# The efficacy of chloroquine treatment of *Giardia duodenalis* infection in calves

## *De werkzaamheid van chloroquinebehandeling van* Giardia duodenalis-*infectie bij kalveren*

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# ABSTRACT

The purpose of the present study was to evaluate the effect of chloroquine treatment on cyst excretion in calves naturally infected with *Giardia duodenalis*. The calves were randomly assigned into two groups based on placebo (group I, n=7 untreated control calves) or treatment (group II, n=7 calves treated orally with 2.5 mg/kg chloroquine twice daily for five consecutive days). The *G. duodenalis* isolates were identified by molecular characterization with β-giardin nested PCR and gene sequence analysis as assemblage A3. Cyst excretion was determined on days 0, 3, 7 and 10, before and after treatment. Geometric means of the number of excreted cysts did not change significantly in the control group during the trial. The reduction in cyst excretion after chloroquine treatment was 99% on day 3 and 100% on days 7 and 10. Chloroquine treatment is most probatly practically applicable, relatively inexpensive and highly effective against giardiosis in calves.

## SAMENVATTING

Het doel van deze studie was om het effect van chloroquinebehandeling te evalueren op de cyste-excretie van kalveren met een natuurlijke infectie met *Giardia duodenalis*. De kalveren werden willekeurig ingedeeld in twee groepen op basis van placebo (groep I, n = 7 onbehandelde controlekalveren) of behandeling (groep II, n = 7 kalveren behandeld met chloroquine). De behandeling gebeurde door orale toediening, tweemaal daags gedurende vijf opeenvolgende dagen aan een dosis van 2,5 mg/kg lichaamsgewicht. Door middel van moleculaire typering met ß-giardin geneste PCR en gensequentie-analyse werden de *G. duodenalis*-isolaten geïdentificeerd als behorende tot het A3-assemblage. Cyste-uitscheiding werd bepaald op dag 0, 3, 7 en 10, zowel voor als na de behandeling. De geometrische gemiddelden van het aantal uitgescheiden cysten waren niet significant veranderd tijdens het experiment in de controlegroep. De cyste-excretie was met 99% verminderd op dag 3 en met 100% op dag 7 en 10 bij de behandelde kalveren. Chloroquine is dus mogelijk een praktisch haalbare, relatief goedkope en zeer effectieve behandeling tegen giardiose bij kalveren.

## INTRODUCTION

Giardiosis is an ubiquitous, intestinal protozoal infection distributed worldwide within the vast majority of domestic/wild mammals and humans. Transmission of *Giardia duodenalis* infection occurs via cysts, which are excreted in the feces of infected humans and animals (O'Handley and Olson, 2006). Livestock infection is also common and has been reported in 9%–73% of fecal samples from cattle, with higher rates of infection in calves. Farm prevalence rates might rise up to 100% (Geurden et al., 2010). Giardiosis in livestock animals is associated with a high morbidity, which may result in significant production losses (Aloisio et al., 2006; Geurden et al., 2010; Sweeny et al., 2011). Typically, chronic or reoccurring infections exist in calves and cyst excretion might continue for months. Clinical signs include diarrhea, weight loss,

lethargy and poor condition of the calves, but subclinical infections are common (Geurden et al., 2006).

Ruminants are most commonly infected by *G. duodenalis* of genetic assemblage E, which is considered to be livestock specific (O'Handley and Olson, 2006). Indeed, mono or mixed infections with genetic assemblage A (Geurden et al., 2008; Feng et al., 2008; Mark-Carew et al., 2012) or infection with genetic assemblage B (Coklin et al., 2007) have been reported. Thus, calves have been thought to have the potential to serve as a reservoir for human giardiosis (O'Handley et al., 2000; Trout et al., 2007; Uehlinger et al., 2007; Winkworth et al., 2008). However, recent molecular studies with multi-locus genotyping of assemblages A and B have shown that animals do not share identical genotypes with humans in most cases, which provides limited support of their role in zoonotic transmission (Sprong et al., 2009; Lebbad et al., 2010; Caccio, 2015).

The high prevalence rates, risk of production losses and zoonotic risk warrant suitable treatment of *G. duodenalis* infections in ruminants. Currently, there are no Food and Drug Administration (FDA)/European Medicines Agency (EMA) approved drugs available for the treatment of giardiasis in cattle. The traditional treatment of giardiosis in calves consists of fenbendazole (Xiao et al., 1996; O'Handley et al., 1997; O'Handley et al., 2000; Garossino et al., 2001; O'Handley et al., 2001; Geurden et al., 2010), albendazole (Xiao et al., 1996; Ragbetli et al., 2014) or paramomycin (Geurden et al., 2006). Uehlinger et al. (2007) evaluated the efficacy of vaccination in the prevention of *G. duodenalis* infection in calves, but no differences were found between vaccinated and non-vaccinated calves in occurrence of giardiosis or cyst shedding.

The literature regarding the efficacy of treatment against giardiosis in ruminants is limited to the aforementioned studies. Taking into account cases refractory to traditional treatment in humans (Nash et al., 2001; Nabarro et al., 2015), there is still a need to establish reasonably priced and novel therapeutic protocols. Benzimidazoles are quite reasonably priced. Moreover, these compounds are already registered for the use in cattle as anthelmintics (O'Handley et al., 1997; O'Handley et al. 2001), which is not the case for chloroquine. Although fenbendazole has shown to be 100% effective and eliminated cysts from the feces within 6 days in calves, reinfection was observed in some of the treated calves within four weeks of trial (O'Handley et al., 1997). Chloroquine, a 4-aminoquinoline compound used for treatment of malaria, has recently been re-identified as an old drug with new perspective against giardiosis in humans (Escobedo et al., 2015).

Therefore, the purpose of this study was to evaluate the effect of chloroquine treatment on the cyst excretion in calves naturally infected with *G. duodenalis*.

## MATERIALS AND METHODS

### Animals, housing and treatment applications

A total of fourteen Holstein Friesian calves of one to three months old and of both sexes were enrolled into the study. A commercially available solid phase immunochromatographic assay (Anigen Rapid Bovid-5 Ag test Kit, Bionote, Korea) was used for the rapid, qualitative detection of Rotavirus, Coronavirus, *Escherichia coli* K99, *Cryptosporidium parvum* and *G. duodenalis* in calf feces. Initial diagnosis of mono infection with *G. duodenalis* was confirmed microscopically by detection of cysts in the fecal samples. The samples were withdrawn at different times of the year from several farms.

Calves naturally mono-infected with *G. duodenalis* were selected and randomly assigned into two groups that received either a placebo (group I, n=7 untreated control calves) or treatment (group II, n=7 calves treated with chloroquine). Enrolled calves were weighed prior to drug application via a cattle weighing scale having a calibration of 1 kg. The weighing was performed by the animal keepers not involved and informed within the trial. The calves in the treatment group received chloroquine (Kutlu tablet® 250 mg, Keymen, Turkey) twice daily at a dosage of approximately 2.5 mg/kg body weight orally (by the investigator applied directly into the mouth, followed by 10 ml water) for five consecutive days, whereas the calves in the control group received a placebo. The treatment dose was rounded off to the nearest weight. Calves weighing up to 50 kg received 125 mg compound, i.e. half a tablet, while calves weighing 50-100 kg received one tablet. The placebo included water with an equivalent volume as in the treatment group. Systematic clinical examinations were carried out and fecal samples were collected on days 0, 3, 7 and 10 after the first administration.

The calves were housed in individual boxes, which were cleaned and disinfected daily with a commercially available quaternary ammonium product (Derdevice Plus Y, Deren Ilac, Turkey) against re-infection with environmental contamination. Strict biosecurity measures were implemented to prevent possible transmission and contamination between groups. The animals were fed with commercially available milk replacer (Eurolac Blue, Agrovit, Turkey) and had access to concentrate (Ilke, Abalim, Turkey) according to their body weight and age. Water and hay were provided ad libitum during the study period. No other medications were administered. After the completion of the study, the control calves were treated with chloroquine at the same dosage as the previously treated animals. The study protocol was approved by the institutional laboratory animals ethics committee of Adnan Menderes University HADYEK (with no: 2016/039 and date 25.02.2016). Prior to enrolment in this study, informed written consent was obtained from all of the owners/animal care takers.

### Examination of fecal samples

The day of the first administration was determined as day 0. Collections from each calf were obtained on days 0 (before treatment), 3, 7 and 10 (after treatment). Fecal samples were collected manually from the rectum of all calves. Fecal material (1.5 g) was mixed through 33% $ZnSO_4$ solution (15 ml) and centrifuged at 880 x g for 5 minutes (Wilson et al., 2009). Fifty µl of fluid from the surface was emitted on a microscope slide containing Lugol iodine, which was covered by a slip. The slide was microscopically examined (400x power) for visualization of *Giardia* cysts. This procedure was repeated two times from different samples belonging to each calf by a single blinded researcher. The number of cysts per gram of feces (CPG) was calculated by [(number of cysts identified × 100)/1.5].

### Assessment of efficacy of treatment

The efficacy of chloroquine treatment in the present study was assessed by microscopic examination of fecal samples collected on days 0, 3, 7 and 10 and measured based on the reduction in the number of CPG for the calves in the treatment group in comparison to the calves in the control group. The reduction in cyst excretion was calculated using the Henderson–Tilton formula, involving the geometric mean of the CPG similar to those of Geurden et al. (2010):

$$100\times \left[ 1 - \frac{Ta \times Cb}{Tb \times Ca} \right]$$

Ta and Tb represented the geometric mean CPG in the chloroquine treatment group before (Tb) and after (Ta) treatment with chloroquine, respectively; whereas Ca and Cb denoted the geometric mean CPG in the control animals before (Cb) and after (Ca) placebo treatment, respectively.

The Henderson-Tilton formula is considered as the most appropriate method as described and used previously by Geurden et al. (2010).

### Molecular characterization of Giardia isolates

DNA was extracted directly from feces with QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's manual. Polymerase chain reaction (PCR)-based methods were employed to genotype using the procedures of Cacciò et al. (2002) and Lalle et al. (2005). Molecular characterization was carried out using PCR amplification and sequencing of the 511 bp region of the *β-giardin* gene.

### Statistical analysis

The variables were tested for normality using the Shapiro-Wilk test. The group, time and group by time interactions were tested with repeated measures ANOVA. Related samples Friedman's two-way analysis of variance was done to control the statistical results of CPG before (day 0) and after the start of treatment (days 3, 7, 10) for each group. A Mann-Whitney-U test was used to compare differences between groups for each day. P values <0.05 were considered to indicate a significant difference. Software package (SPSS 22.0, SPSS Inc., Chicago, USA) was used for all tests.

## RESULTS

### Clinical sings

Calves in both groups presented clinical signs compatible with naturally occurring giardiosis, involving diarrhea on day 0. No other pathogens were detected in the feces of any of the calves during the study period. No observable and significant adverse reactions related with chloroquine treatment were noticed in the present study. All clinical signs including diarrhea had resolved on day 7 in all calves of the treatment group. Comparatively 5 out of 7 calves exhibited diarrhea on day 10, whereas diarrhea stopped in another 2 calves without treatment.

### PCR amplification and sequence analysis of the β-*giardin* gene

Fecal samples of fourteen calves diagnosed *Giardia*-positive by the rapid test kits and microscopy, were positive by nested PCR (Figure 1). PCR products from each of the 14 *Giardia*-positive calf fecal samples were selected for molecular characterization. The β-giardin nested PCR assay was successfully applied to 14 isolates. The obtained DNA sequence from the selected isolates was compared with reference sequences (GenBank® accession number: M36728 for sub-assemblage A1, AY072723 for sub-assemblage A2 AY072724 for sub-assemblage A3). The sub-assemblage of all 14 isolates was determined as A3.

### Cyst excretion

The geometric mean number of excreted cysts and reduction in cyst shedding are presented in Table 1. In the calves of the control group, cyst excretion on day 10 (ranging from 8000-140,000 CPG) was comparable to the initial excretion (ranging from 4000-340,000 CPG). Following chloroquine treatment, the reduction in cyst excretion calculated based on geometric mean was 99% on day 3 and 100% on days 7 and 10. The mean number of excreted cysts was significantly decreased (p<0.001) on days 7 and 10 after treatment.

## DISCUSSION

*G. duodenalis* is an important intestinal pathogen of livestock. The number of studies on the prevalence
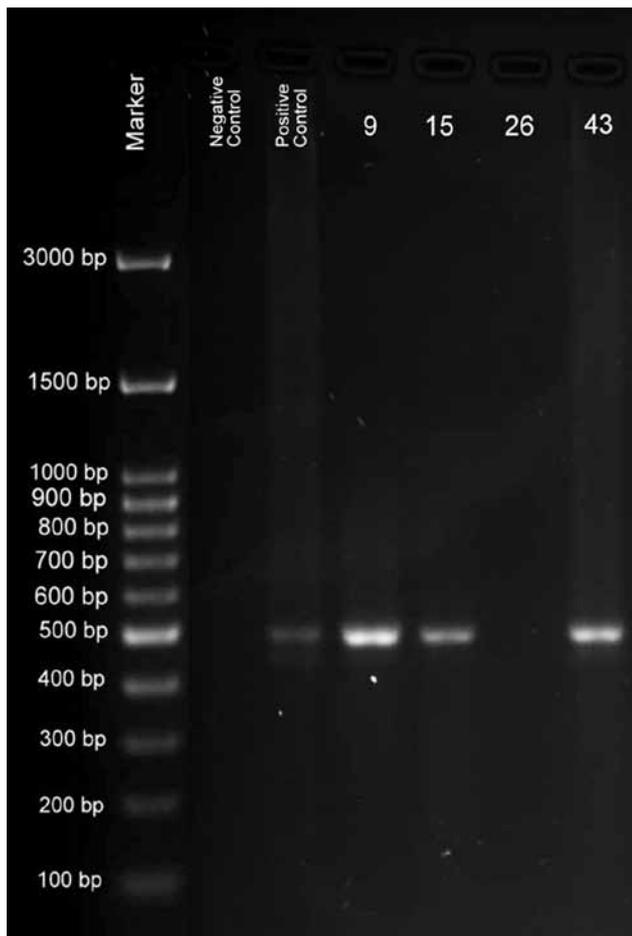
**Figure 1. 2% Gel electrophoresis image of some of the positive samples in the nested PCR process (511 bp).**

and molecular characterization of *G. duodenalis* in cattle in different parts of the world is increasing (Coklin et al., 2007; Gillhuber et al., 2013; Huang et al., 2014; Liu et al., 2015). However, only limited studies have been performed in Turkey on giardiosis in calves, with prevalence rates between 4.1% and 14.7% (Degerli et al., 2005; Goz et al., 2006; Gul et al., 2008).

Molecular characterization of *G. duodenalis* isolates in cattle has not yet been reported in Turkey. The vast majority of the previous studies detected assemblage E as the predominant genotype in livestock (O'Handley et al., 2000; Trout et al., 2007; Santín et al., 2009; Dixon et al., 2011), but shedding of assemblages A and B have also been reported in dairy cattle with mono or mixed infections (Cacciò et al., 2005; Geurden et al., 2008) and a recent study from different parts of the New York City watershed identified 100% of specimens from calves under 84 days of age, as assemblage A (Mark-Carew et al., 2012). Similarly, the present study revealed that all 14 of the *G. duodenalis* isolates collected from the calves, belonged to the sub-assemblage A3. Interestingly, the calves included in the study were from different farms in the Aydin region, but all samples belonged to same sub-assemblage. Assemblages of *G. duodenalis* may vary due to the geographical location and age as has been

reported in previous studies (Winkworth et al., 2008).

*Giardia* assemblages A and B have been associated with their potential zoonotic role but recent molecular studies have demonstrated that the genetic structure of *Giardia* is more complex than thought before, and most of the sub-assemblages from animals do not share identical genotypes with humans (Sprong et al. 2009; Lebbad et al., 2010; Caccio, 2015). Epidemiologic studies have shown that most of the human isolates belong to sub-assemblages A2, whereas sub-assemblages A1 are found less often, and sub-assemblage A3 is not found at all (Sprong et al., 2009; Feng and Xiao, 2011). In this study, the sub-assemblage of *Giardia* isolates from the calves were described as A3 with the limited zoonotic risk. Only one gene (β-giardin) was targeted for genotyping the *G. duodenalis* positive samples, which is similar to what has been described elsewhere (Lee et al., 2016). Multilocus genotyping at the SSU rRNA, β-giardin, glutamate dehydrogenase (*gdh*), and triosephosphate isomerase (*tpi*) loci (Wang et al., 2014) was not performed.

Giardiosis should be treated with a practically applicable, safe, low-priced and highly effective protocol due to its high prevalence rates in calves, the risk of production losses by cause of clinical signs and the zoonotic potential, which is related with the genetic assemblage of the infection (Uehlinger et al., 2007). However, until this moment, relatively few options, specifically benzimidazole derivatives such as fenbendazole and albendazole, have been recommended for therapy against giardiosis in ruminants (Xiao et al., 1996; O'Handley et al., 2001; Geurden et al., 2010). With regard to the limited efficacy and therapy failure reports in human medicine (Argüello-García et al., 2009; Nabarro et al., 2015) and the lack of FDA approved drugs for the treatment of giardiosis in ruminants, there is clearly a need for alternative treatment options.

Chloroquine, an old but promising agent, has been re-identified as a possible treatment of giardiosis (Escobedo et al., 2015). This synthetic 4-aminoquinoline compound has been used as first-line treatment against malaria for many years (WHO, 2010). Currently, the compound is still widely used for uncomplicated malaria cases and is recommended as a second-line treatment option for several infectious and non-infectious diseases in humans (Escobedo et al., 2015).

The use of chloroquine in the treatment of giardiosis was first described by Basnuevo and Sotolongo, reporting the successful treatment of two human patients in 1946. Several case reports and studies with high success rates were published confirming the antigiardial efficacy of chloroquine until 1965 (Lamadrid-Montemayor, 1954; Benetazzo and Tronca, 1955). After 1965, chloroquine was not used as a treatment choice for giardiosis anymore (Escobedo et al,. 2015). However, since 2000, two randomized clinical trials have been published in which chloroquine was given in a dose of 10 mg/kg twice daily for five days leading

**Table 1. The geometric means of *G. duodenalis* cyst excretion in the control and chloroquine treated groups at each sampling day (before treatment [day 0] and after treatment [days 3, 7, 10]). The percentage of reduction calculated based on geometric means are presented.**

|  | Day 0 | Day 3 | Day 7 | Day 10 |
|---|---|---|---|---|
| Control | 27307.94[a] | 17904.42[a] | 15853.16[a] | 32042.65[a] |
| Treatment | 29777.96[a] | 91.51[a,b] | 0[b] | 0[b] |
| p value | **0.902** | **0.128** | **0.001** | **0.001** |
| Reduction in cyst shedding |  | **%99** | **%100** | **%100** |

*Different letters indicate significant differences between rows and columns (p<0.05).

up to 86% cure rate of giardiosis in children (Escobedo et al., 2003; Canete et al., 2010).

Antigiardial activity of chloroquine against *G. duodenalis* trophozoites has been demonstrated in in vitro studies (Gordts et al., 1985; Baveja et al., 1998; Nava-Zuazo et al., 2010). Although the mechanism of action of chloroquine is not completely understood, the efficacy of that compound is probably to be attributed to a reduced ability of the *Giardia* trophozoites to attach to the surface of the enterocytes (Baveja et al., 1998). Additionally, another hypothesis has been suggested that chloroquine might inhibit the function of the peripheral vacuoles in *Giardia* trophozoites (Tai et al., 1993).

Paramomycin, fenbendazole and albendazole have shown efficacy against giardiosis in calves (Xiao et al., 1996; O'Handley et al., 1997; Geurden et al., 2006). However, in a ninety-day study, it has also been reported that calves are mostly re-infected following fenbendazole treatment with *G. duodenalis* cysts in their environment (O'Handley et al., 2000). *Giardia* cysts may survive up to seven weeks in soil (Olson et al., 1999). Therefore, short treatment protocols might not be enough to prevent reinfection and should be combined with disinfection of the environment (Geurden et al., 2006). In the present study, the individual boxes of the calves were cleaned and disinfected every day of the study with a quaternary ammonium product. No reinfection occurred in the treatment group on days 7 and 10. However, based on other studies, it may be suggested that a long term follow-up is necessary in order to evaluate reinfection in calves (O'Handley et al., 2000; Geurden et al., 2006).

Chloroquine has only been studied in a few experiments in calves. Long term use of intramuscular chloroquine against *Onchocerca gutturosa* has shown promising results (Husna et al., 2010). Contrarily, a much earlier study revealed that it was ineffective against *Eimeria bareillyi* coccidiosis in buffaloes (Sanyal et al., 1985). However, no side effects were noted in both studies after parenteral chloroqine use in calves. Similarly, in the present study, no side effect was noticed related with oral chloroquine administration at a dose of 2.5 mg/kg, twice daily for five consecutive days.

To the authors' knowledge, the efficacy of an oral treatment with chloroquine against naturally occurring giardiosis in calves has been demonstrated for the first time. In the present clinical trial, chloroquine reduced the cyst excretion by 100% on days 7 and 10 after the start of the treatment without side effects. Additionally, chloroquine is an easily available and relatively cheap drug. In the present study, the cost per calf for a five-days therapy was 0.85 dollars (as was calculated by the total dose used) and thus would be a cost-effective alternative for the use against giardiosis in calves.

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