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Systemic acute phase proteins response in calves experimentally infected with *Eimeria zuernii*

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ABSTRACT

Acute phase proteins (APPs) have been demonstrated to be useful in evaluating general health stress and diseases in cattle. Serum amyloid A (SAA) and haptoglobin (Hp) are APPs that are produced during inflammation, and likely play a role in host immunological defence against *Eimeria* infection and the associated intestinal tissue damage. We investigated the involvement of SAA and HP in an experimental study, including three groups of calves: a control group (group 0, $n = 11$), and two groups infected with either 150,000 or 250,000 *Eimeria zuernii* oocysts (group 1 ($n = 11$) and group 2 ($n = 12$), respectively). The calves were monitored for 28 days and data was collected on oocyst excretion, faecal score, animal weight, and SAA and Hp serum concentrations. Generalized linear mixed models showed that the clinical symptoms, indicated by an increase in the number of oocysts in the faeces and severe diarrhoea, manifested at patency for group 1 and 2. Serum Hp and SAA levels also increased during this period. Hp appeared to be a more sensitive marker than SAA, and differences between groups 1 and 2 were observed only for Hp. Linear regression models showed a negative association between weight gain and Hp concentrations, calculated as the area under the curve (AUC) during the overall experimental period and the patency period. A similar result was seen for SAA only during the patency period. This result supports the assumption that reduced weight gain due to *E. zuernii* infection is an immunologically driven process that involves an increase in APPs. A random intercept regression model of oocyst shedding groups showed that calves shedding 1–500 oocysts had reduced concentrations of Hp, indicating that a different immunological reaction occurs during mild shedding of *E. zuernii* oocysts than during more intensive shedding. A similar model was used to examine associations between faecal scores and Hp concentrations for each group. Group 2 calves with haemorrhagic diarrhoea displayed higher Hp levels than calves in that group with lower faecal scores, which may be in response to an increased demand for Hp in the repair process as a result of haemolysis. APPs seem to play an important role in determining the course of *E. zuernii* infection in calves, which may enhance our understanding of the immunological reaction and development of this disease.

1. Introduction

The acute phase response is an early immune response that is crucial for early immune defence against events such as inflammation, neoplasia, and infections (Cray et al., 2009). Early in infections, or during tissue damage, pro-inflammatory cytokines are released: IFN-γ and IL-6 from affected cells and TNF-α and IL-1β from mononuclear cells (van Miert, 1995; Heinrich et al., 1990). When cytokines reach the liver, they stimulate the hepatocytes to release acute phase proteins (APPs) into the blood stream (Heinrich et al., 1990). APPs circulate in the blood and help to stabilize the internal environment and speed the healing process (Cray et al., 2009). In response to inflammation, APPs may increase (positive APP) or decrease (negative APP) plasma protein levels. APPs in cattle include serum amyloid A (SAA) and haptoglobin (Hp).

In humans, APPs are well established as markers for several physical illnesses, whereas their application in veterinary medicine has developed at a slower pace (Murata et al., 2004; Petersen et al., 2004; Ceron et al., 2005). In calves, APPs have been used to measure the general health of calves, and as indicators of stress, inflammation related to hoof diseases, and various bacterial and viral infections, including coronavirus, bovine respiratory syncytial virus, *Escherichia coli*, bovine adenovirus, and mastitis related infections (Gånheim et al., 2007; Saco et al., 2008; Suojala et al., 2008;

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Angen et al., 2009; Kujala et al., 2010; Orro et al., 2011; Pyörälä et al., 2011). Recently reference values of Hp and SAA for clinically healthy dairy calves under natural condition up to 2 months of age have been published (Seppä-Lassila et al., 2013).

Bovine *Eimeria* species are common and important coccidia of calves that can lead to severe disease (eimeriosis). The clinical disease and subclinical effects can potentially result in long-term losses regarding animal health and production (Fitzgerald, 1980; Stockdale, 1981; Daugschies et al., 1986; Lassen and Ostergaard, 2012). After a sporulated oocyst has excysted in the gut, the parasite undergoes several asexual and sexual cycles of invasion and destruction of the intestinal cells. Eimeriosis due to *Eimeria (E.) zuernii* manifests as massive tissue damage accompanied by the release of oocysts 2–3 weeks after infection (Daugschies and Najdrowski, 2005). Changes in APP profiles may serve as a useful tool for estimating the health of calves by determining of the impact of pathogens, such as *Eimeria*, in the clinical and subclinical stages, and may potentially serve as a tool for prognosis of the severity of disease (Hashemnia et al., 2011).

Previous experimental studies investigated the variations in blood chemistry, electrolyte concentrations, acidity, and blood gases over the course of a 28-day experimental period after infecting groups of calves with single doses of 0, 150,000, or 250,000 *E. zuernii* oocysts (Bangoura and Daugschies, 2007a,b; Bangoura et al., 2007). In this study, we assessed the expression of SAA and Hp in the serum of these experimental groups to test the hypothesis that these levels are unchanged in the early immune response of calves to *E. zuernii* infection.

2. Materials and methods

2.1. Study design

Samples originated from 41 Holstein-Mix calves. The calves were vaccinated against Bovine Rota and Corona virus via the dam. The animals were between 10 and 28 days old and were determined to be healthy by a veterinarian at the beginning of the experimental period based on observation of general animal behaviour, good overall health, body temperature between 38.5 °C and 39.5 °C, normal breathing, skin turgidity and heart auscultation, and presence of wounds and diarrhoea. All animals were weighed individually on a mobile scale once weekly in 7-day intervals starting on day 0 or 1 of the study, respectively, until the end of the study. Calves were divided into three infection groups: group 0 ($n = 14$), uninfected control calves; group 1 ($n = 11$), calves infected orally with 150,000 sporulated *E. zuernii* oocysts; and group 2 ($n = 16$), calves infected orally with 250,000 sporulated *E. zuernii* oocysts. Interference of *E. coli* K99 and Giardia were excluded at the onset of diarrhoea, or 18 day post infection (dpi) for controls, using antigen tests (FASTest *E. coli* K99 strip, and FASTest GIARDIA strip, Megacor, Hoerbranz, Austria). Samples taken simultaneously with the antigen tests were investigated to confirm absence of *Cryptosporidium* spp. using a carbol fuchsin staining technique (Heine 1982). Calves were kept under the same housing conditions (same feed, two calves per pen, same stable, and air-conditioned rooms). The infective material was isolated from a commercial farm, passaged in calves by experimental infection before use and determined to be comprised of greater than 97% *E. zuernii* oocysts and less than 3% *Eimeria ellipsoidalis* oocysts. Blood samples were collected in vacuum tubes (Vacuette® serum clot activator tubes, Greiner bio-one, Kremsmuenster, Austria) on days 0, 1, 3, 7, 9, 12, and 14–28. Blood was drawn from all animals during the experimental period, except on days 3 and 9, upon which 10 and 30 calves were sampled, respectively. Faecal samples (50–100 g) were collected on the sampling days by digital stimulation of the anus. The consistency was scored as: (1) normal to pasty, (2) semiliquid to liquid, (3) watery, and (4) haemorrhagic and/or with tissue according to Mundt et al. (2005). Oocyst excretion was determined using the quantitative McMaster method, as described previously (Thienpont et al., 1990; Bangoura and Daugschies, 2007b).

As the APP response is not disease-specific and diseases other than eimeriosis can influence the results, seven calves were removed from statistical analysis because of clinical signs unrelated to eimeriosis. As a result, the total number of calves in this study was smaller than in the original studies (Bangoura et al., 2007; Bangoura and Daugschies, 2007a,b). From group 0, two calves were removed because of arthritis and one because of conjunctivitis and anorexia. From group 2, four calves were removed because of arthritis. The final number of calves in group 0, group 1 and group 2 were 11, 11 and 12, respectively.

2.2. Acute phase proteins detection

Serum concentrations (mg/l) of Hp were determined using the haemoglobin binding assay described previously by Makimura and Suzuki (1982) and with modifications described by Seppä-Lassila et al. (2013). The working range of the assay was 60–1900 mg/l. The inter-assay and intra-assay coefficients of variation (CV) for Hp analysis were <10% (mean concentrations of control samples 112 mg/l; $n = 20$ and 458 mg/l; $n = 20$) and <13% (mean concentrations of control samples 125 mg/l; $n = 27$ and 471 mg/l; $n = 25$), respectively.

The serum concentrations of SAA were measured with a commercial multispecies ELISA kit (Phase SAA Assay, Tridelta Development Ltd., Dublin, Ireland) according to the manufacturer's instructions for cattle. Serum samples were initially diluted 1:1,000. If the concentration was over of the range of standard curve (greater than 150 mg/l), they were diluted as necessary and re-assayed. Intra- and inter-assay CVs were <9% (mean concentrations of control samples 16.4 mg/l; $n = 20$ and 104.5 mg/l; $n = 20$) and <12% (mean concentrations of control samples 15.1 mg/l; $n = 18$ and 119.2 mg/l; $n = 18$), respectively.

2.3. Statistics

Generalized linear mixed models (GLMM) were used to explore overall time trend differences in APP concentrations, faecal scores, and oocyst shedding between the control and infected groups. The same models allowed investigation of associations between APP concentrations and daily oocyst excretion and faecal scores. Calves were included as random intercepts and polynomials of time (days), with interactions with the infection group added as fixed effects in increasing order. The overall time trend differences between groups were tested with the *F*-test. Bonferroni coefficients for multiple comparisons were used to correct *P*-values. As the time between sampling was not the same, an isotropic spatial exponential covariance structure was used for modelling serial correlations of repeated measurements at the within-calf level in all models.

The general equation of the linear mixed models for evaluating changes over time between the infection groups was defined as follows:

$$Y_i = \beta_0 + \beta_1 X_{1(i)} \times X_{4(i)} + \beta_2 X_{2(i)} \times X_{4(i)} + \beta_3 X_{3(i)} \times X_{4(i)} + u_{\mathrm{calf}(i)} + \epsilon_i$$

in which Y is the outcome variable, β_0 is the intercept, $\beta_{1\text{-}3}$ are the sizes of the effects of independent variables $X_{1\text{-}3}$ (day, day^2 and day^3, respectively), and X_4 (infection group), $u_{\mathrm{calf}(i)}$ is the random effect (calf) with the spatial exponential covariance structure for repeated measures and ϵ_i is an error term.

Initially, the APP models included calf age (days of age) at the beginning of the experiment as a covariate and faecal score (4-level

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