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Characterisation of serum IgG(T) responses to potential diagnostic antigens for equine cyathostominosis

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Running title: serum IgG(T) levels to candidate diagnostic antigens for cyathostominosis

Abstract

Cyathostomins are ubiquitous parasitic nematodes of horses. These worms spend substantial periods as intestinal wall stage encysted larvae, which can comprise up to 90% of the total burden. Several million larvae have been reported in individuals. Emergence of these larvae from the gut wall can lead to life-threatening colitis. Faecal egg count tests, increasingly used by horse owners to inform anthelmintic treatments, do not correlate with the intra-host burden of cyathostomins; this represents an key gap in the diagnostic toolbox. Previously, a cyathostomin Gut Associated Larval Antigen (Cy-GALA) was identified as a promising marker for the intra-host stages of infection. Here, Cy-GALA and an additional protein, Cyathostomin Immuno-diagnostic (Cy-CID) antigen, were investigated to examine their value in providing information on cyathostomin burden. ELISA analyses examined serum IgG(T) responses to recombinant proteins derived from individual cyathostomin species. Receiver Operator Characteristic (ROC) curve analysis was performed on the ELISA data; proteins with the highest Area Under the Curve (AUC) values were selected to test protein combinations to investigate which were the most informative in identifying the infection status of individuals. Three cocktail (CT) combinations were tested, comprising: a) Cy-GALA proteins from two species and a Cy-CID protein from a third species (CT3), b) Cy-GALA proteins from five species (CT5), and c) all CT5 components, plus a Cy-CID protein from an additional species (CT6). The best predictive values for infection were obtained using CT3 and CT6, with similar values achieved for both. Proteins in CT3 are derived from the most commonly reported species, *Cyathostomum catinatum, Cylicocyclus nassatus* and *Cylicostephanus longibursatus*. This combination was selected for future development since it represents a more commercially viable format for a diagnostic test.

Key words: nematode, cyathostomin, ELISA, ROC analysis, diagnostic test

♦ New nucleotide sequences reported in this manuscript have been submitted to GenBank under accession numbers KC759138 (Cy-CID-ash), KC759134 (Cy-CID-gol), KC759133 (Cy-CID-lon), KC759139 (Cy-CID-nas), KC759131 (Cy-CID-pat).

1. Introduction

Cyathostomins are highly prevalent pathogenic equine nematodes (Matthews, 2008). Approximately 50 individual species are classified in this group (Lichtenfels et al., 2008); however, in individuals, the majority of the burden comprises 5-10 common species with other species having a low abundance (Mfitilodze and Hutchinson, 1990; Bucknell et al., 1995; Lyons et al., 1999; Chapman et al., 2002a; Collobert-Laugier et al., 2002). Across regions, similar species proportions are found. Globally, the commonest species are *Cyathostomum catinatum*, *Cylicostephanus longibursatus* and *Cylicocyclus nassatus* (Ogbourne, 1976; Reinemeyer et al., 1984; Krecek et al., 1989; Mfitilodze and Hutchinson, 1990; Bucknell et al., 1995; Gawor, 1995; Lichtenfels et al., 2001; Collobert-Laugier et al., 2002). Cyathostomins spend a sizeable part of their life cycle as encysted larvae in the mucosa/sub-mucosa of the caecum and colon. Experimental studies demonstrate that these larvae can persist for many months (Murphy and Love, 1997). In natural infections, encysted larvae have been found to comprise over 90% of the total burden (Collobert-Laugier et al., 2002; Dowdall et al., 2002), with counts in excess of 5 million larvae recorded (Dowdall et al., 2002). Mass emergence of larvae from the intestinal wall causes larval cyathostominosis leading to sudden onset colitis, which has a case fatality rate of up to 50% (Giles et al., 1985; Love et al., 1992).

Effective cyathostomin control is complicated by a high prevalence of anthelmintic resistance to benzimidazole and pyrantel compounds (Kaplan, 2002; Matthews, 2014). There are also reports of emerging resistance to the commonly used macrocyclic lactones, measured as a reduced strongyle egg reappearance period after treatment (Rossano et al., 2010; Canever et al., 2013; Geurden et al., 2014; Relf et al., 2014; Tzelos et al., 2017). As no new anthelmintic classes are under development for use in horses in the short to

81 medium term, it is important that the efficacy of currently effective compounds is
82 preserved. Recommendations for control now place a strong emphasis on reducing
83 anthelmintic treatment frequency (Matthews, 2014; Tzelos and Matthews, 2016). Because
84 cyathostomin infections exhibit a negative binomial distribution amongst hosts (Lester et
85 al., 2013; Relf et al., 2013; Wood et al., 2013), substantial reductions in treatment levels
86 can be achieved by targeting anthelmintics based on worm egg shedding levels. Faecal
87 egg count (FEC)-directed treatments are thus recommended (Sangster, 2003; Kaplan and
88 Nielsen, 2010; Nielsen et al., 2014; Tzelos and Matthews, 2016), with good uptake in
89 some regions (Easton et al., 2016, Tzelos et al., 2019). A main disadvantage of FEC
90 analysis is the lack of information on total intra-host burden; in terms of targeting larvae
91 to avoid disease, FEC tests therefore do not provide the relevant information. Horses with
92 sizeable larval burdens often have no or low worm egg shedding (Dowdall et al., 2002).
93 Previously, two native antigen complexes, demonstrated to be targets of serum IgG(T),
94 were identified as promising markers of cyathostomin infection (Dowdall et al., 2002;
95 Dowdall et al., 2003; Dowdall et al., 2004). As antigen production requires large
96 quantities of material from infected horses and purification is technically challenging,
97 steps were taken to identify genes that encode protein components of these complexes to
98 develop a recombinant protein-based test. Cyathostomin Gut Associated Larval Antigen-1
99 (Cy-GALA-1) was identified by immuno-screening a complementary (c)DNA library
100 using sera from experimentally infected horses (McWilliam et al., 2010). Cy-GALA-1,
101 derived from *Cyathostomum pateratum*, was shown to be a strong target of serum IgG(T)
102 in infected individuals and did not exhibit reactivity to serum from horses infected with
103 non-cyathostomin nematodes (McWilliam et al., 2010). Recombinant Cy-GALA proteins
104 were generated from four additional species, *Cylicocyclus ashworthi* (Cy-GALA-ash),
105 *Cyathostomum catinatum* (Cy-GALA-cat), *Cylicostephanus goldi* (Cy-GALA-gol) and
106 *Cylicostephanus longibursatus* (Cy-GALA-lon) (Mitchell et al., 2016). Antibody

responses to each protein were assessed to identify if these could detect the presence of infection and level of burden and, when used as markers to discriminate infected *versus* un-infected animals, these performed well (Mitchell et al., 2016). Here, because of possible genetic restriction of antibody responses against a single recombinant antigen (Else and Wakelin, 1989, McKeand et al., 1994), an additional protein, Cyathostomin Immuno-Diagnostic (Cy-CID) antigen, was isolated from five common species and serum antibody responses assessed in infected and non-infected horses. Serum IgG(T) responses to individual Cy-CID and Cy-GALA proteins and three multi-antigen cocktails comprising variations of the antigens were then tested for their ability to predict the presence of infection and level of burden.

2. MATERIALS AND METHODS

2.1. *Parasite material*

Cyathostomins were obtained from large intestinal luminal contents of naturally infected horses and encysted larvae recovered by pepsin-HCl digestion or by manual removal from the mucosa and submucosa at post-mortem as described previously (Dowdall et al., 2002). Worms were identified to species on morphology based on Lichtenfels et al. (2008).

2.2. *Serum samples*

For immunoblotting and ELISA studies, sera used here were as described in Mitchell et al. (2016), and summarised here in Supplementary Table 1. Samples were included from horses maintained under helminth-free conditions (102, 103, 106) and horses (101, 104, 105) subjected to multiple experimental cyathostomin infections (Murphy and Love, 1997). Serum collected at 'Day 0' of the experiment before the infections of 101, 104 and 105 were considered as negative samples. Details of parasite burdens and species found

133 post-mortem in these animals are reported in Murphy and Love (1997). Pools of this sera
134 were used as cyathostomin-negative (helminth-free, HF) and -positive control sera
135 (cyathostomin infected, CI). The CI pool comprised samples taken 12-16 weeks post-
136 infection. These time points were selected due to high serum IgG(T) reactivity to native
137 larval antigen complexes identified in previous studies (Dowdall et al., 2002). Sera used to
138 examine cross-reactivity of each recombinant protein to other species were available from
139 horses with mono-specific experimental infections of *Strongylus edentatus* or *Strongylus*
140 *vulgaris* (Klei et al., 1982) or with *Parascaris* spp. or *Strongyloides westeri* (Dowdall et
141 al., 2003). Sera from additional infected horses from the UK and US were used to further
142 investigate immunogenicity of the antigens. For some animals, enumerated nematode
143 burden data were available allowing comparison of serum antigen-specific IgG(T) levels
144 with cyathostomin burden.
145
146 2.3. *Selection of immunogenic antigens for the diagnostic test*
147 Previously, Cy-GALA-pat protein was selected by immune-screening a cyathostomin
148 cDNA library using sera from CI horses (McWilliam et al., 2010). During screening, a
149 second protein was identified as strongly reactive to IgG(T) in CI serum. This was
150 designated Cyathostomin Immuno-Diagnostic (Cy-CID) antigen as no significant identity
151 to other characterised proteins was identified by BLAST searching. Similar to the strategy
152 used to ascribe species identity to *Cy-gala-pat* (Mitchell et al., 2016), nucleotide sequences
153 in the *Cy-cid* library clone were compared with *cid* sequences obtained by polymerase
154 chain reaction (PCR) amplification from individual identified worms of various
155 cyathostomin species. On the basis of a comparison of the resultant sequences, the library
156 clone sequence was identified as derived from *Cylicocyclus nassatus* (Cy-CID-nas;
157 KC759139.1). Using *Cy-cid-nas* sequence as template, conserved gene-specific primers
158 were designed (Fwd 5'-GGTCACACCACAAGCTCAGGA-3', Rev: 5'-

159 AGGTGAGCGAACTTT-CTGAA-3') and a region of *cy-cid* amplified from DNA
160 extracted from single identified adult worms of *Cyathostomum pateratum*, *Cylicocyclus*
161 *ashworthi*, *Cylicostephanus goldi* and *Cylicostephanus longibursatus* employing the
162 methodology used for amplifying *Cy-gala* sequences from single worms (Mitchell et al.,
163 2016). For size determination, PCR products were analysed on 1.1% w/v agarose TAE
164 gels using a TrackIt 100bp DNA Ladder (Invitrogen) and stained with 1 X GelRed
165 (Biotium). PCR products were cloned into pGEM®-T Easy, plasmid preparations made
166 and inserts sequenced as per Mitchell et al. (2016). To generate PCR products for sub-
167 cloning for recombinant expression, species-specific primers, incorporating *SacI* and *NotI*
168 restriction sites (Supplementary Table 2), were designed using *cy-cid* sequences obtained
169 from individual identified worms. *Cy-cid* sequences were amplified from pGEM®-T and
170 sub-cloned into pET-22b(+) (Novagen) as described in McWilliam et al. (2010). Colonies
171 were examined by PCR for an insert of the correct estimated size using vector-specific
172 primers and plasmid preparations made from two colonies, which were sequenced using
173 the same primers. Clones of the correct sequence were transformed into BL21-
174 CodonPlus(DE3)-RIL cells and expressed (McWilliam et al., 2010). Cy-CID proteins were
175 purified from the soluble fraction using HisTrapHP columns (GE Healthcare), eluted in
176 increasing concentrations of imidazole, dialysed with 20 mM sodium phosphate, 0.5 M
177 NaCl (pH 7.4) and stored at -20°C. Nucleotide and amino acid sequence alignments were
178 performed using ClustalW2 (Larkin et al., 2007) and sequence identity levels examined
179 using MegAlign 10.0.1 (DNASTAR) based on the ClustalW2 alignments. All sequences
180 were translated and molecular mass estimations made using the Sequence Manipulation
181 Suite (http://www.bioinformatics.org/sms2/protein_mw.html). Cy-GALA recombinant
182 proteins were prepared as described in Mitchell et al. (2016).
183

184 2.4. *Immunogenicity and specificity of recombinant Cy-CID proteins assessed by*
185 *immunoblotting*

186 To assess the immunogenicity and cyathostomin specificity of recombinant Cy-CID
187 proteins, IgG (T) reactivity to each protein was assessed by immunoblotting using sera
188 pooled from three CI horses at 12-16 weeks post-infection; these time points being
189 selected due to high serum IgG(T) reactivity to native larval antigen complexes (Dowdall
190 et al., 2002). Reactivity in the CI pool was compared to IgG(T) in control sera prepared
191 from three HF animals (Section 2.2). Cross-reactivity to other helminth species was
192 investigated using serum from individuals mono-specifically infected experimentally with
193 either *Parascaris* spp., *Strongylus edentatus*, *Strongylus vulgaris* or *Strongylus westeri*
194 (McWilliam et al. 2010). For blotting, 0.1 μg of each protein was loaded per lane onto 15-
195 well, 12% NuPAGE gels with SeeBlue Plus2 protein standards used for size estimations
196 (Invitrogen). An additional lane was loaded with 0.1 μg of each recombinant protein. After
197 electrophoresis, this lane was removed and stained with Coomassie blue for comparison
198 with the immunoblots. Blocking, primary, secondary and tertiary antibody steps and blot
199 development were as described in McWilliam et al. (2010).
200
201 2.5. *Immunoreactivity of the recombinant Cy-CID proteins and Cy-CID/Cy-GALA*
202 *protein combinations assessed by ELISA*

203 Once the immunogenicity and specificity of each Cy-CID protein was confirmed by
204 immunoblotting, ELISA was used to evaluate serum IgG(T) levels to each in uninfected
205 and infected equids. Antigen-specific IgG(T) was measured in samples from naturally-
206 infected horses from an abattoir in the UK (n=26) for which the cyathostomin burden was
207 known and cyathostomin-infected horses from the US (n=48, including 10 naturally-
208 infected horses and 38 horses administered with experimental infections, Monahan et al.,
209 1997; Monahan et al., 1998; Chapman et al., 2002b). Antigen-specific IgG(T) levels in

these groups were compared with those in true cyathostomin-negative horses (US cyathostomin-free horses infected with *S. vulgaris, P. equorum* or *S. westeri* and UK non-infected horses raised in a helminth-free environment [n=6]). Wells of ELISA plates (96-well flat bottomed Microlon High binding plates, Greiner Bio-One) were coated with recombinant CID antigens at 2 µg/ml, diluted in 100 µl coating buffer (0.1 M carbonate coating buffer, pH 9.6) and incubated overnight at 4°C. Plates were washed six times with 0.05% Tween-20 in 1X PBS (PBS-T), then blocked using 200 µl block buffer (2% soya infant formula [Wysoy, SMA nutrition], w/v in 1X PBS), per well for 1 h at 37ºC. All serum dilutions were made in block buffer. Plates were washed six times (as above) and 100 µl sera (diluted 1:800) added to each well and incubated for 2 h at 37ºC. Each sample was tested in triplicate. Plates were washed six times, incubated for 1 h with (100 µl per well) goat anti-horse IgG(T) HRP (AbD Serotec, AAI38P), diluted 1:5,000 in block buffer. Reactions were developed by adding 100 µl *o*-Phenylenediamine dihydrochloride (OPD) solution prepared from SIGMA*FAST* OPD tablets (Sigma Aldrich) to each well. After 15 min at room temperature, 50 µl 2.5 M H_2SO_4 were added to stop reactions and absorbance in each well read at an optical density (OD) of 490 nm. On all plates, aliquots from the same pool of CI and HF sera were tested in triplicate as positive and negative controls, respectively, for inter-plate variation. Results were expressed as the percentage OD of the CI sample mean for each plate. Minitab 17 Statistical Software for Windows was used for statistical analysis. For cyathostomin infected populations, group medians of the percentage positivity were compared to those of true cyathostomin-negative horses by the Mann-Whitney test. A p value <0.05 was taken to indicate statistical significance. Combinations of Cy-CID and/or Cy-GALA proteins were subsequently assessed for diagnostic performance based on Receiver Operator Characteristic (ROC)-curve analysis (see below) of ELISA data obtained using each Cy-GALA (Mitchell et al. 2016) and Cy-CID protein. Protein combinations (cocktails, CT) were then tested as follows:

- CT3: Cy-GALA-cat, Cy-GALA-lon, Cy-CID-nas,
- CT5: Cy-GALA-pat, Cy-GALA-cat, Cy-GALA-ash, Cy-GALA-gol, Cy-GALA-lon,
- CT6: Cy-GALA-pat, Cy-GALA-cat, Cy-GALA-ash, Cy-GALA-gol, Cy-GALA-lon, Cy-CID-nas.

2.6. *Data analysis*

ROC curve analysis is frequently used in diagnostic test development to demonstrate the connection between sensitivity and specificity, allowing an assessment of trade-off of diagnostic sensitivity against specificity over a range of cut-offs to inform test design. The area under the ROC curve provides a value relating to test performance. As an estimate of test accuracy, the area under the curve (AUC) may be interpreted such that; an AUC=0.9-1.0 demonstrates excellent discrimination between positive and negative results; an AUC=0.8-0.9, good discrimination; an AUC=0.7-0.8, fair discrimination; an AUC=0.6-0.7, poor discrimination and an AUC=0.5-0.6, no discrimination (Swets, 1988). Here, ROC curve analysis was undertaken to indicate the diagnostic accuracy of each protein or protein combination ELISA result relating to cyathostomin infection (postive or negative) and also to cyathostomin total mucosal burden (TMB) and total worm burden (TWB). Data were subjected to ROC analysis using Prism 6 (Graphpad Software Inc, USA). In addition to this analysis, Spearman's rank correlations were performed on the antigen combination (CT3, CT5, CT6) data. The variables examined were ELISA percentage positivity values *versus* TMB and TWB. Minitab 17 Statistical Software for Windows was used for analysis. P-values <0.05 were considered significant.

3. Results

3.1. *Analysis of Cy-CID sequences*

Following identification of antigen Cy-CID by screening a cyathostomin cDNA library using infected sera (McWilliam et al., 2010), a region of *Cy-cid*-encoding orthologous sequences was amplified from single adult worms of the following species, *C. nassatus* (n=2), *C. pateratum* (n=3), *C. ashworthi* (n=1), *C. goldi* (n=2) and *C. longibursatus* (n=4). The sequences from the two *C. nassatus* adults were 96-99% identical to the original cDNA library clone, confirming its identity as *C. nassatus*. Comparative sequence analysis (Fig. 1) of the Cy-CID orthologs revealed a high degree of inter-species sequence conservation over the transcript. The Cy-CID-ash sequence demonstrated 98% identity with the consensus Cy-CID-nas sequence. The consensus sequence from *C. goldi* worms demonstrated 95.3% amino acid identity to the Cy-CID-nas consensus sequence. The consensus Cy-CID-lon sequence displayed 95.1% to the Cy-CID-nas consensus sequence, while the Cy-CID-pat consensus sequence demonstrated 91.2% identity to the consensus sequence from *C. nassatus*. No functional domains were identified in any sequence, nor were significantly matching orthologous sequences found in non-cyathostomin species.

3.2. *Immunoreactivity and cyathostomin specificity of the recombinant Cy-CID proteins and time course dynamics of IgG(T) levels in experimentally infected horses*

Subsequent to production and purification of recombinant proteins, Cy-CID-ash, Cy-CID-gol, Cy-CID-lon, Cy-CID-pat and Cy-CID-nas, a Coomassie blue stained 12% SDS-PAGE gel (Fig. 2A) confirmed that the approximate size observed for each antigen corresponded to its calculated molecular mass. Immunoblot analysis (Fig. 2B), demonstrated that all Cy-CID recombinant proteins were specifically bound by IgG(T) in

286 sera pooled from three equids experimentally infected with cyathostomins (CI sera),
287 whereas IgG(T) in sera from six HF horses lacked reactivity. In terms of cross reactivity to
288 other helminth species, there was negligible IgG(T) binding to each Cy-CID protein in sera
289 obtained from individual horses that were mono-specifically infected with the species,
290 *Parascaris* spp., *S. edentatus*, *S. vulgaris* or *S. westeri* nematodes.

291 To evaluate specific serum IgG(T) responses to each of the five recombinant Cy-CID
292 proteins, an ELISA time-course study over a repeated cyathostomin larval infection series
293 was performed. Antigen-specific IgG(T) levels of infected equids (101, 104, 105) were
294 analysed prior to infection and until 16 weeks after initial larval challenge and compared to
295 IgG(T) levels in uninfected equids (102, 103, 106). Comparable with the previously
296 observed IgG(T) responses in the same horses to Cy-GALA antigens (Mitchell et al.,
297 2016), Fig. 3 demonstrates an increase in antigen-specific IgG(T) to Cy-CID antigens
298 around 4-5 weeks after initial challenge. However, when examining serum antibody
299 response dynamics between horses to the different Cy-CID proteins, this was observed to
300 be more variable when compared to the observed IgG(T) responses to Cy-GALA proteins
301 in the same horses (Mitchell et al., 2016). Specifically, the dynamics of the serum IgG(T)
302 response to the Cy-CID-ash and Cy-CID-gol antigens varied between different infected
303 animals. The pattern of IgG(T) responses to Cy-CID-lon, Cy-CID-pat and Cy-CID-nas
304 were more consistent across infected individuals, reaching a plateau after an initial
305 increase in IgG(T) at 4-6 weeks post challenge, before a subsequent decline in antigen
306 specific antibody. Despite an initial slower serum IgG(T) response in animal 101 to Cy-
307 CID-nas, equivalent IgG(T) levels were measured in all infected horses by the end of the
308 time-course. Antigen-specific IgG(T) levels in the uninfected controls were negligible
309 across the entire experiment.

310

3.3. *ELISA and ROC curve analysis of serum IgG(T) responses to recombinant Cy-CID proteins in cyathostomin-infected horses with enumerated worm burdens*

Serum IgG(T) levels to individual Cy-CID proteins were compared using samples from equids with enumerated burdens, allowing comparison of parasitological parameters with specific IgG(T) levels in matched end-point sera. Serum IgG(T) levels of cyathostomin-infected horses were compared with cyathostomin-negative horses raised helminth-free. Antigen-specific IgG(T) was significantly higher ($P<0.05$) in cyathostomin-positive horses (n=74) than in cyathostomin-negative horses (n=9) for Cy-CID-ash ($P<0.0001$), Cy-CID-nas ($P=0.0001$) and Cy-CID-pat ($P=0.0275$). Signficant differences in antigen-specific serum IgG(T) levels between the two groups were not observed for ELISA experiments performed with the Cy-CID-gol ($P=0.2105$) and Cy-CID-lon ($P=0.0579$) proteins.

To further evaluate the potential of individual Cy-CID antigens for discriminating between cyathostoin-negative and -positive horses, ELISA results from equids for which worm burden data was available were subjected to ROC curve analysis (Table 1). For all proteins, antigen-specific IgG(T) levels in cyathostomin-negative equids were compared to those in cyathostomin-infected horses. Likelihood ratios and percentage sensitivity and specificity values were generated by the software package; these values were used to calculate cut-off percentage positivity thesholds for each protein. The cut-off values were selected on the basis of the highest sum of percentage sensitivity and specificity and a likelihood ratio generated for each protein. High AUC values (i.e. >0.9) were obtained for Cy-CID-ash and Cy-CID-nas proteins. The highest level of sensitivity was demonstrated for Cy-CID-nas (90.41%), with 100% specificity observed for Cy-CID-gol. Next, AUC values at different thresholds of TMB and TWB levels were compared to assess if the outputs were likely to be confounded by the half-life of Cy-CID-specific serum IgG(T) responses; for example, in equids where there had been recent emergence of previously encysted larvae or in those horses that had recently been treated with an effective larvicidal

compound. For all proteins, the AUC, sensitivity and specificity values were higher for TWB than TMB (Table 1). For example, for a '0' burden threshold, AUC values for Cy-CID-ash and Cy-CID-nas were 0.91 and 0.92 compared to 0.73 and 0.79 for TWB and TMB, respectively. Horses were also grouped on the basis of a threshold of 5,000 TMB or TWB. At a cut-off of a TMB of 5,000 larvae, AUC values of individual Cy-CID proteins ranged from 0.58 (Cy-CID-gol) to 0.75 (Cy-CID-nas). When taking luminal burden into account at a cut-off of 5,000 TWB, Cy-CID-nas (AUC value = 0.78) gave the highest value. When cyathostomin worm burden data were partitioned at a level of 10,000 TMB /TWB or above, AUC values generated by the ROC curve analysis were <0.7 (data not shown), indicating poor discrimination between the positive and negative groupings at these higher burden thresholds.

3.4. *ELISA and ROC curve analysis of serum IgG(T) responses to combinations of recombinant Cy-CID and Cy-GALA proteins in cyathostomin-infected horses*

To take account of the fact that cyathostomin infections are multi-species, the next step was to test combinations of antigens from common species. Analysis of Cy-CID and Cy-GALA antigen combinations were assessed as antibody responses to single recombinant helminth antigens have been shown to be genetically restricted such that some individuals are non-resposive despite being infected (Trenholme et al., 1994). ROC curve AUC values obtained previously for Cy-GALA proteins were generally higher (Mitchell et al., 2016) than those observed for Cy-CID proteins; for example, when studying horse serum IgG(T) responses grouped as infected *versus* non-infected, Cy-GALA antigen ROC AUC values range from 0.91-0.93 (Mitchell et al., 2016), compared to a range of ROC AUC values of 0.65-0.92 for Cy-CID antigens (with the highest value measured for Cy-CID-nas). Likewise, when assessing a 5,000 TWB threshold, Cy-GALA proteins give a range of

362 ROC AUC values from 0.75 (Cy-GALA-ash) to 0.82 (Cy-GALA-lon, Cy-GALA-cat).
363 Similar results were obtained for a TMB threshold of 5,000 worms. In these cases, ROC
364 AUC values were higher for all Cy-GALA proteins, with the exception of Cy-CID-nas.
365 First, all five Cy-GALA proteins were tested in a single combination, CT5, representing
366 the species *C. catinatum*, *C. pateratum*, *C. ashworthi*, *C. goldi* and *C. longibursatus*. These
367 five Cy-GALA proteins were then tested in combination with Cy-CID-nas. This protein
368 gave the highest ROC curve AUC value of the CID proteins tested and represents one of
369 the commonest reported species. This combination was designated CT6. Finally, a
370 combination of three proteins was assessed. These proteins were selected on the basis of
371 previous species prevalence reports for cyathostomins (for example, Bucknell, et al., 1995;
372 Chapman et al., 2002a; 2002b; Collobert-Laugier, et al., 2002), which indicate that the
373 commonest species observed across regions are *C. nassatus* (Cy-CID-nas), *C.*
374 *longibursatus* (Cy-GALA-lon) and *C. catinatum* (Cy-GALA-cat). This combination was
375 designated CT3.

376 Levels of serum IgG(T) to the three cocktails were compared by ELISA using serum
377 samples as per the single-protein analysis above. Specific serum IgG(T) levels were
378 significantly higher ($p<0.05$) in the cyathosotmin-infected than in the -uninfected
379 population for CT3, CT5 and CT6. ROC curve AUC values were calculated for the three
380 cocktails at different burdens of total worms (TWB) or total mucosal worms (TMB, Table
381 2). As with the individual antigens, the chosen cut-off values for each cocktail were based
382 on the highest sum of percentage sensitivity and specificity and a likelihood ratio present.
383 At the 0 TWB threshold, specificity was 90% for all three cocktails. A sensitivity of
384 87.67%, 86.30% and 91.78% was reported for CT3, CT5 and CT6, respectively. In terms
385 of ROC curve outputs, at the 0 TWB threshold, CT3 and CT6 performed best, both
386 achieving an AUC value of 0.94. For a 5,000 TWB threshold, AUC values were 0.84 for
387 CT3 and CT6, and 0.80 for CT5. Thus, the use of antigen cocktails improved the AUC

values at each of the selected worm burden thresholds, with the highest values obtained when the Cy-GALA antigens were combined with a Cy-CID antigen from *C. nassatus*. When cyathostomin burden data were partitioned at a level of 10,000 TMB or TWB or above, most AUC values generated by ROC curve analysis were <0.7 (data not shown), indicating poor discrimination between the positive and negative groupings on the basis of these higher worm burden thresholds.

Spearman's rank correlations were performed using the ELISA data for each protein combination for all cyathostomin infected horses (Table 3). Significant positive correlations between the ELISA data and the cyathostomin worm burden parameters were observed for CT3 and CT6, which included the CID proteins, but not for the CT5, which comprised only GALA proteins.

4. Discussion

The lack of a diagnostic test for cyathostomin intra-host stages, including encysted larvae, means that in some regions, whole-group administration of an annual anthelmintic treatment is commonly recommended in autumn/winter (Tzelos and Matthews, 2016; Rendle et al., 2019). With no new equine anthelmintic compounds on the horizon, a reduction in unnecessary treatments is paramount to preserve efficacy, in particular, of moxidectin, currently the only compound widely effective against encysted cyathostomin larvae. The availability of a test that provides information on the presence, or burden, of cyathostomin infections could benefit specific targeting of equine anthelmintics to lower treatment frequency and hence reduce selection pressure for anthelmintic resistance (Matthews, 2014). The current study describes key steps that identified two cyathostomin proteins, Cy-GALA and Cy-CID, that could be used in such a test to inform on the presence or level of infection of cyathostomins in horses.

Due to the complexity of cyathostomin infections, development of a test for these nematodes is challenging (Lichtenfels et al., 2008). The studies here sought to define serum IgG(T) responses in infected horses to two antigens from the most commonly reported species. Cy-CID antigen was identified by immuno-screening a mixed mucosal stage cDNA library. The *Cy-cid* transcript was detected by reverse transcriptase PCR in late mucosal larval stages and in luminal stage parasites (unpublished data). The developmental expression profile of *Cy-cid* is therefore complementary to that of *Cy-gala*, shown previously to be expressed in early and late mucosal larval stages, but undetectable by reverse transcriptase PCR in luminal stage worms (McWilliam et al., 2010). When Cy-CID sequences were compared among cyathostomin species, it was observed that, similar to Cy-GALA, the Cy-CID sequences demonstrated a high degree of conservation and high intra-specific identity. Since heterologous species seemingly lack orthologous Cy-CID sequences, these proteins may be cyathostomin-specific. The genomes and transcriptomes of rarer nematode species are yet to be analysed; ongoing sequencing projects could reveal the presence of Cy-CID orthologs outside the *Cyathostominae* group.

Here, Cy-CID proteins representing five common species were expressed as recombinant proteins and cyathostomin specificity of each confirmed by an absence of immune-reactivity of each protein to IgG(T) in sera from horses harbouring non-cyathostomin helminths. Immuno-blotting studies also demonstrated that each Cy-CID protein was strongly reactive with serum IgG(T) from cyathostomin-infected horses. For the Cy-CID-ash, Cy-CID-pat and Cy-CID-nas proteins, these results were further supported by ELISA data that demonstrated significantly higher antigen-specific serum IgG(T) levels to each protein in cyathostomin-infected equids compared to equids that had no prior exposure to cyathostomin infection. The time-course study provided additional support for the diagnostic potential of the CID antigens, demonstrating consistent specific

serum IgG(T) responses, in particular to the Cy-CID-pat and Cy-CID-nas proteins, in all infected horses, with lowest background reactivity observed to the Cy-CID-nas protein.

The CID proteins were thereafter assessed for their ability to define the presence of infection, or infection level, at increasing cyathostomin burden thresholds in a larger cohort of horses. The ROC curve analysis demonstrated high AUC values generated from the ELISA analysis using the *C. nassatus* and *C. ashworthi* CID proteins, but lower AUC values when analysing IgG(T) responses to CID proteins representing *C. goldi, C. longibursatus* and *C. pateratum*. In previous studies using sera from the same cohort of horses, all cyathostomin GALA proteins consistently achieved AUC values in excess of 0.9 (Mitchell et al., 2016). Here, only Cy-CID-nas and Cy-CID-ash ELISA data generated similar AUC scores, in agreement with the significantly higher specific IgG(T) response to Cy-CID-nas and Cy-CID-ash in comparison to the other Cy-CID proteins. *Cylicocyclus nassatus* is reported as one of the most prevalent and abundant species infecting domesticated equids (Krecek et al., 1989, Bucknell et al., 1995; Kuzmina et al., 2005, Traversa et al., 2009). *Cylicocyclus ashworthi* is also common, despite previous reports underestimating its prevalence due to misidentification because of high morphological similarity to *C. nassatus* (Chapman et al., 2002a). An explanation for the observed differences in IgG(T) response to the various Cy-CID antigens could be that some recombinant proteins might be more inherently immunogenic than others.

Variation in individual's immune responses is a feature of helminth infections and, to account for potential heterogeneity in the host antibody repertoire, antibody responses to combinations of proteins are generally deemed to be more informative in defining worm infection status (Bradley et al., 1991; Li et al., 2011). Hence, the next step in this study was to examine three combinations of the best-performing antigens from the individual-protein analysis, also taking into account previous literature on individual species abundance. Antigens were thus selected from the three commonest species reported, *C. longibursatus,*

464 *C. nassatus* and *C. catinatum*, and from two further common species, *C. ashworthi* and *C.*
465 *pateratum* (Krecek et al., 1989; Mfitilodze and Hutchinson, 1990; Bucknell et al., 1995;
466 Gawor, 1995; Lichtenfels et al., 2001; Kuzmina et al., 2005) Both ROC curve and
467 statistical correlation analysis of the performance of the three antigen combinations
468 indicated that CT3 and CT6 gave the best discriminatory information when comparing
469 cyathostomin-negative to cyathostomin-positive individuals. For both antigen
470 combinations, derived AUC values were >0.9, representing excellent accuracy for a
471 diagnostic test (Swets, 1988). Stratification of the horses based on TWB, rather than on
472 mucosal worm burden, resulted in higher AUC scores. A potential explanation for this
473 observation could be the presence of residual specific IgG(T) to recently emerged larvae.
474 In terms of worm burden thresholds, the test performed well up to a burden of 5,000
475 cyathostomins. One reason for this is that, as evidenced by the experimental-infection
476 time-course study where IgG(T) levels to both Cy-CID and Cy-GALA antigens were
477 measured, IgG(T) levels displayed a plateau pattern 8-10 weeks after initial infection,
478 despite continuous larval challenge. Inclusion of three additional Cy-GALA antigens in
479 CT6 did not appear to substantially increase the overall accuracy of the test compared to
480 the CT3 combination. Therefore, to balance the financial resource required for antigen
481 generation with the diagnostic value provided by the protein combination, CT3 was
482 selected for downstream development in a commercial setting.

483 Determination of a reliable cut-off value for diagnosis of this disease is not a trivial task
484 and factors other than test performance need to be considered, i.e. the overall benefit to the
485 animal as well as the entire co-grazing population. Clinical larval cyathostominosis is a
486 serious condition but is relatively uncommon; however, to minimise potential
487 misdiagnosis, a suitable worm burden theshold needs to be selected to avoid such a
488 scenario occurring. The threshold of burden selected must also take into account owner
489 perception as to what level represents a “substantial” worm burden; otherwise they may

not engage in using a test if they consider the parasite threshold selected for treatment as too high. Unfortunately, there is no data published that indicates the level of cyathostomin burden that can lead to disease. Further, the cost of incorrectly classifying an infected animal as non-infected would be conceived by most owners to outweigh the cost (i.e. anthelmintic treatment) of a potential false-positive diagnosis. Potential residual IgG(T) from past infection can have confounding effects on assay accuracy. Since the serum half-life of equine IgG(T) has been reported as 21 days (Sheoran et al., 2000), this also needs to be taken into account in applying the test in practice. Thus, the test result must be interpreted alongside the clinical and treatment history of the individual/population under assessment. Monitoring of antigen-specific serum IgG(T) responses in individuals after moxidectin administration will provide further insight into temporal dynamics of antibody responses post-treatment and will inform application of this test in practice. Equine parasitology experts advocate application of moxidectin in moderate-high risk animals at the end of the grazing season to reduce the risk of larval cyathostominosis (Rendle et al., 2019). Thus, the timing of use of this test is likely to provide most value if used in northern temperate regions in autumn/early winter, when it can be employed to inform on the need for a larvicidal treatment.

This serum-based test has subsequently been validated in a commerical setting (Austin Davis Biologics Ltd., UK) and optimised for use in a robotic system. Where matching serum samples could be assessed, outputs were compared with the prototype test described here; significant correlations were demonstrated between the two methods (Matthews, Austin, et al., unpublished data). The commercial version of the test was launched in the UK in September 2019 (www.austindavis.co.uk/small-redworm-blood-test). In selecting thresholds for treatment, the requirement for high sensitivity to minimise false negatives was made. The veterinarian's decision to use the test and apply its outputs to inform the application of anthelmintic treatment are defined in guidelines developed to be used in

516 interpreting the outputs of the ELISA and are based on the CT3-specific antibody level
517 measured, expressed as a 'serum score', taking into account the grazing management and
518 historic parasitological (FEC) parameters of the individual or group being tested.
519
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527

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687
688 **Table 1.** Receiver operator characteristic (ROC) curve analysis of ELISA data relating to
689 antigen-specific IgG(T) levels comparing cyathostomin-positive (Pos; n=73) to negative
690 (Neg; n=10) horses. Horses stratified at 0 or 5,000 worm threshold of cyathostomins in the
691 mucosa plus lumen (total worm burden, TWB) or larvae in the mucosa (total mucosal
692 burden, TMB). Area under the curve (AUC), 95% confidence intervals (CI) and P values
693 for data generated by ROC curve analysis for each protein are shown. The cut-off value
694 indicated is based on the value calculated as the highest sum of percentage sensitivity and
695 specificity values with a likelihood ratio present obtained in the ROC analysis.

	Antigen	AUC (95% CI)	P value	Cut-off	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
	Cy-CID-pat	0.74 (0.61-0.87)	0.015	>28.44	60.27 (48.14-71.55)	90.00 (55.50-99.75)
TWB 0	Cy-CID-ash	0.91 (0.84-0.98)	< 0.001	>26.51	84.93 (74.64-92.23)	90.00 (55.50-99.75)
Neg: 10	Cy-CID-gol	0.65 (0.49-0.82)	0.114	>42.80	32.88 (22.33-44.87)	100 (69.15-100.0)
Pos: 73	Cy-CID-lon	0.67 (0.53-0.81)	0.082	>36.09	46.58 (34.80-58.63)	90.00 (55.50-99.75)
	Cy-CID-nas	0.92 (0.85-0.99)	< 0.001	>5.970	90.41 (81.24-96.06)	80.00 (44.39-97.48)
	Cy-CID-ash	0.73 (0.59-0.87)	0.003	>35.20	74.24 (61.99-84.22)	70.59 (44.04-89.69)
TMB 0	Cy-CID-gol	0.60 (0.46-0.74)	0.210	>52.58	30.30 (19.59-42.85)	100 (80.49-100.0)
Neg: 17	Cy-CID-lon	0.70 (0.58-0.82)	0.011	>33.40	56.06 (43.30-68.26)	82.35 (56.57-96.20)
Pos: 66	Cy-CID-pat	0.74 (0.62-0.86)	0.003	>28.44	65.15 (52.42-76.47)	88.24 (63.56-98.54)
	Cy-CID-nas	0.79 (0.68-0.90)	< 0.001	>12.72	71.21 (58.75-81.70)	76.47 (50.10-93.19)
	Cy-CID-pat	0.73 (0.60-0.85)	0.004	>28.44	64.62 (51.77-76.08)	83.33 (58.58-96.42)
TWB 5,000	Cy-CID-ash	0.72 (0.58-0.86)	0.005	>35.20	73.85 (61.46-83.97)	66.67 (40.99-86.66)
Neg:18	Cy-CID-gol	0.63 (0.50-0.76)	0.102	>52.58	30.77 (19.91-43.45)	100.0 (81.47-100.0)
Pos: 65	Cy-CID-lon	0.73 (0.62-0.84)	0.003	>36.97	49.23 (36.60-61.93)	94.44 (72.71-99.86)
	Cy-CID-nas	0.78 (0.66-0.89)	< 0.001	>16.22	58.46 (45.56-70.56)	88.89 (65.29-98.62)
	Cy-CID-pat	0.64 (0.52-0.77)	0.030	>30.55	60.38 (46.00-73.55)	73.33 (54.11-87.72)
TMB 5,000	Cy-CID-ash	0.65 (0.53-0.78)	0.021	>44.10	67.92 (53.68-80.08)	63.33 (43.86-80.07)
Neg: 30	Cy-CID-gol	0.58 (0.46-0.71)	0.214	>43.46	35.85 (23.14-50.20)	86.67 (69.28-96.24)
Pos: 53	Cy-CID-lon	0.63 (0.50-0.75)	0.054	>36.97	49.06 (35.06-63.16)	76.67 (57.72-90.07)
	Cy-CID-nas	0.75 (0.64-0.85)	<0.001	>18.89	54.72 (40.45-68.44)	83.33 (65.28-94.36)

696

Table 2. Receiver operator characteristic (ROC) curve analysis of ELISA data relating to antigen-specific IgG(T) levels comparing cyathostomin-positive (Pos; n=73) vs. cyathostomin-negative individuals (Neg; n=10). Horses stratified on at 0 or 5,000 worm threshold of cyathostomins in the mucosa plus lumen (total worm burden, TWB) or larvae in the mucosa (total mucosal burden, TMB). Area under the curve (AUC), 95% confidence intervals (CI) and P values for data generated by ROC curve analysis are shown. Cut-off based on the value calculated as the highest sum of percentage sensitivity and specificity values with a likelihood ratio present obtained in the ROC analysis. Cocktails comprised the following: CT3 – Cy-GALA-lon, Cy-GALA-cat and Cy-CID-nas; CT5 – Cy-GALA-pat, Cy-GALA-cat, Cy-GALA-ash, Cy-GALA-gol and Cy-GALA-lon; CT6 – Cy-GALA-pat, Cy-GALA-cat, Cy-GALA-ash, Cy-GALA-gol, Cy-GALA-lon and Cy-CID-nas.

	Antigen	AUC (95% CI)	P value	Cut-off value	Likelihood ratio	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
TWB 0 Neg: 10 Pos: 73	CT3	0.94 (0.88-0.99)	<0.001	>17.04	8.77	87.67 (77.88-94.20)	90.00 (55.50-99.75)
	CT5	0.90 (0.83-0.97)	<0.001	>15.06	8.63	86.30 (76.25-93.23)	90.00 (55.50-99.75)
	CT6	0.94 (0.88-0.99)	<0.001	>14.44	9.18	91.78 (82.96-96.92)	90.00 (55.50-99.75)
TMB 0 Neg: 17 Pos: 66	CT3	0.75 (0.62-0.88)	0.002	>19.74	2.23	78.79 (66.98-87.89)	64.71 (38.33-85.79)
	CT5	0.73 (0.59-0.86)	0.004	>15.21	2.06	84.85 (73.90-92.49)	58.82 (32.92-81.56)
	CT6	0.76 (0.63-0.89)	0.001	>14.44	1.93	90.91 (81.26-96.59)	52.94 (27.81-77.02)
TWB 5,000 Neg:18 Pos: 65	CT3	0.84 (0.73-0.95)	<0.001	>18.06	3.1	86.15 (75.34-93.47)	72.22 (46.52-90.31)
	CT5	0.80 (0.68-0.91)	<0.001	>16.56	2.94	81.54 (69.97-90.08)	72.22 (46.52-90.31)
	CT6	0.84 (0.73-0.95)	<0.001	>18.52	3.16	87.69 (77.18-94.53)	72.22 (46.52-90.31)
TMB 5,000 Neg: 30 Pos: 53	CT3	0.70 (0.58-0.82)	0.003	>21.66	1.87	81.13 (68.03-90.56)	56.67 (37.43-74.54)
	CT5	0.68 (0.56-0.80)	0.007	>23.22	2.01	73.58 (59.67-84.74)	63.33 (43.86-80.07)
	CT6	0.70 (0.58-0.82)	0.003	>21.63	1.93	77.36 (63.79-87.72)	60.00 (40.60-77.34)

709
710 **Table 3.** Spearman's rank correlations of cyathostomin infected individuals (n=83)
711 comparing % positivity values for each of three protein combinations (CT3, CT5, CT6)
712 with cyathostomin burden (total worm burden (TWB) and total mucosal burden (TMB).
713 The variables examined, Spearman's rank correlation coefficient (Spearman rho) and P-
714 values are shown.

715

Cocktail (% positivity values)	Cyathostomin burden measure	Spearman rho	P-Value
CT3	TWB	0.337	0.002
CT5	TWB	0.27	0.013
CT6	TWB	0.348	0.001
CT3	TMB	0.267	0.015
CT5	TMB	0.206	0.061
CT6	TMB	0.27	0.013

716

Supplementary Tables

Supplementary Table 1. Details of serum samples used to test IgG(T) responses to the recombinant antigens including, sample numbers (n) and the original article (Reference). The populations were from the UK or US as described in Materials and Methods.

Serum population	n	Reference
UK cyathostomin infected (weeks 12 and 16 post infection)	3	Murphy and Love, 1997
UK helminth free	3	Murphy and Love, 1997
UK abattoir	26	Dowdall et al. 2004
US naturally infected	10	Monahan et al. 1996
US experimentally infected	38	Chapman et al. 2002b; Monahan et al. 1997, Monahan et al. 1998
US cyathostomin-free	3	Dowdall et al. 2003; Klei et al. 1982

Supplementary Table 2. Primers used for recombinant protein expression (restriction enzymes' sites, *SacI* and *NotI* are highlighted in bold in forward and reverse primers, respectively).

Gene	Forward primer 5' – 3'	Reverse primer 5' – 3'
Cy-CID-pat	ATTC**GAGCTC**CGGAGCGAAGGCTAGAATTATTC	**GCGGCCGC**AGCTTTGAGAGCTTCACTGAG
Cy-CID-ash	ATTC**GAGCTC**CCCAGGTCACACCACAAGCTCA	**GCGGCCGC**GAGGTGAGCGAACTTTCTGGCTT
Cy-CID-gol	ATTC**GAGCTC**CCTGGCCAAGTGGGAAAAGAC	GCTT**GCGGCCGC**TTCCGGATCCTCAGCTAAATCT
Cy-CID-lon	ATTC**GAGCTC**CCAGGTCACACCACAAGCTCA	GCTT**GCGGCCGC**GAGGTGAGCGAACTTTCTG
Cy-CID-nas	ATTC**GAGCTC**CCAATTCCTGGCTGGGAGAAAAGAGA	**GCGGCCGC**TTCCGGATCCTCAGCTAAGTCC

Legend to Figures

Fig. 1. Cy-CID orthologs share extensive sequence similarity. ClustalW alignment of Cy-CID orthologs isolated from *C. pateratum* (Cy-CID-pat; GenBank Accession number: KC759131), *C. ashworthi* (Cy-CID-ash; GenBank Accession number: KC759138), *C. nassatus* (Cy-CID-nas; GenBank Accession number: KC759139), *C. longibursatus* (Cy-CID-lon; GenBank Accession number: KC759133), *C. goldi* (Cy-CID-gol; GenBank Accession number**:** KC759134). Identical residues are shaded in black and similar amino acids are denoted by a grey background. Multiple sequence alignment was constructed using MUSCLE EBI (Edgar, 2004) and Boxshade V3.2 used for alignment annotation.

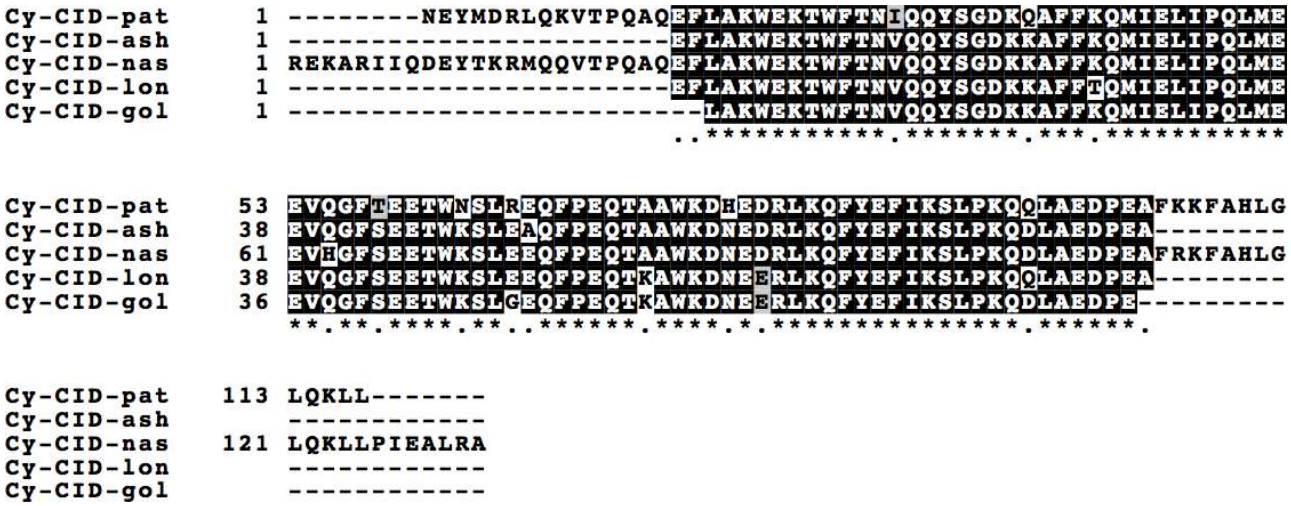
Fig. 2. Specificity of the five recombinant Cy-CID proteins. A) Coomassie stained SDS-PAGE gel depicting molecular marker (kDa) on the left and recombinant versions of Cy-CID-ash, Cy-CID-gol, Cy-CID-lon, Cy-CID-pat and Cy-CID-nas in lanes 1-5 respectively. **B - F)** Immunoblots of each recombinant protein (Cy-CID-ash, -gol, -lon, -nas and -pat) probed for IgG(T) reactivity using sera from helminth free (HF) horses, experimental cyathostomin-infected (CI) horses and horses infected experimentally with *Parascaris* spp. (Ps), *Strongylus edentatus* (Se), *Strongyloides westeri* (Sw) or *Strongylus vulgaris* (Sv). M (kDa) = molecular weight marker.

Fig. 3. Time course of IgG(T) responses in experimentally infected horses to the five Cy-CID antigens over time. Protein-specific IgG(T) responses (Cy-CID-ash, Cy-CID-gol, Cy-CID-lon, Cy-CID-pat and Cy-CID-nas) were measured over an experimental trickle infection (Murphy and Love, 1997). Six British native-breed equids (6-12 months at initial infection) were reared as helminth-naïve prior to the start of the trial. Three foals

759 (Ponies 101, 104 and 105; represented by markers ●, ■ & ▲) were infected with a total of
760 3.9 million cyathostomin third stage larvae (L3), administered as a trickle infection of
761 150,000 L3 by nasogastric tube, three times a week. Foals 102, 103 and 106 (represented
762 by ○, □ & ∆) were maintained as uninfected controls. Results are expressed as the
763 percentage positivity OD of the CI sample mean for each plate.

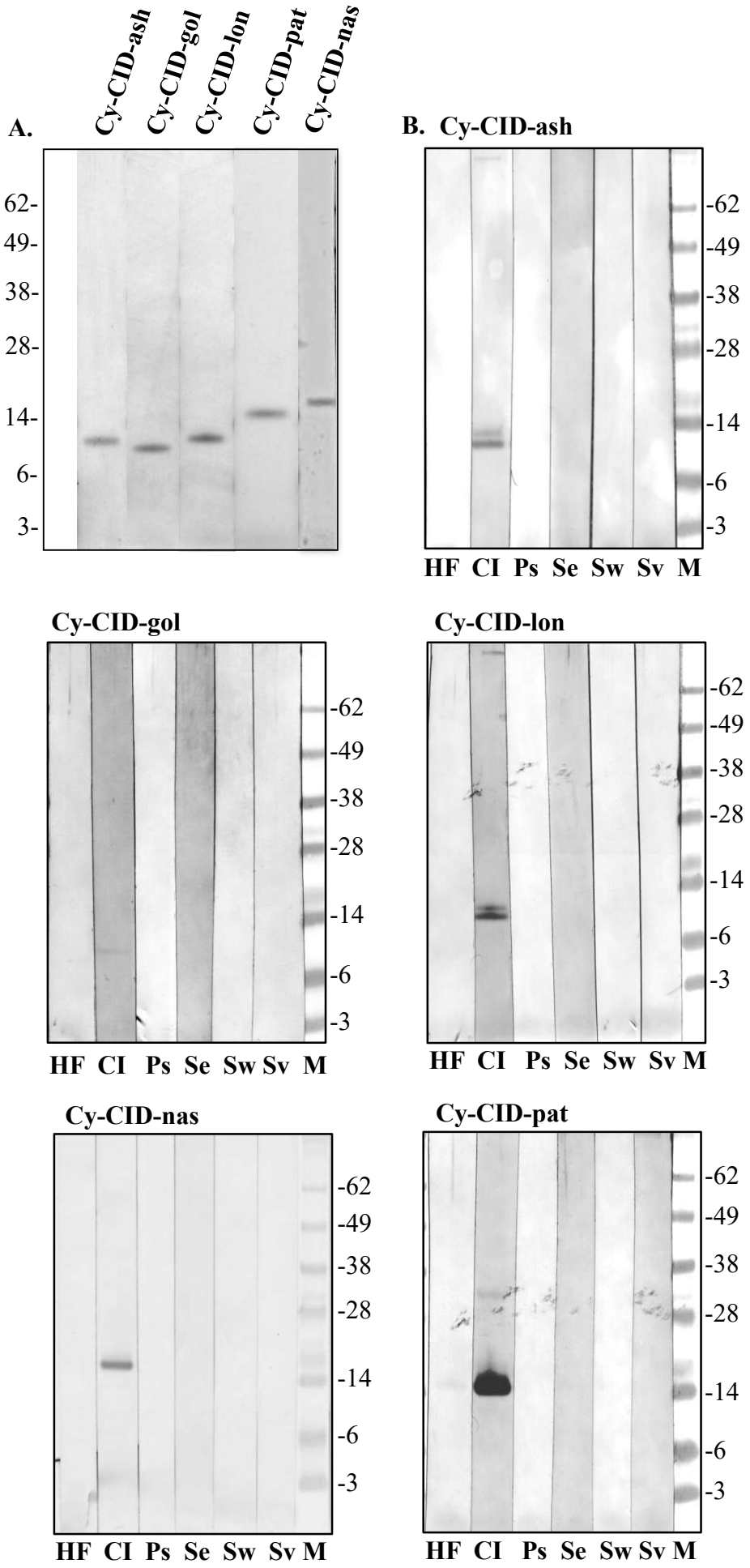
Figure

Fig.1


Cy-CID-pat 1 --------NEYMDRLQKVTPQAQEFLAKWEKTWFTNIQQYSGDKQAFFKQMIELIPQLME
Cy-CID-ash 1 ------------------------EFLAKWEKTWFTNVQQYSGDKKAFFKQMIELIPQLME
Cy-CID-nas 1 REKARIIQDEYTKRMQQVTPQAQEFLAKWEKTWFTNVQQYSGDKKAFFKQMIELIPQLME
Cy-CID-lon 1 ------------------------EFLAKWEKTWFTNVQQYSGDKKAFFTQMIELIPQLME
Cy-CID-gol 1 --------------------------LAKWEKTWFTNVQQYSGDKKAFFKQMIELIPQLME
..***********.*******.***.***********
Cy-CID-pat 53 EVQGFTEETWNSLREQFPEQTAAWKDHEDRLKQFYEFIKSLPKQQLAEDPEAFKKFAHLG
Cy-CID-ash 38 EVQGFSEETWKSLEAQFPEQTAAWKDNEDRLKQFYEFIKSLPKQDLAEDPEA--------
Cy-CID-nas 61 EVHGFSEETWKSLEEQFPEQTAAWKDNEDRLKQFYEFIKSLPKQDLAEDPEAFRKFAHLG
Cy-CID-lon 38 EVQGFSEETWKSLEEQFPEQTKAWKDNEERLKQFYEFIKSLPKQQLAEDPEA--------
Cy-CID-gol 36 EVQGFSEETWKSLGEQFPEQTKAWKDNEERLKQFYEFIKSLPKQDLAEDPE---------
..****.**..******.****.*.***************.******.
Cy-CID-pat 113 LQKLL-------
Cy-CID-ash ------------
Cy-CID-nas 121 LQKLLPIEALRA
Cy-CID-lon ------------
Cy-CID-gol ------------

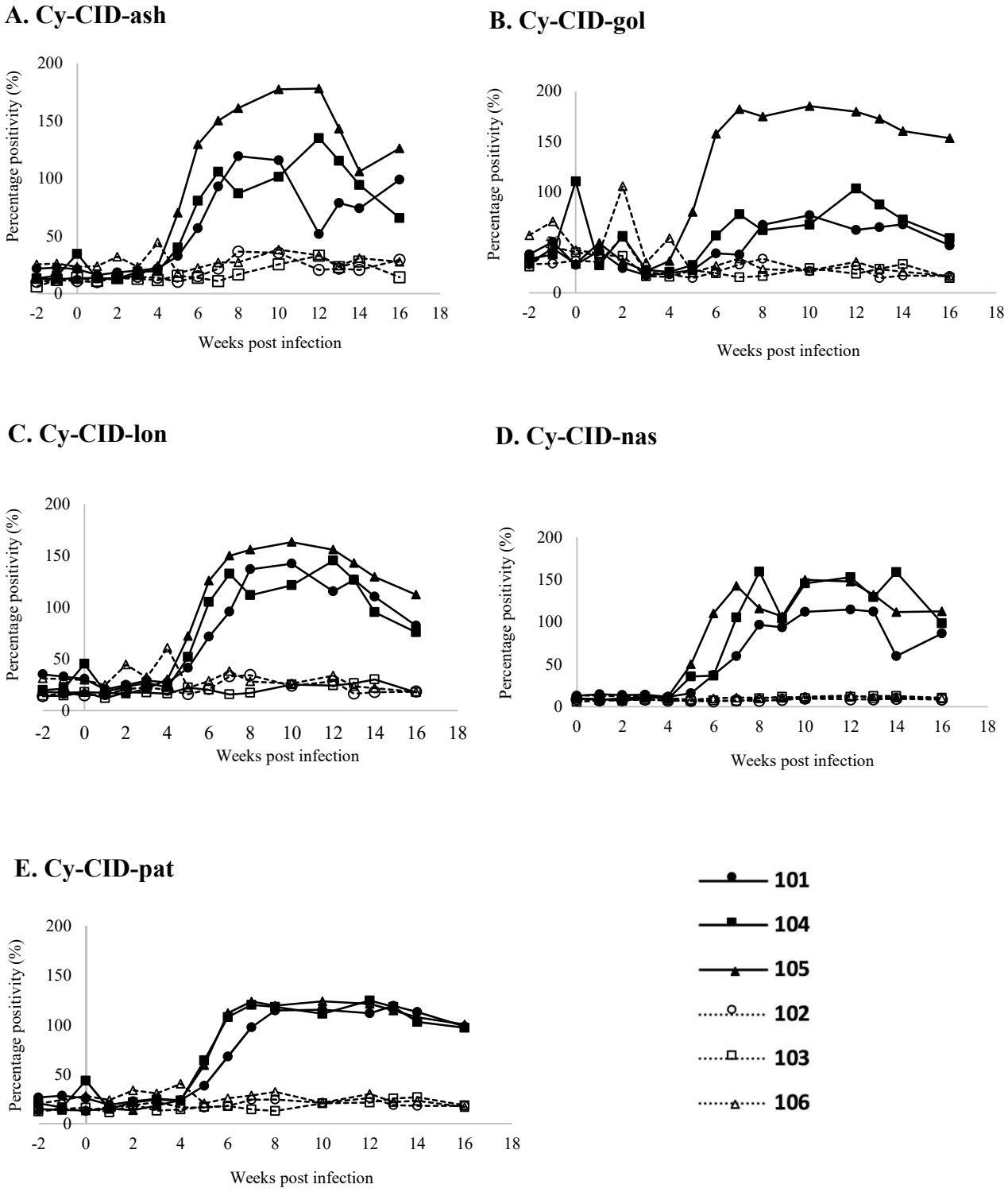
Figure

Fig.2


A.
Cy-CID-ash
Cy-CID-gol
Cy-CID-lon
Cy-CID-pat
Cy-CID-nas
62-
49-
38-
28-
14-
6-
3-
B. Cy-CID-ash
-62
-49
-38
-28
-14
-6
-3
HF CI Ps Se Sw Sv M
Cy-CID-gol
Cy-CID-lon
Cy-CID-nas
Cy-CID-pat

Figure

Fig.3


A. Cy-CID-ash
B. Cy-CID-gol
C. Cy-CID-lon
D. Cy-CID-nas
E. Cy-CID-pat
Percentage positivity (%)
Weeks post infection
101
104
105
102
103
106