Inter-laboratory optimization and evaluation of a serological assay for diagnosis of human baylisascariasis

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Abstract

A Western blot assay using a recombinant protein, rBpRAG1, was developed for the diagnosis of human baylisascariasis concurrently by the Centers for Disease Control and Prevention (CDC) in Atlanta, GA and the National Reference Centre for Parasitology (NRCP) in Montreal, Canada. The assay performance was assessed by testing 275 specimens at CDC and 405 specimens at NRCP. Twenty specimens from 16 cases of baylisascariasis were evaluated. Eighteen were positive, with the assay correctly identifying 14 of 16 patients. The rBpRAG1 Western blot showed no cross-reactivity with *Toxocara* positive serum and had an overall sensitivity of 88% and specificity of 98%.

Human baylisascariasis, caused by the raccoon roundworm *Baylisascaris procyonis*, results from infection of a human host with *B. procyonis* L3 larvae. Subsequent neural, visceral, and ocular larva migrans syndromes may occur. The severity of disease is dependent on the number of infective eggs ingested and the tissues affected by larval migration (1). *B. procyonis* has emerged in recent years as one of the most serious causes of larva migrans in humans (2). Pathogenesis is similar to that of toxocariasis, but usually the disease is more severe and the migration of *B. procyonis* larvae through tissues is more aggressive than that of *Toxocara* spp. (3). Unlike *Toxocara* spp., *B. procyonis* larvae continue to grow while migrating, increasing the likelihood of severe neurological disease. Infected patients present with symptoms ranging from mild to severe neurologic dysfunction which may deteriorate rapidly to marked postural and locomotor problems, blindness, seizures, coma, and death, especially if larvae invade the brain in large numbers (2,4). Due to the limited timeframe between onset of symptoms and severe neurological disease, early detection is imperative for patient outcome and survival. Currently, definitive diagnosis is dependent on identification of larvae in biopsy or autopsy specimens (4). Although CT and MRI scans are an adjunct for diagnosis, they cannot definitively differentiate between *Baylisascaris* infection and infections with other eosinophilic meningitis-causing parasites. In most cases, changes visualized by neuroimaging lag behind severe clinical manifestations (3,4). The most efficient and least invasive method for diagnosis is serology. Diagnostic assays using native excretory-secretory (ES) antigen from *B. procyonis* infective larvae were useful, but cross-reactivity, particularly by *Toxocara* specific antibodies, remained a problem in enzyme-linked immunosorbent assays (ELISA) (5). Better results were obtained using Western blot tests, which could differentiate *Baylisascaris* from *Toxocara* infection, although both tests had to be performed (6). The

differential diagnosis of baylisascariasis must rule out toxocariasis because both parasites can cause similar clinical problems and have overlapping geographical distributions (5).

Recently, serological assays for baylisascariasis have improved greatly with the use of a recombinant protein (5, 7). An ELISA was developed based on the *Baylisascaris procyonis RAG1* recombinant protein (*rBpRAG1)* and early testing showed no cross-reactivity with *Toxocara spp.* infections (7). However, in a more recent study testing a total of 384 human patient serum specimens, including 20 patients with clinical *Baylisascaris* larva migrans, 137 patients with other parasitic infections (8 helminth and 4 protozoan), and 227 with unknown/suspected parasitic infections, the rBpRAG1 ELISA showed a sensitivity of 85% and specificity of 87% with 25% cross-reactivity with *Toxocara* sp. infections (5). Previously, diagnostics for baylisascariasis were only available at Purdue University, but recently the rBpRAG1 reagents were transferred to two separate national centers for assay development and independent optimization. In the present study, a sensitive and specific Western blot for the diagnosis of *B. procyonis* infections in humans based on the rBpRAG1 recombinant protein was developed and optimized for use in both the Centers for Disease Control and Prevention (CDC) Parasitic Diseases Reference Diagnostic Laboratory in Atlanta, Georgia and the National Reference Centre for Parasitology (NRCP) in Montreal, Canada.

Materials and Methods

Sera for assay optimization. Both institutions used a positive control anti-*B. procyonis* serum from a baboon that developed severe NLM after experimental infection with *B. procyonis* embryonated eggs. A normal human serum pool was constructed from 5 serum samples from negative, presumably healthy United States residents with no history of international travel. At CDC, a *Toxocara*-positive serum pool was prepared by combining 10 EIA positive serum samples.

Serum panels for determinations of diagnostic sensitivity and specificity. To determine diagnostic sensitivity and specificity of the assay at CDC, a total of 275 human serum specimens were tested

including 15 sera and one CSF from patients diagnosed with *B. procyonis*, 109 sera from patients with other diseases, and 150 sera from North Americans with no history of international travel. At NRCP, a total of 405 samples were used for an independent evaluation of sensitivity and specificity, consisting of nine sera and two CSF specimens from seven patients with baylisascariasis, 264 sera from patients with other diseases, and 130 sera from negative, presumably healthy individuals. All serum samples were used in compliance with protocols approved by the ethical review boards of the participating institutions.

Expression and purification of rBpRAG1 and development of Western blot assays. The pRSET C/*RAG1* plasmid was obtained by CDC and NRCP in 2012 from Purdue University. Expression, purification, and Western blot optimization were performed independently at each center based on the methods of Dangoudoubiyam et al. (7).

Expression and purification of rBpRAG1 at CDC. At CDC, the pRSETC/*RAG1* plasmid was transformed into Bl21 (DE3) pLysS and the recombinant colonies were selected from LB agar plates containing 34µg/mL chloramphenicol and 100µg/mL ampicillin. Colonies were grown in LB Broth containing 34µg/mL chloramphenicol and 100µg/mL ampicillin overnight at 37°C. Expression of rBpRAG1 was achieved 3 hours after adding 0.1 mM isopropyl-β, D-thiogalactopyranoside (IPTG) to the overnight culture. Samples of the induced and non-induced cultures were collected and separated using SDS-PAGE under non-reducing conditions. The gels were visualized and analyzed using Western blotting following previously described methods (8) with anti-HIS and anti-Xpress antibodies (Life Technologies), *B. procyonis* infected baboon serum, and a normal human serum pool. All primary antibodies were diluted in PBS/0.3% Tween-20 (PBS-Tw)/5% milk with the anti-HIS and anti-Xpress antibodies at a 1:5,000 dilution and the baboon and normal human serum pool at a 1:100 dilution. Human serum and CSF specimens were tested using a 1:100 or 1:10 dilution, respectively, in PBS/0.3% Tween-20 (PBS-Tw)/5% milk. The secondary antibody used for anti-HIS and anti-Xpress antibodies was a HRP-

conjugated goat anti-mouse IgG at a 1:8,000 dilution in PBS-Tw and HRP-conjugated goat anti-human IgG at a 1:8,000 dilution in PBS-Tw for the baboon and normal human serum pools. The membranes were incubated with 3,3□-diaminobenzidine (DAB) and H_2O_2 solution for band detection.

Purification of the poly-histidine-tagged rBpRAG1 fusion protein was performed using 6M urea as the denaturing agent and TALON metal affinity resins (Clontech, Inc., Mountain View, CA) (7). Eluted fractions were collected and analyzed by SDS- PAGE and Western blotting with anti-HIS antibodies. Protein concentration was measured using the BCA Protein Assay (Pierce, Rockford, IL).

For determination of antigen purity and optimal concentration, four concentrations of the rBpRAG1 protein, 18.3, 37.5, 75, 150 ng/well, were run on 4-12% NuPage Bis-Tris gradient gels (Life Technologies), blotted, and probed with anti-*B. procyonis* baboon serum, anti-*Toxocara* positive serum pool, and a normal human serum pool using the previously described method. For high-throughput testing of the CDC panel of sera, 2.75 µg f rBpRAG1 antigen was loaded and electrophoresed on a Criterion XT 4-12% Bis-Tris gel with an 11cm IPG well (Bio-Rad Laboratories, Hercules, CA), transferred to nitrocellulose, and cut into 2.5 mm strips.

Expression and purification of rBpRAG1 at NRCP. At the NRCP, expression and purification of the rBpRAG1 protein was modified from the protocol described by Dangoudoubiyam et al. (4). One liter of SOB medium with final concentrations of 34 *u*g/mL chloramphenicol and 100 *u*g/mL ampicillin was inoculated with an overnight culture of BL21(DE3)/pLysS competent *E. coli* transformed with the pRSET C/*RAG1* expression plasmid. The cultures were incubated at 37°C with shaking at 225 rpm until the optical density (OD) at 600nm reached 1.0 to 1.2. Isopropyl-1-ß-D-thio-1-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the cultures further incubated at 37°C with shaking for 3.5 hours. The cells were harvested by centrifugation at 3,000 x g for 10 minutes at room temperature (RT) and stored at -80°C. Cell pellets were lysed under denaturing conditions with purification buffer (20 mM Tris-HCl, pH 8.5, 8 M urea, 500 mM NaCl, 10 mM ß-mercapthoethanol, 20 mM imidazole and 5

144 *u*g/mL DNAseI) and incubated on a rotary shaker for 30 minutes at RT. After centrifugation at 24,000 x g
145 for 20 minutes at RT, the recombinant 6xHis-tagged RAG1 protein was purified using Superflow Ni-NTA
146 resin (Qiagen, Venlo, NL). Five mL of 50% saturated resin was equilibrated with 10 column volumes of
147 purification buffer. The supernatant containing the rBpRAG1 protein was added to the equilibrated resin
148 and allowed to bind on a rotary shaker for 1 hour at RT. The resin-lysate mixture was transferred to a
149 polypropylene column and packed under gravity flow. Six wash buffers of decreasing concentrations of
150 urea (8, 7, 6, 5, 4 and 3 M) in 20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 10 mM ß-mercapthoethanol and 20
151 mM imidazole were applied to the column and drained by gravity flow. The bound protein was eluted in
152 8 column volumes (20 mL) of elution buffer (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 3 M urea, 10 mM ß-
153 mercapthoethanol, and 150 mM imidazole). The eluted protein was dialyzed against TE buffer (10 mM
154 Tris-HCl, pH 7.5, 1 mM EDTA) using Zeba Desalt Spin Columns (Pierce, Rockford, IL) and protein
155 concentration was measured by BCA assay (Pierce) according to the manufacturer's protocols.
156 Expression and purification of the rBpRAG1 antigen was verified by SDS-PAGE and immunoblot.
157 Non-transformed BL21 (DE3) pLysS competent *E. coli* cells were used as a control to assess for rBpRAG1
158 expression. Aliquots of both transformed and non-transformed, induced and non-induced cultures were
159 collected at various time-points and separated on Mini-PROTEAN 4-15% TGX gradient gels (Bio-Rad
160 Laboratories) under reducing conditions. Separated proteins were transferred to nitrocellulose
161 membranes by iBlot dry transfer (Life Technologies) using the default 7-minute protocol. Non-specific
162 sites were blocked with 5% skim milk in 0.05% PBST (0.05% Tween 20 in PBS) for 1 hour at RT.
163 Membranes were incubated overnight at 4°C with a mouse monoclonal anti-poly-histidine serum at
164 1:6,000 (Sigma, St-Louis, MI) followed by incubation with HRP-conjugated anti-mouse IgG at a 1:50,000
165 dilution (GE Healthcare, Little Chalfont, UK). The purified and desalted rBpRAG1 protein was assessed
166 for reactivity with human serum. The nitrocellulose membranes were probed with either a pool of
167 human serum specimens (n =3) positive for *Baylisascaris procyonis* at a dilution of 1:100 or a pool of

healthy human sera (n=3) at 1:100 and incubated with HRP-conjugated anti-human IgG at a 1:100,000 dilution (Perkin-Elmer, Waltham, MA). All membranes were incubated with the chemiluminescent substrate, in SuperSignal West Pico, (Pierce) and exposed to autoradiography film.

For determination of optimal antigen concentration, four amounts of the rBpRAG1 protein (128, 256, 512 and 1024 ng/well) were run on 4-12% NuPage Bis-Tris gradient gels (Life Technologies) under reducing conditions and transferred to nitrocellulose membranes as described above. The membranes were probed with the same pools of *B. procyonis* positive and healthy human sera used above at a dilution of 1:100 but probed with HRP-conjugated anti-human IgG at a dilution of 1:25,000. The membranes were incubated with DAB/H_2O_2 solution.

For high-throughput testing of sera, 6 μg of rBpRAG1 protein was separated on 1D well 4-12% NuPage Bis-Tris gradient gels (Life Technologies) and transferred onto nitrocellulose for Western blot analysis using a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad Laboratories). Human serum and CSF specimens were tested at a dilution of 1:100 and incubated with anti-human IgG-HRP at 1:5,000. Membranes were developed with DAB/H_2O_2 solution.

Results

Expression of rBpRAG1 was monitored by the presence of a 37 kDa band detected by the anti-His antibody only in cells transformed with the expression clone. At the NRCP, recombinant RAG1 was expressed at basal level at all times but reached optimal expression levels after 3.5 hours of induction (data not shown). To improve the diagnostic potential of the rBpRAG1 antigen a few changes were made in the composition of the purification and wash buffers by adding 10 mM ß-mercaptoethanol and 20 mM imidazole. The goal was to eliminate all the bands detected in the immunoblot when probed with healthy human sera but to keep a strong signal with the 37 kDa rBpRAG1 antigen when *B. procyonis* positive samples are tested. The purified and desalted rBpRAG1 antigen (37 kDa) showed a strong reaction to the human *B. procyonis* positive antisera pool (Figure 1). When probed with individual or

pooled healthy human sera no band was detected at 37 kDa confirming the specificity of the rBpRAG1 antigen for *B. procyonis*. However, a single unidentified band slightly larger than 75 kDa was observed (Figure 1). The presence of this 75 kDa band suggested that an optimal test would still require separation of proteins, thus eliminating ELISA as a potential assay format. To further simplify the assay, the detection method was switched from ECL to DAB keeping in mind the facility and rapidity of use of the latter method. The next step was to find the optimal rBpRAG1 antigen amount to use for detecting the 37 kDa rBpRAG1 antigen in *B. procyonis* positive individuals (but not in negative patients), and to eliminate reactivity with the 75 kDa band in negative patients sera. We found that any amount between 128 and 256 ng/well of rBpRAG1 antigen was sufficient to achieve that outcome (Figure 2A).

Experiments were also conducted at CDC to determine antigen purity and optimal concentration; four concentrations of the rBpRAG1 protein were evaluated, 18.3, 37.5, 75, 150 ng/well, using Western blot (Figure 2B). *Baylisascaris*-specific antibodies reacted with the rBpRAG1 protein with an approximate molecular mass of 37 kDa. No cross-reactivity was seen with the normal human serum pool or *Toxocara* strong-positive serum pool. Based on protein resolution and band intensity, the 75 ng/well concentration of rBpRAG1 was chosen for further evaluation. Experiments were also performed to determine if a pre-blocking step was necessary. Pre-blocking the membrane for 1 hour with PBS-Tw/5% milk prior to the addition of serum or CSF improved assay performance and was therefore incorporated into the final protocol.

Assay performance was evaluated independently at both CDC and NRCP (Tables 1 and 2). Sensitivity was assessed using specimens from 16 patients with baylisascariasis. A total of 15 sera and one CSF were tested at CDC and nine sera and two CSF from patients at NRCP. Of the 16 *B. procyonis* positive patients tested, 14 were positive using the rBpRAG1 Western blot, resulting in a sensitivity of 88%. Serum specimens from seven patients with baylisascariasis were tested at both laboratories and all tests were positive. It was not possible to test all specimens at both institutions due to insufficient

quantities of some sera and CSF. A panel of 373 sera was tested from patients with varying diseases, including 63 *Toxocara* positive samples. No sera from patients with toxocariasis were positive (Figure3A); three sera from patients with malaria, and single sera from patients with giardiasis, filariasis, and strongyloidiasis reacted with rBpRAG1 (Figures 3B). Eight sera from presumed normal individuals were also reactive. Overall 14 sera reacted with rBpRAG1, resulting in a specificity of 98%.

Discussion

The performance of the rBpRAG1 Western blot greatly improves laboratory diagnosis for baylisascariasis. Assays based on native ES antigen showed cross-reactivity with antibodies from patients with toxocariasis which necessitated additional testing with *Toxocara* antigens to determine if antibodies specific to *Toxocara* were present; if so, a diagnosis of baylisascariasis could not be definitively made. The lack of cross-reactivity in the rBpRAG1 assay eliminates the necessity for additional testing to rule out toxocariasis and increases the confidence of a baylisascariasis diagnosis. The inter-laboratory evaluation of the assays showed that the results from both centers were concordant and demonstrated the robustness of the assay. Our results indicated a sensitivity and specificity of the assay of 88% and 98%, respectively. However, they may actually be higher than this, since we do not know the full infection history of test sera, e.g., if positive cross-reactors with other parasites or conditions were also infected with *Baylisascaris* as covert dual infections. Sera from two patients gave false negative results in our assay. This may be related to infection levels or the kinetics of the immune response to the parasite. Future studies to examine the kinetics of the immune response and appearance of detectable antibody could provide insight into the false-negative results seen with our assay.

The availability of a more sensitive and specific assay will also facilitate serosurveys to better examine the epidemiology of baylisascariasis. Covert infections with *B. procyonis*, showing mild or no symptoms, may be expected based on the widespread distribution of raccoons in North America, the

prevalence of *B. procyonis* in raccoons (68-90%), and the level of human exposure to *B. procyonis* eggs (2,4,5). Covert infections have been shown in previous reports, e.g., one study showed 8% seroprevalence in children in the Chicago area (1), and the parents of a child with severe NLM symptoms tested positive for *B. procyonis* antibodies without showing signs or symptoms of infection (10). Although an assay in an ELISA platform would be more efficient for a service diagnostic laboratory and for serosurveys, there are several bands seen by Western blotting that seem to be reactive when testing any human serum sample, including normal human serum and *Toxocara*-positive serum (Figure 2). The presence of these bands suggests there would be a lower performance if used in the ELISA format and could explain the slightly lower specificity seen with the BpRAG1 ELISA which shows cross-reactivity to toxocariasis at 25% (5); therefore, further modification must be performed on the rBpRAG1 antigen before an ELISA assay can be developed.

Currently, the rBpRAG1 diagnostic Western blot is being used for testing at the CDC and NRCP. *B. procyonis* infection should be considered based on the presence of NLM, VLM, or OLM symptoms with supporting epidemiological evidence such as a history of pica/geophagia and exposure to raccoons or raccoon latrines. With a high specificity and no cross-reaction in *Toxocara* infections, a positive rBpRAG1 test result should be considered indicative of infection with *B. procyonis* in patients with consistent clinical presentation and risk factors.

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288 **Figure Legends**

289 **Figure 1.** Purified and desalted rBpRAG1 antigen produced at NRCP was tested for reactivity against
290 human serum pools: (+) *B. procyonis* positive sera or (-) healthy control sera. The membranes were
291 detected by ECL. A 37 kDa band corresponding to rBpRAG1 was only detected when probed with sera
292 from individuals with baylisascariasis. A single unidentified band running slightly above 75 kDa was
293 detected by both the positive and negative serum pools.

294 **Figure 2.** Determination of the optimal rBpRAG1 concentration. **(A)** At NRCP, a titration of the purified
295 rBpRAG1 antigen was performed with 128, 256, 512 and 1024 ng/well and the blots were probed with
296 (+) *B. procyonis* positive human serum pool and (-) healthy human serum pool. **(B)** At CDC, a titration of
297 the purified rBpRAG1 antigen was performed at concentrations of (1) 150 ng/well, (2) 75 ng/well, (3)
298 37.5 ng/well, and (4) 18.3 ng/well. Western blots were probed with (A) normal human serum pool, (B)
299 anti-*B. procyonis* baboon serum, and (C) *Toxocara* strong-positive serum pool.

300 **Figure 3. (A)** At CDC, reactivity of serum specimens from patients with *B. procyonis* (A) and *Toxocara* (B)
301 were tested using rBpRAG1 Western blot strips. Strip A1 is a sample from a patient diagnosed with
302 baylisascariasis; strip A3 is a sample from the same patient 3 years post-infection. Patients positive for
303 *Toxocara* by EIA (CDC) were tested using the rBpRAG1 strips (B1-B10). The positive and negative
304 controls were an anti-*B. procyonis* baboon serum (Strip B11) and a normal human serum sample (B12),
305 respectively. **(B)** At NRCP, reactivity of serum specimens to rBpRAG1 from patients with Chagas (1-3),
306 trichuriasis (4-5), strongyloidiasis (6-8), toxocariasis (9-11), echinococcosis (12-14) and filariasis (15-16).
307 Western blot controls included healthy human serum pool (-) and *B. procyonis* baboon serum (+).
308 Positive reactions are observed in lane 15.

309

310 Table 1. Reactivity of sera from patients with baylisascariasis in the rBpRAG1 assays tested at CDC and
311 NRCP.

Patient #	Collection Date	CDC Result	NRCP Result
1	10/17/1990	Positive	Positive
2	10/23/1993	Positive	Positive
3	1/24/1997	Positive	Positive
4	8/7/1997	Negative	N.T.
5	10/20/1997	Positive	N.T.
6	9/1/1998	Positive	Positive
7	1/27/2000	Positive	Positive
	1/31/2000	N.T.	Positive
8	7/17/2000	Positive	Positive
	07/24/2000 (CSF)	N.T.	Positive
	7/26/2000	N.T.	Positive
9	5/28/2002	Positive	Positive
	07/17/2002 (CSF)	N.T.	Positive
10	4/12/2004	Negative	N.T.
11	9/24/2004	Positive	N.T.
12	8/13/2005	Positive	N.T.
13	8/15/2007	Positive	N.T.
14	1/17/2008	Positive	N.T.
15	9/16/2008	Positive	N.T.
16	10/23/2008 (CSF)	Positive	N.T.

312 N.T., not tested. CSF, cerebral spinal fluid. All specimens are serum/plasma unless indicated.

313 Table 2. Analysis of the specificity of the rBpRAG1 assays determined at CDC and NRCP.

	CDC		NRCP	
Disease	# Samples	Positives	# Samples	Positives
Normal	150	6	130	2
Amebiasis	NT	NT	15	0
Ancylostomiasis	1	0	NT	NT
Ascariasis	18	0	11	0
Cancer	2	0	NT	NT
Chagas Disease	NT	NT	25	0
Cysticercosis	2	0	8	0
Echinococcosis	3	0	24	0
Eosinophilic Myalgia	2	0	NT	NT
Fascioliasis	1	0	35	0
Filariasis	NT	NT	7	1
Giardiasis	NT	NT	17	1
Gnathostomiasis	4	0	NT	NT
Hookworm	11	0	NT	NT
Hymenolepiasis	1	0	NT	NT
Malaria	NT	NT	10	3
Schistosomiasis	4	0	21	0
Strongyloidiasis	8	1	25	0
Toxocariasis	40	0	23	0
Toxoplasmosis	4	0	12	0
Trichinellosis	4	0	22	0
Trichuriasis	4	0	9	0
Totals (n)	259	7	394	7
Specificity (%)	**97**		**98**	

314