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SHORT COMMUNICATION

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Sequence and immunogenicity of the *Taenia saginata* homologue of the major surface antigen of *Echinococcus* spp.

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Abstract A clone (R-Tso18) was isolated from a *Taenia saginata* oncosphere cDNA library by screening with sera from rabbits immunised with oncosphere extract. It contained a full-length cDNA sequence of 1893 bp with an open reading frame of 1680 bp, corresponding to 559 amino acids with a deduced molecular mass of 65.173 kDa and an isoelectric point of 6.08. The R-Tso18 protein showed 80–84% nucleotide identity with the major protoscolex surface antigens of *Echinococcus multilocularis* (EM10) and *E. granulosus* (EG10). Preliminary immunogenicity studies employing the radiolabeled R-Tso18 protein in immune co-precipitation assays indicated sero-positivity for *T. saginata*-infected calf sera (6/13), *T. solium* cysticercosis human (7/22) and pig (2/2) sera and *E. multilocularis* (6/10)- and *E. granulosus* (1/12)-infected human sera, whereas other helminth-infection sera were negative. As immuno-precipitation is a relatively insensitive assay, it was concluded that further studies on the diagnostic potential of the purified recombinant R-Tso18 antigen, or its peptides, are merited.

The nucleotide sequence data reported in this paper are available in the EMBL, Genbank and DDJB data bases under the accession number X97000

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Introduction

The cestodes *Taenia saginata* and *T. solium* are of medical and veterinary importance, typically passing their metacestode developmental stages in cattle and pigs, respectively. In addition, *T. solium*, but not *T. saginata*, metacestodes may also develop in humans, causing cysticercosis, with neurocysticercosis occurring as a frequent and sometimes fatal complication (Flisser et al. 1980). In spite of this important biological difference, the two species exhibit a high level of antigenic cross-reactivity; thus diagnostic and therapeutic tools developed for one species are directly applicable to the other (Harrison and Parkhouse 1989; Parkhouse and Harrison 1987).

Differential diagnosis of human taeniasis is currently based on adult morphology, parasite antigen detection or DNA detection in faeces (Allan et al. 1990; Harrison et al. 1990). Diagnosis of *T. saginata* cysticercosis and *T. solium* cysticercosis of either pigs or humans, on the other hand, is a continuing problem and in many cases the detection is carried out only at post-mortem examination. Therefore, the development of a reliable and specific serological diagnosis is urgently needed. Several approaches have been used, including preparation of different parasite extracts (Diaz et al. 1992; Parkhouse and Harrison 1987), isolation of purified products from heterologous species (Larralde et al. 1990; Rhoads et al. 1985) and the use of monoclonal antibodies (Correa et al. 1989; Harrison et al. 1989). The scarcity of parasite material handicaps the development of suitable diagnostics and, in addition, the crude parasite extracts that may be employed for diagnosis are not universally standardised. However, an expressed recombinant antigen (Felleisen and Gottstein 1993) would circumvent such limitations. With this in mind, a *T. saginata* oncosphere cDNA library was prepared for the expression of *Taenia* gene products with relevance for diagnosis and/or protection. In the present report we describe the cloning and characterisation of a *T. saginata* recombinant antigen, R-Tso18, and examine its diagnostic potential.

Materials and methods

Taenia saginata oncospheres

T. saginata eggs were extracted from mature proglottides obtained from infected hosts in Kenya. Eggs were hatched in sodium hypochlorite solution (Stevenson 1983), cleaned of egg-wall keratin blocks and debris by centrifugation on Isopaque 400 (Onyango-Abuje 1984) and stored in liquid nitrogen in 50-µl aliquots at 5×10^6 oncospheres/ml until use.

cDNA expression library

mRNA was prepared as previously described (Benitez et al. 1996a, 1996b) from 3×10^6 *T. saginata* hatched-activated oncospheres using the Fast-Track System (Invitrogen, Ltd.) according to the manufacturer's instructions. A sample containing 1.2 µg mRNA was converted into double-stranded cDNA with the ZAP-cDNA synthesis kit (Stratagene, La Jolla, Calif.) and then ligated to the ZAP expression vector employing the Uni-ZAP II library kit (Stratagene). The original library was amplified and contained 1.2×10^{11} pfu/ml and a 5% proportion of non-recombinant phages.

Preparation of T. saginata oncosphere extract for immunisation

Hatched oncospheres were recovered from liquid nitrogen storage, diluted in 20 m*M* TRIS/HCl-PI buffers, (pH 8.3) to 1×10^6 oncospheres/ml and sonicated on ice as described above. An aliquot of this preparation was added to an equal volume of 4% (w/v) NaDOC in 20 m*M* TRIS/HCl-PI buffer, (pH8.3) and left to extract on ice for 5 min. The suspension was then centrifuged at 15,000 *g* for 5 min at 4 °C and the supernatant was retained. This NaDOC extract was equivalent to 5×10^5 oncospheres/ml and 500 µg protein/ml. The intramuscular priming inoculations (week 0) were made up to 1.25×10^4 oncospheres/ml (12.5 µg protein/ml) in 20 m*M* TRIS/HCl buffer (pH 8.3) with 1.25 mg saponin/ml (Wells et al. 1982). The intramuscular boosting inoculations (week 6) contained half the number of extracted oncospheres, respectively, but no saponin.

Serum samples

Serum samples were obtained from the following:

1. Rabbits primed (week 0) with 0.5 ml *T. saginata* oncosphere/saponin extract (6.25 µg) and boosted (week 6) with oncosphere extract (3.25 µg). The serum samples were collected 1–2 weeks later (RTSE). Pre-bleeds were taken prior to immunization for use as negative controls in immuno-precipitation assays.
2. Cattle primed (week 0) with 4.0 ml *T. saginata* oncosphere/saponin extract (25 µg) and boosted (week 6) with oncosphere extract (12.5 µg). The serum samples were collected 1–2 weeks later (CTSE).
3. Cattle (13) orally infected with 10,000 *T. saginata* eggs and killed at 4–16 weeks post-infection. The cattle were shown to be infected with viable *T. saginata* metacetodes at post-mortem examination.
4. Porcine sera pooled from pigs orally infected with *T. solium* eggs taken at 38 and 72 days post-infection.
5. Human patients (22) identified as sero-positive for *T. solium* cysticercosis by a commercial cysticercosis diagnosis kit (Melotest, Barcelona) and sero-positive human patients with clinically diagnosed *Echinococcus granulosus* hydatidosis (12) or cystic echinococcosis, *E. multilocularis* hydatidosis (10) or alveolar echinococcosis, *Fasciola hepatica* fasciolosis (5) and *Schistosoma mansoni* schistosomiasis (3).

Normal bovine, porcine and human sera were included as negative controls.

Screening of the cDNA library

The library was differentially screened using rabbit anti-*T. saginata* oncosphere extract serum versus normal rabbit serum. Bound rabbit total Igs were revealed with goat anti-rabbit Ig-alkaline phosphatase (Southern-Biotechnology Associates, Birmingham, Ala.). Positive clones were purified using the same serum selection procedure and finally subjected to in-vivo excision by helper phage rescue (Short et al. 1988) to generate pBluescript plasmids. For the removal of anti-*Escherichia coli* antibodies and, thus, the reduction of background, both sera were pre-adsorbed by incubation with *E. coli* XL1-B lysates (Sambrook et al. 1989).

DNA sequencing

DNA from recombinant plasmids was sequenced automatically using fluorescence-base labeling with the ALF System (Pharmacia, Uppsala, Sweden) and ABI PRISM (Perkin-Elmer, Langen, Germany). Comparisons of DNA sequences and predicted amino acid sequences were carried out with the EMBL and SWISS-PROT data banks using software packages from the Genetic Computer Group (Devereux et al. 1984). The antigenicity of the product was deduced from the Jameson-Wolf antigenic index (Jameson and Wolf 1988).

In vitro transcription/translation and immuno-precipitation of radiolabeled R-Tso18 antigen

In-vitro translated proteins were obtained from a coupled transcription/translation system (TnT Coupled Reticulocyte Lysate System, Promega, Madison, Wis.) employing plasmid DNA directly obtained from pBluescript positive clones. Plasmid DNA (1 µg) prepared with the Qiagen plasmid kit (Qiagen, Surrey, England) was eluted into diethylpyrocarbonate-treated water and translated in a 50-µl final volume employing T3 RNA polymerase and 40 µCi [^{35}S]-methionine of cell-labeling grade (Amersham, Little Chalfont, England) according to the manufacturer's recommendations. The reaction was incubated at 30 °C for 90 min. A 5-µl sample of the translated product was retained as a control. The rest of the preparation was incubated (15 min, 20 °C) in a rotator with 30 µl of a suspension of ProteinA-Sepharose 6MB in TBS (Sigma). After incubation the sample was diluted with 255 µl TBS and divided into 30-µl aliquots. Individual aliquots were then incubated with 1 µl serum for 1 h at room temperature in a rotator. The immune complexes were captured by the addition of 20 µl of a suspension of ProteinA-Sepharose 6MB and incubated for 1 h. The Sepharose was washed three times (5 min each) with RIPA buffer [150 m*M* NaCl; 1% Nonidet P-40; 0.5% Na-deoxycholate; 0.1% Sodium dodecyl sulfate (SDS); 50 m*M* TRIS-HCl, pH 7.5] and one more time (5 min) with buffer 2 [10 m*M* TRIS-HCl (pH 7.5); 0.1% Nonidet P-40]. The immune complexes were dissociated by boiling of the Sepharose in 10 µl electrophoresis buffer containing 5% β-mercaptoethanol. Translation products and immuno-precipitated proteins were separated by 10% SDS-polyacrylamide gel electrrophoresis (SDS-PAGE; Laemmli 1970). Gels were fixed (40% methanol; 10% acetic acid), placed in Amplify (Amersham) for 15 min and then dried under vacuum. X-ray films (Agfa-Gevaert, Spain) were exposed for 16 h at −70 °C.

Results

Sequence of the *Taenia saginata* homologue of the *Echinococcus* spp. surface antigen

Screening of 10^5 clones from the *T. saginata* oncosphere cDNA library with the serum from rabbits immunised with *T. saginata* oncosphere extract yielded 50 promising signals. Of these, two with molecular weights of 1.1 (R-Tso10) and 1.9 (R-Tso18) kb, respectively were

sequenced and found to be overlapping. Thus, R-Tso10 was a truncated cDNA, corresponding to a fragment of 1449 bp from clone R-Tso18 (see data bank). The clone R-Tso18 was a complete cDNA of 1893 bp with an open reading frame of 1680 bp, corresponding to 559 amino acids, a 65.173-kDa molecular mass, and an isoelectric point of 6.08.

Comparison of the R-Tso18 sequence with the EMBL data bank revealed a high degree of homology (80–84% nucleotide identity) with EM10 and EG10, two immunodominant major protoscolex surface antigens from *E. multilocularis* and *E. granulosus* (Felleisen and Gottstein 1994; Frosch et al. 1991), respectively (Fig. 1A.). The GC percentage of the R-Tso18 codifying region (47.9%) was very similar to the percentage described for the EM10 gene (47.4%; Felleisen and Gottstein 1994), and the polyadenylation signal (ATTAAA) described for EM10 and EG10 was also maintained in *T. saginata* R-Tso18 cDNA. At the protein level there was 89–90% identity between the R-Tso18 *T. saginata* gene and the *Echinococcus* sequences. In addition, a search of the sequences included in the SWISS-PROT library revealed homology with genes from the ERM family, consisting of ezrin, radixin and moesin molecules (Lankes and Furthmayr 1991; Sato et al. 1992).

Study of the predicted secondary structure revealed that R-Tso18 was a hydrophilic protein (Fig. 1B), with small stretches of hydrophobic residues and mixed α-helices, β-sheets, turns and random coils. Neither a signal peptide nor a transmembrane domain was observed.

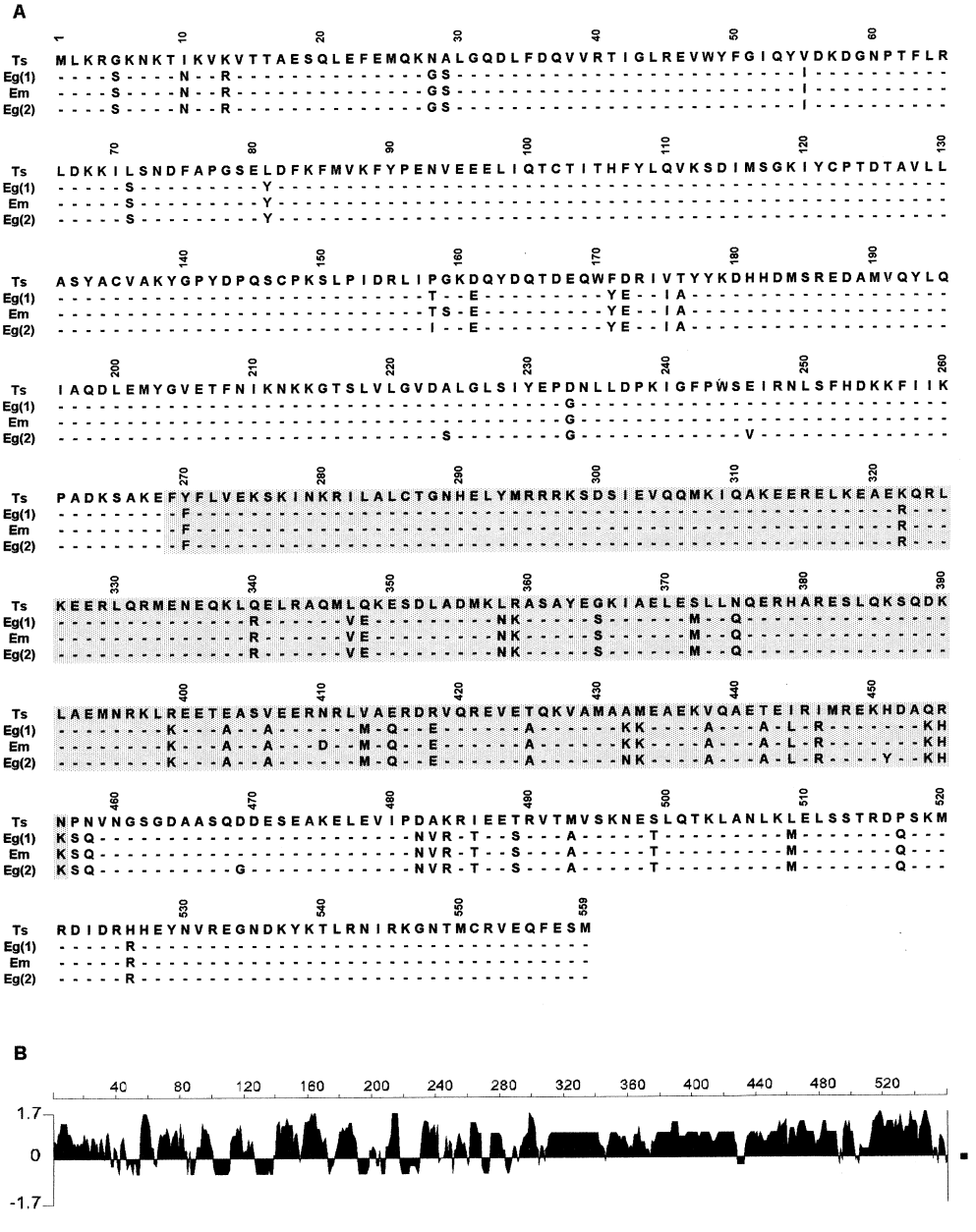

Fig. 1 **A** Alignment of the R-Tso18 deduced amino acid sequence with EM10 (*Echinococcus multilocularis*) and EG10 (*E. granulosus*). *Eg(1)* corresponds to the EG10 gene described by Frosch et al. (unpublished data; AC Z29489); *Eg(2)*, to the EG10 gene described by Felleisen and Gottstein (1994; AC U05574); and *Em*, to the EM10 gene described by both Frosch et al. (1991; AC M61186) and Felleisen and Gottstein (1994; AC U05573) *Hyphens* show identical residues. Antigen II/3 (Phe^{269}-Met^{559}, Gottstein et al., 1993) corresponds to a fragment of the complete sequence including the 3′-end of the molecule and the poly (A) stretch). Antigen II/3-10 (Vogel et al., 1988) is indicated by the *grey shaded area* and is a fragment of II/3. The R-Tso10 clone from *Taenia saginata* comprises the region extending from Asp^{172} to the end of the sequence. **B** The antigenic index according to Jameson and Wolf (1988)

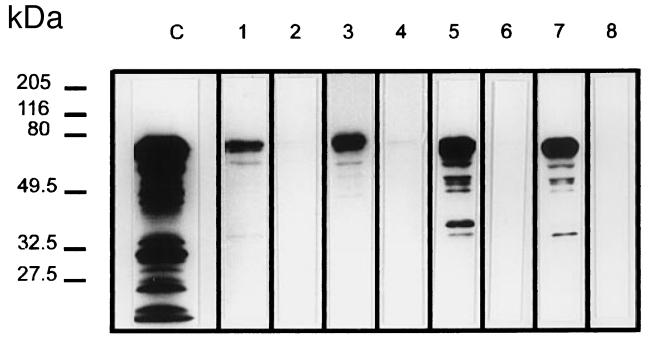

Fig. 2 Immune co-precipitation of the in vitro translated R-Tso18 protein (*track C*) by rabbit anti-*T. saginata* oncospheres (*track 1*), normal rabbit serum (*track 2*), *T saginata*-infected cattle serum (*track 3*), normal bovine serum (*track 4*), *T solium*-infected porcine serum (*track 5*), normal porcine serum (*track 6*), *T. solium* cysticercosis patients (*track 7*) and normal human serum (*track 8*). Molecular-mass markers in kDa are on the *left*

Immunogenicity of in vitro expressed *T. saginata* 65-kDa oncosphere protein

The R-Tso18 clone contained a full open reading frame with a start codon at the 5′-end of the cDNA insert. This was therefore used in an in vitro expression system to provide a radioactive expressed transcription product for immuno-precipitation and SDS-PAGE analysis of sera. The expressed transcription product contained a principal component of 65-kDa (Fig. 2, track C), in agreement with the predicted size of the protein derived from the DNA sequence, although a number of other minor components were also present. Serum from pigs and humans with *T. solium* cysticercosis (Fig. 2, tracks 5, 7) reacted with this 65-kDa component and with some of the minor components, but all the other sera tested, including the RTSE sera, reacted only with the 65-kDa protein (Fig. 2 tracks 1, 3). None of the negative control sera reacted with the product or with sera from humans infected with fasciolosis or schistosomiasis. Six of the calves orally infected with *T. saginata* eggs reacted with the 65-kDa product, but two of these reacted relatively weakly. It was also noted that sera from 6/10 of the *E. multilocularis*-infected patients reacted with the 65-kDa molecule, as opposed to only 1/12 of the *E. granulosis*-infected patients' sera (data not shown). Table 1 summarises the reactivity of the various sera tested.

Table 1 Reactivity as judged by an immune co-precipitation assay of various antisera with the 65-kDa in-vitro expressed transcription protein

Serum source	Antibody to	Sample size	Specific co-precipitation (%)
Bovine	*Taenia saginata*	13	6 (46)
Human	*T. solium*	22	8 (31.8)
Human	*Echinococcus granulosus*	12	1 (8.3)
Human	*E. multilocularis*	10	6 (60)

Discussion

The screening of a cDNA expression library of oncospheres from *Taenia saginata* with sera from rabbits immunised with the *T. saginata* oncosphere extract yielded a full-length cDNA sequence (R-Tso18) with a high degree of homology with metacestode genes from *Echinococcus multilocularis* (EM10) and *E. granulosus* (EG10); Felleisen and Gottstein 1994; Frosch et al. 1991). Thus, the presence of this gene in taeniids had been inferred from these previous studies. Interestingly, both EM10 and EG10 are protoscolex surface antigens. An incomplete sequence (R-Tso10) lacking the initial 171 residues found in R-Tso18 was also identified. As the EM10 expression product is regarded as an excellent diagnostic reagent for alveolar echinococcosis (Helbig et al. 1993; Vogel et al. 1988), it seemed appropriate to study the potential of R-Tso18 as a diagnostic antigen for *Taenia* spp. infections.

As expected, at the structural level the similarity between the molecules was lower at the nucleotide than at the amino acid level. Even so, the homology at the amino acid level between R-Tso18 and the corresponding *Echinococcus*, spp. genes was 90%, whereas the homology between EG10 and EM10 was 98.6% (Felleisen and Gottstein 1994). Prediction of their secondary structure suggested that all three proteins (R-Tso18, EM10, EG10) are hydrophilic. Although the function of the R-Tso18 antigen is unknown, the striking homology with EM10 and EG10 raises the possibility that it could be a tegumental/surface protein, perhaps associated with parasite growth (Felleisen and Gottstein 1993; Frosch et al. 1991). An alternative possibility, based on its homology with ezrin, radixin and moesin molecules, the ERM family (Lankes and Furthmayr 1991; Sato et al. 1992), is that it is a component related to cell junctions or, perhaps, to interactions between the host and the parasite. The ERM family are functionally implicated in cell-to-cell adherence junctions, cleavage furrows, microvilli and ruffling membranes (Sato et al. 1992) and have been postulated to serve as structural links between the plasma membrane and the cytoskeleton (Lankes and Furthmayr 1991). High priorities for future work in our laboratory are therefore the expression of the R-Tso18 product in *T. saginata* metacestodes and, crucially, its localisation in the different parasite stages.

Both R-Tso18 and the truncated R-Tso10 expression products were recognised by the RTSE sera on Western blot analysis (data not shown) despite the finding that R-Tso10 has an incomplete cDNA sequence lacking the first 171 residues. This was taken to indicate, at least to some extent, that the epitope(s) recognised by sera from rabbits immunised with the *T. saginata* oncosphere extract were present in the C-terminal end of the molecule. It would perhaps be relevant to note that shorter fragments from the EM10 gene (II/3 and II/3–10), which also did not contain the N-terminal part of the molecule, showed immuno-reactivity identical to that of the full

sequence (Vogel et al. 1988; Müller et al. 1989). This commonality between R-Tso18, EG10 and EM10 may indicate that the main antigenic regions are located at the middle and/or near the C-terminal end of the molecule. Interestingly, the highest degrees of variability between EG10/EM10 were concentrated at the final region of the consensus sequence, where there was also the greatest variability between R-Tso18 and EG10/EM10. It is therefore possible that selection of appropriate peptides in this region could be exploited for differential diagnosis between these parasites.

For the study of the immunogenicity of the R-Tso18 antigen, expression of radioactive material was achieved by eukaryotic translation of R-Tso18 in vitro. This yielded a prominent labeled protein of 65 kDa as well as minor components of smaller size. Such a pattern could be explained by the presence of numerous ATG codons and, thus, potential ribosomal attachment sites along the sequence. Upon immune co-precipitation and subsequent SDS-PAGE analysis of the total translated product, the positive sera yielded predominantly the 65-kDa polypeptide. Some additional small components were detected by pigs experimentally infected with *T. solium*, which may be explained by degradation or incomplete translation of the 65-kDa product or messenger RNA, respectively.

The in vitro system described in the current work, although not suitable for testing of numerous sera, is certainly useful for preliminary studies of novel recombinant antigens. As can be seen from these results, the R-Tso18 expression product clearly exhibited its immunogenicity and specificity for taeniids, including the reactivity observed with sera from *E. granulosus*- and *E. multilocularis*-infected patients. The difference in reactivity of these two groups of patients' sera may be due to the differing morphology of these two parasites, resulting in differences in the level of exposure of the host to the parasite, this being greater for *E. multilocularis*. Even although immune co-precipitation was observed in only 46% of *T. saginata*-infected cattle and 31.8% of *T. solium* sero-positive cysticercosis patients, these results obtained with such an insensitive test as compared with enzyme-linked immuno-sorbent assay using purified recombinant antigen make R-Tso18 a good diagnostic candidate for further validation, including, as mentioned above, the selection of terminally located peptides. In the future it may be possible to combine the use of R-Tso18 or selected peptides with other antigens to improve the assay performance, as has been done with EM10 and II/3-10 antigens (Gottstein et al. 1993; Helbig et al. 1993).

In conclusion, the *T. saginata* homologue of an *Echinococcus*, spp., surface antigen has been cloned, sequenced and shown to warrant further study as a potential diagnostic reagent. Its possible surface localisation needs to be confirmed as this also raises the possibility of its suitability as a protective antigen.

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