CLINICAL AND VACCINE IMMUNOLOGY, Sept. 2011, p. 1462–1466 Vol. 18, No. 9
1556-6811/11/$12.00 doi:10.1128/CVI.05189-11


# Isolation of Potentially Useful Antigens from Cyathostomin Third-Stage Larvae by Using a Fast Protein Liquid Chromatography One-Step Method[▽]

A. Paz-Silva,* R. Francisco, I. Rodríguez, I. Francisco, C. F. Cazapal-Monteiro, M. S. Arias, J. L. Suárez, and R. Sánchez-Andrade

*Equine Diseases Study Group (Epidemiology, Parasitology and Zoonoses), Animal Pathology Department, Veterinary Faculty, Santiago de Compostela University, 27002-Lugo, Spain*

Received 23 May 2011/Returned for modification 13 June 2011/Accepted 7 July 2011

**Three major protein complexes (51, 29, and 15 kDa, named P1 to P3, respectively) were resolved by gel filtration of the excretory/secretory antigens collected from a mixture of horse cyathostomin third-stage larvae (L3s). The potential application for the detection of infected horses was assessed with an enzyme-linked immunosorbent assay (ELISA) by the comparison of the serological and copromicroscopical results. The value of the area under the receiver operating characteristic (ROC) curve was higher than 0.9 when the three peaks were used. Elevated values (>90%) for the sensitivity, specificity, and the positive-likelihood ratio were also observed for all the antigen complexes. A significant increment in the IgG antibody levels 4 weeks prior to the observation of eggs in the feces of weanlings naturally infected was recorded. Our results indicate that the evaluation of chemotherapy is possible by using immunoenzymatic probes and fast protein liquid chromatography (FPLC)-purified antigens. Data collected in the present investigation indicate that FPLC isolation offers a very helpful one-step method for collecting antigens with diagnostic potential to be employed in immunoenzymatic probes.**

The horse is host to a great number of helminths, of which nematodes of the family Strongylidae, the roundworm *Parascaris equorum* and the cestode *Anoplocephala perfoliata*, are the most important (11). Several investigations demonstrated the high prevalence of strongyles, which are ubiquitous parasites responsible for clinical disease in horses (13, 19). With the decline of infections caused by large strongyles as a result of widespread use of modern anthelmintic compounds, the clinical importance of the small stronglyes has been underscored, and these nematodes have been recognized as an important cause of digestive diseases in horses, including weight loss, hypoalbuminemia, and diarrhea as well as colic, poor growth, anemia, and rough hair coat (17).

Cyathostominae are a subfamily of the strongylid roundworms of horse with a continuous life cycle, and infection occurs when horses consume grass contaminated by infective larval stages (L3), which can undergo a period of inhibited development as early third-stage larvae (L3) in the wall of large intestine (12, 18). These inhibited larvae can constitute up to 90% of the total cyathostomin burden and become very significant in cyathostomin-associated disease because large numbers of larvae can accumulate and reactivate to provoke a syndrome known as larval cyathostominosis (25).

Routinely, detection of infection by cyathostomins is based on fecal egg count analysis, which indicates the presence of adult organisms in the host. Only a few methods using serological probes for the detection of infection by cyathostomins have been developed, based on the use of somatic complexes obtained after the sonication of L3 and L4 cyathostomin larval stages, electrophoresis in SDS-PAGE gels, and posterior elution (1, 3). In this way, several antigens with diagnostic potential for estimating mucosal larval burdens were found, which could be helpful for the control of this parasitosis (2). More recently, the characterization of an immunodiagnostic marker for cyathostomin developing-stage larvae has been reported (14).

The composition of excretory/secretory antigens is less complex than that of somatic products, so these products have been used for the diagnosis of several parasitic diseases (8, 22, 24, 26). The study presented here analyzes the potential use of protein fractions collected after the incubation of a mixture of cyathostomin third-stage larvae and then run under an automated system of gel exclusion chromatography.

## MATERIALS AND METHODS

**Experimental design.** Two experiments were developed in the current work using indigenous Pura Raza Galega (PRG) horses. The first consisted of assessing the suitability of the antigens collected from cyathostomin third-stage larvae for the detection of cyathostomin infection by using horse serum samples. On May 2009, one sample of blood and feces was individually collected from 46 4-month weanlings (G-W) and 53 1-month suckling foals (G-S). These animals were sold 2 weeks after the sampling.

Prior to the sampling, the weanlings were allowed to graze from the time of their foaling to allow the natural infection by cyathostomins, and their sera were employed as positive controls. In contrast, the suckling foals remained stabled without access to herbage to prevent the possibility of parasite infection. The sera from these animals were used as negative controls in the enzyme-linked immunosorbent assays (ELISAs).

In the second experiment, the kinetics of the humoral antibody response against fast protein liquid chromatography (FPLC)-isolated antigens from L3 in

* Corresponding author. Mailing address: Equine Diseases Study Group, Animal Pathology Department, Veterinary Faculty, Santiago de Compostela University, 27002-Lugo, Spain. Phone: 34 982 822 126. Fax: 34 982 252 195. E-mail: adolfo.paz@usc.es.

▽ Published ahead of print on 20 July 2011.

TABLE 1. Gel filtration chromatography protocol

| Step | Vol (ml) | Description |
|---|---|---|
| 1 | 0.00 | Collection of 4-ml fractions during entire run |
| 2 | 0.00 | Lamp (UV detector) turned on |
| 3 | 0.00 | Zero baseline |
| 4 | 0.00 | Isocratic flow |
| 5 | 5.00 | Load/inject 2.5-ml sample |
| 6 | 7.5 | Isocratic flow |
| 7 | 600 | End of protocol |

naturally infected horses was assessed. Between June and October 2009, blood and fecal samples were taken from one herd of 16 3-month weanlings. These equines fed on pastures from the date of their foaling to ensure the infection. All the animals were bled by jugular venous puncture, and the sera were kept at −35°C until used (7).

**Copromicroscopical analysis.** Five grams of each fecal sample was processed (in duplicate) by the quantitative McMaster flotation technique (16), with a specific gravity of 1.2 and a sensitivity of 10 eggs per gram (EPG). The laboratory technician conducting the microscope analysis was blinded to the study design and the selection of the stool specimens.

The genus identification of strongyle eggs was done by culturing fecal samples for 10 to 14 days at 20 to 25°C to allow the development of L3, which were collected by means of the Baermann procedure and then identified according to previously described methods (6, 10).

**Analysis of the IgG humoral response.** By taking into account previous investigations about the usefulness of metabolic (excretory/secretory) antigens for the detection of different parasite infections by means of immunoenzymatic probes (22, 23), we prepared excretory/secretory antigens from cyathostomin third-stage larvae (L3CES).

**Antigen preparation.** To obtain a large quantity of L3 cyathostomins, feces from horses passing strongyle eggs were collected and cultured according to previous investigations (5). All these larval cultures were examined under the microscope as stated previously, and only cyathostomins were detected. Once larvae reached the third stage (9), they were incubated for 24 h at 37°C and 5% $CO_2$ in RPMI medium, using a ratio of approximately 1,000 larvae/1.5 ml of RPMI medium. During a 3-day period, the medium was removed every 6 h and then centrifuged, dialyzed extensively against water, and concentrated.

**Gel filtration chromatography.** The native L3CES were fractionated under nonreducing conditions by size exclusion fast protein liquid chromatography (FPLC) on a Duo-Flow (Bio-Rad, Madrid, Spain) system with a Sepharose S-200 HR 10/30 column (Pharmacia, Madrid, Spain) (15). The applied protocol is reflected in Table 1.

Fractionation of the L3CES was performed by the injection of 2.5 ml of antigen at a protein concentration of 5 mg $ml^{-1}$. The buffer (pH 7.4) was composed of bisodium phosphate (50 mM), monosodium phosphate (50 mM), and sodium chloride (15 mM). All the antigens and the buffers were passed through a 0.22-μm-pore-size filter before the introduction into the FPLC system.

One pool of several standards (67, 43, 25, and 13.7 kDa) (Pharmacia) was also run to allow the estimation of the molecular mass of the purified proteins.

The heat stability for each antigen was assessed by incubation at 65°C, and no differences were reached in respect to nonheated proteins.

**ELISA.** ELISAs were performed on serum samples by using U-bottom microtiter plates (Costar, Barcelona, Spain). The antigen concentration, the sera, and immunoconjugate dilutions were assessed by a checkerboard titration. The plates were coated with 100 μl per well of L3CES at a concentration of 0.5 μg $ml^{-1}$ in a phosphate-buffered saline (PBS) coating buffer (pH 7.4) for 10 to 12 h at 4°C. Serum was added at 1:200. A dilution of horseradish peroxidase-conjugated rabbit anti-equine IgG(T) (Sigma, Madrid, Spain) was added at 1:1,000 and incubated at 37°C for 1 h. Five minutes after s substrate composed of OPD (*ortho*-phenylen-dienamine; Sigma), citrate buffer (pH 4), and $H_2O_2$ was added, the absorbances were read using a Titertek Multiskan spectrophotometer at 492 nm.

A pool of sera from the grazing weanlings (G-W) was used as a positive control in each plate, and another from suckling foals (G-S) was used as a negative control.

**Statistical analysis.** Statistical analysis was first performed using Levene's test of homogeneity, and then analysis of variance (ANOVA) was performed for the analysis of the egg output and the absorbances. Differences were considered significant at a $P$ value of $<0.05$.

The copromiscroscopical flotation technique was utilized as the gold standard. The percentages of the predictive values and the likelihood ratios were estimated according to Thrusfield (27), by considering the respective sensitivities and specificities obtained with the receiver operating characteristic (ROC) curves for each cutoff value.

The existence of correlations among the different parameters was assessed by using the Pearson test. The kappa statistic was used to quantify agreement between the flotation test and the ELISA.

All tests were done using SPSS for Windows (version 15.0).

**Cutoff estimation.** A ROC analysis, or ROC curve, was performed to determine a cutoff value for each isolated antigen by using the sera from naturally infected and uninfected foals (experiment 1) (see Fig. 2). Levels of sensitivity were plotted against 1 minus specificity at each cutoff point on a ROC curve. Threshold values used were those that gave the highest values of sensitivity (S), specificity (SP), positive-likelihood ratio (PLR), and the area under the curve (AUC), while lower values for the negative-likelihood ratio (NLR) were expected (27).

ROC analysis is a useful tool for evaluating the performance of diagnostic tests and more generally for evaluating the accuracy of a statistical model (e.g., logistic regression or linear discriminant analysis) that classifies subjects into one of two categories, diseased or nondiseased. Its function as a simple graphical tool for displaying the accuracy of a medical diagnostic test is one of the most well-known applications of ROC curve analysis (28).

**Analysis of the cross-immunity.** To determine the possible development of cross-immunity, sera from the horses in experiment 1 were challenged with excretory/secretory antigens of second-stage *Parascaris equorum* larvae.

## RESULTS

**Copromicroscopical findings.** Only eggs belonging to intestinal Nematoda were observed in the feces, whereas no coccidian oocysts or eggs from Trematoda, Cestoda, or lungworm larvae were obtained. By using the flotation copromicroscopical technique, strongyle eggs were recorded in the feces of G-W only, and the examinations of the feces of G-S were negative.

The coprocultures showed the presence of larvae belonging to *Cyathostomum* and *Poteriostomum* (6, 10).

**Experiment 1: analysis of the FPLC-isolated antigens.** Three major protein complexes were resolved by gel filtration of the L3CES (Fig. 1). The fractions corresponding to each complex were collected separately (peak 1 [P1] to P3), and their molecular masses were estimated at 51, 29, and 15 kDa, respectively.

Table 2 summarizes some data obtained from the antigen

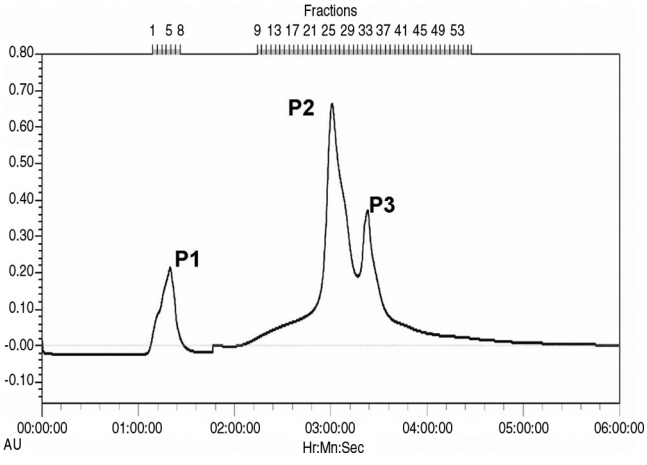



FIG. 1. Chromatogram of the FPLC protein complexes isolated from a mixture of cyathostomin third-stage larvae. AU, arbitrary units.

TABLE 2. Analysis of the liquid chromatography (FPLC) protein complexes isolated from a mixture of cyathostomin third-stage larvae[a]

| Antigen peak and size (kDa) | Cutoff value | AUC | *P* | S (%) | SP (%) | PLR | NLR |
|---|---|---|---|---|---|---|---|
| P1 (51) | 0.4850 | 0.982 | 0.001 | 92 | 98 | 46 | 0.08 |
| P2 (29) | 0.4965 | 0.985 | 0.001 | 92 | 94 | 15 | 0.08 |
| P3 (15) | 0.4815 | 0.982 | 0.001 | 92 | 91 | 10 | 0.09 |

[a] The cutoff point for each antigen was established on the basis of the highest values achieved for the AUC (maximum 1), S (sensitivity, 0 to 100), SP (specificity, 0 to 100), PLR (positive-likelihood ratio; elevated numbers are expected), and NLR (negative-likelihood ratio; low values are expected).

analysis by establishing a comparison between the ELISA probe and the copromicroscopical one.

The estimation of cutoff values of 0.485, 0.4965, and 0.481 for the P1, P2, and P3 antigens, respectively, yielded a high diagnostic value (area under the ROC curve, AUC, of >0.9; $P < 0.05$) for the P1, P2, and P3 peaks.

Elevated values (>90%) for the sensitivity, specificity, and the positive-likelihood ratio were obtained for all the antigen complexes used (Fig. 2).

The concordance in the diagnostics of cyathostomin infection by using the ELISA and the copromicroscopy was established by the estimation of the kappa statistic, and a value of 0.7 ($P = 0.001$) was achieved for P1, P2, and P3.

**Analysis of cross-immunity.** Sera from 6 out of 46 weanlings (13%) did react to the *P. equorum* L2 excretory/secretory antigens, as did sera from 5 out of 53 suckling foals (9%).

**Experiment 2: IgG kinetics against FPLC-purified antigens.** As shown in Fig. 3, strongyle eggs were observed at the 12th week of the study. The IgG(T) antibodies against P1 and P2 exhibited similar patterns (Fig. 4), increasing significantly from the 8th week, when values above the cutoff point were recorded. The antibody kinetics increased again at the 12th week (P2) and 14th week (P1). Higher absorbances against P1 were achieved.

Figure 4 shows that antibody values exceeding the cutoff point against P3 were obtained from the 16th week.

Positive correlation between the egg counts and the antibody values against the P1 ($\rho = 0.708$, $P = 0.001$), P2 ($\rho = 0.720$, $P = 0.001$), and P3 ($\rho = 0.517$, $P = 0.001$) were recorded.

## DISCUSSION

Grazing equines ingest cyathostomin infective third-stage larvae (L3) with the grass, and after exsheathing in the small intestine, the larvae penetrate the mucosa or the submucosa and molt to fourth-stage larvae (L4) in the intestinal wall within a fibroblastic cyst (4).

Knowledge of the host-parasite relationships is essential to the consideration of efficient strategies for their control based on the appropriate diagnosis. By using an FPLC one-step method, three protein complexes (51, 29, and 15 kDa) were identified in the excretory/secretory antigens collected from a mixture of cyathostomin third-stage larvae. After their use in a serological probe (ELISA), all were recognized by IgG(T) in serum from naturally infected foals. The presence of two antigen complexes of 25 and 20 kDa which were bound by IgG(T)

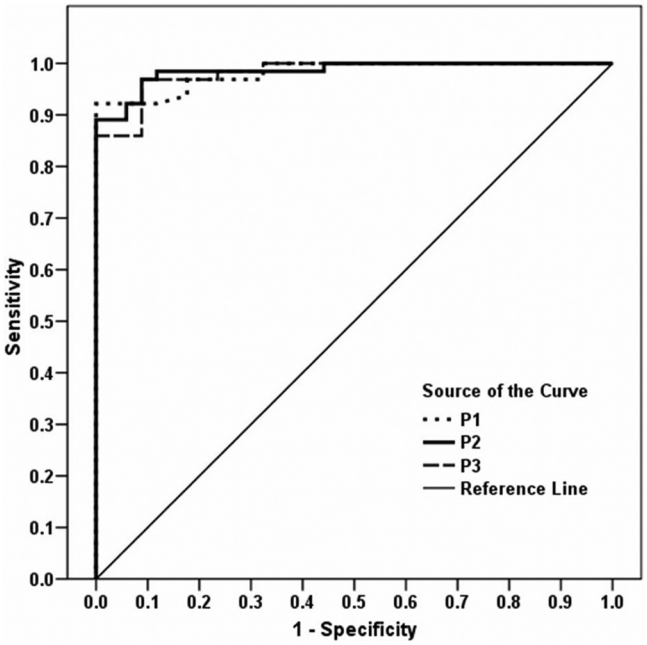



FIG. 2. ROC analysis of the results achieved by using the FPLC protein complexes isolated from a mixture of cyathostomin third-stage larvae and sera from infected and uninfected horses.

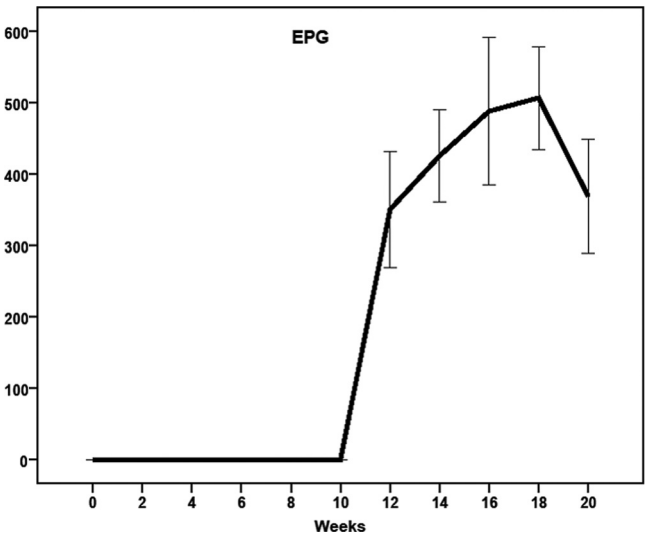
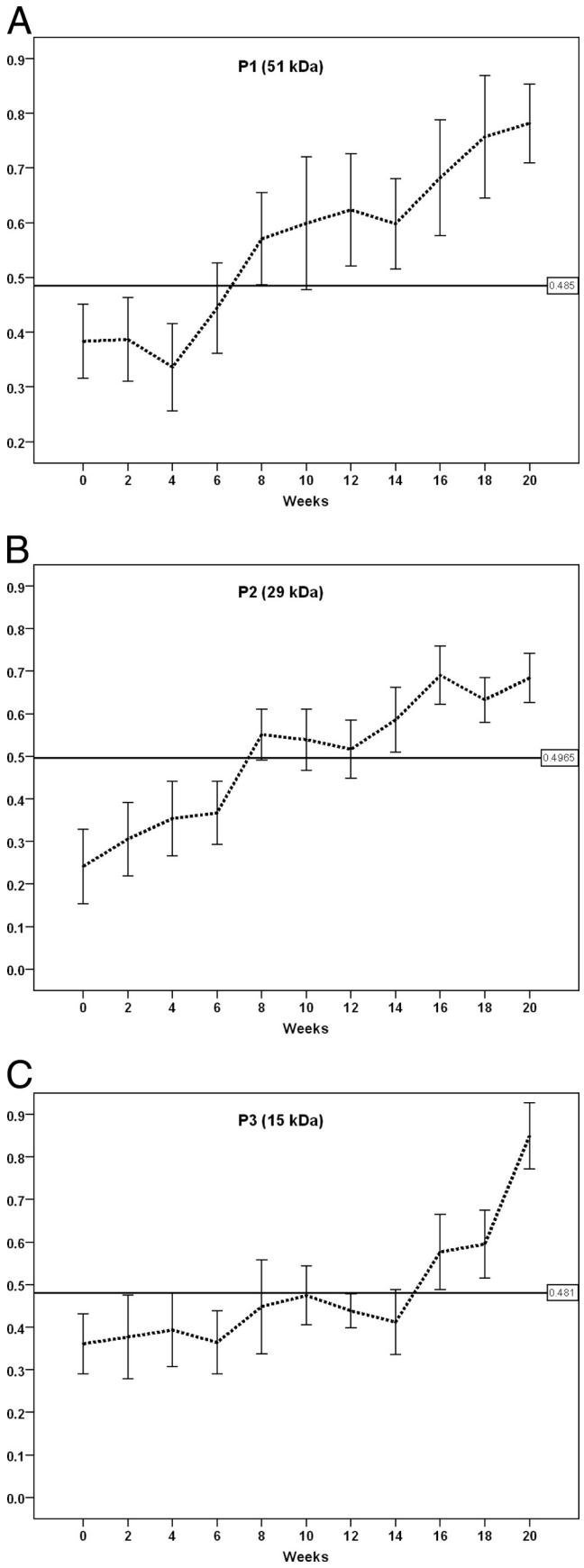



FIG. 3. Kinetics of cyathostomin egg output in naturally infected weanlings. Values along the *y* axis represent numbers of cythostomin eggs per gram of feces (EPG). Data represent the mean values plus 2 standard deviations.




in the somatic products from larval cyathostomins (L3 and L4) has been previously reported (2).

The diagnostic value of the P1, P2, and P3 fractions for the detection of cyathostomin infection was demonstrated by an elevated estimation for the area under the curve and for the kappa statistic also. An elevated correlation between the egg counts and the levels of antibodies against the 15-kDa complex was also recorded. There have been only a few studies, but a significant correlation between anti-25 kDa IgG(T) responses and the burdens of L3 in experimentally infected horses has been reported (3).

A detailed knowledge of the antibody response that the host develops against parasites would foster an understanding of the development of infection and thus its detection. One of the main features in the relationship between the host and the parasite concerns the immune response developed by hosts against the antigens released by the parasites during their endogenous cycle.

By considering that diagnostics of cyathostomin infection is routinely detected by the copromicroscopical flotation probe, a second experiment was developed to gain information on the usefulness of the FPLC-isolated antigens for the detection of infection. Values above the cutoff points against both the 51-kDa and 29-kDa fractions at the 8th weeks were obtained, i.e., 4 weeks prior to the observation of the egg output. Likewise, positive antibody values from the 16th week were observed by using a 15-kDa protein.

By using sera from experimentally infected ponies, it has been demonstrated that IgG(T) was raised against two protein complexes (25 and 20 kDa) in somatic antigens from larval cyathostomins by 5 to 7 weeks after the infection (2, 3).

The difference in the source of the antigens employed in the current investigation seems to explain the discrepancy between the proteins identified in previous and current investigations. As affirmed previously, the somatic antigens used by Dowdall et al. (2, 3) were collected by the sonication of L3 and L4 cyathostomin larval stages and posterior elution from SDS-PAGE gels. The use of this kind of antigen reveals the possible interaction between the immune system and antigens localized at the parasite surface or in its internal structures.

In contrast, metabolic products (excretory/secretory antigens) were collected by incubating L3 cyathostomins in RPMI liquid culture medium and used in the present work. Their application makes it possible to detect the humoral immune response developed against the products released for the larvae. The infective cyathostomin larvae develop inside a fibrous capsule in the mucosa or submucosa, and the excystment from this location is accompanied by the release of the excretory and secretory products that have accumulated over a period of weeks up to more than 2 years (21). The main advantage provided by using an FPLC system for antigen purification

FIG. 4. Kinetics of IgG(T) antibodies against antigenic peaks P1 (A), P2 (B), and P3 (C) isolated from L3 cyathostomin excretory/secretory antigens by means of an FPLC system. Values along the *y* axis represent optical density at 492 nm. Data represent the mean values plus 2 standard deviations.

relies in the possibility of automating this procedure, improving the collection of purified antigens.

Equine cyathostomin infections are the main targets of parasite control programs based mostly on the administration of anthelmintics (20). By means of the copromicroscopical flotation test, the period of the appearance of eggs in feces has been established as more than 12 weeks after infection; thus, the convenience of probes providing an earlier diagnostic seems essential.

The ELISA is very valuable for the detection of many parasitic infections and provides the possibility for the simultaneous processing of many samples, reducing the cost for the analyses; however, appropriate antigens are required. In the current research, three protein complexes have been purified by means of liquid chromatography (FPLC). The early detection of infected horses has been achieved by using two antigens of 29 and 15 kDa and the immunoenzymatic procedure.

Data collected in the present investigation indicate that FPLC purification offers a very helpful one-step method for collecting antigens with a diagnostic potential to be employed in immunoenzymatic probes. Further studies are in progress to get more information on the composition of these antigens and on the host-parasite relationships to improve their usefulness.

## ACKNOWLEDGMENTS

We are in debt to B. Valcárcel for her valuable assistance in the preparation of the manuscript.

This work was partly sponsored by the Research Project XUGA 10MDS261023PR (Xunta de Galicia, Spain). This sponsor was not involved in the experimental design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

None of the authors has established any financial or personal relationships with other people or organizations that could inappropriately influence (bias) the current work.

This project underwent ethical review and was given approval by the Ethics Committee of the University of Santiago de Compostela, Spain, and the care and use of the horses complied with Spanish animal welfare laws, guidelines, and policies.

## REFERENCES

1. **Dowdall, S. M. J., et al.** 2002. Antigen-specific IgG(T) responses in natural and experimental cyathostominae infection in horses. Vet. Parasitol. **106:**225–242.
2. **Dowdall, S. M. J., C. J. Proudman, T. R. Klei, T. Mair, and J. B. Matthews.** 2004. Characterisation of IgG (T) serum antibody responses to two larval antigen complexes in horses naturally—or experimentally—infected with cyathostomins. Int. J. Parasitol. **34:**101–108.
3. **Dowdall, S. M. J., C. J. Proudman, S. Love, T. R. Klei, and J. B. Matthews.** 2003. Purification and analyses of the specificity of two putative diagnostic antigens for larval cyathostomin infection in horse. Res. Vet. Sci. **75:**223–229.
4. **Eysker, M., J. Jansen, and M. H. Mirck.** 1984. Inhibited development of cyathostominae in the horse in the early third stage. Res. Vet. Sci. **37:**355–356.
5. **Francisco, I., et al.** 2009. Silvopastoralism and autochthonous equine livestock: analysis of the infection by endoparasites. Vet. Parasitol. **164:**357–362.
6. **Francisco, I., et al.** 8 April 2009. Intrinsic factors influencing the infection by helminth parasites in horses under an oceanic climate area (NW Spain). J. Parasitol. Res. doi:10.1155/2009/616173.
7. **Francisco Vázquez, I.** 2005. Epidemiology and control of the main parasitism affecting horses in NW Spain. Ph.D. thesis. Veterinary Faculty of the Santiago de Compostela University, Lugo, Spain.
8. **Kjaer, L. N., M. M. Lungholt, M. K. Nielsen, S. N. Olsen, and C. Maddox-Hyttel.** 2007. Interpretation of serum antibody response to *Anoplocephala perfoliata* in relation to parasite burden and faecal egg count. Equine Vet. J. **39:**529–533.
9. **Lichtenfels, J. R., V. A. Kharchenko, and G. M. Dvojnos.** 2008. Illustrated identification keys to strongylid parasites (Strongylidae: Nematoda) of horses, zebras and asses (Equidae). Vet. Parasitol. **156:**4–161.
10. **Lichtenfels, J. R., V. A. Kharchenko, R. C. Krecek, and L. M. Gibbons.** 1998. An annotated checklist by genus and species of 93 species level names for 51 recognized species of small strongyles (Nematoda: Strongyloidea: Cyathostominea) of horses, asses and zebras of the world. Vet. Parasitol. **79:**65–79.
11. **Lind, E. O., et al.** 2007. Parasite control practices on Swedish horse farms. Acta Vet. Scand. **49:**25.
12. **Love, S., D. Murphy, and D. Mellor.** 1999. Pathogenicity of cyathostome infection. Vet. Parasitol. **85:**113–122.
13. **Mair, T., T. Divers, and N. Ducharme.** 2002. Manual of equine gastroenterology, 1st ed. W. B. Saunders, London, United Kingdom.
14. **McWilliam, H. E., A. J. Nisbet, S. M. Dowdall, J. E. Hodgkinson, and J. B. Matthews.** 2010. Identification and characterisation of an immunodiagnostic marker for cyathostomin developing stage larvae. Int. J. Parasitol. **40:**265–275.
15. **Mezo, M., M. González-Warleta, and F. M. Ubeira.** 2003. Optimized serodiagnosis of sheep fascioliasis by Fast-D protein liquid chromatography fractionation of *Fasciola hepatica* excretory-secretory antigens. J. Parasitol. **89:**843–849.
16. **Ministry of Agriculture, Fisheries and Food of Great Britain.** 1986. Manual of veterinary parasitological laboratory techniques. Her Majesty's Stationary Office, London, United Kingdom.
17. **Murphy, D., and S. Love.** 1997. The pathogenic effects of experimental cyathostome infections in ponies. Vet. Parasitol. **70:**99–110.
18. **Osterman, L. E.** 2005. Prevalence and control of Strongyle nematode infections of horses in Sweden. Ph.D. thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden.
19. **Osterman, L. E., M. Eysker, O. Nilsson, A. Uggla, and J. Höglund.** 2003. Expulsion of small strongyle nematodes (cyathostomin spp) following deworming of horses on a stud farm in Sweden. Vet. Parasitol. **115:**289–299.
20. **Ramsey, Y. H., et al.** 2004. Seasonal development of Cyathostominae larvae on pasture in a northern temperate region of the United Kingdom. Vet. Parasitol. **119:**307–318.
21. **Reinemeyer, C. R., and M. K. Nielsen.** 2009. Parasitism and colic. Vet. Clin. North Am. Equine Pract. **25:**233–245.
22. **Romasanta, A., et al.** 2003. Diagnosis of parasitic zoonoses by immunoenzymatic assays—analysis of cross-reactivity among the excretory/secretory antigens of *Fasciola hepatica*, *Toxocara canis*, and *Ascaris suum.* Immunol. Invest. **32:**131–142.
23. **Sánchez-Andrade, R., et al.** 2005. Comparison of *Oestrus ovis* metabolic and somatic antigens for the immunodiagnosis of the zoonotic myasis oestrosis by immunoenzymatic probes. Immunol. Invest. **34:**91–99.
24. **Sánchez-Andrade, R., et al.** 2010. A novel second instar *Gasterophilus* excretory/secretory antigen-based ELISA for the diagnosis of gasterophilosis in grazing horses. Vet. Parasitol. **171:**314–320.
25. **Smets, K., D. Shaw, J. Deprez, and J. Vercruysse.** 1999. Diagnosis of larval cyathostominosis in horses in Belgium. Vet. Rec. **144:**665–668.
26. **Suárez, J. L., et al.** 2005. Analysis of the humoral immune response to *Oestrus ovis* in ovine. Vet. Parasitol. **134:**153–158.
27. **Thrusfield, M.** 2005. Veterinary epidemiology, 3rd ed. Blackwell Science, Oxford, England.
28. **Zou, K. H., A. J. O'Malley, and L. Mauri.** 2007. Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. Circulation **115:**654–657.