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Evaluation of Endotoxaemia in the Prognosis and Treatment of Scouring Merino Lambs

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Summary

This study looked at measurement of endotoxaemia as a tool in determining prognosis and probable response to treatment in scouring lambs. One hundred eighty-three lambs in the first 15–20 days of life, from eight Merino sheep farms located in the region of La Serena, south-west Spain, were used in this experiment. Scouring and normal/control lambs were selected following a clinical examination, the scouring group was further divided into subgroups, specifically those that did or did not survive 72 h following treatment. At the time of the clinical examination, faecal and blood samples were taken. Faecal culture and commercial faecal antigen tests for detection of enteropathogens in faeces and serum endotoxin measurement using chromogenic lymulus amoebocyte lysate (LAL) were carried out. Scouring lambs received 0.07 mg/kg liveweight halofuginone once a day for 3 days, a single oral dose of 0.20 mg/kg liveweight of spectinomycin and oral rehydration fluid. The pathogens isolated were *Cryptosporidium* spp. and *Escherichia coli*. The case fatality rate was 51% in the scouring lambs. Postmortem findings were consistent with enterotoxigenic *E. coli* infection. The concentration of endotoxin was 0.18 ± 0.12 ng/ml in the control group, 0.35 ± 0.17 ng/ml in the surviving lambs and 0.46 ± 0.14 ng/ml in the non-surviving lambs. Significant differences between groups were found. Case fatality rate of the scouring lambs with endotoxaemia below 0.30 ng/ml was 0%, while it was 100% above 0.50 ng/ml. These results may be utilized as a prognostic indicator in lambs affected by *E. coli* and *Cryptosporidium* that will help aid in decision-making as to whether to treat a lamb or not based on its chances of survival.

Introduction

It is well known that *Escherichia coli* and *Cryptosporidium* spp. are involved in the multiple aetiology of the scouring syndrome (Muñoz et al., 1996). This syndrome is an important cause of neonatal deaths or losses pre-weaning in sheep, especially in housed lambs. In the three most prominent farmer associations in the region of the study, losses are estimated to be approximately 3.14 million Euros per year (Agriculture Research Service, personal communication). These significant financial losses are attributed to the costs of treatment, the loss in production and the death of lambs, often by endotoxaemia. Endotoxaemia is defined as the presence of endotoxin in blood and is characterized by signs caused by the organic reaction against the lipopolysaccharide component of the outer leaflet of the outermembrane of Gram-negative bacteria (MacKay, 2002). If serum endotoxin concentration could be used to predict the probable success of treatment, then this measurement could be used to avoid unnecessary costs associated with treating lambs with a very reduced likelihood of survival.

Chromogenic lymulus amoebocyte lysate (LAL) assay is a quantitative method to determine the concentration of endotoxin (Hurley, 1995). This test has been used to measure endotoxaemia in humans and animals suffering from different disorders (Hodgson et al., 1999; Haussman et al., 2000; Wittek et al., 2004). It has been shown that this method is more sensitive than other available methods for the measurement of endotoxin (Fujiwara et al., 1999). The chromogenic LAL test is as much as 300 times more sensitive than the rabbit pyrogen test (Hurley, 1995). Endotoxaemia measurements obtained by chromogenic LAL assay have been used to predict non-survival or a favourable outcome in horses with acute abdominal disease (Barton and Collatos, 1999). The aim of this study was to investigate the role of endotoxaemia, assessed by LAL assay, in determining the prognosis for the survival of lambs with severe diarrhoea in order to avoid unnecessary treatment expenses in lambs with a very poor chance of survival.

Materials and Methods

Trials were performed according to an approval issued by the Regional Government and European Funds for Regional Development (Project 2PR03B024).

Eight Merino sheep commercial farms were selected for this experiment, which was conducted in autumn as it is the season with the increased incidence of scouring syndrome in this area. At the time of the trial, all the flocks presented natural outbreaks of diarrhoea affecting lambs in the first 15–20 days of life, with a prevalence of 10–75%. The farms were located in the same geographical area (La Serena, south-west Spain) and they were managed under similar health, nutrition and husbandry practices.

On each of the eight farms, lambs were examined to identify 'scouring' lambs with active diarrhoea but maintaining good general state and 'healthy' lambs without signs of diarrhoea, dehydration or fever. The number of lambs selected in each farm was proportional to the prevalence of the scouring syndrome in the flock (Table 1) with a total of 54 healthy

Table 1. Number of animals in the experiment, proportion of lambs scouring, case fatality rate of the farms and serum endotoxin level (mean ± standard error) in the control and the scouring lambs

Farm	Proportion of lambs scouring	Control lambs	Scouring lambs		Case fatality rate (%)
			Recovered	Dead	
1	20/180	3	6	–	0*
2	100/130	9	18	30	62.5
3	42/90	3	6	12	66.6
4	30/80	9	3	9	75
5	25/100	9	3	6	66.6
6	20/150	6	9	–	0*
7	34/90	9	12	3	20*
8	36/100	6	6	6	50
Total		54	63	66	51
Serum endotoxin level (ng/ml)		0.18 ± 0.12[a]	0.35 ± 0.09[b]	0.46 ± 0.14[c]	

*Case fatality rates in these farms are significantly different ($P < 0.05$) from the rest of the farms.
[a, b, c]Mean values with different superscripts are significantly different ($P < 0.05$).

lambs (control group) and 129 lambs with diarrhoea included in the study.

At the time of the physical examination, samples of faeces were taken from the rectum of the lambs using sterile culture swabs (Eurotubo®, IASA, Barcelona, Spain) and 2 ml blood were withdrawn from the jugular vein into sterile clotting tubes (Eurotubo®). The scouring lambs were treated with an oral dose of 0.07 mg/kg liveweight halofuginone (Halocur®, Intervet, Salamanca, Spain) once a day for 3 days, a single oral dose of 0.20 mg/kg liveweight spectinomycin (Spectamporcelet®, Ceva, Barcelona, Spain) and 250 ml of an oral rehydration solution (Rehydion®, Ceva).

All the animals were under veterinary supervision and experienced teams of observers monitored lambs at regular intervals during the 72 h following clinical examination and treatment to assess the outcome of treatment. At the end of the 72-h period, the scouring lambs were identified as 'recovered' ($n = 63$) or 'deceased' ($n = 66$) (Table 1). All the treated lambs that did not die stopped scouring and became clinically normal. The dead lambs were examined post-mortem for gross evidence of disease.

Detection of enteropathogens

The infectious agents involved in scouring were investigated by culture in specific media, immune assay and polymerase chain reaction (PCR). Enteric bacteria were cultured on Agar MacConkey® (Oxoid, Madrid, Spain) and Agar XLT4® (Merck, Barcelona, Spain). Specific culture media were used for the culture of *Clostridium* spp. (Blood Agar®, Oxoid) and *Campylobacter* spp. (Modified Brucella Agar®, Oxoid). The cultures were carried out according to the procedures of Carter and Chengappa (1990). Commercial immunochromatographic faecal antigen detection tests, validated for use in ruminants, were performed as per manufacturer's instructions and used to detect antigens of Rotavirus spp. (Rota Vet Uni-Strip®, Coris Bioconcept, Brussels, Belgium), Coronavirus spp. (Corona Vet Uni-Strip®, Coris Bioconcept), *Crytosporidium* spp. (Crytpo Vet Uni-Strip®, Coris Bioconcept) and enterotoxigenic *Escherichia coli* K99 (K99 Vet Uni-Strip®, Coris Bioconcept) in faeces. A PCR technique validated for use in sheep was carried out according to Rey et al. (2003). This method was used to identify virulent *E. coli* genes (primers and PCR mixture, Amersham Biosciences, Barcelona, Spain).

Endotoxin assays

Serum was preferred to avoid the inhibitory effects of anti-coagulants on the LAL assay (Hurley, 1995). Following serum extraction, samples were treated by dilution with pyrogen-free water (Cambrex Iberia Products, Barcelona, Spain) and heated at 70° for 10 min to remove non-specific inhibitors present in blood products (Friberger et al., 1982). Samples were assayed for endotoxin with the chromogenic LAL test according to the manufacturer's instructions (chromogenic LAL lysate test, QCL 1000®, Cambrex Iberia Products). Colour released was measured by photometry (spectrophotometer Shimazdu UV 160 A®, Pacisa, Spain) at 405 nm after the reaction was stopped.

Statistical analysis

After checking the normality of the distribution of data, the differences between the concentrations of endotoxin in the serum of the lambs in the control group, the recovered subgroup and the deceased subgroup were tested for statistical significance by using ANOVA. Contingency tables, calculation of odds ratio (OR) and chi-squared test were employed to evaluate the statistical significance of any differences for case fatality rates among farms and between the proportion of lambs dead or recovered in the scouring group at different levels of endotoxaemia. The statistical package G-Stat 2.0 was used (Letón and Marino, 2002).

Results

Cultures from all the scouring lambs in all the farms were positive for *E. coli* and *Cryptosporidium* spp. However, enterotoxigenic K99 strains were not isolated. The rest of enteropathogens included in the study, i.e. *Campylobacter jejuni*, Rotavirus spp., Coronavirus spp. and *Salmonella* spp. were not detected.

Case fatality rates are shown in Table 1. The mean case fatality rate was 51% in scouring lambs but varied significantly between farms (range 0–75%). It was observed that case fatality rate was higher than 50% in five of eight farms. All the dead lambs showed the following postmortem findings: inflamed gastrointestinal tract with multiple haemorrhages on the mucosal surfaces distended with fluid and gas, and enlarged and reactive mesenteric lymph nodes.

The concentration of endotoxin in the serum of the lambs in the control group was 0.18 ± 0.12 ng/ml (mean ± SE) with values of zero in 17 of 54 healthy lambs (range 0–0.36 ng/ml). In the lambs suffering from diarrhoea, the mean concentration of endotoxin in serum were 0.35 ± 0.17 ng/ml for the lambs recovered after treatment (range 0.24–0.45 ng/ml) and 0.45 ± 0.14 ng/ml for the dead lambs (range 0.31–0.72 ng/ml). Significant differences ($P < 0.05$) were found between all three groups.

The percentage of lambs that recovered or died in the scouring group at different concentrations of endotoxins, from

Table 2. Relationship between serum endotoxin level (ng/ml) and recovery chance in the scouring group

Endotoxin concentration	Deceased (%)	Recovered (%)	OR (95% CI)	χ^2-value	*P*-value
>0.20	51.16	46.51	3.2927	1.0726	0.3004
≤0.20	0	2.33	0.12–85.4		
>0.25	51.16	44.19	5.7692	2.1974	0.1382
≤0.25	0	4.65	0.26–127		
>0.30	51.16	37.21	15.000	5.9216	0.0149
≤0.30	0	11.63	0.77–290		
>0.35	48.84	23.26	23.100	12.224	0.0005
≤0.35	2.33	25.58	2.60–204		
>0.40	46.51	16.28	20.000	15.248	0.0001
≤0.40	4.65	32.56	3.60–110		
>0.45	37.21	4.65	25.333	17.632	0.0001
≤0.45	13.95	44.19	4.47–143		
>0.50	30.23	2.33	28.889	14.444	0.0001
≤0.50	22.93	46.51	3.26–255		

<0.20 ng/ml to >0.50 ng/ml, and OR and chi-square values are included in Table 2.

Discussion

Endotoxins are normally present in the intestinal contents. Although gut wall is an efficient barrier preventing these substances from crossing, a small quantity is absorbed into the portal circulation. The liver eliminates these toxins and only a small concentration reaches the peripheral circulation (MacKay, 2002). This explains the presence of low quantities of endotoxin in the serum of the healthy lambs of this experiment. The concentrations detected were 0 in the 31% of the control lambs and very low in the rest (range 0–0.36 ng/ml). These results are consistent with those reported by Barton and Collatos (1999) in healthy horses; and by Nadhazi et al. (2002) in human blood donors, with <0.1 ng/ml (0.001–0.1 ng/ml).

The concentrations of endotoxin recorded in the scouring lambs were similar to those registered in horses affected by colic (Barton and Collatos, 1999) and in human patients suffering from septic shock (Danner et al., 1991). High concentrations of endotoxin may be attributed to the ischaemic injury caused to the digestive mucous barrier by bacterial overgrowth, decrease of intestinal pH or inflammatory intestinal disorder. In this instance, large quantities of endotoxin are absorbed from the intestine, overwhelming the detoxifying ability of the liver and increasing the concentration of endotoxin in the peripheral circulation (King and Gerring, 1988; Haussman et al., 2000).

The significantly higher serum endotoxin concentration in the scouring lambs compared with the healthy lambs was consistent with the findings of other authors that compared serum endotoxin concentration in horses with colic and healthy horses (Barton and Collatos, 1999). The increase in circulating endotoxin concentration may be attributable to the adverse effects of gastrointestinal disease on the integrity of the gut wall.

The microbiology results were consistent with typical findings in lambs affected by 'scouring syndrome' in southwestern Spain (Muñoz et al., 1996). Halofuginone has been reported to be highly effective against *Cryptosporidium* spp. infection in calves (Lefay et al., 2001). Spectinomycin has been shown to be an effective treatment for *E. coli* infections in lambs (Hodgson et al., 1999). In the current experiment, combined spectinomycin and halofuginone treatment, together with an oral rehydration solution, was an effective treatment for scouring in lambs with a serum endotoxin concentration ≤0.30 ng/ml but not in lambs with a serum endotoxin level ⩾0.50 ng/mL. Similarly, lambs that recovered had a significantly lower average serum endotoxin measurement compared with lambs in which treatment was unsuccessful. In addition, the deceased lambs showed gross pathological changes consistent with enterotoxigenic *E. coli* infection. This suggests the potential prognostic value of serum endotoxin measurement and the possibility of using serum endotoxin measurement to predict the likely success of treatment. Treatment of lambs with serum endotoxin levels above an established reference range would carry no guarantee of success.

The association between the presence of endotoxin in blood and the unfavourable prognosis of severe digestive disorders has been suggested previously (Brandtzaeg et al., 1989; Danner et al., 1991). In the present study, no lamb in the scouring group with a serum endotoxin concentration in serum lower or equal to 0.30 ng/ml died, and no lamb with a endotoxin concentration above 0.50 ng/ml survived (Table 2).

The increasing significance of the OR values (Table 2) pointed at an inverse relationship between the probability of survival of the lambs affected by the scouring syndrome and the degree of endotoxaemia. This supports the concept of measurement of serum endotoxin using chromogenic LAL as a tool for predicting prognosis for survival and likely response to treatment in lambs affected by *E. coli* and *Cryptosporidium* spp.

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