Recombinant Antigen-Based Enzyme-Linked Immunosorbent Assay for Diagnosis of Baylisascaris procyonis Larva Migrans

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Baylisascaris larva migrans is an important zoonotic disease caused by Baylisascaris procyonis, the raccoon roundworm, and is being increasingly considered in the differential diagnosis of eosinophilic meningoencephalitis in children and young adults. Although a B. procyonis excretory-secretory (BPES) antigen-based enzyme-linked immunosorbent assay (ELISA) and a Western blot assay are useful in the immunodiagnosis of this infection, cross-reactivity remains a major problem. Recently, a recombinant B. procyonis antigen, BpRAG1, was reported for use in the development of improved serological assays for the diagnosis of Baylisascaris larva migrans. In this study, we tested a total of 384 human patient serum samples in a BpRAG1 ELISA, including samples from 20 patients with clinical Baylisascaris larva migrans, 137 patients with other parasitic infections (8 helminth and 4 protozoan), and 227 individuals with unknown/suspected parasitic infections. A sensitivity of 85% and a specificity of 86.9% were observed with the BpRAG1 ELISA, compared to only 39.4% specificity with the BPES ELISA. In addition, the BpRAG1 ELISA had a low degree of cross-reactivity with antibodies to Toxocara infection (25%), while the BPES antigen showed 90.6% cross-reactivity. Based on these results, the BpRAG1 antigen has a high degree of sensitivity and specificity and should be very useful and reliable in the diagnosis and seroepidemiology of Baylisascaris larva migrans by ELISA.
and excretory/secretory (ES) antigens, have demonstrated high sensitivity but often show lower specificity, related to various levels of cross-reactivity (8, 9, 11, 13). Western blot assays have some advantage over ELISAs in separating cross-reacting from parasite-specific antigens (11, 20) but are logistically more difficult and time-consuming to perform. Currently available serodiagnostic tests for Baylisascaris larva migrans include a combination of a highly sensitive BPES antigen-based ELISA with a Western blot assay; in the latter, Baylisascaris-specific 30- to 45-kDa ES antigens are recognized by sera from B. procyonis infected individuals (6). Serodiagnostic tests using recombinant antigens have shown increased specificity in the diagnosis of different parasitic infections, including Toxocara larva migrans (21, 26). In addition to possessing high specificity, these recombinant antigens overcome the various limitations involved in the preparation of ES antigens and obviate the possible infection risk to those generating this material.

Toxocara larva migrans is known to occur commonly in the United States, where the national seroprevalence is currently 14% (25). Toxocariasis is the most important parasitic infection that needs to be serologically differentiated from B. procyonis, because these two parasites overlap with similar epidemiologies in temperate regions, and the infections show similar nonspecific as well as clinical symptoms. Recently, a recombinant B. procyonis larval excretory-secretory antigen, RAG1 (rRAG1), with considerable diagnostic potential was reported for use in the development of improved serological assays for the diagnosis of Baylisascaris larva migrans (7). This BpRAG1 antigen did not cross-react with anti-Toxocara canis or anti-Ascaris suum antibodies raised in rabbits, and it showed great potential for use in ELISAs. Since this BpRAG1 antigen does not cross-react with antibodies to Toxocara infection, it will also overcome the problem of one-way cross-reactivity observed with the BPES antigen and should be of great utility in the diagnosis of Baylisascaris larva migrans.

In the present study, we examined the use of this BpRAG1 antigen in a diagnostic ELISA for Baylisascaris larva migrans. We determined the diagnostic sensitivity and specificity of this BpRAG1 ELISA, based on the reactivities of serum samples from patients with Baylisascaris larva migrans, Toxocara larva migrans, and a variety of other parasitic infections. In addition, we report the results of testing of 227 serum samples from patients with unknown or suspected parasitic infections.

**MATERIALS AND METHODS**

**Preparation of BPES and BpRAG1 antigens.** Collection, preservation, and *in vitro* embryonation of *B. procyonis* eggs were performed according to the work of Kazacos et al. (18). Second-stage larvae (L2) were hatched aseptically from *B. procyonis* eggs (6). The *B. procyonis* eggs (6). The embryonated *B. procyonis* eggs were collected and dialyzed against 0.1 M ammonium bicarbonate solution. The dialyzed antigen was concentrated by lyophilization, aliquoted, and stored at −80°C until use.

The BpRAG1 antigen was prepared according to the protocol described previously (7). Briefly, the polyhistidine-tagged BpRAG1 protein was expressed in *Escherichia coli* BL21(DE3)pLysS cells and was purified under denaturing conditions. The eluted protein fractions were extensively dialyzed against phosphate-buffered saline at 4°C, aliquoted, and stored at −80°C.

**Serum samples.** (i) Positive- and negative-control sera. The positive-control serum was obtained from the Division of Parasitic Diseases, CDC, Atlanta, GA, and consisted of serum from an experimentally infected baboon that developed severe NLM following infection with embryonated *B. procyonis* eggs (6). The negative-control serum was from a healthy adult human with no history of exposure to raccoons and no clinical signs of infection.

(ii) Human serum samples. (a) Sera from patients with clinical Baylisascaris larva migrans. Serum samples from 20 individuals who were diagnosed with clinical Baylisascaris larva migrans and were determined to be seropositive by a BPES Western blot assay (6) were used as Baylisascaris-specific human sera to evaluate the sensitivity of the BpRAG1 ELISA. The criteria on the basis of which these sera were considered Baylisascaris specific have been described previously (6).

(b) Sera from patients with other parasitic infections. A total of 115 serum samples from patients with 12 different parasitic infections, viz., *Toxocara*, Strongyloides, and Trichinella infections, filariasis, and Schistosoma, Fasciola, Taenia, Echinococcus, Trypanosoma, Entamoeba, Leishmania, and *Phaeocystis* infections, were obtained from the National Reference Centre for Parasitology, McGill University Health Centre, Montreal, Quebec, Canada, and were used to assess the specificities of both the BpRAG1 and BPES ELISAs. The cross-reactivity of the BPES antigen to different parasitic diseases (except those caused by *Toxocara* spp.) is not known and therefore was evaluated during this study. In addition to these 115 samples, 22 serum samples from patients positive for *Toxocara* larva migrans (identified by testing by the *Toxocara* enzyme immunoassay (rRAG1), previously described from the CDC and tested by the BPES Western blot assay (6), were also used in this study.

(c) Unknown/suspected parasite serum samples submitted for serology. A total of 227 serum samples from human patients (either sex and different age groups) primarily from the United States and Canada were submitted to the Parasitology Laboratory, Purdue University, West Lafayette, IN, during the period from 1986 to 1990 for testing for Baylisascaris larva migrans using the BPES antigen ELISA (6). The results were compared to the specificities of Baylisascaris specific (a) and (b) sera.

**Enzyme-linked immunosorbent assay and Western blotting.** Checkboard titrations were done to determine optimum well-coating amounts of the antigen and the blocking agent, as well as dilutions of primary and secondary antibodies in the ELISAs (5). The Baylisascaris procyonis ES antigen and the BpRAG1 antigen at concentrations of 0.1 μg and 0.125 μg per well, respectively, were used to coat the wells of Immulon 2 HB flat-bottom microtiter plates (Thermo Scientific, Asheville, NC), and ELISA was performed as described previously (7) with a few modifications. The primary antibody (human patient sera) was used at a 1:200 dilution in the BPES ELISA and at a 1:100 dilution in the BpRAG1 ELISA. Alkaline phosphatase-conjugated goat anti-human IgG (H+L) (Bethyl Laboratories, Inc., Montgomery, TX) was used as the secondary antibody and para-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) as the substrate. Microtiter plates were read in a ThermoMax absorbance microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. All sera were run in duplicate and results averaged.

Western blot assays using the BPES antigen were performed on representative patient sera that tested positive by the BPES ELISA, according to the protocol described previously (6), to further determine the specificity of the BPES antigen for the diagnosis of Baylisascaris larva migrans as well as to identify cross-reacting ES components.

**Determination of cutoff values and parameters.** Cutoff values for the BPES ELISA were based on an analysis of multiple sets of sera obtained from children and adults, including (i) 251 random sera collected from 5- to 7-year-old children in Chicago, IL, in 2001, (ii) 84 sera collected primarily from children in Southern California in 2002, associated with a case of NLM, and (iii) 201 sera from all age groups submitted to our laboratory in the 1990s to early 2000s for testing purposes, which included some of the 227 sera from patients with unknown or suspected parasitic infections. By using calculated means and standard deviations (SD) for negative to low optical density (OD) groupings (in increments up to an OD of <0.250) as “negative” populations, and by employing the customary practice of setting cutoffs for a 99% confidence interval at the mean ± 3 SDs (5), a cutoff OD of approximately 0.200 was determined for all three sera groups. Since a true cutoff is never known, a prudent and conservative practice involves bracketing the calculated cutoffs as indicating a suspect or indeterminate reactor group (5); this was done with a bracket of ±0.050. The following parameters were thus set for the BPES ELISA for use in this study and until such time as this ELISA is in more routine use, better negative-population sera are obtained, and/or other analyses, such as receiver operating characteristic (ROC) curves or
J-index analysis (27), can be done: serum samples with an OD of <0.100 were considered negative; those with 0.100 to 0.150, probably negative; those with 0.150 to 0.250, suspect reactors; and those with >0.250, positive. Similar determinations were conducted for the rRAG1 ELISA, using 207 sera submitted to our laboratory, which included the 201 sera of the third group mentioned above. Based on its greater sensitivity and specificity, average mean 3 SD values were lower for this ELISA, and a cutoff OD of approximately 0.175 was determined for incremental OD groupings of >0.250. Taking this into account, and setting a slightly narrower suspect reactor bracket (5), the following parameters were set for the rRAG1 ELISA: samples with an OD of <0.100 were considered negative; those with 0.100 to 0.150, probably negative; those with 0.150 to 0.200, suspect reactors; and those with >0.200, positive.

### RESULTS

**Diagnostic sensitivity of the BpRAG1 ELISA.** All 20 _B. procyonis_ -specific sera reacted with moderate to high OD values in the BPES ELISA, with an average OD of 1.811 (range, 0.326 to 3.132) and 16 samples with an OD of >1.000. When these 20 sera were tested by the BpRAG1 ELISA, 17 samples reacted as positive or suspect, resulting in 85% sensitivity. One of the 20 samples was determined to be negative by the BpRAG1 ELISA, and 2 samples were considered probably negative. Of the 17 samples that reacted strongly, 6 had ODs between 0.500 and 1.000, while 5 had ODs above 1.000 (Table 1; Fig. 1).

### Comparison of the diagnostic specificities of the BpRAG1 and BPES ELISAs. A high diagnostic specificity of 86.9% was obtained for the BpRAG1 ELISA, compared to 39.4% for the BPES ELISA, when serum samples from individuals positive for different helminth and protozoan diseases were examined by the two ELISAs. Cross-reactions in the BpRAG1 ELISA were observed mainly with other nematode (19.4% [12 of 62 samples]) and trematode (15% [3 of 20 samples]) infections (Table 2). On the other hand, although the cross-reactions observed in the BPES ELISA were also mainly due to nematode and trematode infections, the degree of cross-reactivity was much higher, with 77.4% (48 of 62 samples) and 85% (17 of 20 samples) positive reactions with different nematode and trematode infections, respectively (Table 2). The BPES ELISA also showed cross-reactivity (45%) with serum samples from patients with cestode infections, while no cestode cross-reactivity was observed in the BpRAG1 ELISA. Although only 25.7% cross-reactivity with sera from patients with protozoan infections was observed in the BPES ELISA, the BpRAG1 ELISA was comparatively more specific, with only 8.6% cross-reactivity. BPES Western blot assays, carried out on representative serum samples from patients with different parasitic infections that showed high ODs on the BPES ELISA, confirmed that their reactions were due to cross-reactivity (data not shown).

### Cross-reactivity with _Toxocara_ infections in the BpRAG1 and BPES ELISAs. Among the different nematode infections tested by the two ELISAs, a high degree of cross-reactivity occurred with _Toxocara_ infections. Cross-reactivity with _Toxocara_ infections in the BPES ELISA was known previously, and the BpRAG1 ELISA showed much lower cross-reactivity by comparison. For the 32 toxocariasis samples tested by the two ELISAs, the rRAG1 ELISA showed only 25% (8/32 samples) cross-reactivity, as opposed to 90.6% (29/32 samples) cross-reactivity for the BPES ELISA, and almost all of the OD values were low or borderline positive (Table 2; Fig. 1). A single sample showed a very high OD (2.821) by the BpRAG1

### TABLE 1. Sensitivity of the BpRAG1 ELISA for the diagnosis of _Baylisascaris_ larva migrans

| Category       | Optical density at 405 nm | No. of samples<sup>a</sup> |
|----------------|---------------------------|-----------------------------|
| Negative       | <0.100                    | 1                           |
| Probably negative | 0.100–0.150               | 2                           |
| Suspect reactor | >0.150–0.200              | 2                           |
| Positive       | >0.200                    | 15                          |
|                | 0.200–0.500               | 4                           |
|                | 0.500–1.000               | 6                           |
|                | 1.000–1.500               | 2                           |
|                | >1.500                    | 3                           |

<sup>a</sup> A total of 20 samples were tested.
ELISA, and since this sample also recognized 30- to 45-kDa proteins on a BPES Western blot (6), it represented a patient with a dual infection with both parasites.

**Reactivities of unknown/suspected serum samples in the BpRAG1 and BPES ELISAs.** A total of 227 unknown/suspected serum samples were run in the BpRAG1 and BPES ELISAs. Based on the cutoffs that were set for these two ELISAs, a large proportion (89%) of samples was negative by the BpRAG1 ELISA, compared to 59.5% by the BPES ELISA. Sixty-three samples with an OD of 0.250 were determined to be positive by the BPES ELISA, with a mean OD of 0.693, while only nine samples with an OD of 0.200 were considered positive by the BpRAG1 ELISA, with a mean OD of 0.235. Twenty-nine samples were considered suspect reactors by the BPES ELISA, with ODs from 0.150 to 0.250, whereas 16 samples were considered suspect reactors by the BpRAG1 ELISA, with ODs from 0.150 to 0.200 (Table 3).

**DISCUSSION**

Cross-reactivity is a major hurdle in the development of serological tests with high specificity for the diagnosis of parasitic diseases. In this study, we have demonstrated the high sensitivity and very low cross-reactivity of a recombinant *Baylisascaris* antigen, BpRAG1, and its utility in the diagnosis of *Baylisascaris* larva migrans in human patients. The BpRAG1 antigen showed a sensitivity of 85%, with 17 of 20 samples reacting in this ELISA. Obtaining gold-standard parasite-specific human sera is difficult; however, great efforts were made in defining the *Baylisascaris*-specific human sera used in this study. Although multiple parameters, such as exposure history, clinical symptoms, autopsy or biopsy findings, epidemiology, and positive serology in the BPES ELISA, were used to define the samples as true positives, a high positive reaction by the BPES ELISA but not the BpRAG1 ELISA is attributed to

**TABLE 2. Comparison of the specificities of the BPES and BpRAG1 ELISAs for the serodiagnosis of Baylisascaris larva migrans**

| Infection group | No. of serum samples tested | BPES ELISA result | BpRAG1 ELISA result |
|-----------------|----------------------------|-------------------|---------------------|
|                 |                           | No. negative | No. positive | % Positive reactions | No. negative | No. positive | % Positive reactions |
| Nematode        |                            |              |             |                    |              |             |                    |
| Strongyloidesis | 10                         | 4            | 6           | 60                  | 7            | 3           | 30                  |
| Filarisis       | 10                         | 4            | 6           | 60                  | 9            | 1           | 10                  |
| Trichinellosis  | 10                         | 3            | 7           | 70                  | 10           | 0           | 0                   |
| Toxocariasis    | 32                         | 3            | 29          | 90.6                | 24           | 8           | 25                  |
| Subtotal        | 62                         | 14           | 48          | 77.4                | 50           | 12          | 19.4                |
| Trematode       |                            |              |             |                    |              |             |                    |
| Fasciolisis     | 10                         | 0            | 10          | 100                 | 9            | 1           | 10                  |
| Schistosomiasis | 10                         | 3            | 7           | 70                  | 8            | 2           | 20                  |
| Subtotal        | 20                         | 3            | 17          | 85                  | 17           | 3           | 15                  |
| Cestode         |                            |              |             |                    |              |             |                    |
| Cysticercosis   | 10                         | 7            | 3           | 30                  | 10           | 0           | 0                   |
| Echinococcus    | 10                         | 4            | 6           | 60                  | 10           | 0           | 0                   |
| Subtotal        | 20                         | 11           | 9           | 45                  | 20           | 0           | 0                   |
| Protozoa        |                            |              |             |                    |              |             |                    |
| Amebiasis       | 10                         | 6            | 4           | 40                  | 10           | 0           | 0                   |
| Leishmaniasis   | 5                          | 5            | 0           | 0                   | 5            | 0           | 0                   |
| Malaria         | 10                         | 8            | 2           | 20                  | 10           | 0           | 0                   |
| Chagas’ disease | 10                         | 7            | 3           | 30                  | 7            | 3           | 30                  |
| Subtotal        | 35                         | 26           | 9           | 25.7                | 32           | 3           | 8.6                 |
| Total           | 137                        | 54           | 83          | 60.6                | 119          | 18          | 13.1                |

**TABLE 3. Reactivities of unknown/suspect samples in the BpRAG1 and BPES ELISAs**

| Category        | BpRAG1 ELISA | BPES ELISA |
|-----------------|--------------|------------|
|                 | OD           | No. of samples | Mean OD at 405 nm | OD           | No. of samples | Mean OD at 405 nm |
| Negative        | <0.100       | 144         | 0.065              | <0.100       | 101           | 0.058              |
| Probably negative | 0.100-0.150 | 58          | 0.125              | 0.100-0.150 | 34            | 0.127              |
| Suspect reactor | >0.150-0.200 | 16         | 0.165              | >0.150-0.250 | 29            | 0.193              |
| Positive        | >0.200       | 9           | 0.235              | >0.250       | 63            | 0.693              |

* A total of 227 unknown/suspect samples were tested. BpRAG1, *Baylisascaris procyonis* recombinant RAG1 antigen; BPES, *Baylisascaris procyonis* larval excretory-secretory antigen.
cross-reactivity of the BPES antigen with the antigens of other, coinfesting geohelmiths, as well as with those of some other parasites. Larval ES antigen is a heterogeneous mixture of glycoproteins released by metabolically active larvae. Protein sharing, epitope sharing, and/or the presence of similar sugar moieties on the proteins are some reasons for the cross-reactivity seen with the use of ES antigens (19). The recombinant antigen, on the other hand, is a single protein that is nonglycosylated when produced in E. coli, resulting in less or no cross-reactivity.

The BpRAG1 antigen had minimal reactivity with sera from patients with other parasitic diseases and demonstrated a high degree of specificity (86.9%) compared to that of the BPES antigen (39.4%) in the respective ELISAs. A similar study evaluating a recombinant Toxocara antigen (26) demonstrated 44.4% specificity using Toxocara ES antigen, while the use of the recombinant antigen at the same concentration showed almost no cross-reactivity. The BpRAG1 antigen still needs to be evaluated for potential cross-reactivity with Ascaris lumbricoides infections, anisakid infections, and some others; however, since BpRAG1 did not cross-react with anti-Ascaris suum antibodies raised in rabbits (7), we would expect it to show minimal cross-reactivity to these other parasites. One drawback of using recombinant antigens for serodiagnostic assays could be lower sensitivity than that of the ES antigen, because the recombinant antigen is a single protein. However, combinations of recombinant antigens are being used successfully to improve the sensitivities of recombinant antigens for the serodiagnosis of parasitic infections (21). High cross-reactivity with the BPES antigen might not be of much concern, considering the absence or very low prevalence of other helmith infections in the United States, Canada, and Europe, etc. However, there is a possibility that background titers of antibodies to other geohelmith infections are present in immigrant populations and travelers, as well as in areas of the world where Ascaris and other geohelmiths are prevalent. Therefore, the BpRAG1 ELISA would have greater utility than the BPES ELISA in any geographical region where serodiagnosis of Baylisascaris is sought.

Some of the parasite-infected serum samples used for specificity testing in the BpRAG1 ELISA were thought to be cross-reacting with the BpRAG1 antigen; however, we speculate that these could be false-positive reactions to copurified E. coli antigens in the purified fraction of the BpRAG1 antigen. Similar false-positive reactions were observed in our previous study (7) involving sera raised against different ascidian species in experimentally infected rabbits. Adsorbing these sera with E. coli antigens prior to their use in ELISA testing eliminated these reactions. Owing to the large number of sera involved, this adsorption was not performed in the present study. In the long term, this issue can be overcome either by using improved purification techniques or perhaps by using RAG1-based peptide antigens in the ELISA. Occasional false-positive reactions in the BpRAG1 ELISA were also evident by the fact that the same serum samples were not simultaneously positive in both BpRAG1 and BPES ELISA specificity testing, indicating discrepancies. In addition, when unknown or suspected serum samples were run in the BpRAG1 ELISA, as expected, a fairly large percentage of these samples (89%) were negative compared to the proportion in the BPES ELISA (59.5%). Similarly, 92 of the unknown/suspected samples tested in the BPES ELISA were positive, compared to only 25 samples in the BpRAG1 ELISA. Although there is a low prevalence of other helmith infections in the U.S. population, a 14% national seroprevalence of Toxocara spp. was documented recently (25). Considering the facts that the unknown/suspected samples used in this study were submitted to our laboratory from across the United States, that background titers of anti-Toxocara antibodies are common in the population, and that the BPES antigen is known to cross-react with anti-Toxocara antibodies, the percentage of positive reactors observed in the BPES ELISA (40.5%) (Table 3) should be interpreted with caution. The BpRAG1 antigen ELISA showed 11% positive reactors, much more in line with what would be expected, and in agreement with what is known for Toxocara spp. (25), the level of exposure to which is similar to that of Baylisascaris.

In areas of endemicity, there is a real possibility that people will be exposed to infective eggs of both Baylisascaris and Toxocara from the same environments, due to the commonality of their respective hosts and their close association with humans (10, 22). In addition, dogs sometimes develop patent Baylisascaris infections and could contaminate domestic environments and neighborhoods with the eggs of both parasites (12, 14, 15). People also could be exposed due to infected kinkajous, which are related procyonids sometimes kept as exotic pets (4, 14). Luckily, the prevalence of patent Baylisascaris infections in dogs appears to be low, and pet kinkajous are relatively uncommon, so despite the possibility of occurrence, the main concern will continue to be contamination from peridomestic or pet raccoons. The BpRAG1 ELISA showed high specificity and little cross-reactivity to Toxocara infection in humans and therefore can be used in the differential serodiagnosis of larva migrans caused by these two parasites. The BpRAG1 ELISA will be a superior test in cases of larva migrans caused by concurrent infections with these two parasites, compared to the combination of the BPES ELISA, Toxocara ELISA, and Western blot assay recommended previously (6). In a previous study, a high-titer Toxocara ELISA-positive serum sample was suspected of dual infection with Baylisascaris and Toxocara, based on its recognition of 30- to 45-kDa BPES antigen components in a Western blot assay (6). Given the high cross-reactivity observed with the BPES ELISA, it was not unexpected that we obtained a strong reaction with this sample (OD, 2.249); however, this particular serum sample also showed strong reactivity in the BpRAG1 ELISA (OD, 2.821) (the outlying sample in Fig. 1), confirming dual infection of this patient and the utility of the BpRAG1 ELISA.

In conclusion, this study clearly showed the high sensitivity and specificity of the BpRAG1 antigen for the serodiagnosis of Baylisascaris larva migrans, with low or no cross-reactivity with other parasites, including Toxocara spp. In areas of endemicity, all patients suspected of larva migrans should be tested for antibodies against both Baylisascaris and Toxocara spp. Testing for anti-B. procyonis antibodies using the BpRAG1 antigen ELISA is much easier than performing a combination of BPES ELISA and Western blotting, and the BpRAG1 antigen holds great promise for use in diagnostic applications and seroepidemiological investigations.

Finally, we wish to inform the scientific community that
ELISA testing for *Baylisascaris* has been discontinued by our laboratory at Purdue University and that, in the public interest, such testing, including the BpRAG1 antigen ELISA, has been transferred to the U.S. Centers for Disease Control and Prevention in Atlanta, GA, and the Canadian National Reference Centre for Parasitology in Montreal, QC, both of which will undertake serologic testing for *Baylisascaris* in the near future.

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