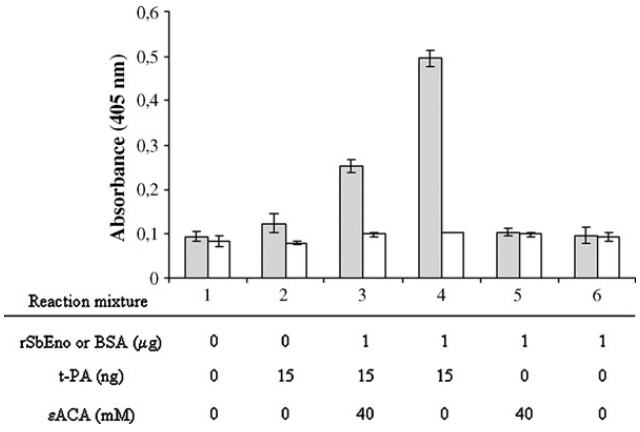

Fig. 3. Plasminogen-activation assay with rSbEno (grey bars) or BSA (negative control, white bars) alone, or together with t-PA and/or εACA. The quantities and combinations of each reagent are shown under the abscissa axis.

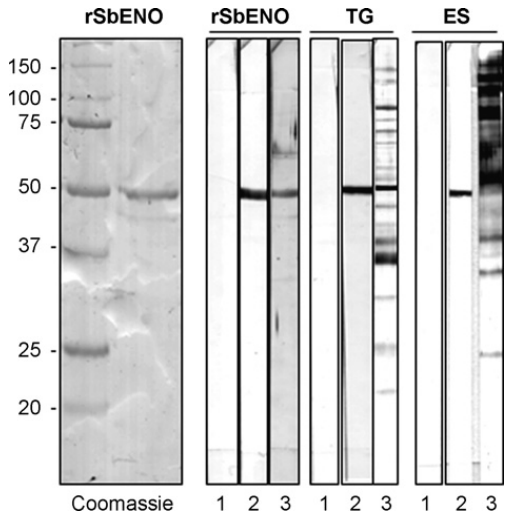

Fig. 4. Immunoblot for study of the specificity of an anti-rSbEno serum on the rSbEno, a tegument extract (TG), and an excretory-secretory product extract (ES) from *S. bovis* adult worms. 1, negative control serum; 2, anti-rSbEno rabbit serum at 1:6400 dilution; 3, serum from a sheep infected with *S. bovis* at 1:50 dilution.

3.4. Specificity of the anti-rSbEno hyperimmune serum

The reactivity and specificity of the hyperimmune serum against rSbEno was tested in ELISA against the recombinant protein prior to its use in the immunolocalization studies. The antibody titre of this serum was higher than 1/3200, with an optical density (OD) of 1.13 at this dilution, while a negative serum showed an OD of 0.03 at the same dilution.

The specificity of the serum was assessed by Western blot against the rSbEno and the TG and ES native extracts from *S. bovis* adult worms. As shown in Fig. 4, the anti-rSbEno reacted strongly with the recombinant protein and specifically recognized the native enolase in the TG and ES extracts. The serum obtained from an infected sheep also reacted with the recombinant protein and with several proteins in the native parasite extracts. The negative serum showed no reactivity with any of the proteins tested.

3.5. Immunolocalization of enolase in adult worms and lung-stage schistosomulae of S. bovis

Paraffin-embedded sections from adult worms incubated with the negative serum plus the phalloidin-Alexa Fluor 495 (an actin ligand) only resulted in green fluorescence (Fig. 5A). In the left image some egg autofluorescence can be observed (arrowhead).

Sections incubated with the specific anti-rSbEno serum plus phalloidin-Alexa Fluor 495 showed actin reactivity (in green) as well as specific enolase reactivity (in red) to be abundantly distributed in internal tissues and also in the most superficial areas of the worm tegument (Fig. 5B).

Whole parasites were also subjected to immunolocalization assays with a confocal microscope, using the same sera and reagents as those used employed for the paraffin sections. The corresponding assays in schistosomulae revealed a lack of specific enolase reaction on their tegumental surface (Fig. 6A), but resulted in a specific reaction on whole adult worms (Fig. 6B). In this figure, an intense green fluorescence due to actin can be observed, together with some red enolase-specific spots on the tegument surface (Fig. 6B 1). At 60×, the presence of enolase on the surface of the adult worms was clearly detected in the protuberances of the tegument and its folds, together with the green actin spines in the protuberances (Fig. 6B 2 and 3).

4. Discussion

Schistosomiasis due to *S. bovis* usually results in a chronic long-lasting disease, adult worms remaining in the host blood vessels for many years. The host rarely shows haemostatic alterations, either thrombotic or haemorrhagic, which is striking taking into account that the mere presence of worms 1.5 cm in length in the vessels should result in the impairment of blood circulation and in thrombotic alterations. In addition, exposure of the bloodstream to a live non-self structure – the tegument of adult worms – should induce platelet activation and aggregation, leading to thrombus formation, as well as the triggering of the specific reactions leading to blood coagulation. None of the above-mentioned phenomena seems to occur in chronic schistosomiasis and hence the worms have become adapted to their intravascular environment, probably by developing mechanisms aimed at modulating the haemostatic system of the host (Wu et al., 2007). Nevertheless, putative schistosome anti-haemostatic molecules have been little studied. We have previously shown that *S. bovis* interacts with the fibrinolytic system of its host through protein receptors expressed on its tegumental surface, proposing that such an interaction would be used by the parasite to avoid blood clot formation on its surface (Ramajo-Hernández et al., 2007).

In the present work, our aim was to check whether the enolase identified in the tegument of adult *S. bovis* worms is involved, as a plasminogen receptor and activator, in interactions with the fibrinolytic system of the host. This meant that we had to demonstrate not only that the enolase binds to and activates plasminogen but also that it is expressed on the surface of the tegument in contact with the host's blood.

The experimental approach used to achieve this objective relied first on the cloning and sequencing of *S. bovis* cDNA and its production as a recombinant protein. The rSbEno was then used for plasminogen-binding and -activation assays, as well as for the collection of a specific hyperimmune serum to determine the tissue expression of the native protein by immunohistochemistry and confocal microscopy of the parasite.

After cloning and sequencing, a deduced polypeptide of 434 amino acids was obtained, showing high sequence identity with enolases from other schistosomes – *S. mansoni* (93%) and *S. japonicum* (89%) – and helminths (ranging from 80% to 69%). This confirms the high degree of enolase conservation, like other glycolytic enzymes, in different organisms (Pancholi, 2001).

Similar to what has been described for other enolases, the *S. bovis* enzyme did not show motifs for its transport or expression at the cell surface (signal peptide, transmembrane motifs, or GPI anchors). This and other proteins have

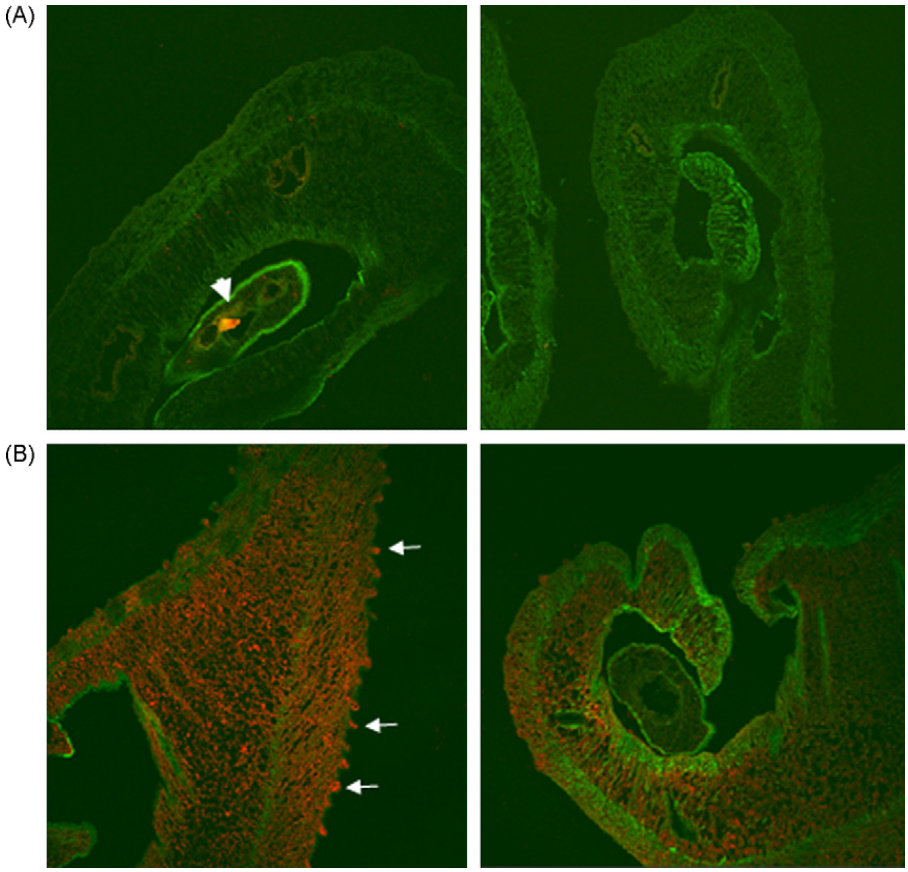

Fig. 5. Detection of enolase in sections from *S. bovis* adult worms. (A) Images of parasite sections incubated with phalloidin (reactivity in green) and a negative rabbit serum. Arrowhead indicates non-specific red autofluorescence due to the presence of parasite eggs. (B) Images of parasite sections after incubation with phalloidin (in green) plus an anti-rSbEno rabbit serum (in red). Arrows indicate the presence of enolase at the surface of the parasite tegument (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

been included in a new category of surface-located proteins that reach their location through a hitherto unknown mechanism (Pancholi, 2001; Pancholi and Chhatwal, 2003), but one which could be associated with secretion and re-association with surface structures (Bergmann et al., 2001). This re-association of secreted proteins without typical membrane anchors seems to be a general phenomenon and has been observed for a number of other such proteins in bacteria (Pancholi and Chhatwal, 2003). Nevertheless, there is no evidence of the existence of such a mechanism in schistosomes.

The ability of the rSbEno to bind and activate plasminogen was studied using ELISA and Western blot. We were able to demonstrate that the recombinant enolase binds plasminogen, like the enolase from other parasites (Avilan et al., 2000; Almeida et al., 2004; Marcilla et al., 2007; Mundodi et al., 2008). We were also able to show that this binding is mediated by lysine residues in the rSbEno, as described previously for enolase and for other plasminogen receptors, in which the main plasminogen-binding motifs consist of carboxy-terminal lysine residues (Plow et al., 1995; Bergmann et al., 2001; Jones and Holt, 2007). Recently, Bergmann et al. (2003) reported that enolase from *Streptococcus pneumoniae* has an additional internal plasminogen-binding motif (FYDKERKVYD). *S. bovis* enolase does not show this motif, and hence the protein could bind plasminogen through lysines at positions 433 and 434, although further studies should be performed to rule out the presence of additional internal binding motifs in rSbEno.

We have also shown that rSbEno enhances the generation of plasmin induced by the physiological plasminogen activator t-PA, like the TG extract from whole worms. However, the TG extract is also able to activate plasminogen by itself (Ramajo-Hernández et al., 2007), while this activity was lacking in rSbEno. This suggests that on its tegument *S. bovis* displays proteins other than enolase that participate in the activation of the host fibrinolytic system. The presence of several plasminogen receptors represented by different molecules in a given species has been already demonstrated for other pathogens (Xolalpa et al., 2007).

As mentioned, a protein that binds plasminogen in schistosomes could be of *in vivo* relevance when expressed in contact with host blood on the surface of the parasite (Hawley et al., 2000). Enolase has been localized in the tegument and tegumental membranes from *S. mansoni* adult worms, and in tegumental extracts from *S. bovis* (Van Balkom et al., 2005; Braschi et al., 2006; Pérez-Sánchez et al., 2006). However, the fact that it forms part of the outer cover of the parasite does not imply that the protein is exposed on the surface. Until now, the only data concerning the expression of enolase on the tegumental surface of schistosomes come from

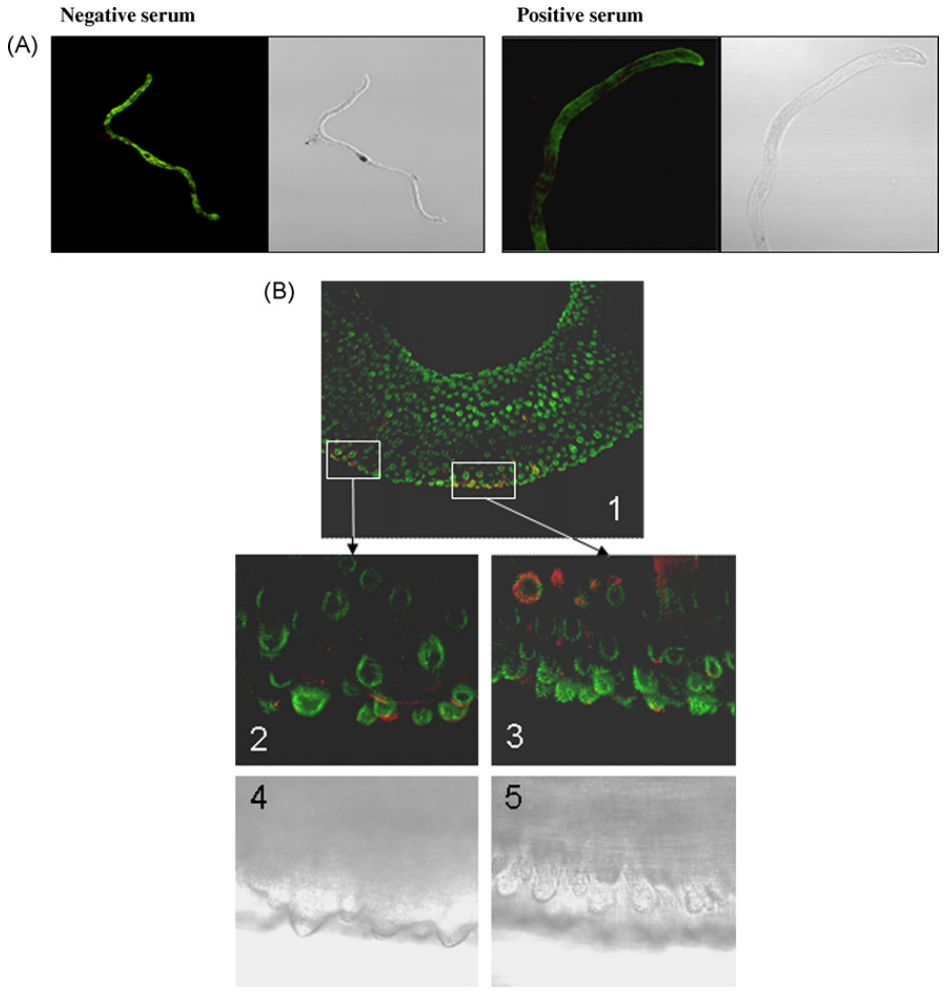

Fig. 6. Detection by confocal microscopy of *S. bovis* enolase in whole parasites. (A) schistosomula images after incubation with phalloidin (in green) plus the negative or positive (anti-rSbEno) rabbit sera. B, the reactivity of adult worms was also assessed with the anti-rSbEno serum, resulting in a specific reactivity shown in red with the 10× objective in (1), and in greater detail (60× objective) in (2) and (3). The corresponding transmitted light images can be seen in (4) and (5) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

the work of Mulvenna et al. (2010) on *S. japonicum* and that of Pérez-Sánchez et al. (2008) on *S. bovis*

Mulvenna et al. (2010) purified the surface proteins from *S. japonicum* adult worms after biotin labelling, achieving the identification of 54 proteins, among them enolase. Nevertheless, those authors pointed out that although enolase is putatively exposed on the surface, this cytosolic protein may have been pulled down by protein-protein interactions with *bona fide* membrane proteins during incubation of the solubilised tegument with streptavidin. Further, limited tegumental damage may have led to the biotinylation of internal proteins. In *S. mansoni*, using a similar approach Braschi and Wilson (2006) failed to identify enolase.

In *S. bovis,* enolase has previously been identified in a tegument extract from adult worms and also in an enriched fraction of the surface-most proteins from the tegument of male worms (Pérez-Sánchez et al., 2006, 2008). Its tegumental expression was also evidenced by immunofluorescence and confocal microscopy using a commercial anti-enolase antibody (Pérez-Sánchez et al., 2008). With a view to checking those results and performing a more detailed study, here we obtained a specific serum against the rSbEno protein. This serum was also reactive against the native enolase in TG and ES extracts. We were also able to show that the sera from experimentally infected sheep reacted with the rSbENO, as expected, since we had already shown that infected animals recognize four native isoforms of the parasite enolase (Pérez-Sánchez et al., 2006).

The immunofluorescence study performed here revealed that enolase is localized in the cytoplasm of *S. bovis* cells, as expected for a glycolytic enzyme, but also on the outermost surface of adult worms, but not schistosomulae, demonstrating its direct contact with the blood of its host.

In sum we have shown that *S. bovis* male adult worms express enolase at their tegumental surface and that this protein binds host plasminogen and enhances its activation in the presence of the host-specific serine protease t-PA. This suggests that the surface-associated enolase found here is a physiological receptor of plasminogen that is involved in the activation of the host fibrinolytic system, most probably to avoid blood clot formation on the worm surface.

Further and more in-depth studies addressing this and other parasite molecules involved in anti-haemostatic

mechanisms could be of great help for the definition of new therapeutic and vaccine targets in schistosomes.

Acknowledgements

This research was funded by project AGL2007-60413/GAN, granted by the Spanish Ministry of Science and Innovation. E. de la Torre-Escudero is a holder of a Ministry of Science and Innovation predoctoral grant.

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Veterinary Parasitology 184 (2012) 25–36

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Molecular and functional characterization of a *Schistosoma bovis* annexin: Fibrinolytic and anticoagulant activity

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ARTICLE INFO

Article history:
Received 3 May 2011
Received in revised form 26 July 2011
Accepted 8 August 2011

Keywords:
Annexin
Schistosoma bovis
Plasminogen
Anticoagulant activity
Tegument

ABSTRACT

Annexins belong to an evolutionarily conserved multigene family of proteins expressed throughout the animal and plant kingdoms. Although they are soluble cytosolic proteins that lack signal sequences, they have also been detected in extracellular fluids and have been associated with cell surface membranes, where they could be involved in anti-haemostatic and anti-inflammatory functions. Schistosome annexins have been identified on the parasite's tegument surface and excretory/secretory products, but their functions are still unknown. Here we report the cloning, sequencing, *in silico* analysis, and functional characterization of a *Schistosoma bovis* annexin. The predicted protein has typical annexin secondary and tertiary structures. Bioassays with the recombinant protein revealed that the protein is biologically active *in vitro*, showing fibrinolytic and anticoagulant properties. Finally, the expression of the native protein on the tegument surface of *S. bovis* schistosomula and adult worms is demonstrated, revealing the possibility of exposure to the host's immune system and thus offering a potential vaccine target for the control of schistosomiasis in ruminants.

1. Introduction

Schistosomiasis is a parasitic disease affecting man and domestic and wild animals that represents an important health and veterinary problem in many tropical and subtropical areas of the world. *Schistosoma bovis* is a cosmopolitan trematode of ruminants that can produce significant economic losses in endemic areas (Vercruysse and Gabriel, 2005). In addition, studies on *S. bovis* are interesting from the perspectives of both veterinary and human medicine because this species represents the genetic and immunological analogue of the important human pathogen *Schistosoma haematobium* (Agnew et al., 1989).

Like all schistosome species, *S. bovis* adult worms can survive in host blood vessels for many years. In order to achieve such long survival times, parasites have molecules that allow them to modulate immune and haemostatic host responses to their own benefit (Pearce and MacDonald, 2002; Mountford, 2005; Secor, 2005; Ramajo-Hernández et al., 2007; De la Torre-Escudero et al., 2010). It is well known that a significant part of schistosome evasion mechanisms are achieved by the parasite's inner and outer tegument surface (Abath and Werkhauser, 1996). The tegument, besides the gut, constitutes one of the most important host–parasite interchange surface, playing a role in the parasite's nutrient uptake, excretion, osmoregulation, sensory reception, signal transduction, and interaction with the host's immune and haemostatic systems (Jones et al., 2004; van Hellemond et al., 2006; Ramajo-Hernández et al., 2007; De la Torre-Escudero et al.,

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doi:10.1016/j.vetpar.2011.08.013

2010). Thus, the tegument is a key parasite compartment to be mined for target molecules in the development of new anti-schistosome vaccines and drugs. With this aim, in the last years numerous investigations have focused on the identification and characterization of molecules expressed by schistosomes in their tegument and surface membranes (van Balkom et al., 2005; Braschi et al., 2006; Braschi and Wilson, 2006; Pérez-Sánchez et al., 2006, 2008; Skelly and Wilson, 2006; Mulvenna et al., 2010a; Castro-Borges et al., 2011). As a result, annexins have been one of the molecules frequently identified in the tegument of schistosomes.

Annexins are Ca^{2+}- and phospholipid-binding proteins that form an evolutionarily conserved multigene family, its members being expressed throughout the animal and plant kingdoms. Structurally, annexins are characterized by a highly α-helical and tightly packed protein core domain, considered to represent a Ca^{2+}-regulated membrane-binding module. Human annexins, designated A1–A13 (except annexin A12, which is unassigned), have been implicated in a broad range of biological processes such as membrane trafficking and fusion, plasma membrane repair, anticoagulation, interaction with cytoskeletal proteins and signal transduction (Gerke and Moss, 2002; Moss and Morgan, 2004; Draeger et al., 2011). These soluble cytosolic proteins lack signal sequences that direct them to the classical secretory pathway. Nevertheless, some members of the family have consistently been identified in extracellular fluids. Binding sites for extracellular annexins exist on the cell surface and several possible functions for these proteins have been proposed. They include a role of annexin A5 as an anticoagulant protein, a function of annexin A2 as an endothelial cell-surface receptor for plasminogen and tissue-type plasminogen activator (t-PA), and the anti-inflammatory activities of annexin A1 (Hajjar and Krishnan, 1999; Gerke and Moss, 2002; Cederholm and Frostegård, 2007).

Regarding schistosome annexins, several proteomic studies have identified them on the tegument surface of *Schistosoma mansoni* (Braschi and Wilson, 2006; Castro-Borges et al., 2011) and *Schistosoma japonicum* (Mulvenna et al., 2010a), as well as in the excretion-secretion products of *S. bovis* (Pérez-Sánchez et al., 2006). Moreover, in a recent survey of draft genomes for *S. mansoni* and *S. japonicum* (http://www.schistodb.org), Hofmann et al. (2010) identified 14 annexins from *S. mansoni*, 6 from *S. japonicum* and 1 from *S. haematobium*. Of these, it is known that schistosome annexins 1, 3 and 5 are expressed on the tegument of the adult parasite (Braschi and Wilson, 2006; Mulvenna et al., 2010a; Castro-Borges et al., 2011). Hofmann et al. (2010) reported that annexins are particularly noteworthy as surface-associated molecules of adult schistosomes and are likely to be an abundant surface-related molecule of digeneans such as the human liver fluke, *Opistorchis viverrini*, which also expresses abundant surface annexins (Mulvenna et al., 2010b). The physiological roles of these schistosome annexins are still unknown although it has been speculated that they could play important roles in surface maintenance, such as by ensuring the integrity of the membrane-membranocalyx complex (Braschi and Wilson, 2006).

Unveiling the functions of schistosome annexins, and particularly whether they exhibit extracellular activities such as those reported for the annexins of other organisms (i.e., anticoagulant and fibrinolytic activities), is important as these extracellular activities could be vital for schistosome development and survival. Accordingly, the aims of the present work were to determine the physiological role of the annexin of *S. bovis* adult worms regarding its potential fibrinolytic and anticoagulant activities and to demonstrate its expression on the parasite surface at the host–parasite interface.

2. Material and methods

2.1. Parasite material

The life cycle of *S. bovis* was maintained at the laboratory by routine passage through sheep, golden hamsters, and the intermediate snail host *Planorbarius metidjensis*. Adult worms and lung schistosomula were recovered, respectively, from infected sheep and hamsters as described in De la Torre-Escudero et al. (2010). Briefly, lambs were infected with 2000 cercariae and 4 months later they were sedated with 10 mg of ketamine per kg of live weight and sacrificed by bleeding through the jugular vein.

Adult worms were recovered from mesenteric veins and washed in warm phosphate buffered saline (PBS) pH 7.2 at 37 °C. Worms were inspected microscopically to verify their integrity and vitality, and immediately processed for RNA extraction, the collection of a tegument extract (TG), and immunolocalization studies. The tegument extract was obtained as described by Ramajo-Hernández et al. (2007).

Lung schistosomula were obtained from hamsters, following the method of Gui et al. (1995). The animals were infected through the skin with 1000 cercariae by bathing them individually (in a solution containing the cercariae) for 1 h. Six days after infection, the hamsters were euthanatized and their lungs were removed, minced and incubated in RPMI medium at 37 °C for 2 h on a rocker-shaker. The suspension was sieved and live, intact schistosomula were collected with a 20 μl pipette. After three washes in warm PBS, they were fixed in 4% formalin and stored at 4 °C until use.

Animal experimentation was done according to the rules from the ethical and animal welfare committee from the institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. RNA isolation, RT-PCR and cloning

Total RNA from adult worms was isolated using the NucleoSpin RNA II kit (Macherey-Nagel), following the manufacturer's instruction, and preserved at −80 °C. Reverse transcription was performed from total RNA using the first Strand cDNA Synthesis kit (Roche). For PCR amplification of *S. bovis* annexin cDNA, primers were designed from the *S. mansoni* and *S. japonicum* annexin sequences (GenBank AF065599 and AY813612, respectively). The forward primer (ANXFw, 5′-ATGGCYAAWRTTTCTGRATTTGG) was designed from the *S. mansoni* and *S. japonicum* annexin

consensus sequence. This primer was used in two amplifications with two different reverse primers designed, respectively, from the *S. mansoni* and *S. japonicum* annexin sequences (ANXRev1, 5′-TTATTGTTCTTCATTATATATTTC; ANXRev2, 5′-TTATTCACCTAGACCACATAATG).

PCR amplifications were performed in 35 cycles of 94 °C for 40 s, 42 °C for 40 s, and 72 °C for 1 min 30 s for the 5 first cycles, and 94 °C for 40 s, 48 °C for 40 s, and 72 °C for 1 min 30 s for the remaining 30 cycles. PCR products were electrophoresed in agarose gel and the cDNA band corresponding to the ANXFw/ANXRev2 amplification was purified from the gel using the StrataPrep DNA Gel Extraction kit (Stratagene). This PCR product was cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene), following the manufacturer's instruction, and sequenced on both strands. At least three different clones of the insert were sequenced to check for errors caused by PCR amplification.

2.3. Bioinformatic analysis

The deduced amino-acid sequence of *S. bovis* annexin (SbANX) was analysed as follows: BLAST searching of the most similar sequences were conducted in the Swissprot/Uniprot and SchistoDB databases (http://www.uniprot.org/ and http://www.genedb.org/Homepage/Smansoni); analysis of conserved domains was performed using SMART at http://smart.embl-heidelberg.de; theoretical isoelectric point (p*I*) and molecular weight (MW) calculations at http://www.expasy.org/tools/pi_tool.html; prediction of transmembrane helices using the TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM-2.0; prediction of signal peptides with SignalP 3.0 (Bendtsen et al., 2004) at http://www.cbs.dtu.dk/services/SignalP, and search for glycosyl-phosphatidyl anchors in the sequence with the big-PI Predictor (Eisenhaber et al., 2000) at http://mendel.imp.ac.at/sat/gpi/gpi_server.html.

The amino acid sequence and secondary structure of the SbANX were compared to those of four parasite and human annexins known to possess fibrinolytic or anticoagulant activities, namely, the *Taenia solium* B1 and B2 (ANX B1, ANX B2) and the human A2 and A5 (ANX A2, ANX A5). Multiple sequence alignment of all these five annexins was done with ClustalW 2.1 at http://www.ebi.ac.uk/Tools/msa/clustalw2/. For the prediction of the secondary structures and three-dimensional modelling, the amino acid sequences were submitted to the Swiss-Model server (Arnold et al., 2006) at http://swissmodel.expasy.org/. The 3-D models were visualized using the Pymol package (DeLano, 2002).

2.4. Expression and purification of recombinant annexin and polyclonal antibody production

The full-length cDNA sequence of SbANX was subcloned into the expression vector pQE-30 (Qiagen) and expressed in *Escherichia coli* M15 cells (Qiagen). For this, two new primers were designed from the *S. bovis* complete annexin sequence that contained KpnI adapters to assist in the subclonning (sense primer; 5′-GGGGTACCATGGCTAATGTTTCTGAATTTGG; antisense primer, 5′-GGGGTACCTTATTCACCAAGTAGAACAC) (underlined letters represent the restriction enzyme sites). The complete cDNA coding sequence of *S. bovis* annexin was amplified from the SbANX-pSC-A construction. PCR amplification was accomplished under the following conditions: 35 cycles of 94 °C for 15 s, 64 °C for 30 s and, 72 °C for 40 s. The amplified product was purified using the DNA Gel extraction kit (Stratagene) and digested with KpnI restriction endonuclease (Roche). After digestion, the PCR product was purified again from agarose gel and ligated to the KpnI predigested pQE-30 vector. This construct was used to transform *E. coli* M15 cells. Single recombinant clones were selected and plasmid DNA extracted and sequenced to confirm the presence and the correct orientation of the SbANX cDNA insert.

Expression was then induced in correctly transformed *E. coli* M15 cells by adding isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37 °C for 3 h. The induced cells were harvested and lysed by sonication in a lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.9. After a 15 min centrifugation step at 40,000 × *g*, the lysate pellet was solubilised in binding buffer containing 100 mM NaH_2PO_4, 10 mM Tris–Cl, and 8 M urea, and recentrifuged as above. The new supernatant was passed through His-Bind resin (Qiagen) according to the manufacturer's instructions. Before elution of the recombinant, urea was eliminated by washing the column with wash buffer (100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 6.3) containing decreasing concentrations of urea (6 M, 4 M, 2 M). Then, the recombinant protein was eluted with elution buffer (250 mM imidazole, 0.5 M NaCl, 50 mM NaH_2PO_4, pH 7.9). The eluted rSbANX was dialysed against PBS for 24 h at 4 °C and stored at −80 °C until use. The concentration of the recombinant protein was measured using the DC Protein assay kit (Bio-Rad) and its purity was checked by SDS-PAGE.

Once the recombinant protein had been obtained, and in order to have a specific probe for immunofluorescence studies, a polyclonal rabbit serum against *S. bovis* annexin (rSbANX) was obtained. To accomplish this, two rabbits (New Zealand) were immunized subcutaneously with three doses of 100 μg of rSbANX, together with Freund's adjuvants. The first dose was administered with the complete adjuvant, the second one with incomplete adjuvant and the third one only in PBS. The rabbits were bled at 15 days after the third dose and the antibody titre was determined by ELISA, using a standard protocol (Oleaga-Pérez et al., 1994).

The reactivity and specificity of the polyclonal serum was tested by Western blotting against rSbANX and a tegument extract (TG) from adult worms following the protocol described by De la Torre-Escudero et al. (2010). In this case the anti-rSbANX rabbit serum was used at a dilution of 1/1600 and the peroxidase-conjugated anti-rabbit antibody (Sigma) was diluted at 1/1000.

2.5. Bioassays with rSbANX

2.5.1. Plasminogen binding assays

The binding of rSbANX to plasminogen was assessed by ELISA in a similar way to that described for the *S. bovis*

enolase (De la Torre-Escudero et al., 2010). Briefly, plate wells were coated with 0.5 μg of rSbANX and non-specific binding sites were blocked with 1% BSA in PBS (PBS-BSA). After washing, the different wells were incubated with increasing amounts (from 0 to 3 μg) of human plasminogen (Acris Antibodies) diluted in PBS-BSA. After a new wash, the wells were incubated with peroxidase-conjugated goat anti-human plasminogen IgG (Cedarlane Laboratories) diluted 1/2000 in PBS-BSA and finally orthophenylenediamine (OPD) was used as a chromogenic substrate for peroxidase. The plate included negative control wells, coated only with BSA, and positive control wells, coated with 1 μg of TG extract.

To assess whether plasminogen binding to rSbANX occurs through lysine residues, a similar assay was performed in which the plate wells were coated with 0.5 μg of rSbANX and incubated with 0.5 μg of plasminogen and increasing concentrations (from 0 to 60 mM) of ε-aminocaproic acid (εACA, Sigma). εACA is a lysine analogue that competitively inhibits plasminogen from binding to its receptor. Then, bound plasminogen was developed by incubating with peroxidase-conjugated goat anti-human plasminogen IgG and OPD. Similarly, BSA-coated wells were used as negative controls.

2.5.2. Plasminogen activation assays

The effect of rSbANX on plasminogen activation and plasmin generation was evaluated by measuring the amidolytic activity of newly generated plasmin on the chromogenic substrate D-Valyl-L-leucyl-L-lysine 4-nitroanilide dihydrochloride (Sigma). This assay was performed on ELISA plates according to the protocol described previously (Mundodi et al., 2008).

In each plate well, 2 μg of plasminogen, 3 μg of chromogenic substrate, 1 μg of rSbANX, and 15 ng of tissue-type plaminogen activator (t-PA) (Acris antibodies) were mixed in a total volume of 150 μl of PBS and incubated at room temperature. In a parallel assay, plasmin generation was measured in the absence of t-PA, in the absence of rSbANX, and substituting annexin by BSA or by the buffer in which the rSbANX had been dissolved. Plasmin generation was monitored by quantifying the hydrolysis of the substrate through absorbance measurements at 405 nm every 30 min over 2 h as from the time the reaction began. Each reaction was analysed in quadruplicate and the whole assay was repeated three times.

2.5.3. Prothrombin time and activated partial thromboplastin time of rSbANX

The anticoagulant activity of rSbANX was analysed in two coagulation tests for general screening such as the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) tests. The PT and aPTT tests respectively detect disturbances in the extrinsic and intrinsic coagulation pathways. These tests measure the time elapsed between the addition of the corresponding coagulation initiation reagent (STA neoplastin plus or STA aPTT; Roche Diagnostics) to the plasma sample and clot formation. The PT results can also be expressed as a percentage of the normal activity.

To carry out these tests, plasma samples were obtained from lamb blood extracted in polypropylene tubes containing sodium citrate. The samples analysed consisted of 500 μl of plasma with increasing amounts of rSbANX (from 0 to 80 μg/ml). Two series of control samples were included in the analysis: one series contained BSA instead of rSbANX, and the other one contained no protein but equivalent volumes of the buffer in which the rSbANX had been dissolved.

Each sample was analysed in duplicate and the assays were repeated twice. Samples were analysed with the STA-R Evolution® analyser (Diagnóstica Stago, Inc.).

2.6. Immunolocalization of annexin in adult worms and lung-stage schistosomula

Tissue expression of annexin was determined in adult worms and schistosomula by indirect immunofluorescence and later analysis by confocal microscopy. The study was carried out on sections of adult worms, on whole adult worms, and on 6-day-old lung schistosomula. The worms were fixed in formaldehyde buffered at 4% for either 5 h (schistosomula) or 24 h (adults).

For assays on adult sections, the worms were dehydrated and embedded in paraffin following standard protocols. Microtome-cut 5-μm sections were placed on microscope slides, deparaffinised in xylene, and rehydrated. The sections were then blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBST) for 1 h at 37 °C, after which they were incubated with the anti-rSbANX rabbit serum diluted 1/50 in blocking buffer for 1 h at 37 °C. Samples were washed three times with PBST and incubated at 4 °C overnight with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Invitrogen) diluted 1/400 in blocking buffer containing phalloidin-Alexa Fluor 488 (Invitrogen) diluted 1/200, which binds to actin microfilaments. The samples were then washed four times and mounted in antifade reagent (Prolong Gold, Invitrogen). All incubations were performed in a humid chamber.

A similar protocol was followed for whole-mount assays. The reactions were performed in 1.5-ml test tubes containing 100 schistosomula or 5 adult pairs each. Fixed parasites were blocked for 2 h at room temperature, incubated with the anti-rSbANX serum overnight at 4 °C, and then with the above-mentioned Alexa Fluor reagents, both at a 1/300 dilution, for 4 h at room temperature. Whole parasites were washed five times and then mounted in antifade reagent (schistosomula) or in PBS (adult worms). In each assay, serum from a non-immunized rabbit was used as a negative control. Samples were analysed with a Leica TCS-NT confocal microscope.

2.7. Statistics

The results of the PT and aPTT coagulation tests were first analysed with the one-way ANOVA test, and when significant differences were found a *post hoc* analysis with the HDS Turkey test was conducted to determine the causes of the significance. A similar procedure was applied to check whether there were any significant differences as a function of the amount of rSbANX added to the plasma samples

and, if so, to establish which rSbANX concentration provided significantly different results.

3. Results

3.1. Cloning and in silico characterization

The ANXFw/ANXRev2 primer pair amplified a 1080 bp cDNA fragment compatible with the expected size for a schistosome annexin and an open reading frame coding for a protein of 359 amino acids, with a predicted MW and p*I* of 40,663 Da and 5.85, respectively (GenBank accession number EU595758).

BLAST searching of the SWISS-Prot database with the deduced amino acid sequence of SbANX retrieved numerous annexins from different species. *S. bovis* annexin showed the highest identity percentages with the *S. mansoni* (Q9XY89) and *S. japonicum* (AAW25344) annexins (91% and 86%, respectively). Similarly, BLAST searching of the SchistoDB database retrieved several annexin sequences including the 14 annexins sequences of *S. mansoni* reported by Hofmann et al. (2010). Among them, the sequence showing the highest identity (91%) was Sm_074150, which corresponds to sequence Q9XY89 from the SWISS-Prot database; the remaining 13 sequences retrieved showed lower identities (from 37% to 23%).

Regarding non-schistosome helminths, SbANX showed the greatest identity to *Taenia solium* ANX B1 and B2 (42% and 36%, respectively). Finally, SbANX showed between 37% and 23% sequence identity to human annexins A1–A13, in particular 37% to ANX A5 and 32% to ANX A2.

Fig. 1A shows the alignment of SbANX with *T. solium* B1 and B2 and human A2 y A5 annexins. The SbANX contained a 309-amino acid core domain and a 50-amino acid tail at its N-terminus. The core domain contained the four typical annexin repeats and each repeat harboured type II and III Ca^{2+}-binding sites (Fig. 1A, yellow-shaded and underlined residues, respectively). Type II calcium-binding sites were characterized by the M-K/R-G/R-X-G-T-(38 residues)-D/E sequence motif, and type III sites by the G-X-G-T-D/E sequence motif. In SbANX, each annexin repeat contained between 52 and 60 amino acid residues, mapping in the following sequence segments: 51–103, 123–183, 229–281 and 304–356 amino acid. The *E*-values for the four annexin repeats ranged from $2.70e^{-20}$ to $113e^{-04.}$ (Fig. 1A).

The SbANX contains the KGLGT sequence motif within repeat II of the core domain as *T. solium* ANX B1 and human A2 annexins. In human A2 annexin, this motif and the aspartic acid located at position162 (D^{162}) appear to be required for the interaction of annexin with cell surface phospholipids (Fig. 1A) (Hajjar and Krishnan, 1999).

The SbANX also had the ^{100}LCQL^{103}/^{114}SL^{115} sequence motif, which showed 50% identity with the ^{8}LCKLSL^{13} sequence motif of human ANX A2. This motif is responsible for the interaction between human ANX A2 and t-PA (Hajjar et al., 1998). A similar motif (^{38}LCK^{40}/^{69}SL^{70}) is also displayed by the *T. solium* ANX B2. In Fig. 1B, the above-mentioned motif is highlighted only in the 3D model of SbANX, but not in that of ANX A2 because this molecule was modelled from the 32nd amino acid onwards not including the N-terminus fragment in with the motif is located.

SbANX and *T. solium* ANX B1 and B2 have an insert fragment in the linker region between repeats II and III, which is absent from human annexins (Fig. 1A and 1B).

In silico secondary structure prediction revealed that SbANX was mainly made up of α-helices although four beta sheets are also included. The model does not predict secretory signal, transmembrane helices or glycosylphosphatidyl inositol anchors. The secondary and tertiary structures of these annexins are highly conserved even though their amino acid sequence identity only ranges between 32% and 42% (Fig. 1). The alignment in Fig. 1A shows that the four typical annexin repeats and the predicted α-helices match in the five sequences. In Fig. 1B it can be observed that the four repeats are packed in a structure that resembles a flattened disc, with a slightly convex surface on which the Ca^{2+}-binding loops are located, together with a concave surface where the amino and carboxyl termini come into close opposition.

3.2. Expression and purification of recombinant S. bovis annexin

The full length SbANX cDNA was sub-cloned into the expression vector pQE-30 and transformed into M15 *E. coli* host cells. The his-tagged recombinant protein (rSbANX) was expressed abundantly, although it was 100% insoluble. After solubilisation and purification under denaturing conditions, 5.7 mg of protein per litre of cell culture was obtained. The purified rSbANX migrated as a single band of 39 kDa by SDS-PAGE.

3.3. Plasminogen binding and activation assays

The plasminogen-binding assays showed that plasminogen bound to rSbANX and that the amount of plasminogen bound increased with the rising amount of plasminogen added to the reaction medium. Likewise, in the plate wells in which tegument extract (TG) was included as a positive control in the assay the binding of the plasminogen to such proteins was observed. In the negative control wells, coated only with BSA, unspecific binding of plasminogen did not occur (Fig. 2A). Competition experiments with εACA revealed that plasminogen binding occurred through lysine residues, and that 10 mM of εACA completely inhibited the binding of plasminogen to rSbANX (Fig. 2B).

Fig. 3 shows the results of the assays on plasminogen activation, in which it may be seen that rSbANX enhanced plasminogen activation in the presence of t-PA. It can also be observed that rSbANX was unable to activate plasminogen and generate plasmin in the absence of t-PA. In the controls, in which rSbANX was replaced by BSA or by buffer, no reactivity was observed (not shown).

3.4. Anticoagulant activity

To investigate the anticoagulant effect of rSbANX, we evaluated the inhibition of blood clotting in PT and aPTT assays (Fig. 4A and B).

Both assays revealed significant differences ($p < 0.01$) between the samples with rSbANX and the controls with

A

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SbANX          MANVSEFGITRSLIHAFDPHGKHYTP-----TIKPTTGFSASADAERLHRAMKGPGTDET 55
ANX_B1_Ts      ------MAYCRSLVHLYAPNGEKYKP-----TITPTPGFSPTADAEHLKRAMRGLGTNER 49
ANX_B2_Ts      -----MAKNTRSPSQYFDCNGKPFRP-----TLKPNPNFDVNADVEALCKSMRCWGTDEE 50
ANX_A2_Human   ------MSTVHEILCKLSLEGDHSTPPSAYGSVKAYTNFDAERDALNIETAIKTKGVDEV 54
ANX_A5_Human   ------MAQVLR-----------------GTVTDFPGFDERADAETLRKAMKGLGTDEE 36
                                             ::.  ..*.   *.  :  :::  *.:*

                                Annexin repeat I
SbANX          AIINILARRTNYERQELCRSYKSLYKHDLKDDLKSETSGDFRKVLCQLVVDTPYMLAKSL 115
ANX_B1_Ts      AIIDILGNRTSAERMAIRDAYPSISSKTLHDALTSELSGKFRRFALLLIQSPWQVMAEAL 109
ANX_B2_Ts      TITKILGKRTSEERLQIVSLYKQKYGRELAHDLDGDLKGHFRDCTILLTEDPIYLMAKSL 110
ANX_A2_Human   TIVNILTNRSNAQRQDIAFAYQRRTKKELASALKSALSGHLETLILGLLKTPAQYDASEL 114
ANX_A5_Human   SILTLLTSRSNAQRQEISAAFKTLFGRDLLDDLKSELTGKFEKLIVALMKPSRLYDAYEL 96
               :*  :*  *:. :*  :   :     : *   * .  .*.:.     *   .    *  *

                                Annexin repeat II
SbANX          YYAMKGLGTNDRVLIEIFTTLWNDETRAVADAYKQVLKDKGIEESERSLVTDMKKEISGD 175
ANX_B1_Ts      YDAMKGAGTKERVLNEIIAGCSKDDIPQLKKAFEEVSGGETLDDA-------IKGDTSGD 162
ANX_B2_Ts      YYAMKGVGTNENTIIEIIVGCTNEEINKLKQFYIYVLRDKGIKDPKRTLETDIRTETTGY 170
ANX_A2_Human   KASMKGLGTDEDSLIEIICSRTNQELQEINRVYKEMYKTD--------LEKDIISDTSGD 166
ANX_A5_Human   KHALKGAGTNEKVLTEIIASRTPEELRAIKQVYEEEYGSS--------LEDDVVGDTSGY 148
                 ::** **.:  : **:     ::   :   :      .            :  : :*

SbANX          YEYALLCLVQADRDEIPVLQLKAIPEKGINSIINHELAEADAKDLYASGVGRVGTSEKRI 235
ANX_B1_Ts      YREALLLALAGQADEPQAMQLKNLTPSTLSQVVNPGLAETDAKELYACGEGRPGTAESRF 222
ANX_B2_Ts      FCKMLLQLLKGDIPDPTPEQLRTIQQKGGDLMVNQKEVTAAVKQIVEALAKPKNSTNSVL 230
ANX_A2_Human   FRKIMVALAKGR-------------RAEDGSVIDYELIDQDARDLYDAGVKRKGTDVPKW 213
ANX_A5_Human   YQRMLVVLLQAN-------------RDPD-AGIDEAQVEQDAQALFQAGELKWGTDEEKF 194
               :   ::    .                        ::       .: :  .     .:

                                Annexin repeat III
SbANX          TRVICNRTPYQLYLTSEIYFKMYGKTLLEHIESETSGDYRKLLVAILRYAIDRPGLIAEW 295
ANX_B1_Ts      MRPIVNRSFLQLNATNEAYNRAYGHPLIDAIKKETSRDLEDFLITRVRYATDRASLFAEL 282
ANX_B2_Ts      LNAFQHKNVWEIAAMDKEYKKASGKGLISAISEAVEGEFGTLLMAMVQHAVDRPKFYSEA 290
ANX_A2_Human   ISIMTERSVPHLQKVFDRYKSYSPYDMLESIRKEVKGDLENAFLNLVQCIQNKPLYFADR 273
ANX_A5_Human   ITIFGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIRSIPAYLAET 254
                  :  :.  .:    . *      : . *   .. :    ::  ::   . .   ::

                                Annexin repeat IV
SbANX          LHDSMAGLGTKDYAIMRLLITRSEIDLQDIMNPYESIYGKSLLNAVIDDTSGDYRRTLCV 355
ANX_B1_Ts      LHFAMRGAGTKDSTLQRVLALRADTDLGSIKEKYAELYGETLEAAIKGDTSGDYEALCLK 342
ANX_B2_Ts      LYQSMVGQGTRDFLIMRVLILRSEIDLLDIKETFDKDH-KSLAEWIKGETSGYYEQLLLA 349
ANX_A2_Human   LYDSMKGKGTRDKVLIRIMVSRSEVDMLKIRSEFKRKYGKSLYYYIQQDTKGDYQKALLY 333
ANX_A5_Human   LYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGDYKKALLL 314
               *: :* * ** *  * *::  *:: *: .* . :   .  :*   :  :*.* *.

               Identity (%)
SbANX          LLGE— 359      -
ANX_B1_Ts      LIGPA- 347     42
ANX_B2_Ts      LLNES- 354     36
ANX_A2_Human   LCGGDD 339     32
ANX_A5_Human   LCGEDD 320     37
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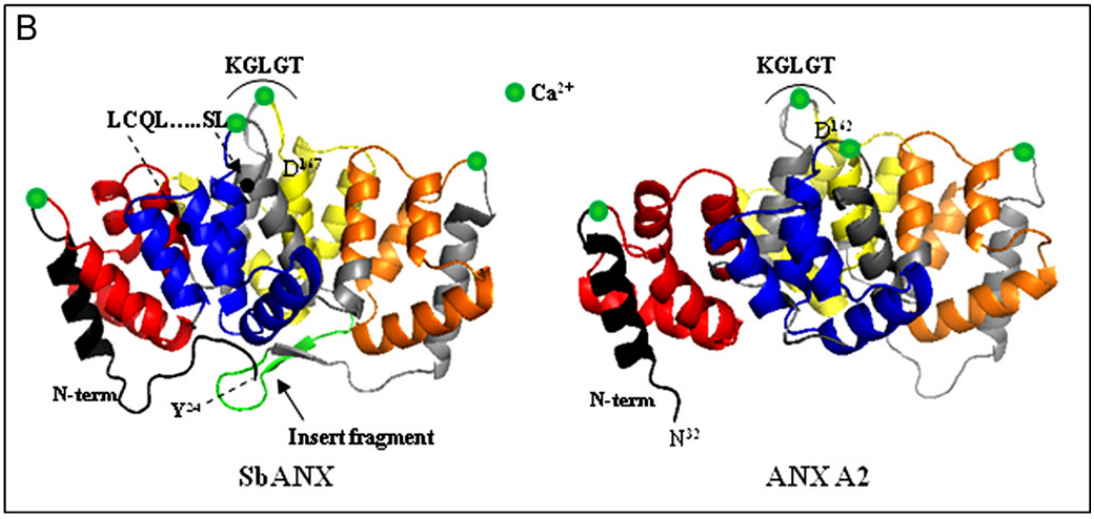
B



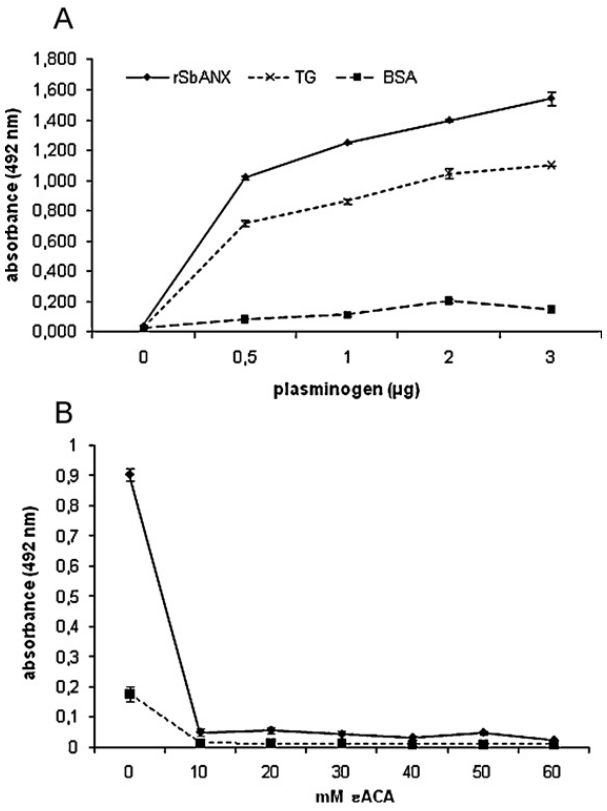


Fig. 2. (A) Plasminogen-binding assay to 0.5 μg of rSbANX and incubation with increasing amounts of plasminogen, 0–3 μg (♦). Positive control using 1 μg of tegument extract (TG) instead of rSbANX (×). Negative control coated with BSA (■). (B) Competition assay. Wells coated with 0.5 μg of rSbANX (♦) or BSA (■) were incubated with 0.5 μg of plasminogen and increasing amounts of εACA. Each point is the mean of three replicates ± standard deviation.

BSA or buffer. Additionally, there were no significant differences between either type of control sample (those containing BSA or buffer).

The coagulant activity in the PT assay was lower in the plasma samples with rSbANX than in the controls and clearly decreased as the concentration of annexin increased. This difference was statistically significant ($p < 0.01$) in the samples containing a concentration of 80 μg/ml of annexin with respect to the controls without it. Likewise, coagulation time increased in parallel with the concentration of rSbANX. This increase in coagulation time was significant at rSbANX concentrations equal to or higher than 60 μg/ml (Fig. 4A).

The anticoagulant effect of rSbANX was considerably stronger in the aPTT assay. In this assay, the coagulation time was significantly longer ($p < 0.01$) as from an rSbANX concentration of 10 μg/ml in plasma (Fig. 4B).

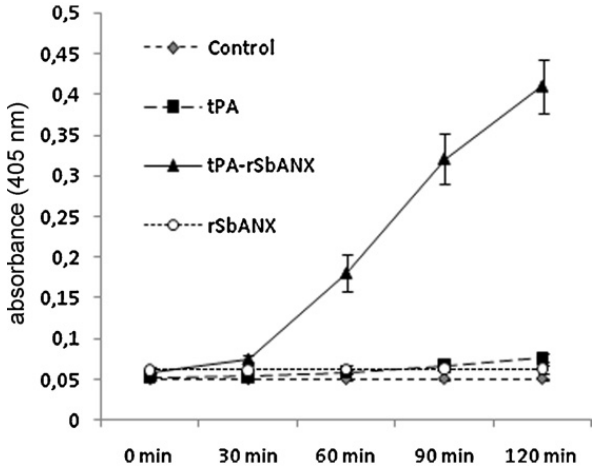


Fig. 3. Plasminogen activation assay with rSbANX or t-PA alone, rSbANX together with t-PA, and the negative control with the reaction substrate alone. Each point is the mean of four replicates ± standard deviation. The experiments were performed three times.

3.5. Reactivity and specificity of the anti-rSbANX rabbit hyperimmune sera

The reactivity of the anti-rSbANX hyperimmune serum was tested in ELISA against the recombinant protein prior to its use in the immunolocalization assays. The antibody titre was 1/1600. The specificity of the serum was assessed by Western blotting against the rSbANX and the native TG extracts from *S. bovis* adult worms. As shown in Fig. 5, the anti-rSbANX serum reacted strongly with the recombinant protein and specifically recognized the native annexin in the TG extract. The negative serum showed no reactivity with any of the proteins tested.

3.6. Immunolocalization of S. bovis annexin

Annexin localization in the parasite was analysed in paraffin sections from adult worms, as well as in intact adult worms and 6-day-old lung schistosomula.

The schistosomula incubated with the negative rabbit serum plus phalloidin (an actin ligand) only resulted in green fluorescence, indicating a lack of non-specific reactivity. The schistosomula incubated with the specific anti-rSbANX serum plus phalloidin showed actin reactivity in green, as well as the specific annexin reactivity (red) distributed on the tegument surface (Fig. 6). This figure shows the images obtained at each of the laser wavelengths – 594 (red) and 488 (green) – since the merged image was difficult to interpret due to the co-localization of the specific reaction and the actin fluorescence.

Fig. 1. (A) Alignment of the SbANX (ACC78610) with annexins B1 (AAD34598) and B2 (AAY17503) from *Taenia solium* and human A2 (AAH09564) and A5 (NP_001145). The conserved amino acids are labelled with asterisks; the conservative and semi-conservative substitutions are labelled with two and one points, respectively. Predicted α-helices are indicated in grey and β-sheets in green. The four-repeat domains of the annexin sequence are indicated with a horizontal line over the conserved zone. The type II Ca^{2+}-binding sites are shaded in yellow, and the type III Ca^{2+}-binding sites are underlined. The t-PA-binding motif of human A2 annexin and other similar motifs identified in the SbANX and in the *T. solium* B2 annexin are in red. The phospholipid-binding motif of the cell surface identified in the human A2 annexin is boxed in. (B) Molecular modelling of *S. bovis* annexin and human A2 annexin. The variable N-terminal end is in black; and the annexin repeat domains I, II, III and IV are in red, yellow, orange and blue respectively. The insert fragment in the linker region between repeats II and III is in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

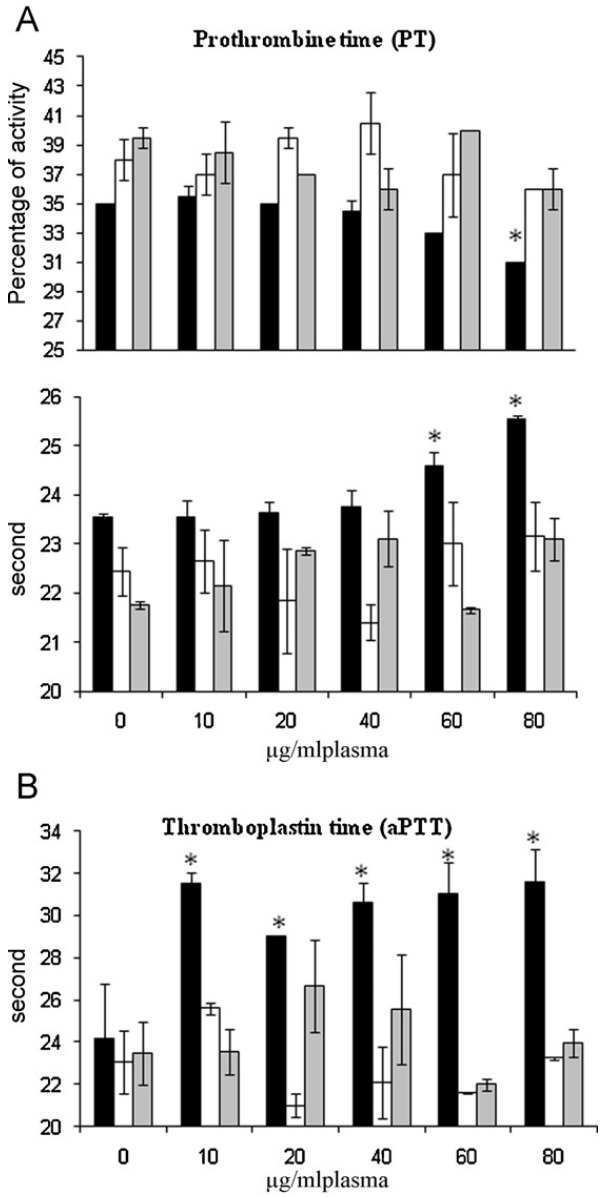


Fig. 4. Coagulation assays in plasma samples with increasing concentrations of rSbANX (0–80 μg/ml). (A) Prothrombin time assay (PT) expressed in time and percentage of activity. (B) Activated partial thromboplastin time (aPTT). (■) rSbANX, (□)BSA and (▭) buffer. Each point is the mean of two replicates ± standard deviation. The experiments were performed two times. $^{*}p < 0.001$.

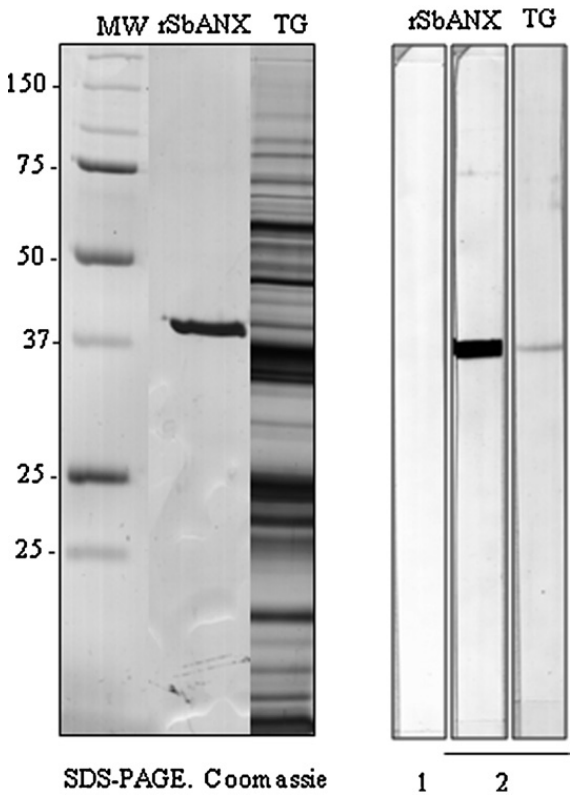


Fig. 5. Western blot for study of the specificity of an anti-rSbANX serum on the rSbANX and a tegument extract (TG) from *S. bovis* adult worms. (1) Negative control sera; (2) anti-rSbANX rabbit serum at 1:1600 dilution.

Paraffin-embedded sections from adult worms incubated with the negative serum plus phalloidin resulted in green fluorescence on the outermost part of the tegument. Sections of male and female worms incubated with the specific anti-rSbANX serum plus phalloidin showed besides the actin reactivity in green the specific annexin reactivity, in red, abundantly distributed in internal tissues and also in the tegument surface. In male tegument, the red signal appears as discontinuous patches (arrows) and in female tegument, apparently, as a more continuous signal (Fig. 7A and B).

Whole adult worms incubated with negative serum only showed the green florescence from actin. By contrast, adult worms incubated with the anti-rSbANX serum showed a red fluorescence pattern on the outermost part of the tegument. In males this pattern consisted of low abundant scattered tiny patches, and in females these patches were similarly distributed but more abundant (Fig. 7C).

4. Discussion

It is well known that schistosomes have adapted to the intravascular habitat of their hosts by developing mechanisms to modulate the haemostatic response. However, despite the relevance of the molecules involved in the biology of the parasite and their potential interest as vaccine antigens, these haemostatic molecules have aroused little attention.

This prompted us to undertake studies of the interaction of *S. bovis* with the host's haemostatic system. In previous work, we observed that adult worms activate the fibrinolytic system, presumably as a strategy to prevent the formation of clots around them, and that they did this through protein receptors for plasminogen expressed on the surface of their tegument, one of which has been determined to be enolase (Ramajo-Hernández et al., 2007; De la Torre-Escudero et al., 2010). Here we wished to determine whether the annexin of *S. bovis* possesses any anti-haemostatic activity. In this sense, for some time it has been known that other annexins, such as the human A2 and A5 and the B1 and B2 annexins of *T. solium* show anticoagulant and fibrinolytic activities (Hajjar et al., 1998; Gerke and Moss, 2002; Wang et al., 2006; Winter et al., 2006). So we cloned and sequenced the *S. bovis* annexin

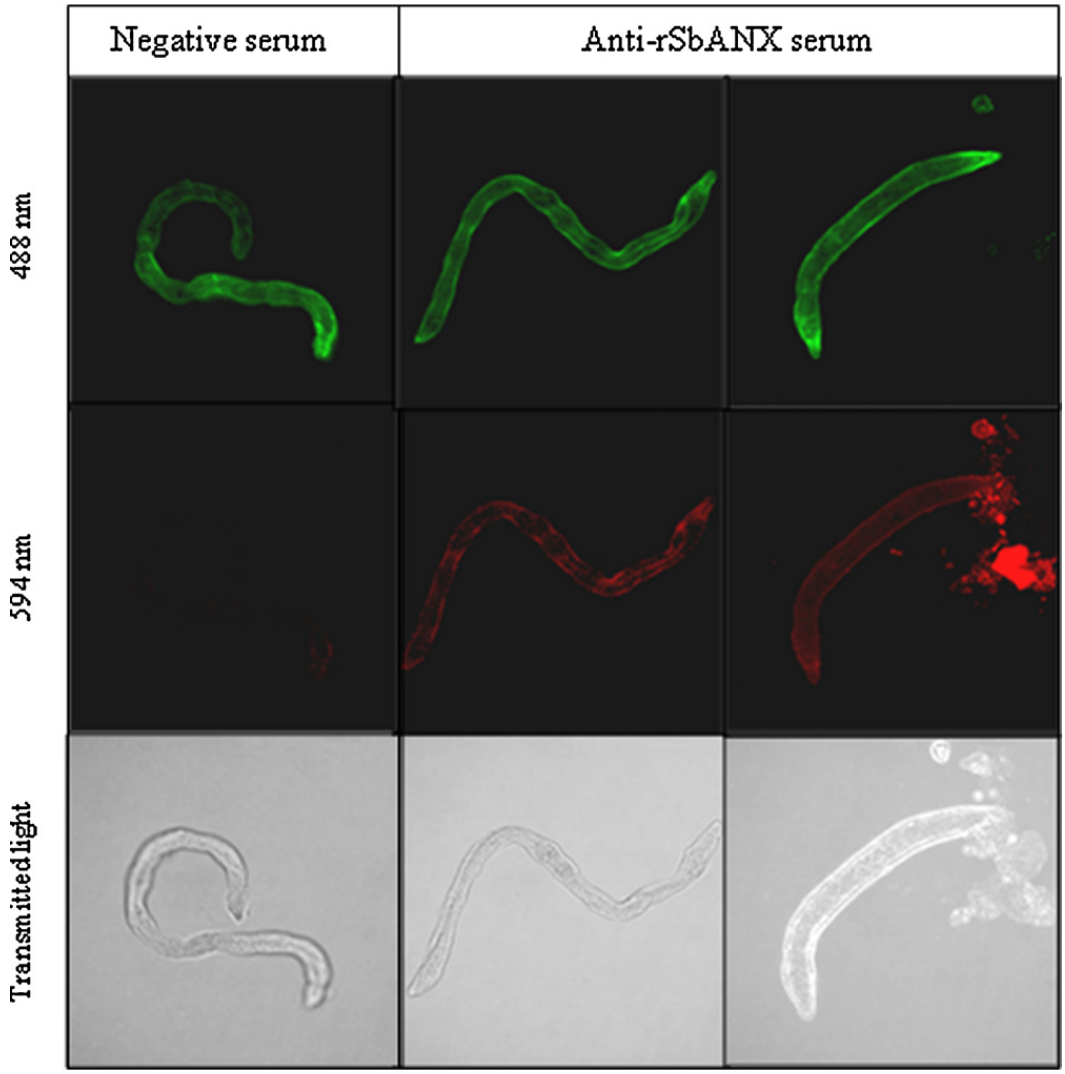


Fig. 6. Immunolocalization of *S. bovis* annexin in lung-schistosomula. Confocal microscope images of schistosomula after incubation with phalloidin-Alexa Fluor 488 (green) plus the negative or the anti-rSbANX rabbit sera and an anti-rabbit IgG-Alexa Fluor 594 (red). Magnification 600×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cDNA and obtained a peptide sequence of 359 amino acids that displayed structural characteristics typical of the annexin family. That is, a core domain containing four typical annexin repeats characterized by alpha-helices and a variable N-terminal region (Gerke and Moss, 2002). In this way, we confirmed the fact, already observed in vertebrate annexins, that the secondary and tertiary structures of annexins are highly conserved despite not showing high amino acid sequence identity among themselves (Moss and Morgan, 2004).

One particularity of the SbANX sequence, that human annexins do not have, is the long linker region between repeats II and III. This characteristic is also seen in other schistosome annexins and in those of *T. solium* (Wang et al., 2006; Hofmann et al., 2010; Tararam et al., 2010). As reported by Hofmann et al. (2010), these unique structural features combined with the immunogenic properties of several parasite annexins and unique epitopes not present in mammalian annexins may provide an opportunity to pursue these antigens as vaccine targets while preventing cross-reactivity with host annexins.

Sequence analysis also revealed that SbANX lacks motifs for its transport or expression on the cell surface (signal peptide, transmembrane motif or GPI anchors), even though it is present on the surface of schistosomula and adult worms. This is not an isolated phenomenon since the same occurs with other annexins, and in particular with human A2 annexin, which is constitutively expressed on the endothelial cell surface and the mechanism of its export from the cell is unknown. It has been described that human annexin A2 interacts with cell surface phospholipids via a calcium-dependent binding site that includes ^{119}KGLGT^{123} residues and the coordinating D^{162} of core repeat 2 (Hajjar and Krishnan, 1999). In the *S. bovis* annexin we found the same motif in residues 120–124 (^{120}KGLGT^{124}) as well as a D^{167}. It is therefore possible that this motif could be responsible for the interaction between the *S. bovis* annexin and the phospholipids of the plasma membrane at the surface of the tegument of the worms, although confirmation of this point requires additional studies.

The plasminogen binding and activating assays indicated that the *S. bovis* annexin, like the human A2 annexin

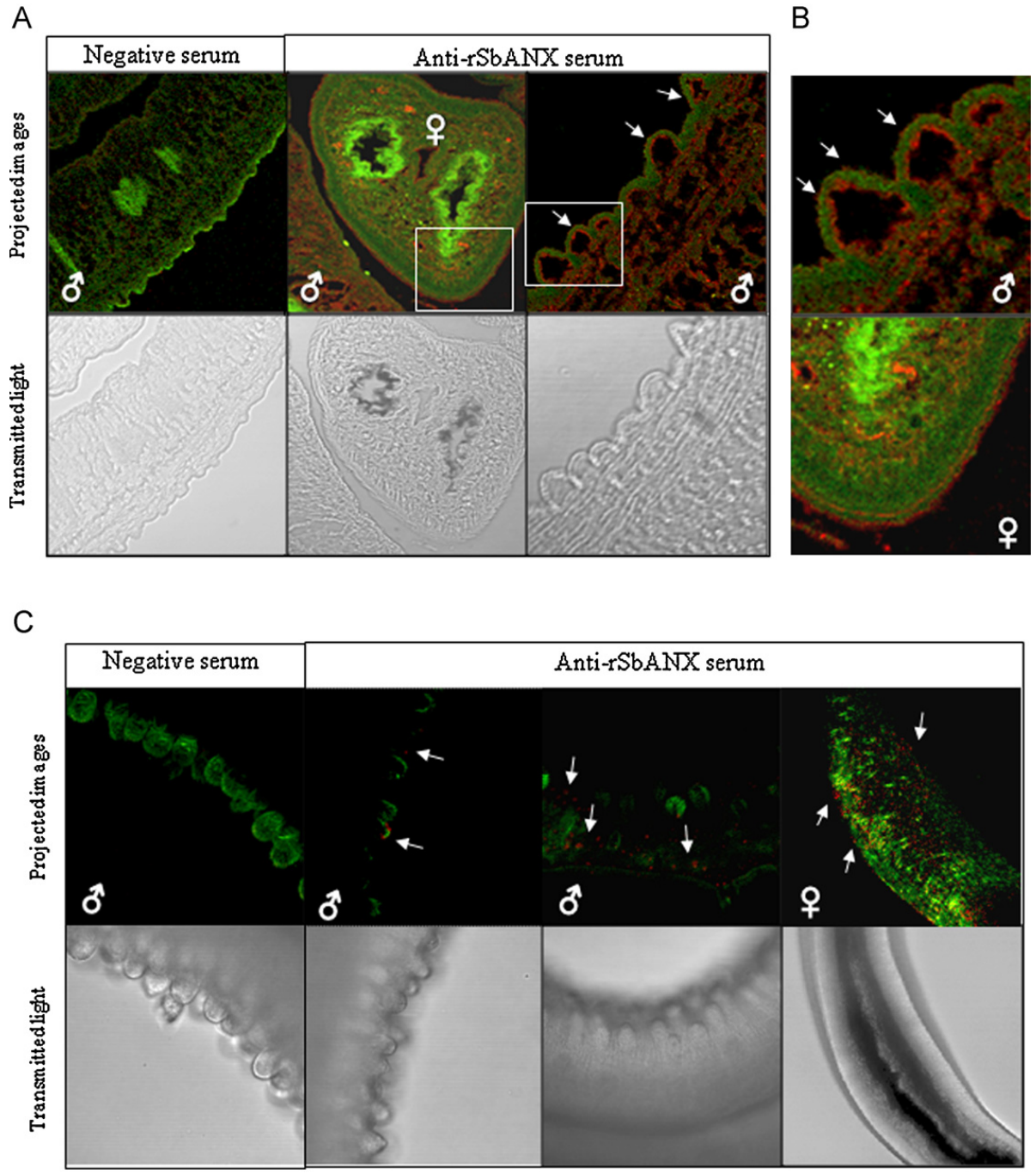


Fig. 7. Immunolocalization of *S. bovis* annexin in adult worms. (A) Images of parasite sections, (B) magnification of the boxes highlighted in (A) showing the male and female tegument, and (C) images of whole parasites. Both, the sections and whole parasites were incubated with phalloidin-Alexa Fluor 488 (green) plus the negative or the anti-rSbANX rabbit sera and an anti-rabbit IgG-Alexa Fluor 594 (red). The upper row of panels (A) and (C) shows the merged projected images derived from sections generated by the confocal microscope and the lower row shows the corresponding transmitted light images. Magnification 600×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Hajjar et al., 1998), had fibrinolytic activity. It was observed that rSbANX binds plasminogen through lysine residues and that it enhanced t-PA mediated plasminogen activation. This is the first time that profibrinolytic activity has been demonstrated in an annexin of parasite origin, and particularly in a schistosome species.

Human A2 annexin interacts with t-PA through the ^{8}LCKLSL^{13} motif present at the N-terminal end (Hajjar et al., 1998). In the rSbANX sequence, a similar motif has been identified with an identity of 50% (^{100}LCQL^{103}/^{114}SL^{115}). However, despite this similarity it does not seem likely that this motif would be responsible for the interaction with t-PA since, as seen from the three-dimensional model of rSbANX, it is localized in a zone of the molecule that is not very accessible (Fig. 1B).

The A5 extracellular annexin shows anticoagulant properties that depend on its Ca^{2+}-regulated binding to anionic phospholipids; those exposed on the surface of activated platelets or endothelial cells. In the presence of Ca^{2+} annexin competitively binds to phospholipids with very

high affinity and disturbs the form of some activated coagulation complexes (Gerke and Moss, 2002). Regarding this type of activity, in known parasite annexins anticoagulant properties have only been demonstrated for the annexins B1 and B2 of *T. solium* (Wang et al., 2006). In *S. bovis*, the results of the PT and aPTT assays clearly demonstrate that rSbANX inhibits the extrinsic coagulation pathway and, more strongly, the intrinsic pathway. Inhibition of the extrinsic coagulation pathway was achieved with high concentrations of rSbANX (60–80 μg/ml), which were higher than those of *T. solium* annexin B2 producing the same effect (40 μg/ml); by contrast, inhibition of the intrinsic pathway was achieved with quite lower rSbANX concentrations (10 μg/ml) as compared to *T. solium* annexin B1 (60 μg/ml) (Wang et al., 2006).

Thus, this would be the first demonstration of anticoagulant activity by an annexin from a Schistosoma species. Additionally, bearing in mind the different functions of vertebrate annexins, it cannot be ruled out that *S. bovis* annexin could have additional activities, in particular some type of immunomodulatory activity, as has been demonstrated for the B1 *T. solium* annexin (Gao et al., 2007; Yan et al., 2008).

It is evident that for these anticoagulant and fibrinolytic activities of SbANX to have relevant physiological roles, the SbANX should be exposed on the surface of the worm into contact with the host blood. As reported in different studies addressing the tegument of schistosomes, annexin has been identified on the tegument surface of adult worms and schistosomula of *S. mansoni* and S. *japonicum* (Braschi and Wilson, 2006; Tararam et al., 2010; Castro-Borges et al., 2011). The SbANX immunolocalization studies performed in adults and schistosomula of *S. bovis* indicate that both developmental stages express this protein on the surface of their tegument. The schistosomula of *S. bovis* shows an annexin expression pattern similar to that of *S. japonicum* schistosomula, whereas on the surface of *S. bovis* adults the annexin is expressed less abundantly than in *S. japonicum* adult worms (Tararam et al., 2010). Additionally, the SbANX described in this work is homologous to Smp_074150. This *S. mansoni* annexin was detected by mass spectrometry of material released by trypsin shaving of live *S. mansoni* (Castro-Borges et al., 2011), providing additional support to the notion that SbANX, as Smp_074150, might also be surface-located.

Bearing in mind these results, it is somewhat surprising that annexin, despite being expressed on the surface of the tegument and also found in the secretion-excretion products, should not be immunogenic in natural infections by *Schistosoma* (Mutapi et al., 2005; Pérez-Sánchez et al., 2006). It would be interesting to test if vaccination with this protein will lead to its recognition by the host immune system allowing the evaluation of its protective potential against infections in ruminant schistosomiasis.

In sum, here we have cloned, sequenced and characterized a *S. bovis* annexin. We show that, *in vitro,* the corresponding recombinant protein displays fibrinolytic and anticoagulant activities; that the native protein is expressed on the tegument surface of adult worms and schistosomula, and that it is therefore in direct contact with the host's blood. All this suggests that this annexin could be used by *S. bovis*, together with other tegument proteins of proven fibrinolytic activity, such as enolase, to prevent thrombus formation and other haemostatic disturbances that could be lethal for the survival of the parasite in the bloodstream. The anti-haemostatic activities observed, and other potential immune modulatory functions make the *S. bovis* annexin and its homologues promising antigenic targets for development of new anti-schistosome vaccines.

Acknowledgements

This research was funded by project AGL2007-60413/GAN, granted by the Spanish Ministry of Science and Innovation. E. De la Torre-Escudero is holder of a Ministry of Science and Innovation predoctoral grant and R. Manzano-Román is funded by the JAEDoc program (CSIC-FSE).

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Veterinary Parasitology 190 (2012) 530–540

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Molecular cloning, characterization and diagnostic performance of the *Schistosoma bovis* 22.6 antigen

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ARTICLE INFO

Article history:
Received 24 January 2012
Received in revised form 14 June 2012
Accepted 19 June 2012

Keywords:
Schistosoma bovis
22.6 antigen
Diagnosis
Cattle

ABSTRACT

Animal schistosomiasis caused by *Schistosoma bovis* is a veterinary problem in many areas of the world. It affects a large number of animals and causes important economic losses in livestock production. The 22.6 kDa antigen is a tegumental protein of unknown function, restricted to schistosomes. In *S. bovis* it has been identified in the tegument and in an excretion–secretion extract, consisting of several, non-glycosylated isoforms that are recognised by the sera of animals infected with *S. bovis*. The aims of the present work were to clone, sequence, express and characterize at molecular level the *S. bovis* 22.6 antigen (Sb22.6), as well as to assess the usefulness of the corresponding recombinant protein as a diagnostic antigen in ELISA tests for the detection of free-range cattle farms infested with *S. bovis*. Immunolocalization studies revealed that Sb22.6 is expressed in the tegument and some internal tissues of the adult worms, but it is not exposed on the surface of the adult worms and schistosomula. The reactivity of the recombinant Sb22.6 (rSb22.6) in ELISA against antibodies in sera from *S. bovis* experimentally infected hamsters and sera from free-range cattle from a *S. bovis* endemic area showed that the recombinant protein and the soluble extract of adult worms (SbC) exhibited a similar diagnostic performance. In addition, rSb22.6 did not show cross-reactions with antibodies against *Fasciola hepatica*, also a frequent trematode parasite in cattle. The rSb22.6 antigen can be readily produced in large amounts and in a highly reproducible fashion, avoiding the types of problem that arise upon using crude extracts such as the SbC. In conclusion, this protein represents a promising epidemiological tool for the surveillance of *S. bovis* and may help to implement control measures in the areas and farms were the parasite is present.

1. Introduction

Schistosomiasis is a disease caused by trematodes of the genus *Schistosoma* that may affect both human beings and animals and it is an important public health and veterinary problem in many tropical and subtropical areas of the world. There are three main species that parasitize humans—*Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium*, and at least another 10 that parasitize domestic ruminants, among which *Schistosoma bovis* is outstanding owing to its pathogenicity (Vercruysse and Gabriel, 2005).

S. bovis, closer phylogenetically to *S. haematobium* (Agnew et al., 1989), lives in the mesenteric veins of its host. The parasite is found in whole Africa and south western Asia, where it has been estimated that there are 165 million of infected animals. In Europe it is present in the Mediterranean islands and in Spain (Moné et al., 1999). In endemic areas, most *S. bovis* infections are subclinical, although more severe outcomes may occasionally occur,

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http://dx.doi.org/10.1016/j.vetpar.2012.06.023

leading to animal death. The subclinical infections cause important economic losses in livestock farms as a result of their long-term effects on animal growth and productivity and also because they increase the susceptibility of infected animals to other parasitic and bacterial diseases (De Bont and Vercruysse, 1998).

The diagnosis of this infection can be accomplished by direct techniques, such as the detection of parasite eggs in excreta and the detection of parasite-derived material in blood, but also by indirect techniques such as those based on the detection of schistosome-specific antibodies (Jin et al., 2010). More recently, molecular techniques that use the polymerase chain reaction (PCR) have been developed, showing high sensitivity and specificity in the diagnosis of human-affecting schistosomes, although they have some disadvantages, such as their high cost and technical complexity (Rabello et al., 2002; Sandoval et al., 2006a, 2006b).

Coprology-based diagnosis is the most frequently used approach, having important advantages over other procedures, such as its high specificity and low equipment requirements and cost. However, they are laborious, time-consuming and have low sensitivity, which results in an underestimation of cases of infection, with frequent false negatives in areas where schistosomiasis has a low incidence and the parasitic burden is low (Gonçalves et al., 2006). In comparison with coprology, the detection of specific antibodies against parasite antigens is more sensitive for detecting light infections and can be applied in large-scale examinations, making serology advantageous over other parasitological techniques. However antibody-based serological assays do not discriminate between active and prior infections and cannot be used to evaluate therapeutic efficacy (Alarcón de Noya et al., 2007; Jin et al., 2010).

One of the greatest pitfalls for the success of this kind of serological assays is the identification and production of highly specific antigens. In the particular case of the diagnosis of schistosomal infections, the crude extracts or soluble protein fractions used as diagnostic antigens contain high levels of glycoproteins, whose glycans are responsible for many cross-reactions with other parasite infections (Alarcón de Noya et al., 2000).

For *S. bovis* diagnosis, the soluble extract of adult worms (SbC) has proved to be useful in cattle and sheep (López-Abán et al., 2004; Oleaga et al., 2004) and also for the detection of human infections by *S. mansoni*, *S. haematobium* and *S. intercalatum* (Roffi and Lemasson, 1981; Pardo et al., 2004, 2007; Belo et al., 2009). However, this protein extract has the drawbacks that its production is limited and time-consuming and, additionally, it has a high proportion of glycoproteins, which may give rise to cross-reactions with other helminths (Ramajo-Hernández et al., 2007).

The 22.6 kDa antigen is a tegumental protein unique to the genus *Schistosoma*. Several authors have indicated that the protein has interest as a vaccine candidate because the anti-22.6 IgE antibody levels correlate with resistance to re-infection after chemotherapy (Fitzsimmons et al., 2004). However, several vaccine trials carried out with this molecule yielded variable results, so its applicability for the prevention of schistosomiasis remains to be confirmed (Li et al., 2000; Pacífico et al., 2006a, 2006b). The 22.6 antigen is found in all the infective stages of the parasite and it displays conserved calcium-binding domains, in spite of its experimentally proven lack of calcium affinity (Dunne et al., 1997; Lin and He, 2006; Pacífico et al., 2006b). Although this protein has been studied in some depth in the main schistosome species that affect humans, such as *S. mansoni*, *S. japonicum* and *S. haematobium*, to date its function is still not clear. It has been described to inhibit thrombin and delay clotting time, such that it could be involved in the manipulation of haemostasis in the host (Lin and He, 2006). Owing to its tegumental expression, it has also been suggested that it could be involved in apical membrane movement (Mulvenna et al., 2010).

In *S. bovis*, two isoforms of the 22.6 antigen have been identified, both in an excretion–secretion extract and in the tegument, and it has been shown that they are recognized by the sera of lambs infected with the parasite (Pérez-Sánchez et al., 2006). Additionally, the study of the glycoproteome of these two fractions has shown that the 22.6 antigen of *S. bovis* is not glycosylated, which suggests that the anti-22.6 antibodies developed during infection are directed against peptide epitopes (Ramajo-Hernández et al., 2007).

In sum, the 22.6 antigen is a protein recognized by the sera of animals infected by *S. bovis* and is not glysosylated, which prevents the possibility of cross-reactions due to the existence of antibodies against glycan groups shared with other parasites. Bearing this in mind, we were prompted to address the potential of this molecule in its recombinant form as a diagnostic antigen, overcoming the above-described drawbacks shown by crude extracts of the parasite.

Here we first report the molecular cloning, characterization and tissue expression of the 22.6 antigen by the *S. bovis* schistosomula and adult worms. Then, we describe the production and purification of the 22.6 antigen as a recombinant protein and evaluate its diagnostic performance in detecting experimental infections in hamsters and natural infections in free range cattle, while further demonstrating the lack of cross-reactivity of the rSb22.6 with anti-*Fasciola hepatica* antibodies from experimentally infected animals.

2. Material and methods

2.1. Parasite material

The life cycle of *S. bovis* was maintained in the laboratory by routine passage through sheep, golden hamsters, and the intermediate snail host *Planorbarius metidjensis*. Adult worms and lung schistosomula were respectively recovered from infected sheep and hamsters as described in De la Torre-Escudero et al. (2010).

Briefly, lambs were infected with 2000 cercariae and 4 months later they were sedated with 10 mg of ketamine per kg of live weight and sacrificed by bleeding through the jugular vein. Adult worms were recovered from the mesenteric veins and washed in warm phosphate buffered saline (PBS) pH 7.2 at 37 °C. Worms were inspected microscopically to verify their integrity and vitality, and immediately processed for RNA extraction, collection of the tegument extract (TG) and the soluble extract (SbC), and for

immunolocalization studies. The TG and SbC extracts were obtained as described respectively by Pérez-Sánchez et al. (2006) and Oleaga and Ramajo (2004).

Lung schistosomula were recovered from hamsters according to the procedure described by Gui et al. (1995). The animals were infected percutaneously with 3000 cercariae by bathing them individually over 1 h. Six days after infection, the hamsters were euthanized and their lungs were removed, minced, and incubated in RPMI medium at 37 °C for 2 h on a rocker-shaker. The suspension was sieved and intact live schistosomula were collected with a micropipette. After three washes in warm PBS, they were fixed in 4% formalin and stored at 4 °C until use.

Animal experimentation was carried out according to the rules from the ethical and animal welfare committee of the institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. PCR amplification of the 22.6 antigen coding sequence

Total RNA from adult worms was isolated using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instruction and preserved at −80 °C. Reverse transcription was performed from total RNA using the 1st Strand cDNA Synthesis Kit (Roche) as recommended by the manufacturer. The cDNA sequence of the *S. bovis* 22.6 antigen (Sb22.6) was amplified using the following set of degenerate primers: 22.6Fw (5′-ATGKCAACCGARACGARATTRAG) and 22.6Rev (5′-TTACTGAGATGGTGTTCTCC). These primers were designed on the consensus sequence resulting after the alignment of 22.6 cDNA sequences from *S. haematobium* and *S. mansoni* (GenBank AY851615 and DQ059818, respectively).

PCR amplifications were performed in 35 cycles of 94 °C for 40 s, 42 °C for 40 s, and 72 °C for 90 s for the 5 first cycles, and 94 °C for 40 s, 48 °C for 40 s, and 72 °C for 90 s for the remaining 30 cycles. PCR products were electrophoresed in an agarose gel and the cDNA band was purified from the gel using the StrataPrep DNA Gel Extraction Kit (Stratagene). This PCR product was cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene), following the manufacturer's instructions, and sequenced on both strands. At least three different clones of the insert were sequenced to verify sequence correctness.

2.3. Recombinant protein expression and purification

The full-length cDNA sequence of Sb22.6 was subcloned into the pGEX-4T-1 expression vector (GE Healthcare), which adds a glutathione S-transferase (GST) tag to the cloned protein. The pSC-A-Sb22.6 construction was digested with EcoRI (Roche Applied Science), and the digestion product was purified from agarose gels and ligated to the predigested pGEX-4T-1 vector. This construct was used to transform *Escherichia coli* BL21 codon plus cells. Single recombinant clones were selected and plasmid DNA was extracted and sequenced to confirm the sequence and the correct orientation of the Sb22.6 cDNA insert.

For protein expression, the selected transformants were grown overnight at 37 °C with shacking in 2x yeast extract tryptone medium (2xYT) supplemented with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. Then, the cultures were diluted to 1/100 in fresh 2xYT with antibiotics and grown again at 37 °C with shacking until their ODs at 595 nm reached 0.8–1. Recombinant protein expression (rSb22.6) was then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Following this, the cells were pelleted and sonicated following standard procedures (De la Torre-Escudero et al., 2010). The cell lysates were centrifuged and the soluble fraction recovered and incubated over 30 min with Glutathione Sepharose 4B (GE Healthcare). The resin was then treated with thrombin for 16 h and the excised rSb22.6 was eluted. The concentration of rSb22.6 was measured using the DC Protein Assay Kit (Bio-Rad) and its purity was checked by SDS-PAGE.

2.4. Bioinformatic analysis

The deduced amino-acid sequence of Sb22.6 was analysed as follows: analysis of conserved domains was performed using Pfam (http://pfam.sanger.ac.uk); theoretical isoelectric point (p*I*) and molecular weight (MW) calculation at http://www.expasy.org/tools/pi_tool.html; prediction of transmembrane helices using the TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM-2.0; prediction of signal peptides with SignalP 3.0 (Bendtsen et al., 2004) at http://www.cbs.dtu.dk/services/SignalP; search for glycosyl-phosphatidyl anchors in the sequence with big-PI Predictor (Eisenhaber et al., 2000) at http://mendel.imp.ac.at/sat/gpi/gpi_server.html. For the prediction of secondary structures and three-dimensional modelling, the amino acid sequences were submitted to the Swiss-Model server (Arnold et al., 2006) at http://swissmodel.expasy.org/, and the resulting 3-D models were visualized using the Pymol package (DeLano, 2002).

The Sb22.6 amino acid sequence was also used to search the non-redundant Swissprot/Uniprot database (http://www.uniprot.org/) by BLASTP analysis, to search for orthologues from other helminth species. The retrieved sequences were aligned using ClustalX. Using this alignment, neighbour-joining analysis was performed using the Mega5.05 package (Tamura et al., 2011). Gaps were treated as pairwise deletions, amino acid distances were calculated using the Poisson model, and branch supports were estimated using bootstrap analysis (10,000 bootstraps).

2.5. Immunolocalization of 22.6 antigen in S. bovis adult worms and lung-stage schistosomula

The tissue expression of the 22.6 antigen was analysed by immunofluorescence and confocal microscopy on adult worms and lung schistosomula.

The specific probe used in this assay was an anti-rSb22.6 polyclonal rabbit serum obtained *ad hoc* following the protocol described in De la Torre-Escudero et al. (2010). The anti-rSb22.6 serum titre was assessed by standard ELISA, and serum reactivity and specificity were checked against the rSb22.6 and the TG extract, which contains the native

isoforms of the 22.6 antigen (Pérez-Sánchez et al., 2006), by conventional Western blot. Thus, samples of 10 μg of TG and 2 μg of rSb22.6 per lane were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membranes. The sheets were post-coated in 2% BSA and incubated with rabbit anti-rSb22.6 serum diluted 1/600. After washing, the sheets were incubated with 1/1000 diluted peroxidase-conjugated anti-rabbit IgG (Sigma). Finally, the reactive bands were developed using 4-chloro-1-naphthol.

The tissue expression study was performed on adult worm sections, and on whole adult worms and 6-day lung schistosomula previously fixed in buffered 4% formalin for 5 h (schistosomula) or 24 h (adults).

For assays addressing parasite sections, the worms were dehydrated and embedded in paraffin following standard protocols. Microtome-cut 5 μm sections were placed on microscope slides, deparaffinized in xylene, and rehydrated. Sections were then blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBST) for 1 h at 37 °C, and incubated with the anti-rSb22.6 rabbit serum diluted 1/50 in blocking buffer for 1 h at 37 °C. Samples were washed three times with PBST and incubated overnight at 4 °C with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Invitrogen) diluted 1/400 in blocking buffer containing 1/200 Phalloidin-Alexa Fluor 488 (Invitrogen), which binds to actin microfilaments. The samples were then washed four times and mounted in antifade reagent (Prolong Gold, Invitrogen). All incubations were performed in a humid chamber.

A similar protocol was followed for whole-mount assays. The reactions were performed in 1.5 ml test tubes containing 100 schistosomula or 5 adult pairs each. The fixed parasites were blocked for 2 h at room temperature, incubated with the rabbit anti-rSb22.6 serum overnight at 4 °C, and then with the above-mentioned Alexa Fluor reagents, both at 1/300 dilution, for 4 h at room temperature. Whole parasites were washed five times and mounted in antifade reagent (schistosomula) or in PBS pH 7.2 (adult worms). In each assay, serum from a non-immunized rabbit was used as a negative control. Samples were analysed with a Leica TCS-NT confocal microscope.

2.6. Diagnostic performance of rSb22.6

The diagnostic performance of rSb22.6 was compared in ELISA to that of the SbC extract using a panel of sera obtained from *S. bovis* experimentally infected hamsters and from naïve or naturally infected free-range cattle. The rSb22.6 specificity was tested against a panel of sera obtained from lambs experimentally infected with *F. hepatica*.

2.6.1. Sera analysed.

2.6.1.1. Sera from lambs experimentally infected with F. hepatica. These sera were obtained in a previous work (Martínez-Fernández et al., 2004) from six animals that were infected with 100 *F. hepatica* metacercariae per lamb and bled at several times post-infection. The sera were analysed by ELISA against the excreted/secreted antigen of *F. hepatica* (E/S Fh) prepared as described by Casanueva et al. (2001), and against the SbC and rSb22.6 antigens.

2.6.1.2. Sera from hamsters experimentally infected with S. bovis. Six hamsters were infected percutaneously with 150 cercariae per animal by individual bathing for 1 h. At 16 weeks post-infection, all the hamsters were sacrificed and their infection viability and intensity were confirmed by the collection of fully developed worms from the portal system. Serum samples from all these animals were collected before infection and at 4, 8, 12 and 16 weeks post-infection and analysed for anti-*S. bovis* antibodies (see below).

2.6.1.3. Sera from free-range cattle. The sera analysed in this study were selected from among those previously collected by Oleaga et al. (2004) from free-range cattle farms, both free of and infected with *S. bovis*, in the province of Salamanca (Spain). By ELISA, those authors analysed up to 5.665 serum samples (from 191 farms) for anti-*S. bovis* antibodies using the SbC extract as antigen.

For the present analysis, the sera from 46 SbC-negative, non-infected animals from *S. bovis*-free farms were selected as negative controls, and the sera from 35 infected cattle presenting high anti-SbC reactivity (high optical density) were selected as positive controls.

In addition, both antigens were used to analyse the serum from 281 free-range cattle from 11 infected and non-infected farms.

2.6.2. ELISA and statistics

The diagnostic performance of the rSb22.6 and SbC antigens for the detection of anti-*S. bovis* IgG antibodies were compared in ELISA. The reactivity of the anti-*F. hepatica* antibodies with the E/S Fh, rSb22.6 and SbC antigens was also analysed by ELISA.

Polystyrene plates (Corning) were coated with E/S Fh, SbC or rSb22.6 overnight at 4 °C in 100 μl of carbonate buffer (pH 9.6). The SbC and E/S Fh antigens were used at 1 μg/well, and the rSb22.6 was used at 0.2 μg/well for the bovine and sheep sera and at 0.2 and 0.5 μg/well for the hamster sera. Plates were washed three times with 0.05% Tween 20 in PBS (TPBS) and post-coated with 200 μl/well of 1% BSA in PBS for 1 h at 37 °C. After a new wash, sheep and hamster sera (1/100 dilution) and bovine sera (1/50 dilution) were added in duplicate (100 μl/well) and incubated at 37 °C for 1 h. After a further wash, peroxidase-labelled anti-bovine IgG (Sigma), anti-sheep IgG (Sigma), and anti-hamster IgG (Serotec) was added at 1/1000, 1/6000 and 1/4000 dilution, respectively, and the plates were incubated for 1 h at 37 °C. After a final washing step, the reaction was developed with 100 μl/well of citrate buffer, pH 5, plus orthophenylene diamine (0.6 mg/ml) and hydrogen peroxide (0.4 μl/ml). The reaction was stopped with 100 μl/well of 3 N sulphuric acid, and the plates were read at 492 nm in an ELISA reader (Multiskan GO, Thermo Scientific).

The serological index (SI) was calculated for each optical density (OD) and was used to establish a common cut-off for all of the ELISA plates using the following formula: $[(NC - S)/(NC - PC)] \times 100$, where NC and PC represent the negative control and positive control, respectively, and S stands for each serum (Hernández-González et al., 2008).

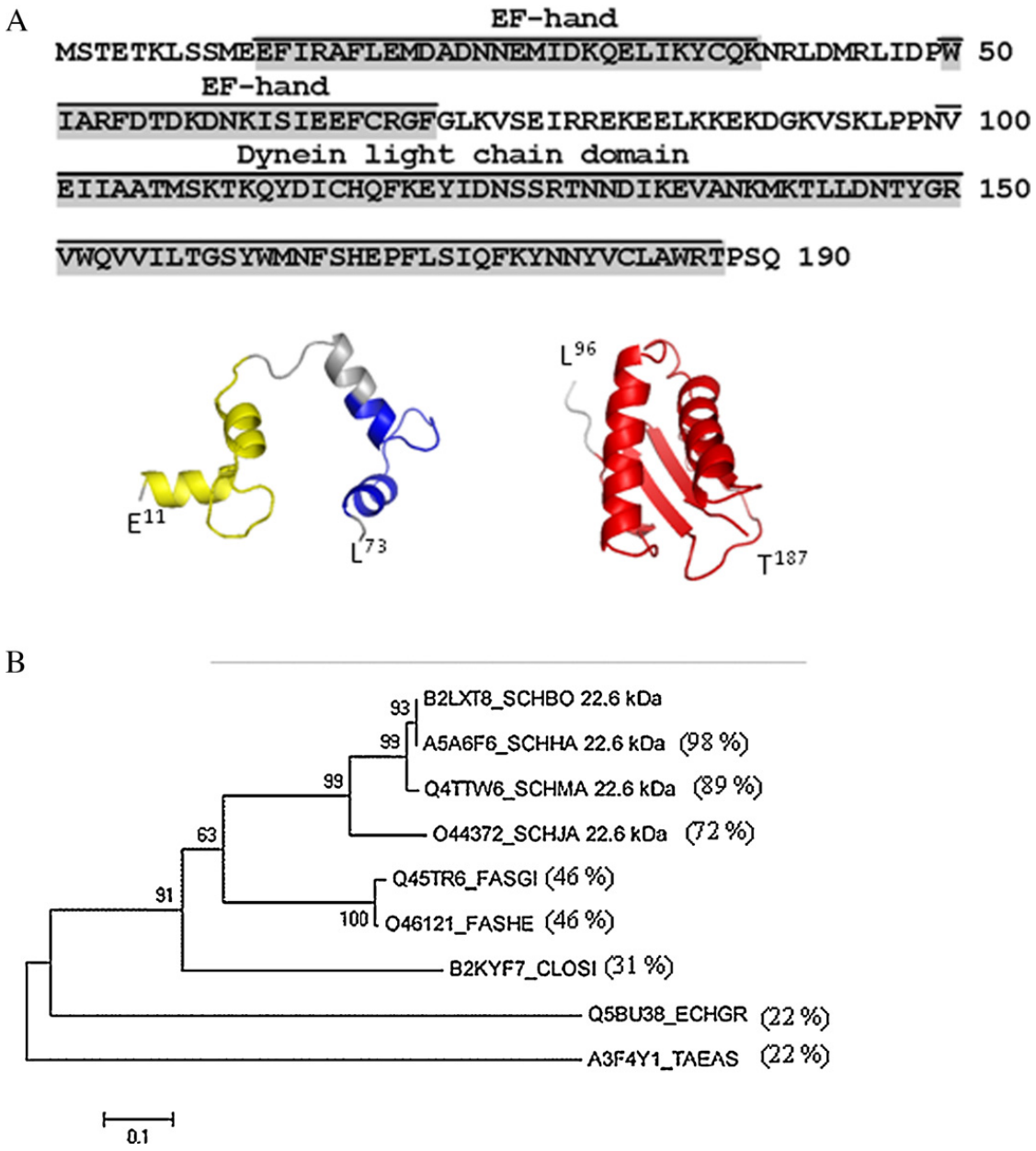

Fig. 1. (A) Amino acid sequence and molecular modelling of the 22.6 kDa antigen from *Schistosoma bovis* (Sb22.6). The two EF-hand motifs are highlighted in yellow and blue, and the dynein light-chain motif in red. (B) Phylogenetic analysis of the amino acid sequences of Sb22.6 (B2LXTB), its orthologues from *Schistosoma haematobium* (A5A6F6), *Schistosoma mansoni* (Q4TTW6) and *Schistosoma japonicum* (O44372), and its homologous sequences retrieved from *Fasciola gigantica* (Q45TR6), *Fasciola hepatica* (O46121), *Clonorchis sinensis* (Q2PMV7), *Echinococcus granulosus* (A3F4Y1) and *Taenia asiatica* (Q5BU38). In brackets, percentage of identity with the amino acid sequence of Sb22.6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Mean SI was calculated for each type of bovine sera and antigen.

Receiver–operator characteristic (ROC) curves were built for each antigen using the SI from the 35 positive and 46 negative bovine control sera and used to establish the cut-off values. The cut-off value selected was that giving the highest diagnostic performance for each antigen, which was calculated as the sum of the sensitivity and specificity divided by two. These values were used to establish the positive/negative state of the 281 bovine sera analysed with each of the antigens: rSb22.6 and SbC.

A *Z*-test was used to compare the ratio among positive and negative sera between both antigens.

All statistical analyses were performed with the SPSS v17 package.

3. Results

3.1. Amplification, cloning, sequencing and expression of the Sb22.6 cDNA

The Sb22.6 cDNA molecule contained a 573-nucleotide open reading frame coding for a protein of 190 amino acids, with a predicted MW and p*I* of 22,535 Da and 6.35, respectively. This sequence was submitted to GenBank and received the accession number EU595756.

The bioinformatics analyses of the deduced amino acid sequence did not reveal a signal peptide, transmembrane helices or glycosyl-phosphatidyl inositol anchors but they did show two EF-hand domains located in its N-terminus (residues 12–39 and 50–71) and a dynein light-chain domain located in its C-terminus (residues 100–187). *In silico* three-dimensional modelling of the Sb22.6 molecule predicted the 3D structures for two sequence fragments (from amino acids 11–72 and 96–187, respectively), showing the presence of 6 α-helices and 4 β-sheets (Fig. 1A).

A BLAST search of the SWISS-Prot database with the deduced amino acid sequence of Sb22.6 revealed a strong identity with the homologous genes of *S. haematobium*, *S. mansoni* and *S. japonicum* (98%, 89% and 72%, respectively), as well as with the other members of the 20–23 kDa schistosome tegument-associated antigen family (not included in the ensuing alignment and phylogenetic analysis). With other non-schistosomal helminth proteins, the sequence of Sb22.6 showed the greatest homologies with the calcium-binding tegumental proteins of *Fasciola gigantica*, *F. hepatica*, *Clonorchis sinensis*, *Taenia asiatica*, and a partial sequence of *Echinococcus granulosus*, annotated as a tegumental antigen, with identity percentages ranging from 46% to 22% (Fig. 1B).

The alignment and the phylogenetic analysis of these nine amino acid sequences (Fig. 1B) indicated that these proteins were grouped within two major clades, corresponding to the genus *Schistosoma* (support value 99%) and *Fasciola* (support value 100%). A third clade could be established that would include all the trematode sequences (support value 91%).

The *S. bovis* 22.6 cDNA was cloned into the expression vector pGEX-4-T1. After the induction of expression in *E. coli*, the GST-tagged recombinant protein was purified under native conditions using glutathione affinity chromatography. The purified recombinant protein rSb22.6 had a molecular weight of approximately 23 kDa in a polyacrylamide gel (Fig. 2), in accordance with that calculated theoretically.

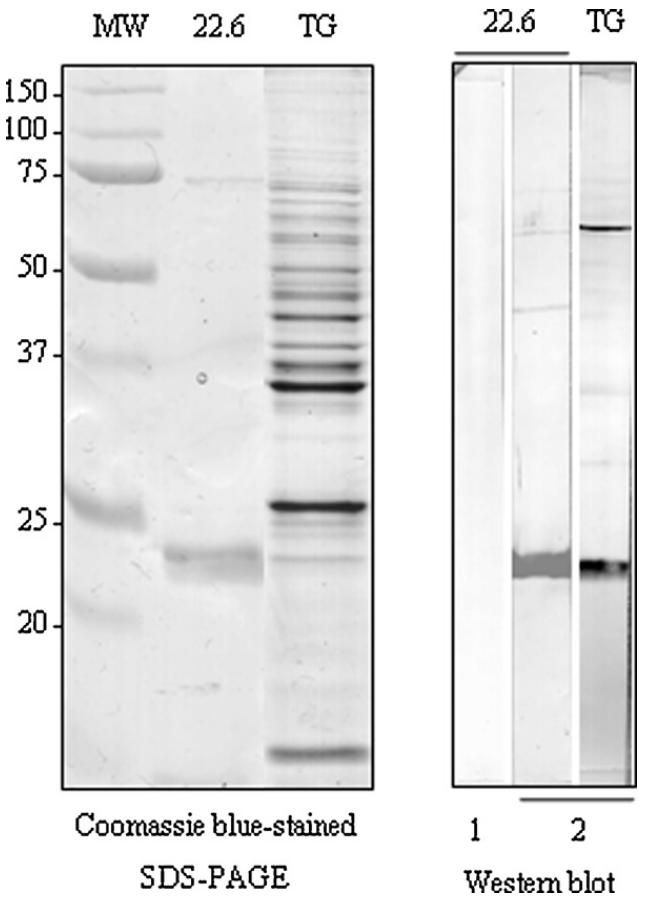

Fig. 2. Coomassie blue-stained SDS-PAGE loaded with 2 μg of rSb22.6 and 10 μg of tegument extract (TG). Western blot showing the specificity of the anti-rSb22.6 serum on the rSb22.6 and TG. 1, negative control serum; 2, anti-rSb22.6 rabbit serum; MW, molecular weight standards.

3.2. Reactivity and specificity of the anti-rSb22.6 rabbit hyperimmune serum

The reactivity of the anti-rSb22.6 hyperimmune serum was tested in ELISA against the recombinant protein prior to its use in the immunolocalization assays. The antibody titre was 1/3200 (not shown). The specificity of the serum was assessed by Western blotting against rSb22.6 and the native TG extract from *S. bovis* adult worms. As shown in Fig. 2, the anti-rSb22.6 serum reacted strongly with the recombinant protein and specifically recognized the native 22.6 antigen in the TG extract.

3.3. Immunolocalization of Sb22.6 in adult worms and lung-stage schistosomula of S. bovis

Paraffin-embedded sections from adult worms incubated with the specific anti-rSb22.6 serum plus phalloidin-Alexa Fluor 495 revealed actin reactivity (in green) as well as specific Sb22.6 reactivity (in red) to be distributed in internal tissues and also in the external areas of the worm tegument, although no expression of the Sb22.6 protein was observed on the outer surface of the tegument (Fig. 3A). Sections incubated with the negative serum plus the phalloidin-Alexa Fluor 495 only resulted in green fluorescence (not shown).

Whole parasites were also subjected to immunolocalization assays using the same sera and reagents as those used employed for the paraffin sections. The assays in whole adult worms and schistosomula revealed a lack of specific Sb22.6 reaction (red) on their tegument surface. The images from these adult worms and schistosomula incubated with the anti-rSb22.6 serum only showed the green signal from actin (Fig. 3B and C).

3.4. Cross-reactivity of rSb22.6 and SbC with anti-F. hepatica antibodies

The sera from sheep experimentally infected with *F. hepatica* show detectable and increasing levels of anti-*F. hepatica* antibodies between weeks 2 and 12 post-infection. These sera did not react with the SbC or rSb22.6 antigens (Fig. 4).

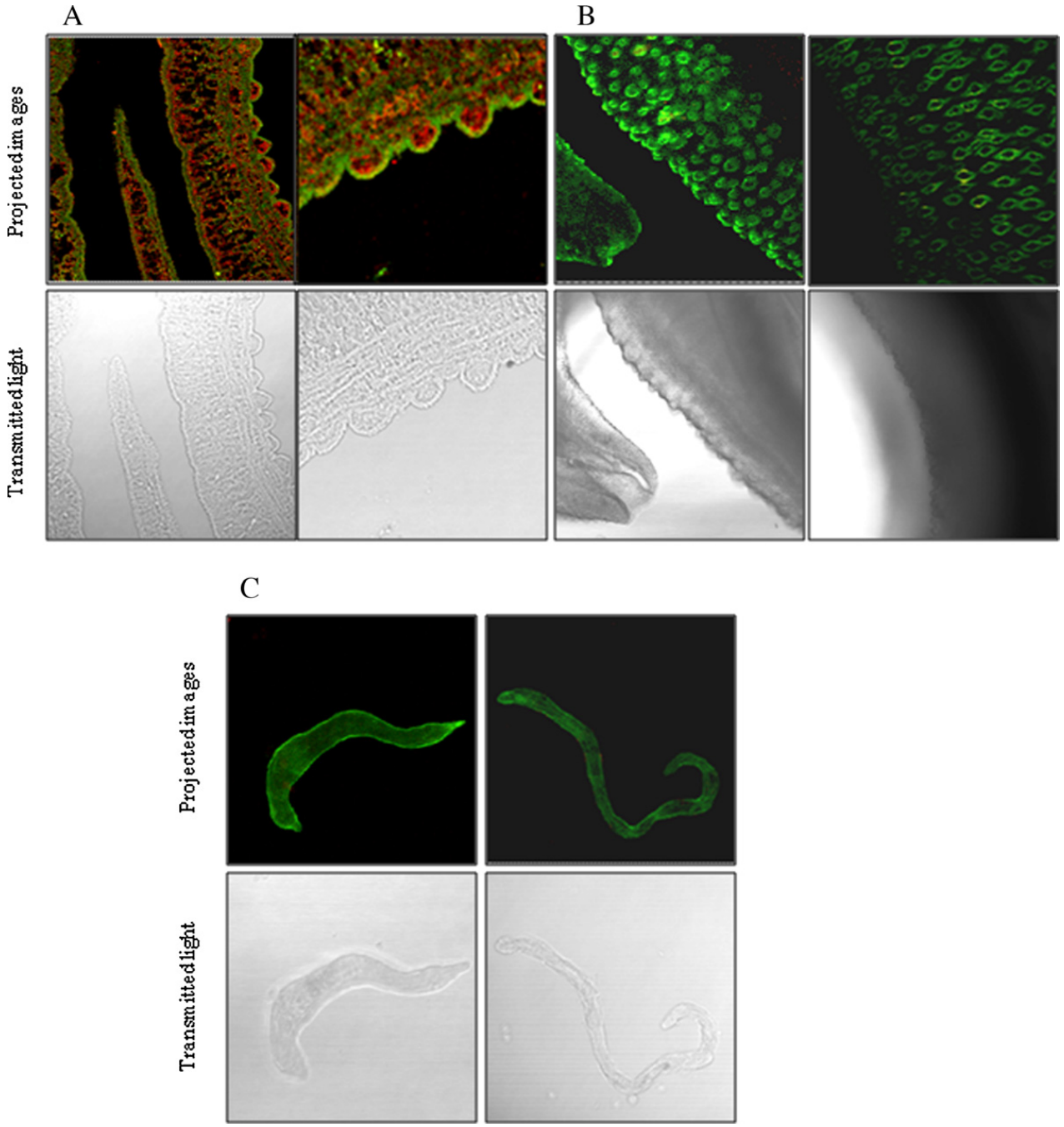

Fig. 3. Immunolocalization of *S. bovis* 22.6 antigen (Sb22.6). (A) Images of adult worm sections. (B) Images of whole adult worms. (C) Images of lung schistosomula. Confocal microscopy images after incubation with phalloidin-Alexa Fluor 488 (green) plus the anti-rSb22.6 rabbit sera and an anti rabbit IgG-Alexa Fluor 594 (red). The upper row of panels shows the merged projected images derived from sections generated by the confocal microscopy and the lower row shows the corresponding transmitted light images. Magnification 600×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.5. Comparative performance of rSb22.6 and SbC in ELISA

In the ELISA, the sera from the hamsters experimentally infected with *S. bovis* showed almost the same reactivity with the crude extract SbC and with the rSb22.6 (Fig. 5). It was also observed that the rSb22.6 antigen provided a slightly higher reactivity when used at the lower concentration tested (0.2 *vs*. 0.5 μg/well). Thus, 0.2 μg/well of rSb22.6 was used for the analysis of the bovine sera.

Once the usefulness of rSb22.6 for the detection of anti-*S. bovis* antibodies in hamsters had been confirmed, we checked its diagnostic performance in natural *S. bovis* infections in free-range cattle. Owing to their free-range regime, these animals may enter into contact with many other parasites and pathogens, providing an excellent natural model to assess the diagnostic performance of the rSb22.6 antigen and compare it to that of SbC.

As can be observed in Fig. 6, the SI values obtained for the 35 positive and 46 negative bovine control sera

Table 1
ROC curves of the diagnostic performance of rSb22.6 and SbC antigens in ELISA for the detection of anti-*S. bovis* IgG antibodies: area under the curve ± standard error (AUC ± SE), confidence interval (CI), cut-off value, sensitivity, specificity, and diagnostic performance for each antigen.

Antigen	AUC ± SE	CI (95%)	Cut-off	Sensitivity (%)	Specificity (%)	Diagnostic performance
SbC	1.000 ± 0.000	1	23.56	100	100	100
rSb22.6	0.999 ± 0.001	0.000–1.000	23.84	100	94.9	97.5

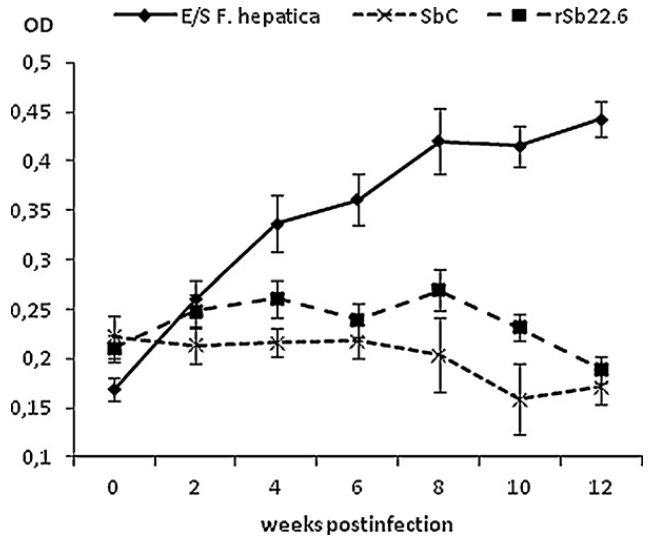

Fig. 4. ELISA using the excreted/secreted *F. hepatica* antigen (E/S Fh, 1 μg/well), adult worm soluble extract (SbC, 1 μg/well) and rSb22.6 (0.2 μg/well) as antigens for the detection of anti-*F. hepatica* antibodies in serum samples from lambs experimentally infected with 100 metacercariae of *F. hepatica*. Each point in the plot represents the mean optical density from 6 animals ± SE.

were very similar with both SbC and rSb22.6 antigens. ROC analysis of these SIs allowed the establishment of positivity/negativity cut-off values at 23.56% for SbC and at 23.84% for rSb22.6 (Table 1). Using these control sera, SbC afforded, as expected, 100% sensitivity and specificity, and rSb22.6 gave 100% sensitivity but 94.9% specificity.

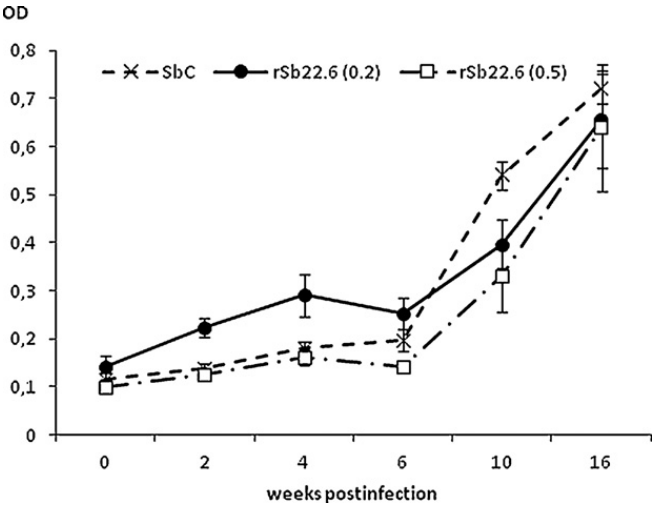

Fig. 5. ELISA using the adult worm soluble extract (SbC, 1 μg/well) and rSb22.6 (0.2 and 0.5 μg/well) as antigens for the detection of anti-*S. bovis* antibodies in serum samples from hamsters experimentally infected with *S. bovis*. Each point in the plot represents the mean optical density from 6 animals ± SE.

Application of these cut-off values to the 281 samples of bovine sera showed that the ELISA results using SbC and rSb22.6 coincided in 96.1% of the sera analysed. The SbC and rSb22.6 antigens respectively detected 38 and 41 infected animals, all of them from the same 5 farms. These differences in the proportion of positive animals detected with each antigen were not significant ($p > 0.05$) (Fig. 7A). The regression straight line established between the SIs obtained after the analysis of the 281 problem sera with both antigens revealed a good correlation ($r = 0.858$) between both tests (Fig. 7B).

4. Discussion

Bovine schistosomiasis, caused by *S. bovis*, is a serious veterinary problem, affecting large numbers of animals and causing significant economic losses to farmers in many areas of the world. In addition, *S. bovis* may have also an impact on human health, as suggested in recent studies carried out by Huyse et al. (2009). Those authors found that *S. bovis* and the human schistosome *S. haematobium* have hybridized in the Senegal River Basin in Africa, and hybrid parasites have been detected in human urine and faeces. Hybridization between schistosomes under laboratory conditions has been shown to result in heterosis (higher fecundity, faster maturation time, a broader intermediate host spectrum), with important implications regarding the prevalence of disease, its pathology and its treatment. If this new hybrid in Senegal exhibits the same hybrid vigour, it may develop into a new emerging pathogen, demanding

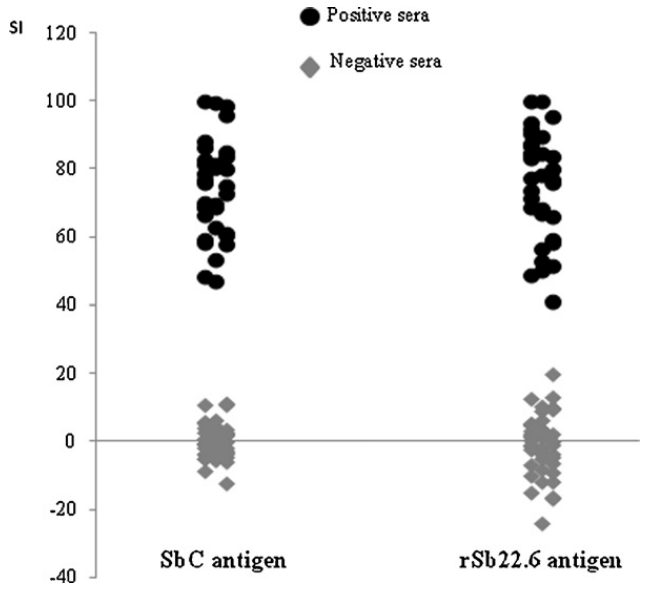

Fig. 6. Serological indexes (SI) of the 35 positive and 46 negative control sera from free-range cattle after analysis by ELISA using the soluble extract from adult worms (SbC) and the recombinant 22.6 (rSb22.6) as antigens.

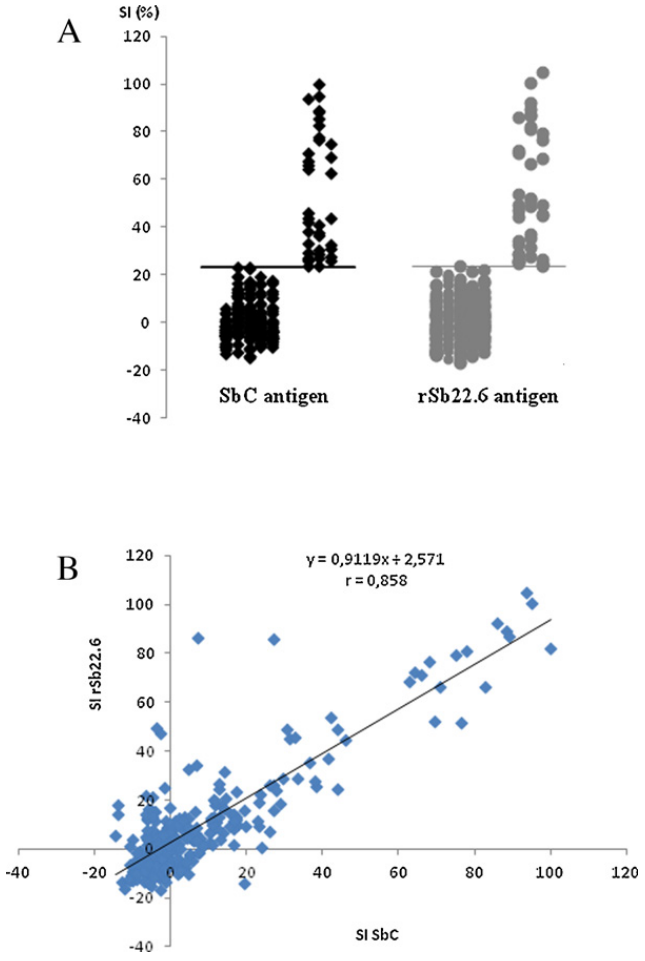

Fig. 7. (A) Serological indices (SI) of the 281 sera from free-range cattle after analysis by ELISA using the soluble extract from adult worms (SbC) and the recombinant 22.6 (rSb22.6) as antigens. (B) Correlation trend line between the SI values obtained with each antigen for the 281 problem sera analysed.

new control strategies in zones where both parental species overlap (Huyse et al., 2009). This situation poses the need to promote more surveillance programs, for which it is necessary to develop sensitive diagnostic tools able to be applied in large-scale explorations.

Bearing in mind the importance of *S. bovis*, some years ago we began to study the molecular and functional characterization of its proteins; in particular, those present in the parasite-host interface (Pérez-Sánchez et al., 2006, 2008; Ramajo-Hernández et al., 2007; De la Torre-Escudero et al., 2010, 2011). One of the proteins identified in the tegumental proteome and in an excretion–secretion extract of adult *S. bovis* worms was the 22.6 antigen (Sb22.6) (Pérez-Sánchez et al., 2006, 2008). This protein has been fairly well studied in other schistosome species, but to date it has not been known in *S. bovis*. After several different studies we observed in the Sb22.6 the following: (i) its isoforms are recognized by IgG antibodies from the serum of animals infected with the worm (Pérez-Sánchez et al., 2006), (ii) it is not glycosylated, which reduces the possibility of cross-reactions due to the presence of antibodies against carbohydrate epitopes (Ramajo-Hernández et al., 2007) and (iii) its sequence, like the sequence of the homologous proteins from other species of schistosomes, has low similarity with other helminthic proteins. Bearing in mind these findings, we surmise that the Sb22.6 antigen could be a good candidate for the diagnosis of *S. bovis* infections.

It is well known that the antibody-based serological assays do not discriminate between ongoing and previous infection and cannot be used to evaluate therapeutic efficacy (Jin et al., 2010). The diagnosis of human schistosome infections through the detection of parasite-derived antigens in the blood or urine can be more adequate, as this kind of methods allow detection of an active infection and the rate of cure after drug treatment (Gentile et al., 2011). In animal schistosomiasis, however, this issue is not as critical since livestock suffer from chronic infections, which are usually not treated. In this scenario the most important would be to identify the foci where the parasite is in order to apply there the adequate control measures.

Accordingly, the objectives of the present work were to clone, sequence, and characterize the Sb22.6 at molecular level, as well as to undertake its production as a recombinant protein in order to assess its usefulness as a diagnostic antigen in an ELISA tests for detecting cattle farms infected with *S. bovis*.

After cloning and sequencing the Sb22.6 cDNA, the analysis of its deduced amino acid sequence revealed a high identity with the orthologous proteins in other species of schistosomes. This identity was higher within certain recognized domains such as the two EF-hand motifs and the dynein light-chain homology domain, a fact that has been already highlighted by Fitzsimmons et al. (2004). The almost full sequence identity of Sb22.6 with the *S. haematobium* 22.6 antigen (they only differ in two amino acids) confirms the phylogenetic closeness of these two species. The three-dimensional model of the protein (Fig. 1B) shows, as expected, that the EF hand is a helix-loop-helix domain (Fitzsimmons et al., 2004). As observed in its orthologues of *Schistosoma* sp. and in the tegumental calcium-binding proteins from other trematodes, Sb22.6 lacks a signal peptide and membrane-anchoring motifs, suggesting it may be a soluble cytoplasmic protein (Vichasri-Grams et al., 2006).

The immunolocalization studies carried out here showed that Sb22.6 is expressed in the tegument and internal tissues of the adult worms but not on their tegument surface. These results are in good agreement with those observed for *S. japonicum* and *S. mansoni* in similar studies (Dunne et al., 1997; Li et al., 2000), which demonstrated the expression of Sj22.6 and Sm22.6 in the tegumental cytoplasm and also the expression of Sm22.6 in the gastrodermis and protonephridial system but not on the tegument surface. Other studies performed on *S. mansoni* also support these results (Braschi and Wilson, 2006). In that work the authors did not find the Sm22.6 on the tegument surface even after treating the worms with a membrane-impermeant probe to biotinylate the most externally-accessible proteins and recover the tagged molecules by affinity chromatography. In contrast, Mulvenna et al. (2010) applied the same methodology to *S. japonicum* adult worms and identified

Sj22.6 among the biotinylated proteins. Notwithstanding, those authors suggested that limited tegumental damage may have led to a low-level biotinylation of internal proteins.

Our immunolocalization studies also revealed the absence of Sb22.6 from the surface of lung schistosomula, in accordance with the result of a recent proteomic analysis of the *S. bovis* schistosomulum surface performed by De la Torre-Escudero et al. (2011), who failed to identify Sb22.6 among the peptides released by soft digestion with trypsin of intact larvae not fixed with methanol.

We obtained Sb22.6 as a recombinant protein in order to compare its diagnostic performance to that of SbC in parallel ELISA tests. The results with the sera from experimentally infected hamsters clearly showed that rSb22.6 detects the anti-*S. bovis* IgG antibodies, with a sensitivity comparable to that of SbC. In addition, the results of the analysis of the sera taken from free-range cattle from farms with and without *S. bovis* indicated that both antigens performed similarly. It should be noted that the ROC analysis carried out here is not valid for quantifying the real sensitivity and specificity of the SbC and rSb22.6 tests, but it has allowed us to establish cut-off values to compare the positive sera detected with each antigen.

The results of the analysis of the sera from lambs infected with *F. hepatica* showed that the rSb22.6 antigen did not cross-react with antibodies against this parasite. We performed this analysis because we found some sequence homology between Sb22.6 and the orthologues in *Fasciola* spp. and because in many areas, *F. hepatica* is present alongside *S. bovis* and can also infect cattle (Dalton, 1999; Moné et al., 1999).

The application of rSb22.6 show the advantage over the SbC of being produced more easily in large amounts and in a highly reproducible fashion, avoiding the hindrances of using crude extracts, such as the SbC.

Moreover, it should not be forgotten that the objective of *S. bovis* surveillance and control programs is not the individual diagnosis of infected animals but the identification of endemic foci and livestock farms where the parasite is present.

Keeping this in mind, it may be concluded that rSb22.6 represents a promising epidemiological tool for *S. bovis* surveillance that may help to implement control measures in areas and farms harbouring the parasite.

Acknowledgements

This research was funded by projects AGL2007-60413 and AGL2010-18163, granted by the Spanish Ministry of Science and Innovation. E. De la Torre-Escudero is a holder of a predoctoral fellowship from the Ministry of Science and Innovation. R. Manzano-Román was funded by the JAEDoc program (CSIC-FSE).

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