

Acute Phase Proteins in Response to *Dictyocaulus viviparus* Infection in Calves

By C. Gånheim¹, J. Höglund², and K. Persson Waller³

¹Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, Swedish University of Agricultural Sciences, ²Department of Parasitology (SWEPAR), National Veterinary Institute and Swedish University of Agricultural Sciences, and ³Department of Ruminant and Porcine Diseases, National Veterinary Institute, Uppsala, Sweden

Gånheim C, Höglund J, Persson Waller K: Acute phase proteins in response to *Dictyocaulus viviparus* infection in calves. Acta vet. scand. 2004, 45, 79-86. – Three experiments were carried out to examine the acute phase response, as measured by the acute phase proteins (APP) haptoglobin, serum amyloid A (SAA) and fibrinogen, in calves infected with lungworm, *Dictyocaulus viviparus*. In addition, eosinophil counts were analysed. Three different dose models were used in 3 separate experiments: I) 250 *D. viviparus* infective third stage larvae (L3) once daily for 2 consecutive days, II) 100 *D. viviparus* L3 once daily for 5 consecutive days, and III) 2000 L3 once. All 3 dose regimes induced elevated levels of haptoglobin, SAA and fibrinogen, although there was considerable variation both between and within experiments. A significant increase was observed in all 3 APP at one or several time points in experiment I and III, whereas in experiment II, the only significant elevation was observed for fibrinogen at one occasion. The eosinophil numbers were significantly elevated in all 3 experiments. The results show that lungworm infection can induce an acute phase response, which can be monitored by the selected APP. Elevated APP levels in combination with high numbers of eosinophils in an animal with respiratory disease may be used as an indicator of lung worm infection, and help the clinician to decide on treatment. However, high numbers of eosinophils and low levels of APP do not exclude a diagnosis of lungworm. Thus, lungworm infection may not be detected if measurements of APP are used to assess calf health in herds or individual animals.

lungworm; *Dictyocaulus viviparus*; acute phase proteins; calves; respiratory disease; haptoglobin; serum amyloid A; fibrinogen.

Introduction

Respiratory diseases in young cattle are often caused by viral and/or bacterial infections, but can also be due to the lungworm *Dictyocaulus viviparus*. *D. viviparus* is a pathogenic parasitic nematode of cattle. It causes parasitic bronchitis, also known as dictyocaulosis, which is a disease that typically affects young cattle during their first grazing season in temperate areas. In Sweden, clinical signs are primarily observed in August. In a recent Swedish survey lungworm-infected (seropositive) animals were

found in approximately 80% of organic dairy herds examined in late autumn (Höglund *et al.* 2001). The costs of outbreaks in such herds can be considerable (Wooley 1997).

D. viviparus has a direct life-cycle with infective third stage larvae (L3) that are ingested with herbage (Eysker 1994). Following penetration of the intestinal mucosa the larvae reach the mesenteric lymph nodes about one week after ingestion of L3, moult into L4 stage and follow lymph and blood to the lungs where they

penetrate the alveoli. In the lung, as the larvae mature and move up the bronchi, the parasite induces alveolitis, followed by bronchiolitis and bronchitis. This generates clinical signs such as coughing and dyspnoea of varying degrees. The prepatent phase is 24 days, and after a patent phase of approximately 60 days the recovery phase follows and clinical signs are vanishing slowly (Urquhart et al. 1996).

The acute phase response (APR) is a series of physiologic reactions initiated early in the inflammatory process (Baumann & Gauldie 1994). It includes the release of inflammatory mediators, such as the cytokines interleukin-1 and interleukin-6, which stimulate hepatocytes to produce acute phase proteins (APP). The APP are involved in many events during inflammation e.g. tissue repair, binding of bacterial components, and activation of complement (Gruys et al. 1994), and play a role in the balancing of the immune responses (Uhlar & Whitehead 1999, Arredouani et al. 2003). Haptoglobin and serum amyloid A (SAA) are important APP in cattle (e.g. Alsemgeest et al. 1994, Gruys et al. 1994) as is fibrinogen, which is a commonly used marker of the APR (McSherry et al. 1970, Eckersall & Conner 1988).

It has been suggested that the APR can be used for assessment of calf health (Gånheim et al. 2003). In support for this, increased blood levels of haptoglobin, SAA and fibrinogen were detected during viral and/or bacterial respiratory infections in calves (e.g. Conner et al. 1988, Gånheim et al. 2003). In many of these cases, the calves show no, or only mild, clinical symptoms, that could easily be missed in a group of calves in a farm (Gånheim et al. 2003). Screening for elevated APP values could therefore be useful to identify animals that are, or have recently been, clinically or sub-clinically diseased. However, it is, to our knowledge, not known if lungworm infection in cattle

also can elicit an APR. In comparison, an increase in serum fibrinogen in red deer was observed 7 days after inoculation with the related lungworm *D. eckerti*, suggesting that the tissue migration of larvae evoked a host response (Johnson 2002). To further evaluate the benefit of APP measurements in assessment of calf herd health, it is of value to know if respiratory disease due to lungworm infections also can be detected using this tool. Therefore, the aim of this study was to elucidate if an APR can be detected after *D. viviparus* infection of calves. Blood levels of haptoglobin, SAA and fibrinogen were studied during 3 separate experiments using different doses of infective larvae administered with varying frequency.

Materials and methods

Animals and parasite strain

Male calves (n=22) of Swedish dairy breeds (Swedish Red and White breed, or Swedish Holstein) were used. They were purchased from conventional dairy farms at the age of 2-3 months. The dairy farms were all declared free from bovine viral diarrhoea virus (BVDV) and enzootic bovine leucosis (EBL), according to the Swedish eradication programmes for these diseases. Before the start of the experiments the animals were allocated in smaller groups and were given an adaptation period of at least 4 weeks. They were kept on straw beddings and fed hay *ad libitum* and supplement according to weight. The infective *D. viviparus* third larvae (L3) used in the experiments were obtained from Intervet Nederland bv (Boxmeer, Netherlands). The L3s used for inoculation were fresh and obtained from donor calves. These larvae were incubated at 15 °C and they were less than 3 weeks old when they were used for experimental infection.

Experimental design

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Animals, Uppsala, Sweden approved the 3 experimental studies (I-III) performed. The average age of the animals at the start of the experiments was 21 weeks (range 19-23) in experiment I, 14 weeks (range 12-15) in experiment II and 27 weeks (range 23-31) in experiment III. In experiment I, 11 penned calves were inoculated orally once daily with 250 *D. viviparus* L3 on day 0 and 1 of the experiment. On day 35 post inoculation (p.i.) the animals were slaughtered and the lungs examined for gross lesions and presence of adult lungworms. These calves had 10 weeks earlier been inoculated with 500 larvae for 2 consecutive days, but the infection never reached patency as determined by faecal larval counts. This was likely due to that the L3s had been stored in water in tissue culture flask at 4°C for almost a year. In experiment II, 5 animals were inoculated once daily with 100 *D. viviparus* L3 on 5 consecutive days (day 0-4) at the start of the experiment. This experiment was finished on day 30. The calves in experiment II were not slaughtered, but were kept for other studies. In experiment III, 6 calves were inoculated with 2000 *D. viviparus* L3 on day 0. The experiment was finished on day 28 p.i., when the animals were slaughtered and the lungs examined for gross lesions and presence of adult lungworms.

Rectal temperatures were recorded daily throughout the adaptation periods and the experiments. Clinical signs such as coughing and depression were also recorded daily. Blood samples were taken day 0, i.e. before inoculation with *D. viviparus* larvae, and at 8 (experiment I) or 6 (experiment II and III) occasions after inoculation. Blood samples were obtained from the jugular vein in Venoject tubes with EDTA and without additive (Terumo Europe N.V., Leuven, Belgium). Samplings were always performed between 8 and 11 a.m. Faeces samples were collected from the rectum at the start of the experiments and then once weekly.

Analyses

The EDTA samples were analysed at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences (SLU), for eosinophil numbers, using Cell-Dyn 3500 (Abbot Diagnostic Division, Abbot Park, IL, USA), and for fibrinogen concentrations by a kinetic method according to *Becker et al.* (1984), using an automated analyser (Konelab 30, Konelab Corporation, Espoo, Finland). The values of eosinophils measured by Cell-Dyn were 2% higher compared to manual differential count, the correlation was 0.86. Samples without additive were centrifuged and the serum was collected and kept at -20°C until analysis of haptoglobin and SAA using Tridelta Phase™ Range Haptoglobin Assay and Phase™ Range Serum Amyloid A Assay (Tridelta Development Limited, Greystones, Co. Wicklow, Ireland). For haptoglobin and SAA, the intra- and inter-assay coefficients of variation were <4% and <10%, respectively. The specific antibody response reflecting patent *D. viviparus* infection was measured in serum using a diagnostic ELISA kit (Ceditest, IDO-DLO, Lelystadt, The Netherlands). In experiments I and III, infections were also confirmed by demonstration of lungworms at slaughter of the calves according to procedures described by *Andrews & James* (1994) and *Borgsteede et al.* (1998) and/or by demonstration of larvae in faeces according to *Höglund et al.* (2003).

Statistical evaluation

A general linear model (GLM) for repeated measures was used in SAS for making statistical inferences of the dependent variables, namely: eosinophils, haptoglobin, SAA and fibrinogen. The values of APPs and eosinophils of the different days were also tested pairwise with the values day 0 using Dunnett adjustment to avoid mass significances.

Table 1. The experimental design and results of larval count at slaughter and serological confirmation

Experiment	Number of calves	Average age of calves (weeks)	Dose of L3 x days	Mean (SD) number of adults at slaughter	Mean (SD) % seropositivity ^b
I	11	21	250 x 2	47 (46)	120.3 (49.3)
II	5	14	100 x 5	— ^a	61.0 (23.5)
III	6	27	2000	350 (150)	4.2 (3.2)

^a The animals were not slaughtered.

^b Analyses were made day 35, 37 and 28 p.i. for experiment I, II and III, respectively.

Results

Clinical observations

All calves showed a varying degree of respiratory distress such as coughing and/or dyspnoea from 1 week p.i. and onward. Several animals had fever and their general appearance was affected. These animals were treated with benzylpenicillin procaine (Ethacillin vet.[®], Intervet, Stockholm, Sweden) at a dose rate of 20 mg/kg bodyweight once daily for 5 consecutive days to prevent secondary bacterial infection.

Coughing was still a common sign at the end of the 3 studies although the more severe respiratory signs had subsided at that time.

Parasitology

Larvae were found in faeces from all calves from day 24-28 p.i. and onwards. From day 28 p.i. we also observed a specific antibody response. However, seroconversion was not apparent at 28 days p.i. in experiment III. The

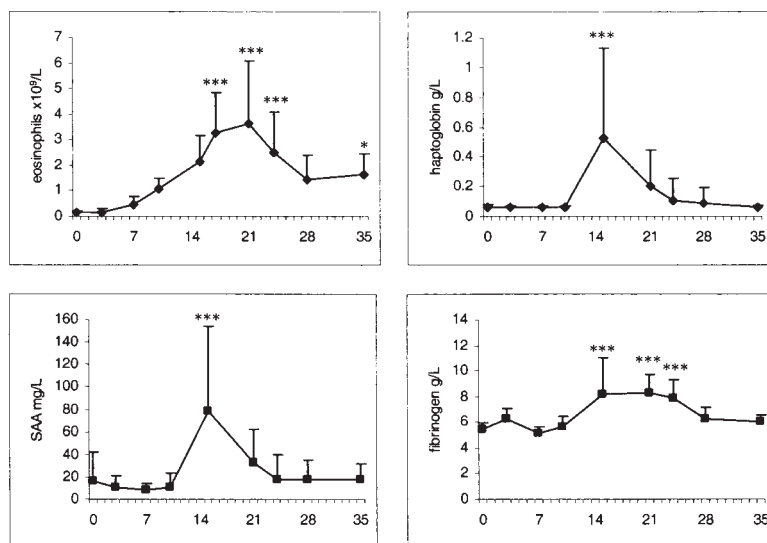


Figure 1. Mean (SD) numbers of eosinophils, and concentrations of haptoglobin, serum amyloid A (SAA) and fibrinogen in calves in experiment I after inoculation with 250 L3 larvae of *Dictyocaulus viviparus* on days 0 and 1. The value differs significantly from day 0 at * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

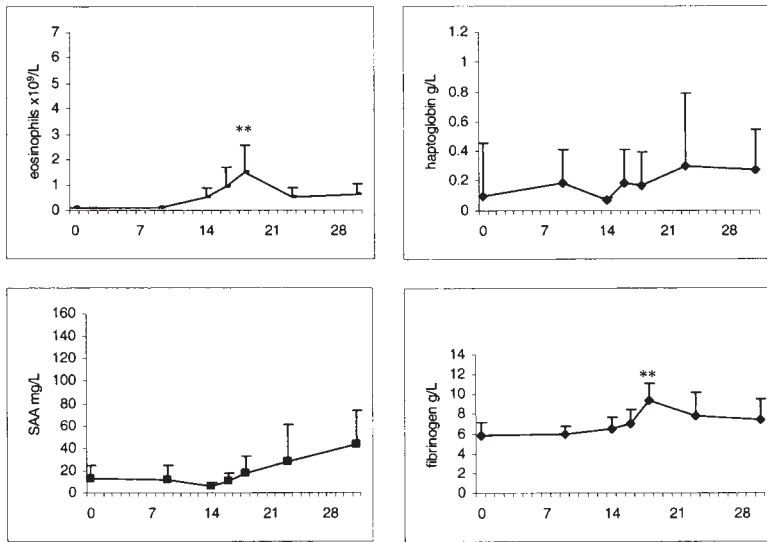


Figure 2. Mean (SD) numbers of eosinophils, and concentrations of haptoglobin, serum amyloid A (SAA) and fibrinogen in calves in experiment II after inoculation with 100 L3 larvae of *Dictyocaulus viviparus* on days 0-4. The value differs significantly from day 0 at *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

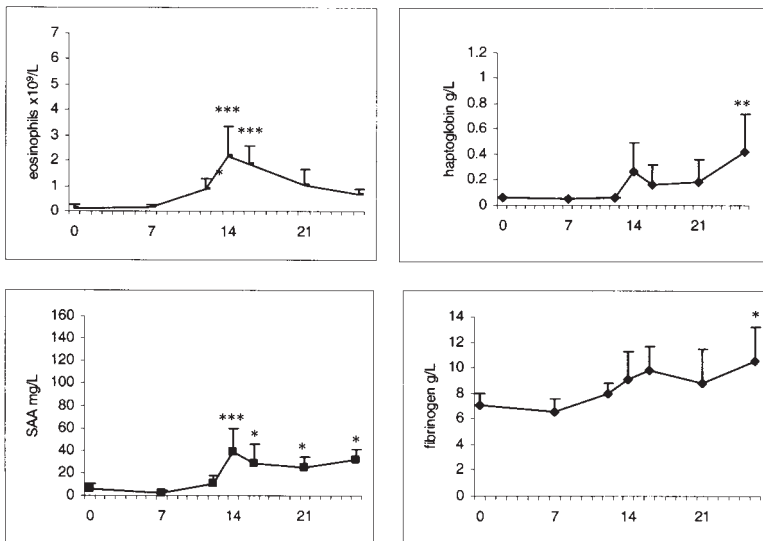


Figure 3. Mean (SD) numbers of eosinophils, and concentrations of haptoglobin, serum amyloid A (SAA) and fibrinogen in calves in experiment III, after inoculation with 2000 L3 larvae of *Dictyocaulus viviparus* on day 0. The value differs significantly from day 0 at *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

mean (SD) number of adults and seropositivity at different endpoints are shown in Table 1. The calves in experiment II were not slaughtered, as they were included in another study following the present one.

Eosinophil numbers and acute phase proteins

The eosinophil numbers were elevated in all 3 experiments (Figs. 1-3). A significantly higher level compared to pre-inoculation, was first observed at day 17 ($p < 0.001$, experiment I), day 18 ($p = 0.003$, experiment II) and day 12 ($p = 0.018$, experiment III). The numerically highest maximal numbers were found in experiment I, and the numerically lowest maximal numbers in experiment II. In experiment I, the eosinophil numbers were still significantly ($p = 0.031$) elevated at the end of the study periods.

In all 3 experiments, inoculation with *D. viviparus* L3 induced a rise in the levels of haptoglobin, SAA and fibrinogen, although there was a considerable variation both between and within experiments. A significant increase was observed in the concentrations of all 3 APP at one or several time points in experiment I and III (Figs. 1 and 3). However, in experiment II, the only significant ($p = 0.009$) elevation was observed for fibrinogen at day 19 p.i. (Fig. 2). The numerically highest mean values for haptoglobin and SAA were observed in experiment I, whereas the numerically highest mean value for fibrinogen was observed at the end of experiment III. In experiment I, where the animals were followed for a longer period compared to experiments II and III, the SAA and haptoglobin levels had decreased markedly already at d 21 p.i., while the fibrinogen levels were still significantly elevated d 24 p.i. A different pattern was observed in experiment III where the SAA concentration was significantly elevated from d 14 p.i. until the end of the study, i.e. d 26 p.i. Moreover, both haptoglobin

($p = 0.004$) and fibrinogen ($p = 0.015$) were significantly increased day 26.

Discussion

According to the present results, lungworm infection of calves induced an APR as measured by an increase in SAA, haptoglobin and fibrinogen. The APP kinetics varied depending on inoculation dose and administration routine. The time of onset of clinical symptoms and the APP reaction coincided, which is consistent with results from experimental viral and/or bacterial infections (Gånheim et al. 2003). However, the present results indicate that no signs of an APR was evident during the prepatent phase day 1-7, and that it was not initiated until the larvae had reached the lungs. Thus, the tissue damage caused by migrating larvae did not evoke enough inflammatory reaction to give a systemic increase in APP. Instead, the APR coincided with the early lung parasitic phase, and was probably related to the more severe tissue damage, which occurred when the parasite had been established for some time in the lungs. These findings are somewhat different from a similar study in red deer, where elevated fibrinogen values were observed already at day 7 after infection with *D. eckertii* (Johnson 2002). A considerable individual variation in APP response to lungworm infection was observed. Unfortunately, this variation was not correlated to the individual burden of established worms in the lungs, as measured indirectly by the number of adults at slaughter, and/or to the antibody response as measured by the ELISA.

In the 3 experiments included in the study, different numbers of larvae was administered using different inoculation routines. The most marked response in APP and eosinophil numbers were observed in experiment I after inoculation with a daily dose of 250 larvae for 2 days, while a dose of 100 larvae daily for 5 days (experiment II) gave the smallest reactions. A sin-

gle high dose, or a moderate dose given twice, is not very likely to occur under field conditions. In practice, animals at pasture are more likely to be exposed to low numbers of parasites for a prolonged time, making the dose alternative in experiment II the one most relevant for field conditions. As mentioned, this model gave the smallest APP response, and a significant increase compared to pre-inoculation was only observed for fibrinogen at day 18 p.i. The previous, unsuccessful inoculation of the calves in experiment I may have influenced the results in that group. The viability of the larvae first used was probably very low, giving a very low inoculation dose, explaining why the infection did not reach patency. However, the inoculation may have induced an immune response that made the animals respond stronger at the inoculation in experiment I. This is in line with a study by *Kooyman et al.* (2002), where a specific IgE response was recorded after inoculation of a very low dose of L3.

The eosinophil counts were significantly elevated in all 3 experiments, which was in accordance with previous reports (*Johnson* 2002). Eosinophilia is a fairly constant finding in response to lungworm infection, although it is not considered to be pathognomonic (*Radostits et al.* 2000), and it appears also in sub-clinical lungworm infections (*Schnieder & Daughshies* 1993.) Interestingly, elevated eosinophil numbers appeared earlier, in general, than the APP increase, indicating that the larvae were recognised by the immune system before they had caused enough tissue damage to induce an APR.

In conclusion, the present study showed that lungworm infection in calves can induce an APR that can be measured as an increase in the blood concentrations of the APP SAA, haptoglobin and fibrinogen. However, considerable individual variations were detected as well as variations depending on dose and administra-

tion routine. Moreover, the observed increases in APP occurred when clinical signs were already present. The APP response is similar to what has been observed earlier during viral and bacterial respiratory diseases making differentiation between lungworm infection and those other respiratory infections not possible based solely on measurements of the APP response. However, an increase of APP in combination with eosinophilia may be of some help in the diagnosis of respiratory disease caused by lungworm. Thus, lungworm infection may not be detected if measurements of APP are used to assess calf health in herds or individual animals.

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Sammanfattning

Akutfasproteiner vid infektion med Dictyocaulus viviparus hos kalvar.

Tre experiment utfördes för att undersöka akutfasreaktionen, mätt med hjälp av akutfasproteinerna (APP) haptoglobin, serum amyloid A (SAA) och fibrinogen, hos kalvar infekterade med lungmask, *Dictyocaulus viviparus*. Dessutom räknades antalet eosinofiler i blodet. Tre olika infektionsmodeller användes i 3 separata experiment: I) 250 infektiösa larver (L3) *D. viviparus* inokulerades en gång dagligen 2 dagar i rad, II) 100 *D. viviparus* L3 inokulerades en gång dagligen 5 dagar i rad, och III) 2000 L3 inokulerades vid ett tillfälle. Alla 3 modellerna resulterade i förhöjda nivåer av haptoglobin, SAA och fibrinogen även om det fanns avsevärd variation i svaret både inom och mellan experimenten. En signifikant ökning observerades i alla 3 APP vid en eller flera tidpunkter i experiment I och III medan en signifikant ökning bara observerades för fibrinogen vid en tidpunkt i experiment II. Antalet eosinofiler var signifikant förhöjt i alla 3 experimenten. Resultaten visar att lungmaskinfektion kan inducera en akutfasreaktion som kan mätas med hjälp av de utvalda APP. Förhöjda APP-nivåer i kombination med högt antal eosinofiler hos ett djur i samband med lungsjukdom skulle kunna användas som indikator för lungmaskinfektion och vara till hjälp vid beslut om behandling. Lungmaskinfektion kan dock inte uteslutas även om APP-nivåerna är låga men antalet eosinofiler är högt. Det är därför möjligt att man inte upptäcker lungmaskinfektion om APP används för att utvärdera kalvhälsan på besättnings- och/eller individnivå.

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Reprints may be obtained from: C. Gånheim, Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, P.O. Box 7039, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden. E-mail: Charina.Ganheim@og.slu.se, tel: +46 (0) 18 671927, fax: +46 (0) 18 673545.