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Research paper

Haptoglobin and serum amyloid-A concentrations and their relationship with oocyst count in neonatal lambs experimentally infected with *Cryptosporidium parvum*

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ABSTRACT

This study aimed to evaluate the acute phase response (APR) through haptoglobin (Hp) and serum amyloid A (SAA) concentrations in serum and to examine the correlation between these acute phase proteins (APPs) and oocyst shedding using experimental *Cryptosporidium parvum* (*C. parvum*) infection model in neonatal lambs. Twenty lambs were divided into two equal groups: group CON remained uninfected as negative control and lambs of the group EXP were inoculated orally with 1×10^6 *C. parvum* oocysts. Blood and faecal samples were obtained from both groups before colostrum intake and prior to inoculation (day-1), and at 2, 6, 13, and 20 days post-inoculation (dpi). The serum concentrations of SAA increased following the experimental infection of lambs with *C. parvum*, the difference being statistically significant from pre-inoculation levels at 2 dpi, while significant increases in serum concentration of Hp were observed at 2 and 6 dpi. At the same occasions, serum concentrations of both APPs were significantly higher in the *C. parvum*-infected lambs compared to the healthy control lambs. A moderate positive correlation ($rho = 0.67$; $p < 0.001$) was observed between serum Hp concentration and oocyst count (OPG),whereas the serum SAA concentration didn't significantly correlate with OPG ($rho = 0.18$; $p > 0.05$). In conclusion, the results of the study shed some light on APR due to *C. parvum* infection in neonatal lambs.

1. Introduction

Neonatal diarrhoea in lambs is one of the most important problems with worldwide distribution in sheep enterprises. It results in the greatest economic losses due to the costs of labour, treatment, and prophylaxis, increasing susceptibility to other infections, poor growth and sometimes death (de Graaf et al., 1999; Olsen et al., 2015). Aetiology of neonatal lamb diarrhoea is diverse and involves a wide range of factors related to the animal, conditions of the environment and husbandry, and various infectious agents. As in calves, *C. parvum* is now recognised as one of the main causes of diarrhoea in neonatal lambs resulting in significant morbidity (up to 85%) and economic losses all over the world (Munoz-Fernandez et al., 1996; de Graaf et al., 1999; Ulutas and Voyvoda, 2004; Robertson et al., 2014; Olsen et al., 2015). Furthermore, this species of *Cryptosporidium* have zoonotic potential, and treatment options are extremely limited (de Graaf et al., 1999; Robertson et al., 2014; Olsen et al., 2015; Certad et al., 2017). Cryptosporidiosis is usually self-limiting, but infection among immunocompromised hosts can be more severe (de Graaf et al., 1999; Olsen et al., 2015; Certad et al., 2017). Cryptosporidiosis caused by *C. parvum* is of great public and animal health concern (de Graaf et al., 1999; Certad et al., 2017) and leads to significant economic burden (Munoz-Fernandez et al., 1996; Robertson et al., 2014). *Cryptosporidium parvum* invades epithelial cells primarily in the distal small intestine and causes destruction of intestinal epithelia resulting in a reduction of enzymatic activity and a decrease in the absorptive surface, finally leading to maldigestion and malabsorption followed by osmotic diarrhoea (de Graaf et al., 1999; Certad et al., 2017). Dehydration and even death as consequences of diarrhoea occur in affected lambs (de Graaf et al., 1999; Robertson et al., 2014). In this content, Thomson (2016) reported that the infection in lambs appears to cause more mortality than in calves, perhaps because lambs can become dehydrated much more quickly than calves.

The APR is a prominent and non-specific systemic reaction of the

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innate immune system to local or systemic disturbances caused by trauma, infection, stress, surgery, neoplasia or inflammation (Murata et al., 2004; Gruys et al., 2005; Kjelgaard-Hansen and Jacobsen, 2011; Ceciliani et al., 2012). This early reaction of the host to tissue damage or infection consist a large number of behavioural, physiological, biochemical, and nutritional changes (Murata et al., 2004; Gruys et al., 2005; Ceciliani et al., 2012). One of the most important metabolic changes in the APR is the production of APPs predominantly by the liver and their release into the circulation following stimulation by pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Murata et al., 2004; Gruys et al., 2005; Ceciliani et al., 2012). The biological functions of APPs, although variable, generally relate to defence to pathological damage and restoration of homoeostasis (Murata et al., 2004; Gruys et al., 2005; Ceciliani et al., 2012). APPs whose plasma concentrations increase 10-fold or more in response to stimulation are termed as major positive APPs (Murata et al., 2004). In ruminants, Hp and SAA are known to be major APPs, and the circulating concentrations of these APPs are usually related to the severity of the disorder and the extent of the tissue damage (Murata et al., 2004; Ceciliani et al., 2012; Iliev and Georgieva, 2016). Therefore, quantification of serum Hp and SAA concentrations in ruminants can provide diagnostic and prognostic information and assessment of the response to the triggering event (Murata et al., 2004; Gruys et al., 2005; Ceciliani et al., 2012; Tothova et al., 2014; Iliev and Georgieva, 2016).

The APR and the APPs changes that occur in cattle in response to disease have been documented in a large number of studies as reviewed by Ceciliani et al. (2012) and Tothova et al. (2014). In comparison to cattle, the APR in small ruminants is poorly described (Ceciliani et al., 2012; Tothova et al., 2014; Iliev and Georgieva, 2016). Most of the studies performed on sheep have been focused on the role of APPs after several inflammatory stimuli and in natural or experimental bacterial infections whereas studies on the APR in parasitic infections are quite limited (Ceciliani et al., 2012; Tothova et al., 2014). Monitoring changes in APPs levels in a natural infection can have disadvantages for diagnostic and prognostic information. Although experimental infection models often differ from the natural infection in having greater severity due to the large infectious dose used, they have a number of advantages over natural infection including control of time and dose of pathogen inoculation, controllable confounding pathogens, predictability time of symptom onset and close monitoring of clinical parameters. Clinical examination and sample timing plans for clinical and laboratory findings are particularly important to infections with short prepatent (between 2 and 7 days) and patent (at least 13 days) periods such as cryptosporidiosis in lambs (de Graaf et al., 1999; Robertson et al., 2014). For this reason, relevant animal models are critically important for discovery of cryptosporidiosis mechanism targets for diagnostic and prognostic information, medical interventions and the development of new therapies. Serum Hp and SAA concentrations have been investigated in calves with naturally occurring diarrhoea with or without *C. parvum* (Pourjafar et al., 2011; Balikci et al., 2014). To determine the severity of cryptosporidiosis due to *C. parvum* the correlation between oocyst count and diarrhoea severity in an experimental model (Operario et al., 2015) and the correlation between the above-mentioned APPs and faecal score in natural infection (Pourjafar et al., 2011) have also been reported in calves. To our best knowledge, there has been no previous attempt to characterise the dynamics of the APPs like Hp and SAA and their relationship with oocyst count in experimentally *C. parvum*-infected neonatal lambs.

The objectives of present study were therefore to elucidate the APR of Hp and SAA in neonatal lambs experimentally infected with 1×10^6 oocysts of *C. parvum*, and to evaluate whether a significant correlation between these APPs and oocyst count over the course of the disease would be possible.

2. Material and methods

All study procedures were reviewed and approved by the Animal Research Ethics Committee of the Adnan Menderes University, under protocol number B.30.2.ADÜ.0.00.00.00/050.04/2012/042. The study was carried out between March 2014 and December 2015 at the Clinic for Large Animal Internal Medicine of the University.

2.1. Animals and experimental design

A total of 20 Kivircik cross-breed female or male lambs were selected and enrolled in the study as they were born. The lambs were born as singles or twin from estrus-synchronized dams with pregnancies of normal length and uncomplicated births at the Clinic for Large Animal Internal Medicine of the Adnan Menderes University in Aydin-Turkey. They were separated from their dams' immediately after birth and subsequently an overall clinical examination on neonatal vitality including evaluations of the Apgar system (heart rate, respiratory rate and effort, muscle tone, irritability reflex and mucous color), rectal temperature and suckle reflex along with body weight (BW) measurement was performed to determine health status prior to admitting each animal to the study. During the experimental trial, the lambs were housed in two separate, isolated rooms in the same facility, one for the CON and one for the EXP lambs. In each isolated room with restricted access, lambs were kept individually in concrete sided isolation boxes bedded with straw. The isolated rooms were cleaned with boiling water and then sprayed with 20% hydrogen peroxide. Moreover, the study personnel used single-use equipment when entering each lamb isolation room. Thus, the possibilities of oocysts spread from EXP lambs to CON lambs and extraneous infections were minimised throughout the study. All the lambs were bottle fed with whole colostrum at 10% of (BW) per day during the first two days, with the first colostrum intake at 2 h of age, and thereafter with milk replacer twice daily (10% body weight for the first 5 days and thereafter 50% more) until termination of the experiment at 20 dpi.

After clinical examinations and BW measurements, twenty clinically healthy lambs were randomly allotted to two equal groups based on birth weight and gender (day −1): group CON remained uninfected as negative control and group EXP were infected orally with 1×10^6 *C. parvum* oocysts. The CON group contained six female and 4 male lambs while there were equal numbers of females and males in the EXP group.

The *C. parvum* isolates used in the present study was obtained from a naturally infected lamb with diarrhoea, confirmed by microscopy and molecular characterization. Oocysts were isolated from faeces (in sterile distilled water/diethyl ether), purified on a discontinuous Percoll gradient, and quantified with a Neubauer hemacytometer as described previously (Lorenzo et al., 1993). *Cryptosporidium* oocysts were detected by staining methods, namely modified Ziehl-Neelsen (Henriksen and Pohlenz, 1981) and carbol fuchsin (Heine, 1982), and a conventional PCR. For conventional PCR, DNA was extracted from 200 µl of faecal samples after homogenisation (Promega Wizard genomic DNA extraction kit; Madison, WI, USA) following the manufacturer's instructions. PCR was performed using primers from the β-tubulin gene region of *C. parvum* (Cacciò et al., 1999) as described previously (Kar et al., 2010).

The infective dose of 1×10^6 oocysts of *C. parvum* was expected to provoke clinical cryptosporidiosis according to previous experimental studies in lambs (Ortega-Mora and Wright, 1994; Bukhari and Smith 1997) and to compare clinical and laboratory results obtained with those published in the literature. The day prior to inoculation of *C. parvum* oocyst in the EXP group or an equal volume of physiologic saline in the CON group was designated as day (d)-1, and all data collected through the d −1 were considered as baseline values. Lambs at 24 h of age in EXP group were infected by oral inoculation of 1×10^6 *C. parvum* oocyst suspended in 5 ml of sterile distilled water at 24 h of age as reported by Bukhari and Smith (1997), whereas lambs in