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Effect of *Cryptosporidium parvum* infection on the absorptive capacity and paracellular permeability of the small intestine in neonatal calves

Pavel Klein [a,*], Tereza Kleinová [a], Zdeněk Volek [a], Jiří Šimůnek [b]

[a] *Institute of Animal Science, Pratelstvi 815, Praha-Uhrineves, CZ-10401, Czech Republic*
[b] *Institute of Animal Physiology and Genetics, Academy Of Sciences of The Czech Republic, Praha, CZ-14220, Czech Republic*

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Abstract

Cryptosporidium parvum is recognized as one of the most important pathogens causing enteritis and severe diarrhoea in calves up to 1 month of age. Although the infection may be responsible for some mortality, its impact is mainly associated with the impairment of intestinal functions and lower performance of animals. The aim of this study was to determine the effect of cryptosporidiosis on the intestinal functions in neonatal experimentally infected Holstein calves. Absorption tests with D-xylose and retinyl-palmitate, and the lactulose/mannitol test of intestinal permeability were simultaneously performed in 1-week intervals from challenge to full recovery. In infected animals, reduced intestinal absorptive capacity for both D-xylose and retinyl-palmitate was observed on day 7 post-infection (p.i.). At the same time, a more than 100% elevation of intestinal permeability was observed in the infected calves. All intestinal functions, except absorption of retinyl-palmitate, were significantly affected and changes were detected up to day 14 p.i. In contrast, results of all tests obtained on day 21 p.i. suggest full recovery of the infected intestine. Significantly, growth of the calves which had recovered from cryptosporidiosis was still affected between days 14 and 21 p.i.

Keywords: Calves; *Cryptosporidium parvum*; Intestinal absorption; Intestinal permeability

1. Introduction

Diarrhoeal diseases of infectious aethiology represent one of the major health problems in calves in the postnatal period. Since the infection is localized at the site of food digestion and absorption of nutrients, performance and nutritional status of the infected host is often negatively affected (Gookin et al., 2002).

One of the most frequent pathogens responsible for outbreaks of severe diarrhoea, mainly in calves up to 1 month of age, is the apicomplexan parasite *Cryptosporidium parvum*. For lack of effective drugs, this zoonotic, cosmopolitan coccidiosis is difficult to cure. On farms with endemic occurence of *C. parvum*, morbidity of calves up to 1 month of age may reach 100% (de Graaf et al., 1999). Calf mortality due to cryptosporidiosis, especially among animals receiving good zoohygiene and care, is usually low. The biggest losses are thus mainly indirect, due to the extra costs associated with the care of sick animals, and due to growth impairment.

The pathogenesis of cryptosporidiosis is not fully explained, but it has been found that the parasite causes destruction of intestinal epithelia resulting in a

* Corresponding author. Tel.: +420 267 009 547; fax: +420 267 710 779.
E-mail address: pavel.klein@seznam.cz (P. Klein).

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reduction of villi and microvilli. This morphological damage leads to a decrease in enzymatic activity and reduction of the absorptive surface, which finally leads to maldigestion and malabsorption followed by diarrhoea (Argenzio et al., 1990; Gookin et al., 2002). Studies on infected children, moreover, indicate that mucosal damage caused by cryptosporidia is associated with higher paracellular permeability of the intestine and an impairment of the mucosal barrier function (Kukuruzovic et al., 1999).

Both the absorptive and barrier function of the intestinal epithelia may be assessed by simple non-invasive tests based on the transepithelial transport of appropriate markers. The D-xylose absorption test is used to mirror absorption of sugars (Hammon and Blum, 1997), whereas vitamin A absorption is similar to that of fat, as factors affecting lipid digestion and absorption are also likely to affect vitamin A absorption (Legerton et al., 1953).

Mucosal integrity may be well measured by the lactulose/mannitol test (Uil et al., 1997; Hall, 1999). The principle of the test is based on assumption that D-mannitol passes intestinal epithelia transcellulary, through small pores on the apical surface of enterocytes, whereas lactulose passes epithelia paracellulary, through tight junctions between the epithelial cells. Higher uptake of lactulose and its higher ratio to D-mannitol in urine therefore reflects impairment of the intestinal barrier function, i.e. a ''leaky gut'', as seen in many pathological processes in gut (Hall, 1999).

Although the negative effect of cryptosporidiosis on the function and morphology of the calf intestine has been described, these data are almost exclusively related to the acute phase of the disease. The aim of the present study was therefore to describe the changes in intestinal permeability and absorptive capacity in calves experimentally infected with *C. parvum* from the time of challenge to their complete recovery. The lactulose/mannitol intestinal permeability test, the load absorption tests with D-xylose, and the absorption test with retinyl-palmitate were used in parallel for non-invasive monitoring of the status of the small intestine.

2. Materials and methods

2.1. Animals, housing, diet

A total of 19 male Holstein calves aged 6 days (±36 h) at the beginning of the experiment were allocated to (A) an uninfected control group (9 animals) and (B) an infected group (10 animals). All calves were obtained from the same commercial dairy farm (890 dams) near Prague, Central Bohemia, from groups of cows with synchronized oestrus and insemined at the same time. The cows were vaccinated with a combined vaccine against rotaviruses, coronaviruses and enteropathogenic *E. coli* (Rotakol, Mevak, Slovakia). Rigorous hygienic conditions during the calving and transport were maintained. Both groups were created from all healthy calves born live during the 72 h of the expected calving period.

Immediately after birth, calves were isolated from other animals, fed with colostrum, and moved to the experimental facility at the Institute of Animal Science in Prague, where they were adapted to the new environment for at least 2 days.

Calves were penned individually in boxes with concrete floor and polypropylene washable walls, excluding contact between animals. Wood shavings were used as bedding and changed daily.

The first four feedings were colostrum from each calve's own mother. Next, two feedings of a 1:1 mixture of colostrum from a frozen pool with milk replacer were given. The colostrum was purchased from the native farm. Milk replacer (Telasan AA, Bodit, Czech Republic) was then fed exclusively throughout the study at a quantity equivalent to 15% body weight, divided into two equal daily feedings. Between days 5 and 6 post-infection (p.i.), after onset of the symptoms of cryptosporidiosis in the infected group, four calves exhibited apathy, inappetence and signs of acute dehydration. These animals were therefore drip-fed by infusion (Rehyvet, Infusia, Czech Republic). An infusion was connected to a permanent intravenous catheter as described below. The duration of drip-feeding did not exceed 1 day.

In order to prevent transmission of the infection between controls and infected animals, the experiment was initiated with the nine uninfected control animals. Immediately after the observation of control calves was terminated, the same experiment was carried out on 10 infected animals using the same protocol.

2.2. Experimental infection and coprological examinations

Oocysts of *C. parvum*, bovine isolate VUZV-N maintained in Institute of Animal Science in Prague by propagation in calves, were refreshed in a neonatal Holstein calf 1 week before the experimental infections. Oocysts were isolated from faeces on secondary sucrose gradient (Arrowood and Sterling, 1987), then treated in 1% sodium hypochlorite (20 min, 4 °C) and finally washed three times in sterile PBS by centrifugation

(1500 × *g*, 10 min). Oocysts were stored in sterile PBS at 4 °C until used. Each of 10 calves of the infected group received 5×10^5 oocysts orally at the beginning of the study.

Faeces from experimentally infected and control calves were taken directly from rectum twice a day during the prepatent period, and every morning thereafter. Each sample was homogenized and approximatelly 50 mg of faeces were transferred to a 5-mL tube, shaken with 1.5 mL of deionized water and 1.5 mL of petroleum ether, then adjusted to 5 mL with deionized water and centrifuged (1500 × *g*, 10 min, 4 °C). The layers above the pellet were discarded and the pellet was resuspended in 1 mL of water. Forty microliter of the suspension was smeared onto a 25 mm × 40 mm area on a slide, air-dried, fixed in absolute methanol, and acid-fast stained with Ziehl–Neelsen's carbol fuchsin (Casemore et al., 1985). Oocysts were counted in polychromatic light at 500× magnification and the intensity of infection was expressed as follows: 0, negative sample; 1, up to 1 oocyst; 2, 1–4 oocysts; 3, 5–12 oocysts and 4, more than 13 oocysts per field in average. The morning fecal samples were also examined daily for the presence of blood using a commercial guaiac test (hemoCARE®, CARE-diagnostica, Germany).

The examinations were performed daily in uninfected control calves as well to ensure absence of *C. parvum*. On days 9 and 16 of the experiment all calves were examined for the occurence of other enteropathogens which were previously found on their home farm, namely rotavirus, coronavirus, *E. coli* K99, *Clostridium* and *Campylobacter*.

2.3. Catheterisation of calves

One day before initiation of the study, all calves were catheterised with a permanent intravenous catheter as follows: after venepuncture of the *vena jugularis* with a 2.50 mm × 70 mm needle (Injecta, Germany), a sterilised 20 cm long tube (Tygon S-54-HL, Norton Performance Plastic, USA) was put through the needle into the lumen of vein. A protruding 5 cm section of tubing was fitted with a cut-off detrited injection needle Luer 1.2 × 40 and covered with a screw cap. This external part of the catheter was closed with a piece of water-proof adhesive tape and fixed with two sutures to the skin of the neck. The place of venepuncture was treated with iodine unguent (Betadine, Egis Pharmaceuticals, Hungary) throughout the study. After each blood sampling 0.5–1 mL of 1% heparin in physiological solution was introduced into the catheter. Moreover, the tube was preventively rinsed each 2–3 days with 1 mL of 5% spectinomycine (Spectam, Sanofi, France). When blood was not taken, the catheter was covered with a sterile protective bandage to avoid damage or inflammation. This catheter enabled rapid blood sampling throughout the study by one person without causing excessive stress to the calves.

2.4. Intestinal tests

The intestinal absorptive capacity for D-xylose and for retinyl-palmitate, as well as the test of intestinal permeability with lactulose and D-mannitol, were assesed in all calves on day 0 (just before experimental infection in group B) and then on days 7, 14 and 21 p.i. or on equivalent days in the control calves. All three tests were performed in parallel, always on an empty stomach and after an overnight fasting period. Water was available *ad libitum* throughout the tests. Before testing, calves were always weighed and a blank sample of blood and urine was taken.

2.4.1. Assessment of the intestinal absorptive capacity for D-xylose

The implementation of the D-xylose test for calves (Hammon and Blum, 1997) was used as follows: calves received a load of 0.5 g of D-xylose (Merck, Germany) per kg of body weight. The marker was prepared as a 10% solution in deionized water and administered perorally using a drencher. Blood samples were taken in 30-min intervals from the catheter into a sterile syringe for a consecutive 3.5-h period. Blood was centrifuged immediately in heparinized tubes (2500 × *g*, 25 min, 4 °C) and the plasma frozen at −28 °C until analyzed. The plasma D-xylose concentration was determined spectrophotometrically by the method of Eberts et al. (1979). Time curves of the D-xylose plasma concentrations between 0 and 3.5 h post-administration were constructed, and each animal's absorptive capacity was expressed as the area under the curve ($AUC_{0–3.5}$).

2.4.2. Assessment of the intestinal absorptive capacity for retinyl-palmitate

In the last day of acclimatisation, all calves were given an intramuscular injection of 12,500 IU of retinyl acetate per kilogram body weight (Axetocal, Biotika, Slovakia) in order to saturate the tissues, especially the liver, thus preventing enhanced uptake of vitamin A during testing.

A modified test originally designed for calves by Holland et al. (1992) was used as follows: on the day of test all calves received 7500 IU of vitamin A per

kilogram of body weight orally. The test doses were prepared by dissolving 20.625 g of pure retinyl-palmitate (Sigma–Aldrich, Praha) into 1 L commercial sunflower oil under a protective atmosphere of argon and in the absence of light. The test doses (0.2 mL of the oil solution per kilogram body weight) were immediately administered directly to the oral cavity using a syringe to ensure correct dosage. The blood samples for vitamin A analysis were taken from the catheter for a consecutive 8-h period in 60-min intervals. The heparinized tubes with blood samples were placed on ice, protected from light, immediately transferred to laboratory, and centrifuged (2500 × *g*, 25 min, 2 °C). Plasma was frozen at −80 °C until analyzed using HPLC (Van Vliet et al., 1991). The plasma concentrations of total vitamin A (a sum of retinol and its detectable esters) was recorded. A curve of the gain of pure vitamin A in plasma between 0 and 8 h was constructed and $AUC_{0–8}$ was calculated for each animal.

2.4.3. Assessment of the intestinal permeability

The lactulose/D-mannitol intestinal permeability test was administered as described previously (Klein et al., 2007). Briefly, all animals received a solution of 5 g D-mannitol (Lachema, Czech Republic) and 10 g lactulose (Duphalac®, Solvay Pharmaceuticals, The Netherlands) in 100 mL of distilled water. The solution was administered *via* a drencher, together with the D-xylose solution. Thereafter all urine was collected for five consecutive hours using urine collection bags. Urine volume was measured and 100 mL aliquots were stored at −28 °C until analyzed using gas liquid chromatography. The index of intestinal permeability (IP) was calculated as the ratio of the recovery (%) of lactulose to D-mannitol in the postprandial 5-h urine production.

2.5. Data evaluation

The AUC for both D-xylose and vitamin A were calculated using the trapezoidal rule after the baseline value obtained in time zero was subtracted. Student's *t*-test or Mann–Whitney *U*-test were used for the comparison of AUC values, weight gains, and IP indexes. The statistical package QC Expert, version 2.5 (TriloByte, Czech Republic) was used.

The experiment was approved by The Ethics Committee of the Institute of Animal Science in Praha-Uhrineves and by Central Commission for Animal Welfare at The Ministry of Agriculture of The Czech Republic (Project No. 01/99).

3. Results

The length of the prepatent period in experimentally infected calves was 3 days in one animal, 4 days in seven animals and 5 days in two animals (Table 1). Onset of watery diarrhoea was observed in all infected animals at the beginning of the patent period. Faeces of most of these animals contained clumps of mucus, sometimes with blood visible by naked eye. This severe diarrhoea, in most animals followed by apathy and inappetence, lasted 2–5 days. Maximal counts of oocysts were observed between the 6th and 7th day p.i. Presence of blood in faecal samples, as determined with the guaiac test, was recorded in all infected animals in the acute stage of infection. On day 7 p.i. all *C. parvum* inoculated animals shed oocysts and had profuse watery diarrhoea. In contrast, on day 14 p.i. all faecal samples were free of *C. parvum* (Table 1) and the consistency of faeces in *C. parvum* inoculated animals was normal. No other enteropathogens were detected in any of the control

Table 1
Intensity of infection in calves orally inoculated with 5×10^5 *Cryptosporidium parvum* oocysts on day 0

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Calf 1	0	0	0	3 B	4 B	4 B	4 B	3 B	3	2	1	1	0	0	0
Calf 2	0	0	0	3 B	4 B	4 B	4 B	2	1	1	1	0	0	0	0
Calf 3	0	0	0	4 B	4 B	4 B	4	3 B	2	1	1	1	0	0	0
Calf 4	0	0	0	0	3 B	4 B	4 B	3	3	1	1	1	1	0	0
Calf 5	0	0	0	4 B	4 B	4 B	4 B	3 B	3	1	1	1	0	0	0
Calf 6	0	0	0	0	1 B	4 B	4 B	4 B	4 B	3	1	1	1	0	0
Calf 7	0	0	0	1 B	3 B	3 B	4	3	3	2	1	1	0	0	0
Calf 8	0	0	0	4 B	4 B	4 B	2	2	2	1	1	1	0	0	0
Calf 9	0	0	0	1 B	4 B	4 B	4 B	4 B	4	3	3	1	0	0	0
Calf 10	0	0	1	4 B	4 B	4 B	4 B	4	4	4	4	2	1	0	0

B indicates presence of blood in the faeces; (0) negative sample; (1) sporadic finding of oocysts in faeces; (2) middle infection; (3) strong infection; (4) very strong infection.

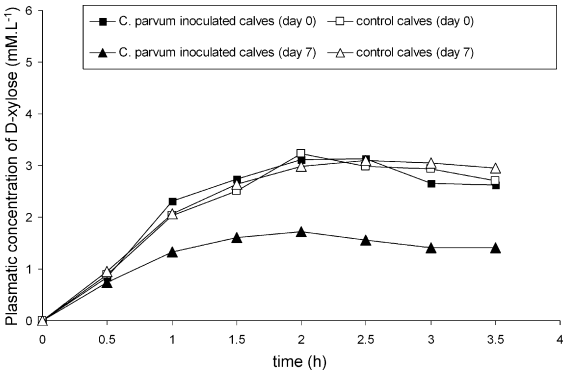

Fig. 1. Plasma concentration of D-xylose in *Cryptosporidium parvum* inoculated and control calves on the day of infection and on day 7 p.i.

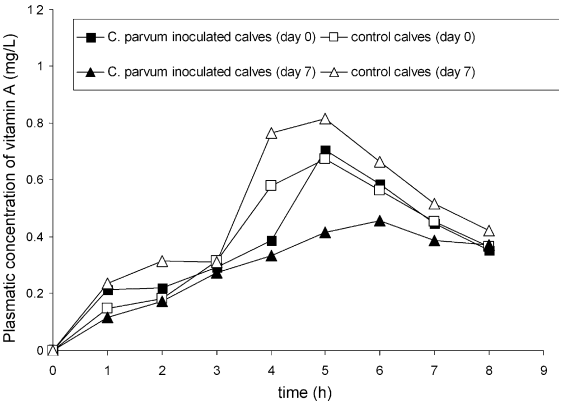

Fig. 3. Plasma concentration of vitamin A in *C. parvum* inoculated and control calves on the day of infection and day 7 p.i.

and *C. parvum* inoculated calves throughout the study and control calves stayed clinically healthy for all the time.

The change in D-xylose concentration in plasma over time, as well as $AUC_{0–3.5}$ values, showed that the intestinal absorptive capacity in the infected animals was lower on days 7 and 14 p.i. in comparison with the control animals (Figs. 1 and 2). The results of the vitamin A test followed the same trend as observed with D-xylose (Figs. 3 and 4). The indexes of intestinal permeability were significantly affected by the infection on days 7 and 14 p.i., whereas values recorded on day 21 p.i. suggest that intestinal permeability in the infected group had returned to a normal range as seen in the healthy controls (Table 2). The infection strongly affected growth of the animals. Daily weight gain in *C. parvum* inoculated and control animals was 107 ± 49 versus 204 ± 55; -15 ± 187 versus 355 ± 28 and 111 ± 98 versus 395 ± 30 g during the 1st, 2nd and 3rd week of the experiment, respectively.

4. Discussion

Results of this study show that absorption of D-xylose in the infected group was strongly affected by the clinical development of the infection. Whereas on day 7 p.i. all clinically healthy animals had absorption curves with a plasma concentration peak of 3.09 ± 0.51 mM L^{-1} at 2.5 h, the curve in sick animals receiving the same load had maximal concentration of only 1.72 ± 0.39 mM L^{-1} at 2 h. These results may be influenced also by the severity of the infection. In fact, at day 7 p.i. most of infected calves showed severe diarrhoea, which in addition, may contribute to reducing the time of D-xylose exposure to the site of absorption, and thus lead to an underestimate of absorption. The D-xylose test results of Perin et al. (2001) obtained with HIV positive children partially support our observation in calves, since the highest impairment of the intestinal absorption was observed just in children with cryptosporidiosis. Interestingly, the authors of that study did not find a direct association between diarrhoea and xylose absorption.

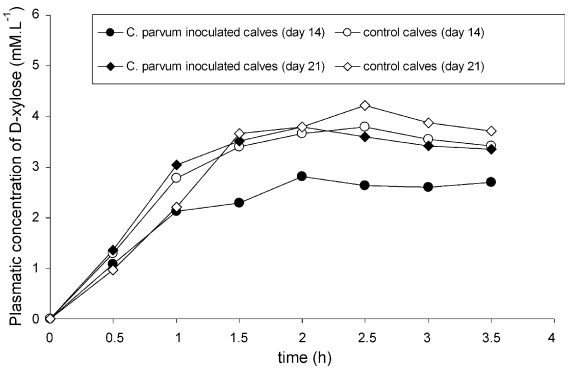

Fig. 2. Plasma concentration of D-xylose in *C. parvum* inoculated and control calves on the days 14 and 21 p.i.

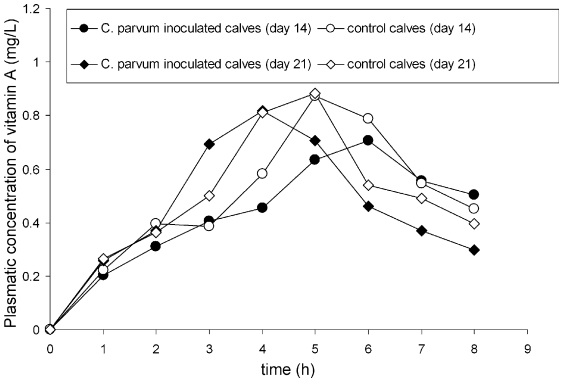

Fig. 4. Plasma concentration of vitamin A in *C. parvum* inoculated and control calves on days 14 and 21 p.i.

Table 2
Absorption of vitamin A and D-xylose, and intestinal permeability index in calves orally inoculated with 5×10^5 *C. parvum* oocysts and in control calves

	Day of infection							
	7th day p.i.				14th day p.i.		21st day p.i.	
	C. parvum	Control	*C. parvum*	Control	*C. parvum*	Control	*C. parvum*	Control
Absorption of vitamin A (AUC_{0-8}; mg min L^{-1})	185.8 ± 18.7	181.9 ± 35.3	140.7b ± 32.7	230.3b ± 39.8	211.4 ± 29.1	241.2 ± 33.4	229.4 ± 19.1	242.9 ± 23.3
Absorption of D-xylose ($AUC_{0-3.5}$; mM min L^{-1})	482.9 ± 25.6	478.2 ± 35.6	271.6c ± 38.7	487.3c ± 38.2	446.7d ± 41.5	605.4d ± 57.4	612.1 ± 37.7	617.2 ± 36.2
Intestinal permeability index	0.261 ± 0.04	0.254 ± 0.04	0.588e ± 0.12	0.262e ± 0.05	0.375a ± 0.09	0.276a ± 0.03	0.303 ± 0.04	0.261 ± 0.03

Values with the same letters differ significantly from each other: (a) $p < 0.01$; (b–e) $p < 0.001$.

On day 14 p.i. all animals of the infected group were already clinically healthy and shed no oocysts, but absorption of D-xylose was still significantly lower than in control calves (Table 1, Fig. 2). Histological examinations of gnotobiotic calves infected by *C. parvum* confirmed negative alterations in the epithelial architecture (Heine et al., 1984; Ruest et al., 1997); our findings from day 14 p.i. are thus consistent with the results of Rolston and Mathan (1989), which found that the D-xylose test mirrors primarily the intestinal absorptive surface rather than the performance of membrane transporters. The persisting malabsorption of D-xylose show that the small intestine in *C. parvum* infected calves requires more than 2 weeks to fully recover its absorptive surface. The results obtained on day 21 p.i. clearly show that intestinal functions probed by the D-xylose test were already fully reestablished.

The absorption of vitamin A in the present study clearly reflected the clinical status of the calves. As was observed with D-xylose, the absorption of vitamin A was also strongly impaired in the acute phase of the disease on day 7 p.i. (Table 2, Fig. 3). The difference in AUC values on day 14 p.i nevertheless suggests that absorption of vitamin A in recovered animals is affected probably to a lesser extent than absorption of D-xylose. Comparison of the absorption of vitamin A in both groups on day 21 p.i. clearly indicates full recovery of the infected gut epithelia as also shown by the D-xylose test.

Our results obtained with vitamin A confirm previous conclusion of Holland et al. (1992) that calves with cryptosporidiosis are not able to absorb sufficient dietary vitamin A, and that they are at higher risk of vitamin A deficiency. Indeed, it seems that this risk is associated only with the acute phase of the disease. On all accounts, extra application of vitamin A, preferably injected, should be considered to reduce the negative impact of cryptosporidiosis in calves.

Results of the lactulose/mannitol test show that IP of infected calves on day 7 was more than 100% higher in comparison to the control animals (Table 2). Such elevated IP values indicate severe impairment or even failure of the mucosal protective barrier in the acute stage of disease. Higher intestinal permeability to Cr-EDTA in cryptosporidial calves in the acute stage of disease was previously observed by Hunt et al. (2002), but intestinal uptake of this probe was only elevated by about 25%. Similarly Moore et al. (1995) found only about 20% higher transepithelial mannitol flux in the *C. parvum* infected porcine ileum in an early stage of the infection. In contrast, the urinary lactulose/rhamnose ratio in the Australian aboriginal children with cryptosporidiosis described by Kukuruzovic et al. (1999) reflected

impairment of the IP in a comparable extent as observed in this study on calves.

Moreover, the positive results of the guaiac test in all infected animals at the beginning of the patent period indicate a damage of the small intestine followed by desquamation of the mucosa and haemorrhage. On the other hand, the guaiac test may give false positive results due to damaged vessels near the rectum which may be a consequence of more frequent defecation and mucosal irritation.

Impairment of the mucosal barrier in the acute stage of infection is of clinical relevance. Although cryptosporidiosis is not characterized by high mortality in calves, it rarely occurs alone (de Graaf et al., 1999). Disruption of the intestinal mucosa resulting from *C. parvum* infection thus may substantially increase the risk of death or serious complications in the presence of concurrent viral, and especially bacterial infections. Since cryptosporidiosis is not easily curable, results of this test show that optimal care of sick calves promote protection and quick regeneration of the intestinal mucosa.

5. Conclusion

The results of these experiments show that cryptosporidiosis in calves causes a significant impairment of both, intestinal absorption and barrier function, mainly in the acute phase of the disease, followed by diarrhoea and high oocysts output. Significant differences in absorption of D-xylose as well as increased intestinal permeability were still detectable 14 days p.i., whereas on day 21 p.i. no alterations in monitored intestinal function was found. Smaller weight gains were nevertheless observed up to 21 day p.i., suggesting that the negative impact of cryptosporidiosis on the performance of animals persist for at least 3 weeks after challenge.

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