Interpretation of serum antibody response to *Anoplocephala perfoliata* in relation to parasite burden and faecal egg count

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**Keywords:** horse; *Anoplocephala perfoliata*; ELISA; parasite burden; faecal egg count

**Introduction**

*Anoplocephala perfoliata* is the most common equine tapeworm globally (Gasser *et al.* 2005) and is found at the ileo-caecal junction and adjacent areas of the ileum, caecum and colon (Owen *et al.* 1988). High levels of infection have been associated with clinical cases of intestinal intussusception, perforation and peritonitis (Barclay *et al.* 1982; Beroza *et al.* 1983; Owen *et al.* 1989; Gaughan and Hackett 1990). Moreover, heavy burdens may be associated with an increased risk of spasmodic colic and ileal impaction (Proudman and Edwards 1993; Proudman *et al.* 1998; Little and Blikslager 2002).

The gross pathological lesions in gut mucosa are related significantly to intensity of infection and may include ulceration, diphtheric membranes, oedema and local thickening of the mucosa (Pearson *et al.* 1993; Fogarty *et al.* 1994; Nilsson *et al.* 1995; Williamson *et al.* 1997).

The traditional diagnostic approach has been detection of eggs in faeces using modifications of the centrifugation/flotation method, the reliability of which has been highly variable with sensitivities ranging 0.08–0.61 (Proudman and Edwards 1992; Nilsson *et al.* 1995; Meana *et al.* 1998; Williamson *et al.* 1998). However, using a modified centrifugation/flotation technique, with a sensitivity of 0.61 for detecting at least one tapeworm, the sensitivity rose to 0.92 when elevating the detection level to above 20 tapeworms (Proudman and Edwards 1992). A high correlation between egg counts and number of tapeworms would provide a desirable possibility to predict the risk of disease; but hitherto the best reported correlation has been 0.37 (Williamson *et al.* 1998).

Proudman and Trees (1996a,b) developed an enzyme-linked immunosorbent assay (ELISA) that detects serum IgG(T) against *A. perfoliata* 12/13 kDa excretory/secretory (ES) antigens. These authors reported a sensitivity of 0.62 at a cut-off of optical density (OD) = 0.103, and a correlation with infection intensity of 0.63. A case control study (Proudman *et al.* 1998) demonstrated an odds ratio (OR) of 15.46 for spasmodic colic at OD values above 0.6, whereas OD values of 0.2–0.6, although yielding an OR > 1, could not be related significantly to an increased risk of spasmodic colic when using conditional logistic regression. In a parallel study, an association was found between increasing OD values and risk of ileal impaction starting at OD = 0.103 (Proudman *et al.* 1998). These findings have lead to...
the current cut-off values of OD = 0.2 for moderate and 0.6 for high infection intensities, as suggested by Diagnosteq1.

In Denmark, due to concerns of anthelmintic resistance, use of drugs is restricted to prescription-only and must be based on a diagnosis by the veterinarian, therefore accentuating the need for accurate diagnostic tests. Recent availability of the Diagnosteq ELISA test has initiated its widespread use in equine practice in Denmark, although recommendations regarding treatment indicative antibody levels have not been assessed under Danish conditions.

The purpose of this study was, therefore, to validate and determine sensitivity and specificity of the ELISA and the sensitivity of a modified faecal egg count technique by comparing the findings with actual tapeworm burdens determined at slaughter.

Materials and methods

Horses at abattoir

During winter 2005/2006 (November–January), a total of 84 horses randomly selected of mixed type and age were examined at an abattoir in Denmark. The age of the horses was estimated by dental examination. No history of the horses was available, but all had passed a veterinary inspection before slaughter.

Within 20 min of death, the caecum, distal 30 cm of ileum and proximal 30 cm of colon were cut open to examine the contents and the adjacent mucosa for the presence of macroscopically visible A. perfoliata. All tapeworms were placed in 70% ethanol until examination at the laboratory, where the tapeworms were counted and classified as mature (triangular) or immature (lancet shaped) (Schuster 1991).

Visible pathological lesions of the mucosa in the caecum and the ileo-caecal opening were scored into 4 groups: 0 = no pathological changes; 1 = hyperaemia and slight mucosal thickening; 2 = moderate mucosal thickening with scattered necrotic ulcers; and 3 = severe lesions with multiple confluent necrotic ulcers (Nilsson et al. 1995).

Faecal egg count

Faecal samples were collected from the horses with visible A. perfoliata infection, and were examined subsequently by a modified faecal egg count technique modified after Beroza et al. (1986). In brief, 30 g faeces were mixed with 60 ml tap water and pressed through a piece of gauze (28 thread, 15 x 15 cm). The filtrate was distributed in four 15 ml tubes and centrifuged 10 min at 1000 g. The supernatant was removed and the pellet resuspended in the remaining liquid before filling the tubes with a saturated suspension of saline with 50% glucose (specific gravity = 1.27 g/ml) until the surface tension created a convex surface at the rim of the tube. A cover slip (24 x 24 mm) was placed on top of each tube before centrifugation 5 min at 210 g (no brake). The cover slips were transferred to microscope slides and examined at 40x magnification and the total egg count was recorded. To minimise the potential of bias, each microscope slide was examined only once. For confirmatory purposes the A. perfoliata egg and embryophore were measured at 400x magnification.

ELISA

Blood was collected from each horse, immediately after death, and placed in a cool box at 5–10°C until further processing at the laboratory. Within 24 h, serum was extracted by centrifugation for 10 min at 1000 g, and subsequently stored at -25°C until all samples could be forwarded simultaneously to Diagnosteq1 for ELISA analysis of serum-IgG(T) against A. perfoliata 12/13 kDa ES antigens (Proudman and Trees 1996a,b).

Statistical analyses

The validity of a test is described by comparing it to a gold standard, which is perceived as being the true finding. In the present study, macroscopically visible tapeworms in the gut or the mucosa score, respectively, were used as gold standard.

The ability of the ELISA test to predict tapeworm burdens or mucosa score was evaluated using the relative operating characteristic (ROC) analysis. The ROC curve displays the relationship between the true positive ratio (sensitivity) and the false positive ratio (1-specificity) over the range of possible cut-off values. The area under the ROC curve (AUC) is a measurement of the validity of the test, with a value of 1 representing the perfect test (Jensen and Poulsen 1992). The ROC curves were programmed using logistic regression (SAS version 9.1)3.

As the ELISA test results (OD values) did not follow a normal distribution, when tested with Shapiro-Wilk test, the relationships between mucosa score, egg count, worm burden and OD values were evaluated using Spearman’s rank correlation coefficient.

Results

Macroscopically visible A. perfoliata were found in 24 (29%) of the 84 horses (Table 1). Infection intensity varied from 1–1324

<table>
<thead>
<tr>
<th>Age</th>
<th>No. of horses</th>
<th>Infected horses</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–3 years</td>
<td>10</td>
<td>20% (2)</td>
<td>68.5 (5–132)</td>
</tr>
<tr>
<td>4–9 years</td>
<td>35</td>
<td>22% (10)</td>
<td>24 (1–988)</td>
</tr>
<tr>
<td>10–15 years</td>
<td>9</td>
<td>33% (3)</td>
<td>44 (32–1324)</td>
</tr>
<tr>
<td>16+ years</td>
<td>30</td>
<td>30% (9)</td>
<td>3 (1–15)</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>29% (24)</td>
<td>8.5 (1–1324)</td>
</tr>
</tbody>
</table>

Fig 1: Faecal Anoplocephala perfoliata egg count as a function of the tapeworm burden of 24 horses. One horse that had 1324 tapeworms identified post mortem and had a faecal egg count of 33 is not shown.
tapeworms, with no relation to age. Among the infected horses, 63% harboured <21 tapeworms, 26% had 21–100 tapeworms and 21% had >100 tapeworms. Five horses with infection intensities of 1–15 worms harboured exclusively immature infections. This was not seen in horses with >15 tapeworms.

Considerable pathological lesions (mucosa scores 2 and 3) were found only in A. perfoliata infected horses. Eight horses with mucosa score 2 had median intensity (MI) = 8.5 tapeworms, while 7 horses with mucosa score 3 had MI = 132 tapeworms. In contrast, 5 of the infected horses with MI = 1 tapeworm had no gross pathological lesions in the mucosa (mucosa score 0), and 12 horses, of which only 4 were infected (MI = 3 tapeworms), had mucosa score 1.

Eggs of A. perfoliata were recovered from 11 (46%) of the 24 infected horses (Fig 1). The sensitivity of the coprological technique was 0.46 for detecting parasite burdens of ≥1 tapeworm, but increased to 0.89 if the detection level was ≥20 tapeworms. For all of the 8 horses harbouring >30 tapeworms, faecal egg counts were positive.

Serum antibody responses against A. perfoliata were detected at varying levels in the majority of the horses (Fig 2). Twenty-four percent of the horses had an OD value <0.2, while 27% had OD values 0.2–0.6; and the remaining 49% had OD values ≥0.6.

The relationship between sensitivity and specificity is illustrated for different OD cut-off values in relation to the 3 gold standards of: 1) detecting any tapeworm infection; 2) the presence of >20 tapeworms; or 3) mucosa score ≥2 (Fig 3). Sensitivity and specificity for the gold standard 1) of detecting any tapeworm infection, cross at OD = 0.7 (sensitivity = 0.71, [95% confidence interval = 0.53; 0.89] and specificity = 0.68 [95% confidence interval = 0.57; 0.80]). The AUC was 0.82, which is significantly different from 0.5 (P<0.0001). By changing the gold standard to either ‘the presence of >20 worms’ or ‘mucosa score ≥2’, sensitivity and specificity changed considerably (Table 2).

Using Spearman’s rank correlation coefficient, significant correlations were found between egg counts, number of tapeworms, ELISA OD values, mucosa score and number of mature tapeworms (Table 3).

**Discussion**

The present study indicated a prevalence of A. perfoliata infected horses (29%) similar to that found previously in Denmark (22%: Hansen and Mansa 1997). While the Danish level apparently remains below that reported for Wales/England (69%: Owen et al. 1988) Ireland (51%: Fogarty et al. 1994) and Sweden (65%: Nilsson et al. 1995), it is similar to prevalence levels described in

**Table 2: Sensitivity and specificity at different cut-off values of antibody responses expressed as optical density (OD), using 3 different gold standards: macroscopic detection of tapeworms, more than 20 tapeworms, or mucosa score ≥2.**

<table>
<thead>
<tr>
<th>OD Cut-off</th>
<th>Tapeworms &gt;0</th>
<th>Tapeworms &gt;20</th>
<th>Mucosa score ≥2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se</td>
<td>Sp</td>
<td>Se</td>
<td>Sp</td>
</tr>
<tr>
<td>0.2</td>
<td>1</td>
<td>0.33</td>
<td>1</td>
</tr>
<tr>
<td>0.3</td>
<td>1</td>
<td>0.43</td>
<td>1</td>
</tr>
<tr>
<td>0.4</td>
<td>0.96</td>
<td>0.55</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.96</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>0.6</td>
<td>0.83</td>
<td>0.65</td>
<td>1</td>
</tr>
<tr>
<td>0.7</td>
<td>0.71</td>
<td>0.68</td>
<td>1</td>
</tr>
<tr>
<td>0.8</td>
<td>0.58</td>
<td>0.73</td>
<td>1</td>
</tr>
<tr>
<td>0.9</td>
<td>0.54</td>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.50</td>
<td>0.83</td>
<td>0.89</td>
</tr>
<tr>
<td>1.1</td>
<td>0.46</td>
<td>0.83</td>
<td>0.78</td>
</tr>
<tr>
<td>1.2</td>
<td>0.46</td>
<td>0.9</td>
<td>0.78</td>
</tr>
</tbody>
</table>

**Table 3: Spearman’s rank correlation coefficients for the relationship between Anoplocephala perfoliata egg count, worm burden, ELISA optical density (OD) values, mucosa score and number of mature worms.**

<table>
<thead>
<tr>
<th>Spearman</th>
<th>Worm burden</th>
<th>OD value</th>
<th>Mucosa score</th>
<th>Number of mature worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg count</td>
<td>0.713</td>
<td>0.365</td>
<td>0.702</td>
<td>0.715</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Worm burden</td>
<td>0.536</td>
<td>0.536</td>
<td>0.754</td>
<td>0.892</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>OD value</td>
<td>0.536</td>
<td>1</td>
<td>0.520</td>
<td>0.528</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>Mucosa score</td>
<td>0.754</td>
<td>0.520</td>
<td>1</td>
<td>0.767</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>84</td>
</tr>
</tbody>
</table>

5 N = Number of horses examined.
Germany (38%: Beelitz and Gothe 1997), Norway (20%: Ihler et al. 1995) and the Netherlands (21%: Borgsteede and Beek 1996). Comparisons between different diagnostic methods corroborated the usefulness of both faecal egg count technique and serological assay for identification of potentially treatment-demanding horses infected with A. perfoliata. However, the present findings also suggested that OD values below 0.7 should not be interpreted as indicative of need of treatment in individual horses.

Considering the correlation between infection intensity and the risk of disease caused by A. perfoliata (Proudman et al. 1998), it is pertinent to employ a diagnostic test with a sensitivity correlating to infection intensity. It has been demonstrated, in this and other studies, that the degree of mucosal damage is significantly related to the level of infection and that a tapeworm burden <20 probably is a conservative estimate of a nonpathogenic infection intensity (Bain and Kelly 1977; Pearson et al. 1993; Fugarty et al. 1994; Nilsson et al. 1995).

For the faecal egg count, a significant correlation (0.71) was found between egg count and parasite burden. This is in contrast to other studies where no correlation (Proudman and Edwards 1992; Nilsson et al. 1995) or a correlation of 0.37 (Williamson et al. 1998) has been demonstrated. Certain aspects of the presently employed method may have increased the recovery of eggs and may therefore explain the high correlation: the use of 30 g faeces instead of 5 g, as Williamson et al. (1998); performing flotation with centrifugation rather than without as described by Proudman and Edwards (1992); and the examination of 4 cover slips instead of 2 (Proudman and Edwards 1992). Additionally, only faecal samples from horses with visible A. perfoliata infections were examined, which could cause a potential bias when searching the microscope slide for eggs. However, it was ensured that the microscopist was not informed of the tapeworm burden level and that each microscope slide was inspected only once. Therefore, the investigation suggests that this centrifugation/flotation technique is a reliable diagnostic test for detecting tapeworm eggs, particularly in horses infected with ≥20 tapeworms. Nonetheless, studies should be performed on even larger data sets to further corroborate this.

Because sensitivity of the methods depends on the level of infection, it is difficult to compare overall sensitivities between studies. When the detection level in the present study was increased to ≥20 tapeworms, sensitivity increased from 0.46 to 0.89 comparable to the 0.92 found by Proudman and Edwards (1992). The low sensitivity of the faecal egg count in horses with <21 tapeworms could be due partly to the fact that 5 of the 15 horses with 1–20 tapeworms were infected exclusively with immature tapeworms. Furthermore, fluctuation in egg excretion or an unequal distribution of eggs in faeces may contribute to the low sensitivity (Nilsson et al. 1995). It has been reported that 75% of a mature tapeworm population can be sterile (Schuster 1991), perhaps due to inhibition among tapeworms or inhibitory host immune responses. Our study indicated a linear relationship between egg count and low to moderate tapeworm counts, possibly followed by stagnation in egg counts at a maximum level with further increasing burdens, but more data are needed to investigate this tendency. In the present study, faeces were examined only from A. perfoliata positive horses; hence the exact specificity of the flotation/centrifugation technique is unknown, but is probably similar to that reported by others, i.e. specificity = 0.98–1 (Proudman and Edwards 1992; Nilsson et al. 1995; Williamson et al. 1998).

There was a considerable variation in antibody levels between individual horses with similar tapeworm burdens, rendering a moderate correlation of 0.54 between OD values and tapeworm burden similar to the correlation of 0.63 found by Proudman and Trees (1996a). This wide variation confirms that the test is not able to determine exact worm burdens but rather to identify horses with treatment-demanding infection levels (i.e. OD ≥0.7) or can be applied in epidemiological investigations, as previously suggested (Proudman and Trees 1996a; Morgan et al. 2005).

When a cut-off value is determined for a continuous outcome, such as OD levels, the value reflects the wish to optimise sensitivity, specificity or both, in order to provide a clinically useful guide to interpretation of the diagnostic test. In this study population, a cut-off of OD = 0.7 would optimise both sensitivity and specificity to detect horses with ≥20 tapeworms, i.e. for detection of A. perfoliata infection of assumed clinical importance. The conservatively chosen cut-off value of 0.7 is considerably higher than the 0.2 recommended by others (see below).

In a study of ileal impactions, 38 of 40 control horses had OD values ≤0.2 (Proudman et al. 1998). Similarly, none of the 72 A. perfoliata negative horses examined in the validation study of this ELISA had OD values above 0.2 (Proudman and Trees 1996a). However, in the present study, only 24% of the 84 horses had OD values below 0.2, whereas 66% of the horses harbouring no visible parasites had OD values >0.2, suggesting that background OD levels in Denmark, and perhaps elsewhere, may be higher than in the UK. This discrepancy could, in theory, be explained by our horses having recently received anthelmintic therapy; information that unfortunately could not be obtained in the present study. However, considering the prescription-only legislation in Denmark for anthelmintic drugs, recent treatment is considered unlikely for horses destined for slaughter.

Only few studies have evaluated the decline of antibody levels after treatment. Proudman and Trees (1996a) reported that the decline occurred immediately post treatment in 4 examined horses, whereas Abbott et al. (2003) described a significant decline in IgG(T) occurring 3 months after treatment in 26 horses. Similarly, Barrett et al. (2004) found a mean decline in OD values from 0.64 to 0.23 12 weeks after treatment of 62 horses with praziquantel. Microscopic immature stages of A. perfoliata might also cause apparently false positive responses, as they would not be detected post mortem. The level of antibody response to such immature stages remains unclear, although Höglund et al. (1998) reported varied antibody responses between premature and mature infections when measuring anti-scolex antibodies.

The short time span between killing and gut examination (10–20 min) should preclude the loss of tapeworms, considering also that the observed forms were attached to the mucosa, and none were found in the gut contents.

Cross-reactions with other infectious agents could give a false positive antibody response, Proudman and Trees (1996a) confirmed that the ELISA assay had no cross-reactivity with strongyle infections, Parascaris equorum or Paranoplocephala manillana. Possible cross reactivity with Anoplocephala magna has not been examined. In Denmark, A. magna is considered to be very uncommon.

In conclusion, the present data suggest that the centrifugation/flotation technique is a sensitive test for the diagnosis of clinically important A. perfoliata infection. However, a limited number of horses were examined and further studies are needed. The considerable variation in the results for
the ELISA assay between antibody level and tapeworm burden confounds the interpretation in individual animals. However, as shown by the ROC analysis, OD cut-off values of ≥0.7 are clinically useful for identification of horses in need of anti-A. perfoliata treatment.

Acknowledgements

The authors thank Gerth Hansen’s abattoir, Christiansfeld, for enabling data collection. Bent Andersen, ChemVet, is acknowledged for financial support of serum analyses. Cynthia D. Juel and Leif Eiersted are acknowledged for technical assistance.

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References


