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Integration of halofuginone lactate treatment and disinfection with p-chloro-m-cresol to control natural cryptosporidiosis in calves



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ABSTRACT

Two field studies were accomplished on a dairy farm in Saxony to compare different strategies for the control of natural cryptosporidiosis in newborn calves. In the first study, 96 newborn calves were allocated to four different groups immediately after birth. Calves of group H and of group HN were treated orally with 120 µg/kg body weight (BW) of halofuginone lactate daily during the first seven days of life. Calves of group C and of group CN were treated with a same volume of tap water. As an additional measure, the pens of groups HN and CN were disinfected with 3% Neopredisan 135-1® (p-chloro-m-cresol), the pens of groups C and H remained non-disinfected. Faeces were examined semi-quantitatively for oocyst excretion using carbofuchsin-staining and the clinical course was recorded. While disinfection alone (group CN) had no effect on oocyst shedding and diarrhoea, treatment with halofuginone lactate (groups H and HN) reduced oocyst shedding and diarrhoea significantly. Combination of treatment and disinfection (group HN) controlled cryptosporidiosis completely during the first two weeks after birth. However, prevalence of diarrhoea and oocyst shedding was higher in the third week of life in group HN than in any of the other groups. This delayed occurrence of cryptosporidiosis was not seen in study 2 when all calves were similarly protected by treatment and specific disinfection. Oocyst shedding was not observed in periparturient cows either by carbofuchsin staining, ELISA or PCR.

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1. Introduction

The intestinal protozoan parasite *Cryptosporidium parvum* occurs in calves mainly in the first three weeks of life with the highest prevalence in the second week after birth (Pavlasek, 1982; Fayer et al., 1998; de la Fuente et al., 1999; Naciri et al., 1999; Santin et al., 2004). The clinical signs of the disease are mucous to watery diarrhoea, inappetence, depression and dehydration (Pavlasek, 1982; Heine et al., 1984; Fayer and Ungar, 1986).

Infection may result in high mortality or mild diarrhoea, depending on the isolate the calves are exposed to and the susceptibility of the individual animal (Pozio et al., 1992; Luginbühl und Pfister, 1996; Fayer et al., 1998). In piglets, there is evidence for a synergistic pathogenic effect of co-infection with *Cryptosporidium parvum* and rotavirus (Enemark et al., 2003).

Many drugs (e.g. lasalocid, paromomycin, sulfonamides) have been tested for therapy or metaphylactic use against cryptosporidiosis but most failed to sufficiently prevent clinical disease or were toxic to calves (Fayer, 1992; Luginbühl und Pfister, 1996; Grinberg et al., 2002; Sahal et al., 2005). Pro- or metaphylactic treatment with 120 µg/kg body weight (BW) halofuginone lactate

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significantly decreases oocyst shedding and controls diarrhoea, although not equally effective in all calves (Naciri et al., 1993; Villacorta et al., 1991; Joachim et al., 2003a).

Neopredisan 135-1[®] (p-chloro-m-cresol) inactivates *Cryptosporidium* oocysts *in vitro* (Joachim et al., 2003b) and this observation is supported by field experience. However, the effect of disinfection on infection pressure under field conditions has not been critically evaluated so far. In this study, metaphylactic treatment with halofuginone lactate and disinfection with Neopredisan 135-1[®] were analysed as sole measures and in combination in terms of control of natural cryptosporidiosis in calves.

2. Materials and methods

2.1. Study farm

The study was performed on a large dairy farm (approximately 2000 cows) in eastern Saxony. The male calves are sold at an age of 14 days, the female calves are raised as replacement heifers. All cows are vaccinated against *Escherichia coli* before calving, the heifers are additionally vaccinated against rotavirus and coronavirus. The newborn calves are housed in single crates with straw bedding during the first two weeks of life (K0) and are supplied with colostrum in the first two days after birth. From the third day of life on, milk replacer is fed three times a day from buckets. From the third week of life on, the female calves are stabled in groups of 24 animals in large pens with deep litter (K1) and milk replacer is fed from an automatic feeder. Hay and concentrates are offered *ad libitum*. The farm has a persistent history of clinical cryptosporidiosis in young calves, mainly in the second week of life. The field isolate was determined as *C. parvum* bovine genotype (Najdrowski et al., 2007).

2.2. Faecal consistency and oocyst detection

Faecal consistency was scored as follows: 0: normal, 1: pasty, 2: semi-liquid, 3: liquid. Only scores 2 and 3 were considered as diarrhoea. For the counting of the oocysts, samples were smeared, stained with carbolfuchsin and examined under the microscope (400-fold magnification) as described by Heine (1982). For each sample, the number of oocysts in 20 randomly chosen fields of vision was determined and divided by 20 to calculate the average score. Since the examination technique was semi-quantitative, a score was generated to differ between low, moderate and high oocyst counts. The average oocyst score was indexed as none, low (>0, <5 oocysts), moderate (5–25 oocysts) or high (>25 oocysts).

2.3. Study 1

In the first field study, 96 newborn female calves were randomly allocated to one of four study groups (24 calves per group): H, C, HN and CN. Calves of groups H and HN were treated metaphylactically with 8 ml Halocur[®] (approx. 120 µg/kg BW of halofuginone lactate; Intervet Deutschland GmbH, Unterschleissheim, Germany) on 7 consecutive days, beginning 24–48 h after birth

(according to the manufacturer's instructions). Calves of groups CN and C were sham treated on the same days with 8 ml of tap water.

All calves were kept under identical conditions, except for disinfection of single crates (K0 period). The crates of groups H and C were conventionally cleaned with water whereas those of groups CN and HN were additionally disinfected with 3% Neopredisan 135-1[®] (Menno Chemie, Norderstedt, Germany) according to the manufacturer's protocol the day before the calves were brought in.

During the first two weeks of life, faecal consistency and oocyst shedding were determined daily from D4 to D13 for each calf. In the third week of life, when the calves were housed in groups (K1 period), the faecal consistency was determined daily from D14 to D20 by rectal sampling and parasitological examination was performed on D16 and D20.

Differences between groups regarding faecal consistency and oocyst shedding were calculated separately for K0 period and K1 period. Calculations were based upon faecal scores and oocyst scores of all samples in the corresponding time period. Additionally, percentage of animals with oocyst shedding and diarrhoea per study day was calculated for each study group to show the course of clinical coccidiosis in each group.

For differential diagnosis 30 randomly selected samples of liquid consistency were examined with "bovine strip tests" (BioX, Netherlands) for *E. coli* K99, rotavirus and coronavirus.

2.4. Study 2

Deviating from study 1, the groups of study 2 were not run at the same time. Initially, nine untreated calves (control group), housed in non-disinfected stables were sampled from D6 to D22 (K0, K1). Oocyst excretion and faecal scoring were done as in study 1. Thereafter, 44 female calves (study group) were all treated with halofuginone lactate as described above and kept in single crates (until D14, K0) and group pens (until D28, K1) disinfected with 3% Neopredisan 135-1[®] the day before the calves were brought in. From D6 to D28, faecal samples were taken individually and scored for oocyst shedding and consistency every second day. Percentage of animals with oocyst shedding and diarrhoea per day was calculated to show the course of clinical coccidiosis in each group.

2.5. Examination of cows

To evaluate whether cows have to be considered as an infection source, 62 cows were examined for excretion of oocysts within one week before/after calving. All samples were examined by carbolfuchsin staining as described above and with a commercial ELISA (ProSpect[®] *Cryptosporidium* Microplate Assay, Remel, Lenexa, USA). Moreover, 19 samples were also examined by PCR. DNA was extracted with QuiAmp[®] Stool Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The final PCR mixture contained 3.5 mM magnesium chloride, 1 mM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer (CP 3.4-3' and CP 3.4-5'; Petry et al., 1998;

Joachim et al., 2003b) and 1 U of Taq polymerase (Promega, Mannheim, Germany). 2.5 µl DNA was added to 22.5 µl of the master mix. The PCR programme (except 'program' in computers) consisted of an initial denaturation step at 94°C for 3 min followed by 35 cycles at 94°C for 45 s, 57°C for 45 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. The products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed using a digital camera. Presence or absence of the expected DNA band (650 base pairs in size) was recorded.

2.6. Statistical analysis

Data were analysed using the SPSS statistical software package version 14.0 (SPSS Inc., Chicago, IL, USA) by Kruskal–Wallis-test and Mann–Whitney-*U*-test (for differences between groups). *P* values of 0.05 or lower were considered significant.

3. Results

3.1. Study 1

In group C (controls) and in group CN (Neopredisan®135-1 disinfection) oocyst shedding started on D7 and D6, respectively. Both groups reached the highest prevalence on D12 (79% in group C and 75% in group CN). After D12, the proportion of positive samples decreased in both groups and on D20 only 4% of the animals in group C and none in group CN passed oocysts (Fig. 1).

The animals in group H (halofuginone lactate) began to excrete oocysts on D9. The highest prevalence was observed on D13 and D16 (38%). On D20, 25% of the samples were still positive. In group HN (treatment and disinfection) oocysts were first diagnosed on D11 with less than

10% of samples being positive. Prevalence remained comparatively low albeit showing an increasing trend in this group until D13. In contrast to the other groups highest prevalence was seen on D16 (63%). 42% of samples were positive on D20 (Fig. 1).

Until D13 (K0, single crates) the intensity of excretion was significantly lower ($P < 0.05$) in group HN as compared to groups H, C and CN and also significantly lower ($P < 0.05$) in group H compared to group C and CN. However, no significant differences were observed between group C and group CN ($P > 0.05$). After movement to group housing (K1), no significant differences ($P > 0.05$) between groups H, C and CN were seen, whereas oocyst shedding was significantly more intense in group HN in comparison to groups H, C and CN ($P < 0.05$ – $P < 0.001$, Table 1). The cumulative prevalence was 96% for groups C and CN, 83% for group HN and 75% for group H.

As for oocyst excretion, no significant differences regarding faecal consistency between group C (controls) and group CN (Neopredisan® 135-1) were obvious during the whole study period (Fig. 2). Both groups started to display diarrhoea (liquid/semi-liquid faeces) at the end of the first week of life (D6/D7) and some calves showed diarrhoea until the end of the study period (D26).

In group H (halofuginone lactate) diarrhoea occurred for the first time on D8 and reached the highest percentage of approx. 20% on D20. The number of calves with diarrhoea decreased until D23 and diarrhoea had disappeared by D26. In group H, calves displayed significantly less frequently diarrhoea than those of groups C and CN over the whole study period ($P < 0.001$). Corresponding to oocyst excretion, no significant differences of diarrhoea prevalence between groups C and CN were recorded ($P > 0.05$). The percentage of calves with diarrhoea in group H during group housing (K1) was significantly lower ($P < 0.001$, Table 1) when compared to the values in the other three groups.

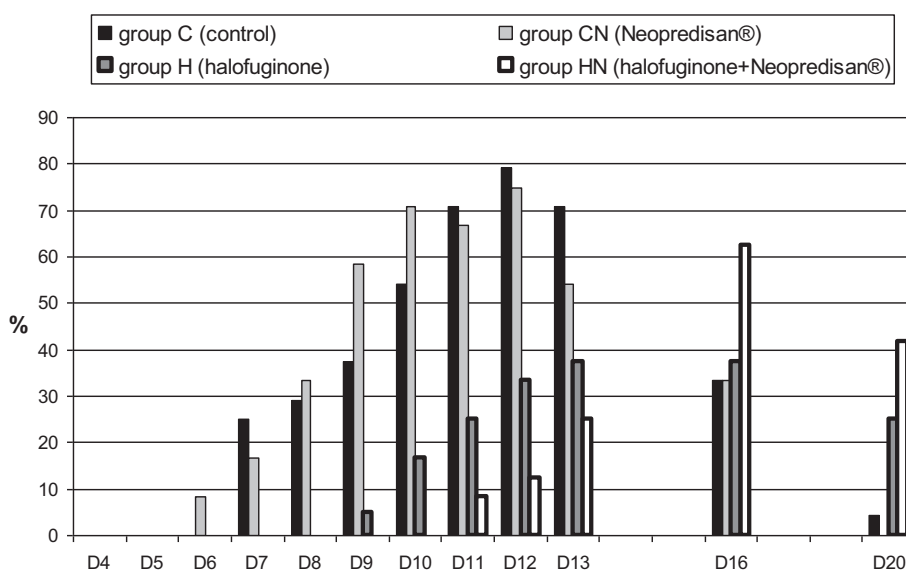


Fig. 1. Prevalence of *Cryptosporidium parvum* excretion.

Table 1

Study 1: group related comparison of oocyst excretion and faecal score in K0 (single crates) and K1 (group housing).

	All groups ^a	Group H/group C ^b	Group H/group HN ^b	Group H/group CN ^b	Group K/group HN ^b	Group K/group CN ^b	Group HN/group CN ^b
Oocyst excretion (K0)	0.000**	0.000**	0.003**	0.000**	0.000**	0.904	0.000**
Oocyst excretion (K1)	0.000**	0.112	0.048*	0.53	0.001	0.749	0.000**
Faecal score (K0)	0.000**	0.001**	0.000**	0.000**	0.000**	0.337	0.000**
Faecal score (K1)	0.000**	0.000**	0.000**	0.000**	0.181	0.323	0.716

^a Kruskal–Wallis-test.^b Mann–Whitney–U-test.* $P < 0.05$.** $P < 0.01$.

In group HN diarrhoea appeared with a striking delay (D13), on the other hand the proportion of calves suffering from diarrhoea later on (D16 and D20: approx. 40%) was higher than in any of the other groups. On D23, diarrhoea prevalence dropped below 20%. By the end of the study period (D26) diarrhoea had completely disappeared in group HN (Fig. 2). The differences between group HN and each of the other groups were highly significant ($P < 0.001$) during the first two weeks (single crates, K0), however, in the following observation period (group housing, K1) group HN did not differ in terms of diarrhoea prevalence from groups C and CN ($P > 0.05$) and samples were significantly more often of liquid or semi-liquid character than in group H ($P < 0.001$).

All tested samples were negative for *E. coli* K99 and coronavirus, while three samples were weakly positive for rotavirus.

3.2. Study 2

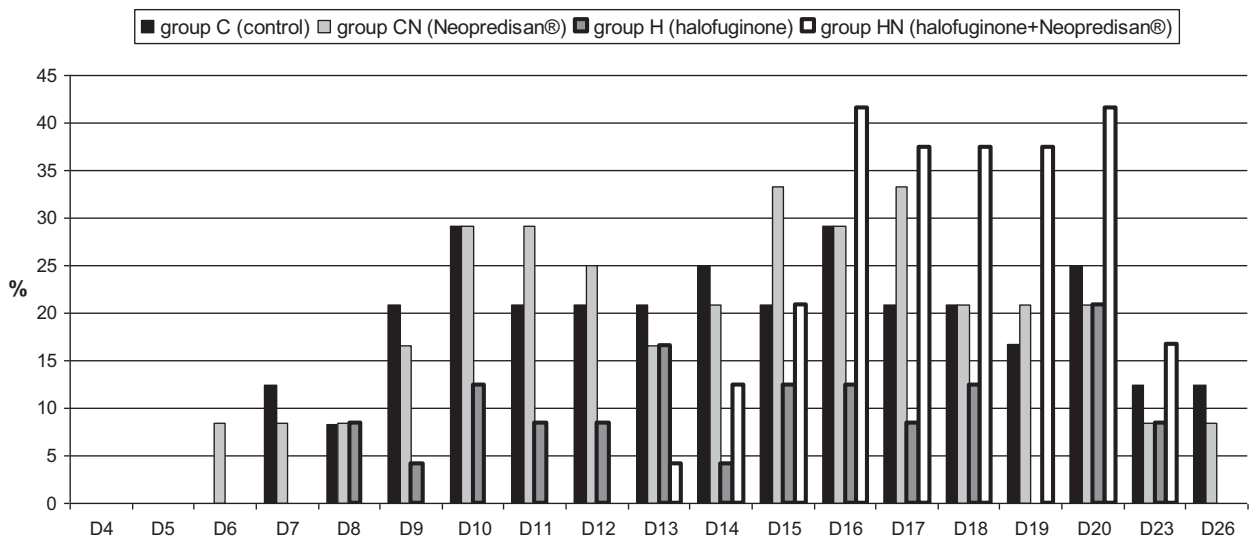
56% of the control calves started to excrete oocyst on D8. The highest prevalence was seen on D10 when 78% of the animals were positive. Excretion prevalence decreased thereafter and on D22 (the last day of examination for this

group), only one animal out of nine shed oocysts. All control calves suffered from diarrhoea during the study period. Diarrhoea (liquid or semi-liquid) started on D6, prevalence was highest (>50%) on D12, D14 and D16 and decreased thereafter with only one animal suffering from diarrhoea on D22 (Fig. 3).

In the study group ($n = 44$, halofuginone lactate and disinfection of K0 and K1 premises) oocyst excretion also started on D8, but the proportion of positive calves was low (2%) and remained below 20% until D16. Thereafter a steady increase to peak prevalence of 55% on D22 was observed. At the end of the study period on D28, 30% of calves were still positive.

Calves of the study group showed diarrhoea for the first time on D6 (11%). Highest prevalence of 23% was recorded on D14. Diarrhoea prevalence was comparably low (<20%) thereafter. Diarrhoea prevalence was low (5%) at the end of the study period (D28) (Fig. 3).

Altogether, 23% of the animals of the study group did not show diarrhoea over the whole study period. Of the diarrhoeic samples 13% appeared liquid and 87% semi-liquid whereas in the control group 52% were liquid and 48% semi-liquid reflecting the more severe nature of cryptosporidiosis in the controls.

**Fig. 2.** Percent of calves with diarrhoea.

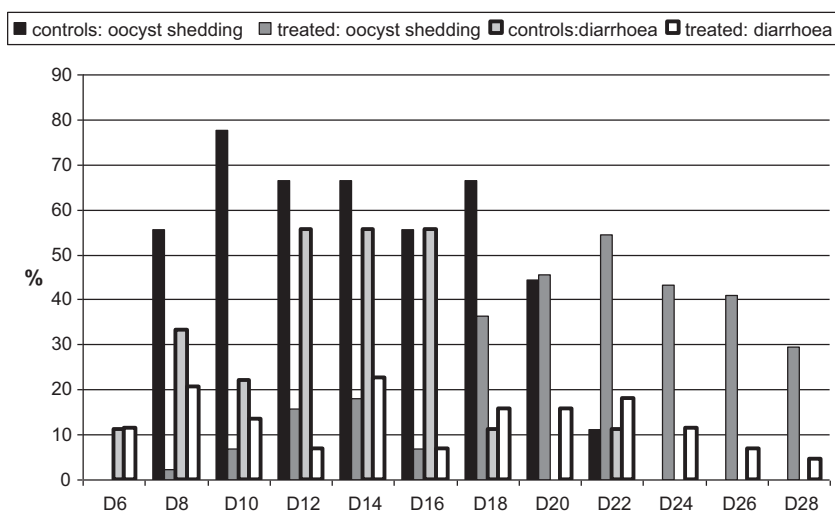


Fig. 3. Study 2: diarrhoea and oocyst shedding in controls and in the treated and disinfected group.

3.3. Examination of cows

All samples taken from cows were negative for cryptosporidia in all three diagnostic methods.

4. Discussion

Disinfection of the single crates (K0 period) with 3% Neopredisan® 135-1 alone had no effect on oocyst excretion or diarrhoea. It has been reported that this product is active against *C. parvum* *in vitro* (Joachim et al., 2003b). However, in the field it appears difficult if not impossible to reduce the oocyst number in a presumably heavily contaminated environment below the level needed to infect calves. No reliable data regarding the infective dose is available for cattle, but the median infective dose of *C. parvum* oocysts in men is as low as 132 oocysts (DuPont et al., 1995). Considering that millions of oocysts can be shed in one gram of calf faeces and that several hundreds of oocysts are sufficient to infect calves, it is obvious, that even inactivation of 99% of oocysts (which is defined as sufficient under *in vitro* conditions) may not absolutely prevent infection with the consequence of rapid recontamination of calf stables. In addition to remains of previous contamination in cleaned and disinfected crates and rapid increase of infection pressure by droppings of diseased calves kept in the same environment, animal keepers and other mechanical vectors (e.g. flies) may help to distribute oocysts into appropriately cleaned and disinfected premises (Graczyk et al., 2000).

Cows are not considered to serve as an important source of infection, since all respective samples examined were negative for *C. parvum*. This is attributable to immunity in adult animals (Akili and Harp, 2000). Besides, elder cattle are more frequently infected with *Cryptosporidium andersoni* and *Cryptosporidium bovis* than with *C. parvum* (Fayer et al., 2005). Nevertheless, no *Cryptosporidium*-oocysts at all were found in the carbolfuchsin-stained faecal samples of cows in this study.

As expected, metaphylactic oral treatment with halofuginone lactate was suitable to reduce both oocyst shedding and incidence of diarrhoea significantly, although no complete control can be expected from metaphylactic application of this drug. This was also observed by other authors (Naciri et al., 1993; Lefay et al., 2001; Joachim et al., 2003a). However, oocyst excretion may reappear after withdrawal of the drug, particularly if improved control is aspired by applying higher doses of the drug (Villacorta et al., 1991).

In fact, combination of halofuginone treatment and disinfection together controlled diarrhoea almost completely during the first two weeks of life. However, intensity and prevalence of oocyst shedding and diarrhoea was even higher in these calves afterwards (K1) than in calves only partially protected by halofuginone treatment or not protected at all during the critical period (K0). It can be speculated that optimal protection during the first two weeks after birth may prolong susceptibility of calves to infection due to insufficient stimulation of immunity. Similar observations were reported for calves treated with a high dose of halofuginone (Villacorta et al., 1991).

The design of study 1 was chosen to compare efficacy of different measures, however, this does not necessarily reflect a situation where all calves of the respective age group are subject to a certain control strategy. The combination of disinfection and treatment in study 1 (group HN) gave the best results during the period of highest susceptibility of calves to cryptosporidiosis, i.e. the first 2 weeks after birth. Therefore, this strategy was applied on all calves of the respective age group in study 2, supplemented by additional disinfection of the K1 area (group housing) to avoid exposure of previously protected calves to infection after movement to a potentially contaminated group pen. Under farm conditions the simultaneous keeping of non-protected controls would bear the risk of accidental carryover of oocysts into the experimental premises and thus might alter the interpretability of results. On the other hand, without a control group it is difficult to estimate the

course of infection if disinfection and treatment were not applied. We attempted to resolve this dilemma by examining a control group and performing the control strategy trial subsequently, assuming that the overall conditions are comparable and results obtained from the controls can be extrapolated to the study period.

Study 2 proved that the combined disinfection-treatment strategy is suitable to keep cryptosporidiosis at a low level for at least 4 weeks. Diarrhoea was either prevented or mild in most cases. Increase of oocyst shedding was observed from D18 to D20 and remained, although gradually decreasing, relatively high thereafter. However, this was not associated with increased prevalence of clinical disease. It is most probable that immunity is sufficiently stimulated under these conditions paralleled by lower clinical susceptibility to *C. parvum* in older calves e.g. due to a mature intestinal flora (Ortega-Mora and Wright, 1994; Harp, 2003). On the other hand, recontamination of a disinfected environment remains a constant threat and therefore proper hygiene measures, including suitable disinfection, are an important aspect of successful cryptosporidiosis control.

5. Conclusion

As previously demonstrated treatment of calves with 120 µg halofuginone lactate/kg BW in the first seven days of life is suitable to reduce oocyst excretion and severity and prevalence of diarrhoea due to field outbreaks of cryptosporidiosis. The combination of pro-/metaphylactic treatment within the 1st week of age and specific disinfection of single crates substantially improved control during the most critical period (1st and 2nd week of life). Properly protected young calves may remain more susceptible to cryptosporidia afterwards and therefore it is advisable to also disinfect group pens before previously protected calves are brought in.

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