The efficacy of a combination of azithromycin and toltrazuril for the treatment of calves naturally infected with cryptosporidiosis: a randomised, double-blind, placebo-controlled comparative clinical trial

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ABSTRACT: *Cryptosporidium* spp. are important emerging pathogens that can cause infections in humans, especially in immunosuppressed patients. Treatment of diseased calves that shed the infectious stage of the protozoon is critically important to prevent contamination by *Cryptosporidium* spp. oocysts. The objective of this study was to determine if a combination of azithromycin and toltrazuril provides a better treatment option for calf cryptosporidiosis compared to the use of either agent alone. A total of 55 Holstein calves with cryptosporidiosis were randomly assigned into four groups. Group A (*n* = 15) received azithromycin at a dose of 20 mg/kg/day *per os* for six days, group T (*n* = 15) received toltrazuril at a dose of 20 mg/kg *per os* every other day on a total of three occasions, and group AT (*n* = 15) received the combination of both drugs. The control group (*n* = 10) received purified water. A randomised, placebo-controlled, double-blinded clinical study was designed. The number of oocysts in faeces and clinical parameters were followed daily. Selected haematological and biochemical parameters were measured at the beginning and end of the study. The calves receiving the combination of azithromycin and toltrazuril exhibited a better clinical score as well as the lowest number of oocysts at all time-points. In conclusion, the combination of azithromycin and toltrazuril promotes rapid clinical recovery in calves infected with cryptosporidiosis and stops oocyst shedding. Thus, the combination of azithromycin and toltrazuril is an effective alternative treatment option for calf cryptosporidiosis.

Keywords: neonatal; cattle; diarrhoea; apicomplexan; therapy

Cryptosporidium spp. are apicomplexan, zoonotic and cyst-forming protozoa that cause diarrhoea, especially in neonatal calves. Belonging to the order of *Coccidia*, *Cryptosporidium* spp. are related to *Toxoplasma*, *Isopora*, *Eimeria* and *Sarcocystis* spp. They inhabit the microvillus of epithelia lining the respiratory and gastrointestinal systems of humans, mammals and other vertebrates (Current and Garsia 1991). Like *Toxoplasma* spp., *Cryptosporidium* spp. are not species-specific, but they infect a wide range of animals and are transmitted through the intake of contaminated water or through direct contact with oocyst-containing faeces (Uip et al. 1998). *Cryptosporidium* oocysts, the infective form of the parasite, are environmentally resistant and may survive in soil or even water for six months or longer; further, they are resistant to most decontaminants, including chlorine (Harp et al. 1996).

Cryptosporidiosis causes significant economic losses in cattle breeding due to slower growth rates and the death of calves (Azami 2007). The disease is clinically characterised by depression, anorexia, a change in faeces colour ranging from orange to white-grey with frequent discharge and watery fae-

ces. The colour and viscosity of the faeces vary from yellow and watery to white and pasty. Importantly, faeces often contain undigested milk, blood, bile and mucus (Tzipori et al. 1982).

A number of partially effective compounds have been tested for the treatment of cryptosporidiosis (Harp et al. 1996). Halofuginone, paromomycin, decoquinate and bovine hyper immune colostrum are among the drugs that have showed some effects in experimental studies; however, their efficacy has not been fully validated in natural infections (Lefay et al. 2001). Triazine-based anti-protozoal drugs such as toltrazuril are also used in the treatment and prophylaxis of apicomplexan diseases. Toltrazuril, a triazinetrione derivate, is used in treatment of coccidiosis in poultry, goats and cattle (Darius et al. 2004; Ocal et al. 2007). Toltrazuril acts on the entire range of intracellular developmental stages of all *Eimeria* and *Isospora* spp., but not on their oocysts. Although the exact mechanisms of action of these anti-protozoal drugs are unknown, they may exert selective toxicity for apicomplexan parasites (Dirikolu et al. 2009). Toltrazuril interferes with the intracellular development of apicomplexan parasites; for instance, it causes swelling in mitochondria and the endoplasmic reticulum (Gottstein et al. 2001). Toltrazuril has been shown to be effective in acute coccidiosis in goat kids with no side-effects (Ocal et al. 2007).

In recent years, azithromycin has been used to treat cryptosporidiosis in animals and humans. Azithromycin is effective in the prevention and treatment of experimental infections in the ileum of immunocompromised rats and naturally infected calves. Importantly, azithromycin acts in a dose-dependent manner (Rehg 1991; Vargas et al. 1993; Elitok et al. 2005). Azithromycin, an azalide antibiotic, is similar to the macrolides, which exhibit immune-modulating effects, including a strengthening of humoral and cellular immune responses, a reduction in the production of inflammatory mediators and an increase in the production of Interleukin 10. The macrolide antibiotics also exhibit anti-inflammatory effects which include dramatic induction of *COX-2* gene expression and of nitrite oxide synthases (Baba et al. 1998). Studies conducted in numerous species have, taken together, demonstrated that azithromycin has good oral bioavailability and exhibits an extensive tissue distribution with very slow elimination; thus, it exerts a prolonged anti-bacterial effect at the site of action. Furthermore, azithromycin has

an augmented stability at acid pH, and reaches a high tissue concentration due to direct uptake and delivery via phagocytes (Davis et al. 2002).

The hypothesis of this study was that a combination of azithromycin and toltrazuril may provide a better effective treatment option for calf cryptosporidiosis compared to the use of either agent alone. Therefore, in this study we evaluated the efficacy of azithromycin and toltrazuril separately, as well as in combination, in natural cryptosporidiosis in calves.

MATERIAL AND METHODS

Animals and study design. A randomised, placebo-controlled, double-blinded clinical trial was conducted. A total of 55 Holstein calves with cryptosporidiosis were included in the study. The calves with cryptosporidiosis were randomly assigned to one of the following four groups: group A (azithromycin-receiving calves), group T (toltrazuril-receiving calves), group AT (azithromycin- and toltrazuril-receiving calves) and group C. As the animals used as controls in group C received no treatment, this group had a reduced sample size (n = 10). Calves were randomly allocated into the four groups after being assigned predetermined randomisation codes. The investigators as well as the animal owners were blinded to the groups. The sample size in experimental groups was determined by power analysis, based on the effect size between the best and the worst group with f = 0.50, a = 0.05 Type I error, β = 0.05 Type II error and 0.95 power. For the sample size and power calculation, the Power Ver 3.00.10 software was used.

Evaluation of clinical signs and laboratory work-up. All the calves included in the study were physically examined and tentatively diagnosed. Furthermore, total blood count and venous blood gas monitoring were conducted. Cases were diagnosed based on faecal examination for the presence of cryptosporidium oocysts.

For examination, 10–20 g of fresh faeces were collected directly from the rectum using digital evacuation daily for 15 days. Faecal samples were transferred into sterile plastic containers, stored at 4 °C and analysed within 24 h. Faeces were smeared onto glass slides, stained according to the negative staining technique of Heine and then examined under a light microscope for the presence of cryptosporidium oocysts. Using a 100× microscope

objective, 20 randomly selected fields were analysed for the number of oocysts. The mean of the 20 fields was used as the final score. The average oocyst count (final score) was categorised as follows: 0 (no oocysts), 1 (≤ 1 oocysts), 2 (2–5 oocysts), 3 (6–10 oocysts), and 4 (> 10 oocysts).

Blood samples were collected from the jugular vein for the analysis of selected biochemical parameters and venous blood gasses. pH, PCO_2, PO_2, HCO_3, base excess, haematocrit (Hct), haemoglobin (Hb), Na, K, glucose and blood urea nitrogen (BUN) were measured using a portable blood gas analyser (GASTAT-Mini; sensor card No. 983-984).

Allocation to treatment group and evaluation by owners. Once enrolled, calves were randomly allocated to four groups according to predetermined randomisation codes. Both owners and investigators were blinded for the allocation to groups. The 1st group (group A) received azithromycin suspension at a dosage of 20 mg/kg *per os* every 24 h for six days. The 2nd group (group T) received toltrazuril at a dose of 20 mg/kg *per os* every other day for three-times. The 3rd group (group AT) received a combination of azithromycin and toltrazuril at the above-mentioned dosages. The placebo control group (group C) received purified water for six days. All affected calves received fluid therapy based on their haematological and biochemical values.

Investigator evaluation of clinical signs. All calves enrolled in the study were examined and clinically scored (Table 1) according to the protocol previously described by Sahal et al. (1994).

Statistical analysis. The measured and scored data were transferred to a computer and controlled for errors, which were corrected. Graphic analysis and Shapiro-Wilk normality test were applied. As some parameters were not normally distributed, median and IQR (interquartile range) values were used along with mean ± SD for descriptive statistics. One-way variance analysis (ANOVA) was used for comparison of normally distributed data of blood gases and biochemical and clinical scores. If a significant difference was found, the Bonferroni test was applied as a post-hoc analysis. Kruskall-Wallis variance analysis was conducted for the comparison of abnormally distributed data. In the case of a significant difference, a Bonferroni-corrected Mann-Whitney test was applied to determine the source of the difference. For comparison of clinical scores collected at different time-points (Days 1, 8 and 15), repeated measures variance analysis was used. Kruskall-Wallis variance analysis was applied to compare the oocyst numbers among groups. If any significant difference was found in Kruskall-Wallis analysis, a Bonferoni-corrected Wilcoxon test for related samples was used to determine the different group/groups. MS-Excel and SPSS for Windows Ver. 15.00 software were used for all statistical analyses. A *P*-value of ≤ 0.05 was considered significant.

Table 1. Clinical score table of diarrhoeic calves

Parameters	Clinical Scores						
Posture	normal	0	gibbous	1	recumbency	2	paresis	3
Temperament	active	0	sedate	1	aphetic	2	coma	3
Body condition	good	0	normal	1	weak	2	cachexia	3
Appetite	good	0	slow sucking	1	few sucking	2	no sucking	3
Body temperature (°C)	38–39.5	0	39.6–40	1	40.1–41	2	< 38 to > 41	3
Bronchopneumonia	no sign	0	mild	1	moderate	2	several	3
Hair coat	normal	0	confused	1	dirty	2	very dirty	3
Heart rate	90–130/dk	0	111–130/dk	1	131–150/dk	2	> 151/dk	3
Lack of skin elasticity	no sign	0	mild	1	several	2	moderate	3
Mucosa	normal	0	pale	1			anaemic	2
Capillaries	normal	0	filling	1			over filling	2
Eye position in the orbita	normal	0	moderately sunken	1			severe sunken	3
Fossa paralumbalis	normal	0	moderately sunken	1			severe sunken	2
Joints	normal	0	moderately swollen	1			severely swollen	3
Jugular veins filling	immediately	0	short time	1			long time/collapsed	3
Stool consistency	solid	0	soft	1			liquid	3
Stool colour	normal	0	yellowish-greenish	1			bloody	5
Smell of stool	normal	0	bad	1			very bad	2

Table 2. The mean clinical scores of the four groups at different time-points

	Group A		Group T		Group AT		Group C	
	mean	SD	mean	SD	mean	SD	mean	SD
Day 1	23.31	6.75	25.25	6.15	26.73	8.56	27.86	4.63
Day 8	7.54	3.95	8.08	3.85	2.93	2.74	13.43	4.28
Day 15	1.15	1.63	1.83	1.85	0.13	0.35	5.86	3.89

RESULTS

Blood gases and biochemical parameters measured at the beginning of the study were not within the physiological limits due to metabolic acidosis that had developed as a consequence of fluid and electrolyte losses. Such a condition is typical for diarrhoea. Although these parameters were not within the normal limits in any of the diseased calves, there were no differences among groups ($P > 0.05$). Repeated Measures variance analysis was used to evaluate the relationship between treatment groups and measurement time (the 1st, 8th and 15th days). There was significant effect of time ($F = 367.041$; $P < 0.001$), and of group ($F = 5.566$; $P = 0.003$) as well as time-group interaction ($F = 3.092$; $P = 0.022$) in the total clinical score. There were significant differences among all time points (1st–8th days, 1st–5th days and 8th–15th days) ($P < 0.001$). On the 1st day, group A had the lowest clinical score (23.31 ± 6.75), while group C had the highest clinical score (27.86 ± 4.63). On the 8th day, there were significant declines in clinical scores in all groups, except for in the control group. The decrease in the control group was negligible. The

highest decline was observed in group AT. The mean clinical scores based on the time-points are given in Table 2 and Figure 1.

Starting from the 8th day, calves, especially in group AT, exhibited lower clinical scores and clinical parameters improved quickly. Importantly, the clinical scores of the calves in group AT on the 15th day ($X = 0.13$) were comparable to those of healthy calves.

Analysing the change in clinical scores over time, the order of the clinical scores from the highest to the lowest was as follows: group C, group T, group A and group AT. The score difference (5.05) between group A and group C was significant ($P = 0.010$). Similarly, the score difference (5.78) between group AT and group C was statistically significant ($P = 0.002$). The differences among the other groups varied from 0.73 to 3.99 without any significance ($P > 0.05$).

The change in the number of shed oocysts by time was also significant ($\chi^2 = 79.609$; $P < 0.001$). The number of oocysts was 3.15 ± 0.75 (median = 3.00) on the first day. This number decreased to 1.53 ± 1.00 (median = 2.00) on the 8th day, and then down to 0.49 ± 0.62 (median = 0.00) on the 15th day. The oocyst scores at different time-points are presented in Table 3.

On the 1st day, the number of oocysts was similar among groups ($\chi^2 = 2.899$; $P = 0.407$). On day eight, the number of oocysts was significantly lower ($\chi^2 = 32.153$; $P < 0.001$) in group AT compared to the other groups while it was similar among the remaining groups. At day 15, the number of oocysts decreased in all groups except for group C; how-
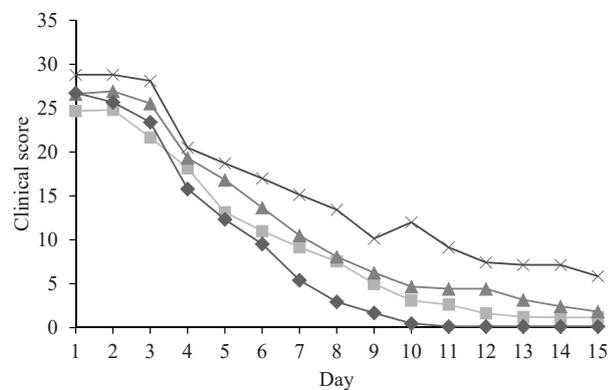

Figure 1. The mean clinical scores of the four groups (■ = group A, ▲ = group T, ◆ = group AT, × = group C) on different study days

Table 3. The oocyst scores of the four groups at different time-points

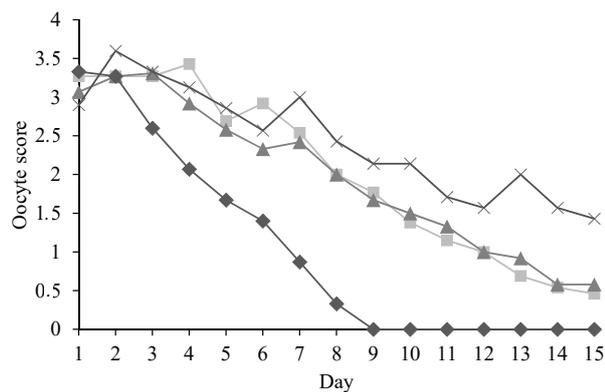|	Group	*n*	Mean	SD	Median
Day 1	A	15	3.27	0.80	3.00
	T	15	3.07	0.80	3.00
	AT	15	3.33	0.72	3.00
	C	10	2.90	0.57	3.00
Day 8	A	13	2.00	0.71	2.00
	T	12	2.00	0.43	2.00
	AT	15	0.33	0.49	0.00
	C	7	2.43	0.53	2.00
Day 15	A	13	0.46	0.52	0.00
	T	12	0.58	0.51	1.00
	AT	15	0.00	0.00	0.00
	C	7	1.43	0.53	1.00

Figure 2. The mean oocyte scores of the four groups (■ = group A, ▲ = group T, ◆ = group AT, ✕ = group C) on different study days

ever, the decrease was most evident in group AT in which no oocysts were detected at all (Figure 2).

DISCUSSION

Cryptosporidiosis is one of the life-threatening diseases affecting neonatal calves, and causes malabsorptive and secretory diarrhoea. As also indicated by the present study, neonatal calves are quite susceptible to cryptosporidiosis and they are at maximum risk for the disease at 9–12 days of age (Castro-Hermida et al. 2002). Likewise, cryptosporidium oocysts are shed extensively during days 10–19 of age (O'Handley et al. 1999).

Diarrhoea in calves with cryptosporidiosis causes clinical findings and metabolic changes. Dehydration (haemoconcentration), acidosis, electrolyte imbalance, negative energy balance and/or hypoglycaemia are the common findings in diarrhoeic calves. In our study, all enrolled calves had hypoglycaemia along with other common signs. Acidosis in diarrhoeic calves develops as a consequence of loss of bicarbonate and cations via faeces and deposition of lactic acid in tissues as a result of insufficient renal excretions. In neonatal diarrhoeic calves, HCO_3, Cl^-, Na^+, K^+, and H^+ losses occur along with fluid loss; consequently, the electrolyte balance of the body is impaired. The severity of electrolyte imbalance and acidosis increases due to the fact that losses in HCO_3, Na^+, and K^+ levels exceed the losses in Cl^- and H^+ levels. Subsequently, the transport of extracellular H^+ into intracellular fluid and K^+ transport into extracellular fluid are increased. Such a situation induces Na^+ and Cl^- passage into

intracellular fluid. This ion exchange causes a paradoxical condition, in which plasma K^+ levels increase despite the significant decline in total K^+ levels. In our study, all diseased calves had lower bicarbonate levels, hyperkalaemia and metabolic acidosis prior to treatment. Thus, we administered a common fluid treatment to all calves, even those in the control group.

Haematological parameters in neonatal calves vary greatly compared to values in adults. For instance, normal haematocrit and haemoglobin levels are lower in neonatal calves compared to adults (Mohri et al. 2007). As also confirmed by the presented study, Hct and Hb levels can increase in diarrhoeic calves as a consequence of haemoconcentration. The diarrhoeic calves in the present study exhibited a moderate increase in BUN levels. The increase in BUN levels occur due to a lower glomerular filtration rate as a consequence of hypovolemia and the subsequent decrease in renal blood perfusion.

The treatment of cryptosporidiosis is often tedious since a safe and effective treatment protocol has not yet been established (Lefay et al. 2001; Joachim et al. 2003; Ocal et al. 2007). The ideal treatment protocol should aim to improve the systemic condition and to minimize or even stop oocyte shedding. Furthermore, the drug should have a better margin of safety as some effective drugs such as lasalocid (Benson et al. 1998) can easily harm the animals as a result of overdosing. In our study, infected calves in all groups had faecal oocyst scores of 3.16 ± 0.739 prior to treatment, which is comparable to the results of Castro-Hermida et al. (2001), who reported 3 ± 0.8. In addition to common fluid therapy, we administered toltrazuril that has been used for the treatment of parasitic diseases such as neosporosis and coccidiosis, caused by other apicomplexan parasites (Gottstein et al. 2001; Cuteri et al. 2005; Epe et al. 2005; Mundt et al. 2005; Ocal et al. 2007). Azithromycin, a macrolide antibiotic used for treatment of toxoplasmosis (Degerli et al. 2003), was also tested in this study. Elitok et al. (2005) administered azithromycin alone for the treatment of naturally occurring cryptosporidiosis in calves and obtained successful results. In our study, azithromycin alone also reduced the number of faecal oocyst with some fluctuations during the 15-day period. Importantly, however, faecal oocyst shedding still continued after the use of azithromycin alone. Furthermore, improvements in clinical parameters were slower

than expected. Like azithromycin alone, toltrazuril alone caused some reduction in the number of faecal oocysts compared to the beginning of the treatment protocol. The faecal oocyst counts during the 15-day period were slightly higher compared to the azithromycin alone group; however, the difference was not significant. As in the azithromycin alone group, the faecal oocyst shedding was decreased, but not completely eliminated after the administration of toltrazuril alone.

Azithromycin and toltrazuril have different mechanisms of action (Baba et al. 1998; Gottstein et al. 2001; Davis et al. 2002; Dirikolu et al. 2009). Thus, we also tried a combination of azithromycin and toltrazuril as a treatment option. The azithromycin and toltrazuril combination was revealed to be the better treatment option. The calves recovered faster with improved clinical parameters and significant decreases in oocyst shedding. Importantly, no oocyst shedding was detected by the 9th day of treatment, and the stool consistency returned to normal. Evidently, this combination reduced the time needed for healing of microvillus damage that causes impaired intestinal absorption. Normally, a time period of two weeks or longer is needed to repair such inflammatory damage in cryptosporidiosis (Fahey 2003; Klein et al. 2008). To our knowledge, therefore, the combination of azithromycin and toltrazuril seems to be the best treatment option for cryptosporidiosis in calves. The combined effect can be explained by the complementary mechanisms of action of azithromycin and toltrazuril. As previously discussed, toltrazuril targets all intracellular stages of apicomplexan parasites, while azithromycin targets the sporulated forms of such parasites. Furthermore, azithromycin has anti-inflammatory effects and augments cellular immune responses.

In conclusion, the combination of azithromycin and toltrazuril is an effective treatment option for cryptosporidiosis in calves as it stops oocyst shedding as rapidly as within nine days. Compared to azithromycin or toltrazuril alone, the combination provided a quicker recovery time in naturally occurring calf cryptosporidiosis.

Acknowledgements

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