REPRODUCTION OF PROLIFERATIVE ENTEROPATHY IN FOALS USING PORCINE INTESTINAL MUCOSAL HOMOGENATE


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Summary


Proliferative enteropathy (PE) is an intestinal disease that affects equine and several other species. The goal of this study is to determine whether proliferative enteropathy could be experimentally reproduced in foals. Seven foals were divided into two groups: A (n=5), and B (n=2). The foals were inoculated intragastrically with porcine intestinal mucosal homogenate (group A), or a placebo challenge (group B). Ante mortem and post mortem tests were performed. All foals were euthanised on day 22 post challenge. Physical examination detected variable signs of depression, colic, decreased appetite, diarrhoea, dehydration and emaciation in three foals in group A. At necropsy, one foal (foal 5, group A) was severely emaciated, the wall of the ileum and distal jejunum was thickened and the mucosa was corrugated and hyperaemic. Histologically, hyperplasia of immature enterocytes and reduced number of Paneth and goblet cells were observed. PCR confirmed the presence of Lawsonia intracellularis. The foals in groups A were positive for L. intracellularis by immunohistochemistry. The control foals (group B) remained physically normal, had no pathologic lesions and were negative by immunohistochemistry. PE was reproduced in foals using intestinal mucosal homogenate. Physical, gross and histopathologic alterations typical of PE were detected in some foals. The presence of L. intracellularis was confirmed by immunohistochemistry and/or PCR. The role of L. intracellularis from different species in the etiopathogenesis of PE in the horse was established.

Key words: experimental reproduction, foals, Lawsonia intracellularis, proliferative enteropathy

INTRODUCTION

Proliferative enteropathy (PE) is an enteric disease that affects weanling foals (Lavoie et al., 2002). The disease has been described in multiple animal species including horses, pigs, hamsters, deer, dogs, blue foxes, guinea pigs, rats, ferrets, monkeys, rabbits, emus and ostriches (Williams et al., 1996; Cooper et al., 1997; Lawson & Gebhart, 2000). In horses, PE was described for the first time in
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L. intracellularis is an intracellular bacterium that is non-culturable in conventional media (Lawson et al., 1993; McOrist et al., 1995). To our knowledge, there is one report of experimental reproduction of PE in horses in which the infection was reproduced using a pure culture (Pusterla et al., 2010). The disease has been reproduced successfully in swine and hamsters (Roberts et al., 1977; Stills, 1991). In swine, challenge models using pure culture of L. intracellularis usually produce clinical and macroscopic lesions comparable to the chronic form, porcine intestinal adenomatosis (PIA), of the disease in growing pigs (McOrist et al., 1993). Hamsters experimentally infected with a porcine source of L. intracellularis do not usually present any clinical signs of PE other than reduced growth rate (Gebhart, 1987).

The goal of this study was to determine whether PE can be experimentally induced in horses with a porcine mucosal homogenate containing L. intracellularis. Experimental induction of PE in foals, clinical monitoring and laboratory testing of blood parameters will provide us with valuable information concerning clinical signs and pathology of the disease.

MATERIALS AND METHODS

Sample population

Seven two-month-old foals were obtained from a herd with no history of PE in the past two years. One week prior to challenge, the foals were weaned and screened for L. intracellularis using serology and faecal PCR utilising previously described methods (Cooper et al., 1997, Guedes et al., 2002). The foals were also screened for other intestinal bacteria (Salmonella spp., Clostridium difficile), and parasitic (using faecal flotation) diseases of horses. One day before challenge, the foals were divided into two groups, challenged group A (n=5) and control group B (n=2). The foals were kept off feed overnight before the challenge day. Animals in group A were inoculated intragastrically with intestinal mucosal homogenate derived from experimentally Lawsonia-infected pigs that tested Salmonella spp. negative using PCR and culture enrichment. Each foal received 4.5×10⁹ L. intracellularis organisms, scraped from the affected mucosa of the pigs and diluted in a proportion of 1:1 (w/v) in sucrose potassium glutamate (SPG) with 10% foetal bovine serum (FBS) solution (McOrist et al., 1993). The control foals received a placebo challenge of SPG with 10% FBS only. Each foal was incubated with 250 mL of the challenge material. The foals were housed in the Research Animal Resources Facility at the College of Veterinary Medicine, University of Minnesota. The study protocol was conducted according to the guidelines of the Animal Care and
Sampling Procedure

Whole blood, serum and faecal samples were collected at days 0, 7, 14, and 21 post challenge. The foals in groups A and B were humanely euthanised on day 22 with the exception of one foal (No 5), which was euthanised on day 19 due to severe signs of depression, decreased appetite, diarrhoea, dehydration, emaciation and hypoalbuminaemia. Tissues from duodenum, jejunum, ileum, large colon, caecum, small colon and mesenteric lymph nodes were formalin-fixed, processed for routine histopathology by the hematoxylin and eosin (H&E) method and by immunohistochemistry specific for L. intracellularis. Sections from stomach, liver, kidney, lung, spleen and heart were also formalin-fixed and processed for routine histopathology (H&E).

Ante mortem testing

Physical examination, including evaluation of body temperature, appetite, attitude, lung sounds, respiratory and heart rates, gut sounds and motility, digital pulse, and mucus membranes, was performed every day. Complete blood counts (CBC) and serum chemistry profiles were performed on days 0, 7, 14, and 21. Ultrasound examination of the abdominal cavity was performed on days –1 and 21. The ultrasound examination evaluated thickness, content and smoothness and motility of the small intestine, large colon and caecum. Serology, including a slide immunoperoxidase assay (slide-IPX), an immunoperoxidase monolayer assay (IPMA) and faecal PCR testing were performed on days 0, 7, 14, and 21 post-infection.

Slide immunoperoxidase assay (slide-IPX)

Glass slides with 15 wells, coated with L. intracellularis, were prepared as described elsewhere (Guedes et al., 2002). Briefly, L. intracellularis were harvested from the supernatant of infected cell culture monolayers, concentrated by centrifugation and inactivated via addition of 0.2% formalin. Ten µL of the suspension, containing 1.5×10⁶ bacteria/mL, was added to each well on 15-well slides. The slides were dried and fixed with cold acetone for 20 s. Serum samples were diluted in PBS at two fold dilutions (1:15, 1:30, 1:60 and 1:120) and 10 µL of each diluted sample was added to each well. Slides were incubated for 30 min at 37 °C and washed with PBS. Horseradish peroxidase conjugated rabbit anti-horse IgG diluted 1:500 in 4% FBS was added and slides were incubated for 45 min at 37 °C. Excess fluid was discarded and slides were incubated at room temperature with 3-amino-9-ethyl-carbozole (AEC) for 20 min.

Immunoperoxidase monolayer assay (IPMA)

The IPMA serologic test used for pig serum was modified for horse serology (Guedes et al., 2002). Fifty µL of 1% bovine serum albumin (BSA) was added to rehydrate the antigen and as blocking buffer and plates were incubated for 30 minutes. Serum samples were diluted 1:15 to 1:480 in PBS and 30 µL were added to each well. Plates were incubated for 30 min at 37 °C and then washed 4 times with PBS. Horseradish peroxidase conjugated rabbit anti-horse IgG diluted 1:500 in 1% BSA was added to wells and
incubated for 45 min at 37 °C in a wet chamber. After washing, AEC was added and incubated at room temperature for 20 min.

**Faecal PCR**

The QIAamp DNA stool mini kit was used to extract DNA from feces. Primers 878F 5’-TAA-CGC-GTT-AAG-CAY-C-3’ and 1050R 5’-GTC-TTG-AGG-CTC-CCC-GAA-AGG-CAC-CTC-TTA-ATC-3’ that were developed from the 16S rDNA sequence of *L. intracellularis* (GeneBank accession number L15739) and primers A (5’-TAT-GGC-TGT-CAA-ACA-CTC-CG-3’) and B (5’-TGA-AGG-TAT-TGG-TAT-TCT-CC-3’) that amplify a random genomic fragment of *L. intracellularis* (GenBank accession number L08049) were used (Jones et al., 1993; Cooper et al., 1997). PCR was performed in 25 μL volumes containing PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dATP, dTTP, dCTP and dGTP, 1 U Taq polymerase, 45 μM of each of primers 878F and 1050R and 30 μM of each of primers A and B and 5–10 ng of extracted sample DNA. PCR was performed in a thermal cycler 9800. Samples were subjected to 35 cycles of denaturation at 93 °C for 30 s, annealing temperature of 45 °C for 1 min, and an extension temperature at 72 °C for 1 min. PCR products were electrophoresed on 2% agarose gel for 2 h at 85 V. Samples were considered positive only when they produced two bands: a band of 182 bp using primers 878F and 1050R and a band of 319 bp using primers A and B.

**Post mortem examination**

Foals in groups A and B were euthanised 22 days after inoculation. The jejunum, ileum, caecum and colon were evaluated in each foal for lesions typical of PE.

**Histology and immunohistochemistry**

The formalin-fixed samples were processed routinely for histology, embedded in paraffin, and sectioned 5 μm thick. All tissue sections were stained by hematoxylin and eosin. Selected tissue sections were stained by the immunoperoxidase method of labelled streptavidin with polyclonal antibodies to *L. intracellularis* as well as with Warthin-Starry silver stain (Guedes et al., 2003a).

**RESULTS**

**Ante mortem testing**

In group A, the daily physical examination revealed signs suggestive of PE in three foals. Two foals, foal 1 and 2, had mild signs of depression, reduced appetite, colic and loss of body weight. One foal, foal 5, had severe clinical signs of PE. This foal gradually lost weight from day 8 through day 19 and had decreased gut motility on days 11 to 13 after which the motility increased until day 19 (Fig. 1). The foal passed soft faeces on day 14 that progressed to profuse watery diarrhoea until day 19. On days 17 and 18, the foal was very weak, severely emaciated and recumbent and on
day 19 it was euthanised for humane reasons. Physical examination of two other challenged foals, foal 3 and 4 detected no unusual findings.

Foal 5 had elevated fibrinogen, 8 g/L (normal range 1–5 mg/dL), on day 14. On day 7, the albumin was slightly low, 28 g/L (normal range 29–37), with normal total protein but on day 14 the total protein was 55 g/L (normal range 58–75) and the albumin was 24 g/L. The total plasma protein averaged 54 g/L in the challenged foals and the albumin averaged 24.2 g/L. Ultrasound examination of the foals in group A showed no significant findings. Foal number 5 was not evaluated during the second examination since it was euthanized on day 19. Serologic testing failed to detect antibody titer to *L. intracellularis* in any foal up to day 21 post challenge. Faecal PCR was negative in all the foals with the exception of foal 5 which was positive on days 14 and 19 (Fig. 2).

Daily physical examination of the control foals (group B) detected no abnormal findings. In addition, CBC and serum chemistry analysis showed no significant deviations. The foals were negative using the serologic tests and faecal PCR.

**Post mortem testing**

At necropsy, gross pathologic examination of foal 5 indicated that it was severely emaciated and had soft, non-formed faeces in the small colon. The wall of the ileum and distal jejunum up to 2 metres from the ileocecal valve was thickened and the mucosa was corrugated and hyperaemic (Fig. 3). Histopathologic examination of foal 5 showed that the crypts of the ileum were elongated, with marked proliferation of the enterocytes.
resulting in formation of multiple layers of cells (Fig. 4). The villi of the ileum were severely atrophied. The number of Paneth and goblet cells was reduced in the hyperplastic crypts. Similar pathologic changes were detected in the jejunum up to 4.7 m proximal to the ileocecal valve. No abnormal pathologic lesions were detected in the duodenum or the large intestines. Two additional foals in group A, foals 1 and 2, had signs of poor body condition during gross pathologic examination however histopathologic examination revealed no significant findings.

Immunohistochemistry staining of foal 5 intestinal tissue detected abundant L. intracellularis-specific antigen in the apical part of the cytoplasm of enterocytes in the hyperplastic crypts and villi (Fig. 5). Mononuclear cells in the lamina propria also had bacterial antigen in the cytoplasm. The bacteria were detected in the ileum, jejunum and duodenum, but only in the lamina propria of the duodenum.

Bacterial antigen was also detected in the cytoplasm of the mononuclear cells in the ileocecal lymph nodes (Fig. 6). Scattered bacteria were detected in the surface epithelium of the large and small colon and in the lamina propria in the caecum. IHC testing of the other 4 challenged foals detected L. intracellularis antigen in focal areas in the lamina propria in the ileum and jejunum.

The foals from the control group (B) had no abnormal findings during gross and histopathologic examinations. In addition, no Lawsonia-specific staining was detected in the tissue from the foals.

DISCUSSION

L. intracellularis has been associated with PE in horses (Williams et al., 1996). In this study, intestinal mucosa homogenate obtained from pigs that were experimentally infected with L. intracellularis was successfully used to reproduce the disease in foals. The use of porcine intestinal mucosa homogenate provides great advantage since it is available for larger scale studies while pure culture may be available for limited studies. Although the disease has been reported to occur in foals up to 7 months of age (Duhamel & Wheel- don, 1982; Williams et al., 1996; Frank et al., 1998; Brees et al., 1999; Schumacher et al., 2000; Lavoie et al., 2002) our pre-
previous experience with reproducing PE in foals 4–6 months of age that weighed 150 to 200 kg failed (Al-Ghamdi, unpublished data). There is a possibility that these foals were previously exposed to *L. intracellularis* and, therefore, developed natural immunity to the challenge. In pigs, successful challenge with *L. intracellularis* was possible in animals between 4–5 weeks of age and weighing 10–15 kg (McOrist *et al.*, 1993; Guedes & Gebhart, 2003b). This suggests that experimental challenge with *L. intracellularis* is more likely to induce pathologic disease in very young foals that have no previous exposure to *L. intracellularis*.

Severe clinical signs suggestive of PE were observed in one foal only. Similar
signs of anorexia, lethargy and loose faeces were reported during the experimental reproduction of the disease by Pusterla (Pusterla et al., 2010). Some of these signs were observed during the second week. This foal was the smallest in size and probably the youngest among challenged foals in group A. This may indicate that the disease is more severe in young foals. A change in the normal flora of the intestine induced by weaning may have resulted in an increased susceptibility of the foals to the disease. An additional reason might be the elimination of milk from the diet which may have provided some intestinal protection to the unweaned foals through molecular components such as secretory IgA, cytokines, lactoferrin, oligosaccharides, nucleotides, lysozyme and other protein molecules that may be found in milk (Kelleher & Lonnerdal, 2001). The presence of immune cells including macrophages, lymphocytes and neutrophils may also be present in milk (Riedel-Caspari, 2001). However, since all foals were weaned at the same time these factors may not explain the severity of the disease in foal 5. Variation in size and possibly age is the most logical explanation since foal 5 was apparently the youngest in the group and therefore may have had the least mature immune system and less chance to develop acquired immunity. Two additional foals in the challenge group A exhibited minor clinical changes such as sporadic episodes of colic, failure to gain weight and hypoproteinaemia. These signs are consistent with reported symptoms of natural PE cases in horses as well as experimentally reproduced disease (Frank et al., 1998; Lavoie et al., 2000; Pusterla et al., 2010).

Although foal 5 showed severe signs of the disease and extensive pathologic lesions, serologic tests failed to detect any positive antibody response to *L. intracellularis*. Therefore, this foal was probably unable to mount a normal immune response to *L. intracellularis*. The other 4 foals in the challenge group also failed to mount detectable immune response despite fairly normal clinical conditions. Our previous experience with *L. intracellularis* challenge in older foals, 4-6 months of age, showed that foals may produce an antibody response by day 14 postchallenge without showing clinical symptoms of PE (Al-Ghamdi, unpublished data). The fast immune response in these foals might be due to a previous exposure to *L. intracellularis* and therefore by the time of challenge the immune system was more mature. In this study, the immune response might require more time in the course of the disease to be detected. In pigs, challenged animals had detectable antibody titre as early as day 14 and cell mediated immune response at 21 days postchallenge (Guedes & Gebhart, 2003b). However, on occasion, challenged pigs may not develop a detectable immune response (McOrist et al., 1993). Since *L. intracellularis* is an intracellular organism, the bacteria may be capable of evading normal body immune defense through establishing a sheltered environment inside the host cells.

Gross and histopathologic examinations detected no abnormal lesions in the control and challenged foals in group A except foal 5. The lesions typical of PE observed in these two foals reflected the poor clinical condition of the foals. Faecal PCR testing of foal 5 detected *L. intracellularis* shedding on days 14 and 19, similar to shedding time in a previous report (Pusterla et al., 2010). PCR testing of the other challenged foals detected no positive cases. Clearly, PCR results reflected the clinical and pathological status of the foals. Finally, the use of IHC
and Warthin-Starry silver stain confirmed the physical and pathologic findings. Large numbers of bacteria were detected in the ileum, and jejunum of foal 5 (group A). In addition, the four other challenged foals (group A) tested positive using IHC, however to a lesser degree. These foals may not have been infected with sufficient number of bacteria to produce clinical changes. Nonetheless this indicates that IHC may detect \textit{L. intracellularis} in horses that have no obvious clinical disease.

In conclusion, PE was reproduced in foals using intestinal mucosa homogenate obtained from experimentally \textit{Lawsonia}-infected pigs. Signs consistent with PE were detected using physical examination, chemical profile, gross and histopathologic examination. In addition, the causative agent of PE, \textit{L. intracellularis}, was detected with faecal PCR. IHC not only confirmed these finding but also detected the bacteria in clinically normal challenged foals.

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