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## The effect of halofuginone lactate on experimental *Cryptosporidium parvum* infections in calves

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### ABSTRACT

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The chemoprophylactic effects of halofuginone lactate were tested against calf experimental cryptosporidiosis. Twenty 2-day-old calves, divided into four groups, were orally inoculated with  $1 \times 10^6$  oocysts of *Cryptosporidium parvum*. The infected control group was unmedicated whereas the three other groups were medicated with the drug at 30, 60 and 120  $\mu\text{g kg}^{-1} \text{day}^{-1}$ , respectively, for 7 days, from Day (D)2 to D8 post-inoculation (D 0 was inoculation day). The calves were weighed twice weekly and disease development and drug efficacy were assessed daily from D0 to D30 from consistency of feces, shedding of oocysts and mortality. Experimental *C. parvum* infection caused a severe clinical disease with profuse watery diarrhea, high oocyst shedding and mortality (3 out of 5) in the unmedicated group. The results clearly demonstrated the efficacy of halofuginone lactate in reducing the severity of clinical cryptosporidiosis. This efficacy was dose-dependent. The lowest dose (30  $\mu\text{g kg}^{-1} \text{day}^{-1}$ ) was not able to prevent clinical disease and mortality (3 out of 5). No clinical signs were observed with the 60 and 120  $\mu\text{g kg}^{-1} \text{day}^{-1}$  doses, but the animals shed oocysts after drug withdrawal. This shedding was more delayed the higher the dose of drug administered, but the delayed shedding had no effect on the growth of the animals.

### INTRODUCTION

The protozoan *Cryptosporidium parvum* is an important coccidial parasite of intestinal and respiratory tracts in several species of vertebrates, including humans. Although concurrent infections with enteropathogenic *Escherichia coli* K99, rotaviruses and coronaviruses can occur in neonatal calves, *C. parvum* alone may cause severe diarrhea, weight loss, dehydration and mortality.

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More than 80 antimicrobial drugs, including coccidiostats or antiprotozoal drugs (Moon et al., 1982a,b; Tzipori et al., 1982; Angus et al., 1984; Kim, 1987; Lindsay et al., 1987; Naciri and Yvoré 1989; Regh and Hancock, 1990; Villacorta et al., 1991), broad-spectrum antibiotics (Sloper et al., 1982), sulphamids (Nagy and Pohlenz, 1982; Fischer, 1983; Gunther, 1983, 1984; Naciri et al., 1984) and even anthelmintics (Stemmermann et al., 1980; Sloper et al., 1982) were shown to be inactive against *Cryptosporidium* in humans and animals. At this moment, only treatment with lasalocid-sodium (15 mg kg<sup>-1</sup> body weight once a day for 3 days) in experimentally or naturally infected calves (Göbel, 1987) or with halofuginone lactate (500 µg kg<sup>-1</sup> day<sup>-1</sup> for 3 days) in experimentally infected lambs (Naciri and Yvoré, 1989) has been shown to be effective. Halofuginone lactate is an anticoccidial drug initially used in the treatment of bovine theileriosis. Villacorta et al. (1991) established that halofuginone lactate at a dosage of 500 µg kg<sup>-1</sup> day<sup>-1</sup> was too close to the toxic concentration. They tested lower concentrations to try to avoid toxic effects while guaranteeing a security of about five. The drug was effective at a practical dose of 60 to 100 µg kg<sup>-1</sup> day<sup>-1</sup> for 7 consecutive days in neonatal calves naturally infected with *C. parvum*.

The aim of this study was to determine the chemoprophylactic effect of halofuginone lactate at daily doses of 30, 60 or 120 µg kg<sup>-1</sup> day<sup>-1</sup> for 7 consecutive days in experimentally infected neonatal calves.

## MATERIALS AND METHODS

### *Animals*

Twenty male and female colostrum-fed calves of mixed (Holstein-Friesian) breed obtained from traditional calf breeders were separated from their dams at 1 day of age and individually fed a commercial milk replacer twice a day. The milk replacer did not contain antibiotics, vitamins or other nutritional supplements and the animals were not vaccinated against bovine pathogens. When delivered, the calves were divided into four experimental groups of five animals and reared in two rooms (ten calves per room). Strict hygienic measures were taken, including filtering air, to avoid extraneous infection.

Each animal in the four groups of calves was inoculated with  $1 \times 10^6$  sporulated oocysts of *C. parvum* at 24–48 h old. The control group remained unmedicated whereas the three other groups were medicated with halofuginone lactate at 30, 60 or 120 µg kg<sup>-1</sup> day<sup>-1</sup>, respectively, for 7 days, from Day (D)2 to D8 post-inoculation (p.i.).

### *Inoculation*

The strain of *C. parvum* used was free of microbial or viral pathogenic agents, and was isolated from a child (Arnaud-Battandier et al., 1982) and serially propagated for 8 years in lambs or calves without exposure to any anticoccidial treatment. Fecal samples containing *Cryptosporidium* oocysts were washed through a graded series of metal sieves with meshes measuring from 1000 to 100  $\mu\text{m}$  to remove large debris. The resulting suspension was then centrifuged for 10 min at 2000  $g$ . The pellet was resuspended in 1/5 (V/V) ether–water and centrifuged at 1000 $\times g$ . The resulting pellet containing the oocysts was washed three times with water and treated with 10% sodium hypochlorite for 10 min. After three further washings, the pellet was suspended in 2.5% aqueous potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ ), the number of oocysts was counted with a hemacytometer (cell of Thoma) and the pellet was stored at +4°C for 3 weeks before use. Each calf was then inoculated with  $1 \times 10^6$  oocysts of *C. parvum* suspended in 20 ml of distilled water.

### *Drug*

The halofuginone lactate used is a derivative of febrifugine and comprises a solution of 25 mg active substance per milliliter. The required dose for each animal was diluted in 50 ml of water and the drug was administered orally to the calves just before milk feeding.

### *Assessment of infection*

The calves were weighed at the start of the experiment (D0) and then 3, 6, 10, 13, 17, 24 and 31 days after infection. Fecal samples were examined for the presence of rotavirus, coronavirus, *E. coli* and *Salmonella* before experimental infection. Disease development and drug efficacy were assessed by evaluating the consistency of feces, shedding of oocysts and calf growth and mortality. Fresh fecal material was collected daily from D0 to D31 p.i. by rectal sampling. Elimination of *Cryptosporidium* oocysts was detected by flotation using Sheather's sucrose solution. Oocyst numbers were scored semi-quantitatively by light microscopy at a magnification of  $\times 250$  on a scale from 0 to 4: 0, no oocyst; 1, less than one oocyst per field; 2, 1 to 5 oocysts; 3, 6 to 10 oocysts; 4, more than 10 oocysts.

### *Statistics*

The results were analyzed by analysis of variance (Fisher test) and comparisons of means (Newman–Keuls test). Statistical significance of the variables was tested at the 0.05 and 0.01 levels of confidence.

## RESULTS

*C. parvum* caused severe clinical symptoms in the non-treated group: diarrhea, emaciation and/or dehydration and mortality (3 animals out of 5). In some animals other etiological agents of neonatal diarrhea were detected (Table 1). No coronaviruses were detected but seven animals distributed over the different experimental groups eliminated rotaviruses in the feces before inoculation. *E. coli* K99 was established in four calves of which one animal was also positive for *Salmonella*. The effects of halofuginone lactate on weight gain and oocyst shedding are summarized in Figs. 1 and 2.

*Clinical observations*

After infection, non-medicated calves and calves medicated with the lowest dosage ( $30 \mu\text{g kg}^{-1} \text{ day}^{-1}$ ) showed first anorexia, followed by diarrhea and oocyst shedding within 24 h. All calves exhibited diarrhea for several days. In these two groups, the two surviving calves exhibited profuse diarrhea for 6 days (D5 to D10). The fecal stain varied from brown to orange, yellow or white-grey. Consistency varied from pasty to watery. Feces contained mucus,

TABLE 1

The presence of potential pathogenic agents before inoculation of the calves with *C. parvum*

Group	Animal	Rotavirus	Coronavirus	<i>E. coli</i> K99	<i>Salmonella</i>
Infected non-medicated	1	+	-	-	-
	2	-	-	-	-
	3	+	-	-	-
	4	-	-	-	-
	5	-	-	-	-
Infected medicated $30 \mu\text{g kg}^{-1} \text{ day}^{-1}$	6	-	-	+	+
	7	-	-	-	-
	8	+	-	-	-
	9	+	-	+	-
Infected medicated $60 \mu\text{g kg}^{-1} \text{ day}^{-1}$	10	+	-	-	-
	11	-	-	-	-
	12	-	-	-	-
	13	-	-	-	-
	14	-	-	-	-
Infected medicated $120 \mu\text{g kg}^{-1} \text{ day}^{-1}$	15	-	-	+	-
	16	+	-	-	-
	17	-	-	-	-
	18	+	-	-	-
	19	-	-	-	-
	20	-	-	+	-

+, positive finding; -, negative finding.

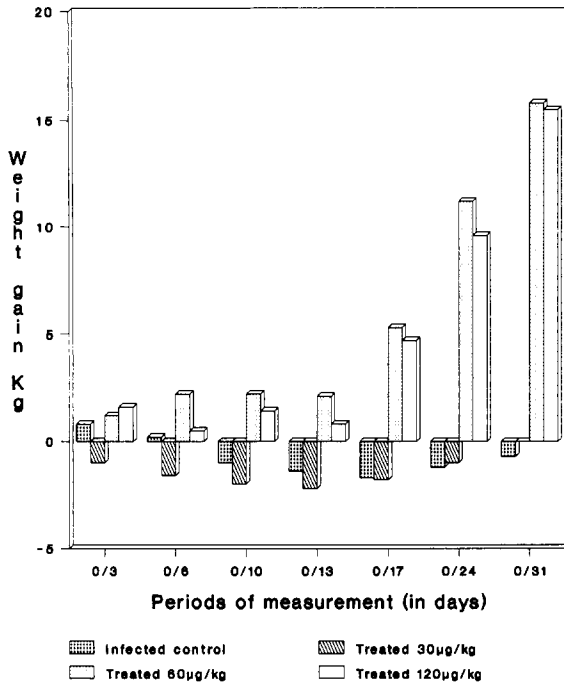


Fig. 1. Effect of halofuginone lactate on weight gain. (Treatment from D2 to D8 p.i.).

undigested milk and sometimes strings of blood, and the emission frequency was increased. In both groups, *Cryptosporidium* infection greatly impaired the growth of the calves (Fig. 1).

In the  $60 \mu\text{g kg}^{-1}$  medicated group, the calves did not exhibit watery diarrhea. Only slightly pasty feces were observed in four animals for a few days. In the  $120 \mu\text{g kg}^{-1}$  medicated group, no diarrhea was established. In both these groups, weight gain was apparently impaired during medication. Until D13, no significant difference was noted between the weight gains of the four groups. For the D0–D17 period ( $P < 0.05$ ) and for the D0–D24 and D0–D31 periods ( $P < 0.01$ ), the weight gains of the  $60$  and  $120 \mu\text{g kg}^{-1}$  medicated groups were significantly higher than those of the two other groups.

#### Parasitological findings

The kinetics of *C. parvum* oocyst output are shown in Fig. 2. In non-medicated calves, the prepatent period was 5 days with the exception of one animal that shed a small number of oocysts from D3. In surviving calves, oocyst shedding was important from D5 to D10 p.i., then decreased and stopped at D14.

In the  $30 \mu\text{g kg}^{-1}$  medicated group, slight oocyst shedding was noted

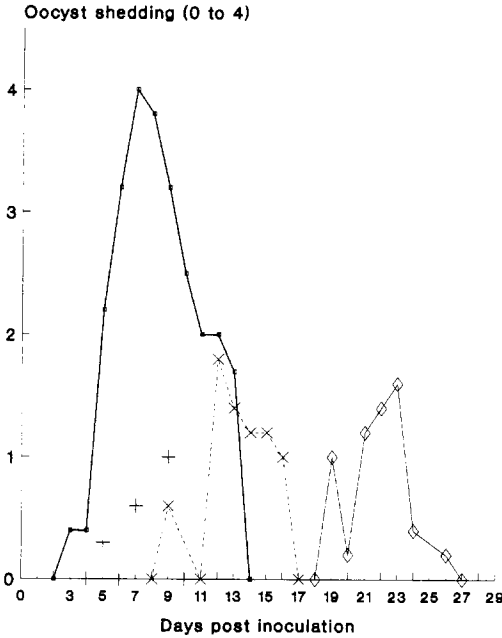


Fig. 2. Effect of halofuginone lactate on *C. parvum* oocyst shedding. (Treatment from D2 to D8 p.i.). —■—, Infected control; ..... + ....., Treated 30 µg kg<sup>-1</sup> day<sup>-1</sup>; ---×---, Treated 60 µg kg<sup>-1</sup> day<sup>-1</sup>; —◇—, Treated 120 µg kg<sup>-1</sup> day<sup>-1</sup>.

in some animals during halofuginone lactate administration. In the 60 µg kg<sup>-1</sup> day<sup>-1</sup> medicated group, no oocyst shedding was observed during treatment. Nevertheless four animals started to shed oocysts immediately after drug withdrawal, with particularly high oocyst output (Score 4) from D9 to D16 in two animals. In the 120 µg kg<sup>-1</sup> day<sup>-1</sup> medicated group, the oocyst shedding started longer after drug withdrawal from D18 to D27, in four calves.

### DISCUSSION

The importance of the clinical symptoms after experimental infection, associated with the death of three calves in the control group, clearly demonstrates the pathogenicity of *C. parvum*. The presence of other neonatal enteric pathogens in some of the calves apparently did not interfere with the results.

The results clearly demonstrate the efficacy of halofuginone lactate, as medication reduced the severity of clinical cryptosporidiosis in calves. The efficacy of the drug, administered from D2 to D8 p.i., before the appearance of clinical symptoms, is dose-dependent. A dose of 30 µg kg<sup>-1</sup> day<sup>-1</sup> was not able to prevent clinical disease as three calves out of five died as a result of the infection. With 60 or 120 µg kg<sup>-1</sup> day<sup>-1</sup> no clinical symptoms were ob-

served in any calf. Until D13, all medicated groups showed negative weight gain or no weight gain at all. In our first experiments (Naciri and Yvoré, 1989) a dose of  $500 \mu\text{g kg}^{-1} \text{day}^{-1}$  for 3 days was closely associated with a toxic side-effect. With  $60$  or  $120 \text{ kg}^{-1} \text{day}^{-1}$  for 7 days, no sign of toxicity was observed, with the exception of the depression of weight gain during the medication period and until D13. The depression of weight gain during the first days of life may be ascribed in part to the stress suffered by the animals after changes in rearing and feeding conditions. After D13, both  $60$  and  $120 \mu\text{g kg}^{-1} \text{day}^{-1}$  dosages allowed good growth in comparison with the unmedicated or  $30 \mu\text{g kg}^{-1} \text{day}^{-1}$  medicated groups. Over a period of 31 days, the weights of the survivors of the two last-named groups did not increase further.

None of the three doses of halofuginone lactate tested stopped oocyst shedding completely. Apparently, oocyst shedding was delayed longer the higher the dose of drug administered. With  $30 \mu\text{g kg}^{-1} \text{day}^{-1}$  a weak development of the parasite was noted during medication but not afterwards. With  $60 \mu\text{g kg}^{-1} \text{day}^{-1}$  shedding occurred just after drug withdrawal (D9), and with  $120 \mu\text{g kg}^{-1} \text{day}^{-1}$  it started at D18, but had no detrimental consequences on weight gain in the two last-named groups. Villacorta et al., (1991) observed similar effects after treatment of naturally infected calves. The number of animals which restarted shedding was also closely linked with the dose of halofuginone lactate administered. These authors argued that lower doses do not completely prevent development of the parasite and thus facilitate development of immunity and prevention of reinfection, while larger doses, which stop the cryptosporidia life cycle completely, prevent development of immunity and leave the animals susceptible to reinfection. In practical conditions, it is possible that calves become reinfected, but in our experiment the animals received only one dose of *C. parvum* oocysts and were housed separately, so the chances of reinfection were limited. Perhaps the delayed oocyst shedding reflects the pharmacodynamics and the mode of action of halofuginone lactate in the host: it seems to indicate that the drug is cryptosporidiostatic and only inhibits parasite development. With other coccidia (e.g. *Eimeria*), Long and Millard (1968) observed that sporozoites of *Eimeria tenella* and *Eimeria acervulina* can develop following drug withdrawal even after treating with meticlorpindol for as long as 60 days.

In the absence of reinfection, we have previously recorded, in experimentally infected lambs showing profuse watery diarrhea and oocyst shedding (Score 4), that a large dose of this drug ( $1 \text{ mg kg}^{-1} \text{day}^{-1}$ ) for only 1 day immediately stopped oocyst shedding, with reinfection following 4 days later. This recurrence of oocyst shedding had no consequence on either weight gain or growth (Naciri and Yvoré, 1989). This period of 4 days corresponds to the prepatent period of *C. parvum*. All these data from lambs and calves might indicate that the drug may destroy the schizogonic and gamogonic stages but only inhibit the sporozoites which restart infection after drug withdrawal. In



avian coccidiosis, it has been shown that other anticoccidial drugs are clinically effective against *Eimeria* even when they do not completely stop elimination of oocysts. Unfortunately, the infectivity of the recovered oocysts has not been tested.

In conclusion, medication with halofuginone lactate at doses from 60 to 120  $\mu\text{g kg}^{-1} \text{ day}^{-1}$  for a period of 7 consecutive days prevents mortality induced by *C. parvum* and guarantees normal weight gain. Moreover, it guarantees a security factor of about five.

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