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Characterisation and serodiagnostic evaluation of a recombinant 22.6-kDa tegument protein of *Schistosoma spindale*

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Research Paper

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Abstract

Schistosomosis in animals due to *Schistosoma spindale* significantly burdens India's livestock economy because of high prevalence and morbidity and is mostly underdiagnosed from the lack of sensitive tools for field-level detection. This study aimed to clone, express the 22.6-kDa tegument protein of *S. spindale* (rSs22.6kDa) and to utilise it in a dot enzyme-linked immunosorbent assay for serodiagnosis. RNA was extracted from adult worms recovered from the mesenteries of slaughtered cattle to amplify the gene encoding the 22.6-kDa protein. *In silico* analysis revealed the protein's secondary structure, consisting of 190 amino acids forming alpha helices (47.89%), extended strands (17.37%), beta turns (8.95%), and random coils (25.79%), with α helices and β sheets in the tertiary structure. Two conserved domains were noted: an EF-hand domain at the N-terminus and a dynein light-chain domain at the C-terminus. Phylogenetic studies positioned the *S. spindale* sequence as a sister clade to *Schistosoma haematobium* and *Schistosoma bovis*. The gene was cloned into a pJET vector and transformed into *Escherichia coli* Top 10 cells, with expression achieved using a pET28b vector, BL21 *E. coli* cells, and induction with 0.6 mM isopropyl-β-d-thiogalactopyranoside. The protein's soluble fraction was purified using nickel-chelating affinity chromatography, confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting, identifying a distinct immunodominant 22.6-kDa protein. The diagnostic utility was validated using a dot enzyme-linked immunosorbent assay which demonstrated a of sensitivity of 89.47% and specificity of 100%. The study records for the first time the prokaryotic expression and evaluation of the 22.6-kDa tegumental protein of *S. spindale*, highlighting its potential as a diagnostic antigen for seroprevalence studies in bovine intestinal schistosomosis.

Introduction

Schistosomosis, a significant snail-borne blood fluke infection, affects approximately 200 million people and 165 million cattle across tropical and subtropical regions (Torre-Escudero *et al.*, 2012; Colley *et al.*, 2014; Frahm *et al.*, 2019; Anisuzzaman and Tsuji, 2020; Hossain *et al.*, 2023). Within Asia, the species *Schistosoma spindale*, *S. indicum*, *S. nasalis*, and *S. japonicum* predominantly affect cattle (De Bont and Vercruysse, 1997; Labony *et al.*, 2022; Anisuzzaman *et al.*, 2023). Particularly in India, *S. spindale* leads to substantial economic losses in livestock in terms of animal health and productivity due to visceral schistosomosis in ruminants (Sumanth *et al.*, 2004). Despite its prevalence, infections often remain undetected in field conditions due to factors such as the low fecundity of female schistosomes, the trapping of eggs in tissues, the masking of eggs by mucus, the uneven egg distribution in faeces, and the low egg output during mild infections (Lakshmanan *et al.*, 2018). The infection exacerbates economic burdens by reducing growth, conception, and pregnancy rates in cattle, increasing vulnerability to other diseases and necessitating liver condemnation post-slaughter (McCauley *et al.*, 1984; De Bont and Vercruysse, 1997). Given these challenges, there is an urgent need for the development of enhanced and sensitive diagnostic methods that can detect low-intensity infections and facilitate widespread screening (Torre-Escudero *et al.*, 2012; Xu *et al.*, 2014). Current diagnostics rely heavily on crude schistosome antigens, like egg and somatic antigens, which often suffer from specificity issues because of cross-reactivity linked to glycoprotein presence (Alarcon de Noya *et al.*, 2000). Consequently, research has shifted towards more reliable alternatives such as purified and recombinant antigens, which demonstrate greater diagnostic efficacy (Doenhoff *et al.*, 2004; Li *et al.*, 2012). This focus highlights the potential of these innovations to significantly improve disease control strategies.

Approximately 43 tegumental proteins have been identified in schistosomes, most of which show no sequence similarity to proteins from other parasites (Fonseca *et al.*, 2012). The distinctiveness of these schistosome proteins, which are primarily located at the tegument—

the initial point of contact with the host—plays a crucial role in the survival of the parasite (van Balkom *et al.*, 2005; Hellemond *et al.*, 2006). Additionally, the schistosome tegument is vital for host interaction, signal transduction, nutrition, excretion, osmoregulation, immune evasion, and modulation (Han *et al.*, 2009; Mulvenna *et al.*, 2010; Fonseca *et al.*, 2012; Anisuzzaman *et al.*, 2021). The antigenic properties of various schistosome tegumental proteins have been confirmed. Recently, a number of recombinant peptide antigens, including rSj23, rSm21.6, rSj29, and rSb22.6, have been developed to diagnose both animal and human schistosomosis (Li *et al.*, 2012; Torre-Escudero *et al.*, 2012; Ren *et al.*, 2017; Lv *et al.*, 2016). The 22.6-kDa tegument protein from *S. bovis* and *S. mansoni* has been evaluated and recognised as a promising tool for epidemiological surveillance. This protein has been noted to delay clotting by inhibiting thrombin, thereby altering host haemostasis. It is also unique in that it is non-glycosylated, reducing the likelihood of cross-reactions with related helminths (Pacifico *et al.*, 2006; Torre-Escudero *et al.*, 2012).

Diagnosis of *S. spindale* in India has traditionally relied on whole worm antigens and excretory-secretory antigens (Sumanth *et al.*, 2003; Divya *et al.*, 2012; Murthy *et al.*, 2013; Lakshmanan *et al.*, 2016). Given the high prevalence of *S. spindale* in Indian dairy cattle, there is a need to identify, produce, and characterise a suitable diagnostic protein candidate and analyse its immunogenicity. This study was therefore designed to clone, express, purify, and assess the diagnostic potential of the recombinant 22.6-kDa tegument protein of *S. spindale* (rSs22.6).

Materials and Methods

Sample collection and RNA isolation

Adult schistosome worms were recovered from the mesentery samples of cattle slaughtered and were morphologically identified as *S. spindale* as per Kumar (1999). The worms were washed several times in phosphate-buffered saline and were kept in RNA later solution at -20°C. RNA was isolated from *S. spindale* worms using TRI reagent (Sigma-Aldrich, Bangalore) according to the standard protocol suggested by the manufacturer. Reverse transcription was performed from total RNA extracted using Revert Aid First strand cDNA synthesis kit (Thermo Scientific, USA).

Amplification and cloning of 22.6-kDa tegument protein coding gene of S. spindale

The sequence of 22.6-kDa tegument protein coding gene of *S. spindale* (Ss22.6) was not available in GenBank and hence degenerate primers *viz.* ATGKCAACCGARACGARATTRAG (SS F), TTACTGAGATGGTGTTCTCC (SS R) used by Torre-Escudero *et al.* (2012) for amplifying corresponding gene of *S. bovis* was used. Polymerase chain reaction (PCR) was performed in 25 µL of the reaction mix at an annealing temperature of 50°C for 30 sec. The amplified products were electrophoresed in 2% agarose gel and purified by elution method using GeNei TM Gel Extraction Kit according to the manufacturer's protocol and was confirmed by dideoxy Sanger's sequencing at AgriGenome Labs Private Limited, Cochin. The sequences were aligned and blasted using NCBI BLASTn tool for further confirmation.

The eluted product was then cloned into pJET cloning vector using cloneJET cloning kit (Thermo Scientific, USA) following the manufacturer's instructions where blunt-end ligation was carried out. The transformation was then done in competent *E. coli* Top10 cells (Sambrook and Russell, 2001). Appropriate negative controls with unligated vector were also processed simultaneously. After ascertaining the presence of positive clone by colony PCR using pJET primers, the positive clones were grown in Luria Bertani (LB) tubes containing LB broth with ampicillin (100 mg/ mL). The cloned plasmids were isolated using kit (Thermo Scientific, USA) and sequenced at AgriGenome Labs Private Limited, Cochin. The sequences were aligned using the BioEdit sequence alignment editor and blasted using NCBI BLASTn tool for further confirmation.

In silico analysis of 22.6-kDa tegument protein coding gene of S. spindale

The nucleotide sequences obtained was first subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/blast) to ascertain the similarities of Ss22.6 sequences with the nucleotide sequences of the corresponding genes of different schistosomes. 'Translate' tool (http://web.expasy.org/translate/) was used to translate the rSs22.6 coding genes to predicted amino acid sequences. The predicted protein sequences obtained from translate tool were then aligned with those of other schistosomes using BLASTp to assess interspecies similarity. The protein's secondary structure and key characteristics were predicted using the self-optimised prediction method (SOPMA), which evaluated the proportions of alpha helices, beta turns, random coils, and extended strands in the rSs22.6 protein.

Parameters such as molecular weight and theoretical isoelectric point (pI) were predicted using the ProtParam tool (http://web.expasy.org/protparam/). The tool predicted the molecular weight of the protein theoretically by adding the average isotopic masses of amino acids in the protein sequence with the average isotopic mass of one molecule of water. The pI was calculated using previously described pK values of the amino acids. The SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/) predicted the presence and location of signal peptide cleavage sites in the amino acid sequences. The TMHMM Server v. 2.0, a membrane protein topology prediction method based on a hidden Markov model, was used to predict transmembrane helices and discriminate between soluble and membrane proteins with high accuracy. The Immune Epitope Database and Analysis Resource tool (www.iedb.org) was employed for linear epitope prediction, listing the possible number and composition of epitopes. The NCBI conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) identified conserved domains in the rSs22.6 protein sequences, providing insights into the structure, sequence, and function relationships (Marchler-Bauer *et al.*, 2015). The tertiary structure was predicted using the SWISS MODEL Prot Param based on amino acid sequences (https://swissmodel.expasy.org/interactive) and the Pymol package was utilised for visualising the three-dimensional models generated (DeLano, 2002). The model developed in SWISS MODEL, available in pdb format, was utilised to predict parameters related to the protein's geometry and stability. A Ramachandran plot of the predicted protein structure was generated using the PROCHECK tool in SAVES v 6.0 (https://saves.mbi.ucla.edu/). The MolProbity bioinformatics server validated three-dimensional atomic models of macromolecules, providing essential information on the Ramachandran plot and the protein's geometric parameters such as favourable angles and bonds (http://molprobity.biochem.

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duke.edu/) offering insights into their conformational stability and overall quality.

A phylogenetic tree was constructed to elucidate evolutionary relationships between various schistosome species, based on multiple sequence alignment. The length of each pair of branches in the tree indicates the distance between sequence pairs, and the units at the bottom specify the number of substitution events. The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model in Mega 5.2 (Tamura and Nei, 1993; Tamura *et al.*, 2004; Tamura *et al.*, 2011). The tree with the highest log likelihood (-1659.3968) was displayed, showing the percentage of trees in which the associated taxa clustered together, with standard error estimates obtained by a bootstrap procedure (1000 replicates).

Recombinant protein expression and purification

The amplification of the confirmed sequence of Ss22.6 for protein expression was carried out by PCR using primers which was custom designed to introduce a restriction site for *BbsI* restriction enzyme at its 5′ end. The primer sequences were designed such that BbsI digestion would create a staggered cut compatible with *NcoI* restriction digestion in the forward primer (5′-AATATTCTTATGAAGACACCATGGCAACCGAGACGAAATTAAGTTCAA-3′) and a staggered cut compatible with *XhoI* restriction digestion in the reverse primer (5′- TTAAAGTTTTGAAGACACTCGAGCTGAGATGGTGTTCTCCATGCT-3′). Amplification and purification were done per the previous protocol adopted for amplification of Ss22.6. The gel eluted PCR product was digested with the restriction enzyme, Bbs1 (New England BioLabs, USA). The expression vector, pET 28b (Novagen) was digested with the restriction enzymes *NcoI* and *XhoI* (Thermo Scientific, USA).

The digested Ss22.6 and the pET28b vector were ligated using T4 DNA Ligase (MBI Fermentas). Transformation was then carried out by mixing 5 µL of ligation reaction mixture with 50 µL of *E. coli* BL-21 competent cells that was prepared by $CaCl_2$ method (Sambrook and Russell, 2001). Colony PCR followed by agarose gel electrophoresis was done to confirm the presence of positive colonies.

The bacterial clones with the insert were grown in LB - Kanamycin (100 mg/ mL) broth overnight at 37°C under constant shaking and 1 mL was inoculated in 50 mL of LB medium and incubated until an OD_{600} of 0.6 was attained. Recombinant protein (rSs22.6) expression was then optimised with different concentrations (0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1.0 mM) of isopropyl-β-d-thiogalactopyranoside (IPTG) (Sigma-Aldrich) for a period of 1 to 5 h. The induced BL21 cells were pelleted and lysed using cell lytic B-cell reagent (Sigma) according to the manufacturer's protocol. The lysate was centrifuged, and the supernatant containing the soluble fraction of the expressed recombinant protein was collected for further purification using His-affinity chromatography. The pellet which contained the insoluble fraction of the expressed protein was resuspended in 50 µL of distilled water and kept at -20°C until use. Further, all the four portions (uninduced, induced, induced soluble, and induced insoluble) were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The supernatant containing the soluble fraction of expressed protein was loaded onto a Ni-NTA agarose column (Qiagen, Germany). The recombinant protein was then eluted under native conditions using a step gradient of increasing imidazole concentrations (20 mM to 250 mM).

Immunodiagnostic analysis of rSs22.6

The purity of the newly expressed rSs22.6 was analysed using SDS-PAGE (Laemmli, 1970) in a vertical electrophoresis apparatus (Biorad, USA) followed by silver staining using kit (BioLit, Sisco Research Laboratories, Mumbai). The protein concentration of the most pure elute was estimated by spectrophotometer (Nanodrop 2000, Thermo Scientific, USA). Immunoprofiling was conducted using the Western blotting technique as described by Towbin *et al.* (1979), with slight modifications. Primary antibodies (known schistosome positive and negative bovine serum samples) were used at a dilution of 1:20 in blocking buffer, whereas rabbit anti-bovine IgG HRP conjugate was used as the secondary antibody at a dilution of 1:10,000 with blocking buffer. Sera from animals harbouring adult amphistomes in the rumen were used to verify the specificity of the antigen by Western blotting.

Evaluation of diagnostic potential of rSs22.6 using dot ELISA

Standardisation of *rSs22.6* based dot ELISA was carried out using different dilutions of rSs22.6 antigen in carbonate bicarbonate buffer (500 ng/µL, 250 ng/µL, 100 ng/µL) and three different dilutions of bovine sera in blocking buffer (1:20, 1:50, and 1:100). Antibovine IgG-HRP conjugate at a dilution of 1:5000 was used as the secondary antibody and then subjected for chromogenic visualisation. Development of brown-coloured spots indicated a positive reaction. Further, the specificity and sensitivity of the assay were determined using the optimum dilutions of antigen and sera from specificity controls. Known schistosome positive, known schistosome negative, and amphistome positive sera available in the Department of Veterinary Parasitology, CVAS, Mannuthy were utilised as controls. Further field validation was done using the sera from cattle that harboured schistosomes in the mesentery (n = 50), known schistosome-negative animals using the sera from cattle that did not harbour schistosomes in the mesentery (n = 30) and from animals in which rumen harboured adult amphistomes (n = 30).

Results

Amplification of 22.6-kDa tegument protein coding gene of S. spindale

Amplification of the Ss22.6 produced a 541 bp product at an annealing temperature of 50°C. The nucleotide sequence displayed 96% similarity with the corresponding tegument protein coding gene of other schistosomes, as determined by the BLASTn tool (AB114680.1; EU595756.1; AY851615.1; XM_018788634.1). Further cloning of the sequence in pJET 1.2 blunt cloning vector resulted in approximately 10 colonies. Colony PCR and sequencing of these amplicons revealed a product size of 573 bp (Figure 1). BLASTn analysis of these amplicon sequences (accession number MN534767) showed 91 to 96% similarity with the tegument protein coding gene of other schistosomes (EU595756.1; AB114680.1; XM_051209117.1; AY851615.1; XM_018788634.1).

In silico analysis of 22.6-kDa tegument protein coding gene of S. spindale

Nucleotide sequence analysis using BLASTn indicated a maximum similarity of 95.81% with the *S. bovis* 22.6-kDa protein mRNA and a similarity of about 95% and 91% with *S. haematobium* and *S. mansoni* sequences, respectively. Protein

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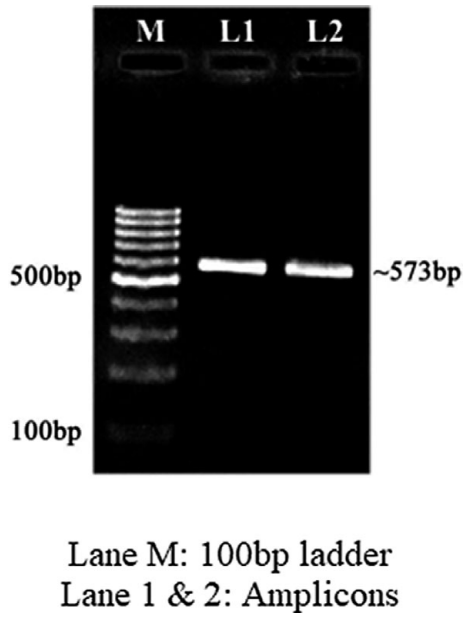

Figure 1. Amplicons of Ss22.6 gene. Agarose (2%) gel electrophoresis of colony PCR products of Ss22.6 gene (573 bp) of *S. spindale* isolated from bovine mesentery samples. M: 100-bp ladder, L1 and L2 amplicons.

sequence prediction tools deduced 190 amino acids and the predicted protein sequence revealed structural similarity to *S. bovis, S. mansoni*, and *S. haematobium* 22.6 kDa sequences. (ACC78608.1; XP_018654157.1; XP_051074544.1). The predicted secondary structure showed that among the 190 amino acids majority formed alpha helices (47.89 %) involving 91 amino acids, 17 amino acids (8.95%) involved in the formation of beta turns, and 33 amino acids (17.37%) formed extended strands; the remaining 49 (25.79%) were associated with random coil structures (Figure 2). The molecular weight and pI of the rSs22.6 protein were determined to be 22628.92 Da and 6.76, respectively. The analysis of predicted amino acid composition highlighted lysine as the most common amino acid, constituting 10% of primary structure of the protein, followed by glutamate (9.5%), and isoleucine (7.9%). Histidine was the least prevalent amino acid accounting for 1.1% of the composition. The calculated grand value of hydropathy (GRAVY) score of -0.651 indicated the non-polar nature of the protein. Additionally, both the aliphatic index (71.84) and instability index (36.88) suggested the protein is stable. Subcellular location of rSs22.6 could not be confirmed as signal peptides or transmembrane helices were not detected (Supplementary file 1). Three possible linear antigenic epitopes were predicted out of which, one (having a score >0.8) was recommended by the server. The sequence that had the highest possibility to become a linear antigenic epitope was a 20 amino acid long sequence which obtained a score of 1.00. The protein exhibited two conserved domains, an EF-hand, a calcium binding domain at the N-terminus (residues 12–71) and a dynein light-chain domain at the C-terminus (residues 99–186) (Supplementary file 2). Analysis of the tertiary structure showed the presence of α helices and β sheets, with an EF-hand forming a helix-loop-helix domain (Figure 3). Of the total 168 residues in the selected model on the Ramachandran plot, 156 (92.9 %) were found in the most favoured regions, 10 residues (6.0 %) in additional allowed regions, and two serene residues in generously allowed regions and no residues in the disallowed regions (Figure 4).

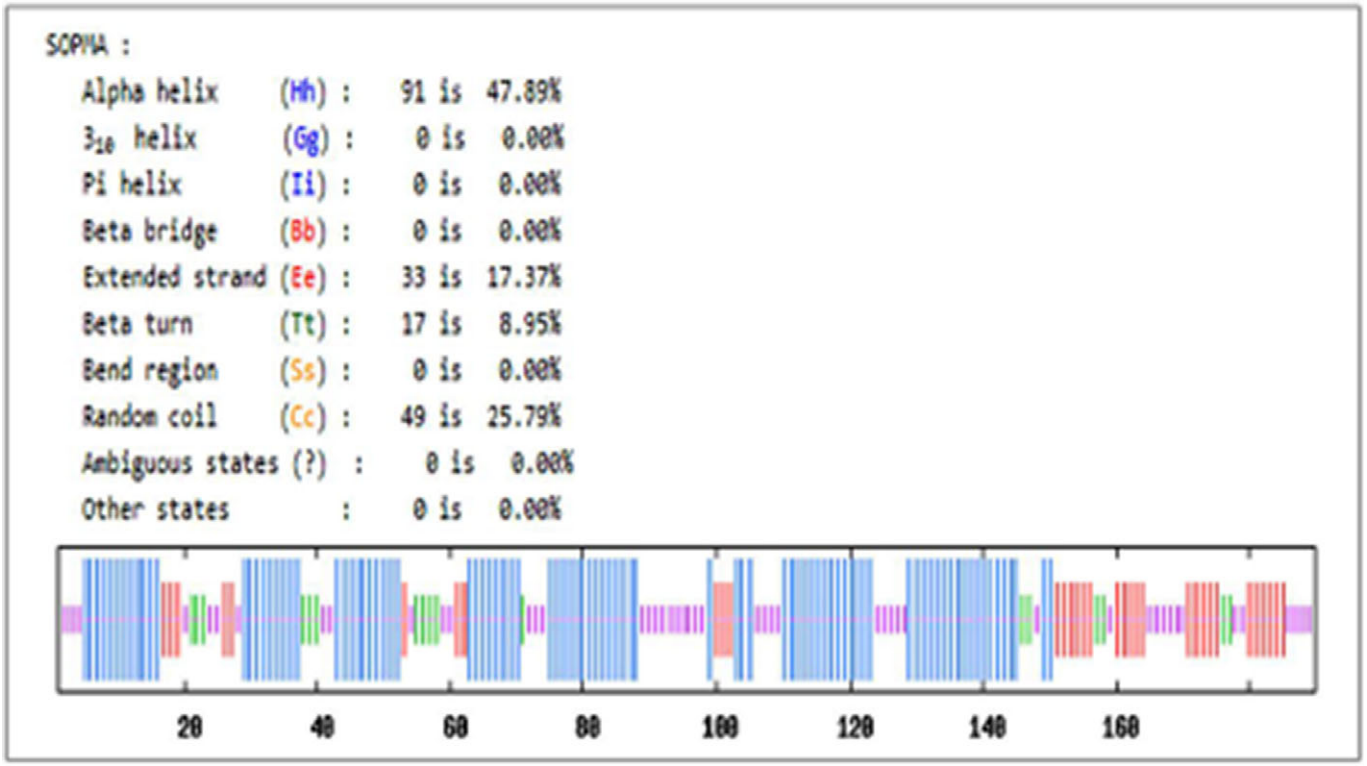

Figure 2. Predicted secondary structure of rSs 22.6 protein. The predicted secondary structure of rSs22.6 with 190 amino acids, the majority of which formed alpha helices (47.89%) involving 91 amino acids, 17 amino acids (8.95%) involving in the formation of beta turns, 33 amino acids (17.37%) forming extended strands, and the remaining 49 (25.79%) involving in the formation of random coil structures.

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MATETKLSSMEEFIRAFLEMDADNNEMIDKQELIKYCQKHRLDM
KLIDPWIARFDTDKDNKISIEEFCRGFGFKVSEIRREKEELKRERE
GKISKLPPNVEIIAATMSKTKQYDICYQFKEFIDSTSRTNNDVKEVA
NKMKTLLDNNYGRVWQVVILTGSYWMNFSHEPFLSIQFKYNNYV
CLAWRTPSQ

Figure 3. Tertiary structure of rSs22.6 protein. Amino acid sequence and molecular modelling of the 22.6 kDa antigen from *Schistosoma spindale* (Ss22.6). One EF-hand motif highlighted in gold yellow and the dynein light-chain motif highlighted in olive green.

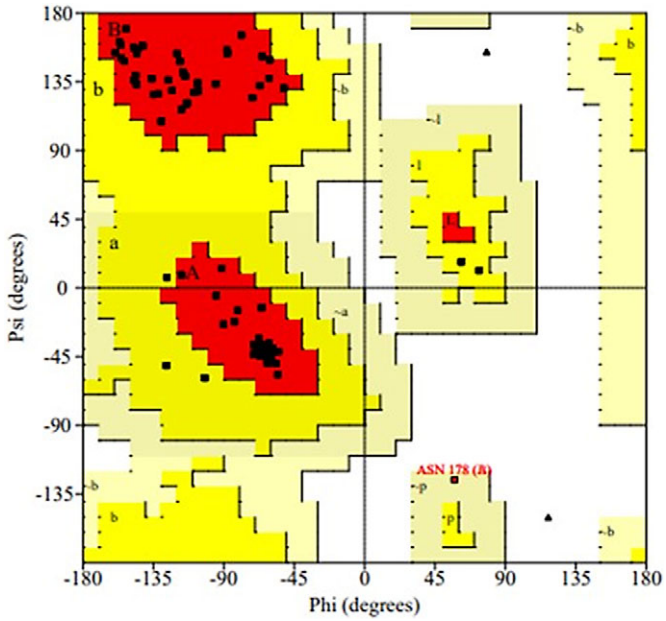

Figure 4. Ramachandran plot of predicted rSs22.6. Ramachandran plot of the selected model of predicted rSs22.6. Of the total 168 residues, 156 (92.9%) were in the most favoured regions (marked as A, B, L), 10 residues (6.0%) in additional allowed regions (marked as a, b, l, p), and two residues (1.2%) in generously allowed regions (marked as -a, -b, -l, -p) without any residues in the disallowed regions.

Phylogenetic analysis of the 22.6-kDa coding region of the newly expressed protein, using 1000 bootstraps, identified a sister clade to *S. haematobium* and *S. bovis*, and distinct from the clade containing *S. japonicum.* The phylogenetic tree showed robust bootstrap support, with values ranging from 70 to 100 (Figure 5). The distance matrix used to estimate evolutionary divergence between sequences indicated that the analysed sequence of *S. spindale* was closest to the gene sequence of *S. bovis* and most distant from *S. japonicum.*

Expression of 22.6-kDa tegument protein coding gene of S. spindale

Expression of the confirmed sequence in *E. coli* BL21 cells resulted in the development of about 100 colonies. Colony PCR of these samples revealed a distinct band of approximately 573 bp, indicating the presence of the confirmed sequences after expression. The optimal induction of the rSs22.6 occurred with 0.6 mM IPTG over 4 h and hence the harvesting of the induced recombinant cells was done after 4 h of incubation.

Analysis of the newly expressed protein

The SDS-PAGE analysis confirmed the expression of rSs22.6 in the induced cells, whereas no expression was detected in the uninduced cells. The presence of the protein was detected in both insoluble and soluble fractions after centrifugation of the cell lysate. The soluble fraction was further subjected to purification. The most pure and concentrated fraction of rSs22.6 was observed in the elute with 150-mM concentration of imidazole buffer (Figure 6). A bright single band of approximately 22.6-kDa size without any nonspecific bands in silver-stained gels indicated the high purity of the protein (Figure 6). The concentration of rSs22.6 was estimated to be 2.5 mg/mL. Upon Western blotting with known schistosome positive sera, a single immunogenic band of 22.6-kDa size was observed. The absence of reactivity with known amphistome positive sera and known schistosome negative sera confirmed the specificity of rSs22.6 (Figure 7).

Diagnostic performance of rSS22.6 dot-ELISA

To further validate the diagnostic utility the protein was employed in dot ELISA for antibody detection from field samples. Antigen concentration of 500 ng/µL and sera dilution of 1:100 were found to be optimum for dot-ELISA using rSs22.6. Of the 50 known positive samples, rSs22.6 dot-ELISA revealed positive signals in 45, indicating a sensitivity of 90%. All the known schistosome-negative sera samples (n = 30) collected from cattle that did not harbour schistosomes in the mesentery were detected as negative by rSs22.6 dot-ELISA, indicating a 100% specificity for the diagnostic assay. In addition, rSs22.6 did not show any cross reaction with amphistome positive sera samples (Figure 7).

Discussion

Animal schistosomosis, a highly prevalent blood fluke infection caused by *S. spindale*, imposes significant financial burden on livestock farmers in India. The lack of sensitive parasitological techniques highlights the urgent need to develop improved diagnostics for this schistosome species. Among an array of immunogenic proteins, 22.6-kDa tegument protein is unique to the genus *Schistosoma.* Besides its homologues in *S. mansoni* (Sm22.6), *S. japonicum* (Sj22.6), and *S. bovis* (Sb22.6) have been shown to play a crucial role in resistance to reinfection in endemic areas, thus offering promise as an epidemiological tool for monitoring these infections (Dunne *et al.*, 1997; Santiago *et al.*, 1998; Torre-Escudero *et al.*, 2012). Functionally, 22.6-kDa tegument protein was reported to delay clotting by inhibiting thrombin, thus modulating haemostasis of the host. Additionally, the protein's non-glycosylated nature reduced the likelihood of cross-reactions with related helminths. However, its presence and potential as a recombinant diagnostic antigen had not been previously explored in Indian schistosome species. In this study, the 22.6-kDa tegument protein of *S. spindale* (Ss22.6) was expressed in a prokaryotic system, characterised, and its diagnostic potential was validated through dot-ELISA for the first time

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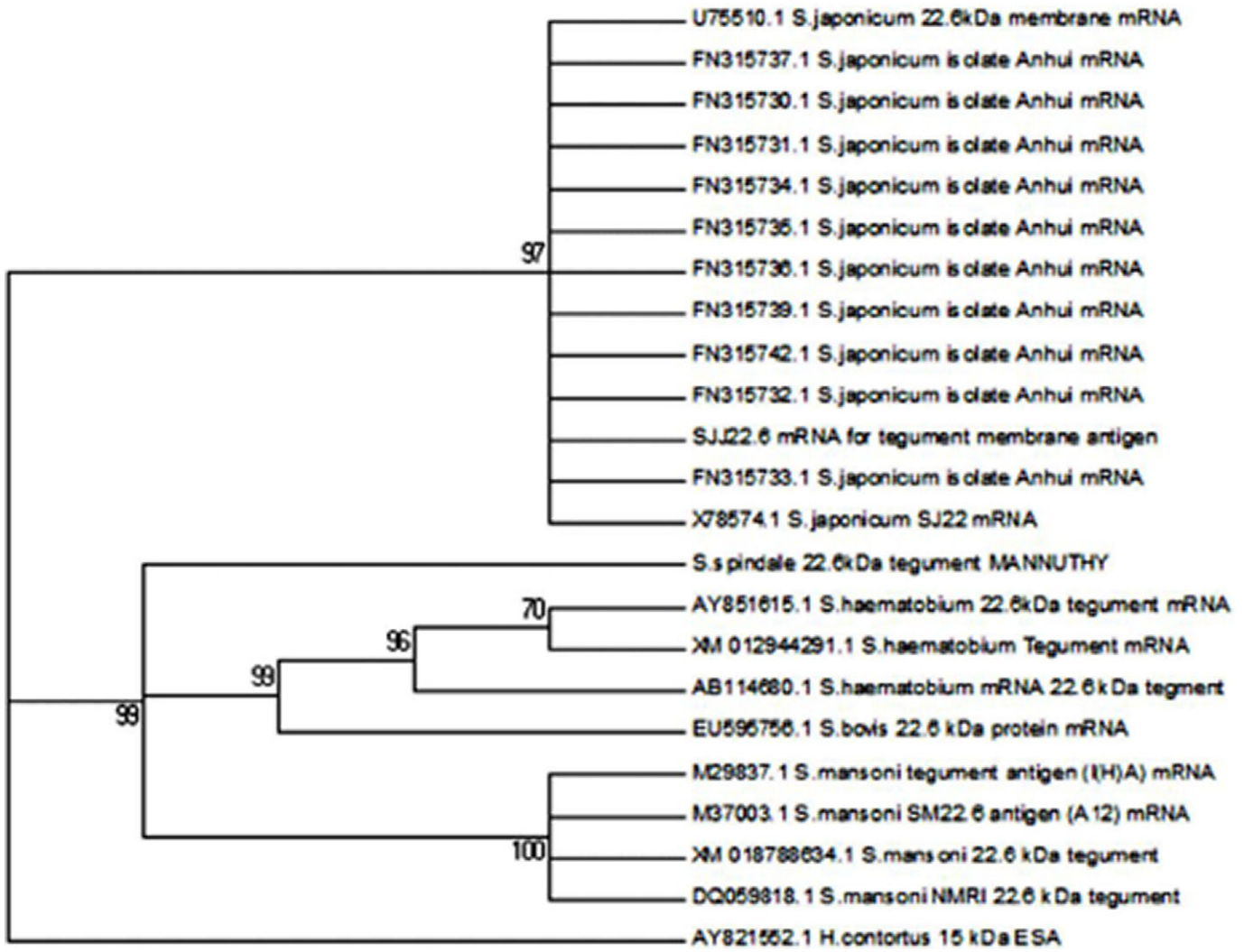

Figure 5. Phylogenetic analysis of Ss22.6 gene. Phylogenetic tree using maximum likelihood method of *S. spindale* based on the analysis of 22.6-kDa coding gene sequences of the newly expressed protein. *Haemonchus contortus* is the outgroup. Bootstrap values (ranging from 70 to 100) are indicated in each node after 1000 replicates. GenBank accession numbers are given in front of each entry.

A 573-bp region corresponding to 22.6-kDa tegument protein coding gene of *S. spindale* amplified using cDNA of *S. spindale* was ascertained to be similar to the corresponding gene of other schistosomes. Further, *in silico* analysis was done to predict the characteristics of protein including pI, amino acid composition, functional properties, structural stability, secretory nature etc. Similarity analysis of nucleotide sequence and the predicted protein sequences of rSs22.6 confirmed the identity of this sequence with that of the related schistosomes *viz. S. haematobium, S. bovis*, and *S. japonicum.* Analysis of the secondary structure of rSs22.6 revealed high percentage of alpha helices suggestive of the involvement of hydrogen bonds in folding, stabilisation, and functioning of protein. The pI is an important factor that estimates the protein solubility, its electrophoresis, and electrophoretic separation. Proteins with a pI near the pH of their environment are typically electrically neutral and demonstrate reduced aggregation and precipitation. The pI of the rSs22.6 was determined to be 6.76, suggesting an average protein solubility and electrophoretic separation (Sawal *et al.*, 2023). The predicted aliphatic index of rSs22.6 suggested that this protein could remain stable across a wide range of temperatures (Sivakumar *et al.*, 2018). A GRAVY score of -0.651 indicated the non-polar nature of rSs22.6, suggesting increased hydrophilic interactions (Magdeldin *et al.*, 2012). Antigenic epitopes are specific regions on the protein surface preferentially recognized by B-cell antibodies. Predicting these antigenic epitopes provide critical insights for designing vaccine components and immunodiagnostic candidates (Ponomarenko and Regenmortel, 2009). One potential epitope within the rSs22.6 protein was identified by the server suggesting its suitability as diagnostic/vaccine candidate.

Analysis of the rSs22.6 using Signal P 5.0 indicated the absence of signal peptides. Typically, molecules that possess signal peptides or anchors are either excreted, secreted, membrane-anchored, or may interact directly with the host immune system (Dias *et al.*, 2014). The lack of a signal peptide in the newly expressed protein implies that it is likely not secreted. Proteins lacking signal peptides might be secreted through alternate pathways like exosome release and direct translocation across the plasma membrane (Samoil *et al.*, 2018). Consistent with this, Zhang *et al.* (2012) noted the absence of a signal peptide in the SjTP22.4 tegumental protein, suggesting its role as a transmembrane protein at the tegument surface. Analysis

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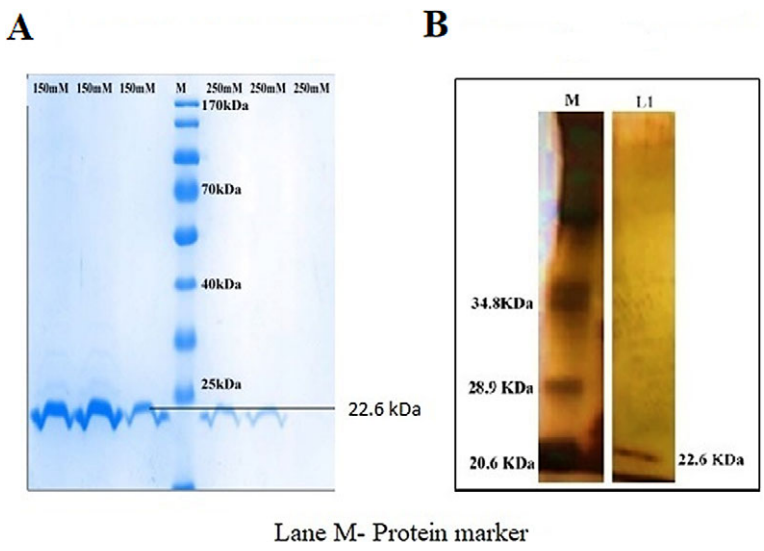

Figure 6. Antigen analysis by SDS PAGE and silver staining. (A) SDS-PAGE analysis of soluble fractions of rSs22.6 protein resolved in 12% gel after Coomassie Brilliant Blue Staining. Presence of the most pure and concentrated fraction of rSs22.6 in the elute with 150-mM concentration of imidazole buffer. M, Broad range protein marker. (B) SDS-PAGE analysis of most pure soluble fraction rSS22.6 protein resolved in 12% gel after silver staining. A bright single band of approximately 22.6-kDa size without any nonspecific bands indicating the high purity of the expressed protein. M, Broad range protein marker; L1, *S. spindale* sample.

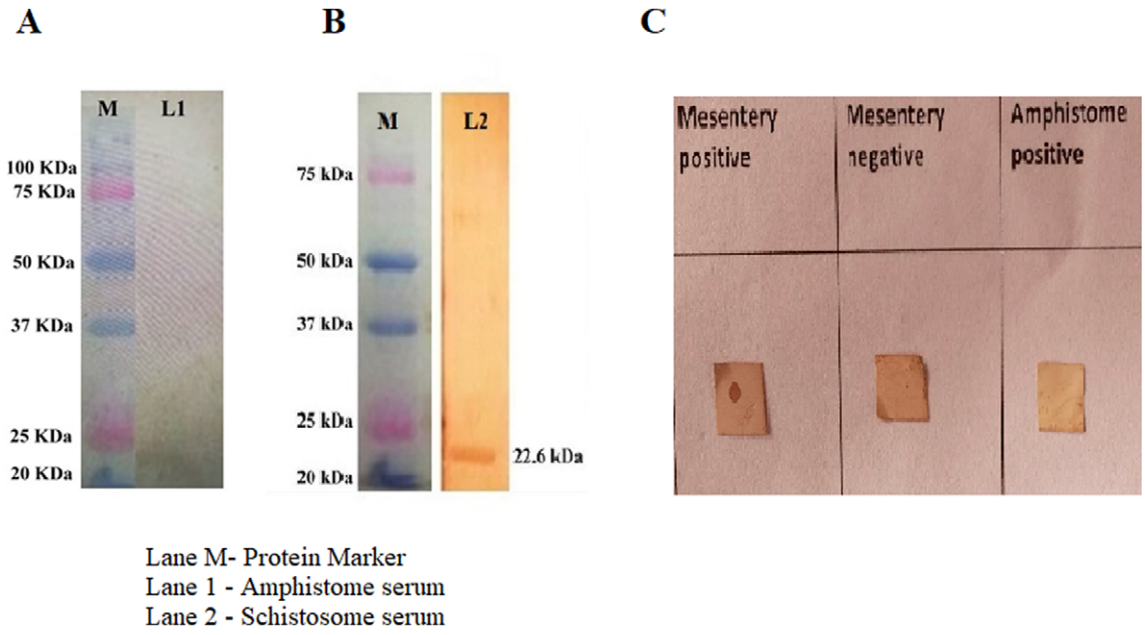

Figure 7. Western blotting and dot ELISA of rSs 22.6 protein. (A) Immunoprofiling of rSs22.6 protein using the western blotting technique. L1, The absence of reactivity with known amphistome positive serum showing the specificity of rSs22.6. Lane M, Broad range protein marker. (B) Immunoprofiling of rSs22.6 protein using the western blotting technique. L2, A single immunogenic band of 22.6-kDa size showing reactivity of the protein with known schistosome positive serum. Lane M, Broad range protein marker. (C) Evaluation of diagnostic potential of rSs22.6 using dot ELISA. Known schistosome positive, known schistosome negative, and amphistome positive sera were utilized as controls. Development of brown-coloured spots in reaction with known schistosome positive serum indicated a positive result whereas absence of development of brown spots in reaction with known schistosome negative and amphistome positive sera samples indicated the absence of cross-reaction and specificity of the protein.

using TMHMM Server v. 2.0 confirmed the absence of transmembrane helices in the predicted protein sequence. The absence of a signal peptide and membrane anchoring motifs in rSs22.6 indicated that it might be a soluble cytoplasmic protein as was previously observed in its orthologues of *Schistosoma* spp., other schistosome tegument protein sequences *viz.* of Sm21.6 and Sb22.6 (Lopes *et al.*, 2009; Torre-Escudero *et al.*, 2012) and in tegumental calcium-binding proteins in other trematodes (Vichasri-Grams *et al.*, 2006).

The presence of an EF-hand domain, a calcium-binding domain, and a dynein light-chain domain in the conserved region of rSs22.6 allows for the modulation of protein conformation and activity in response to calcium signalling. Proteins with EF-hand domains are involved in various biological processes, including signal transduction, muscle contraction, and calcium homeostasis. Similarly, dynein light-chain motifs are crucial for protein-protein interactions, facilitating intracellular

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transport, cytoskeletal organisation, and vesicle trafficking. These motifs can impart specific functional properties to recombinant proteins, such as calcium sensitivity or interactions with cytoskeletal components (O'Connell *et al.*, 2018; Struk *et al.*, 2019). The presence of dynein-related proteins such as Sm21.7 and Sm22.6 primarily localised just beneath the plasma membrane forming a macromolecular complex were documented in tegument extracts (Braschi *et al.*, 2006). These proteins provide structural scaffolding for the parasite tegument and play a crucial role in vesicle transport to the plasma membrane surface. Observations of the EF-hand domain and dynein light chain in various protein molecules of other schistosomes (Lopes *et al.*, 2009; Torre-Escudero *et al.*, 2012; Zhang *et al.*, 2012) are consistent with those in rSs22.6.

Structural validation of the structure of rSs22.6 predicted using Ramachandran plot illustrated the statistical distribution of ϕ (phi) and ψ (psi) dihedral angles in a protein backbone. These angles, along with the omega angle formed by peptide bonds, characterise each residue in a polypeptide chain. This plot classifies regions into generously allowed, moderately allowed, and disallowed regions. A large number of dihedral angles found in the disallowed regions would indicate structural instability in the model. A good quality theoretically rendered protein structure would be expected to have over 90% of its residues in generously allowed region (Motamedi *et al*, 2023; Sawal *et al*, 2023). The presence of a high % (92.9%) of amino acid residues in the most favoured regions without any residues in the disallowed region of Ramachandran plots and those geometrical parameters predicted by molprobity revealed that the constructed model of rSS22.6 is a structurally stable good quality model.

The evolutionary history deduced through phylogenetic analysis using the maximum likelihood method showed that a coding region of the rSS22.6 occurred as a sister clade to *S. haematobium* and *S. bovis* and was distinct from the clade containing *S. japonicum*. The phylogenetic analysis of mitochondrial gene sequences from a Kerala isolate of *S. spindale* had been previously reported to be distantly related to *S. japonicum* (Lakshmanan, 2014). Barker and Blair (1996) had documented through phylogenetic analysis that *S. spindale* may have been brought to India and Southeast Asia from Africa by early humans.

After *in silico* characterisation of the coding sequences and ascertaining its structural stability, it was cloned initially in a cloning vector and then in expression vector. Although many expression studies opt to clone sequences directly into expression vectors (Peng *et al.*, 2008; Lopes *et al.*, 2009; Zhang *et al.*, 2015a), we cloned the rSs22.6 sequence initially in pJET cloning vector using Top10 *E.coli* cells (Torre-Escudero *et al.*, 2012; Zhang *et al.*, 2012). The robust transcription and translation of *E. coli* machinery supports the high-level production of heterologous proteins, with the system's simplicity enabling rapid screening of expression constructs and optimisation of culture conditions (Rosano and Ceccarelli, 2014). Despite these benefits, challenges such as protein misfolding, aggregation, and host cell toxicity from overexpression of foreign proteins can affect yield and solubility. Strategies to address these challenges include the use of fusion tags, co-expressing chaperones, optimising culture conditions, and exploring alternative *E. coli* strains (Baneyx and Mujacic, 2004). Alternative expression systems such as mammalian vectors (e.g., pCMV-Myc), eukaryotic HeLa cells, and yeast cells (e.g., *Pichia pastoris*) also provide options, although they come with challenges like instability and protein overexpression (Andrell and Tate, 2013; Qiu *et al.*, 2013; Damasceno *et al.*, 2017).

The expressed protein was purified using nickel affinity chromatography. The versatility of nickel affinity chromatography allows for the efficient purification of a broad spectrum of recombinant proteins from complex mixtures, including bacterial lysates and cell culture supernatants (Vieira Gomes *et al.*, 2014). Factors such as resin selection, buffer composition, pH, and the concentration of imidazole in the elution buffer significantly influences the efficiency of purification. Imidazole based elution buffers are widely adopted for purification of soluble fractions (Cai *et al.*, 2008; Lopes *et al.*, 2009; Qiu *et al.*, 2013; Zhang *et al.*, 2015a; Zhang *et al.*, 2015b; Lv *et al.*, 2016). In the present study, 2.5 mg/mL of protein was obtained using induction with 0.6 mM IPTG over 4 h followed by purification with 150 mM of imidazole-based buffer. Purification of proteins in their insoluble form requires denaturing conditions using either urea-based or guanidine hydrochloride-based elution buffers (Bornhorst and Falke, 2000). Besides, the design of the histidine tag, including its length and position within the protein sequence, can affect binding affinity and specificity. Optimisation of cell lysis conditions, including the selection of detergents and protease inhibitors, is also critical for minimising non-specific binding and maximising recovery of the target protein (Shi *et al.*, 2018).

Accurate characterisation of expressed proteins was done by SDS-PAGE and Western blot analysis (Laemmli, 1970; Towbin *et al.*, 1979; Sharma *et al.*, 2020). The production of high-quality immunogenic rSs22.6 was indicated by the presence of single specific band by Western blotting using known schistosome positive and the absence of reactivity negative sera. This ensured the repeatability of methodological standardisation and ascertained the immunogenicity of the protein. Cross-reactivity between *Schistosoma* antigens and other trematode antigens is a significant issue in diagnosing various schistosome infections (Raso *et al.*, 2006; Brooker and Clements, 2009; Clements *et al.*, 2010). Here, the absence of reactivity with sera of amphistome positive animals ascertained the specificity of newly expressed protein. This study forms the first documentation of cloning and expression of 22.6 kDa tegumental protein in *S. spindale* and its utility as a diagnostic candidate.

For further validation, we utilised this rSs22.6 in a dot ELISA contemplating the possibility of developing a rapid and field-suitable antibody detection method for diagnosing bovine intestinal schistosomosis. A review of the existing literature revealed that recombinant antigens of *S. spindale* are yet to be employed in a suitable diagnostic assay. Pinto *et al.* (1995) conducted comparative studies using IgM and IgG dot-ELISA with egg and somatic antigens of *S. mansoni* and noted significant sensitivity and specificity, recommending dot ELISA for epidemiological studies to identify specific antibodies against schistosomes. Mafuyai *et al.* (2006) favoured dot ELISA over conventional parasitological methods for epidemiological studies of *S. mansoni* infection in humans in Nigeria. Similarly, Lakshmanan *et al.* (2016) endorsed ESA-based IgG dot-ELISA as a specific and sensitive assay for diagnosing *S. spindale* infections in cattle, suggesting that the use of highly purified antigens could reduce cross-reactivity with other helminths. Dot ELISA employing whole worm antigen has been reported to be less specific in diagnosing visceral schistosomosis in cattle and buffaloes due to potential cross-reactivity (Mott *et al.*, 1987; Montenegro *et al.*, 1999; Noya *et al.*, 2002; Sudhakar *et al.*, 2017). In the current study, a recombinant form of the *S. spindale* antigen was used, offering a more purified version that exhibited no cross-reactivity with amphistome-positive sera, thereby potentially enhancing diagnostic accuracy.

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Conclusion

This study documents the expression and evaluation of a novel recombinant immunogenic antigen of *S. spindale* (rSs22.6). The newly expressed protein demonstrated immunodiagnostic properties in both immunoblotting and *in silico* analyses. The diagnostic potential of rSs22.6 antigen was also effectively validated using dot ELISA, which showed high sensitivity and specificity, highlighting its suitability for diagnosing bovine intestinal schistosomosis under field conditions. Given these attributes, it is reasonable to conclude that rSs22.6 is a promising candidate antigen for the serodiagnosis of bovine intestinal schistosomosis.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000695.

Declaration of Competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Alarcon de Noya, Colmenares C, Lanz H, Caracciolo MA, Losada S and Noya O (2000). *Schistosoma mansoni*: immunodiagnosis is improved by sodium metaperiodate which reduces cross-reactivity due to glycosylated epitopes of soluble egg antigen. *Experimental Parasitology* **95**, 106–112. doi:10.1006/expr.2000.4515

Anisuzzaman MD, Hossain S, Hatta T, Labony SS, Kwofie KD, Kawada H, Tsuji N and Alim MA (2023). Chapter Two - Food- and vector-borne parasitic zoonoses: Global burden and impacts. *Adv. Parasitol.* **120**, 87–136. https://doi.org/10.1016/bs.apar.2023.02.001

Andrell J and Tate CG (2013). Overexpression of membrane proteins in mammalian cells for structural studies. *Molecular Membrane Biology* **30**, 52–63. doi: 10.3109/09687688.2012.703703

Anisuzzaman and Tsuji N (2020) Schistosomiasis and hookworm infection in humans: disease burden, pathobiology and anthelmintic vaccines. *Parasitology International* **75**, 102051. https://doi.org/10.1016/j.parint.2020.102051

Anisuzzaman, Frahm S, Prodjinotho UF, Bhattacharjee S, Verschoor A and Prazeres da Costa C (2021). Host-specific serum factors control the development and survival of *Schistosoma mansoni*. *Frontiers in Immunology* **12**: 635622.

Baneyx F and Mujacic M (2004). Recombinant protein folding and misfolding in *Escherichia coli*. *Nature Biotechnology* **22**, 1399–1408. doi:10.1038/nbt1029

Barker SC and Blair D (1996). Molecular phylogeny of *Schistosoma* species supports traditional groupings within the genus. *Journal of Parasitology* **82**, 292–298.

Bornhorst JA and Falke JJ (2000). Purification of proteins using polyhistidine affinity tags. *Methods in Enzymology* **326**, 245–254. doi: 10.1016/s0076-6879(00)26058-8

Braschi S, Curwen RS, Ashton PD, Verjovski-Almeida S and Wilson A (2006). The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction. *Proteomics* **5**, 1471–1482. https://doi.org/10.1002/pmic.200500368

Brooker S and Clements AC (2009). Spatial heterogeneity of parasite coinfection: determinants and geostatistical prediction at regional scales. *International Journal for Parasitology* **39**, 591–597. doi: 10.1016/j.ijpara.2008.10.014

Cai P, Bu L, Wang J, Wang Z, Zhong X and Wang H (2008). Molecular characterization of *Schistosoma japonicum* tegument protein tetraspanin-2: sequence variation and possible implications for immune evasion. *Biochemical and Biophysical Research Communications* **327**, 197–202. doi: 10.1016/j.bbrc.2008.05.042

Clements AC, Deville MA, Ndayishimiye O, Brooker S and Fenwick A (2010). Spatial co-distribution of neglected tropical diseases in the east African great lakes region: revisiting the justification for integrated control. *Tropical Medicine & International Health* **15**, 198–207. doi: 10.1111/j.1365-3156.2009.02440.x

Colley DG, Bustinduy AL, Secor WE and King CH (2014). Human schistosomiasis. *Lancet* **383**, 2253–2264. doi:10.1016/S0140-6736(13)61949-2.

Damasceno L, Ritter G and Batt, CA (2017). Process development for production and purification of the *Schistosoma mansoni* Sm14 antigen. *Protein Expression and Purification* **134**, 72–81. doi: 10.1016/j.pep.2017.04.002

De Bont J and Vercruysse J (1997). The epidemiology and control of cattle schistosomiasis. *Parasitology Today* **13**, 255–262. doi: 10.1016/s0169-4758(97)01057-0

DeLano WL (2002). The PyMOL Molecular Graphics System. DeLano Scientific, Palo Alto, CA, USA.

Dias SRC, Boroni M, Rocha EA, Dias TL, Souza DL, Oliveira FMS, Bitar M, Macedo AM, Machado CR, Caliari MV and Franco GR (2014). Evaluation of the *Schistosoma mansoni* Y-box-binding protein (SMYB1) potential as a vaccine candidate against schistosomiasis. *Frontiers in Genetics* **5**, 174. doi: 10.3389/fgene.2014.00174.

Divya SP, Lakshmanan B, Subramanian H (2012). Prevalence of schistosomosis in cattle. *The Indian Veterinary Journal* **89**, 81–82.

Doenhoff MJ, Chiodini PL and Hamilton, JV (2004). Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? *Trends in Parasitology* **20**, 35–39. https://doi.org/10.1016/j.pt.2003.10.019

Dunne DW, Webster M and Smith P (1997). The isolation of a 22 kDa band after SDS-PAGE of *Schistosoma mansoni* adult worms and its use to demonstrate the IgE responses against the antigen(s) it contains are associated with human resistance to reinfection. *Parasite Immunology* **19**, 79–89. doi: 10.1046/j.1365-3024.1997.d01-186.x

Fonseca CT, Carvalho GBF, Alves CC and DeMelo TT (2012). Schistosoma tegument proteins in vaccine and diagnosis development: an update. *Journal of Parasitology Research* doi:10.1155/2012/541268

Frahm S, Anisuzzaman A, Prodjinotho UF, Vejzagic N, Verschoor A and Prazeres da Costa C (2019). A novel cell-free method to culture *Schistosoma mansoni* from cercariae to juvenile worm stages for in vitro drug testing. *PLOS Neglected Tropical Diseases* **13**, e0006590. https://doi.org/10.1371/journal.pntd.0006590

Han ZG, Brindley PJ, Wang SY and Chen Z (2009). Schistosoma genomics: new perspectives on schistosome biology and host-parasite interaction. Annual Review of Genomics and Human Genetics **10**, 211–240. doi: 10.1146/annurev-genom-082908-150036

Hellemond JV, Retra K, Brouwers JFHM, van Balkom BWM, Yazdanbakhsh M, Shoemaker, CB and Tielens AGM (2006). Functions of the tegument of schistosomes: clues from the proteome and lipidome. *International Journal for Parasitology* **36**, 691–699. doi: 10.1016/j.ijpara.2006.01.007

Hossain MS, Hatta T, Labony SS, Kwofie KD, Kawada H, Tsuji N and Alim MA (2023). Food-and vector-borne parasitic zoonoses: global burden and impacts. *Advances in Parasitology*, **120**, 87–136.

Kumar V (1999). Trematode infections and diseases of man and animals. Kluwer Academic Publishers, The Netherlands.

Labony SS, Hossain MS, Hatta T, Dey AR, Mohanta UK, Islam A, Shahiduzzaman M, Hasan MM, Alim MA, Tsuji N and Anisuzzaman A (2022). Mammalian and avian larval schistosomatids in Bangladesh: molecular characterization, epidemiology, molluscan vectors and occurrence of human cercarial dermatitis. *Pathogens* **11**, 1213. https://doi.org/10.3390/pathogens11101213

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685. doi: 10.1038/227680a0

Lakshmanan B (2014). Development of improved diagnostics for the detection of bovine intestinal schistosomosis. PhD Thesis, Kerala Veterinary and Animal Sciences University, Pookode.

Lakshmanan B, Devada K, Joseph S, Gleeja VL, Aravindakshan TV, Himachala K and Sankar, S (2018). Seroprevalence of bovine intestinal schistosomosis in different agroecological zones of south India using excretory-secretory antigen based ELISA. *Veterinary Parasitology* **262**, 51–55. doi: 10.1016/j.vetpar.2018.09.012

Lakshmanan B, Devada K, Joseph S and Radhika R (2016). Immunoblot analysis of *Schistosoma spindale* excretory–secretory antigens with sera from naturally infected bovines. *Journal of Applied Animal Research* **44**, 210–214. https://doi.org/10.1080/09712119.2015.1031770

Li Y, Wang L, Fang R, Nie H, Zhou Y and Zhao J (2012). Establishment and evaluation of an iELISA using the recombinant membrane protein LHD-Sj23for the serodiagnosis of *Schistosoma japonicum* infection in cattle in China. *Veterinary Parasitology* **188**, 247–254. doi: 10.1016/j.vetpar.2012.03.047

Lopes DO, Paiva LF, Martins MA, Cardoso FC, Rajão MA, Pinho JM, Caliari MV, Oliveira RC, Mello SM, Leite LCC and Oliveira SC (2009). Sm21.6 a novel EF-hand family protein member located on the surface of *Schistosoma mansoni* adult worm that failed to induce protection against challenge infection but reduced liver pathology. *Vaccine* **27**, 4127–4135.

Lv C, Hong Y, Fu Z, Lu K, Cao X, Wang T, Zhu C, Li H, Xu R, Jia B, Han Q, Dou X, Shen Y, Zhang Z, Zai J, Feng J and Lin J (2016). Evaluation of recombinant multi-epitope proteins for diagnosis of goat schistosomiasis by enzyme-linked immunosorbent assay. *Parasites Vectors* **9**, 135–145.

Mafuyai HB, Uneke CJ, Njoku MO and Chuga G (2006). Dot-ELISA and parasitological examination for diagnosis of *S. mansoni* infection in Nigeria. *Helminthology* **43**, 11–15.

Magdeldin S, Yoshida Y, Li H, Maeda Y, Yokoyama M, Enany S, Zhang Y, Xu B, Fujinaka H, Yaoita E, Sasaki S and Yamamoto T (2012). Murine colon proteome and characterization of the protein pathways. *BioData Mining* **5**, 11. doi: 10.1186/1756-0381-5-11

Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C and Bryant, SH (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Research* **43**, D222–D226.

McCauley EH, Majid AA and Tayeb A (1984). Economic evaluation of the production impact of bovine schistosomiasis and vaccination in Sudan. *Preventive Veterinary Medicine* **6**, 735–754. https://doi.org/10.1016/0167-5877(84)90030-8

Montenegro SML, Silva JDB, Brito MEF and Caravalho LB (1999). Dot ELISA for schistosomosis using Dacron as solid base. *Revista da Sociedade Brasileira de Medicina Tropical* **32**, 139–143.

Motamedi H, Alvandi A, Fathollahi M, Ari MM, Moradi S, Moradi J and Abiri R (2023). *In silico* designing and immunoinformatics analysis of a novel peptide vaccine against metallo-betalactamase (VIM and IMP) variants. *PLoS ONE* **18**, e0275237. https://doi.org/10.1371/journal.pone.0275237

Mott KE, Dixon H, Carter CE, Garcia E, Ishi A, Matsuda H, Mitchell G, Owhashi M, Tanaka H and Tsang VC (1987). Collaborative study on antigens for immunodiagnosis of *Schistosoma japonicum* infection. *Bulletin of the World Health Organization* **65**, 233–244.

Mulvenna J, Moertel L, Jones MK, Nawaratna S, Lovas EM, Gobert GN, Colgrave M, Jones A, Loukas A and McManus DP (2010). Exposed proteins of the Schistosoma japonicum tegument. *International Journal for Parasitology* **40**, 543–554. doi: 10.1016/j.ijpara.2009.10.002

Murthy GSS, D'Souza PE and Isloor KS (2013). Evaluation of a polyclonal antibody based sandwich ELISA for the detection of faecal antigens in *Schistosoma spindale* infections in bovines. *Journal of Parasitic Diseases* **37**, 47–51. doi: 10.1007/s12639-012-0129-9

Noya O, Alarcon de Noya B, Losada S, Colmenares C, Guzman C, Lorenzo MA and Bermudez H (2002). Laboratory diagnosis of Schistosomiasis in areas of low transmission. A review of a line of research. *Memórias do Instituto Oswaldo Cruz* **97**, 167–169.

O'Connell DJ, Bauer MC, O'Brien J, Johnson WM, Divizio CA and O'Kane SL (2018). Protein engineering of Cas9 for improved function. *Methods in Enzymology* **609**, 139–163. doi:10.1016/bs.mie.2018.08.001

Pacifico LG, Fonseca CT, Chiari L and Oliveira SC (2006). Immunization with Schistosoma mansoni 22.6 kDa antigen induces partial protection against experimental infection in a recombinant protein form but not as DNA vaccine. *Immunobiology* **211**, 97–104.

Peng SY, Lee KM, Tsaihong JC, Cheng PC and Fan PC (2008) Evaluation of recombinant fructose-1,6-bisphosphate aldolase ELISA test for the diagnosis of *Schistosoma japonicum* in water buffaloes. *Research in Veterinary Science* **85**, 527–533. doi: 10.1016/j.rvsc.2008.02.005

Pinto PL, Kanamura HY, Silva RM, Rossi CR, de Andrade Júnior HF and Amato NV (1995). Dot-ELISA for the detection of IgM and IgG antibodies to *Schistosoma mansoni* worm and egg antigens, associated with egg excretion by patients. *Revista da Sociedade Brasileira de Medicina Tropical* **37**, 109–15.

Ponomarenko, J, Regenmortel, MHV (2009) B cell epitope prediction. *Structural Bioinformatics* **2**, 849–879.

Qiu C, Fu Z, Shi Y, Hong Y, Liu S and Lin J (2013). A retinoid X receptor (RXR1) homolog from *Schistosoma japonicum*: Its ligand-binding domain may bind to 9-cis-retinoic acid. *Molecular and Biochemical Parasitology* **188**, 40–50.

Raso G, Vounatsou P, Singer BH, N'Goran EK, Tanner M and Utzinger J (2006). An integrated approach for risk profiling and spatial prediction of Schistosoma mansoni – hookworm coinfection. *PNAS USA* **103**, 6934–6939. doi: 10.1073/pnas.0601559103

Ren CP, Liu Q, Liu FC, Zhu FY, Cui SX, Liu Z, Gao WD, Liu M, Ji YS and Shen JJ (2017). Development of monoclonal antibodies against Sj29 and its possible application for schistosomiasis diagnosis. *The International Journal of Infectious Diseases* **61**, 74–78. doi: 10.1016/j.ijid.2017.04.009

Rosano GL and Ceccarelli EA (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers in Microbiology* **5**, 173. doi: 10.3389/fmicb.2014.00172

Sambrook J and Russell DW (2001). *Molecular Cloning: A Laboratory Manual*. (3rd Ed), Cold Spring Harbor Laboratory Press, New York.

Samoil V, Dagenais M, Ganapathy V, Aldridge J, Glebov A, Jardim A and Ribeiro P (2018). Vesicle-based secretion in schistosomes: analysis of protein and microRNA (miRNA) content of exosome-like vesicles derived from *Schistosoma mansoni*. *Scientific Reports* **8**: 3286. DOI:10.1038/s41598-018-21587-4

Santiago ML, Hafalla JCR and Kurtis JD (1998). Identification of the *Schistosoma japonicum* 22.6-kDa antigen as a major target of the human IgE response: similarity of IgE-binding epitopes to allergen peptides. *International Archives of Allergy and Immunology* **117**, 94–104. doi: 10.1159/000023995

Sawal HA, Nighat S, Safdar T and Anees L (2023). Comparative in silico analysis and functional characterization of TANK-Binding Kinase 1–Binding Protein 1. *Bioinformatics and Biology Insights* **17**, 1–5. doi:10.1177/11779322231164828

Sharma G, Kaur A, Goyal D, Nisha N and Vyas M (2020). Western blotting: an introduction. *Biotechnology Journal International* **24**, 27–38. doi:10.9734/bji/2020/v24i330118

Shi D, Yin J, Guo R, Guo L, Liu Y and Li L (2018). Optimization of histidine-tagged protein purification by high-performance immobilized metal affinity chromatography. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **1074**-1075, 33–38. doi:10.1016/j.jchromb.2018.01.029

Sivakumar K and Balaji S (2018). *In silico* characterization of antifreeze proteins using computational tools and servers. *Journal of Chemical Sciences* **16**, 721–730. doi:10.1016/j.jgeb.2018.08.004

Struk A, Boer DR and Rigden DJ (2019). Can dynein light chains help elucidate the evolutionary history of cytoplasmic dynein intermediate chains? *Trends in Cell Biology* **29**, 875–878. doi:10.1016/j.tcb.2019.08.005

Sudhakar K, Murthy GSS and Rajeshwari G (2017). An abattoir study of bovine visceral schistosomiasis in Telengana state, India. *International Journal of Agricultural Sciences* **8**, 3205–3208.

Sumanth S, D'Souza PE and Jagannath MS (2003). Immunodiagnosis of nasal and visceral schistosomosis in cattle by Dot-ELISA. *The Indian Veterinary Journal* **80**, 495–498.

Sumanth S, D'Souza PE and Jagannath MS (2004). A study of nasal and visceral schistosomosis in cattle slaughtered at an abattoir in Bangalore, South India. *Revue scientifique et technique/ Office international des epizooties* **23**, 937–942. doi: 10.20506/rst.23.3.1537

Tamura K and Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512–526. doi: 10.1093/oxfordjournals.molbev.a040023

Tamura K, **Nei M and Kumar S** (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS* **101**, 11030–11035. doi: 10.1073/pnas.0404206101

Tamura K, **Peterson D**, **Peterson N**, **Stecher G**, **Nei M and Kumar S** (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739. doi: 10.1093/molbev/msr121

Torre-Escudero E, **Román RM**, **Sánchez RP**, **Barrera I**, **Siles-Lucas M and Oleaga A** (2012). Molecular cloning, characterization and diagnostic performance of the *Schistosoma bovis* 22.6 antigen. *Veterinary Parasitology* **190**, 530–540. doi: 10.1016/j.vetpar.2012.06.023

Towbin H, **Staehelint T and Gordon J** (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *PNAS* **76**, 4350–4354. doi: 10.1073/pnas.76.9.4350

van Balkom BW, **van Gestel RA**, **Brouwers JFHM**, **Krijgsveld J**, **Tielens AGM**, **Heck AJ and Van Hellemond JJ** (2005). Mass spectrometric analysis of the Schistosoma mansoni tegumental sub-proteome. *Journal of Proteome Research* **4**, 958–966. doi: 10.1021/pr050036w

Vichasri-Grams S, **Subpipattana P**, **Sobhon P**, **Viyanant V and Grams R** (2006). An analysis of the calcium-binding protein 1 of *Fasciola gigantica* with a comparison to its homologs in the phylum Platyhelminthes. *Molecular and Biochemical Parasitology* **146**, 10–23.

Vieira Gomes, AM, **Souza Dias, DC**, **Felli Venancio, K**, **Vicentini, R** (2014). Purification of recombinant proteins: nickel affinity chromatography. *Methods Mol Biol* **1129**, 71–87.

Xu X, **Zhang Y**, **Lin D**, **Zhang J**, **Xu J**, **Liu YM**, **Hu F**, **Qing X**, **Xia C and Pan W** (2014). Serodiagnosis of Schistosoma japonicum infection: genome-wide identification of a protein marker, and assessment of its diagnostic validity in a field study in China. *The Lancet Infectious Diseases* **14**, 489–497. doi: 10.1016/S1473-3099(14)70067-2

Zhang, M, **Fu, Z**, **Li, C**, **Han, Y**, **Cao, X**, **Han, H**, **Liu, Y**, **Lu, K**, **Hong, Y**, **Lin, J** (2015b). Screening diagnostic candidates for schistosomiasis from tegument proteins of adult *Schistosoma japonicum* using an immunoproteomicn approach. *PLOS Neglected Tropical Diseases* **9**. doi:10.1371/journal.pntd.0003454.

Zhang Y, **He Y**, **He L**, **Zong HY and Cai, GB** (2015a). Molecular cloning and characterization of a phospholipidhydroperoxide glutathione peroxidase gene from a blood fluke *Schistosoma japonicum*. *Molecular and Biochemical Parasitology* **203**, 5–13. doi: 10.1016/j.molbiopara.2015.10.001

Zhang Z, **Xu H**, **Gan W**, **Zeng S and Hu X** (2012). *Schistosoma japonicum* calcium-binding tegumental protein SjTP22.4 immunization confers praziquantel schistosomulumicide and antifecundity effect in mice. *Vaccine* **30**, 5141–5150. doi: 10.1016/j.vaccine.2012.05.056

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