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♦ The nucleotide sequences reported in this manuscript have been submitted to GenBank, accession numbers: JN596964 (*Cy-gala-ash*), JN596966 (*Cy-gala-cat*), JN596967 (*Cy-gala-gol*), JN596968 (*Cy-gala-lon*)

ABSTRACT

Cyathostomins are ubiquitous pathogenic nematodes of horses. Once ingested, these parasites can spend a substantial part of their life cycle as encysted larvae in the large intestinal wall. The larvae can comprise up to 90% of the total cyathostomin burden, with up to several million worms reported in some individuals. These developmental stages are pivotal in cyathostomin pathogenicity as they can emerge from the intestinal wall in large numbers to cause a life-threatening colitis. Direct methods for the detection of encysted larval burdens in live horses do not exist. Previously, two native antigen complexes were identified as promising markers for infection. A component of these, cyathostomin gut associated larval antigen-1 (Cy-GALA-1), was subsequently identified following the immunoscreening of a complementary (c)DNA library using sera from infected ponies. Serum IgG(T) responses to recombinant Cy-GALA-1 were shown to inform on encysted larval infection. Sequence analysis of PCR products amplified from DNA from individual identified worms indicated that Cy-GALA-1 was derived from the common species, *Cyathostomum pateratum*. As cyathostomin infections always comprise multiple species, a diagnostic test must account for this. Here, segments of the orthologous *Cy-gala* gene were isolated from four additional common species, *Cyathostomum catinatum, Cylicocyclus ashworthi*, *Cylicostephanus goldi* and *Cylicostephanus longibursatus* and the associated proteins expressed in recombinant form. The cyathostomin specificity and immunogenicity of each recombinant protein was confirmed. Each GALA protein was assessed by ELISA for its predictive ability for informing on the presence of encysted larval infection and the level of burden.

Keywords: horse, nematode, cyathostomin, encysted larvae, ELISA, diagnosis

1. Introduction

Parasitic nematodes of the group Cyathostominae are a potential cause of serious disease in equids of all types and ages. These parasites have a high prevalence and most horses that graze encounter these infections (Matthews, 2008). Cyathostomins exist as a group of around 50 species (Lichtenfels et al., 2008); however, most infections comprise 5-10 common species, with low numbers of rarer species present (Chapman et al., 2002a). The species compositions are similar across regions, with the same group of species appearing as the most prevalent globally (Ogbourne, 1976; Reinemeyer et al., 1984; Mfitilodze and Hutchinson, 1990; Bucknell et al., 1995; Gawor, 1995; Lichtenfels et al., 2001; Collobert-Laugier et al., 2002). For all species, the life cycle involves a period of larval encystment in the wall of the caecum or large colon and these larvae can persist for prolonged periods of up to 2 years (Gibson, 1953; Smith, 1976; Murphy and Love, 1997). In some horses, the encysted larvae can constitute up to 90% of the total burden, with some individuals harbouring millions of worms (Dowdall et al., 2002). Encysted larvae are important in the pathogenesis of cyathostomin infections, because these developmental stages can re-emerge from the intestinal wall in great numbers to cause larval cyathostominosis, a colitis syndrome that can be fatal in up to 50% of cases (Giles et al., 1985; Love et al., 1992). Larval cyathostominosis is most commonly observed in horses of 2 to 5 years of age (Reid et al., 1995); however, animals can have a lifelong susceptibility to infection and disease has been observed in horses of all ages (Mair, 1993). The potential clinical effects of cyathostomins are confounded by the high levels of anthelmintic resistance in these nematodes, with resistance to benzimidazole and pyrantel compounds almost ubiquitous in some regions (Kaplan, 2002; Matthews 2014). The macrocyclic lactones are by far the most commonly used anthelmintics in horses and reduced efficacy against cyathostomins has been reported for ivermectin in Brazil

(Canever et al., 2013), with several studies reporting a shortened strongyle egg reappearance period following ivermectin (Geurden et al 2014; Relf et al., 2014) and moxidectin (Rossano et al., 2010; Relf et al., 2014) treatment. A shortened helminth egg reappearance period is generally regarded as an early indicator of resistance (Sangster, 1999), highlighting the threat of cyathostomin resistance to all available classes of broad spectrum anthelmintic.

Anthelmintic targeting of cyathostomin encysted larvae is now common practice in equine helminth control programmes (Stratford et al., 2014). Moxidectin and fenbendazole (administered over 5 consecutive days) are registered for this purpose (Matthews, 2008); however, because of high levels of resistance to fenbendazole, moxidectin is the only remaining compound effective against encysted larval stages, so its efficacy needs to be preserved. To address this, a reduction in treatment frequency is recommended (Matthews, 2014). This can be achieved by improving grazing practices to reduce cyathostomin transmission via the environment, combined with specific targeting of treatments based on strongyle egg shedding (Nielsen et al., 2006; Nielsen et al., 2014; Lester and Matthews, 2014). Such protocols do not address the presence of encysted larvae within individuals. Indeed, horses with sizeable encysted larval burdens often have no or low egg shedding (Dowdall et al., 2002). Because of these issues, a test that informs on the presence or burden of encysted larvae would facilitate anthelmintic targeting of these stages and would also assist in the definitive diagnosis of larval cyathostominosis, a challenge in practice due to the non-specific nature of the associated clinical signs (Giles et al., 1985). Previously, these authors identified two native antigen complexes that showed promise as diagnostic markers of encysted larval infection (Dowdall et al., 2002, 2003, 2004). The antigen preparations are, however, labour intensive to prepare and rely on a continual source of equine intestinal tissue. For these reasons, steps were taken to identify genes that encode protein components of these complexes to develop a

recombinant protein-based test. One component, cyathostomin gut associated larval antigen-1 (Cy-GALA-1) protein, was identified by immunoscreening a cyathostomin larval complementary (c)DNA library using sera from infected ponies (McWilliam et al., 2010). Sequence analysis of PCR products amplified from DNA from individual identified worms indicated that Cy-GALA-1 was derived from the common species, *Cyathostomum pateratum*, so Cy-GALA-1 was re-designated Cy-GALA-pat (McWilliam et al., 2010). Recombinant Cy-GALA-pat was demonstrated to be the target of serum IgG(T) responses in infected, but not in uninfected horses, and exhibited no reactivity to serum from horses specifically infected with non-cyathostomin helminth species. In experimentally- and naturally-infected horses, antigen-specific IgG(T) levels to the protein were significantly higher than those in cyathostomin-negative animals, with antigen-specific IgG(T) levels shown to have a significant positive correlation with encysted larval burden (McWilliam et al., 2010). As horses invariably harbour a range of cyathostomin species, a diagnostic test may need to take account of the complex nature of these infections. Here, recombinant GALA proteins were generated from four additional common cyathostomin species and the specificity and immunogenicity of each examined. These proteins were then assessed by ELISA for their ability to predict cyathostomin encysted larval infection and the level of burden.

2. Materials and methods

2.1. Parasite material

Individual adult cyathostomins were removed from the large intestinal luminal contents of naturally-infected horses at post mortem (Dowdall et al., 2002). The worms were identified to species by morphological means based on the key of Lichtenfels et al. (2008). Cyathostomin encysted larvae were recovered by pepsin-HCl digestion or by manual removal from the mucosa and submucosa as described previously (Dowdall et al., 2002).

133

134 *2.2. Sub-cloning and recombinant protein expression of Cy-Gala proteins from an*
135 *additional four cyathostomin species*

136 Like Cy-GALA-pat, two of the additional proteins were derived from clones selected
137 by immunoscreening (McWilliam et al. 2010). Similar to the strategy used to ascribe a
138 species identity to *Cy-gala-pat*, nucleotide sequences in these two additional clones were
139 compared to *gala* sequences obtained by PCR amplification from numerous individual
140 identified worms of various species. Based on this analysis, the clones were ascribed to
141 the common species, *Cylicocyclus ashworthi* (sequence named as *Cy-gala-ash)* and
142 *Cyathostomum catinatum* (sequence named as *Cy-gala-cat*). For recombinant protein
143 expression, *Cy-gala-ash* and *Cy-gala-cat* sequences were used to design primers to
144 facilitate PCR amplification of *Cy-gala* encoding sequence (minus the signal peptide
145 sequence) from phage plaque eluates from respective clones selected by immunoscreening
146 (McWilliam et al. 2010). Sequences encoding restriction enzyme sites were incorporated
147 into each primer to facilitate unidirectional cloning. The primer sequences were: *Cy-gala-*
148 *ash* (*SacI*, *NotI* sites underlined) sense 5'-
149 ATTCGAGCTCCCATGAAGAACTTCGTCGTCAC-3', antisense 5'-
150 AGCTTGCGGCCGCATATCTTCTATCCGTGTTGAG-3'; *Cy-gala-cat* (*NcoI*, *NotI* sites
151 underlined) sense 5'-ATGGCCATGGATGAGGATCGTGAAGAACATCGC-3',
152 antisense 5'- AGCTTGCGGCCGCATCTCTTTCATCTGTGTTGAGTCC-3'. The PCR
153 conditions were as follows: 0.5 µM primers, 0.2 mM dNTPs and 1.5 mM $MgCl_2$, with
154 cycling conditions, 94°C for 2 min, 30 cycles at 94°C for 15 sec, with an annealing
155 temperature of 54°C (*Cy-gala-ash*) or 53°C (*Cy-gala-cat*) for 30 sec, and 72°C for 60 sec,
156 with a final cycle of 72°C for 7 min. Amplifications were performed using Platinum Taq
157 (Invitrogen) in combination with 2 µl of each phage plaque eluate in a reaction volume of
158 50 µl. For size determination, PCR products were analysed on 1.1% w/v agarose TAE

159 gels using a TrackIt 100bp DNA Ladder (Invitrogen) and stained with 1 x GelRed
160 (Biotium). Next, the expression plasmid (pET-22b(+), Novagen) and each PCR product
161 were digested with the appropriate restriction enzymes and ligation of the PCR amplicons
162 performed using T4 ligase (Promega). The derived plasmids were transformed into
163 *Escherichia coli* JM109 competent cells (Promega) and selected on ampicillin-LB agar.
164 For each species, one colony containing plasmid with an insert of the correct size was
165 subjected to plasmid purification (Wizard Plus SV Miniprep kit, Promega) and the insert
166 sequenced in full to confirm identity. On confirmation of sequence identity, plasmids were
167 transformed into *E. coli* BL21-CodonPlus(DE3)-RIL competent cells (Stratagene) for
168 expression of recombinant (r) Cy-GALA-ash and Cy-GALA-cat. Recombinant protein
169 expression was induced as described in McWilliam et al. (2010). Similar to rCy-GALA-
170 pat (McWilliam et al., 2010), rCy-GALA-cat was soluble and present in the bacterial
171 lysate supernatant. This protein was purified directly on a HisTrapHP column (GE
172 Healthcare), eluted in increasing concentrations of imidazole, then dialysed with 20 mM
173 sodium phosphate, 0.5M NaCl (pH 7.4), and stored at -20 °C. The rCy-GALA-ash protein
174 was insoluble, so purification and dialysis were carried out as above, with the exception
175 that 8 M urea was added to all buffers. For isolation of *gala* sequences from *Cylicocyclus*
176 *goldi* and *Cylicostephanus longibursatus,* degenerate primers (sense 5'-
177 ACAGTCGTTAGTGCCGTAGTCCT-3', antisense 5'-
178 TTGAGTCCAAACATTCTTCCATT-3') were designed using all of the *gala* sequences
179 described above and used to PCR-amplify a portion of the *gala* gene from single,
180 identified adult *C. goldi* and *C. longibursatus* using cDNA synthesised as described in
181 Lake et al. (2009). The PCR products were cloned into pGEM®-T Easy vector (Promega),
182 plasmid preparations made and the inserts sequenced in both directions. Next, to generate
183 PCR products for sub-cloning for protein expression, species-specific primers were
184 designed using the *C. longibursatus* and *C. goldi* sequences obtained from individual

identified worms. For each, primer sequences (*SacI* and *NotI* restriction sites underlined) were: *Cy-gala-gol* sense 5'-ATTCGAGCTCCCAAGGTGTCATGGCCCTATTTG-3', antisense 5'-ATTAGCGGCCGCCAGGTATCTTCTATCCGTGTTCAG-3', and for *Cy-gala-lon* sense 5'-ATTCGAGCTCCCAAGGTGTCATGGACCTTTTTGG-3', antisense 5'-ATTAGCGGCCGCATATCTTCTATCCGTGTTGATTCCG-3'. PCR conditions were as above, except that 0.25 μM primers and 0.4 μl of Platinum Taq were used in reaction volumes of 100 μl, and 30 amplification cycles performed with an annealing temperature of 58°C. The PCR products and vector were digested with *SacI* (Promega) and *NotI* (Promega) and ligated into pET-22b(+) vector. Plasmids were transformed into *E. coli* JM109 Competent Cells (Promega) and selected on ampicillin-LB agar. Colonies were examined by PCR for the presence of an insert of the correct estimated size using vector-specific primers and plasmid preparations made from two colonies, which were sequenced using the same primers. Clones of the correct sequence were transformed into BL21-CodonPlus(DE3)-RIL cells as above. Recombinant Cy-GALA-lon and Cy-GALA-gol were insoluble so were prepared and stored in the presence of 8M urea as above. Nucleotide and amino acid sequence alignments were performed using ClustalW2 (Larkin et al., 2007) and the levels of sequence identity examined using MegAlign 10.0.1 (DNASTAR) based on the ClustalW2 alignments. Signal peptides were identified using SignalP 4.0 (Petersen et al, 2011). The sequences were translated and their molecular mass estimated using the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/protein_mw.html).

2.3. Immunogenicity and specificity of recombinant GALA proteins tested by immunoblotting

To assess immunogenicity and potential cross reactivity of each recombinant GALA protein, binding of IgG(T) in sera from horses infected only with cyathostomins or with

single-species experimental infections of heterologous helminths was investigated. For immunoblotting, IgG(T) reactivity to each protein was assessed in sera pooled from three experimental cyathostomin-infected (CI) ponies at 12-16 weeks post infection (Murphy and Love, 1997). These time points were selected on the basis of high serum IgG(T) reactivity to native encysted larval antigen complexes identified in Dowdall et al. (2002). Pools of negative control sera were prepared from samples obtained from three control ponies raised and maintained under helminth-free (HF) conditions (Murphy and Love, 1997). Cross reactivity of the GALA proteins was tested using sera from horses mono-specifically infected with either *Strongylus edentatus* or *Strongylus vulgaris* (Klei et al., 1982) and either *Parascaris equorum* or *Strongylus westeri* (Dowdall et al., 2003). Serum IgG(T) reactivity was also assessed in a serum pool from seven naturally-infected horses from the UK (UK+): this material was collected at an equine abattoir and each horse included had a total mucosal burden (TMB) of more than 100,000 cyathostomin encysted larvae (Dowdall et al., 2002). Further, serum IgG(T) reactivity in a pool of sera derived from 11 clinical cases of larval cyathostominosis (UK+Clin) was assessed. These horses were classified as cases based on their presenting signs and the detection of large numbers of cyathostomin larvae in their faeces (Hodgkinson et al., 2003). For immunoblotting, 0.1 μg of each protein was loaded, per lane, onto a 15-well, 12% NuPAGE gel with SeeBlue Plus2 protein standards used for size estimations (Invitrogen). An additional lane was loaded with 0.1 μg protein. After electrophoresis, this lane was removed and stained with Coomassie blue for comparison with the immunoblots. Blocking, primary, secondary and tertiary antibody steps and blot development were as described in McWilliam et al. (2010). To examine if the recombinant proteins were reactive with sera to the native antigen complex (Dowdall et al., 2003) and to sera raised against Cy-GALA-pat (McWilliam et al., 2010), IgG reactivity in rabbit sera to both was assessed as described in McWilliam et al. (2010).

237

238 *2.4. Immunoreactivity of the individual recombinant GALA proteins tested by ELISA*

239 Once the immunogenicity and specificity of each of the four new GALA proteins was
240 confirmed by immunoblotting, the ELISA was used to evaluate serum IgG(T) levels to
241 each. Serum IgG(T) responses to rCy-GALA-pat were compared in these studies also.
242 Antigen-specific IgG(T) levels were assessed in cohorts of horses from the UK (UK+) and
243 from the US (US+) for which the cyathostomin burden was known and in a cohort of
244 larval cyathostominosis cases. The UK+ group comprised 25 horses sampled at a UK
245 abattoir (Dowdall et al., 2004) and three experimentally infected ponies (Murphy and
246 Love, 1997). Cyathostomin burdens in these horses were quantified with the TMB and
247 total worm burden (TWB) calculated as described previously (Dowdall et al., 2004).
248 Antigen-specific IgG(T) levels were also measured in 11 larval cyathostominosis cases
249 from the UK (UK+Clin; Hodgkinson et al., 2003). Serum IgG(T) levels in these cohorts
250 were compared with those in six UK HF ponies (UK-, see Section 2.3 and Murphy and
251 Love, 1997). Several groups of cyathostomin-infected horses from the US (US+) were
252 assessed for serum IgG(T) levels to each antigen. One group comprised 10 naturally-
253 infected horses from Louisiana (Monahan et al., 1996). Another comprised 38 ponies
254 subjected to various cyathostomin experimental infection/treatment regimens (Monahan et
255 al., 1997; Monahan et al., 1998; Chapman et al., 2002b). Serum IgG(T) levels in these
256 cohorts were compared with those in cyathostomin-free ponies that were infected with *S.*
257 *vulgarus, P. equorum* and *S. westeri* (US-, n=3). For studying serum IgG(T) response
258 dynamics in experimentally-infected ponies, antigen-specific IgG(T) levels were measured
259 up to 16 weeks post infection in samples taken weekly from 2 weeks before infection, with
260 the exception of week 9 and 11 post infection (Murphy and Love, 1997). The ELISA
261 plates (96-well flat bottomed Microlon High binding plates, Greiner Bio-One) were coated
262 with the inidivual rGALA antigens at 2 µg/ml. Each well was coated with 100 µl of

263 antigen in coating buffer (0.1 M carbonate coating buffer, pH 9.6) overnight at 4 °C. Plates
264 were then washed six times with 0.05% Tween-20 in PBS (PBS-T), then blocked using
265 200 µl block buffer (2% soya powder (Infasoy™, Cow and Gate Ltd), w/v in PBS), per
266 well for 1 h at 37°C. All serum dilutions were made in block buffer. The plates were
267 washed three times (as above) and 100 µl sera (diluted 1:800) added to each well and
268 incubated for 2 h at 37°C. Each serum sample was tested in triplicate. Plates were washed
269 six times, incubated for 1 h with (100 µl per well) goat anti-equine IgG(T) whole molecule
270 (Serotec), diluted 1:400 in block buffer. The plates were washed six times, then 100 µl
271 rabbit anti-goat Ig:HRP conjugate (Sigma), diluted 1:500 in block buffer, added to each
272 well and incubated for 1 h at 37°C. The reactions were developed by adding 100 µl *o*-
273 Phenylenediamine dihydrochloride solution prepared from SIGMA*FAST* OPD tablets
274 (Sigma) to each well. After 15 min at room temperature, 50 µl 2.5 M H_2SO_4 were added
275 to stop the reactions and the absorbance in each well read at 490 nm. On all plates,
276 aliquots from the same pool of CI sera were tested in triplicate as a control for inter-plate
277 variation. The results derived from all samples were then expressed as the percentage OD
278 of the CI sample mean for each plate. Minitab 17 Statistical Software for Windows was
279 used to analyse the data. For the different infected groups (UK+, UKClin+, US+), the
280 group medians of the percentage positivity were compared to those of the cyathostomin-
281 free horses (UK- and US-) by the Mann-Whitney test. A p value <0.05 was taken to
282 indicate statistical significance.
283
284 *2.5. Receiver Operator Characteristic (ROC) Curve analysis*
285 Receiver operator characteristic (ROC) curve analysis was performed to indicate the
286 accuracy of each rGALA protein ELISA result relating to cyathostomin infection (postive
287 or negative) and to cyathostomin TMB and TWB. As an estimate of test accuracy, the
288 area under the curve (AUC) may be interpreted such that; AUC = 0.9-1.0 demonstrates an

289 excellent level of discrimination between positive and negative results; AUC = 0.8-0.9
290 good discrimination; AUC = 0.7-0.8 fair discrimination; AUC = 0.6-0.7 poor
291 discrimination and AUC = 0.5-0.6 no discrimination (Swets, 1988). The ELISA data were
292 subjected to ROC analysis using Prism 6 (Graphpad Software Inc, USA).

293

294 **3. Results**

295 *3.1. Analysis of GALA sequences from the four additional cyathostomin species*

296 Alignment (Figure 1) of the derived GALA protein sequences demonstrated that, like
297 Cy-GALA-pat, all sequences from the additional four species contained an N-terminal
298 histidine-rich motif and a highly-conserved domain (Marchler-Bauer et al., 2007), the
299 function of which is unknown, and which is termed as Domain of Unknown Function 148
300 in the two most closely related sequences from *Caenorhabditis elegans*. Comparative
301 analysis (Table 1) indicated that the sequences were between 83.3% (Cy-GALA-ash) and
302 92.9% (Cy-GALA-cat) identical to Cy-GALA-pat at the amino acid level. The level of
303 intra-specific identity in the derived amino acid sequences was higher than 90% for all five
304 species examined.

305

306 *3.2. Immunoreactivity and cyathostomin specificity of the rGALA proteins*

307 Coomassie staining of the four new rGALA proteins (Figure 2A) demonstrated that the
308 approximate size observed for each corresponded to the calculated molecular mass. The
309 immunoblot experiments demonstrated that IgG(T) in sera from HF ponies did not bind to
310 any of the four rGALA proteins, whilst IgG(T) in sera from ponies experimentally infected
311 with cyathostomins (CI sera) bound to all four proteins (Figure 2B). In terms of cross
312 reactivity to other helminth species, there was no, or negligible, IgG(T) binding to each of

313 the four rGALA proteins observed in sera from horses infected mono-specifically with *P.*
314 *equorum*, *S. edentatus*, *S. vulgaris* and *S. westeri* (Figure 2B). To further examine the
315 immunogenicity of the proteins in naturally-infected horses, immunoblots were performed
316 using sera pooled from horses that presented at an abattoir and which had high encysted
317 larval burdens. Here, strong reactivity of IgG(T) to all four rGALA proteins was observed
318 (Figure 3A). Likewise, IgG(T) in sera pooled from samples from larval cyathostominosis
319 cases demonstrated strong reactivity to all proteins (Figure 3B). The rGALA proteins also
320 bound IgG in sera from a rabbit immunised with the native 20 kDa complex originally
321 identified in encysted larvae (Dowdall et al. 2003, Figure 3C). Similarly, IgG in sera from
322 a rabbit immunised with rCy-GALA-pat bound each of the four new GALA proteins
323 (Figure 3D). No binding was observed in pre-immunisation sera from either rabbit.

324

325 *3.3. ELISA and ROC curve analysis of serum IgG(T) responses to rGALA proteins in*
326 *cyathostomin-infected horses*

327 A time course study was performed to analyse serum IgG(T) responses to each protein
328 in experimentally-infected ponies across a primary trickle infection (Figure 4). The
329 IgG(T) responses in the infected ponies (Ponies 101, 104, 105) were analysed from before
330 infection until 16 weeks after initial challenge. The levels of antibody in these individuals
331 were compared to those in ponies that remained uninfected throughout the protocol
332 (Ponies 102, 103, 106). Similar to previous observations with rGALA-pat (McWiliam et
333 al., 2010), increases in antigen-specific IgG(T) were observed against all recombinant
334 proteins after infection; however, the dynamics of the IgG(T) repsonse to each protein
335 varied amongst the individuals. Serum IgG(T) responses to rGALA-ash, lon and gol
336 proteins increased more rapidly in Pony 104 than in the other two infected ponies. This
337 was not the case with respect to IgG(T) responses to the rGALA-cat protein, against which

IgG(T) levels increased more slowly than to the other proteins. In Pony 104, IgG(T) levels to rGALA-ash, gol and lon plateaued after 7 weeks post-infection and remained high until the end of the time course. Antigen-specific serum IgG(T) levels in Ponies 101 and 105 increased at a slower rate, but generally reached similar levels to those measured in Pony 104 by the end of the time course, with the exception of Pony 101's IgG(T) response to rGALA-cat. Increases in antigen-specific IgG(T) levels were not observed in any of the HF ponies across the time course.

Next, levels of serum IgG(T) to all four new rGALA proteins and rGALA-pat were analysed in equids for which cyathostomin burden data were available allowing comparison of parasitological parameters with specific antibody levels in matched end-point blood samples. Also analysed were rGALA-specific serum IgG(T) responses in horses that presented with larval cyathostominosis. First, serum IgG(T) levels were compared in cyathostomin-infected groups (UK+, US+, UKClin+) with those in cyathostomin-negative groups (UK-, US-) from the UK and the US. For the UK+ and UKClin+ cohorts, for all five GALA proteins, specific IgG(T) levels were significantly higher than in the cyathostomin-negative horses (Table 2). Likewise, for the US population, the levels of IgG(T) to each recombinant protein were significantly higher in the US+ group than in the US- cyathostomin-free group. The ELISA data from horses for which cyathostomin burdens were available were then subjected to ROC curve analysis. For all five proteins, serum rGALA-specific IgG(T) levels in equids which were known to be truly cyathostomin negative (i.e. the UK- and US- groups) were compared to rGALA-specific IgG(T) levels measured in cyathostomin-infected horses from the UK or the US (Table 3). Here, high ROC curve AUC values (i.e. >0.9) were obtained for all recombinant proteins (AUC values: 'ash' and 'lon' > 'pat' > 'cat' > 'gol'). The highest sum of percentage sensitivity and specificity values generated in this ROC analysis were then used to calculate cut-off percentage positivity thesholds for each rGALA protein

(Table 3). Based on these values, the specificity ranged from 82.43% (ash) to 95.95% (cat) and sensitivity from 77.78% (cat, gol) to 100% (ash and pat). When the data were stratified at different cyathostomin TMB thresholds taking into account that some horses negative for TMB were positive for cyathostmin lumenal parasites (Table 4), the AUC values and the sensitivity and specificity values calculated were lower. Next, TWB thresholds were examined to assess if the outputs were likely to be confounded by the half-life of rGALA-specific serum IgG(T) responses; for example, in equids where there had been a recent emergence of high numbers of previously-encysted larvae or in equids that had been administered recently with an effective larvicidal treatment. Indeed, when the cohort was stratified on the basis of cyathostomin TWB, for all rGALA proteins, the AUC and sensitivity and specificity values were higher than when the group was stratified on the basis of TMB alone (Table 4). For the 0 TWB threshold, the AUC values for IgGT) responses to all all five rGALA proteins was high (i.e. > 0.9). Finally, the cohort was statfied on the basis of a threshold of 5,000 mucosal larvae or TWB. At a cut-off of a TMB of 5,000 larvae, the AUC values ranged from 0.69 (rGALA-ash) to 0.77 (rGALA-cat), so giving 'good' discrimination. At a cut-off of 5,000 total worms, the rGALA-cat and rGALA-lon (AUC values for both = 0.82) gave the highest values, with the AUC values for the other rGALA protiens exceeding 0.7. When the data were partitioned such that the threshold value was 10,000 TMB or TWB, most AUC values generated were <0.7 (data not shown).

4. Discussion

Cyathostomins have high pathogenic potential in equids due to their capacity to cause life-threatening colitis when encysted larvae emerge from the intestinal wall. For this reason, the administration of anthelmintics that have activity against these developmental stages is regarded as an essential component of parasite control programmes (Nielsen

2012; Matthews 2014). As there is no diagnostic test capable of detecting encysted larval stages, current recommendations are to apply whole-group larvicidal treatments at the appropriate time of year (Matthews, 2008; Nielsen et al., 2014). As it is likely that most horses will not carry life-threatening levels of encysted larvae, the availability of a diagnostic test that can inform on the presence or burden of these stages would help in directing the strategic? application of larvicidal anthelmintic treatments.

The development of a diagnostic test for cyathostomins is complicated by the complex nature of this group of nematodes. There are 50 recognised cyathostomin species (Lichtenfels et al., 2008); however, whilst a range of species is found in individuals, the majority of the burden has been found consistently across studies and geographic regions to comprise 5-10 common species. The species investigated here were selected on the basis that they were the most prevalent species identified across multiple studies. In particular, *C. longibursatus* and *C. catinatum* are regularly recorded as the commonest species recovered from horses across different continents (Krecek et al., 1989 [South Africa]; Mfitilodze and Hutchinson 1990 [Tropical Australia]; Bucknell et al., 1995 [Victoria, Australia]; Gawor, 1995 [Poland]; Lichtenfels et al., 2001 [UK], Chapman et al., 2002a [USA]; Boxell et al., 2004 [Western Australia]; Kuzmina et al., 2005 [Ukraine]). A representative protein for *C. ashworthi* was also selected here. This species has been regarded as a synonym of *Cylicocyclus nassatus*; the two are similar morphologically but are now regarded as separate (Lichtenfels et al. 1997). Earlier studies may have misidentified *C. ashworthi* (Chapman et al., 2002a), so this species may not have been represented appropriately in surveys based on nematode morphology. Where *C. ashworthi* has been defined as distinct, it has been found at high prevalence (Lichtenfels et al., 2001; Kuzmina et al., 2005; Kornaś et al., 2009). Likewise, both *C. goldi* and *C. pateratum* are cyathostomin species detected in high abundance in surveys performed across different regions (Reinemeyer et al., 1984; Krecek et al., 1989; Mfitilodze and Hutchinson, 1990;

416 Gawor, 1995; Boxell et al., 2004; Collobert-Laugier et al., 2002; Traversa et al., 2010). As
417 there does not appear to have been an obvious shift in the prevalence ranking of
418 cyathostomin species over time (Chapman et al., 2002a), despite the advent and spread of
419 anthelmintic resistance, it is with confidence that the inclusion of the species here
420 represent common components of cyathostomin infections now as well as in the future.
421 The level of intra-specific variation observed in the GALA sequence was low in all
422 cyathostomin species studied (Table 1). Further, the diversity observed in GALA
423 sequence between these species was far lower than that seen when the GALA sequences
424 were compared to orthologous sequences present in non-cyathostomin species (data not
425 shown) indicating that the test is unlikely to be affected by cross-reactivity to non-
426 cyathostomin infections. In agreement with this, the specificity of each rGALA protein
427 was confirmed in immunoblotting experiments when each protein was probed with sera
428 from horses mono-specifically infected with large strongyle species, *P. equorum* or *S.*
429 *westeri*, and no reactivity was observed. Further immunoblotting experiments
430 demonstrated that all four rGALA proteins bound strongly to IgG(T) in serum pooled from
431 cyathostomin-infected horses and in serum from clinical cases of larval cyathostominosis.
432 These results were substantiated by the ELISA data which demonstrated significantly
433 higher specific serum IgG(T) levels to each rGALA protein in the US and UK
434 cyathostomin-infected groups compared to the respective cyathostomin-negative groups
435 (Table 2). The time course study supported these findings demonstrating robust anti-
436 rGALA serum IgG(T) responses in all infected ponies. Similar to previous results
437 obtained with the rCy-GALA-pat protein (McWilliam et al.,2010) and with the native
438 antigens (Dowdall et al., 2002), specific IgG(T) increased to rGALA-ash, -gol and -lon in
439 Pony 104 earlier than in the other two infected ponies. The more severe clinical
440 parameters in this pony indicated that it had developed the greatest burden of mucosal
441 larvae during the infection period and when this animal was euthanized (at 20 weeks post-

infection), it was found to have a very high (>700,000 cyathostomin larvae) burden, but a negative faecal egg count (Murphy and Love, 1997). The other two ponies had substantially lower cyathostomin burdens, but they were not necropsied until 60 and 62 weeks post-infection so the burdens as enumerated cannot be directly compared with that of Pony 104. The differences in the dynamics in IgG(T) response to the rGALA-cat protein may reflect the relative proportions in species present over the trickle infection period (Murphy and Love, 1997). These observations indicate that the long term goal of this work should still be the development of a test incorporating several rGALA proteins derived from the commonest species, due to the potential risk of false negative results in some individuals in which a single species may be less abundant.

The ROC AUC values obtained indicate that all five rGALA proteins provide excellent discriminatory information when comparing true cyathostomin-negative to true cyathostomin-postive individuals (Table 3). The AUC values obtained were similar for all five rGALA proteins. When horses were stratified on the basis of mucosal larval burden (Table 4), the derived ROC AUC values were lower than the values obtained when the true cyathostomin-negative group was compared to the cyathostomin-positive horses. This is likely due to the fact that some horses in the TMB-negative group harboured lumenal stages of cyathostomins; such horses may have residual GALA-specific IgG(T) in their serum stimulated by a recent previous encysted larval infection. This observation is unlikely to be due to cross-reactivity to antigens in lumenal worms, as it was demonstrated previously that GALA protein and *gala* transcript could not be detected in these stages of cyathostomins (McWilliam et al., 2010). The serum half-life of equine IgG(T) has been measured as 21 days (Sheoran et al., 2000), so a limitation of this test is that, in horses that have had recent emergence of encysted larvae or have received a recent effective larvicidal treatment, rGALA-specific IgG(T) levels will still be elevated. With this in mind, a future objective of ours will be to investigate the dynamics of circulating rGALA protein-specific

serum IgG(T) after anthelmintic treatments known to be effective against encysted larval stages. This issue is not unique to the cyathostomin test and has been a topic of discussion with respect to the commercially-available diagnostic assay for *Anoplocephala perfoliata* in horses. This latter diagnostic is also based on the binding of serum IgG(T) to parasite proteins (Barrett et al., 2004; Abbott et al., 2008). Despite these concerns, the *A. perfoliata* ELISA is still regarded as a useful diagnostic tool (Kjaer et al., 2007; Abbott and Barrett, 2008). In both cases, the output of the test must always interpreted alongside the clinical and treatment history of the individual or population of animals being assessed.

In terms of informing on cyathostomin burden, the rGALA tests performed well when used to discriminate horses at an encysted larval burden threshold of 5,000 worms (Table 4). At higher TMB thresholds (10,000 +), the ROC AUC values were lower (i.e. < 0.7) indicating that the tests could not discriminate horses above and below thresholds > 5,000 larvae. When the ROC curve analysis was repeated with horses segregated on the basis of threshold of TWB (i.e. mucosal and luminal nematode burdens, Table 4), the AUC values obtained were higher at the 5,000 TWB threshold compared to when the horses were grouped according to TMB. Again, with this in mind, the test results will need to be interpretated in the context of the clinical or treatment history of individuals and also with respect to the time of year that the sample is anlysed. In northern temperate climates, experimental studies (Reinemeyer et al., 1986) have shown that there is is a peak of cyathostomin larval luminal stages in spring (presumably derived from recently emerged encysted larvae acquired during the previous grazing season). These larvae develop to mature adults in late spring, which persist through summer and are added to by immature worms, which co-contribute to a second adult worm peak in late summer. In these studies, there was an apparent loss of adult worms and luminal larvae in autumn; hence, the rGALA ELISA is likely to provide most diagnostic value if used in these regions in autumn when it can be used to help inform the need for a larvicidal treatment. A cocktail

of the five rGALA proteins, and combinations thereof, will now be assessed to see which should be combined to optimise the test to commercialisation. A balance will need to be struck between the resource required to generated each recombinant protein and the diagnostic value provided by the various rGALA combinations. Once the final cocktail is selected, a large cohort of sera from naturally-infected horses will be tested to examine how many individuals fall above and below the selected cut-offs for 0 TMB and 5,000 TMB.

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Conflicts of interest

The authors declare no competing interests.

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652

Table 1. Details of the GALA sequences representing the recombinant proteins used in the ELISA. These are derived from the following cyathostomin species: *Cylicocyclus ashworthi* (ash), *Cyathostomum catinatum* (cat), *Cylicostephanus goldi* (gol), *Cylicostephanus longibursatus* (lon) and *Cyathostomum pateratum* (pat).

GALA protein name	Number of worms sequenced per species (range of intraspecific variation in amino acid sequence as a percentage)	Amino acid identity (%) to Cy-GALA-PAT sequence of each clone used for expression	Predicted size of recombinant protein including His tag (kDa)
ash	3 (95.5-97.2)	83.3	25.5
cat	4 (92.9-97.3)	92.9	25.1
gol	4 (90.8-100)	91.3	27.2
lon	2 (97.1-100)	89.8	27.0
pat	5 (94.7-97.6)	100	26.6

Accession numbers of the sequences representative of each clone used for expression for each species are as follows: *Cy-gala-pat* - FJ882059.1, *Cy-gala-cat* - JN596966, *Cy-gala-gol* - JN596967, *Cy-gala-lon* - JN596968, *Cy-gala-ash* - JN596964

Table 2. Comparison of serum IgG(T) levels in cyathostomin-infected *versus* non-infected groups from the UK and the US. Horses were naturally infected or were subjected to experimental infection. The UK+ population comprised 26 cyathostomin naturally-infected horses sampled at an abattoir. The UK- population comprised 6 cyathostomin-negative ponies raised under helminth free conditions. The UKClin+ group comprised 11 equids that presented with larval cyathostominosis in practice. The US+ population (n=48) comprised natuarally- or experimentally-infected equids from the US and the US- population comprised three experimental equids from the US that were riased cyathostomin-free. For the different infected groups (UK+, UKClin+, US+), the group medians of the percentage positivity were compared to that of the cyathostomin-free horses (UK- and US-) by the Mann-Whitney test. A p value <0.05 was taken to indicate statistical significance.

Groups compared	rGALA protein	P value
UK+ (n=26) vs. UK- (n=6)	ash	0.0007
	cat	0.0011
	gol	0.0018
	lon	0.0006
	pat	0.0006
UKClin+ (n=11) vs. UK- (n=6)	ash	0.0011
	cat	0.0077
	gol	0.0011
	lon	0.0022
	pat	0.0104
US+ (n=48) vs. US- (n=3)	ash	0.0172
	cat	0.0356
	gol	0.0392
	lon	0.0213
	pat	0.0192

Table 3. ROC curve analysis of ELISA data relating to antigen-specific serum IgG(T) levels horses from the UK and US: comparing cyathostomin-positive *versus* cyathostomin-negative horses. Horses were naturally infected or were subjected to experimental infection. UK population (n=32) comprised 26 cyathostomin-positive, 6 cyathostomin (true) negative equids. The US population (n=51) comprised 48 cyathostomin-positive and 3 cyathostomin (true) negative equids. The area under the curve, 95% confidence intervals (CI) and P values for the data generated by ROC curve analysis for each rGALA protein are shown. A cut-off percentage positivity value is indicated for each recombinant protein, based on the value calcualated as the highest sum of percentage sensitivity and specificity values obtained in the ROC analysis.

Protein	AUC* (95% CI)	P value	Cut-off	Sensitivity % (95% CI)	Specificity % (95% CI)
ash	0.94 (0.87-0.99)	< 0.0001	> 9.755	82.43 (71.83-90.30)	100.00 (66.37-100.00)
cat	0.91 (0.82-1.01)	< 0.0001	> 5.945	95.95 (88.61-99.16)	77.78 (39.9-97.19)
gol	0.90 (0.82-1.00)	< 0.0001	> 9.245	91.89 (83.18-96.97)	77.78 (39.99-97.19)
lon	0.94 (0.88-1.00)	< 0.0001	> 16.69	75.68 (64.31-84.90)	100.00 (66.37-100.00)
pat	0.93 (0.86-1.00)	< 0.0001	> 7.400	86.49 (76.55-93.32)	88.89 (51.75-99.72)

Table 4. ROC curve analysis of ELISA data relating to antigen-specific serum IgG(T) levels in horses from the UK and US at various thresholds of larvae in the mucosa (total mucosal burden, TMB) and of larvae in the mucosa plus lumen (total worm burden, TWB). Horses were naturally infected or subjected to experimental infection. The area under the curve (AUC), 95% confidence intervals (CI), P values, cutoff values and % sensitivity and specificity are shown.

Protein	AUC* (95% CI)	P value	Cut-off	Sensitivity% (95% CI)	Specificity% (95% CI)
TMB 0 larvae; Negatives: 17, Positives: 66					
ash	0.72 (0.57-0.87)	0.006	>8.88	87.88 (77.51-94.62)	52.94 (27.81-77.02)
cat	0.74 (0.58-0.89)	0.003	>6.72	92.42 (83.20-97.49)	52.94 (27.81-77.02)
gol	0.72 (0.577-0.87)	0.004	>11.36	78.79 (66.98-87.89)	64.71 (38.33-85.79)
lon	0.76 (0.616-0.89)	0.001	>16.69	77.27 (65.30-86.69)	70.59 (44.04-89.69)
pat	0.73 (0.57-0.89)	0.004	>9.78	74.24 (61.99-84.22)	76.47 (50.10-93.19)
TWB 0 larvae; Negatives: 10, Positives: 73					
ash	0.91 (0.83-0.99)	<0.0001	>9.76	82.19 (71.47-90.16)	90.00 (55.50- 99.75)
cat	0.92 (0.83-1.00)	< 0.0001	>6.53	93.15 (84.74- 97.74)	80.00 (44.39- 97.48)
gol	0.91 (0.82-0.99)	< 0.0001	>10.11	90.41 (81.24- 96.06)	80.00 (44.39- 97.48)
lon	0.93 (0.86-0.99)	< 0.0001	>16.69	76.71 (65.35- 85.81)	100.00 (69.15-100.00)
pat	0.92 (0.85-0.99)	< 0.0001	>9.78	72.60 (60.91- 82.39)	100.00(69.15-100.00)
TMB 5,000 larvae; Negatives: 30, Positives: 53					
ash	0.69 (0.57-0.81)	0.004	>10.24	83.02 (70.20-91.93)	50.0 (31.30-68.70)
cat	0.77 (0.66-0.88)	< 0.0001	>11.25	71.7 (57.65- 83.21)	76.67 (57.72-90.07)
gol	0.70 (0.58-0.82)	0.002	>20.90	66.04 (51.73-78.48)	70 (50.60-85.27)
lon	0.74 (0.63-0.86)	0.0003	>31.17	60.38 (46.0-73.55)	83.33 (65.28-94.36)
pat	0.74 (0.63-0.86)	0.0002	>9.780	81.13 (68.03-90.56)	66.67 (47.19-82.71)
TWB 5,000 larvae; Negatives:18, Positives: 65					
ash	0.75 (0.61-0.88)	0.0015	>8.880	89.23 (79.06-95.56)	55.56 (30.76-78.47)
cat	0.82 (0.70-0.94)	< 0.0001	>11.25	66.15 (53.35-77.43)	88.89 (65.29-98.62)
gol	0.79 (0.67-0.91)	0.0002	>19.72	64.62 (51.77- 76.08)	83.33 (58.58-96.42)
lon	0.82 (0.71-0.93)	< 0.0001	>17.89	76.92 (64.81-86.47)	83.33 (58.58-96.42)
pat	0.78 (0.66-0.90)	0.0003	>10.78	64.62 (51.77-76.08)	83.33 (58.58-96.42)

Figure 1. ClustalW alignment of Cy-GALA-pat with its orthologues in other cyathostomin species.

Cy-GALA-pat (Accession Number: FJ882059) is aligned with GALA proteins from four additional cyathostomin species, *Cylicocyclus ashworthi* (Accession number, JN596964), *Cyathostomum catinatum* (Accession number, JN596966), *Cylicostephanus longibursatus* (Accession number, JN596968) and *Cylicostephanus goldi* (Accession number, JN596967). The signal peptide for each sequence is underlined and the domain of unknown function (DUF148) is boxed. The histidine-rich region is highlighted in grey.

Figure 2. Specificity of the four recombinant GALA proteins.

A. Coomassie stained SDS-PAGE gel depicting all four new recombinant GALA proteins. ash = *C. ashworthi* recombinant protein; cat = *C. catinatum* recombinant protein, gol = *C. goldi* recombinant protein, lon = *C. longibursatus* recombinant protein, M = molecular weight marker. B. Immunoblots of each recombinant protein (rCy-GALA-ash, rCy-GALA-cat, rCY-GALA-gol and rCy-GALA-lon) probed for IgG(T) reactivity using sera from helminth free (HF) ponies, experimental cyathostomin-infected (CI) ponies and horses infected monospecifically with either *Parascaris equorum* (Pe), *Strongylus edentatus* (Se), *Strongyloides westeri* (Sw) or *Strongylus vulgaris* (Sv). M = molecular weight marker.

Figure 3. Immunogenicity of the recombinant GALA proteins.

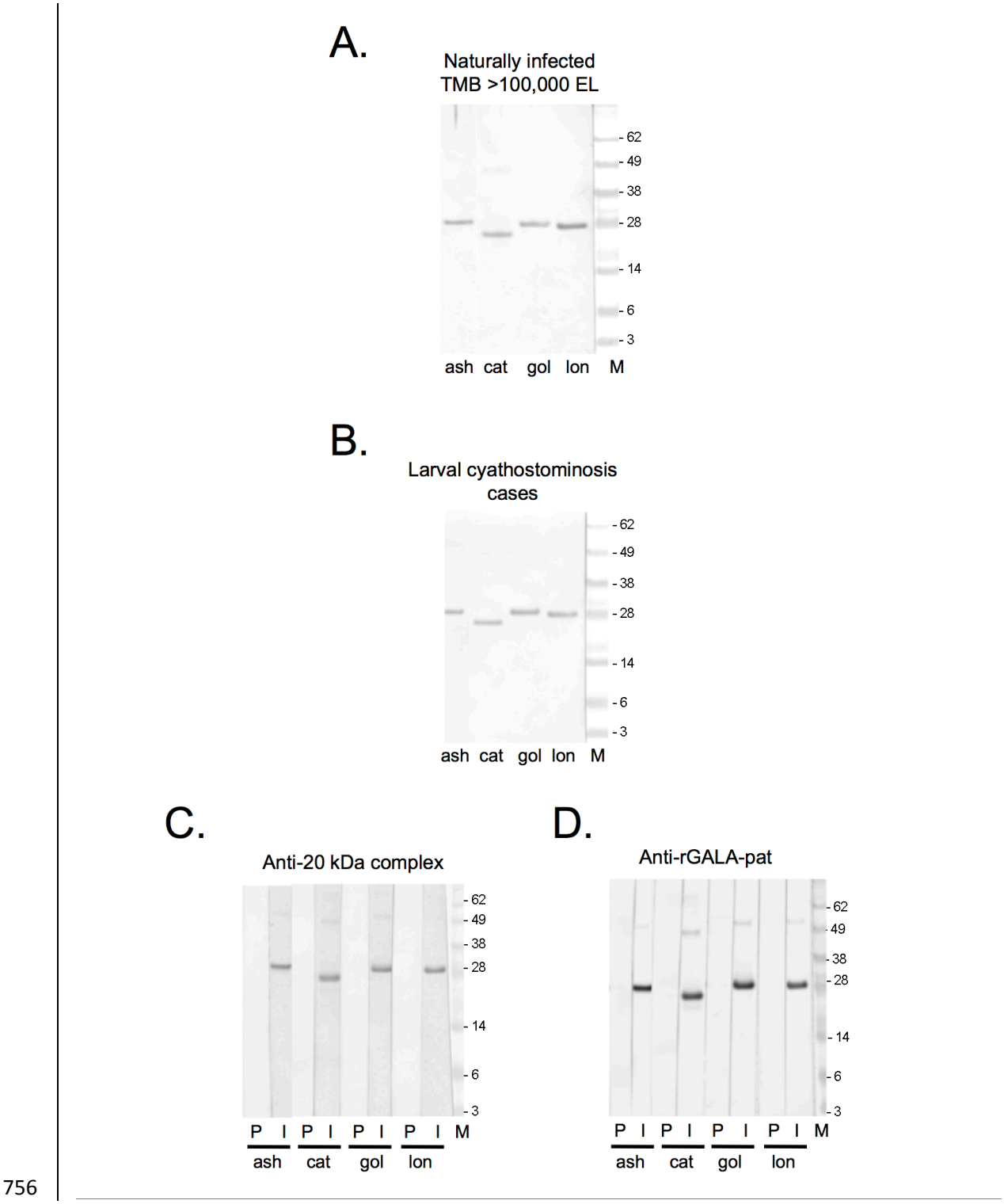
A. Immunoblot of each recombinant protein (rCy-GALA-ash, -cat, -gol and -lon) probed for binding of IgG(T) in a pool of serum from UK naturally infected horses, each of which had a total cyathostomin encysted larval burden of > 100,000 larvae. B. Immunoblot of each recombinant protein (rCY-GALA-ash, -cat, -gol and -lon) probed for binding of

IgG(T) in a pool of serum from larval clinical cyathostominosis cases from the UK. C. Immunoblot of each recombinant protein (rCy-GALA-ash, -cat, -gol and -lon) probed for binding of IgG in sera from a rabbit taken before (P) and after (I) immunisation with the cyathostomin encysted larval 20 kDa complex (Dowdall et al., 2003). D. Immunoblot of each recombinant protein (rCy-GALA-ash, -cat, -gol and -lon) probed for binding of IgG in sera from a rabbit taken before (P) and after (I) immunisation with rCy-GALA-pat (McWilliam et al., 2010).

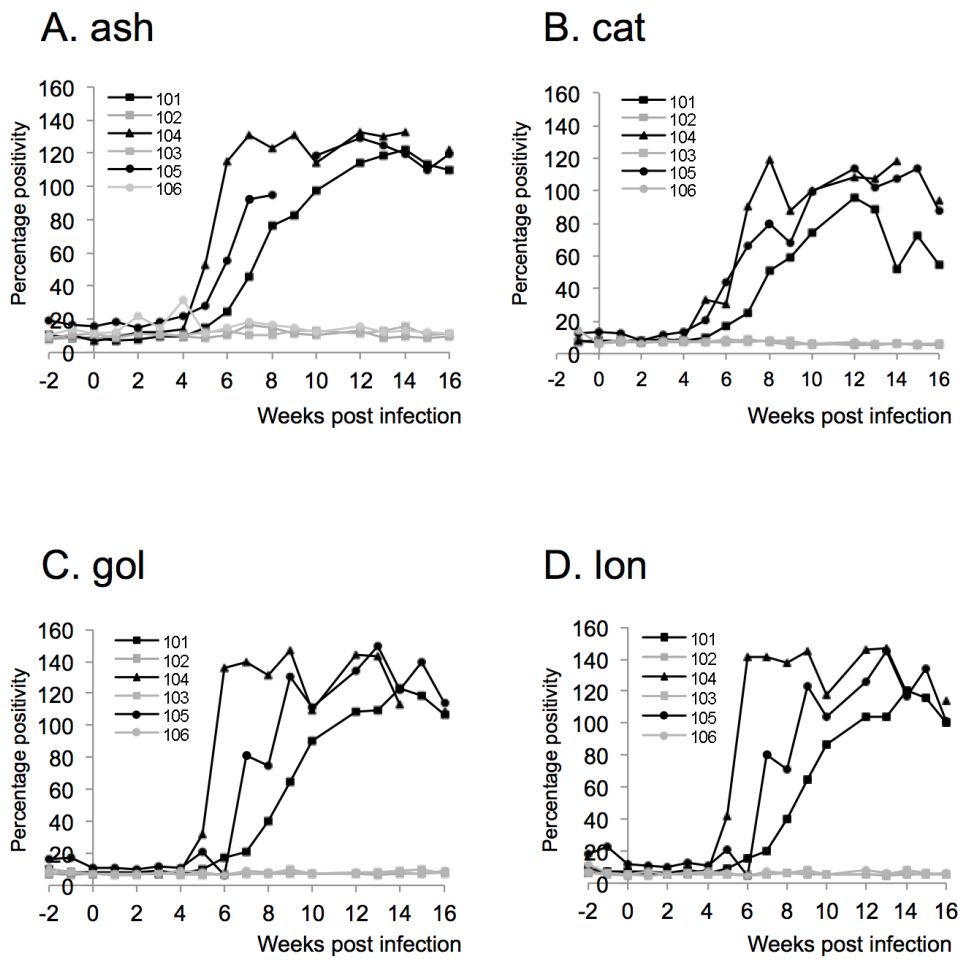
Figure 4. IgG(T) responses in experimentally infected ponies to the four recombinant GALA proteins as measured by the ELISA

Recombinant GALA (A. rGALA-ash, B. rGALA-cat, C. rGALA-gol, D. rGALA-lon) protein-specific IgG(T) responses over an experimental trickle infection (Murphy and Love, 1997). Six British native-breed ponies (6-12 months at the time of initial infection) were reared indoors with their dams and considered to be helminth-naive prior to the start of the trial. Following weaning at 4 months, the ponies were maintained on a high-fibre pelleted ration and bedded on wood shavings. Three ponies (Ponies 101, 104 and 105) were infected with a total of 3.9 million cyathostomin third stage larvae (L3), administered as a trickle infection of 150,000 L3 by nasogastric tube, three times a week. Ponies 102, 103 and 106 were maintained as uninfected controls. Pony 104 was necropsied at 20 weeks post-infection and the remaining ponies at 60-62 weeks post-infection.


Cy-GALA-pat MNKTLTFLTVVSAVALAQGVMDLFGEEGREEHRRHHRHSLLPPYLHNVSCEAKWEYFKIV 60
Cy-GALA-cat ----------EDREEHRRHHRHSLLPPYLHNVSCVAKWEYFRIV 34
Cy-GALA-gol MNKTLTFLTVVSAVVLAQGVMALFGEESREEHRRHHRHSLLPPYLHNVSCVAKWEYFKIV 60
Cy-GALA-lon MNKTLTFLTVVYAVVLAQGVMDLFGEEGREEHRRHHRHSLLPPYLHNVSCVAKWEYFKIL 60
Cy-GALA-ash ----------HEELRRHHRHSLLPPYLHNVSCEAKWEYFKIV 32
** ******:*:
Cy-GALA-pat GNRSLTFAEKKKEISEWAKKYNVVDEVASYNAYREKLKQEHRKNVSELVSALPNAVKKVN 120
Cy-GALA-cat GNRSLTFAEKKKEISEWAKKYNVLDEVASYNAYREKLKQEHRKNVSELVSDLPKAVKKVN 94
Cy-GALA-gol GNRSLTFAEKKKEISEWAKKYNVVDEVASYNAYREKLKQEHRKNVSELVSDLPNAVKKVN 120
Cy-GALA-lon GNRSLTFAEKKEKISEWAKKYNVVDEVASYNACREKLKQEHRKNVSEIVSNLPNAVKKVN 120
Cy-GALA-ash GNRSLTFAEKKGKSSEWAKKYNVVDEVASYNAYREKLKQEHRKNVSELVSGLPGAVKKVN 92
******::: : *:*****:* ******** ******:*******:** ** **::**
Cy-GALA-pat DLLDNENQTPRQLYVALRKLGRQNPALYRIVEYINVAVRLRSEEVDEQEQRRRLSALPFG 180
Cy-GALA-cat DLLDNENQTPRQLYVALRELGRQNPTLYRIVEYINVAVRRRSEELDEQEQGRRLSALPFG 154
Cy-GALA-gol DLLDNENQTSRQLYVALRELGRQNPALYRVVEYINVAVRLRRKEQDEQERQGTLSALPFG 180
Cy-GALA-lon DLLDNENQTPRQLYVALRKLGKQNPALYRVVEYINVLVRLRREEFDE-DQRRSLSALPFG 179
Cy-GALA-ash ELLDNENQTPRQLYVALRKLGKQNPVLYRVVEFVNLVVRFRREDSDEQEQREMLSTLPFS 152
.********.:***:***:**:***.**::**::*: ** : :: *: :: ** *.
Cy-GALA-pat DHNDNLEEQDFGEQDFRYVYGFECARFLLQNGRMFGLNTDERY- 223
Cy-GALA-cat DNNDNLEEQDFGEQDFRYVYGFECARFLLQNGRMFGLNTDERD- 197
Cy-GALA-gol ENNDNLEEQDFGEQDFRYVYGFECARFLLQNGRMFGLNTDRRYQ 224
Cy-GALA-lon DNNDDLEEQDFGEQDFRYIYGFECARFILQNGRMFGINTDRRY- 222
Cy-GALA-ash ENN---EEQDLGEQDFQYIYGFECARFIFQNGRMFGLNTDRRY- 192
::* ****:**:**:*:********::*******:* *..


A.
Naturally infected
TMB >100,000 EL
62
49
38
28
14
6
3
ash cat gol lon M
B.
Larval cyathostominosis
cases
62
49
38
28
14
6
3
ash cat gol lon M
C.
Anti-20 kDa complex
62
49
38
28
14
6
3
P I P I P I P I M
ash cat gol lon
D.
Anti-rGALA-pat
62
49
38
28
14
6
3
P I P I P I P I M
ash cat gol lon

756


A. ash
B. cat
C. gol
D. lon
Percentage positivity
Weeks post infection
101
102
104
103
105
106
0
20
40
60
80
100
120
140
160
-2
0
2
4
6
8
10
12
14
16

757