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Control of cryptosporidiosis in neonatal calves: Use of halofuginone lactate in two different calf rearing systems

V. De Waele[a,b,*], N. Speybroeck[c,d], D. Berkvens[c], G. Mulcahy[b], T.M. Murphy[a]

[a] Central Veterinary Research Laboratory, Department of Agriculture, Fisheries & Food, Celbridge, Co. Kildare, Ireland
[b] School of Agriculture, Food Science & Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland
[c] Department of Animal Health, Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium
[d] Ecole de santé publique, Université Catholique de Louvain, Clos Chapelle-aux-Champs, 1200 Bruxelles, Belgium

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ABSTRACT

To date there is no effective treatment for bovine cryptosporidiosis. This study describes the use of halofuginone lactate in preventing cryptosporidiosis in naturally infected neonatal calves on a dairy farm with a high prevalence of infection. The animals were kept in two different calf rearing systems. A randomized double-blind trial was carried out with 32 naturally infected calves, divided into four groups. The two prophylactic halofuginone lactate treated groups were kept in either individual or group pens. Similarly, the animals receiving the placebo were housed in either individual pens or together in a large pen. A total of ten faecal samples were collected periodically during the 28 days study from each calf and tested for the presence of *Cryptosporidium* spp. using microscopic and molecular methods. Generalized estimating equations models were used to determine if the effects of the various treatments and/or rearing systems on the presence of diarrhoea and infection were statistically significant. Further analysis (classification trees models) was carried out to explore possible risk factors for cryptosporidiosis and interactions between treatments and rearing systems. Halofuginone lactate was shown to be effective in reducing clinical signs of cryptosporidiosis and environmental contamination. However, the treatment did not delay the onset of diarrhoea and did not reduce the risk of infection amongst calves reared together in a highly contaminated environment. The use of halofuginone lactate in combination with good hygienic measures, such as rearing animals in clean individual pens, was the most effective method to reduce the risk of cryptosporidiosis amongst 7–13 days old calves. It was concluded that the control of the parasite could be achieved by the combination of using effective preventive drugs, such as halofuginone lactate and good animal husbandry procedures.

1. Introduction

The zoonotic apicomplexan *Cryptosporidium parvum* is considered the most common enteropathogen of neonatal calves (de la Fuente et al., 1998; Santin et al., 2008). Infected calves can exhibit clinical signs ranging from asymptomatic infection to profuse diarrhoea and dehydration (Fayer et al., 1998; Thompson et al., 2007). These animals readily contaminate their immediate environment as total oocysts

* Corresponding author at: Central Veterinary Research Laboratory, Department of Agriculture, Fisheries & Food, Backweston Campus, Celbridge, Co. Kildare, Ireland. Tel.: +353 16157208; fax: +353 16157253.
E-mail address: valerie.dewaele@agriculture.gov.ie (V. De Waele).

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output per infected calf can be up to 10^{10} over a week (Fayer et al., 2004).

A major problem concerning *C. parvum* is the lack of an effective means for controlling infection and decreasing environmental contamination with oocysts. Because oocysts are highly resistant to environmental stresses and to many disinfectants, hygienic measures on their own are not sufficient to avoid infection and long term contamination of calf rearing facilities (O'Donoghue, 1995). In addition, many drugs and vaccines have been evaluated as potential therapeutic or prophylactic agents for cryptosporidiosis but with little success (Santin and Trout, 2008). Halofuginone lactate is a synthetic quinazolinone with cryptosporidiostatic activity on the sporozoite and merozoite stages of *C. parvum* (Jarvie et al., 2005). It has been recommended for both therapeutic and prophylactic use as it delays the onset of infection, reduces shedding of oocysts, and decreases the severity of cryptosporidiosis in calves (Joachim et al., 2003; Jarvie et al., 2005). Its effectiveness as a prophylactic treatment has not been evaluated for the various calf rearing systems used in Ireland. The primary objective of this study was to evaluate the effect of halofuginone lactate in decreasing the number of diarrhoeic calves kept in two rearing systems on a dairy farm with a high prevalence of cryptosporidiosis amongst neonatal calves. The secondary objective was to test the effect treatment and rearing systems may have on the onset of diarrhoeic signs and oocysts shedding, as well as on the number of calves excreting oocysts and the level of this excretion.

2. Materials and methods

2.1. Study design

A randomized double-blind trial was carried out during the period March to May 2005 in a dairy herd of 400 cows situated in Co. Westmeath, Ireland. The herd was selected on the basis of a previous study in which the prevalence of *C. parvum* was estimated by Bayesian analysis to be 98% (credibility interval: 92–100) in 2-week-old calves (unpublished data). In this herd, calving occurred throughout the year. Cows were moved to a calving pen approximately 1 week before calving and re-introduced to the milking herd 24 h after calving. The straw bedding of the maternity pen was changed every 6 weeks. Calves were fed 2 l of their dam's colostrum and separated from their mother within 12 h of birth. Commercial vaccines were not administered to cows or calves.

All Holstein Friesian calves born during the 3-week period, March 29th–April 19th, 2005, were included in the experiment, with the exception of one calf that died a few hours after birth. Newborn calves ($n = 32$) were allocated to their respective experimental groups using a table of random numbers. The first group consisted of calves treated with halofuginone lactate (Halocur, Intervet Ireland Ltd.) and maintained in individual calf pens; the second group was also treated with halofuginone lactate but the calves were allowed to mix freely in a large loose box; the third group consisted of calves treated with a placebo and maintained in individual calf pens; and, the fourth group was treated with a placebo and the animals were allowed to mix freely in a large loose box (Table 1). During the time calves were allocated to their respective experimental groups, the number of calves in the loose boxes increased from one initially to a final population of six and eight in the placebo and halofuginone lactate treatment groups, respectively.

The individual calf pens were made of aluminum with a slatted wooden base. The loose boxes had concrete floors. Both were washed with disinfectant (Hyperox, DuPont, UK) before introduction of the calves. These floors were covered with fresh straw every day. Every 2 weeks, the old bedding was removed; the floor was washed with disinfectant and covered with fresh straw.

The placebo solution was prepared according to the procedure described by Jarvie et al. (2005) and was similar in consistency, color and composition to the commercial Halocur without the active ingredient (halofuginone lactate). An equal volume of either halofuginone lactate (100 μg/kg) or placebo was administered orally to the calves in the morning just before feeding for the first 7 days of their life. The first dose of halofuginone lactate or placebo was given within 12 h of birth and after colostrum was fed. The animal handlers were not informed of the various treatments administered to calves until all the data had been collected. Each calf received 2.5 l of whole milk twice daily. Water was supplied *ad libitum* with a ration containing soya, wheat and citrus pulp which was mixed on the farm.

2.2. Parameters recorded

Serum was collected on one occasion from each 1-week-old calf. This was analyzed for the transfer of maternally-derived immunoglobulins using the zinc sulfate test (ZST) (McEwan et al., 1970). A total of ten faecal samples (2 g) were taken from each calf. The calves were sampled on days 1 and 2, and thereafter every second day on days 4, 6, 8, 10, 12 and 14. A further two samples were collected on days 21 and 28. The consistency of the faeces was recorded at the time of collection using the following scoring system: 0 for solid or pasty sample, 1 for liquid sample and 2 for watery

Table 1
Cumulative geometric mean and range of the oocysts per gram (OPG) of faeces excreted by untreated control calves and calves treated with halofuginone lactate during their first 4 weeks and reared in either individual or group pens.

Rearing system	Treatment	Number of calves	Cumulative geometric mean of OPG	Range of OPG
Individual pen	Halofuginone	9	28	0–200,384
	Placebo	9	7096	0–1,703,267
Group pen	Halofuginone	8	842	0–576,105
	Placebo	6	66,581	0–2,554,901

sample. The mean faecal score for each experimental group was calculated by taking the mean of the faecal scores of the animals of each group at each sampling point. Diarrhoeic faeces (faeces scored as liquid or watery) were also tested for the presence of other enteropathogens, i.e. *Escherichia coli* K99, *Salmonella* species, rotavirus and coronavirus.

A faecal sample (5 g) was collected from each dam within 24 h of calving. Dust and straw samples (100 g) were collected from five different locations on the floor of the calving pen once a week for the duration of the experiment. Prior to the start of the experiment, five straw samples (100 g) were collected in the two group pens; in addition, dust, faeces and other debris (10 g) were scraped from five different areas of the walls of the group pens. At the same time, dust, faeces and other debris (2 g) were collected from the wooden floors and aluminum walls of all individual calf pens. All these environment samples were collected as aseptically as possible by scraping the surfaces with sterile scalpels.

2.3. Oocysts detection

2.3.1. Oocysts concentration

Concentration of oocysts was initiated by washing sequentially 2 g of calf faeces through a series of stainless steel sieves with diminishing pores sizes until a final mesh size of 45 μm. Oocysts were further concentrated using a diethyl-ether sedimentation method described by Bukhari and Smith (1995). After the last washing step, distilled water was added to the pellet to give a final volume of 1 ml of faecal suspension. In the case of cow samples, the starting amount of faeces was 5 g and this was divided in two 2.5 g aliquots and processed as described for calf faeces.

The environmental samples were initially diluted (1:10) with phosphate buffered saline (pH 7.0); and the suspension stirred for 30 min using a magnetic stirrer. Oocysts were then concentrated using the same protocol as described for faeces.

2.3.2. Immunofluorescent assay (IFA)

A 100 μl aliquot of the concentrated faecal suspension was added to a well (diameter: 14 mm) on a microscope slide and stained with 50 μl of fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* monoclonal antibody (Cellabs Pty Ltd., Australia) using the procedure described by McEvoy et al. (2005). The smears were examined at 400× magnification using a fluorescent microscope (Olympus, Japan) containing a filter cube with an emission of 530 nm and an excitation wavelength of 490 nm. A slide was considered positive if at least one *Cryptosporidium* oocyst was identified. For each positive slide, the approximate number of oocysts per gram of faeces (OPG) was calculated using the mean number of oocysts present in ten randomly chosen fields at 400× magnification and corrected for the total surface of the well and the dilution of the original sample. No correction was made for the consistency of diarrhoeic samples.

The trapezium rule was applied between the successive sampling episodes to estimate the number of oocysts excreted by calves during the period of the experiment (Whittaker and Robinson, 1967). For each calf, the values obtained for each interval of sampling were added to give the cumulative amount of oocysts over the entire period of the experiment. The cumulative amounts of oocysts obtained for each calf were used to calculate the geometric mean cumulative amount of oocysts per gram excreted by the four experimental groups of calves during the period of the study.

2.3.3. Polymerase chain reaction assay (PCR) and sequencing

DNA was extracted with a commercial kit (FastDNA Spin Kit for Soil, Qbiogene Inc., USA) from 500 μl of the faecal suspension obtained after concentrating the oocysts. *Cryptosporidium* spp. DNA was detected using a nested PCR that amplified a segment of the small subunit rRNA gene of approximately 830 b.p. (Xiao et al., 2001). Secondary PCR products of seven randomly chosen positive faecal samples and all positive environmental samples were sequenced to identify the genotype of the isolates present on the farm. In addition, these samples were further tested using another nested PCR that amplified a 850 b.p. fragment of the 60-kDa glycoprotein gene (Alves et al., 2003). Similarly, secondary PCR products from this assay were also sequenced to identify the sub-genotype of the *C. parvum* isolates. A positive *Cryptosporidium* DNA template and a negative control were included in every PCR assay. Purification and sequencing of the amplicons were carried out by a commercial company (MWG Biotech AG, Germany). The amplified PCR products were sequenced in both directions using forward and reverse primers of the secondary PCR. Sequences were assembled, edited and aligned with reference sequences from GenBank using Lasergene software (DNASTAR, Inc., Madison, USA). The recently proposed nomenclature was used in labelling the *C. parvum* subtypes (Sulaiman et al., 2005).

2.4. Statistical analysis

The results were analyzed with STATA/MP 10.0 software (Stata Corporation, College Station, Texas, USA). At each sampling point, correlation between categorical variables (e.g. diarrhoea, infection, treatment, location and sex) was assessed by the Fisher exact test, while correlation between continuous variables, e.g. value of the ZST and level of oocysts excretion, was assessed by the Spearman correlation coefficient. The non-parametric method Kaplan–Meier method was used to create the survivor function to visually compare the groups of calves for the onset of diarrhoea and oocyst shedding (Kaplan and Meier, 1958). Four statistical tests, i.e. the log-rank test, the Wilcoxon test, the Tarone–Ware test and the Peto–Peto–Prentice and Harrington–Flemming tests, were conducted to test for significant differences ($p < 0.05$) between the survivor functions. The differences among the four tests depended on the weights used to combine the estimates derived at each point in time (Dohoo et al., 2003a). Further, generalized estimating equations models (GEE) were used to test for any significant effect ($p < 0.05$) that treatment and rearing systems may have on the number of diarrhoeic calves and calves shedding oocysts and also on the level of oocyst excretion, taking into account repeated

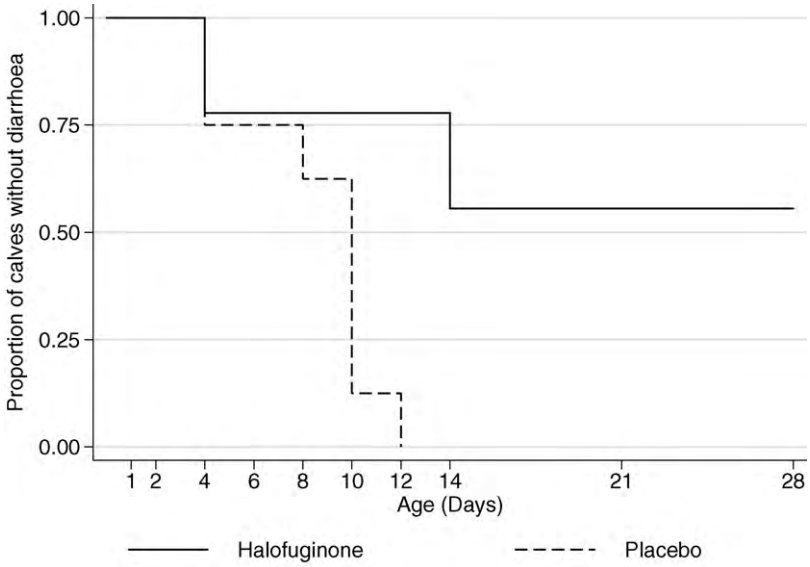

Fig. 1. Kaplan–Meier survival estimates of the proportion of calves maintained in individual pens without diarrhoea and treated either with halofuginone lactate or placebo.

observations for each animal (Dohoo et al., 2003b). For dichotomous outcomes, binomial family, logistic link and exchangeable correlation matrix were assumed; while for continuous outcomes, poisson family, log link and exchangeable correlation matrix were selected (Johnson et al., 2005).

An alternative analytical non-linear and non-parametric approach described by Duc Thang et al. (2008) was also conducted using the classification and regression trees (CART) software to explore possible risk factors for cryptosporidiosis and identify interaction between treatment and calf-rearing regimes. A CART model is fitted by binary recursive partitioning of a multidimensional covariate space, in which the dataset is successively split into increasingly homogeneous subsets until a specified criterion is satisfied (Duc Thang et al., 2008). The one-standard error rule was applied to select the best tree. The CART model was used only as an indicator of interaction between treatment and calf-rearing regimes, as it does not take into account the clustering effect within calves.

3. Results

3.1. General information on calf and cow health and farm environment

A total of 32 calves were used in the study and 18 (56%) of them were male. The results of the zinc sulfate turbidity test were above 20 units in all calves indicating that they had received a sufficient quantity of colostrum. All the animals were negative for *Cryptosporidium* sp. at birth. The results of the microscopy and sequence analyses indicated that the majority of calves were infected with *C. parvum* subtype A18G3R1 at some stage during the experiment, the exception being one halofuginone treated calf kept in an individual pen that remained free of infection for the duration of the study. There was no adverse reaction to the medication in any of the halofuginone lactate treated calves, i.e. no mortality or clinical findings, such as dehydration, prostration, presence of mucus or visible blood in faeces, were recorded from the calves treated with halofuginone lactate during the study.

Table 2
Statistical tests results to evaluate the difference between Kaplan–Meier survival functions for the first indication of cryptosporidiosis (oocysts shedding and/or clinical signs) in neonatal calves either treated with halofuginone lactate or untreated and kept in either individual or in group pens.

Outcome	Population of calves	Variable	Log-rank	Peto–Peto–Prentice
Onset of oocysts excretion detected by PCR[a]	Individual pen	Halofuginone vs placebo	<0.001	0.001
	Group pen	Halofuginone vs placebo	0.008	0.014
	Halofuginone	Individual pen vs group pen	0.007	0.020
	Placebo	Individual pen vs group pen	0.40	0.35
Onset of diarrhoea	Individual pen	Halofuginone vs placebo	0.005	0.024
	Group pen	Halofuginone vs placebo	0.07	0.12
	Halofuginone	Individual pen vs group pen	0.20	0.29
	Placebo	Individual pen vs group pen	0.05	0.26

Significant p-value <0.05.
[a] Polymerase chain reaction.

Table 3
Generalized estimating equation models for risk of cryptosporidiosis (oocysts shedding or clinical signs) in neonatal calves treated either with halofuginone lactate or untreated and maintained in either individual pens or in group pens.

Outcome	Experimental groups	Variable	Coefficient	*p*-value	95% confidence interval	
					Lower limit	Upper limit
PCR[a] detection	Individual pen	Halofuginone vs placebo	−1.36	<0.001	−1.95	−0.78
	Group pen	Halofuginone vs placebo	−0.30	0.12	−0.66	0.07
	Halofuginone	Individual pen vs group pen	−1.16	<0.001	−1.73	−0.59
	Placebo	Individual pen vs group pen	−0.11	0.56	−0.48	0.26
	All calves	Interaction halofuginone and individual pen	−1.02	0.004	−1.72	−0.32
IFA OPG[b]	Individual pen	Halofuginone vs placebo	−3.18	<0.001	−3.18	−3.17
	Group pen	Halofuginone vs placebo	−2.54	<0.001	−2.55	−2.54
	Halofuginone	Individual pen vs group pen	−1.31	<0.001	−1.31	−1.31
	Placebo	Individual pen vs group pen	−0.68	<0.001	−0.68	−0.68
	All calves	Interaction halofuginone and individual pen	−0.63	<0.001	−0.64	−0.63
Diarrhoea	Individual pen	Halofuginone vs placebo	−1.67	<0.001	−2.56	−0.77
	Group pen	Halofuginone vs placebo	−0.83	0.016	−1.50	−0.15
	Halofuginone	Individual pen vs group pen	−0.93	0.08	−1.97	0.12
	Placebo	Individual pen vs group pen	−0.11	0.65	−0.61	0.38
	All calves	Interaction halofuginone and individual pen	−0.82	0.15	−1.94	0.29

Significant *p*-value <0.05.
[a] Polymerase chain reaction.
[b] Number of oocyst per gram of faeces detected by immunofluorescence assay.

E. coli was detected in the faeces of three calves aged 1 week old in the placebo groups, one reared in an individual pen and two others reared in the loose pen. These were also infected with *C. parvum*. Three placebo treated calves over 2 weeks old, one reared in individual pen and two others reared in loose pen, died during the experiment. These animals were subjected to post-mortem examination to determine the cause of dead. Based on gross pathological lesions, two calves had died as a result of respiratory infection and one due to acute enteritis with a concentration of 5×10^5 *Cryptosporidium* oocysts per gram of faeces. No other common enteropathogen was detected from this animal.

The PCR assays carried out on faecal samples collected from cows were negative for *Cryptosporidium* spp. DNA. In addition, there was no evidence that the cows were contaminating the environment with oocysts as the environmental samples from the calving pen were also negative. However, all the environmental samples from the floor and two from the walls of the loose calf pens were positive for *Cryptosporidium* spp. DNA in a PCR assay. In contrast, the majority of samples collected from the individual calf pens were negative with the exception of two pens. These two pens had been blindly assigned to calves of the halofuginone lactate treatment group. The calves in these two pens only excreted oocysts at 21 days of age. Sequencing of these environmental isolates identified them as *C. parvum* subtype A18G3R1.

3.2. Effect of halofuginone lactate on calves kept in individual pens

The majority of the calves receiving the placebo were infected within the first week of age (range 4–8 days) while the mean age for onset of infection in calves treated with halofuginone lactate was 16 days (range 8–28 days) ($p \leq 0.001$). As illustrated by the Kaplan–Meier survival estimates, calves receiving the placebo became diarrhoeic earlier than calves receiving halofuginone lactate at a mean age of 8 days (range 4–12 days) and 17 days (range 4–28 days), respectively (Fig. 1). This difference was statistically confirmed by the four tests, i.e. log-rank, Wilcoxon, Tarone–Ware, Peto–Peto–Prentice and Harrington–Flemming, used to test whether the overall survivor functions in the groups are equal ($p < 0.05$). For the event of diarrhoea, the Peto–Peto–Prentice and Harrington–Flemming test was the most appropriate test when comparing the Kaplan–Meier curves of the treated and the control groups of animals because this test takes into account different censoring patterns between the groups and, in this case, diarrhoea was not detected for five calves treated with halofuginone lactate while all placebo calves had diarrhoea at some stage of the experiment (Table 2). Only one calf was censored for the event of infection; therefore, the log-rank test that assigns equal weight at each time point, was selected (Table 2).

A significant correlation between diarrhoea and infection was found when comparing the experimental groups treated with halofuginone lactate and placebo at the age 10 days ($p = 0.03$). In addition, correlations between infection and treatment received were found for the calves aged 8 days ($p < 0.001$), 10 days ($p = 0.009$) and 12 days ($p < 0.001$).

Analysis of the PCR and IFA results using GEE models indicated that halofuginone lactate significantly reduced the number of diarrhoeic calves, infected calves and oocysts excreted by these calves ($p < 0.001$, Table 3). Fig. 2A illustrates the pattern of the mean faecal scores for the groups of treated and placebo calves. Peak oocysts production (mean circa 500,000 oocysts per gram of faeces) amongst the untreated calves occurred at 10 days of age. In addition, the cumulative geometric means of the number of oocysts per gram of faeces was higher for the placebo calves than the treated calves (Table 1). However, at 21 days, more of halofuginone treated calves (78%) were shedding, albeit low numbers of oocysts, than the untreated calves (33%)

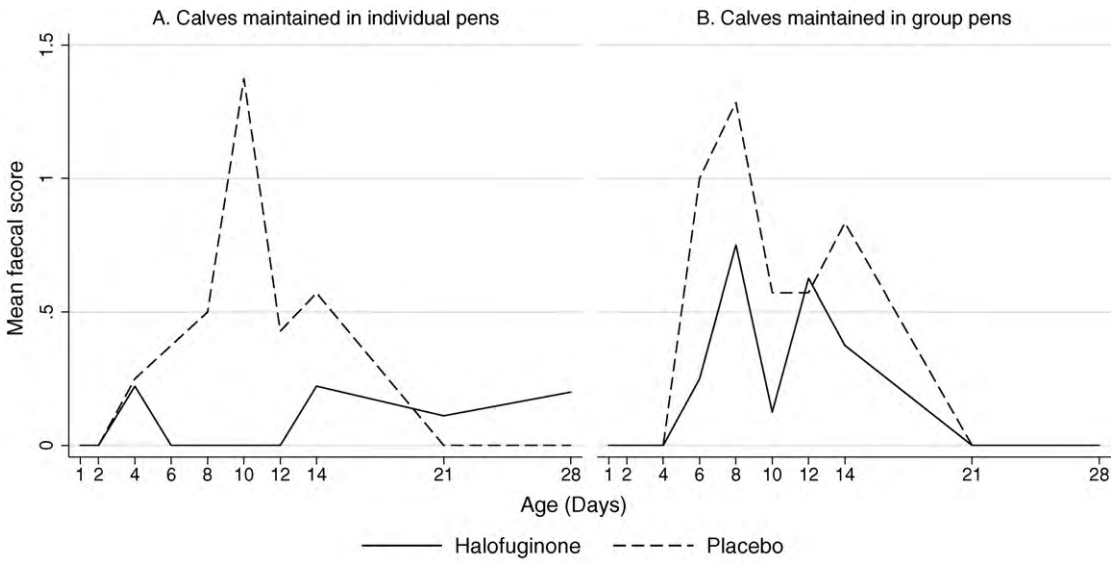

Fig. 2. Comparison of the mean faecal scores for calves maintained in either individual pens or group pens and treated with either halofuginone lactate or a placebo.

(Fig. 3A). At this age, only one of the infected treated calves was diarrhoeic.

3.3. Effect of halofuginone lactate on calves kept in group pens

The halofuginone lactate significantly reduced the number of diarrhoeic calves and oocysts excreted by the calves in the group pens (Table 3 and Fig. 2B). However, the time of onset of diarrhoea was not statistically different between treated and placebo calves. One of the treated calves excreted oocysts while on the medication. All the calves were found to shed oocysts at some stage during the experiment when the nested-PCR assay was used as a detection method (Fig. 3B). Correlation between the number of calves excreting oocysts and the treatment received was found for the calves aged 8 days ($p = 0.026$). The cumulative geometric means of the number of oocysts per gram of faeces was higher for the placebo calves than the treated calves (Table 1).

3.4. Effect of calf rearing systems

When comparing the two placebo groups, no significant differences in the onset of clinical signs and infection and in the number of infected and diarrhoeic calves were

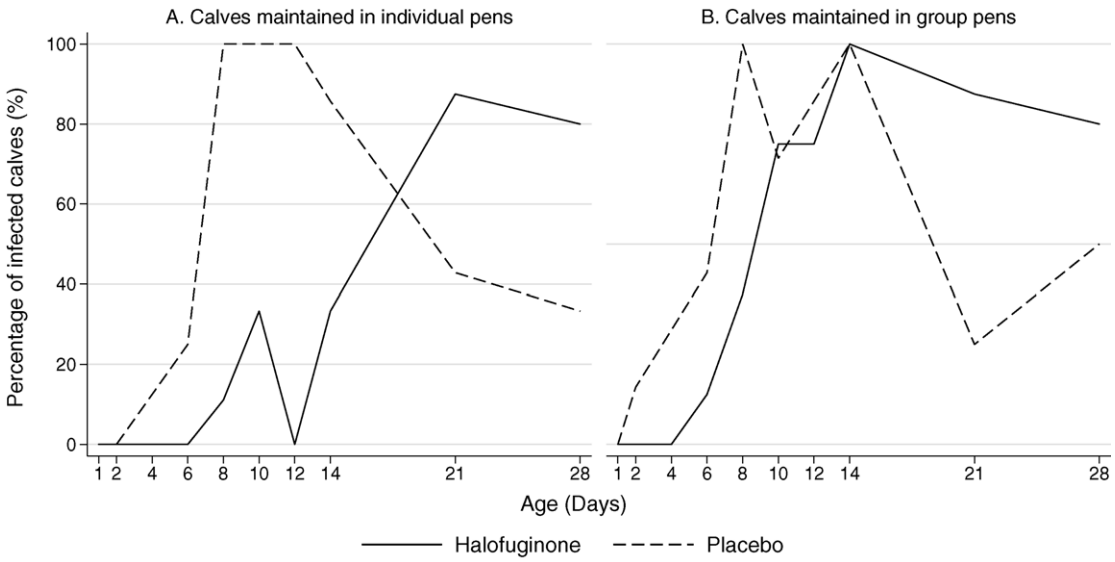

Fig. 3. Comparison of percentage of calves excreting *Cryptosporidium* spp. DNA maintained in either individual pens or group pens and treated with either halofuginone lactate or a placebo.

observed between the two rearing systems (Table 3). One calf from the group pen excreted oocysts as early as 2 days old. In addition, the amount of oocysts excreted by the placebo calves in the group pen was significantly greater than the amount shed by calves in individual pens ($p < 0.001$, Table 3).

When comparing the two halofuginone lactate groups, the time of onset of oocysts excretion was reduced in the calves kept in the individual pen ($p < 0.05$, Table 2). Maintaining treated calves in individual pens significantly reduced the number of animals that were infected with *Cryptosporidium* and the amount of oocysts they excreted ($p < 0.001$, Table 3).

3.5. Combining halofuginone lactate treatment and individual pen rearing system

The GEE model indicated a significant interaction of halofuginone lactate treatment and individual pen in reducing the proportion of infected calves ($p = 0.004$) and the number of oocysts excreted ($p < 0.001$, Table 3). According to the overall discriminatory power in the CART analysis, age was the strongest overall risk factor for cryptosporidiosis, followed by treatment and housing system. The CART model suggested that, among the treated calves aged between 7 and 13 days old, the rearing system was the most important risk factor (Fig. 4).

4. Discussion

An abridged version of this study has been published previously and the abbreviated results were used in a recent review and meta-analyses of the effects of halofuginone lactate in treating and preventing neonatal cryptosporidiosis (De Waele et al., 2007; Silverlas et al., 2009). The present study evaluated the prophylactic benefits of halofuginone lactate against cryptosporidiosis in two different calf rearing systems on the same infected farm. Results were analyzed using Kaplan–Meier and GEE models. In addition, the CART model was used to highlight interactions between various risk factors associated with neonatal cryptosporidiosis.

The primary source of infection for the experimental calves can only be surmised. However, the possibility of initial exposure to infection in calving pen appeared unlikely as oocysts were not detected in any of the faecal and environmental samples taken from the cows and calving pen, respectively. This is in contrast from previous studies which suggested cows as a source of infection for calves due to a peri-parturient rise in oocyst excretion and resultant contamination of the calving pen (Garber et al., 1994; Castro-Hermida et al., 2002).

The results confirmed previous reports that, when calves are reared in good hygienic conditions, such as disinfected individual calf pens, halofuginone lactate was effective in delaying the onset of *Cryptosporidium* infection and diarrhoea, in reducing the number of calves that become infected and exhibit signs of enteritis, and decreasing the level of oocysts excretion (Naciri et al., 1993; Lefay et al., 2001; Jarvie et al., 2005; Klein, 2007). Delaying the onset of diarrhoea is important as newborn calves are susceptible to dehydration and acid–base balance disturbances. Since these risks decrease with age, the preventive use of halofuginone lactate may delay infection and the onset of clinical signs to an age when animals are better able to cope with the pathogenic affects of the parasite. Additional benefits of halofuginone lactate treatment are reduction in the number of infected animals exhibiting signs of enteritis and in the amount of oocysts they excrete (Joachim et al., 2003; Lallemand et al., 2006). As shown in the current study, the efficacy of halofuginone lactate is decreased when calves are maintained in less than optimal conditions such as mixed together in group pens. The benefits of good husbandry were also evident when the placebo groups were compared. Calves reared in the individual pens excreted significantly less oocysts than calves kept in the group pen.

At 3 weeks, the majority of treated calves excreted oocysts albeit at low level. This so-called "rebound phenomenon" has been reported by previous workers (Naciri et al., 1993; Lallemand et al., 2006). As observed in this study, it is generally considered that it has limited clinical consequences and does not markedly increase the cumulative amount of oocysts produced by treated calves. Explanations for its occurrence might include re-infection, activation of an auto-infection phase with thin-wall oocysts and/or re-activation of inhibited stages (Villacorta et al., 1991; Naciri et al., 1993). Another possibility is that the older calves may have been infected with different *Cryptosporidium* species. However, the sequencing of 18S gene and Gp60 gene fragments carried out on isolates from faecal samples collected from some calves exhibiting the rebound phenomenon indicated that they were *C. parvum* subtype A18G3R1, the prevailing *Cryptosporidium* strain amongst the experimental calves. The calves, that received the placebo, did not show any evidence of a recrudescence of oocysts excretion at 21 days. This may be attributable to some degree of acquired resistance from the initial infection (Peeters et al., 1993).

All the statistical models used to analyze the experimental data highlighted the need for an integrated control programme combining good animal husbandry measures with prophylactic use of halofuginone lactate to reduce the risk of neonatal cryptosporidiosis. Hence, as indicated by the CART model, treated calves aged between seven and 13 days were more at risk of infection when reared in group pens than in individual pens. In addition, the CART model identified the age of the calf as the strongest discriminating risk factor for bovine cryptosporidiosis. Calves over 7 days old were more likely to be infected than younger animals whether or not they received halofuginone lactate. Silverlas et al. (2009) came to the same conclusion after their meta-analysis of data from a number of previous studies. Other workers have also reported a similar age predisposition in the susceptibility of calves to cryptosporidiosis (Kvac et al., 2006; Santin et al., 2008). Therefore, it is recommended to separate and rear calves in age-groups based on their level of susceptibility. Thus, newborn calves should not be mixed with calves between seven and 13 days old.

The size of the experimental groups was based on the number of calves born in the farm during the allocation period, feasibility of completing all the laboratory tests in

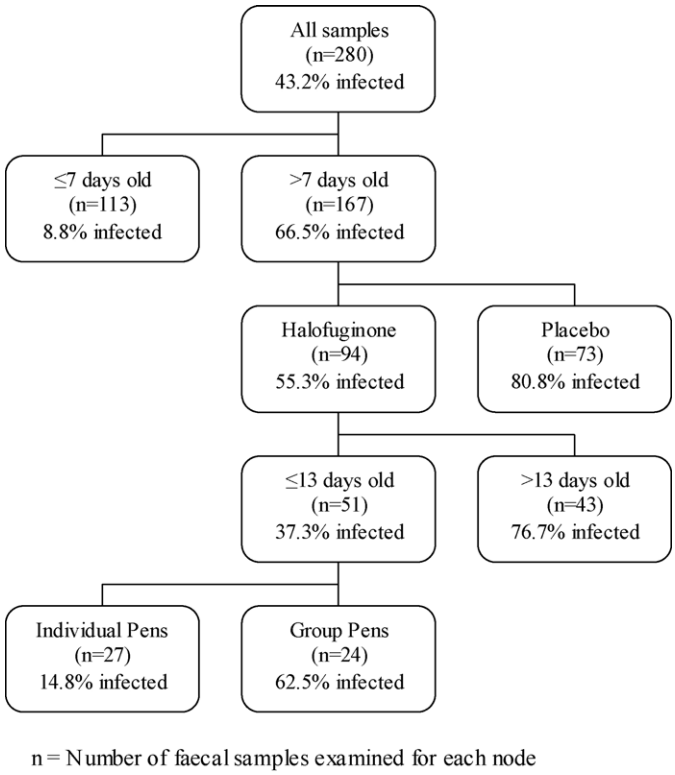

Fig. 4. CART tree indicating risk factors for bovine neonatal cryptosporidiosis.

a reasonable time period and also on the number of animals used in previous studies on the treatment of calves with halofuginone lactate (Naciri et al., 1993; Peeters et al., 1993; Jarvie et al., 2005). The limited number of calves in each experimental group did not affect the statistical power in detecting a beneficial effect of halofuginone lactate treatment.

The husbandry practices, including calf rearing facilities on the farm in which this experiment was carried out, were typical of those on other large dairy herds in Ireland. There was a history of neonatal enteritis and the prevalence of cryptosporidiosis amongst young calves had been estimated over 90% (unpublished data). Throughout the design and implementation of the study, every effort was made to ensure it would be possible to extrapolate from the results to calf rearing practices on the majority of Irish dairy farms and that this study could form the basis of a larger study on a number of farms in the future.

In conclusion, halofuginone lactate was shown to be effective in reducing clinical signs of cryptosporidiosis and environmental contamination with *Cryptosporidium* oocysts. However, control of the parasite can only be achieved by integrated measures such as not mixing animals of different ages, placing newborn calves in an uncontaminated environment and the prophylactic use of drugs such as halofuginone lactate. An effective on-farm control programme in addition to benefiting animal health directly also indirectly benefits public health as the concentration of oocysts in slurry and run-off water from calf houses will be markedly lower thus reducing the risk of surface water contamination with oocysts of zoonotic *Cryptosporidium* species (Fayer et al., 2004).

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