

1 **A recombinant antigen-based enzyme-linked immunosorbent assay for specific diagnosis of**

2 ***Baylisascaris procyonis* larva migrans**

3 **Sriveny Dangoudoubiyam^{*}, Ramesh Vemulapalli, Momar Ndao¹ and Kevin R. Kazacos**

4 Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University,

5 West Lafayette, IN 47907, ¹Department of Medicine, McGill University Health Centre,

6 Montreal, Quebec H3G 1A4

7 **Running title:** *B. procyonis* RAG1 ELISA

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9 *Correspondence

10 Department of Comparative Pathobiology

11 Purdue University

12 Veterinary Pathology Building

13 725 Harrison Street

14 West Lafayette, IN 47907, USA

15 Tel: 765-494-7556

16 Fax: 765-494-9830

17 Email: sriveny.dangoud@uky.edu

18 *Current address: Gluck Equine Research Center, University of Kentucky, Lexington, KY

19 40546-0099, USA

20 **ABSTRACT**

21 *Baylisascaris larva migrans* is an important zoonotic disease caused by *Baylisascaris procyonis*,
22 the raccoon roundworm, and is being increasingly considered in the differential diagnosis of
23 eosinophilic meningoencephalitis in children and young adults. Although *B. procyonis* excretory-
24 secretory (BPES) antigen-based ELISA and Western blot assays are useful in the
25 immunodiagnosis of this infection, cross-reactivity remains a major problem. Recently, a
26 recombinant *B. procyonis* antigen, BpRAG1, was reported for use in development of improved
27 serological assays for the diagnosis of *Baylisascaris larva migrans*. In this study, we tested a total
28 of 384 human patient serum samples in a BpRAG1 ELISA, including 20 patients with clinical
29 *Baylisascaris larva migrans*, 137 patients with other parasitic infections (8 helminth and 4
30 protozoan), and 227 with unknown/suspected parasitic infections. A sensitivity of 85% and
31 specificity of 86.9% was observed with the BpRAG1 ELISA, compared to only 39.4%
32 specificity with the BPES ELISA. In addition, the BpRAG1 ELISA had a low degree of cross-
33 reactivity with antibodies to *Toxocara* spp. infection (25%), while the BPES antigen showed
34 90.6% cross-reactivity. Based on these results, BpRAG1 antigen has a high degree of sensitivity
35 and specificity and should be very useful and reliable in the diagnosis and seroepidemiology of
36 *Baylisascaris larva migrans* by ELISA.

37 **INTRODUCTION**

38 *Baylisascaris larva migrans* is an important zoonotic disease caused by the raccoon roundworm,
39 *Baylisascaris procyonis* (17, 22). The disease manifests as visceral (VLM), ocular (OLM), and/
40 or neural larva migrans (NLM), related to tissue damage and inflammation caused by aggressive
41 migration of *B. procyonis* larvae within the human host (1, 14, 16). Similar to other geohelminth
42 zoonoses, the infection occurs following accidental ingestion of infective *B. procyonis* eggs from

43 areas contaminated with raccoon feces (14). Raccoons typically defecate in preferred sites called
44 latrines. These latrine sites and their surrounding areas become heavily contaminated with
45 infective *B. procyonis* eggs and pose a significant risk of infection to small mammals, birds, and
46 humans. Raccoon latrines are commonly found on logs, at the base of trees, on large tree limbs
47 or rocks, but may also occur on rooftops, in playgrounds, recreational parks, sandboxes, and
48 other areas (23). Children have a higher risk of infection due to their inquisitive nature,
49 exploration of their surroundings and a tendency to put contaminated materials in their mouth.
50 Although this infection is known to occur in Europe and parts of Asia, most reported cases are
51 from North America (United States and Canada), where raccoons are both common and widely
52 distributed (10, 22).

53 Clinical signs and symptoms associated with migrating *B. procyonis* larvae are often non-
54 specific, although there is a greater association with the production of eosinophilic
55 meningoencephalitis (24). Current diagnosis of *Baylisascaris* larva migrans is based on a
56 combination of criteria, including the patient's history of exposure to raccoons or raccoon feces,
57 clinical signs consistent with larva migrans (particularly NLM) and results of clinical laboratory
58 tests. These include eosinophilia in peripheral blood and cerebrospinal fluid, and positive
59 serology (anti-*B. procyonis* IgG detection, performed at Purdue University) using *B. procyonis*
60 larval excretory-secretory (BPES) antigen-based ELISA and Western blot assays (6). Although
61 larval recovery and identification is the gold standard for diagnosis, there is a low probability of
62 detecting larvae in brain biopsy samples, and fewer of these invasive procedures are being done
63 with the availability of serologic testing (10, 22). In conjunction with other criteria listed above,
64 serologic tests are performed to assist the diagnosis of clinical *Baylisascaris* larva migrans,
65 especially NLM and OLM. However, covert infections with *B. procyonis*, showing mild or no

66 symptoms, can also be expected in relatively large numbers based on the widespread distribution
67 of raccoons in North America, the high prevalence of this parasite (68->90%) in raccoon
68 populations, and the level of human exposure to *B. procyonis* eggs (14, 17). Prior to the
69 knowledge of cross-reactivity in the BPES ELISA, an 8% seroprevalence of *B. procyonis*
70 infection was reported in children in the Chicago area (3), and may have been affected somewhat
71 by concomitant *Toxocara* infections in the population.

72 Studies of serologic diagnosis of *Baylisascaris larva migrans*, using BPES antigen-based
73 ELISA and Western blots, have shown that cross-reactivity occurs with *Toxocara* spp. and other
74 ascarid infections (2, 6). Cross-reactivity is a common hurdle in the development of
75 serodiagnostic tests with higher specificity. Serodiagnostic tests developed for various
76 nematodes (including *Toxocara* spp.), using both crude somatic and excretory/secretory (ES)
77 antigens, have demonstrated high sensitivity but often show lower specificity, related to varied
78 levels of cross-reactivity (8, 9, 11, 13). Western blot assays have some advantage over ELISA in
79 separating cross-reacting versus parasite-specific antigens (11, 20), but are logistically more
80 difficult and time consuming to perform. Currently available serodiagnostic tests for
81 *Baylisascaris larva migrans* include a combination of highly sensitive BPES antigen-based
82 ELISA and Western blot assays, the latter in which *Baylisascaris*-specific 30-45 kDa ES
83 antigens are recognized by serum from *B. procyonis* infected individuals (6). Serodiagnostic tests
84 using recombinant antigens have shown increased specificity in the diagnosis of different
85 parasitic infections, including *Toxocara larva migrans* (21, 26). In addition to possessing high
86 specificity, these recombinant antigens overcome the various limitations involved in the
87 preparation of ES antigens, and obviate the possible infection risk to those involved in generating
88 this material.

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

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

107

108 **MATERIALS AND METHODS**

109 **Preparation of BPES and BpRAG1 antigens**

110 Collection, preservation and in vitro embryonation of *B. procyonis* eggs were performed
111 as per Kazacos et al. (18). Second stage larvae (L2) were hatched aseptically from in vitro-

112 embryonated eggs, and larval cultures established and processed at weekly intervals (2, 6).
113 Briefly, the culture medium containing the ES antigen of *B. procyonis* larvae was collected and
114 dialyzed against 0.1M ammonium bicarbonate solution. The dialyzed antigen was concentrated
115 by lyophilization, aliquoted and stored at -20° C until use.

116 The BpRAG1 antigen was prepared as per the protocol described previously (7). Briefly,
117 the polyhistidine-tagged BpRAG1 protein was expressed in BL -21(DE3) pLysS *E.coli* cells and
118 purified under denaturing conditions. The eluted protein fractions were extensively dialyzed
119 against phosphate buffered saline at 4°C, aliquoted and stored at -80°C.

120 Serum samples

121 (i) Positive and negative control sera

122 Positive control serum was obtained from the Division of Parasitic Diseases, CDC,
123 Atlanta GA, and consisted of serum from an experimentally infected baboon that developed
124 severe NLM following infection with *B. procyonis* embryonated eggs (6). Negative control
125 serum was from a healthy adult human with no history of exposure to raccoons or any clinical
126 signs of infection.

127 (ii) Human serum samples

128 a) Sera from patients with clinical *Baylisascaris larva migrans*

129 Serum samples from 20 individuals who were diagnosed with clinical *Baylisascaris* larva
130 migrans and were determined to be seropositive by BPES Western blot assay (6) were used as
131 *Baylisascaris*-specific human sera to evaluate the sensitivity of the BpRAG1 ELISA. The criteria
132 upon which these sera were considered as *Baylisascaris*-specific have been described previously
133 (6).

134 b) Sera from patients with other parasitic infections

135 115 serum samples from patients with 12 different parasitic infections, viz, *Toxocara*,
136 *Strongyloides*, *Trichinella*, filariasis, *Schistosoma*, *Fasciola*, *Taenia*, *Echinococcus*,
137 *Trypanosoma*, *Entamoeba*, *Leishmania*, and *Plasmodium* were obtained from the National
138 Reference Centre for Parasitology, McGill University Health Centre, Montreal, Quebec and used
139 to assess the specificity of both the BpRAG1 and BPES ELISAs. Cross-reactivity of BPES
140 antigen to different parasitic diseases (except *Toxocara* spp.) is not known and therefore was
141 evaluated during this study. In addition to these 115 samples, 22 serum samples from patients
142 positive for *Toxocara* larva migrans (identified by testing in the *Toxocara* EIA), previously
143 obtained from the CDC and tested in the BPES Western blot assay (6), were also used in this
144 study.

145 **c) Unknown/suspected parasite serum samples submitted for serology**

146 227 serum samples from human patients (either sex and different age groups) primarily
147 from the United States and Canada were submitted to the Parasitology Laboratory, Purdue
148 University, West Lafayette, IN during the period 1986 to 2008 to test for *Baylisascaris procyonis*
149 infection/antibodies using BPES antigen-based ELISA or immunofluorescence. These patients
150 had a history of exposure to raccoons or raccoon feces and /or symptoms possibly associated
151 with larva migrans or a clinical laboratory test indicating blood or CSF eosinophilia. Neither
152 exposure history nor clinical symptoms necessarily meant they had *Baylisascaris* infection.
153 Hence, all samples were treated as coming from patients with unknown or suspected parasite
154 infection. These samples were tested in the BpRAG1 ELISA and compared to the BPES ELISA
155 results.

156 **Enzyme-linked immunosorbent assay and Western blot**

157 Checkerboard titrations were done to determine optimum well-coating amounts of
158 antigen, blocking agent, and dilutions of primary and secondary antibodies in the ELISAs (5).
159 *Baylisascaris procyonis* ES antigen and BpRAG1 antigen at concentrations of 0.1 µg and 0.125
160 µg per well, respectively, were used to coat wells of Immulon2HB flat-bottom microtiter plates
161 (Thermo Scientific, Asheville, North Carolina), and ELISA was performed as described
162 previously (7) with a few modifications. Primary antibody (human patient sera) was used at
163 1:200 dilution in the BPES ELISA and at 1:100 dilution in the BpRAG1 ELISA, respectively.
164 Alkaline phosphatase-conjugated goat anti-human IgG (H+L) (Bethyl Laboratories, Inc.,
165 Montgomery, Texas) was used as the secondary antibody and para-nitrophenyl phosphate
166 (Sigma-Aldrich, St. Louis, Missouri) as the substrate. Microtiter plates were read in a
167 THERMOmax absorbance microplate reader (Molecular Devices, Sunnyvale, California) at 405
168 nm. All sera were run in duplicate and results averaged.

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

173 **Determination of cutoff values and parameters**

174 Cutoff values for the BPES ELISA were based on an analysis of multiple sets of sera
175 obtained from children and adults, including (1.) 251 random sera collected from 5-7-year old
176 children in Chicago in 2001; (2.) 84 sera primarily from children in southern California in 2002,
177 associated with a case of NLM; and (3.) 201 sera from all age groups submitted to our laboratory
178 in the 1990s-early 2000s for testing purposes, which included some of the 227 sera mentioned
179 above. Using calculated means and standard deviations (SD) for negative to low OD groupings

180 (in increments up to OD <0.250) as “negative” populations, and the customary practice of setting
181 cutoffs for a 99% confidence interval at the Mean+3SDs (5), a cutoff value of approximately OD
182 0.200 was determined for all three sera groups, respectively. Since a true cutoff is never known,
183 a prudent and conservative practice involves bracketing the calculated cutoff as a suspect or
184 indeterminate reactor group (5), which was done with +/- OD 0.050. The following parameters
185 were thus set for the BPES ELISA for use in this study, and until such time as it is in more
186 routine use, better negative population sera is obtained, and/or other analyses such as Receiver
187 Operating Characteristic (ROC) curves or J-index analysis (27) can be done: Serum samples with
188 OD <0.100 were considered negative, those 0.100-0.150 probable negative, those >0.150-0.250
189 suspect reactors, and those >0.250 were considered positive. Similar determinations were done
190 for the rRAG1 ELISA, using 207 sera submitted to our laboratory, which included the 201 sera
191 of group 3 above. Based on its greater sensitivity and specificity, average mean+3SD values
192 were lower for this ELISA, and a cutoff value of approx. OD 0.175 determined for incremental
193 OD groupings <0.250. Taking this into account, and setting a slightly narrower suspect reactor
194 bracket (+/- OD 0.025) (5), the following parameters were set for the rRAG1 ELISA: Samples
195 with OD <0.100 were considered negative, those 0.100-0.150 probable negative, those >0.150-
196 0.200 suspect reactors, and those >0.200 were considered positive.

197 **RESULTS**

198 **Diagnostic sensitivity of BpRAG1 ELISA**

199 All 20 *B. procyonis*-specific sera reacted with moderate to high OD values in the BPES
200 ELISA, with an average OD of 1.811 (range 0.326 to 3.132) and 16 samples >1.000. When these
201 20 sera were tested in the BpRAG1 ELISA, 17 samples reacted positive or suspect resulting in
202 85% sensitivity. One of the 20 samples was determined negative in the BpRAG1 ELISA and two

203 samples were considered probable negatives. Of the 17 samples that reacted strongly, a majority
204 of 6 samples had ODs between 0.500 and 1.000, while 5 samples had ODs above 1.000 (Table 1,
205 Fig1).

206 **Diagnostic specificity comparison of BpRAG1 and BPES ELISAs**

207 A high diagnostic specificity of 86.9% was obtained for the BpRAG1 ELISA compared
208 to a 39.4% specificity of the BPES ELISA, when serum samples from individuals positive for
209 different helminth and protozoan diseases were examined in the two ELISAs. Cross-reactions in
210 the BpRAG1 ELISA were mainly observed with other nematode [19.4% (12 of 62 samples)] and
211 trematode [15% (3 of 20 samples)] infections (Table 2). On the other hand, although cross-
212 reactions observed in the BPES ELISA were also mainly due to nematode and trematode
213 infections, the degree of cross-reactivity was much higher, with 77.4 % (48 of 62 samples) and
214 85% (17 of 20 samples) positive reactions with different nematode and trematode infections,
215 respectively (Table 2). The BPES ELISA also showed cross-reactivity (45%) with serum from
216 patients with cestode infections, while no cross-reactivity was observed in the BpRAG1 ELISA.
217 Although only 25.7% cross-reactivity was observed in the BPES ELISA with serum from
218 patients with protozoan infections, the BpRAG1 ELISA was comparatively more specific with
219 only 8.6% cross-reactivity. BPES Western blot assays were done on representative serum
220 samples from patients with different parasitic infections that showed high ODs on the BPES
221 ELISA, and confirmed that their reactions were due to cross-reactivity (data not shown).

222 **Cross-reactivity with *Toxocara* spp. infection in BpRAG1 and BPES ELISAs**

223 Among the different nematode infections tested in the two ELISAs, a high degree of
224 cross-reactivity occurred with *Toxocara* spp. infections. Cross-reactivity in the BPES ELISA
225 with *Toxocara* spp. infections was previously known, and the BpRAG1 ELISA showed much

226 lower cross-reactivity by comparison. Of the 32 toxocariasis samples tested in the two ELISAs,
227 the rRAG1 ELISA showed only 25% (8/32 samples) cross-reactivity as opposed to 90.6% (29/32
228 samples) cross-reactivity for the BPES ELISA, and almost all of the OD values were low or
229 borderline positive (Table 2, Fig 1). A single sample showed a very high OD (2.821) on
230 BpRAG1 ELISA, and since this sample also recognized 30-45 kDa proteins on a BPES Western
231 blot (6), it represented a patient with a dual infection with both parasites.

232 **Reactivity of unknown/suspected serum samples in BpRAG1 and BPES ELISAs**

233 A total of 227 unknown/suspected serum samples were run in the BpRAG1 and BPES
234 ELISAs. Based on the cutoffs that were set for these two ELISAs, a large proportion (89%) of
235 samples were negative in the BpRAG1 ELISA, compared to 59.5% samples being negative in
236 the BPES ELISA. Sixty-three samples with an OD > 0.250 were determined as positive in the
237 BPES ELISA, with a mean OD of 0.693, while only nine samples with an OD > 0.200 were
238 considered positive in the BpRAG1 ELISA, with a mean OD of 0.235. 29 samples were
239 considered suspect reactors in the BPES ELISA, with ODs from 0.150-0.250, whereas 16
240 samples were considered suspect reactors in the BpRAG1 ELISA, with ODs from 0.150-0.200
241 (Table 3).

242 **DISCUSSION**

243 Cross-reactivity is a major hurdle in the development of serological tests with high specificity for
244 the diagnosis of parasitic diseases. In this study, we have demonstrated the high sensitivity and
245 very low cross-reactivity of a recombinant *Baylisascaris* antigen, BpRAG1, and its utility in the
246 diagnosis of *Baylisascaris larva migrans* in human patients. The BpRAG1 antigen showed a
247 sensitivity of 85%, with 17 of 20 samples reacting in this ELISA. Obtaining gold-standard
248 parasite-specific human sera is difficult; however, great efforts were made in defining

249 *Baylisascaris*-specific human sera used in this study. Although multiple parameters, such as
250 exposure history, clinical symptoms, autopsy or biopsy findings, epidemiology, and positive
251 serology in the BPES ELISA were used to define the samples as true positives, a high positive
252 reaction in the BPES ELISA but not in the BpRAG1 ELISA is attributed to cross-reactivity of
253 BPES antigen with antigens of other co-infecting geohelminths as well as some other parasites.
254 Larval ES antigen is a heterogeneous mixture of glycoproteins released by metabolically active
255 larvae. Protein sharing, epitope sharing, and/or the presence of similar sugar moieties on the
256 proteins are some reasons for the cross-reactivity seen with the use of ES antigens (19).
257 Recombinant antigen, on the other hand, is a single protein that is non-glycosylated when
258 produced in *E. coli*, resulting in less or no cross-reactivity.

259 The BpRAG1 antigen had minimal reactivity with sera from patients with other parasitic
260 diseases and demonstrated a high degree of specificity (86.9%) compared to the BPES antigen
261 (39.4%) in the respective ELISAs. A similar study evaluating a recombinant *Toxocara* antigen
262 (26) demonstrated 44.4% specificity using *Toxocara* ES antigen while use of the recombinant
263 antigen at the same concentration showed almost no cross-reactivity. The BpRAG1 antigen still
264 needs to be evaluated for potential cross-reactivity against *Ascaris lumbricoides*, anisakid
265 infections and some others; however, since BpRAG1 did not cross-react with anti-*Ascaris suum*
266 antibodies raised in rabbits (7), we would expect it to show minimal cross-reactivity to these
267 other parasites. One drawback of using recombinant antigens for serodiagnostic assays could be
268 lowered sensitivity when compared to ES antigen because the recombinant antigen is a single
269 protein. However, combinations of recombinant antigens are being successfully used to improve
270 the sensitivity of recombinant antigens for serodiagnosis of parasitic infections (21). High cross-
271 reactivity using BPES antigen might not be of much concern considering the absence or very low

272 prevalence of other helminth infections in the United States, Canada, Europe, etc. However,
273 there is a possibility of background titers to other geohelminth infections being present in
274 immigrant populations and travelers, and in areas of the world where *Ascaris* and/or other
275 geohelminths are prevalent. Therefore, the BpRAG1 ELISA would have greater utility than the
276 BPES antigen ELISA in any geographical region where serodiagnosis of baylisascariasis is
277 sought.

278 Some of the parasite infection serum samples used for specificity testing in the BpRAG1
279 ELISA were thought to be cross-reacting with the BpRAG1 antigen, however, we speculate that
280 these could be false positive reactions to co-purified *E.coli* antigens in the purified fraction of
281 BpRAG1 antigen. Similar false positive reactions were observed in our previous study (7)
282 involving sera raised against different ascarid species in experimentally infected rabbits.
283 Adsorbing these sera with *E.coli* antigens prior to their use in ELISA testing eliminated these
284 reactions. Owing to the large number of sera involved, this wasn't done in this study. In the long
285 term, this issue can be overcome by either using improved purification techniques or perhaps by
286 using RAG1-based peptide antigens in the ELISA. Occasional false positive reactions in the
287 BpRAG1 ELISA were also evident by the fact that the same serum samples were not
288 simultaneously positive in both BpRAG1 and BPES ELISA specificity testing, indicating a
289 discrepancy. In addition, when unknown or suspected serum samples were run in the BpRAG1
290 ELISA, as expected a fairly large percentage of these samples (89%) were negative compared to
291 the BPES ELISA (59.5%). Similarly, 92 of the unknown/suspected samples tested in the BPES
292 ELISA were positive compared to only 25 samples in the BpRAG1 ELISA. Although there is a
293 low prevalence of other helminth infections in the U.S. population, recently a 14% national
294 seroprevalence of *Toxocara* spp. was documented (25). Considering the facts that the

295 unknown/suspected samples used in this study were submitted to our laboratory from across the
296 United States, that background *Toxocara* titers are common in the population, and that there is a
297 known cross-reactivity of BPES antigen with *Toxocara* antibodies, then the observed 40.5%
298 positive reactors in the BPES ELISA (Table 3) should be interpreted with caution. The
299 percentage of positive reactors observed in the BpRAG1 antigen ELISA was 11%, which is
300 much more in line with what would be expected, and similar to what is known for *Toxocara* spp.
301 (25), which has a similar level of exposure.

302 In endemic areas, there is the real possibility of exposure of people to infective eggs of
303 both *Baylisascaris* and *Toxocara* from the same environments, due to the commonality of their
304 respective hosts and their close association with humans (10, 22). In addition, dogs sometimes
305 develop patent *Baylisascaris* infections and could contaminate domestic environments and
306 neighborhoods with the eggs of both parasites (12, 14, 15). People also could be exposed from
307 infected kinkajous, which are related procyonids sometimes kept as exotic pets (4, 14). Luckily,
308 the prevalence of patent *Baylisascaris* in dogs appears to be low and pet kinkajous relatively
309 uncommon, so despite the possibility of occurrence, the main concern will continue to be
310 contamination from peridomestic or pet raccoons. The BpRAG1 ELISA showed high specificity
311 and little cross-reactivity to *Toxocara* spp. infection in humans and therefore can be used in the
312 differential serodiagnosis of larva migrans caused by these two parasites. The BpRAG1 ELISA
313 will be a superior test in cases of larva migrans caused by concurrent infection with these two
314 parasites, as compared to the combination of BPES ELISA, *Toxocara* ELISA and Western blot
315 assay recommended previously (6). In a previous study, a high-titered *Toxocara* ELISA-positive
316 serum sample was suspected of dual infection with *Baylisascaris* and *Toxocara*, based on its
317 recognition of 30-45 kDa BPES antigen components in a Western blot assay (6). Given the high

318 cross-reactivity observed with the BPES ELISA, it was not unexpected that we obtained a strong
319 reaction with this same sample (OD 2.249); however, this particular serum sample also showed
320 strong reactivity in the BpRAG1 ELISA (OD 2.821; outlying sample #1 on Fig 1), confirming
321 dual infection in this patient and the utility of the BpRAG1 ELISA.

322 In conclusion, this study clearly showed a high sensitivity and specificity of the BpRAG1
323 antigen for the serodiagnosis of *Baylisascaris* larva migrans, including low or no cross-reactivity
324 to other parasites, including *Toxocara* spp. In endemic areas, all patients suspected of larva
325 migrans should be tested for antibodies against both *Baylisascaris* and *Toxocara* spp. Testing for
326 anti-*B. procyonis* antibodies using the BpRAG1 antigen ELISA is much easier than performing a
327 combination of BPES ELISA and Western blotting, and the BpRAG1 antigen has great promise
328 for use in diagnostic applications and seroepidemiological investigations. Finally, we wish to
329 inform the scientific community that ELISA testing for *Baylisascaris* has been discontinued by
330 our laboratory at Purdue University, and that in the public interest such testing including the
331 BpRAG1 antigen ELISA has been transferred to the U.S. Centers for Disease Control and
332 Prevention in Atlanta, GA and the Canadian National Reference Centre for Parasitology in
333 Montreal, QC, both of which will undertake serologic testing for *Baylisascaris* in the near future.

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408

409 **FIGURE LEGENDS**

410 FIG 1. Reactivity of serum samples from patients with *B. procyonis* and *Toxocara* spp. larva
411 migrans on *B. procyonis* ES antigen and BpRAG1 ELISAs. Serum from patients with *Toxocara*
412 infection showed strong reactivity on the BPES ELISA (0.250 OD cutoff) indicating cross-
413 reactivity, but few of them reacted on the BpRAG1 ELISA indicating low cross-reactivity on
414 that assay (0.200 OD cutoff). The single very strong reactor is considered a dual infection (→)
415 with both parasites, which was also confirmed by Western blotting.

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1 TABLE 1. Sensitivity of BpRAG1 ELISA for diagnosis of *Baylisascaris larva migrans*.

2

Category	<i>Baylisascaris procyonis</i> recombinant RAG1 antigen ELISA	
	Optical Density at 405nm	No. of samples (n=20)
Negative	< 0.100	1
Probable 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

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14 TABLE 2. Specificity comparison of BPES and BpRAG1 ELISAs for serodiagnosis of
 15 *Baylisascaris larva migrans*

Infection group	Number of serum samples tested	BPES ELISA results			BpRAG1 ELISA results		
		No. negative	No. positive	percent positive reactions	No. negative	No. positive	percent positive reactions
Nematode							
Strongyloidiasis	10	4	6	60	7	3	30
Filariasis	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							
Fascioliasis	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
Echinococcosis	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	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. Reactivity of unknown/suspect samples (n=227) in BpRAG1 and BPES ELISAs

Category	<i>Baylisascaris procyonis</i> recombinant RAG1 antigen (BpRAG1) ELISA			<i>Baylisascaris procyonis</i> larval excretory-secretory antigen (BPES) ELISA		
	Optical Density (OD)	Number of samples	Mean OD at 405nm	Optical Density	Number of samples	Mean OD at 405nm
Negative	< 0.100	144	0.065	<0.100	101	0.058
Probable 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

