Molecular Cloning of an Immunogenic Protein of *Baylisascaris procyonis* and Expression in *Escherichia coli* for Use in Developing Improved Serodiagnostic Assays

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Larva migrans caused by *Baylisascaris procyonis* is an important zoonotic disease. Current serological diagnostic assays for this disease depend on the use of the parasite’s larval excretory-secretory (ES) antigens. In order to identify genes encoding ES antigens and to generate recombinant antigens for use in diagnostic assays, construction and immunoscreening of a *B. procyonis* third-stage larva cDNA expression library was performed and resulted in identification of a partial-length cDNA clone encoding an ES antigen, designated repeat antigen 1 (RAG1). The full-length rag1 cDNA contained a 753-bp open reading frame that encoded a protein of 250 amino acids with 12 tandem repeats of a 12-amino-acid-long sequence. The rag1 genomic DNA revealed a single intron of 837 bp that separated the 753-bp coding sequence into two exons delimited by canonical splice sites. No nucleotide or amino acid sequences present in the GenBank databases had significant similarity with those of RAG1. We have cloned, expressed, and purified the recombinant RAG1 (rRAG1) and analyzed its diagnostic potential by enzyme-linked immunosorbent assay. Anti-*Baylisascaris* species-specific rabbit serum showed strong reactivity to rRAG1, while only minimal to no reactivity was observed with sera against the related ascarids *Toxocara canis* and *Acaris suum*, strongly suggesting the specificity of rRAG1. On the basis of these results, the identified RAG1 appears to be a promising diagnostic antigen for the development of serological assays for specific detection of *B. procyonis* larva migrans.

In North America, Europe, and parts of Asia, the raccoon roundworm, *Baylisascaris procyonis*, is responsible for neural larva migrans (NLM) in more than 100 mammal and bird species, including humans (17). Infection occurs upon accidental ingestion of embryonated *B. procyonis* eggs from environmental areas or articles contaminated with raccoon feces (17, 26). During the years from 1975 to 2009, 18 cases of *B. procyonis* larva migrans in children were reported from the United States and Canada, where raccoons are common (4, 13, 24, 27). In addition to causing NLM, the parasite is also a well-known cause of visceral and ocular larva migrans, being considered the primary cause of the large nematode variant of diffuse unilateral subacute neuroretinitis (DUSN) (11, 17). In most patients, NLM caused by heavy infection with *B. procyonis* has resulted in either death or permanent neurological deficits (24). The diagnosis of *B. procyonis* NLM is currently based on clinical symptoms, laboratory findings, and epidemiological investigation, along with corroborating evidence of reactivity of a suspected patient’s serum to *B. procyonis* excretory-secretory (BPES) antigens in an indirect enzyme-linked immunosorbent assay (ELISA) (6, 13, 29). Cases with clinical improvement (4, 13) or apparent recovery (27) following proper diagnosis and aggressive treatment have been documented more recently. Excretory-secretory antigens of helminth parasites are widely used in serological assays, such as ELISA, Western blotting, and multi-immunodot assays, for the diagnosis of parasitic infections in humans (8, 12, 14, 23, 32). Our laboratory currently utilizes BPES antigens in ELISA to test for antibodies in patients suspected of having *B. procyonis* NLM. The test has been a very useful aid in the diagnosis of this infection, especially in children (6, 29). However, antibodies to *Toxocara* spp. cross-react with BPES antigens and thus reduce the specificity of BPES antigen-based ELISA (7). Recently, we have reported that more specific diagnosis of this infection can be made using Western blot assays, with specific recognition of 30- to 45-kDa proteins of BPES antigen by serum from patients with *B. procyonis* larva migrans (7).

Apart from the cross-reactivity factor, the production of ES antigens is cumbersome and time-consuming, is dependent on the availability of *B. procyonis* eggs, and involves the handling of infective eggs and larvae. Identification of *Baylisascaris* species-specific immunogenic proteins can facilitate the production of recombinant antigens, which can be easily purified and which could have increased specificity compared to ES antigens. To date, none of the antigens of the BPES antigen complex have been characterized and no genetic information about *B. procyonis* ES antigens is available.

In this study, in order to identify genes encoding immunodominant ES antigens, we performed immunoscreening of a *B. procyonis* third-stage larva (L3) cDNA expression library using anti-*B. procyonis* baboon sera. We identified and characterized the gene encoding an ES protein, designated repeat antigen 1 (RAG1), the cDNA of which was expressed in *Escherichia coli*. In ELISA, the recombinant RAG1 (rRAG1) showed highly specific reactivity with antibodies to *Baylisascaris* spp. and did...
not cross-react with antibodies to the related ascarids *Toxocara canis* and *Ascaris suum_.

**MATERIALS AND METHODS**

*B. procyonis* bабoon serum and positive- and negative-control sera. Anti-*B. procyonis* serum was used for immunoscreening of the *B. procyonis* L3 cDNA expression library. The protein concentration of the ES antigen was estimated using a bicinchoninic acid protein assay kit (Pierce/Thermo Fisher Scientific, Asheville, NC), and aliquots of the ES antigen were prepared and stored at −20°C until use. ES antigens from *A. suum* and *Toxocara canis* were available in our laboratory from a previous study (3) and were used in Western blot assays to determine the reactivity of anti-RAG1 mouse antibodies to these antigens.

**SDS-PAGE and Western blotting.** *B. procyonis* ES antigens and rRAG1 were analyzed by SDS-PAGE under reducing conditions and Western blotting by standard procedures (20, 33). Western blotting was performed by probing the antigens present in the serum samples were adsorbed prior to application of the serum in ELISA by incubating the serum samples with goat anti-human IgG (H+L) (Jackson Immunoresearch Laboratories, West Grove, PA) or HRP-conjugated anti-mouse IgG. Chromogenic substrate, tetramethyl benzidine (TMB; Thermo Scientific, Rockford, IL), was used to develop the Western blots.

**Construction and immunoscreening of *B. procyonis* L3 cDNA expression library.** *B. procyonis* L3 from 1-week-old in vitro cultures were used for RNA extraction using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA). The cDNA was synthesized using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) and cloned into bacteriophage lambda ZAP II vector digested with EcoRI and XhoI (Uni-ZAP XR vector; Stratagene) according to the manufacturer’s suggested procedures. The titer of the library was determined to be 1.45 × 10^9 PFU ml⁻¹ and to have 94% recombinant phages and with 3/4 of the inserts being either 500 bp or greater in length. Immunoscreening of this expression library was performed to identify a recombinant phage containing *B. procyonis* antigens. Dilutions of the cDNA library were prepared to plate approximately 500 to 1,000 PFU of recombinant phages onto NZY agar (Amresco, Solon, OH) in a 150-by-15-mm petri dish. Recombinant plaques expressing *B. procyonis* antigens were identified by transferring the plaques onto NC membranes and processing as Western blots.

**Preparation of RAG1 antigen-selected monospecific antibodies.** Affinity purification of antibodies specific to RAG1 (expressed by the selected phage Bp1) was performed using NCP. The plaque-purified recombinant phages were plated on NZY agar plates and incubated at 37°C. When the plaques became visible, antigens from the recombinant phages were transferred onto both sides of the NCP and the NCP was carefully removed from the agar plate, washed with TBS, and blocked in 5% skim milk prepared in Tris-buffered saline (TBS). This NCP was then incubated in anti-B. procyonis baboon serum (1-in-100 dilution) at 4°C overnight. After thorough washing of the NCP with TBS containing 0.05% Tween-20, antibodies bound to the RAG1 antigen expressed by the phage were eluted by incubating the NCP in 0.2 M glycine buffer, pH 2.8, and neutralized to pH 7.0 immediately by adding 1.32 M Tris. The eluted RAG1-mono specific antibodies were used to perform Western blots with BPES antigens to identify the reactive native RAG1.

**Sequence analysis of rRAG1 cDNA and genomic DNA.** The single-stranded pBluescript phagemid within the lambda phage carrying the *B. procyonis* rRAG1 cDNA insert was rescued using a rapid excision kit (Stratagene) and sequenced to determine the nucleotide sequence of the rRAG1 cDNA insert. The 5′ region of this partial rRAG1 cDNA was identified by performing 5′ RNA ligase-mediated rapid amplification of the cDNA ends (5′ RLM-RACE) using a FirstChoice RLM RACE kit (Ambion Inc., Austin, TX). Briefly, 5′ RACE adapter-tagged *B. procyonis* cDNA was synthesized from 10 μg of *B. procyonis* L3 total RNA and used as the template in a PCR with a 5′ RACE adapter-specific outer primer (5′-GCTGATGCGATGAAGAACACTG-3′) and rRAG gene-specific primer rRAG/FSP (5′-ATCTGGTCTGTACACTTGTGCACCA-3′) to amplify the unknown 5′ region of rRAG1 cDNA. The full-length gene of rRAG1 from the genomic DNA of the parasite was obtained via PCR using a pair of custom-designed primers. The forward primer rRAG/FLFR (5′-AGATGTTTTCTTATCACAAATTTG-3′) was designed on the basis of the sequence obtained from the 5′ RLM-RACE reaction, and the reverse primer rRAG/FLRL (5′-CCATGTTATTGGCTGTTGAC-3′) was based on the sequence of the rRAG cDNA insert obtained from immunoscreening of the *B. procyonis* L3 cDNA expression library. Products of the RACE PCR and those from the amplification of the rRAG gene from genomic DNA were cloned and sequenced to ascertain the sequence of the 5′ region of rRAG1 cDNA and the full-length gene. All sequencing reactions were performed by Purdue University’s Core Sequencing Facility.

**Cloning, expression, and purification of rRAG1 and generation of anti-rRAG1 mouse antibodies.** The partial rRAG1 cDNA identified from the library was amplified by PCR using the primers rRAG1F and rRAG1R (5′-AGATGTTTTCTTATCACAAATTTG and 5′-CCATGTTATTGGCTGTTGACGAC-3′) and subcloned into BglII- and NcoI-digested pRSETc (Invitrogen) expression vector. BL21(DE3)/pLysS E. coli competent cells (Invitrogen) were transformed with the pRSET/rRAG plasmid and the recombinant colonies were selected. Overexpression of rRAG1 was achieved at 4 h after 0.5 mM isopropyl-β-D-thiogalactopyranoside induction at 37°C. Overexpression of rRAG1 was confirmed by SDS-PAGE and by performing Western blot analysis using anti-Xpress antibodies (Invitrogen) and anti-*B. procyonis* baboon sera.

**Purification of this polyhistidine-tagged rRAG1 fusion protein was achieved under denaturing conditions, according to the manufacturer’s instructions, using Talon superflow metal affinity resins (Clontech, Inc., Mountain View, CA). Briefly, a pH-based purification protocol was used, with all buffers containing 6 M urea as the denaturing agent. The purified protein fractions were extensively dialyzed against phosphate-buffered saline at 4°C, aliquoted, and stored at −80°C until use. Antibodies to the purified rRAG1 raised in BALB/c mice were used to identify the native RAG1 in the BPES antigen complex. Preimmunization serum was used as a negative control.**

**Enzyme-linked immunosorbent assay with rRAG1.** Serum samples from rabbits (New Zealand White) experimentally infected with embryonated eggs of different *Baylascaris* spp. (B. procyonis, B. transiens, B. columbatrix, and B. melis) and related ascarids (*A. suum* and *T. canis*) were available in our laboratory from parasitology studies (2). Prior to use for immunoscreening, the baboon serum was heat inactivated and digested with E. coli X1L-Blue MRF² cell lysates to deplete it of antibodies to the bacterial proteins (30).

**RESULTS**

**Identification and nucleotide sequence determination of a cDNA encoding RAG1.** Several immunoreactive recombinant phages were identified upon screening of the *B. procyonis* L3 cDNA expression library with anti-*B. procyonis* baboon serum.
One of the strongly reactive plaques, Bp1, was selected and analyzed further. Western blot analysis with the monospecific antibodies against this recombinant phage Bp1 showed strong reactivity to *Baylisascaris procyonis* excretory-secretory components of molecular mass 52 kDa and higher and are indicated by arrows. Lane S, protein molecular mass marker; lane 1, RAG1-monospecific antibodies; lane 2, anti-*B. procyonis* baboon serum; lane 3, negative-control human serum.

**FIG. 1.** Western blot reactivity of RAG1-monospecific antibodies to *Baylisascaris procyonis* excretory-secretory antigen. Monospecific antibodies against RAG1 expressed by the recombinant phage Bp1 showed strong reactivity to *Baylisascaris* excretory-secretory components of molecular mass 52 kDa and higher and are indicated by arrows. Lane S, protein molecular mass marker; lane 1, RAG1-monospecific antibodies; lane 2, anti-*B. procyonis* baboon serum; lane 3, negative-control human serum.

The deduced amino acid sequence analysis indicated an open reading frame (ORF) of 741 bp (partial 5′ end) that encoded a 246-amino-acid-long polypeptide, RAG1. A 104-bp untranslated region (UTR) and a poly(A) tail were present at the 3′ end downstream of the stop codon. No start codon was detected at the 5′ end.

**Sequence analysis of full-length rag1 cDNA and genomic DNA.** Sequence analysis of the 5′ RACE PCR-amplified product allowed us to extend the 5′ end of rag1 cDNA by 32 bp and to identify a putative translation initiation codon, which resulted in a combined full-length rag1 cDNA of 877 bp (GenBank accession no. GU811847). Thus, the rag1 cDNA has a 753-bp-long ORF flanked by 5′ and 3′ UTRs of 20 and 104 bp, respectively (Fig. 2A and B). The 753-bp ORF is capable of encoding a protein of 250 amino acids with a predicted molecular mass of approximately 25 kDa (which excludes the signal peptide) (Fig. 2B). The deduced amino acid sequence of this protein is characterized by an N-terminal region spanning 12 tandemly arrayed amino acid repeats consisting of a 12-amino-acid-long invariable sequence (PPAGNQGGAN). Corresponding to the amino acid repeats, the nucleotide sequence of the rag1 cDNA also contained 12 direct repeats of a 36-bp-long sequence. However, the nucleotide sequences of the repeats showed a significant variation in codon usage, suggesting the existence of a selective pressure maintaining the constant amino acid sequence of the repeats. Analysis of the amino acid sequence using the simple modular architecture research tool (SMART) (31) predicted a signal peptide between amino acids 1 and 21 and two copies of cysteine-rich potassium channel-blocking toxin (ShKT) domain at the C terminus of the protein (positions 176 to 212 and 213 to 248) (Fig. 2B). The ShKT domain is also referred to as the NC6 or SXC motif in several nematode proteins and has a consensus sequence pattern XCXDX4–6CX4–8CX12CX2TCX2C (10). Multiple potential O-glycosylation sites in the two ShKT domains were predicted using the NetOGlyc program (version 3.1) (15). Blastn (nucleotide-nucleotide BLAST) (35) searches of the GenBank databases did not reveal any nucleotide sequences with significant similarity to rag1. Blastp analysis with the deduced amino acid sequences showed limited similarity to the sequences of different proteins with repeats rich in proline, glycine, glutamine, and asparagine from multiple organisms.

The *rag1* gene was PCR amplified from the parasite’s genomic DNA using the oligonucleotides rag1FLF and...
rag1FLR. Alignment of the 1,610-bp-long amplicon (GenBank accession no. GU811848) revealed that the 753-bp ORF was separated by one 837-bp-long intron delimited by the canonical splice sites, as determined by the alternate splice site predictor program (ASSP at www.es.embnet.org).

Cloning, expression, purification, and reactivity of rRAG1. The $\text{rag1}$ cDNA insert in the pRSETC expression vector was smaller (615 bp) than the original cloned amplicon (663 bp, which includes all the repeats), and sequence analysis revealed a loss of nucleotide sequences in the repeat region (positions 215 to 359; Fig. 2B), resulting in only 8 repeats in the cloned $\text{rag1}$ cDNA insert. However, the amino acid sequence of the repeats was preserved and did not hinder the expression of rRAG1. This expression clone encoded a protein consisting of a total of 250 amino acids (204 amino acids coded by the $\text{rag1}$ cDNA and 46 amino acids for the vector) and had a predicted molecular mass of approximately 26.1 kDa. However, when analyzed on a 12% SDS-polyacrylamide gel, the protein migrated as an $\approx$35-kDa protein.

Western blots performed by probing the purified rRAG1 with anti-Xpress antibody and anti-$\text{B. procyonis}$ baboon sera showed strong reactivity to the 35-kDa rRAG1 protein in the postinduction fraction as well as in the purified fraction. Lane S, protein molecular mass marker; lane 1, purified rRAG1; lane 2, BL21 transformed postinduction; lane 3, BL21 transformed preinduction; lane 4, BL21 untransformed.

FIG. 3. Western blots showing the reactivity of anti-$\text{B. procyonis}$ baboon serum (A) and anti-Xpress antibody (B) to rRAG1 protein. Both anti-$\text{B. procyonis}$ baboon serum and anti-Xpress antibodies reacted to the 35-kDa rRAG1 protein in the postinduction fraction as well as in the purified fraction. Lane S, protein molecular mass marker; lane 1, purified rRAG1; lane 2, BL21 transformed postinduction; lane 3, BL21 transformed preinduction; lane 4, BL21 untransformed.

Diagnostic potential of rRAG1 protein. Anti-rRAG1 mouse antibodies showed a strong reactivity to rRAG1 in Western blot analysis. The antibodies also reacted with BPES antigenic components of several sizes at $\sim$37 kDa and some above 57 kDa in size, making it difficult to identify the native RAG1 (Fig. 4). Fortunately, the anti-rRAG1 antibodies did not recognize any component of the ES antigens of $\text{T. canis}$ (data not shown), suggesting the absence of cross-reactive epitopes in the ES antigens of the two ascarids evolutionarily related to $\text{B. procyonis}$. To further evaluate the specificity, an ELISA based on rRAG1 was standardized and the results were compared with those of the BPES ELISA. Serum from rabbits experimentally infected with either $\text{B. procyonis}$, $\text{B. columnaris}$, $\text{B. melis}$, or $\text{B. transfuga}$ showed strong reactivity with rRAG1 in ELISA at all three dilutions, although the anti-$\text{B. transfuga}$ serum reaction was weaker than the others (Fig. 5A). In ELISA with BPES antigen serum from all rabbits infected with Baylisascaris spp., including those infected with $\text{B. transfuga}$, showed a similar strong reaction (Fig. 5B). While the anti-$\text{T. canis}$ and anti-$\text{A. suum}$ rabbit sera showed strong reactions in the BPES antigen-based ELISA (Fig. 5B), these serum samples did not react with the rRAG1 antigen and had OD values similar to those of the preinfection serum samples (Fig. 5A).

DISCUSSION

The currently available ES antigen-based ELISA for serological detection of $\text{B. procyonis}$ larva migrans exhibits low specificity, primarily because of antigenic cross-reactivity with related ascarids (such as $\text{T. canis}$ and $\text{A. suum}$) resulting from potentially conserved proteins, a phenomenon well documented in other parasites (9, 28). Despite this, ES antigens are widely used in serological assays in either crude or purified (native or recombinant) form to diagnose parasite infections (23, 32, 34), and recombinant antigen-based serological assays show increased diagnostic specificity (23, 34). However, production of recombinant proteins requires knowledge about the genes encoding the specific antigens. The present study was undertaken to identify genes that code for BPES antigens. To
the best of our knowledge, this is the first report of molecular cloning and expression of an antigenic protein of *Baylisascaris procyonis*.

In the absence of posttranslational modifications, native RAG1 can be expected to have a molecular mass of about 25 kDa, on the basis of full-length *rag1* cDNA (assuming that the signal peptide is cleaved and all 12 repeats are present). However, the presence of multiple cysteines favoring protein-protein interaction and potential sites of glycosylation can result in a protein with a higher molecular mass. In addition, rRAG1 migrated slowly in polyacrylamide gels and showed an unexpected apparent mass, possibly due to the rigidity conferred by proline in polypeptides. This has been documented in proline-rich proteins from different organisms (19, 25). The identification of the native RAG1 antigen was not possible because both monospecific and anti-rRAG1 antibodies reacted with multiple proteins (52 kDa and higher) in the BPES antigen. However, on the basis of the reactivity of monospecific and anti-rRAG1 mouse antibodies to several components of the BPES antigen complex, reactivity of the antibodies to proteins transcribed from a multigene family cannot be ruled out and requires further investigation. In addition to the repeats, rRAG1 contains two tandemly located cysteine-rich motifs at the C terminus, which has been described in several nematodes, including *Toxocara canis* and *Caenorhabditis elegans* (1, 22). This domain has been cited under different names (ShkT domain/NC6 motif/SXC motif) but is commonly recognized as the SXC motif in nematodes. The SXC motif homoology arises only by virtue of the position of the six cysteine residues and not other amino acids. Scientific literature shows that the SXC motif has largely been associated with the functional domain of diverse proteins, *viz.*, phosphatidylethanolamine-binding protein, zinc metalloprotease, tyrosinas, lectins, mucins, etc. The domain is also known to associate with signal peptides or in an extracellular milieu facilitates protein-protein interaction (22). Initially identified in *T. canis*, the SXC motif has been identi-
fied in several other nematode parasites, including *A. suum*, *Brugia* spp., *Trichuris* spp., and *Necator americanus*. In addition, no homology to the 12-mer amino acid repeat could be identified, and therefore, a putative function of this protein is not known. At the least, the presence of a secretory signal and the reactivity of RAG1-monomspecific and anti-rRAG1 mouse antibodies to components of BES antigens suggest that RAG1 is a component of the ES antigen. Excretory-secretory antigens in parasites serve several functions, such as in feeding, penetration of the host, tissue migration, reproduction, and immune evasion (5, 16, 21). Functional assays are required to determine the function of this protein in *B. procyonis*. Also, the *ragl* CDNA described here has been identified from *B. procyonis* L3, a tissue migration stage of the parasite in the paratenic host and also the parasite stage seen in larva migrans. Whether the expression of this gene is parasite stage specific is not known.

Anti-rRAG1 mouse antibodies did not recognize any proteins in *A. suum* or *T. canis* ES antigens, suggesting the absence of RAG1 homologues or cross-reactive epitopes in the ES antigens of *A. suum* and *T. canis*. The antibodies raised against rRAG1 strongly reacted to a similar group of BES antigens (37 to 52 kDa), further supporting the idea of these proteins being specific to *B. procyonis*. The absence of cross-reactivity of this protein in ELISA to serum raised against related ascidans like *Toxocara* and *Ascaris* also suggests that this protein might be unique to *Baylisascaris* spp.

Among the *Baylisascaris* spp., strong reactivity was observed with serum against *B. procyonis*, *B. columbiana*, and *B. melis*. These parasites are closely related and are considered to be highly pathogenic causes of larva migrans compared to the somewhat distantly related and less pathogenic species *B. transfuga*. The anti-*B. transfuga* serum did not react as strongly as serum against other *Baylisascaris* spp. The presence of the RAG1 gene in related parasites remains to be investigated.

The *B. procyonis* RAG1 reported here is a potential diagnostic antigen that appears to be highly specific for *Baylisascaris* spp. and that does not cross-react with antibodies to either *Toxocara canis* or *Ascaris suum*, two common ascidian parasites to which humans may also be exposed. Ongoing studies in our laboratory using this rRAG1 protein for the diagnosis of *B. procyonis* larva migrans in humans by ELISA also suggest that there is no cross-reactivity with antibodies to *Toxocara canis* or other parasites. This recombinant antigen should be useful in various assay formats for the development of improved serological tests for the diagnosis, seroepidemiology, and serosurveillance of *B. procyonis* larva migrans.

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