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Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Anoplocephala perfoliata* in horse sera

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Abstract

A scolex antigen of the horse tapeworm *Anoplocephala perfoliata* containing at least 14 different proteins was employed in an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *A. perfoliata* in equine sera. The assay was applied to sera from 426 slaughtered horses with different numbers of worms and with varying degrees of intestinal lesions. As measured by the ELISA, there was a very strong effect on the antibody levels both from the number of tapeworms present and from the intestinal lesion score. However, considerable individual variation was observed between horses with similar worm counts. The ELISA values of horses that were either negative for strongyles and tapeworms or positive only for strongyles were similar, whereas both of these groups differed significantly from animals that harboured only *A. perfoliata*. Thus it seemed that cross-reactivity with concurrent nematode infections did not occur. Furthermore, a significant seasonal pattern in antibody levels was observed which reflected the establishment of newly acquired infections. No observations were made of confounding effects owing to age and previous anthelmintic treatment of the horses. It is concluded that the ELISA presented here has a potential for monitoring *A. perfoliata* infection on a herd level and can be used as a complementary diagnostic tool in epidemiological studies. The present study indicates that transmission of *A. perfoliata* in Central Sweden occurred during a short period in spring and more consistently in late autumn.

Keywords: *Anoplocephala perfoliata*; Horse-Cestoda; Serodiagnosis-Cestoda

1. Introduction

The horse tapeworm *Anoplocephala perfoliata*, previously regarded as relatively harmless, has been increasingly suspected as a cause of colic and other digestive disorders

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(Proudman and Edwards, 1993). Its life-cycle is indirect and involves oribatid mites as intermediate hosts (for a review, see French and Chapman, 1992). This implies that horses are most likely to acquire the infection orally at pasture during the grazing season. The adult worm measures up to 8 cm in length and 1.8 cm in width, and is characteristically found in the small and large intestines, especially around the ileo-caecal valve. Attachment of the parasite to the gut wall may cause pathological lesions comprising thickening, ulceration and eosinophilic infiltration of the mucosa (Pearson et al., 1993).

A. perfoliata is prevalent in most parts of the world, including the northern hemisphere (Lyons et al., 1987; Owen et al., 1988). In a recent Swedish survey of 470 culled horses, 65% were found to be infected with the parasite (Nilsson et al., submitted for publication). At present, *A. perfoliata* infection is diagnosed by means of faecal examination. However, coprological demonstration of eggs by flotation and sedimentation techniques has shown inadequate sensitivity (Beroza et al., 1987; Proudman and Edwards, 1992; Nilsson et al., 1995). This poses a problem, as the use of anthelmintics should ideally be based on a correct diagnosis. Specific treatment strategies would be of economic and clinical benefit and would prevent the development of drug resistance. Thus, there is a great need for a more sensitive diagnostic test of *A. perfoliata* infection in the horse. One such alternative would be serology.

The aim of the present study was to investigate the potential of an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of *A. perfoliata* in the horse using an assay antigen derived from the scolex of the adult worm. The study is based on a comparison of the antibody levels in sera from slaughtered horses with different levels of infection and severity of intestinal lesions.

2. Materials and methods

2.1. Animals and parasitological examination

A total of 426 horses, slaughtered at the Linköping abattoir in central Sweden between October 1992 and October 1993, were included in the study. As described in more detail by Nilsson et al. (submitted for publication), 50 cm of the terminal ileum, caecum and 50 cm of the proximal colon were checked for the number of tapeworms, and pathological lesions in the intestinal mucosa were arbitrarily scored on a four-grade scale (0, no lesions; 1, slight thickening and hyperaemia of the caecal mucosa; 2, moderate thickening and hyperaemia of the caecal mucosa with scattered necrotic ulcers; 3, severe lesions with multiple confluent necrotic ulcers mainly located at the ileo-caecal orifice). Strongyle faecal egg counts were performed according to a modified McMaster technique (Nilsson et al., submitted for publication). Information on the animals, their age, sex, clinical history and anthelminthic treatment, was collected from farm records. Blood was collected from the stick wound and was allowed to clot overnight at room temperature. Serum was prepared after centrifugation at $800 \times g$ for 5 min and stored at -20°C until required.

2.2. Preparation of A. perfoliata antigen

Adult *A. perfoliata* were collected from the caecum of naturally infected horses. The parasites were cleaned by rinsing several times in 0.1 M phosphate buffered saline, pH 7.4

(PBS), before they were stored at −20°C. After thawing, the scoleces were separated from the proglottides, collected in a small amount of PBS in a glass tissue grinder and homogenized on ice. The homogenized material was then sonicated at 30 s cycles in an ultrasonic disintegrator (MSE, Leicestershire, UK). The supernatant was collected after centrifugation at 800 × *g* for 30 min and used as assay antigen. The protein content was determined with the BCA protein assay reagent (Pierce, Rockford, USA), in accordance with the manufacturer's instructions, using bovine serum albumin (BSA) as standard.

2.3. Characterization of the antigen

The protein banding patterns of the scolex antigen in six preparations/batches were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) in a homogeneous slab gel containing 12% polyacrylamide (Mini Protean II System; Bio-Rad, Hercules, USA). Before the samples were applied to the gel, they were boiled for 5 min in a Tris-HCl buffer, pH 6.8, containing 10% (w/v) SDS and 50 mM dithiothreitol (DTT) (Bio-Rad). Twenty-five mg protein was added per well (5 mm wide), and polypeptide bands were visualised with 0.1% Coomassie blue R-250 solution (CBS) (Merck, Darmstadt, Germany). A broad molecular weight range standard (Bio-Rad) was used as reference.

Enzyme-linked immunoelectrotransfer blot (Western blot; WB) was performed to detect antibodies in horse sera that reacted with the tapeworm antigen. The worm proteins were separated by SDS–PAGE as described above; 250 mg was applied to a single well (73 mm wide). The gel was transferred to a 0.45 micron nitrocellulose (NC) membrane (Bio-Rad) for 80 min at 70 V and 150 mA and then stained with CBS to check that the transfer of proteins was successful. The NC membrane was blocked with 5% skimmed milk powder (Semper, Stockholm, Sweden) in Tris-NaCl buffer, pH 7.5 (TBS), for 30 min and then washed with TBS containing 0.5% Tween-20 (TBS-T) (Kebo, Spånga, Sweden), before it was incubated with sera from six horses with different levels of *A. perfoliata* infection and intestinal lesions as observed at necroscopy. The sera were diluted 1:50 and applied for 2 h at 20°C using a Multi-Screen apparatus (Bio-Rad). The incubated NC membrane was washed twice in TBS-T and incubated for 1 h with horseradish peroxidase (HRP) conjugated affinity purified goat anti-horse IgG antibodies (Cappel, Durham, UK) diluted 1:500 in TBS-T buffer containing 2% skimmed milk powder (Semper). The substrate used was 0.05% hydrogen peroxide and 1% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtitre plates (Greiner F, Alpheen aan de Rijn, Netherlands) were coated with 100 ml antigen diluted to a protein concentration of 5 mg ml^{-1} in 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. After removal of the antigen solution, each well was blocked with 100 ml 1% skimmed milk powder (Semper) in PBS for 1 h at 20°C. The plates were washed three times in 1 mM PBS with 0.5% Tween 20 (PBS-T). The serum samples were diluted 1:100 in PBS-T and added to the wells in duplicates. To account for inter-assay variation, pooled positive and negative control sera were added to fixed

positions on all plates, whereas the sera to be tested were added randomly. After incubation for 1 h at 37°C, the plates were washed as described above and 100 ml HRP conjugated affinity purified goat anti-horse IgG antibodies (Cappel) diluted 1:8000 in PBS-T was added to each well and incubated at 37°C for 1 h. Following three washes in PBS-T, 100 ml 0.1% 5-amino-salicylic acid and 0.05% hydrogen peroxide, pH 5.9 (Merck), were added to each well. After 120 min, the absorbance (OD-value) was recorded at 490 nm in a Titertek Multiscan photometer (Flow Laboratories, Irvine, Scotland, UK). Optimum assay conditions were determined prior to final analysis by routine checkerboard titrations using serial dilutions of the antigen and conjugate as well as the pooled positive and negative control sera.

2.5. Statistical evaluation

The mean OD values of the test sera were converted to indices by dividing with the mean OD value obtained for the negative control sera on the same plate. Statistical analyses were performed using Systat (Systat Inc., Evanstone, USA) for the Macintosh (Apple Computer Inc., Cupertino, USA). Tapeworm numbers were log-transformed according to the formula $Y=\log(x+1)$ owing to a negative binomial distribution including zero counts (Elliot, 1979). The relationship between the number of tapeworms, lesion score and ELISA ratio was determined by analysis of covariance (ANCOVA), while analysis of variance (ANOVA) was performed to analyze the ELISA ratio with reference to the time of year, presence of intestinal strongyle parasites, mucosal lesions and prior anthelminthic treatment. Linear regression analysis was performed to examine the relationship between the age of the horses and the ELISA ratio.

3. Results

3.1. Characterization of the tapeworm antigen

The SDS–PAGE analysis showed that the protein composition of the *A. perfoliata* scolex antigen was very complex, and at least 14 distinct protein bands were identified (Fig. 1(A)). The most prominent bands were observed within the molecular weight ranges 31–45 kD and 66–200 kD. The bands in the lower range seemed to dominate especially. No differences were noted between batches.

The reaction in WB after incubation with sera from *A. perfoliata* negative and positive horses and with different degrees of tapeworm-related intestinal lesions revealed a heterogeneous banding pattern (Fig. 1(B)). No bands were observed in lanes incubated with sera from tapeworm-negative horses with no intestinal lesions (lanes 1 and 2), while three antigens within the molecular weight range 50–70 kD were noted in lanes with sera from horses without demonstrable *A. perfoliata* worms but with slight intestinal lesions (lanes 3 and 4). The strongest reaction of the latter kind of sera was to an antigen with an approximate molecular weight of 70 kD. Although a similar banding pattern was observed in lanes incubated with sera from horses with demonstrable *A. perfoliata* worms with different degrees of mucosal damage (lanes 5–10), additional antigenic components were frequently

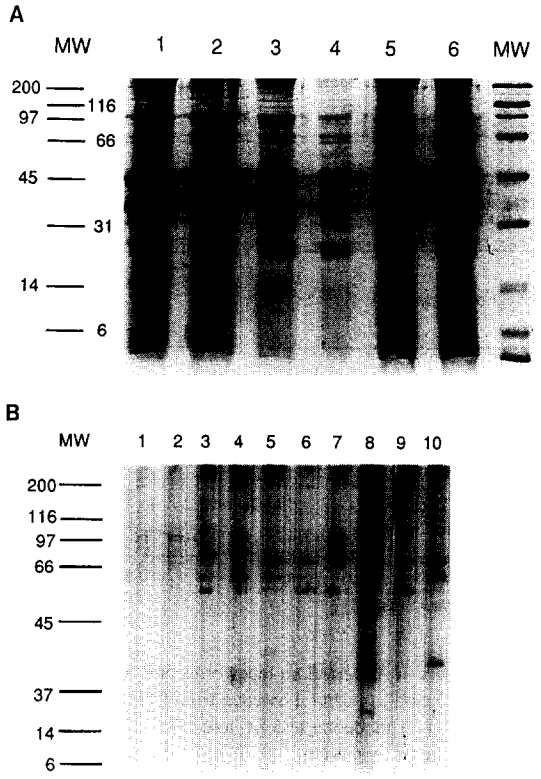

Fig. 1. (A) Polyacrylamide gel electrophoresis of six different preparations of *A. perfoliata* scolex proteins in the presence of sodium dodecyl sulphate (SDS). Molecular weight (MW) standards are indicated. (B). Western immunoblotting of serum samples from horses with different levels of *A. perfoliata* infection and intestinal lesions of the mucosa. Lanes 1–2, non-infected, no lesions; Lanes 3–4, non-infected, slight lesions; Lanes 5–6, infected, slight lesions; Lanes 7–8, infected, moderate lesions; Lanes 9–10, infected, severe lesions.

observed, especially in lanes incubated with sera from animals with moderate to severe lesions (lanes 7–10). It is worth noting that the strongest reaction with the tapeworm-positive sera was in general to an approximate 66 kD antigen, and the reaction to the 70 kD antigen, as seen in lanes incubated with sera from tapeworm-negative horses with slight lesions, was always missing.

3.2. Serological response in ELISA

Great individual variation was found in the ELISA ratio, even in horses with similar numbers of tapeworms (Fig. 2). There was a very strong effect of the number of tapeworms (ANCOVA, $F = 40.26$, $P = 0.000$) and the lesions score (ANCOVA, $F = 4.31$, $P = 0.005$) on the ELISA ratio,but no interaction between the two parameters could be demonstrated (ANCOVA, $F = 1.78$, $P = 0.149$). This means that the slope between the number of worms and the ELISA ratio was similar, regardless of the damage caused. Furthermore, the effect of the number of demonstrable *A. perfoliata* on the ELISA ratio was much stronger than

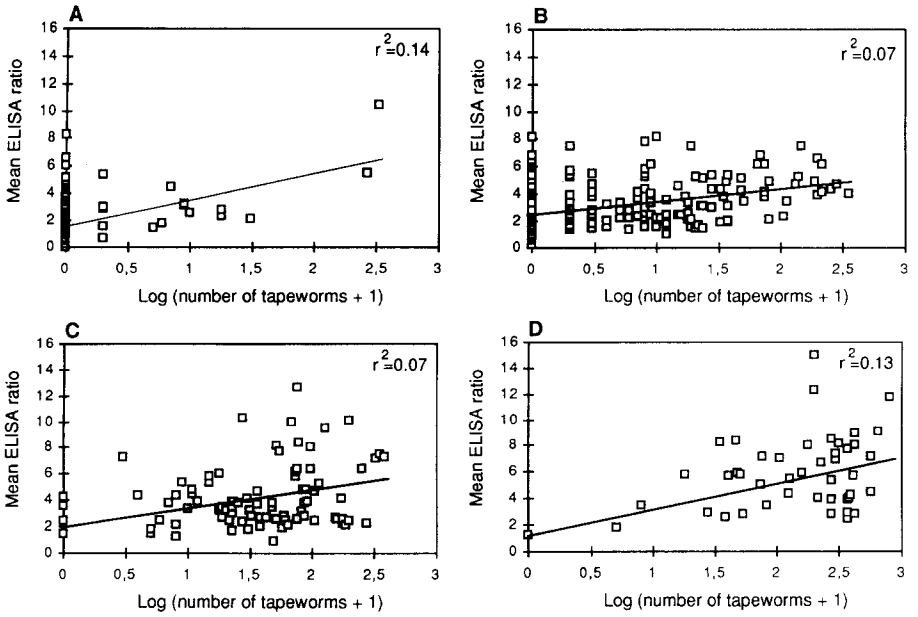

Fig. 2. Relationship between numbers of *A. perfoliata* tapeworms and serum antibody levels to a crude scolex antigen in horses slaughtered at an abattoir in central Sweden from October 1992 to October 1993. Horses with (A) no lesions, (B) slight lesions, (C) moderate lesions, and (D) severe lesions in the intestinal mucosa at necroscopy. The lines represent the linear regression.

that of the lesion score, although the increase in both parameters was significant when the other factor was held constant. There was also a significant monthly variation in the ELISA ratio (ANOVA, $F_{11,416} = 12.35$, $P = 0.000$). A temporary increase in the mean ELISA ratio was observed in June. The rise in the ELISA ratio was followed by an increase in the

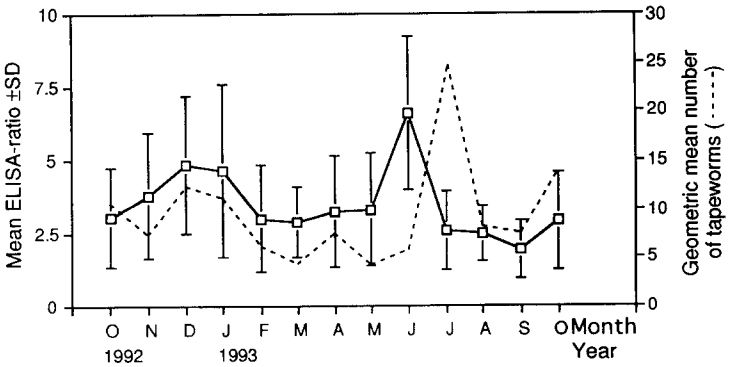

Fig. 3. Seasonal variations in serum antibody levels to a crude scolex antigen of *A. perfoliata* in horses examined at an abattoir in Central Sweden between October 1992 and October 1993. The broken line represents the geometric mean number of tapeworms recorded in the intestine at necroscopy of the individual horses. Results are expressed as mean ± SD for 15–58 horses examined each month.

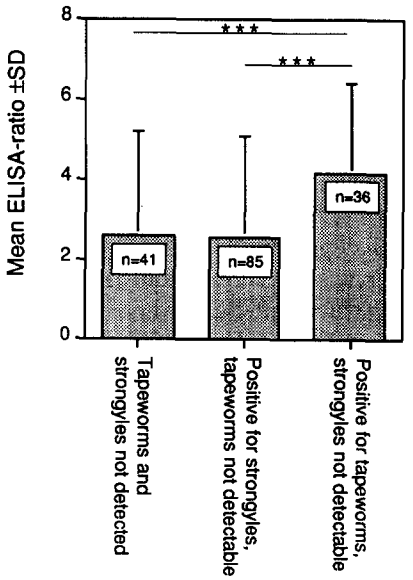

Fig. 4. Comparison of serum antibody levels to a crude scolex *A. perfoliata* antigen in slaughtered horses that were: (1) negative for both strongyles and tapeworms; (2) positive for strongyles but negative for tapeworms; (3) positive for *A. perfoliata* only. The material was collected at an abattoir in central Sweden from October 1992 to October 1993. Results are expressed as mean ± SD. The numbers of horses included are shown in the boxes within the bars, while the stars indicate levels of significance ($P = 0.000$) as calculated by the Tukey HSD-test.

number of intestinal worms first detectable in August, the geometric mean number of worms being 5.8 in June and 29.6 in August. The ELISA ratio soon returned to a low level and remained there until October, after which an increase was observed over a 3-month period (Fig. 3). There was also a significant variation between non-infected horses and those found to be infected with either only strongyles or only tapeworms (ANOVA, $F_{2,162} = 11.42$, $P = 0.000$) (Fig. 4). This was interpreted as meaning that no or only minor cross-reactions were occurring in the present ELISA between the *A. perfoliata* antigen and antibodies directed specifically to concurrent parasitic nematodes in the horses. No correlation was detected between the ELISA ratios and the age of the horses (adjusted multiple $R = 0.000$, $F_{1,373} = 0.24$, $P = 0.620$), nor was there a significant variation caused by previous anthelmintic treatment (ANOVA, $F_{5,374} = 0.50$, $P = 0.774$).

4. Discussion

Although the ELISA technique has been used previously for analyses of humoral antibodies to helminths in the horse, such studies have focused mainly on nematode infections (Soule et al., 1986; Nichol and Masterson, 1987; Smith and Snowdon, 1987; Mezger, 1991). Serological diagnosis of tapeworm infections in the horse seems so far to be limited to the hydatids of the genus *Echinococcus* (Edwards, 1982; Hermann et al., 1988). *A. perfoliata* lives in the intestinal tract, although in close contact with the mucosa, whereas

the hydatids of *Echinococcus* spp. are tissue-dwelling and contain highly antigenic material. This makes comparisons concerning the recognition of parasite antigens by host antibodies complicated.

There are no previous reports available on the attempted serodiagnosis of *A. perfoliata* infection in horses, which is thus described for the first time in this study. Although there was a very strong effect of the number of tapeworms and the intestinal lesions score on the ELISA value, considerable individual variation was seen, even among horses with approximately the same levels of infection. This was probably chiefly a result of the fact that this assay was not capable of distinguishing current from past infections owing to the prolonged persistence of antibodies in the blood after turnover of worms. The nature of this phenomenon needs further clarification. From a practical point of view, this means that we were not able to distinguish false positives from true positives. At present, therefore, it is very difficult to determine any particular cut-off value that would provide information on the level of infection in individual horses. However, when the antigen recognition by antibodies in sera from animals with different degrees of intestinal lesions was characterized by means of immunoblotting, there were indications that the observed banding pattern following WB also reflected the degree of damage caused by the tapeworms. It therefore seems reasonable to purify the crude *A. perfoliata* scolex antigen in future studies to achieve a higher specificity of the assay. Similar work has been carried out for the larval cestode infections of the genera *Taenia* and *Echinococcus* (Hayunga and Sumner, 1991; Brandt et al., 1992; Cheng and Ko, 1992; Gasser et al., 1992). Further research should also be devoted to the dynamics of *A. perfoliata* infections in the horse, especially since it is not known how long IgG antibodies to *A. perfoliata* persist in infected horses.

A crucial problem with ELISAs is the possible cross-reactivity between antibodies to related infections, and it is therefore essential to identify and characterize target antigens before they are used in diagnosis (Voller and De Savigny, 1981). For example, it has been found that antibodies directed to the third-stage larvae of small strongyles cross-reacted with those of other parasitic nematodes in the horse (Mezger, 1991). Consequently, in the present study, we analyzed the antibody levels in the horse sera in order to detect differences between individuals that were: (1) negative both for tapeworms and strongyles; (2) positive for strongyles but negative for *A. perfoliata*; (3) positive for *A. perfoliata* only. As the antibody levels were similar in worm-negative horses and those only positive for strongyles, but differed significantly from those with demonstrable tapeworms, we believe that antibodies directed to the nematodes did not interfere in the present ELISA. However, further studies must be carried out before definitive conclusions on the nature of possible cross-reactivities can be drawn.

A bimodal seasonal pattern in antibody levels was noticed, which showed a minor peak in the period from December to January and a more pronounced peak in June. A similar pattern was also seen for the number of detectable intestinal tapeworms, although the maximum was observed in July. On the basis of these findings, it may be speculated that the antibody response is related more to the establishment of newly acquired infections than to the presence of small or moderate numbers of older worms in the intestine. It thus seems as if there may be a temporary risk of horses becoming infected with *A. perfoliata* during the first part of the grazing season, whereas autumn infections are acquired more consistently. Support for this hypothesis comes from previous studies on foals, which have shown

that they primarily become infected with tapeworms first in the autumn, although infections may occur at a lower frequency in early summer as well (Nilsson et al., submitted for publication). There are also indications that the development of a proportion of the worms may be inhibited over the winter period, and that the prepatent period is longer than 6–8 weeks, as has generally been accepted. Sanda and Tsukada (1985) also reached this conclusion. Consequently, it is possible that a maturation of possibly inhibited larvae, acquired during the preceding season, starts in early June, and that this process is associated with an increase in the antibody levels. Further studies are needed to show the nature of this phenomenon.

This study observed a strong effect of the number of detectable tapeworms and intestinal lesions on specific antibody levels. Based on the present results, it is evident that the ELISA would be a useful tool in monitoring the epidemiology of *A. perfoliata* infection on a herd level. However, as variations in the antibody levels were considerable even in horses with approximately the same number of detectable tapeworms, a further increase in sensitivity is necessary to develop a useful routine diagnostic test which enables the identification of individual horses adversely affected by *A. perfoliata* infection.

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