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Epidemiology of *Anoplocephala perfoliata* infection in foals on a stud farm in south-western Sweden

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Abstract

The egg output and humoral antibody response to scolex antigens of the equine tapeworm *Anoplocephala perfoliata* were monitored in naturally infected foals by an egg flotation/centrifugation method and an indirect enzyme-linked immunosorbent assay (ELISA). The study was performed on a stud farm in south-western Sweden between May 1994 and April 1995. Sequential blood and faecal samples were taken from 21 foals during their first summer on pasture and until tapeworm eggs were detected. Results were expressed separately for 10 and 11 foals born before and after the end of April 1994, respectively. Increased levels of antibodies were noticed from October and onwards in both groups whereas tapeworm eggs were detected in the faeces of all foals about 4 months later. The antibody response was similar in both groups but it was more pronounced in foals born before April 1994. All foals were treated in March 1995 with an oral paste formulation of pyrantel pamoate at a dose rate of 38 mg/kg bodyweight. Most animals responded to the anthelmintic treatment and one month later, tapeworm eggs were only detected in one out of the 18 foals examined one month after treatment. Thus, the treatment reduced the number of *A. perfoliata* egg positive horses by 94%. A concomitant decline in antibody levels

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was also observed. Western immunoblot analysis of sequential individual serum samples showed that at least 10 different scolex antigens in the molecular weight range 10–200 kDa were recognised. Banding intensities, especially of the 10 kDa, 35 kDa, 45 kDa and 66 kDa proteins were different in the sequential sera taken during the course of infection. © 1998 Elsevier Science B.V.

Keywords: *Anoplocephala perfoliata*; Horse—Cestoda; ELISA; Epidemiology—Cestoda

1. Introduction

The tapeworm *Anoplocephala perfoliata* is a common parasite in the terminal part of the ileum and caecum of equines. At the attachment sites the underlying mucosa and lamina propria of the intestinal wall are infiltrated with inflammatory cells and mucosal erosions are frequent (Pearson et al., 1993). The distribution of the parasite is worldwide and in a recent Swedish post-mortem survey 66% of 498 horses were found to be infected with a mean number of 79 worms (Nilsson et al., 1995). The significance of *A. perfoliata* as a pathogen was previously not clear but recent reports have linked high worm burdens to ileocaecal colic and other digestive disorders (Owen et al., 1989; Pearson et al., 1993; Proudman, 1992; Proudman and Edwards, 1993).

Although the life-cycle of *A. perfoliata*, which includes the oribatid mites on pasture as intermediate hosts, has been known for many years (French and Chapman, 1992), knowledge about the parasitic phase of the life cycle and the pathogenesis of the infection is limited. This is probably partly attributed to the difficulties in diagnosing the parasite in the live animal by faecal egg counting techniques (Beroza et al., 1986; Proudman and Edwards, 1992). It has been demonstrated in Sweden that only about 20% of horses harbouring less than 100 tapeworms are detected by the flotation/centrifugation method used, whereas about 70% of those infected with more than 100 worms are identified (Nilsson et al., 1995). Therefore, instead of faecal egg counting methods, attempts have been made to develop immunodiagnostic tools to measure serum antibodies specific for *A. perfoliata* antigens (Höglund et al., 1995; Proudman and Trees, 1996a,b).

Pyrantel pamoate administered at 38 mg/kg bodyweight, is the most commonly used drug for treatment of *A. perfoliata* infection in horses. However, there is a lack of drug activity against immature stages (Lyons et al., 1986). In order to develop effective control strategies for anoplocephalosis there is an increasing need to understand the epidemiology of the tapeworm and how it is influenced by climate and horse management.

In this paper we report on naturally acquired *A. perfoliata* infection in foals kept on a stud farm in south-western Sweden. The aims were to monitor for approximately 1 year the tapeworm egg output and the sequential serum antibody response to the parasite before and after treatment with pyrantel pamoate. By this approach it was possible to further evaluate the diagnostic value of an enzyme-linked immunosorbent assay (ELISA) employing *A. perfoliata* scolex antigens.

2. Material and methods

2.1. Animals and study design

The study was conducted between May 1994 and April 1995 on a stud farm situated in Västergötland in south-western Sweden. The stud was known to be heavily infected with tapeworm because 90% of the resident mares had been infected with *A. perfoliata* the previous year. Twenty-one standardbred foals born between 30 January and 7 July 1994 were included in this study. They were kept with their mares on permanent pastures from mid May to mid October when the horses were housed. In the statistical analysis the foals were confined to two groups according to whether they were born before or after the end of April 1994. In both groups, blood and faecal samples were collected at the same time from each horse at approximately four weekly intervals for 48 weeks. However, it was not always possible to get samples from all foals. Sera were prepared from the blood samples and stored at −20°C until used in the immunodiagnostic tests. When the majority of the foals had detectable numbers of *A. perfoliata* eggs in their faeces they were all treated with 38 mg pyrantel pamoate (Strongid/Banminth® vet., Pfizer, New York, USA) per kilogram bodyweight.

2.2. Faecal examination

The faecal samples were analysed by a centrifugation/flotation method as described previously (Nilsson et al., 1995). Briefly 30 g of faeces was soaked for at least 1 h in 4 dl tap water, homogenised with an electric stirrer and poured through a 250 μm aperture sieve. The filtrate was collected and allowed to stand for at least 3 h. The supernatant was discarded and the sediment transferred to a flat bottomed centrifuge tube and centrifuged at $800 \times g$ for 2 min. The supernatant was siphoned off and the tube refilled to its previous level with saturated NaCl solution and 200 g sucrose/l. After centrifugation at $800 \times g$ for 2 min the suspension from the top of the tube was transferred by a pipette to both sides of a McMaster chamber and after at least 5 min was examined for *A. perfoliata* eggs using microscope at $40\times$–$100\times$.

2.3. Immunodiagnosis

2.3.1. Preparation of scolex antigen

The assay antigen was prepared as previously described (Höglund et al., 1995). Briefly, *A. perfoliata* were isolated from naturally infected horses and thoroughly washed at room temperature in 0.01 M phosphate buffered saline, pH 7.4 (PBS). The scolex including the lappets was dissected from the strobila and stored at −20°C until they were thawed and homogenised on ice in a glass grinder in PBS, ultrasonicated at 30 s cycles and centrifuged at $800 \times g$ for 30 min. The supernatant was collected and filtered through a low protein binding 0.45 μm Aerodisc filter (Gelman Sciences, Ann Arbor, USA) and stored at −20°C until used. The protein concentration in the filtrate was determined by BCA protein assay reagent (Pierce, Rockford, USA) according to the manufacturer's instructions using bovine serum albumin (BSA) as standard.

2.3.2. Enzyme-linked immunosorbent assay (ELISA)

The indirect ELISA used for determination of *A. perfoliata* antibodies has been previously described and characterised (Höglund et al., 1995). Briefly, the wells of polystyrene microtitre plates (Greiner, Frickenhausen, Germany) were coated overnight at 4°C with 100 μl of scolex antigen at a concentration of 5 μg ml^{-1}, diluted in 0.05 M carbonate buffer, pH 9.6 and blocked the following day with 100 μl 1% skimmed milk powder (SMP) (Semper, Stockholm, Sweden) in 1 mM PBS for 1 h at 20°C. All sera to be tested were diluted 1:100 in 1 mM PBS containing 0.05% Tween (T) and added to the coated wells in duplicate 100 μl volumes and then incubated for 1 h at 37°C. The wells were then incubated for 1 h at 37°C with horse-radish peroxidase (HRP) conjugated affinity purified goat anti-horse IgG antibodies (Cappel, Durham, England) diluted 1:8000 in PBS-T. The reaction was visualised by adding 100 μl 0.1% 5-amino-salicylic acid and 0.05% hydrogen peroxide, pH 5.9 (Merck, Darmstadt, Germany). The plates were read after 120 min at 492 nm in a Titertech Multiscan spectrophotometer (Flow Laboratories, Herts, England). Between each incubation step the wells were washed 3 × with PBS-T using a Titertech Handiwash 100 (Flow Laboratories). To control for interplate variation pooled positive and negative control sera were added to fixed positions on all plates.

2.3.3. Western blot analysis (WB)

The scolex proteins were separated under reducing conditions in the presence of sodium dodecyl sulphate (SDS) in prefabricated 10% polyacrylamide Tris–glycine 2/D prep well gels with 4% stacking gel (Bio-Rad, Hercules, USA). Before application of 250 μg of the antigen the proteins 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). A broad molecular weight range standard (Bio-Rad) was used as a reference according to the manufacturer's instruction. After separation of the antigen proteins the gel was transferred to a 0.45 micron nitro-cellulose (NC) membrane (Bio-Rad). After transfer of the proteins for 60 min at 70 V and 150 mA the NC-membrane was blocked with 5% SMP in Tris–NaCl buffer, pH 7.5 (TBS), for 1 h. The sequential sera from each individual and positive control sera were diluted 1:20 in TBS and applied for 2 h at 20°C using a Multi-screen apparatus (Bio-Rad) and then incubated with HRP conjugated affinity purified goat anti-horse IgG antibodies (Cappel) diluted 1:500 in TBS-T containing 2% SMP for 90 min. Finally the reactions on the NC membrane was visualised using 1% 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA) and 0.05% hydrogen peroxide as substrate. Between each of the incubation steps described above the NC membrane was washed twice for 5 min in TBS-T.

2.4. Statistical analysis

The mean OD-values of the sera tested were converted to ELISA-ratios by dividing with the mean OD-value of the negative controls on the same plate. Descriptive statistical analysis was performed using Excel (Microsoft, Redmond, USA) for the Macintosh (Apple, Cupertino, USA). Repeated-measures analysis of ELISA-ratio levels variation over time for both groups of foals was performed with a generalised linear

model (PROC MIXED procedure; SAS Institute, 1992). This type of statistical model accommodates missing values and includes the fixed effects of group, sampling-date and their interaction.

3. Results

3.1. Serological response and faecal egg output

Elevated antibody levels to *A. perfoliata* scolex antigens were noticed from October 1994 to March 1995 in both groups of foals (Fig. 1). There was a significant interaction between group and sampling date (Tests of fixed effects: $F = 5.84$, $p < 0.001$). This means that the ELISA-ratio changes over time in each group of foals were significantly different during the study period and that the increase in antibody levels was more pronounced in the older foals. Antibody levels were observed to increase more than two months earlier than tapeworm eggs could be detected in the corresponding faecal samples in both groups. In the group of older foals tapeworm eggs were detected in late December 1994 and in the younger foals they were not found until in February 1995. However, only two of the younger foals were examined in December 1994. All foals except one that was born after April 1994 responded to the pyrantel treatment. Consequently, 17 out of the 18 foals investigated were tapeworm egg negative at the end

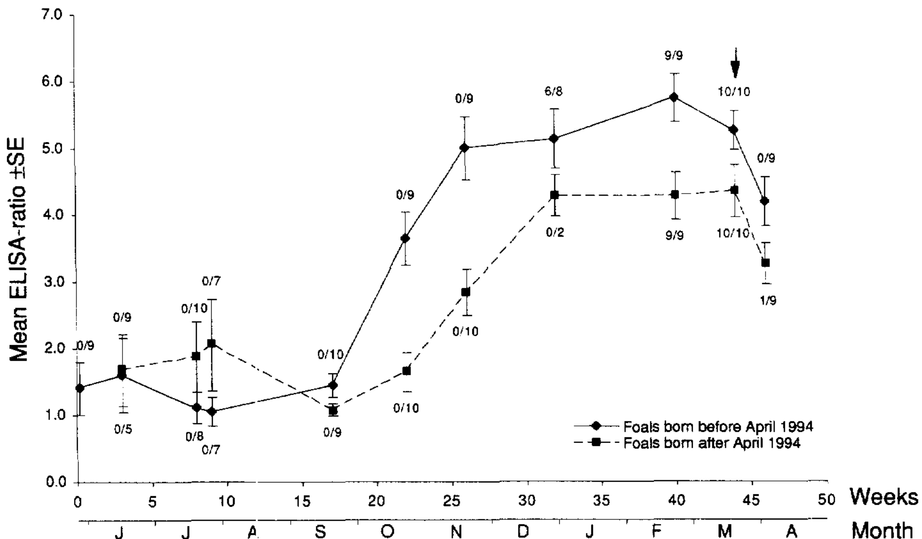

Fig. 1. Serum antibody response to a crude scolex antigen of *A. perfoliata* in groups of foals kept on permanent pastures on a stud farm in south-western Sweden. The straight line represents sequential observations in foals born before April 1994 and the broken line in foals born after April 1994. There was a significant difference in the antibody response among the foal categories (Test of fixed effects: $F = 5.84$, $p < 0.001$). Numerals in the graph denote the number of egg positive horses out of the numbers investigated and the arrow indicate when the horses were treated with pyrantel pamoate at 38 mg/kg bodyweight.

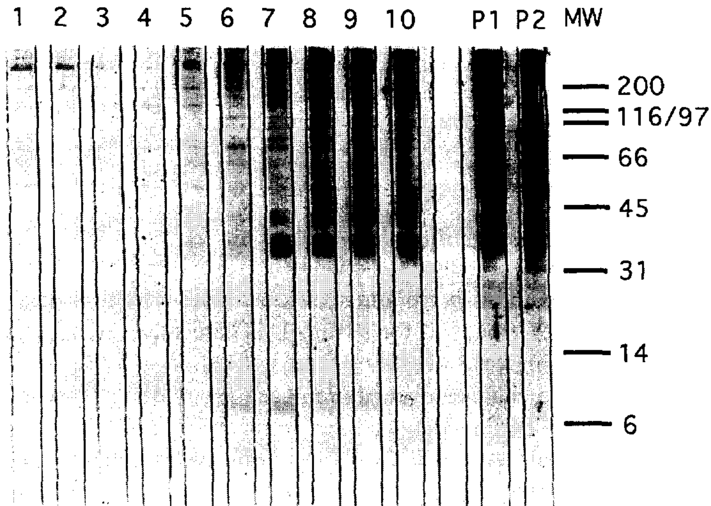

Fig. 2. Western immunoblotting of sequential sera from one foal born after April 1994, to a crude scolex antigen of *A. perfoliata*. Lanes 1–10 reflect the sampling dates shown in Fig. 1. P1 and P2 are positive control sera also used in the ELISA.

of the study in April 1995. Accordingly, the pyrantel treatment reduced the number of *A. perfoliata* egg positive horses by 94%. The interrupted faecal output of tapeworm eggs was also correlated with a significant reduction in antibody levels in both groups (Paired *t*-test, foals born before April 1994: $t = 3.27$, $p = 0.0042$; foals born after April 1994: $t = 22.6$, $p < 0.001$).

3.2. Characterisation of the serological response

Several IgG antibody reactions with scolex antigens within the molecular weight range 10–200 kDa were recognised by immunoblot analysis of sequential sera from an individual foal (Fig. 2). The intensity of the reactions were correlated with the reaction pattern observed in the ELISA. In general no bands were observed before seroconversion occurred as indicated by the ELISA. However, in some foals a 200 kDa protein was recognised by sera sampled in the beginning of the study when the horses had only been on pasture for up to 5 weeks. This band was probably reflecting maternal antibodies to *A. perfoliata*. From the time of elevated antibody levels in October there was a gradual increase in the number of bands and in their intensity. At different occasions strong reactions were seen with the sequential sera to at least four different proteins of molecular weights 10 kDa, 35 kDa, 45 kDa and 66 kDa. Reactions to each of these antigens were noticed more or less simultaneously. However, the strongest reaction to the 10 kDa antigen was noticed about 7 weeks before the most intense reactions to the 35 kDa and 66 kDa antigens were observed. Moreover, the 10 kDa band gradually decreased in intensity and nearly disappeared towards the end of the study. Similarly, it took another 8 weeks from the appearance of the 10 kDa protein until the most intense 45 kDa band was recognised by the sera.

4. Discussion

The present study has shown increasing levels of *A. perfoliata* antibodies in foals naturally infected on permanent pastures on a stud farm in south-western Sweden. This increase, as demonstrated by an ELISA employing crude scolex antigens, was starting from October about 2 months earlier than tapeworm eggs were detected in the faeces of the foals. The specificity of the ELISA has been shown to be satisfactory in relation to other helminths of the horse (Höglund et al., 1995). Antibody levels started to increase at the same time in both group of foals. This verifies that the serological reactions observed were attributed to the actual infection with tapeworms rather than to an unspecific age related stimulation of humoral antibodies. It was also demonstrated that 94% of the foals responded to anthelmintic treatment with pyrantel pamoate at 38 mg/kg bodyweight. The effects of the treatment was also reflected by a significant decrease in serum IgG antibody levels.

Knowledge about the prepatent period of *A. perfoliata* and the turnover of worms is of decisive importance in the design of rational control programmes against anoplocephalosis. Since only adult tapeworms seem to be sensitive to pyrantel (Lyons et al., 1986), it is of particular interest to understand when sexual maturation of the tapeworms occurs. In this study antibodies to *A. perfoliata* started to increase at the end of the grazing period and it then took another 2 months before tapeworm eggs were detected in the faecal samples. Based on these observations the optimal period for treatment against adult *A. perfoliata* using pyrantel pamoate would be during the winter–spring period when the majority of parasites acquired from pasture during the grazing period would have reached maturity.

Although it was not possible to determine the exact time elapsing between primary infection and seroconversion, the foals were most probably exposed to primary infection somewhat earlier than indicated by the antibody responses which started to increase at the end of the grazing period. Consequently, it seems that pasture contamination of overwintering infected mites which developed the previous grazing season was low. Further, since the sensitivity of coprological methods used to detect *A. perfoliata* eggs are low (Nilsson et al., 1995), the egg output probably started earlier than indicated. Thus, it can be concluded that adult *A. perfoliata* in the foals studied were certainly established earlier than in December. This is in accordance with observations in a previous study where it was assumed that the antibody response recorded with the present ELISA was related to the establishment of newly acquired infection (Höglund et al., 1995).

The immunoblot analyses of the sequential sera from the individual foals primarily confirmed the results obtained by the ELISA, but it also revealed the range of antigens involved in the *A. perfoliata* infection. Most of the antigens had a relatively large molecular weight, ranging from 10–200 kDa. The sera with low ELISA-values in the beginning of the observation period recognised no antigen or a lower number of antigens and also showed weaker bands in the blots than the later sera with higher ELISA-values. Of particular interest was the reactions of the sequential sera to the proteins with estimated molecular weights of 10 kDa, 35 kDa, 45 kDa and 66 kDa as their banding intensities were different during the course of infection. For example, the 10 kDa band

was most intense at the time of seroconversion and it then faded. This protein is likely to be equivalent to the 12/13 kDa antigen observed by Proudman and Trees (1996b) and seems to especially reflect the newly established infection. On the other hand, the bands at 35 kDa and 66 kDa were most intense somewhat later. Thus, it seems like these reactions reflect the serological response to different stages in the infection cycle of *A. perfoliata*. Further characterisation of these antigens will give information that might be useful in the development of serological assays capable of differentiating between newly acquired and patent infections.

5. Conclusion

Under Swedish conditions, *A. perfoliata* infection in foals establish on pasture at the end of the grazing season as reflected by an increase in antibody levels followed by faecal egg output some months later. Further, candidate antigens reflecting different stages in the infection cycle of *A. perfoliata* were identified. Following isolation these antigens might be useful in the further development of the ELISA for the diagnosis of *A. perfoliata* infections in horses.

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