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Antibody fusions reduce onset of experimental *Cryptosporidium parvum* infection in calves

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ABSTRACT

Cryptosporidium parvum is one of the main causes of diarrhea in neonatal calves resulting in significant morbidity and economic losses for producers worldwide. We have previously demonstrated efficacy of a new class of antimicrobial antibody fusions in a neonatal mouse model for *C. parvum* infection. Here, we extend efficacy testing of these products to experimental infection in calves, the principal target species. Neonatal calves were challenged with *C. parvum* oocysts and concomitantly treated with antibody–biocide fusion 4H9-G1-LL37 over the course of four days. This resulted in reduced severity of the disease when compared to control animals. Overall clinical health parameters showed significant improvement in treated animals. Oocyst shedding was reduced in treated when compared to control animals. Control of oocyst shedding is a prerequisite for breaking the cycle of re-infection on dairy farms. Antibody–biocide fusion products thus have the potential to reduce the impact of the infection in both individual animals and in the herd.

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1. Introduction

Cryptosporidial infection is one of the most common causes of diarrhea in humans and livestock worldwide (Fayer, 2008b; Fayer et al., 1997; Tzipori and Widmer, 2008; Wyatt et al., 2010). The most prevalent species producing disease in cattle, *Cryptosporidium parvum*, can cause extensive economic loss in young calves, although some cases remain subclinical (Wyatt et al., 2010). *C. parvum* is a primary contributor to the neonatal diarrhea complex, along with rotavirus, coronavirus, and *Escherichia coli*, and is one of the most important zoonotic agents of diarrhea in calves aged below one month (Naciri et al., 1999). Severely affected animals may die, but more often, a self-limiting

diarrhea results in morbidity and increased labor and veterinary costs (deGraaf et al., 1999). Infected animals usually shed large numbers of infective oocysts in their feces (Nydram et al., 2001). In dairy calves, the prevalence of shedding has been reported to be the highest among animals 7–21 days of age, with a prepatent period of 3–6 days (Fayer, 2008a).

In the absence of effective approved drugs in the USA, the control of cryptosporidiosis in calves relies mainly on hygiene measures and good management (Wyatt et al., 2010). A variety of therapeutic, prophylactic, and metaphylactic pharmaceuticals have been tested for efficacy against *C. parvum* in humans and animals with varying results (Silverlas et al., 2009; Stockdale et al., 2008; Zhu, 2008). Halofuginone and paromomycin have been tested for veterinary use. According to a recent review and meta-analysis study investigating multiple field trials, calves did not benefit from Halofuginone treatment (Silverlas et al., 2009). Halofuginone lactate (Halocur[®], Intervet) has been

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approved for use in several European countries, while paromomycin sulphate (Gabbrovet®, Ceva Santé Animale) is only available as an injectable against bacterial infections in a few countries for piglets, calves and poultry. Vaccines made from killed *C. parvum* oocysts (Harp and Goff, 1995, 1998) or repetitively passaged precocious strains of *C. parvum* (Fayer, 1994) have been tested for their ability to protect calves from diarrhea and limit oocyst shedding. Vaccination of day-old calves using gamma irradiated oocysts reduced oocyst shedding and diarrhea when challenged with *C. parvum* on day 21 (Jenkins et al., 2004), however, field trials of killed vaccines have not always convincingly shown protection (Harp and Goff, 1995, 1998). There is increasing awareness of the need to develop new, more efficient and safer products to control cryptosporidiosis in livestock (Stockdale et al., 2008).

Passive immunization against cryptosporidiosis has been investigated using various strategies and was found to be effective at reducing infection (Burton et al., 2011; Imboden et al., 2010; Perryman et al., 1999; Schaefer et al., 2000). A formulation of monoclonal antibodies (MAbs) 3E2 (α CSL), 3H2 (α GP25-200), and 1E10 (α P23) provided significant additive prophylactic efficacy over that of the individual MAbs in neonatal ICR mice (Schaefer et al., 2000). In a study using an adult SCID mouse model to evaluate the therapeutic effect of α CSL MAb 3E2, highly significant efficacy in reducing, but not eliminating, persistent *C. parvum* infection was demonstrated (Riggs et al., 2002). A study to evaluate prophylactic efficacy of MAb 3E2 against oocyst challenge was conducted in calves and the results demonstrated significant reductions in severity of diarrhea and oocyst shedding (Riggs et al., 2004). While these studies demonstrated efficacy of antibody-based treatments for cryptosporidiosis, the effective treatment dose of the bioreactor-produced MAb used was cost prohibitive to developing an economically feasible veterinary product.

In an attempt to improve the treatment efficacy of MAbs, we designed molecules consisting of a MAb “armed” with membrane-disruptive peptides (biocides) from the mammalian innate immunity arsenal (Imboden et al., 2010). The selection of MAbs was based on the work of Schaefer et al. who described significant sporozoite neutralization *in vitro* and reduction of infection in neonatal mice with a panel of MAbs produced against surface-exposed antigens of *C. parvum* sporozoites. Among others, MAb 4H9, which was produced against the GP25-200 antigen complex of *C. parvum*, was chosen for the development of an antibody–biocide fusion (Schaefer et al., 2000). This was done by genetically linking the coding sequence of MAb 4H9 heavy chain with the coding sequence of LL37, a human cathelicidin-derived antimicrobial peptide. The resulting constructs were expressed in a highly efficient retrovector-based mammalian cell culture system. Testing of these novel immunotherapeutics in a neonatal ICR mouse infection (asymptomatic) model revealed that 4H9-G1-LL37 achieved sporozoite neutralization at a 50 fold lower dose than *C. parvum*-specific neutralizing MAb 3E2, the most potent of the neutralizing antibodies identified to date (Imboden et al., 2010). A dose response mouse trial demonstrated efficacy at a dosage as low as 1.5 mg/kg/d given for 5 days (Imboden et al., 2010). This study allowed

establishment of an effective dose for orally administered antibody–biocide fusions in rodents.

The present study assessed the efficacy of 4H9-G1-LL37 against experimental *C. parvum* infection in a calf model. Several clinical and parasitological parameters were used to compare animals treated with the antibody–biocide fusion to controls.

2. Materials and methods

2.1. Parasites

The Iowa *C. parvum* isolate (Heine et al., 1984), which was the source of oocysts for all experiments, has been maintained since 1988 by propagation in newborn *Cryptosporidium*-free Holstein bull calves (Riggs et al., 1989; Riggs and Perryman, 1987). Oocysts were isolated from calf feces by sucrose density gradient centrifugation and stored in 2.5% KCr₂O₇ (4 °C) (Arrowood and Donaldson, 1996; Riggs and Perryman, 1987). For calf and mouse challenge studies, oocysts were used within 30 days of isolation and disinfected with 1% peracetic acid immediately prior to administration (Riggs et al., 1994). Oocyst excystation was determined immediately prior to administration, or to obtain isolated sporozoites, and always exceeded 90%.

2.2. Assembly of genetic constructs

The genetic construct used to produce the recombinant 4H9-G1-LL37 product used in this study was assembled as described earlier (Imboden et al., 2010). Briefly, total RNA from 4H9 hybridoma cells was reverse transcribed and subjected to degenerate primer PCR using an immunoglobulin cloning kit according to the manufacturers instructions (Novagen, EMD Chemicals, Gibbstown, NJ) to isolate the variable region of the IgG1 heavy and light chain genes. The variable regions obtained were linked to murine IgG1 heavy or light chain constant regions respectively. The IgG1 heavy chain constant region was previously engineered (Imboden et al., 2010) to contain the human LL37 sequence attached to the C-terminal end. Fully assembled heavy chain-linker-LL37 and light chain constructs were transduced into CHO (Chinese hamster ovary) cells using a pantropic Moloney murine leukemia virus-based retroviral expression vector system (Clontech, Mountain View, CA). Due to the high efficiency transduction, cell pools secreting the 4H9-G1-LL37 antibody–biocide fusion were subjected to standard limiting dilution cloning to isolate clonal cell lines for pilot scale production in 20 L benchtop fermentors (Wave Bioreactor™ Systems, GE, Piscataway, NJ).

2.3. Production of antibody–biocide fusion in mammalian expression system

For this study, separate 201 batches of 4H9-G1-LL37-producing CHO cells, and control CHO cells not containing any transgenes, were grown for each of the four cohorts of calves described below. Each production batch was treated and processed identically. Upon harvesting the culture via centrifugation, the supernatant was subjected to cross flow filtration using a 50 kDa ultrafiltration cartridge to remove

lower molecular weight range contaminants, to concentrate the product, and dialyse the sample against saline. Following cross flow filtration, the 4H9-G1-LL37 product was quantified by ELISA for detection of heavy and light chain and blind-coded single-dose aliquots of both 4H9-G1-LL37 and control CHO cell supernatant (control substance) were prepared and shipped frozen to the University of Arizona. Aliquots from each production batch were retained and tested in separate neonatal mouse efficacy trials to confirm product consistency (data not shown). No statistical difference in efficacy was observed between the four batches (data not shown); each batch was effective at reducing infection in the neonatal mouse model, consistent with published data (Imboden et al., 2010).

2.4. Animals

A total of sixteen newborn Holstein bull calves were obtained from the same closed herd production unit of approximately 10,000 dairy cows, in 4 cohorts of 4 calves each. To assemble an age-matched cohort of 4 calves, cows calving within a maximum 12 h period were selected. Calving was manually assisted and the calves were delivered onto plastic sheeting to prevent contamination with enteropathogens that might otherwise confound interpretation of the results, and then placed in individual disinfected transport crates containing autoclaved straw. Upon arrival at the University of Arizona Central Animal Facility BSL2 unit, each calf was randomly assigned to groups using the Microsoft Excel Random Number Generation Tool, weighed and then placed into an elevated calf stall (Wenke Manufacturing, Pender, NE) equipped with urine and fecal collection pans. Calf weights were between 37.5 and 48.6 kg. A single ear tag was applied which served as the calf's identifying number in the study. Commercial colostrum replacer (Bovine IgG Colostrum Replacement, Land O'Lakes, Shoreview, MN) from a single production lot for all calves in the study was administered within 2 h of birth per label instructions. Adequacy of passive transfer of colostrum IgG was determined using a commercially available sodium sulfite precipitation test, serum from each calf was collected at 24–36 h of age (Bova-S Bovine FPT Test Kit, VMRD Inc., Pullman, WA). All the calves reached serum levels of immunoglobulin between 800 and 1600 mg/ml which is defined as adequate transfer. Bo-Se[®] vitamin E/Se supplement (3 ml/45 kg) (Merck, Whitehouse Station, NJ) was injected subcutaneously to each calf within 12 h of birth. Beginning at 12 h of age, all calves were maintained on reconstituted milk replacer (Nutrena[®] Snowflakes[®] Calf Milk II-Utiliz Milk Replacer Medicated, Cargill Animal Nutrition, Minneapolis, MN) twice daily (10% body weight at arrival for days 1–5 and thereafter 50% more) until termination of the experiment at day 10 post infection (DPI). At the end of the acclimation period of 36 h, an overall clinical examination was performed to determine health status prior to admitting each animal to the study. Calves were observed for fever (>39.4 °C), diarrhea, respiratory distress, inability to rise on own, and inappetence. No exclusions were needed based on these health parameters. An individual fecal sample was collected from

each calf to determine the presence of other known viral and bacterial enteropathogenic agents (rotavirus, coronavirus, *E. Coli* K99 and/or F41, and *Salmonella*) 2 h post oocyst challenge and on DPI 10. Negative-staining electron microscopy was used for direct examination of feces to identify rotavirus and coronavirus particles. Aerobic cultures were performed on feces to detect the presence of aerobic bacterial pathogens. Sheep blood agar and Tergitol-7 agar (Hardy Diagnostics, Santa Maria, CA) were used for routine aerobic culture, whereas Tetrathionate broth and XLT-4 agar (Hardy) were used for *Salmonella* culture. Enteric bacteria were identified by routine biochemical tests (Anon, 2007). The presence of K99 antigen in *E. coli* isolates was evaluated by the *E. coli* antigen latex agglutination test kit (K-99 Pilitest. VWRD, Inc. Pullman, WA). All samples were found to be negative for these pathogens. The calves were examined once daily by a veterinarian. Each cohort consisted of two groups of two animals, with one group receiving 4H9-G1-LL37 and the other group receiving the control substance. Personnel involved in the calf handling were unaware of the treatment identity (single-blind study). During this 8-month trial, a total of 4 cohorts of 4 animals were recruited. There were two treatment groups: (i) 4H9-G1-LL37 test substance-treated, (ii) control substance-treated animals. All animals belonging to the same treatment group were statistically analyzed as one group, i.e. all data from the same treatment group were averaged across cohorts. The 4H9-G1-LL37 test substance-treated and control substance-treated animals were housed in separate BSL2 rooms. Prior to oocyst challenge at 36 h age, individual fecal samples were collected and examined for *C. parvum* oocysts by acid-fast staining and confirmed to be negative (Smith, 2008).

2.5. Infection and treatment

The day of oocyst inoculation was defined for each cohort as DPI 0, where calves were challenged at 36–48 h of age. Each calf was fed 200 ml of milk replacer in a nipple bottle to close the gastric groove. Immediately thereafter, a 50 ml syringe containing 5×10^7 *C. parvum* oocysts in 25 ml milk replacer was administered orally, followed by up to 500 ml of the pre-aliquoted dose of blind-coded 4H9-G1-LL37 or control substance mixed with milk replacer and fed in a nipple bottle. Two dose regimens were used: (i) cohort 1 was treated with 87 mg of 4H9-G1-LL37 (4 mg/kg/d) concomitant with challenge, and at 4 h, 24 h, 36 h, 48 h and 72 h post infection for a total of 6 treatments, and (ii) cohorts 2–4 were treated with 130 mg of 4H9-G1-LL37 (6 mg/kg/d) concomitant with challenge, and at 4 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h and 96 h post infection for a total of 9 treatments. Equal volumes of control substance, consisting of CHO cell supernatant processed identically to the 4H9-G1-LL37, were given to the control animals at the same time intervals. For the treatment, each calf was fed 200 ml of milk replacer in a nipple bottle to close the gastric groove, followed immediately by 500 ml containing the pre-aliquoted dose of 4H9-G1-LL37 or control substance, and then the appropriate amount of milk replacer (10% of body weight total per day). Potable water (4 l per bucket) was made available ad libitum. Oral electrolytes (Re-Sorb[®],

Pfizer) were available in a bucket to each calf at the onset of diarrhea.

Evaluation of the animals included fecal and parasitological parameters, as well as clinical observation of general health and food intake parameters. Fecal collection pans were positioned beneath the rear of the stall to ensure accurate collection of all feces for subsequent analyses. Contamination of fecal pans with urine was prevented by attaching disposable 8 h urine absorption pads to cover the preputial region and attachment of a full-width angled plastic shield to the underside of the stall floor to divert any urine leakage into a separate collection pan. The shield was placed so that urine was diverted but feces were allowed to pass into fecal collection pans.

2.6. Evaluation criteria

Fecal volume (ml) was measured and fecal consistency was evaluated twice daily for each calf. Feces from each daily collection were thoroughly mixed in an industrial blender (Hamilton Beach, Southern Pines, NC) to ensure sample uniformity and a 50 ml representative sample was then obtained and kept at 4°C for oocyst quantification. Oocysts were quantified by IFA using oocyst-specific MAbs as previously described (Riggs et al., 2002). Clinical parameters were assessed twice daily and assigned a score. Individual scores were recorded for fecal consistency (formed = 1; loose but not diarrheic = 2; diarrheic = 3; severely diarrheic – watery and profuse = 4); willingness to rise (eager to rise or already up = 1; willing to rise on own but needs encouragement = 2; reluctant to rise but able with assistance = 3; unwilling to rise on own, requires lifting = 4); stance when up (stable, normal = 1; somewhat unstable = 2; clearly unstable, loses balance or sways = 3; unable to stand = 4); appetite (ravenous, aggressive = 1; normal = 2; decreased but if coaxed will suckle = 3; anorectic, will not suckle = 4); attitude (bright, alert and responsive = 1; somewhat depressed, dull = 2; clearly depressed and minimally responsive = 3); and hydration status (normal = 1; dehydration evident = 2). Dehydration was tested by the skin tenting test. The care provider grasped the skin on the abdomen or by the rib cage between two fingers so that it is tented up. The skin is held for a few seconds then released. Skin with normal turgor snaps rapidly back to its normal position. Skin with decreased turgor remains elevated and returns slowly to its normal position, indicating dehydration. Intake (ml) of milk replacer, water and electrolytes was recorded twice daily. Bodyweight was recorded at the start and termination of the trial, and was used to compute average bodyweight gain.

2.7. Statistical analysis

Statistical design consisted of randomized complete block design with four animals per cohort (block) and two treatments, measuring the physiological and clinical parameters specified above. Average scores for health parameters were calculated by averaging the scores among all animals of the same treatment and the same day. The best possible score for a healthy animal is 1, accordingly

the worst score for a sick animal is 4. Statistical analysis was carried out using the modeling platform of JMP version 9 (SAS Institute, Cary, NC). A full factorial regression model was used with the following effects and interactions in the model: Cohort, Treatment, Cohort × Treatment, Cohort × Day, Treatment × Day, Cohort × Treatment × Day. In the regression analysis time (Day) was treated as a continuous fixed effect variable. Day is modeled as a fixed effect using a knotted spline as described by Stone and Koo (1986). A regression model with time as a fixed effect was chosen rather than a repeated measures analysis because of the nature of the biological responses which are all non-random time-dependent responses each dependent on the timing of their measurement relative to the parasite challenge. In that regard the study is more akin to a pharmacokinetic/pharmacodynamic study where spline functions are used in the models. Use of the knotted splines makes possible direct area under the curve measures fitting of the biological patterns. Use of knotted splines is similar to the use of polynomial terms in the model but can more closely fit the experimental data. Details of an example output of the modeling platform for the oocyst shedding data can be found in Supplemental Fig. S1.

3. Results

Due to improved production techniques more product became available for the trial after cohort 1 was finished.

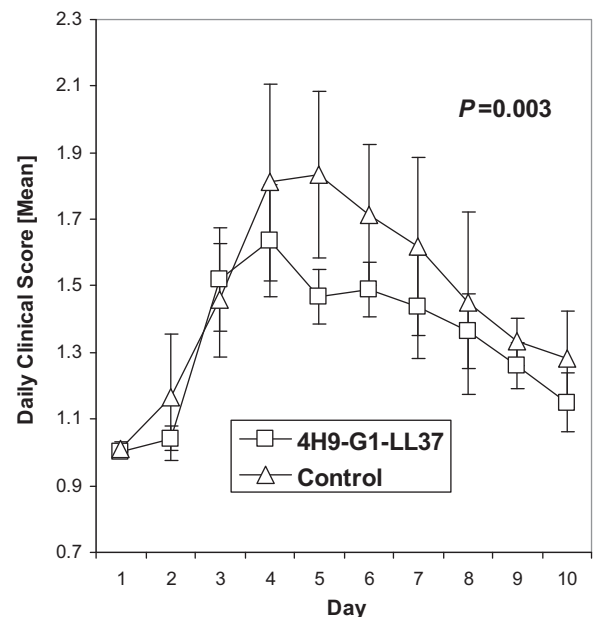


Fig. 1. Daily clinical score. Clinical parameters were scored as described in methods and scores from all animals (cohort 1–4) in the same treatment group were used to calculate the mean daily health score. Error bars represent the daily standard error of all animals in the treatment group (6–8 calves). Statistical analysis of the daily means from treated and control groups for significant differences was done by a full factorial regression model as described in methods. The p-value indicates statistically significantly better health scores for the 4H9-G1-LL37-treated animals when compared to the control-treated animals over the observation period (day 1–10).

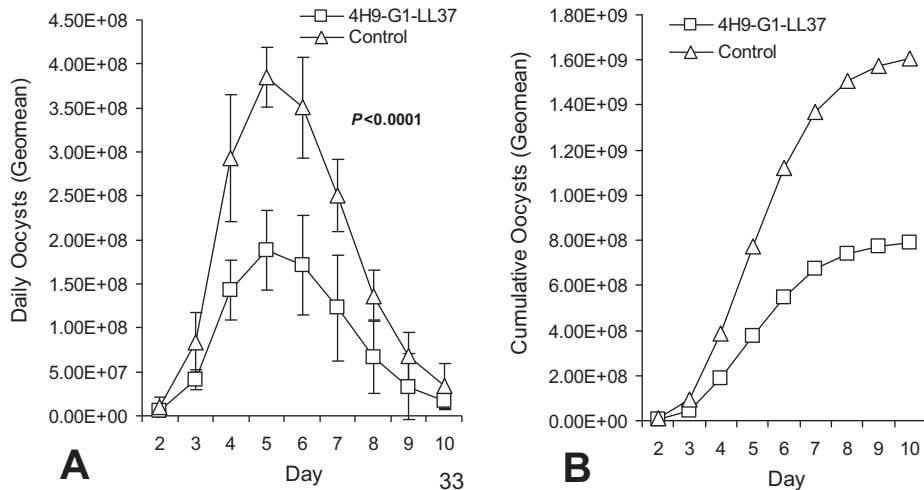


Fig. 2. Daily oocyst shedding. (A) Daily averages of oocysts shed by all animals in each treatment group. (B) Cumulative number of oocysts shed by all animals of each treatment group over the period of the trial. Geometric means (Geomean) were used for graphing and statistical analysis. Error bars represent the standard error of the population.

Despite the fact that the trial had already started, it was decided to increase the dose from 87 mg to 130 mg and from 6 treatments to 9 treatments (for details see Section 2) for all subsequent cohorts. After termination of the trial, statistical analysis comparing the two treatment regimens was performed and no significant difference was observed (data not shown). It was therefore decided to consolidate the data from all cohorts for statistical analysis. Two control calves died, one in cohort 1 on day 4 PI (due to a volvulus of the small intestine) and one in cohort 2 on day 8 PI (due to an acute severe bacterial peritonitis secondary to omphalophlebitis).

3.1. Overall health in antibody fusion-treated animals

As a measurement of overall health, defined clinical parameters were assessed daily using the scoring system described in methods. 4H9-G1-LL37-treated animals had a significantly better mean overall health score (1.30) when compared to the control animals (1.43) (Fig. 1) ($P=0.003$). The difference was most pronounced between days 4 and 6 PI, the period during which clinical signs of cryptosporidiosis are typically of greatest severity: treated animals had a score of 1.53 versus control animals with a score of 1.79 (Fig. 1). When calculated across the duration of the trial, 4 out of 6 individual parameters scored were significantly different between control and 4H9-G1-LL37 treated calves (Table 1). These data indicate that treatment with 4H9-G1-LL37 relieved disease signs of cryptosporidiosis.

3.2. Oocyst shedding in antibody fusion treated animals

One of the most important parameters in measuring positive treatment effects against cryptosporidiosis is reduction in fecal oocyst shedding. Our experimental protocol was designed to accurately measure the total daily oocyst output by multiplying the oocyst count derived

from a homogenized daily sample with the total daily fecal volume. The mean daily oocyst output in the 4H9-G1-LL37-treated animals was significantly lower over the period of 10 days post infection than the mean daily oocyst output in control-treated animals ($P < 0.0001$), with the reduction being greatest during the peak period of shedding days 4–6 PI (Fig. 2A). The cumulative oocyst shedding for the entire trial showed a 52.5% reduction in 4H9-G1-LL37-treated calves compared to the control substance-treated calves (Fig. 2B).

4. Discussion

C. parvum infection is one of the most common causes of diarrhea in livestock worldwide and recognition of the economic impact has grown. There is a clear need to develop more effective, parasite-specific, and safer products for the control of a *C. parvum* infection. Antibody–biocide fusions are novel compounds that have previously shown efficacy against *C. parvum* infection in a neonatal mouse model (Imboden et al., 2010) and are now under development for veterinary and human use. The 4H9-G1-LL37 fusion derives its specificity from the variable region of a monoclonal antibody directed against the GP25–500 complex on the *C. parvum* sporozoite surface (Schaefer et al., 2000) and its parasitocidal function from the human cathelicidin derived peptide LL37, an antimicrobial peptide found to have a neutralizing effect on sporozoites *in vitro* (manuscript submitted).

This clinical pilot study tested the efficacy of 4H9-G1-LL37 in calves, experimentally infected with *C. parvum* oocysts, by comparing several clinical and parasitological parameters between 4H9-G1-LL37-treated and control substance-treated animals. Treatment efficacy was demonstrated by improved health parameters and reduced oocyst shedding in *Cryptosporidium*-infected calves, a reduction of fecal output in treated animals (data not shown) was not detected. From an epidemiologic perspective, reduction of

Table 1

Clinical observations were scored and analyzed by a full factorial regression model according to the experimental model described in methods. 'Treatment' compares all 4H9-G1-LL37-treated animals (cohort 1–4) to all control-treated animals (cohort 1–4) with regard to each health parameter. 'Cohort' compares differences between each cohort (containing both 4H9-G1-LL37- and control-treated animals) with regard to each health parameter. 'Control × Treatment' compares cohorts to each other with regard to treatment and each health parameter. 'Day & Knotted' indicates that a knotted spline transformation was used to accommodate the time-variant responses with regard to treatment, day and each of the health parameters. Data was collected from day 1 to day 10 (conclusion of the experiment). DF = degrees of freedom.

	Treatment		Cohort		Cohort × Treatment		Day & knotted	
	DF	Prob > F	DF	Prob > F	DF	Prob > F	DF	Prob > F
Fecal consistency	1	0.8925	3	<0.0001	3	<0.0001	4	<0.0001
Ability rise	1	0.0005	3	<0.0001	3	<0.0001	4	0.0224
Stance	1	0.0001	3	<0.0001	3	<0.0001	4	0.0454
Appetite	1	0.0079	3	<0.0001	3	0.1643	4	<0.0001
Attitude	1	0.0029	3	<0.0001	3	0.0029	4	<0.0001
Hydration	1	0.3212	3	<0.0001	3	0.0337	4	<0.0001

oocyst shedding is an important factor in helping reduce the parasite pool in the environment. We are aware of the fact that a 50% overall reduction in oocyst shedding might not suffice to control the spread of the disease but it might make a significant contribution to a comprehensive *Cryptosporidium* control program of any given farm. A reduction of the parasite pool would result in reduced dairy herd infection as well as reduced likelihood of human exposure through direct contact at the farm or via drinking and recreational water supply contamination.

The study conducted here was designed to demonstrate efficacy of a novel biotherapeutic in a large animal model for cryptosporidiosis using the least possible number of animals and the highest technically feasible oral treatment dose. Despite the fact that two control animals were lost prematurely due to unrelated complications, the study design was strong enough to demonstrate significance in oocyst shedding and overall health parameters.

This study provides proof of concept for a novel biotherapeutic in a clinical model for cryptosporidiosis. The treatment regimen and dose that were chosen are largely based on previous efficacy data in the neonatal mouse model (Imboden et al., 2010) and based on the assumption that an initial elevated dose during the first 4 h after challenge is important to achieve a therapeutic effect. Whether the subsequent regimen consisting of 12-h interval dosing had a positive or negative impact on the natural, asynchronous life cycle of *Cryptosporidium* and exposure of extracellular stages during the infection is not known. However, this successful proof of concept study serves as the starting point for further development of antibody–biocide fusion efficacy trials. In an effort to test treatment regimens that are more adaptable to field applications on a dairy farm, we will focus on testing less frequent treatment schedules, and lower treatment doses. To generate more field-like conditions, future trials will also include more animals and lower but multiple challenge doses. Lower challenge doses than that used in the present study have been shown by others to induce diarrhea in large numbers of calves with 100% infection rates and lower oocyst numbers shed (Moore et al., 2003; Perryman et al., 1999). As we see a dose-dependent efficacy of our product in mice (Imboden et al., 2010) it is likely that a lower oocyst challenge number which results in a lower

number of shed oocysts would allow a lower treatment dose of our antibody fusion product to achieve efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetpar.2012.02.014](https://doi.org/10.1016/j.vetpar.2012.02.014).

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