Efficacy of halofuginone products to prevent or treat cryptosporidiosis in bovine calves: A systematic review and meta-analyses

[1] Dr. Julii Brainard, j.brainard@uea.ac.uk
[1] Dr. Charlotte C. Hammer, charlottehammer@gmx.net
[1] Prof. Paul R. Hunter, paul.hunter@uea.ac.uk
[2] Dr. Frank Katzer, frank.katzer@moredun.ac.uk
[1] Ms. Georgina Hurle, g.hurle@uea.ac.uk
[1] Dr. Kevin Tyler, k.tyler@uea.ac.uk

[1] Norwich Medical School, University of East Anglia, Norwich NR4 7TJ, UK
[2] The Moredun Research Institute, Pentlands Science Park, Penicuik Midlothian EH26 0PZ, UK

Corresponding author = Julii Brainard, j.brainard@uea.ac.uk

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Abstract

Prior systematic review on the efficacy of halofuginone treatment to prevent or treat cryptosporidiosis in bovine calves was inconclusive. We undertook an updated synthesis and meta-analyses on key outcomes for treatment of calves with halofuginone.

Evaluated outcomes were oocyst shedding, diarrhea, mortality and weight gain. Experiments had to describe results for same age animals in contemporary arms. Most doses were 100 to 150 mcg/kg/day. Results were subgrouped by study design, experiments with lowest risk of bias and lack of industry funding.

Eighteen articles were found that described 25 experiments. Most evidence came from randomized controlled trials in Europe. Significantly lower incidence of oocyst shedding, diarrhea burden and mortality was reported when treatment started before calves were 5 days old. Most studies reported on outcomes for animals up to at least 28 days old. Publication bias was possible in all outcomes and seemed especially likely for diarrhea outcomes. Beneficial results when halofuginone treatment was initiated in calves older than 5 days were also found.

Prophylactic treatment to prevent cryptosporidiosis is effective in preventing multiple negative outcomes and is beneficial to calf health and will result in a reduction of environmental contamination by *Cryptosporidium* oocysts.

Keywords

Halofuginone; bovine calves; cryptosporidiosis; diarrhea; dairy

Introduction

Background

Cryptosporidium parvum is a common protozoan parasite in cattle. It is a frequently diagnosed cause of acute diarrhea (scour) in neonatal calves worldwide leading to delayed growth, considerable morbidity and potentially death (Thomson *et al.*, 2017; Manzoor *et al.*, 2018). Young calves (under six weeks old) are at greatest risk of both catching and spreading pathogenic infections (Silverlås *et al.*, 2009; Wells and Thomson, 2014). Economic costs in Great Britain were estimated in 2014 to be £100-£200 per infected calf (Shaw, 2014), arising mostly from veterinary treatment, need for higher nutritional inputs and reduced weight gain. A more recent study of beef calves in Scotland found that calves with severe cryptosporidiosis in the first 16 days of life were on average 34Kg lighter aged 6 months old, compared to calves in the same housing who had had no clinical signs of the disease (Shaw *et al.*, 2020). The weight difference between the two groups was statistically significant and the authors calculated that the 34Kg in weight difference would account for a loss of £128/head based on market prices of cattle in 2018 (Shaw *et al.*, 2020). Bovine cryptosporidiosis is widespread in Europe and prevalence of *C. parvum* in stool samples of European cattle herds were reported to range from 13-100% (Imre and Dărăbus, 2011). Cattle are recognised as an especially important reservoir for *C. parvum*, which can spread from cattle to other animals or to humans through many routes (Hunter and Thompson, 2005; Wells and Thomson, 2014; Brankston *et al.*, 2018). Higher infectious doses are linked to more severe disease and potentially higher losses (Zambriski *et al.*, 2013). Globally, infections from *C. parvum* and other *Cryptosporidium* species (eg. *C. hominis*) are considered important contributors to combined total human deaths from diarrheal illness (Vermeulen *et al.*, 2017). Large outbreaks in humans (affecting dozens or even hundreds of people) from pathogenic *C. parvum* infection regularly occur in Europe (Cacciò and Chalmers, 2016). Control of *C. parvum* is therefore highly desirable for good animal welfare, to reduce risks to human health and to limit economic losses in affected industries.

An evidence review written for livestock managers (Wells and Thomson, 2014) reiterated that prophylactic and treatment options for *C. parvum* infection are limited. One of the few products widely licensed for the treatment of calves is halofuginone lactate, marketed as Halocur®. Halofuginone (HFG) is a coccidiostat that was identified as having promise for cryptosporidiosis prophylaxis and treatment in calves by the early 1990s (Villacorta *et al.*, 1991). HFG is most often administered as halofuginone lactate. It is a synthetic biological agent derived from the alkaloid Febrifugine isolated from roots and

leaves of *Dichroa febrifuga* plants (Jang *et al.*, 1948; Pines and Spector, 2015). Halofuginone targets several cell processes, including ATP-dependent inhibition of tRNA synthetase (Keller *et al.*, 2012; Zhou *et al.*, 2013). The piperidine ring on HFG binds to both the Proline-AMP binding pocket, stabilised by the quinazolinone group which mimics the terminal adenosine of tRNA. Subsequently the biological activity of tRNA synthetase is inhibited, resulting in arrested cell growth and reduced viability (Francklyn and Mullen, 2019). *In vitro* work by McDonald *et al.* (1990) found that HFG lactate suppressed 90% of *C. parvum* growth using 4μg/ml. *In vitro* studies of HFG lactate using *C. parvum* infected HCT-8 cells, by Shahiduzzaman *et al.* (2009) found that on average, 98.05% of sporozoites were inhibited using 25μM at 27h after exposure. Experiments in Wistar rats demonstrated HFG lactate promoted metalloproteinase (MMP) -3 and -13, mediated by activation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor NFκB, in turn inhibiting T-helper cell-17 activity (Popov *et al.*, 2006). This enables the extracellular matrix to be broken down (Kamberov *et al.*, 2011), reducing fibrosis in the liver and other organs in mammalian systems.

A prior systematic review on the efficacy of halofuginone lactate to prevent or reduce symptoms caused by *C. parvum* infection was published 11 years ago (Silverlås *et al.*, 2009). The meta-analyses in this previous review were based on only 7 comparison groups in 6 publications and concluded that evidence was inadequate and too heterogenous to say if halofuginone (HFG) products were effective. The authors were concerned that the evidence base was limited (just 3-4 studies for most outcomes), heavily influenced by industry funding and suffered from publication bias. Publication bias occurs when researchers decline to publish results due to believing they are not important due to no findings of significant assocation (Deeks *et al.*, 2011). Research may be unlikely to proceed to publication after insigificant findings when the study was at least part-funded by an intervention sponsor. Longer duration studies (with outcomes beyond 3 weeks of age) were also few in the 2009 synthesis. Silverlås et al. (2009) recommended that HFG should only be used in severe cases of existing disease, not least to prevent development of drug resistance. Structured reviews to assess the efficacy of HFG for prevention or treatment have not been published since, although many trials since 2008 have been published. The aim of our study was to investigate the effects of HFG treatment using updated trial data (published up until early 2020) on four outcomes: diarrheal intensity, oocyst shedding intensity, mortality and weight gain when used as prophylactic or therapeutic treatment against calf cryptosporidiosis. This was done by performing a systematic review and meta-analysis of available data.

Materials and Methods

PRISMA literature search reporting guidelines were followed (Toews, 2017). The search strategy is available in Supplemental Material 1.

Population and other inclusion and exclusion criteria

The population of interest were cows (*Bos taurus*). Articles on humans, related species such as buffalo or yaks and other animals were ineligible. Studies on hybrids of cattle with other animals (eg., beefalo) or mixed species herds (of *Bos taurus* mixed with others) were considered individually, in case they provided sufficient cattle-specific information. Selected studies had to address outcomes related to *C. parvum* infection. *C. parvum* infection in the animals was based on clinical diagnosis. Other *Cryptosporidium* spp. infections were excluded because evidence that other *Cryptosporidium* species are likely to be pathogenic in calves is weak (Wells and Thomson, 2014; Thomson *et al.*, 2017; Åberg *et al.*, 2020).

Intervention

The intervention had to be halofuginone treatment, in the form of halofuginone lactate or halofuginone hydrobromide, in an attempt to reduce incidence or severity of cryptosporidiosis in young cows. Interventions could be either relatively prophylactic because very early in life (before 5 days old) or relatively late and therefore more likely to be treatment for existing or developing symptoms (age 5 days +). The vast majority of calves suffering from cryptosporidiosis are under 3-4 weeks old (Castro-Hermida *et al.*, 2002; Wells and Thomson, 2014). The threshold of five days was to distinguish prophylaxis from treatment and was chosen to reflect a likely point of onset of symptoms, typically on day 4 or 5 of life (Erbe, 2010).

Study design and comparators

Only deliberate experiments with concurrent comparison animals were included. Pre-post and case-control designs were not eligible. There were no limits on location or publication date. Studies were

excluded if not available in a language known to the authors (English, German, Spanish or French) or if the article could not be easily translated into English using Google Translate. Articles without abstracts or available full text were excluded.

Outcomes of experiments

To be eligible, studies had to address at least one of these outcomes:

- Clinically detectable infection in (shedding from) live animals, of *C. parvum*.
- Fecal scoring consistency: usually on scales of 1-3, 0-3, 0-4 etc., to describe severity of diarrhea
- Measures of weight gain
- Mortality

Reference Sources

The search was mostly within peer-review research. Literature databases were chosen following recommendations about the most comprehensive bibliographic sources for veterinary science research (Grindlay *et al.*, 2012). Searched databases were: Scopus, CAB International abstracts, Pubmed and Embase. A limited grey literature search was undertaken of three government databases via websites: The UK Dept for Food and Rural Affairs, The US Dept. of Agriculture library (at Cornell University) and The European Commission, Agricultural and Rural Development section. Conference proceedings were not searched.

Search Strategy

From preliminary literature scoping, we selected two exemplar articles that met our inclusion criteria (De Waele *et al.*, 2010; Trotz-Williams *et al.*, 2011). The search terms were developed and validated by making sure searching using the below terms found both exemplar articles with a minimum of extraneous (irrelevant search return results). Within the peer-review bibliographic databases, we searched title/abstract/keywords:

At least one of (*Cryptosporidium , C. parvum*, cryptosporidiosis)

AND

At least one of (calf, cattle, cow, bull, dam, dairy, beef, herd, calves)

Grey literature search terms were *cryptosporidium*, cryptosporidiosis and *parvum*. Some especially thorough and recent review papers about cryptosporidiosis (Silverlås *et al.*, 2009; Johnson *et al.*, 2011; Taylor and Bartram, 2012; Olias *et al.*, 2018; Beaver *et al.*, 2019) were checked for eligible articles missed by our search strategy. Forward and backward citation searches of included articles were not done to look for additional studies.

Study selection, quality assessment and data extraction

After de-duplication, titles and abstracts were independently screened by two investigators (JB and CCH). Items were chosen for full text review or excluded. Selection disagreements were resolved by discussion or on the verdict of a third reviewer (PRH). Full texts were obtained where possible. Decisions about final inclusion or exclusion were made after full text review by one or more authors.

Quality Assessment

The reliability and consistency of the original experimental information was assessed using a customised-quality assessment form. Focused questions were written to assess quality using consistent decision criteria. The standarised extraction form and quality assessment decision criteria are in Supplemental Material 2. Full text review, data extraction and quality assessment were undertaken by a single author (either JB or CCH). Low risk studies were deemed to be those who had low risk of bias assigned for at least 5 of the 7 possible quality checklist questions. Trial quality is shown by colour coding (green = low risk of bias, yellow = unclear, red = high risk of bias). Because *C. parvum* infection in the animals was based on clinical diagnosis, we report narratively what the diagnostic or clinical evidence was for *C. parvum* infection among the calves in each study.

Reporting and Synthesis

Meta-analysis was with random-effects due to expected high heterogeneity, using REVMAN version 5.3 (Deeks *et al.*, 2011). Meta-analyses are described narratively with reference to forest plots. Studies were all either randomized controlled trials (RCT) or controlled clinical trials (CCT) (Deeks *et al.*, 2011). The advantage of RCTs is that animals are assigned randomly to treatment/not treatment, which means

that any co-variates should be randomly distributed and are less likely to bias the experiment. CCTs are structured similarly but animals were not randomized to treatment, which means that there are more likely to be unobserved confounders that explain any differences rather than the treatment regime. The forest plots distinguish results by study design (RCT vs. CCT) for main results with combined study designs in subgroupings. Significance level was set at $p \leq 0.05$. Funnel plots were generated and visually examined for evidence of publication bias, such as missing small trials with negative results. Funnel plots were only generated where at least ten studies provided relevant data (either/both CCTs or RCTs) for any outcome stratified by early/late treatment phase as described below. Funnel plots are discussed narratively in this manuscript and provided in graphic format in Supplemental Material 3.

How to best pool extracted data depended on the specific outcome. Mortality was simple: of the animals that started the trials in each arm, how many died during the monitoring period could be input to calculate pooled risk ratios. However, data for any amount (or severity) of oocyst shedding or diarrhea incidence were reported using a variety of scales. Shedding of oocysts was reported (for instance) as prevalence of animals with any detected oocysts, prevalence of animals shedding above a certain threshold or by average score for the arm on specific dates (scoring from 0 to higher levels, where higher level numbers meant more oocysts detected). Because all these forms of reporting were ways of comparing oocyst detection between groups, we pooled these outcomes into a combined outcome which we describe under the umbrella term “oocyst shedding”. Fecal consistency was typically reported on multi-level scales (from 0 to 2, 3 or 4, where higher levels were more liquid). Weight might be reported as average daily weight gain, total weight at trial end, or weight change since birth. These diverse metrics were measuring the same outcomes but on different scales. They were therefore compared in meta-analysis using *standard mean differences* (SMDs). SMDs standardize for differences between arms rather than rely on the same instrument being used to measure an outcome in all trials. Lower SMD was a better outcome for calves with regard to diarrhea or oocyst shedding but higher SMD value (above 0) was the preferred outcome with respect to weight gain. However, rarely was variance reported with either oocyst counts or diarrhea outcomes. These outcomes were usually presented as simple averages without variance at single points in the monitoring period. For those studies, where an entire-period average and standard deviation value was unavailable, the mean period value was used with standard deviations calculated from the daily averages. Eg., if the only fecal scores supplied for a group of animals for just 3 dates during the monitoring period were daily averages from three scores = 1, 2, 3, then the mean for the monitoring period was assigned to equal 2, sd 1. For transparency, the

data extracted and used to calculate the oocyst/diarrhea scores are available to view in Supplemental Material 4. Comparing transformed scores was valid as the original metrics fundamentally measured the same outcome (eg. weight gain, intensity of oocyst shedding or diarrhea) and were compared between studies using SMDs.

Stratification and subgrouping

Exposure soon after birth for most animals means that most untreated calves are symptomatic by 5 days old (Erbe, 2010). The pathology of disease is worse in young animals (Thomson *et al.*, 2019); older infected animals may be more resilient and have less morbidity and mortality. Results were therefore divided by whether treatment was relatively early (before calves were 5 days old), or relatively 'late': started when animals were age 5 days or older. Where at least four eligible trials were available, we analysed subgroups for when studies were funded by known or likely sellers of HFG or rival products, or had low risk of bias. For diarrhea only, we also tested whether the efficacy of HFG varied depending whether dams received vaccination against other pathogens that can cause diarrhea (eg., rotavirus, coronavirus or *E. coli*). We did not subgroup by daily dosage or duration of treatment because these varied relatively little between trials (see Results, Table 1).

RESULTS

Figure 1 shows the study selection process. There were 2475 mostly unique articles found in scientific databases. There were no hits on the USDA library site and 14 hits on the EC site (none of which were eligible for inclusion). On the UK DEFRA site there were 33 hits, most of which related to prevalence in humans or human disease risk factors; none related to disease prevention in cattle.

Figure 1 Study Selection Procedure

Twenty articles were selected for full text review. Of these reports, one was unavailable. One write-up (available as a thesis, (Erbe, 2010)) was added because it is published online and was mentioned in recent literature reviews. Two articles were excluded after full text review leaving 18 articles that were included in this review. Most articles described randomized controlled trials (RCTs), but some described

experiments where it was not stated that animals had been assigned to treatment randomly (clinical controlled trials, CCTs). Often, more than one experiment was documented within a single report. There were 15 RCT comparison arms described in eleven articles. One article (Keidel and Daugschies, 2013) described two RCTS and a CCT. Seven other articles described ten comparisons in CCTs. Most reports addressed early treatment with HFG, but four articles described tests of HFG when treatment started mostly after 4 days of age. Table 1 lists characteristics of the 18 included scientific articles.

Four trials were assessed to have low risk of bias (De Waele *et al.*, 2010; Trotz-Williams *et al.*, 2011; Al Mawly *et al.*, 2013; Vélez *et al.*, 2019). Diagnosis of *C. parvum* infection was mostly based on clinical presentation, sometimes informed by herd history. Confirmation of *Cryptosporidium* spp. oocyst shedding was done in all studies using microscopy, sometimes enhanced with staining or immunofluorescence (see detection methods for each study listed in Table 1). Two studies (Joachim *et al.*, 2003; Keidel and Daugschies, 2013) also used an immunological test (ELISA) as a diagnosis aid.

All reports described treatment on herds of *Bos taurus* (no mixed species or hybrids). The vast majority were Holstein breed or Holstein crosses, from dairy herds. There was a mix of sexes. Most (14/18) of the included articles described research that took place in Western Europe.

Oocyst shedding, early treatment

Most of the included trials provided data to compare how many animals in each arm shed oocysts. The meta-analysis results for early treatment (Figure 2) are consistent in both RCTs and CCTs in showing significantly less oocyst shedding in the treatment arms (both $p < 0.01$). Similar levels of significance were retained even when the data were subgrouped to only include experiments at low risk of bias (SMD -0.45, 95%CI -0.61 to -0.30, I^2=0%) or those not funded by industry (SMD -0.60, 95%CI -0.87 to -0.32, I^2=35%). The funnel plot (Figure S3.1 in Supplemental Material 3) for these trials showed outliers but not large imbalances either side of the mean effect.

Figure 2. Oocyst shedding following early (prophylactic) treatment with halofuginone products

Oocyst shedding, late treatment

Information about oocyst shedding among animals who started treatment at age 5 days+ was available in four studies. All of the studies reported significantly less oocyst shedding by calves in experimental arms ($p \leq 0.01$; Figure 3). None of these trials had low risk of bias and only one trial (Klein et al 2008) was clearly not funded by industry.

Figure 3. Oocyst shedding following late treatment with halofuginone products

Diarrhea, early treatment

Figure 4 shows outcomes after halofuginone lactate treatment initiation before 5 days old. Diarrheal burden was significantly lower in treated animals in the main results grouped by study design (RCT or CCT) and among studies with stated funders that were not sponsored by industry ($p \leq 0.05$ in all of these; Figure 4). However, among the four trials at low risk of bias, the pooled effect SMD did not reach the significance threshold, SMD -0.39, 95%CI -0.93 to 0.15, I^2 78%, P = 0.16. Heterogeneity was relatively high in all of the comparisons ($I^2 > 50\%$), which indicates lack of consistency in results.

The funnel plot of the early treatment, diarrhea intensity outcome after halofuginone lactate treatment (Supplemental Material Figure S3.2) is strongly not symmetrical and suggests there are many missing small studies with negative results (ie., suggesting publication bias). The funnel plot highlights that the Trotz-Williams *et al.* (2011) study is an outlier; it was especially large and unusually found that untreated calves had less diarrhea. Another trial (Klein, 2008) was relatively large while finding little evidence of differences in diarrhea intensity between experimental arms.

Diarrhea after early treatment, whether dams had any vaccinations against diarrheal diseases

Halofuginone seemed most effective when the studies did not state that dams had relevant vaccinations (SMD -0.57, 95%CI -0.88 to -0.26, I^2 47%, p = 0.0003; Figure 5). However, strong efficacy was also

observed when dams were reported to have had vaccinations relevant to diarrheal disease (second subgroup Figure 5). These findings suggest that vaccination status of dams did not prevent diarrhea in very young calves, but this is caveated with the observation that the pooled data were highly heterogeneous and therefore inconsistent ($I^2 \geq 47\%$).

Figure 4. Diarrhea intensity following early (prophylactic) treatment with halofuginone products, subgroups by industry funding and risk of bias

Figure 5. Diarrhea intensity following early (prophylactic) treatment with halofuginone products, subgroup by vaccination history of dam

Diarrhea, treatment from 5 days of age

Two RCTs reported data about diarrhea in calves following treatment initiation at age 5+ days. The trial by Klein (2008) used halofuginone lactate while the trial by Lallemond *et al.* (2006) used halofuginone hydrobromide. Both seemed at least somewhat effective at reducing the severity of diarrhea. In pooled analysis, diarrheal intensity scores were significantly lower among treated animals (SMD -0.31, 95%CI -0.59 to -0.02, p = 0.03; Figure 6). Neither trial had low risk of bias, while only the trial by Klein (2008) was clearly not funded by industry.

Figure 6. Diarrhea intensity following late treatment (at age 5 days+) with halofuginone products

Mortality, prophylactic (early) treatment

Pooled risk ratios were calculated for mortality outcomes at end of monitoring periods and are presented in the chart below. Mortality was significantly lower in treatment arms (RCT RR 0.64, 95%CI 0.42 to 0.98, I^2 0%; CCT RR 0.24, 95%CI 0.12-0.49, I^2 0%). In Figure 7 a lower risk ratio is good (meaning fewer deaths in that arm). Heterogeneity was low (low I^2) which means that the results from different

trials are fairly similar. The evidence base is somewhat problematic in that many trials made no explicit mention whether there were deaths or not. Many other trials were explicit in saying that no deaths occurred in neither arm. The data below are drawn from trials where at least one death occurred in either arm.

Four studies provided data on five early-treatment comparisons with low risk of bias gave mortality data; the RR almost reached significance threshold, p = 0.07 (RR 0.64, 95%CI 0.40 to 1.03, I^2 0%). The near significance is due to the results of a single large trial (Trotz-Williams *et al.*, 2011). The funnel plot (Supplemental Material Figure S3.3) did not suggest strong publication bias: effects were scattered equally around the mean difference and roughly in a triangular shape.

Figure 7. Mortality following early (mortality) treatment with halofuginone products

Mortality, late treatment

Only two trials, one RCT and one CCT, reported on mortality when HFG treatment was initiated to animals aged ≥ 5 days. Again, mortality was lower in the treatment arms although the risk ratios are less significant than they were for early treatment experiments (see Figure 8).

Figure 8. Mortality following late (age 5 days+) treatment with halofuginone products

Weight gain

Relatively few reports (n=7 providing data on 8 comparisons) gave information about weight gain differences between arms following halofuginone lactate treatment (Figure 9). Weight gain comparison data suitable for meta-analysis were only available when calves had been treated relatively early (no data suitable for meta-analysis were available for when treatment started at age 5 days plus). These data suggest no weight gain benefits for animals in either arm. RCT evidence for weight gain comparisons had SMD 0.13 (95%CI -0.29 to 0.54, I^2 23%). Heterogeneity was relatively moderate among

CCTs or RCTs. It is important to note that the weight comparisons mostly apply to *surviving* animals at the end of the monitoring periods. The monitoring periods were typically 28-33 days long (see Table 1 for specific durations).

There were no studies at low risk of bias with weight gain data. Just two of the studies that reported on weight gain outcomes after halofuginone lactate treatment (Villacorta *et al.*, 1991; Niine *et al.*, 2018) were not sponsored by industry.

Figure 9. Weight gain following early (prophylactic) treatment with halofuginone products

DISCUSSION

This systematic review has shown that the prophylactic use of halofuginone products had significant effects on calf health and welfare as measured by reductions in oocyst shedding, diarrhea and calf mortality. These findings were also upheld when the data were subdivided into randomised control treatment studies or clinical control treatment studies, showing that both study types provided consistent results on the efficacy of the prophylactic treatment of halofuginone. This current systematic review found more conclusive results on these characteristics than the earlier systematic review by Silverlås *et al.* (2009). This was possible because the earlier review could only use the data from 6 publications detailing 7 comparison groups. In contrast, our review had data available from 18 publications involving 25 comparison groups. Our review separated the trials into prophylactic and therapeutic use of halofuginone in order to reduce variation of between studies due to the different treatment regimes. Our analysis offers more conclusive support for the efficacy of halofuginone as a prophylactic treatment against cryptosporidiosis. The previous systematic review by Silverlås *et al.* (2009) also highlighted the effect of potential bias in the available publications due to involvement by the funders. This was addressed in the current review by excluding data from publications which had industry sponsors. Our analysis showed significant differences between groups of calves that received prophylactic halofuginone treatments and the control groups for two outcomes: oocyst shedding and severity of diarrhea. The evidence for reduced mortality was not as conclusive but did approach

significance (p=0.07). A further characteristic that did not give conclusive results was the prophylactic effect of halofuginone treatment on weight gain. This may be because only a few studies provided data suitable for pooled analysis. For example, in an RCT using 60 animals and halofuginone bromide, Lallemond *et al.* (2006) reported nearly identical average daily weight gain in the period from 3 to 28 days old (0.31 kg/day for intervention arm, 0.33 kg/day for controls). Variance units for average daily weight gain in respective groups were unclearly reported in this study which made calculation of an odds ratio unfeasible and the study had to be excluded from meta-analysis. Weight gain as an outcome may be especially sensitive to severity of disease. Shaw *et al.* (2020) found no significant difference in weight gain between severely infected calves and calves that showed mild or moderate symptoms of cryptosporidiosis, by the age of six months. Calves grouped by symptom severity all had significantly lower weight gain by six months old than calves who had never presented symptoms of cryptosporidiosis. This suggests that preventive treatment may need to result in no signs of cryptosporidiosis to achieve significant difference in weight gain results.

This current review also analysed the available data to see if there is any evidence for the efficacy for halofuginone as a therapeutic treatment, as defined by an initiation of treatment date after calves were 5 days of age. Unfortunately, only four studies were available for these analyses. The CCT and the RCT studies did show significant reductions in oocyst shedding by the treated calves but one of the concerns is that only one study (Klein, 2008) did not have funding from industry sponsors. For the subsequent analyses (diarrhea and mortality) only 2 studies provided data and none had data on weight gain, preventing comprehensive investigations into the therapeutic use of halofuginone against cryptosporidiosis. Since the review by Silverlås and colleagues was published in 2009, no new papers have been published with trials that describe the therapeutic use of halofuginone, leaving only four reports (Peeters *et al.*, 1993; Lallemond *et al.*, 2006; Klein, 2008; Pilarczyk *et al.*, 2008). Four studies are unlikely to be sufficient to provide robust conclusions. Other areas that need more research that could not be addressed by the current review were the economic costs/benefits from using halofuginone and the efficacy of other treatments for cryptosporidiosis such as paromomycin-based products, which are licensed in some localities for use against cryptosporidiosis in neonatal calves. The environmental effect of halofuginone treatment was also not assessed in this review but treatment may be beneficial for calves born subsequently, as halofuginone treatment resulted in reduced oocyst shedding. There is evidence that increased infection doses lead to more morbidity due to cryptosporidiosis (Zambriski *et al.*, 2013). This can explain why farmers often see more severe problems with cryptosporidiosis in the

latter stages of the calving period as parasite loads are amplified due to greater environmental contamination.

One of the main limitations of halofuginone treatment is that it cannot be given to calves that have diarrhea for more than 24 hours or that are already weak or dehydrated (European Medicines Agency, 2007). This limits its suitability as a therapeutic treatment. As a result farms may have to rely on a different treatment but still use halofuginone prophylactically for their other calves that do not show any symptoms of infection yet. Another complication with halofuginone treatment is that it is important to get the dose correct for each calf. HFG has been shown to have toxic side at twice the recommended dose, which means that calves should be weighed in order to determine the correct dose (European Medicines Agency, 2007).

Almost all of the studies we found reported on use of halofuginone lactate rather than halofuginone hydrobromide. This is probably because halofuginone hydrobromide (a derivative of halofuginone lactate) is less soluble. HFG products are typically administered as part of liquid feeds to very young calves, so it is impractical to use a poorly soluble product.

It is unlikely that *C. parvum* can be eliminated from an affected farm as infected animals shed oocysts in huge amounts while the infectious dose required for animals to contract cryptosporidiosis is very small (as low as 25 oocysts; Zambriski *et al.*, 2013). However, disease severity seems to be dependent on the infectious dose (Zambriski *et al.*, 2013) which means that many farmers have looked at livestock management strategies to reduce transmission and morbidity. Strategies involve improved hygiene, welfare, segregation and nutritional measures (Wells and Thomson, 2014). Hygiene encompasses maintenance of rigorous cleaning and hygiene routines for both pens and animals with products that are efficacious for use against *Cryptosporidium*. Deep straw bedding may increase cleanliness of the animals and reduce contact with contaminated faeces. Bedding should be kept as dry as possible. Disinfection (buckets or pans) should be available to staff at entrances to calf sheds. Symptomatic treatment includes keeping animals warm and hydrated with rehydration with electrolytes if necessary. Healthy animals should be attended before sick animals as part of daily husbandry routines. Whether higher nutritional planes can make calves more resistant to morbidity from cryptosporidiosis has been tested in

other experiments without conclusive advantages for any specific nutritional strategy (Meganck *et al.*, 2014; Wells and Thomson, 2014; Vélez *et al.*, 2019; Brainard *et al.*, 2020a).

Segregation strategies encompass keeping healthy and sick animals separate as well as segregation by age groups. Young cows are the most at-risk group for developing illness from *C. parvum*. Sick calves should be quarantined as soon as possible after scouring begins and up to seven days after scouring ends. Additionally, as older calves/cows can still shed *C. parvum* oocysts (Thomson *et al.*, 2019), it may be best to keep older and pre-weaned animals separate. Individual housing of neonatal dairy calves does not seem to reduce calf to calf transmission, however (Brainard *et al.*, 2020b). Within Europe the possible management strategies are also governed by EU-side welfare regulations that ban the use of 'veal crates' for example (European Union 2008). Such confinement in contrast to use of large stalls that animals can move freely in was observed to increase morbidity due to cryptosporidiosis (Graef *et al.*, 2018).

This systematic review highlights the need for more targeted research of treatments against cryptosporidiosis. In particular, evidence about the therapeutic use of halofuginone as a treatment for cryptosporidiosis is available from relatively few publications. More studies (either therapeutic or prophylactic in design) not sponsored by industry would be preferable. This review also shows that there is a need for more data on the economic cost/benefit of halofuginone for farmers as well as better measures for evaluating the welfare benefit for the treated animals. The most reliable data available to date focused on oocyst counts and faecal consistency while fewer data on mortality and weight gain were available. Therefore, future drug treatment trials should be better designed to address these outcomes.

CONCLUSION

This systematic review has shown that the evidence in the scientific literature is getting stronger in support of the prophylactic treatment of calves with halofuginone because it has a beneficial impact on their health as it reduces diarrhea, oocyst shedding and mortality. The prophylactic use of halofuginone, as per manufacturer instructions at 12-48 hours old, on farms with confirmed cryptosporidiosis can be justified as it reduces morbidity and mortality. Subgrouping by sponsor or studies with lowest risk of bias does not change the broad conclusion that HFG is protective when administered early. However,

no conclusions could be reached that prophylactic HFG treatment resulted to higher weight gain among surviving animals. In addition, there is evidence of publication bias in the results and a dearth of utility calculations and reporting on adverse events, which means that it is difficult to establish that the treatment has economic benefits.

HFG is less effective in calves if treatment commences after bovine calves are 5 days old, although reduced oocyst shedding and diarrhea intensity are still reduced by relatively late treatment. Evidence is quite limited about whether HFG is effective at reducing mortality when given late. No clear evidence was found whether late-treatment HFG affects weight gain. There is also inadequate evidence about whether other treatments (not halofuginone based) might be equally or even more effective (Brainard *et al.*, 2020a) for improving important outcomes when given prophylactically or to symptomatic animals. Limitations of HFG use due to toxicity means that alternative products may have to be used, such as Paromomycin, which were not evaluated within this systematic review.

Key Findings

Author contributions

PRH and KT conceived the study. JB and CCH designed the study. JB conducted the searches, JB and CCH screened and extracted data and undertook quality assessment. JB undertook analysis, wrote first draft and assembled revisions. GH and KT researched and described *in vitro* and pharmaceutical properties of the drug. FK researched and described the veterinary significance of the treatment. All authors revised draft manuscripts and approve the final manuscript.

Conflicts of Interest

None. On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical Standards

Not appilcable

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725 **Table 1. Included article characteristics**
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Article	Dose* ; Industry sponsored?	Study design Location	Calf age (days) when HFG administered	Oldest age data used in meta-analysis; Detection method	Outcomes used in meta-analysis
Al Mawly *et al.* (2013)	8 ml Halocur®/d Yes	RCT New Zealand	0-6	20 days fluor. microsc.	Diarrhea, Mortality, Oocysts
De Waele *et al.* (2010)	100 µg/kg/d No	RCT Ireland	0-6	28 days fluor. microsc.	Diarrhea, Mortality, Oocysts
Erbe (2010)	8 ml Halocur®/d Yes	RCT Germany	0-6	22+ days stain microsc.	Diarrhea, Oocysts, Weight
Jarvie *et al.* (2005)	5 mg/d Yes	RCT Canada	0-6	26+ days microscopy	Diarrhea, Mortality, Oocysts, Weight
Joachim *et al.* (2003)	2 ml Halocur®/ 10 kg No	RCT Germany	0-6	21 days Stain microsc. & ELISA	Diarrhea, Mortality, Oocysts
Keidel and Daugschies (2013)	120 µg/kg/d Unclear	RCT, CCT Germany	1-7	20 days stain microsc. & ELISA/PCR	Diarrhea, Oocysts
Klein (2008)	100 µg/kg/d No	RCT Czech Rep.	1-7 *and* 8-16	27 days microscopy	Diarrhea, Oocysts
Lallemond *et al.* (2006)	100 µg/kg/d Yes	RCT Canada	8-14	34 days microscopy	Diarrhea, Oocysts, Mortality, Weight †
Lefay *et al.* (2001)	120 µg/kg/d Yes	RCT France	1-7	22 days microscopy	Diarrhea, Mortality
Martins *et al.* (2007)	120µg/Kg/day Unclear	CCT Portugal	0/1 to 6/7	14 days stain microsc.	Oocysts
Naciri *et al.* (1993)	120 µg/kg/d Unclear	CCT Belgium	2-8	31 days microscopy	Oocysts, Mortality, Weight
Niine *et al.* (2018)	Unclear dose No	CCT Estonia	1-7 *or* 3-9	47 days fluor. microsc.	Mortality, Oocysts, Weight
Peeters *et al.* (1993)	120 µg/kg/d Yes	CCT France	2-8 *and* 5-11	29 *and* 53 days microscopy	Mortality, Oocysts
Pilarczyk *et al.* (2008)	2 ml Halocur ®/10 kg Unclear	CCT Germany	0-6 *and* variable to +6 d	18 days Stain microsc.	Mortality, Oocysts
Trotz-Williams *et al.* (2011)	100 µg/kg/d Yes	RCT Canada	0-6	21 days microscopy	Diarrhea, Mortality, Oocysts
Vélez *et al.* (2019)	8 or 12 ml/d Yes**	CCT Germany	0-6	28+ days stain microsc.	Diarrhea, Mortality, Oocysts, Weight
Villacorta *et al.* (1991)	125 µg/kg/d No	CCT Spain	1-7	28 days Stain microsc.	Oocysts, Weight
Wiedemann *et al.* (2012)	100 µg/kg/d Yes	CCT Germany	1-7	35 days stain microsc.	Diarrhea, Oocysts, Weight

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Notes for Table 1: Studies were deemed to be sponsored by industry if they stated an HFG distributor was their funder, a co-author worked for a pharmaceutical company that was possibly a distributor of HFG or potential rival products for controlling cryptosporidium (Intervet, Delimax, Roussel-Uclaf) or if authors stated that a company had "supplied" the HFG. Sponsor was deemed to be "unclear" if no funding statement was made. *Some trials had arms receiving other doses; results from other doses were not used in our pooling. **Vélez et al. 2019 was funded by commercial developer of a potential rival product to HFG that was also trialed in a third arm not summarized in this review. † Weight was reported with unclear variance in Lallemond *et al.* (2006) so not suitable for pooling in meta-analysis. Abbreviations: CCT = controlled clinical trial; ELISA = enzyme linked immunoassay; fluor = fluorescent or immunofluorescence enhanced microscopy; microsc = microscopy; PCR = polymerase chain reaction (test); RCT = randomized controlled trial.