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Coccidiose em ruminantes: tratamento metafilático e diagnóstico molecular

Santa Maria, RS
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COCCIDIOSE EM RUMINANTES: TRATAMENTO METAFILÁTICO E DIAGNÓSTICO MOLECULAR

Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Medicina Veterinária.**

Orientadora: Profª. Dra. Fernanda Silveira Flores Vogel

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RESUMO

COCCIDIOSE EM RUMINANTES: TRATAMENTO METAFILÁTICO E DIAGNÓSTICO MOLECULAR

AUTORA: Caroline Zamperete Reginato
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A infecção por *Eimeria* spp. está entre as principais enfermidades que acometem ruminantes jovens. A eimeriose é responsável por consideráveis perdas econômicas, devido à redução do ganho de peso e ao comprometimento no desenvolvimento de animais jovens, principalmente em decorrência a infecções subclínicas. Esta condição ocorre especialmente em criações intensivas e semi-intensivas e com alta densidade animal, porém surtos em criações extensivas também são relatados, com menor frequência. O controle da coccidiose pode ser realizado com a utilização de fármacos que interrompam o ciclo do parasito e através de práticas sanitárias, visando diminuir a contaminação ambiental. Diante do exposto, esta tese está dividida em dois capítulos, nos quais foram avaliados: (1) a eficácia do tratamento metafilático com toltrazuril a 5% em bezerros de corte naturalmente infectados com *Eimeria* spp. e criados de forma extensiva; (2) seis diferentes protocolos para extração de DNA de *Eimeria* spp. para diagnóstico molecular em bovinos e ovinos. No capítulo 1 apresenta-se um estudo no qual foram avaliados quatro diferentes tratamentos metafiláticos com toltrazuril 5%, em bezerros desde o nascimento até o desmame. Foram utilizados 92 animais, que foram divididos em 4 grupos experimentais: I – tratados no nascimento e tratados no desmame; II – tratados no nascimento e não tratados no desmame; III – não tratados no nascimento e tratados no desmame; IV – não tratados no nascimento e não tratados no desmame (controle). Foi observada uma menor eliminação de oocistos no grupo tratado até aos 75 dias, e aos 150 dias de idade, além de correlação positiva entre a presença de diarreia e contagem de oocistos nas fezes. Não houve diferença no ganho de peso dos animais ao longo do estudo. No capítulo 2 apresenta-se um estudo no qual foram utilizados 20 *pools* de amostras de fezes de bovinos (10 *pools*) e ovinos (10 *pools*), que foram distribuídos em 6 protocolos de extração de DNA: kit comercial, kit comercial com modificação, DNAzol, brometo de cetil-trimetil amônio (CTAB), pérolas de vidro e kit comercial para amostras fecais. Dentre os protocolos testados, o CTAB foi considerado o mais adequado para extração de DNA de oocistos, com 90% de detecção de DNA por PCR.

Palavras-chave: coccidiose, eimeriose, bezerros, *Eimeria* spp., Apicomplexa.

ABSTRACT

COCCIDIOSIS IN RUMINANTS: METAPHYLACTIC TREATMENT AND MOLECULAR DIAGNOSIS

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Eimeria spp. infection is one of the main diseases that affect young ruminants. Eimeriosis is responsible for considerable economic losses, due to the reduction of weight gain and compromising the development of young animals, mainly due to subclinical infections. This condition occurs especially in intensive and semi-intensive livestock with high animal density, but outbreaks in extensive livestock are also reported, less frequently. Coccidiosis control can be accomplished using treatment that interrupt the life cycle of the parasite na with sanitary practices, both aiming decrease the environment contamination. In this context, this thesis presentes two chapters, with evaluated: (1) the effectiveness of metaphylactic treatment with 5% toltrazuril in beef calves naturally infected with *Eimeria* spp. created extensively; (2) Six different protocols for extracting DNA from Eimeria spp. for molecular diagnosis in cattle and sheep. Chapter 1 presents a study in which four different metaphylactic treatments with 5% toltrazuril were evaluated, in calves from birth to weaning. 92 animals were used, which were divided into 4 experimental groups: I — treated at both birth and weaning; II — treated at birth but not treated at weaning; III — not treated at birth but treated at weaning; and IV — neither treated at birth nor at weaning (control). Less oocyst exception was observed in the treated group up to 75 days, and at 150 days of age, in addition to a positive correlation between the presence of diarrhea and oocyst count in the feces. There was no difference in weight gain of animals in any of the study periods. Chapter 2 presents a study in which twenty pools of fecal samples from cattle (10 pools) and sheep (10 pools) were distributed to six DNA extraction protocols: commercial kit, commercial kit with modification, DNAzol, cetyl-trimethyl ammonium bromide (CTAB), glass beads and commercial kit for fecal samples. Among the tested protocols, CTAB was determined to be most suitable for DNA extraction from oocysts, with 90% of DNA detection by PCR.

Keywords: coccidiosis, eimeriosis, calves, *Eimeria* spp., Apicomplexa.

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1. INTRODUÇÃO

A coccidiose bovina é uma doença de distribuição mundial causada por protozoários do gênero *Eimeria*. Estes parasitos são organismos unicelulares, espécie-específicos, que se desenvolvem dentro das células do intestino delgado e grosso de seus hospedeiros (JOLLEY; BARDSLEY, 2006). Embora infecções mistas geralmente sejam observadas em condições naturais, a maioria das espécies não é considerada patogênica. Estes parasitos pertencem à família Eimeriidae, subordem Eimeriorina, ordem Eucoccidiorida, subclasse Coccidiasina, classe Conoidasida, filo Apicomplexa e Reino Protista (TENTER et al., 2002).

A maioria dos casos clínicos de coccidiose é observada em animais jovens, com menos de um ano de idade e a infecção ocorre pela ingestão de oocistos esporulados presentes em ambientes contaminados. (CORNELISSEN et. al, 1995; SNOEP & POTTERS, 2004). O desenvolvimento da doença em ruminantes é dependente de alguns fatores, como espécies envolvidas, a idade dos animais infectados; o número de oocistos ingeridos; presença de outras infecções concomitantes; e o sistema de produção utilizado na propriedade (CORNELISSEN et al., 1995; HOBLET et al., 1992).

A diarreia é o sinal clínico mais comum e pode ser sanguinolenta ou mucoide. A gravidade da doença varia de auto limitante, na qual os animais se recuperam sem tratamento, até casos graves, em que os animais rapidamente sucumbem à infecção e vão a óbito. Em ruminantes, especialmente em bovinos de leite, um adequado desenvolvimento durante os primeiros meses de vida é essencial para um bom desempenho durante sua vida produtiva. Principalmente nessa fase, bezerras são potencialmente mais susceptíveis a coccidiose, associada ou não a outros patógenos (KEETON; NAVARRE, 2017).

A eimeriose é preponderantemente uma doença que acomete animais jovens e em sistemas intensivos de criação, como visto na bovinocultura de leite. Em criações extensivas, baseadas em fornecimento de gramíneas com uso contínuo de pastagens, o parasitismo por *Eimeria* spp. também pode ser um fator limitante na produção de animais jovens (LUCAS, et al. 2014). Nos últimos anos, o interesse na produção de carne bovina exclusivamente a pasto, aumentou consideravelmente. Para sistemas baseados em pastagens nativas ou cultivadas, o parasitismo por *Eimeria* spp. pode

ser um fator limitante de desenvolvimento dos animais nas fases de cria e recria (LUCAS et al., 2014).

Esta tese está composta por uma revisão bibliográfica sobre eimeriose em bovinos, abordando aspectos de epidemiologia, imunologia, diagnóstico, tratamento e controle. O trabalho foi desenvolvido com os objetivos de i. verificar a eficácia do tratamento metafilático com toltrazuril a 5% em bezerros de corte naturalmente infectados com *Eimeria* spp. ii. avaliar as diferentes técnicas de extração de DNA para *Eimeria* spp. para diagnóstico molecular em bovinos e ovinos. Esta tese está dividida em dois capítulos, sendo o primeiro intitulado “DNA extraction methods for molecular detection of *Eimeria* spp. in cattle and sheep” e o segundo: “Eficacy of 5% Toltrazuril in the metaphylactic treatment of coccidiosis in naturally infected and extensively reared beef calves”.

2. REVISÃO BIBLIOGRÁFICA

2.1 ESPÉCIES DE *Eimeria*

A coccidiose bovina ocorre no mundo todo, no entanto, algumas espécies são mais frequentes que outras. Dentre as várias espécies que infectam bovinos, *Eimeria bovis*, *Eimeria zuernii* e *Eimeria alabamensis* são as mais importantes do ponto de vista clínico (BRUHN et al., 2011).

Apenas duas espécies são altamente patogênicas para bovinas e envolvidas em surtos causando manifestações clínicas, sendo elas: *Eimeria bovis* e *Eimeria zuernii* (DAUGSCHIES; NAJDROWSKI, 2005). *Eimeria alabamensis* geralmente tem sua ocorrência relacionada a pastagens e causa doença em doses infecciosas extremamente altas (> 10 milhões de oocistos) (HOOSHMAND-RAD et al., 1994). *E. bovis* e *E. zuernii* são conhecidas por causar doença clínica severa em bezerros estabulados e em confinamento, embora também possam ocorrer em ambientes diferentes do habitual, como em sistemas de pastejo (DAUGSCHIES; NAJDROWSKI, 2005).

No Brasil, em estudos realizados com bovinos de leite, foram identificadas onze diferentes espécies, sendo elas: *E. bovis*, *E. zuernii*, *E. ellipsoidalis*, *E. cylindrica*, *E. subspherica*, *E. canadensis*, *E. alabamensis*, *E. auburnensis*, *E. pellita*, *E. brasiliensis* e *E. bukidnonensis* (ALMEIDA et al., 2011; BRUHN et al., 2011).

2.2 CICLO DE VIDA

Todas as espécies de *Eimeria* possuem ciclo de vida monoxeno. A infecção dos animais ocorre pela ingestão de oocistos esporulados, presentes na água ou alimentos contaminados (VON SAMSON-HIMMELSTJERNA et al., 2006). As espécies que infectam ruminantes desenvolvem-se em um ciclo de vida direto que pode ser dividido em três estágios. Dois estágios ocorrem dentro das células intestinais do hospedeiro e são referidos como merogonia (ou esquizogonia) e gametogonia. O terceiro estágio, chamado de esporogonia, ocorre fora do hospedeiro (LIMA, 2004). Durante a fase exógena, os oocistos são excretados nas fezes pelo animal infectado e para ocorrer a esporulação, eles exigem temperatura, umidade e tensão de oxigênio ideais (DAUGSCHIES; NAJDROWSKI, 2005).

A merogonia envolve dois ou mais ciclos nos quais os merozoítos são produzidos por múltiplas fissões assexuadas. Após a maturação dos merontes, a célula hospedeira parasitada se rompe, liberando merozoítos que entram em outras células e repetem o ciclo ou progridem para a gametogonia (TAYLOR; CATCHPOLE, 1994; ENEMARK et al., 2013).

A gametogonia constitui o estágio sexual do desenvolvimento, que é o estágio terminal no hospedeiro. Os merozoítos produzidos na fase final da merogônia adentram as células intestinais e produzem macrogamontes ou microgamontes, que amadurecem para macrogametas e microgametas, respectivamente (DAUGSCHIES; NAJDROWSKI, 2005). Durante essa fase, aos microgametas fertilizam os macrogametas, produzindo oocistos. Quando os oocistos estão maduros, estes rompem a célula hospedeira, são liberados no lúmen do intestino e excretados pelas fezes como oocistos não esporulados (JOLLEY; BARDSLEY, 2006).

Na fase exógena, os oocistos não esporulados que são excretados nas fezes, sofrem esporulação em condições ambientais favoráveis de oxigênio, temperatura moderada e alta umidade. A esporulação leva de 1 a 4 dias se as condições ambientais forem ideais, mas pode levar várias semanas em condições menos favoráveis. O oocisto esporulado de *Eimeria* contém quatro esporocistos, cada um contendo dois esporozoítos, para um total de oito unidades infecciosas básicas em cada oocisto esporulado. O ciclo se perpetua quando os oocistos infecciosos são ingeridos pelo hospedeiro através de alimentos ou água contaminados (SVENSSON et al., 1997).

O ciclo completo da eimeriose, desde o início da merogonia até a liberação dos oocistos nas fezes, requer aproximadamente 14 a 21 dias, dependendo da espécie envolvida (TAYLOR; CATCHPOLE, 1994). Os sinais clínicos decorrentes da coccidiose geralmente ocorrem durante os estágios finais da gametogonia, quando os oocistos se formam e são liberados no lúmen intestinal (JOLLEY; BARDSLEY, 2006).

Os oocistos não esporulados são mais propensos a mudanças extremas em fatores climáticos do que os esporulados que podem resistir ao congelamento a - 5 °C a - 8 °C por vários meses (SVENSSON et al., 1997).

2.3 EPIDEMIOLOGIA

A eimeriose é considerada uma doença multifatorial, sendo que a severidade e a manifestação clínica variam conforme a imunidade do animal e o sistema de criação. A doença causada por *E. bovis* e *E. zuernii* são primariamente vistas em animais mantidos em sistemas de criação intensivos. Os casos clínicos são frequentemente vistos quando bezerros são transferidos para currais contaminados por fezes de bezerros que estiveram alojados no mesmo local anteriormente (TAYLOR; CATCHPOLE, 1994).

Alguns fatores inerentes às instalações ou condições de pastoreio dos animais, como alta lotação e a presença de umidade nas habitações, predispõem a ocorrência de uma alta taxa de contaminação ambiental e aumento da taxa de translação do parasito, ocasionando uma alta infecção do hospedeiro (KEETON; NAVARRE, 2017). Foi demonstrado que as vacas no período peri-parto também contribuem para a contaminação ambiental através de um aumento na excreção de oocistos nas fezes (FABER et al., 2002).

São variados os fatores que influenciam no aparecimento da doença na sua forma clínica. A faixa etária usual para animais que sofrem de coccidiose é de um mês até um ano de idade aproximadamente, porém bovinos permanecem suscetíveis a coccidiose durante toda a vida ou até desenvolver imunidade adquirida (SNOEP; POTTERS, 2004). A suscetibilidade dos animais é influenciada pelo estado nutricional (fornecimento de colostro), estresse (alta lotação, transporte, clima, higiene, etc.), status imunológico e ocorrência de doenças concomitantes (KEETON; NAVARRE, 2017).

Eimeira spp. é altamente prolífica, sendo que cada oocisto esporulado tem o potencial de produzir até 23 milhões de oocistos durante a fase endógena após apenas 21 dias. Essa capacidade tem como consequência em altos níveis de contaminação ambiental (DENDRICKSON, 2017).

Segundo Svensson et al. (1997), os oocistos de *E. alabamensis* podem permanecer viáveis em pastagens durante o inverno, em número suficiente para causar doença clínica em bezerros, com uma semana após adentrarem em uma pastagem contaminada. A infecção por *E. alabamensis* pode ocorrer em bezerros alojados, por exemplo, se forem alimentados com feno preparado a partir de pastagens contaminadas (NOEP; POTTERS, 2004).

Boughton (1945), sugere que os hospedeiros portadores que excretam relativamente menos oocistos e os hospedeiros multiplicadores (suscetíveis) se infectam e excretam um número alto de oocistos no ambiente. Bezerros expostos a um grande número de oocistos provavelmente desenvolvem coccidiose clínica. Enemark et al. (2013), demonstraram que há uma relação entre o OoPG total e a presença de diarreia em animais.

2.4 SINAIS CLÍNICOS

A coccidiose é uma doença auto limitante e na maioria dos casos a recuperação ocorre quando a reprodução intestinal do parasita é completada, ou seja, quando a excreção de oocistos cessa (DAUGSCHIES; NAJDROWSKI, 2005). A coccidiose pode ocorrer nas formas subclínicas ou clínicas. A infecção subclínica pode causar inibição do apetite, bem como diminuição da conversão alimentar, como consequência de danos no intestino, o que leva a diminuição das taxas de crescimento e ganhos de peso (JOLLEY; BARDSLEY, 2006).

A diarreia é o sinal clínico mais comum e pode ser sanguinolenta ou mucoide. Além disso, os animais acometidos podem apresentar febre, dor abdominal, às vezes tenesmo e anemia, desidratação, anorexia, perda de peso, podendo ocorrer o óbito (SNOEP; POTTERS, 2004). A gravidade da doença varia de autolimitante, na qual os animais se recuperam sem tratamento, até casos graves, em que os animais rapidamente sucumbem à infecção e vão a óbito. A mortalidade é variável e depende da espécie envolvida, podendo atingir 7 - 20% do rebanho (PILARCZYK et al., 1999; JOLLEY; BARDSLEY, 2006).

A velocidade e o grau de recuperação dependem da gravidade da infecção e da área do intestino envolvida. Os animais que sobrevivem a uma infecção podem nunca recuperar totalmente o seu potencial produtivo e ter maior suscetibilidade a outros patógenos intestinais, que pode piorar a condição clínica, por causa da cicatrização permanente do intestino (DAUGSCHIES; NAJDROWSKI, 2005). Os sinais clínicos da coccidiose são quase sempre causados pelo desenvolvimento de gamontes e oocistos no íleo inferior, no ceco e no cólon (JOLLEY; BARDSLEY, 2006).

Eimeria alabamensis geralmente ocasiona diarreia aquosa e sem sangue. A mortalidade de bovinos infectados com esta espécie é baixa e permanece abaixo de 1%, mesmo quando há alta incidência (VON SAMSON-HIMMELSTJERNA et al., 2006). Quando a infeção ocorre por um grande número de oocistos, principalmente de *E. bovis* e *E. zuernii*, provavelmente ocorrerá o desenvolvimento da doença clínica com diarreia sanguinolenta, desidratação, anorexia, letargia, febre, perda de peso e eventuais mortes (STOCKDALE el al., 1981; DAUGSCHIES et al., 1986).

Existe também uma condição clínica referida como coccidiose nervosa, que é descrita em bezerros logo após infecções causadas por *E. zuernii*. É mais frequente em animais com seis meses ou mais de idade do que em bezerros mais jovens. Os sinais clínicos incluem tremores musculares, convulsões, nistagmo e outros sinais do sistema nervoso central e a mortalidade associada à coccidiose nervosa pode chegar 80% (KEETON; NAVARRE, 2017).

2.5 DIAGNÓSTICO

O diagnóstico *ante mortem* de coccidiose clínica é geralmente baseada no histórico do animal ou rebanho e sinais clínicos, associado à observação de oocistos nas fezes. Em propriedades sem histórico de informações, o diagnóstico pode ser difícil, pois os sinais clínicos geralmente precedem a excreção de oocistos nas fezes (KEETON; NAVARRE, 2017).

2.5.1 Diagnóstico microscópico

O diagnóstico definitivo é complexo pela dificuldade na interpretação dos resultados do exame. A coleta de amostras de fezes para diagnóstico por técnicas de flutuação com açúcar ou sal que dão resultados em oocistos por grama de fezes

(OoPG) são mais recomendados, quando comparados a testes não quantitativos (PUGH; NAVARRE, 2001; DAUGSCHIES; NAJDROWSKI, 2005).

A amostragem e o monitoramento da presença de *Eimeria* spp. nas fezes proporcionam uma boa visão geral da presença de coccidiose no rebanho e podem dar uma imagem do curso da infecção no caso de amostragem repetida (DAUGSCHIES; NAJDROWSKI, 2005). A especiação dos oocistos é importante devido ao elevado número de espécies não patogênicas em animais com outras doenças diarreicas (KEETON; NAVARRE, 2017).

Convencionalmente, a identificação é baseada na avaliação de lesões macroscópicas típicas que ocorrem durante a necrópsia, nas características epidemiológicas e na observação de características morfológicas do oocisto (JOYNER; LONG, 1974). Esses métodos são demorados e laboriosos e requerem expertise para interpretação, devido à sobreposição de características morfológicas entre as diferentes espécies de *Eimeria* (LONG; JOYNER, 1984).

2.5.2 Diagnóstico molecular

Atualmente, a observação morfológica de oocistos é o método prático para identificar espécies dentro de coccídios bovinos (DAUGSCHIES; NAJDROWSKI, 2005). As características detalhadas para oocistos de *Eimeria* spp. em ruminantes foram citadas em vários estudos para determinar as espécies (ODA; NISHIDA, 1990, FABER, 2002, SÁNCHEZ, 2008). No entanto, o método morfológico não é totalmente confiável, uma vez que várias espécies apresentam características semelhantes, combinado à presença de variações intraespécie. Além disso, observações morfológicas combinadas com exame fecal são muito trabalhosas e requerem prévio treinamento (KAWAHARA, 2010).

Sabe-se que uma variação interespécies das sequências do gene 18S rRNA em coccídios de ruminantes é rara e isso não é eficiente para a identificação de espécies baseadas em ensaios de PCR que foram relatados (LI, 2007). O conhecimento genômico do filo Apicomplexa tem progredido continuamente e certos métodos moleculares têm sido usados para a identificação das espécies usando a técnica de PCR. Nestes métodos, um alvo de DNA genômico atrativo é a região interna do espaçador transcrito 1 (ITS-1) derivada dos genes de RNA ribossômico (RNAr) (CAI, 1992; SCHNITZLER, 1998). Além disso, a região ITS-1 pertence a uma

família de genes de múltiplas cópias que fornece um grande número de alvos para ensaios de PCR (LEW, 2003).

Em estudos recentes, diversidade interespécies foram demostradas dentro das regiões ITS-1 de *Eimeria* spp. com alta heterogeneidade entre si, sendo que as sequências específicas de DNA têm sido aplicadas para o diagnóstico das espécies (LEW, 2003). Os ensaios de PCR convencionais ou em tempo real foram desenvolvidos usando sequências específicas de espécies da região ITS-1 e tem sido utilizado com sucesso para diferenciar espécies de *Eimeria* spp. que parasitam ruminantes (KAWAHARA, 2010).

2.6 RESPOSTA IMUNE

Após um contato primário do hospedeiro com estágios infectantes, ocorre o desenvolvimento de uma resposta imunológica espécie-específica e infecções posteriores geralmente não estão relacionadas à doença clínica (DAUGSCHIES; NAJDROWSKI, 2005; WITCOMBE; SMITH, 2014). A indução de uma forte resposta imunológica protetora específica para cada espécie, é o que impede o estabelecimento da doença clínica (RUIZ et al., 2014). O nível da imunidade é proporcional a dose infectante que é ingerida pelo hospedeiro, sendo que a exposição a um número irrelevante de oocistos pode não fornecer o estímulo antigênico necessário para desencadear uma resposta suficiente para prevenir infecções e a doença (DAUGSCHIES; NAJDROWSKI, 2005).

A imunidade humoral normalmente não é considerada um fator preponderante no controle da coccidiose por parte do hospedeiro. Em geral, a resolução de infecções primárias por *Eimeria*, assim como o controle de reinfecções homólogas, dependem da resposta imune celular do hospedeiro (SUHWOLD, 2010). Estudos mostram evidências de um papel predominante das células T nas respostas protetoras do hospedeiro em infecções por coccídeos (HERMOSILLA et al., 1999).

Na ocorrência de infecção primária por *Eimeria* spp. em ruminantes, geralmente ocorre um aumento substancial de linfócitos T CD4+ e CD8+, sendo que as células CD4 + parecem ser mais importantes no término de uma infecção primária do que as células CD8 +, enquanto estas podem representar o principal tipo de célula efetora em caso de reinfecções (HERMOSILLA et al., 1999; SUHWOLD et al., 2010). Hermosilla et al. 1999 sugerem que possa ocorrer uma preponderância de células Th1

ativas nos tecidos de animais infectados quando comparados às células Th2. No período pré-patente as respostas imunológicas celulares são do tipo Th1, sendo caracterizadas por produção de IFN-γ.

Embora estas populações de células T ativadas não sejam capazes de interromper o ciclo de vida do parasito em infecções primárias, a resposta imune por células T pode alterar a quantidade e a duração da excreção de oocistos, bem como pode estar relacionada ao controle imunológico em novas infecções (HERMOSILLA et al., 1999).

2.7 TRATAMENTO E CONTROLE

O controle da eimeriose está diretamente relacionado às informações inerentes às características e à evolução da infecção (MUNDT, et. al 2005). Um grande número de fármacos tem sido utilizado para o tratamento da coccidiose em ruminantes. Estes podem ser coccidiostáticos, os quais impedem o desenvolvimento, ou coccidicidas que eliminam os parasitos. Estes produtos podem agir sobre as diferentes fases do ciclo de vida do protozoário, suprimindo o desenvolvimento de fases assexuadas, sexuadas ou de ambas (LIMA, 2004).

Os tratamentos disponíveis para o controle da infecção por *Eimeria* spp. podem ser utilizados na forma metafilática, terapêutica ou preventiva. O tratamento metafilático consiste na aplicação do fármaco no período pré-patente da infecção, ou quando os animais estão expostos ao risco. A forma terapêutica o é realizada quando os animais estão apresentando sinais clínicos e excretando oocistos (ENEMARK et al. 2015; EPE, et. al 2005). Já o tratamento preventivo consiste em administrar o medicamento de forma contínua, para evitar o estabelecimento de uma infecção por *Eimeria* spp. (TAYLOR, 2000).

Em geral, o tratamento terapêutico da coccidiose clínica é considerado de baixa eficácia, pois as lesões da mucosa já estão presentes no intestino, enquanto os tratamentos profiláticos ou metafiláticos são preteridos (VERONESI et al., 2011). Além disso, as perdas de produção por infecções subclínicas, por infecções concomitantes e os potenciais danos permanentes que não respondem ao tratamento, são dispendiosos e têm implicações no bem-estar animal (MUNDT et al., 2005).

No entanto, alguma exposição ao protozoário é necessária para desenvolver imunidade, mas essa deve ser limitada. Onde o controle ambiental não é adequado,

o uso de produtos anticoccidianos pode ser útil tanto para o tratamento como para a prevenção (EPE et al., 2005). A prevenção da coccidiose é geralmente reservada para bezerros após o desmame ou então em fases críticas em que são submetidos a fatores estressantes, quando são mais suscetíveis, principalmente em condições de alta lotação (KEETON; NAVARRE, 2017).

Alguns estudos indicam um efeito positivo no tratamento metafilático com toltrazuril ou diclazuril, frente a infecções naturais de *Eimeria* spp. em bezerros e infecções experimentais em bezerros bubalinos (BOSCO et al., 2015; ROMERO et al., 2013; EPE et al., 2005). O toltrazuril atua em todas as formas intracelulares do parasita, principalmente por interferir na respiração celular e na síntese de pirimidina (HARDER; HABERKORN, 1989). Além disso, não interfere no desenvolvimento da imunidade normal, podendo até demonstrar uma produção de anticorpos aumentada após o tratamento (GREIF, 2000).

3. CAPÍTULO 1 – Artigo científico

Este capítulo originou um manuscrito que foi submetido à revista: Tropical Animal Health and Prodution.

Efficiency of 5% Toltrazuril in the metaphylactic treatment of coccidiosis in naturally infected and extensively reared beef calves

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ABSTRACT

Eimeria spp. infection is one of the main diseases that affect young cattle in beef and dairy herds, leading to economic losses and compromising the performance and growth of animals. This study aimed to evaluate the effectiveness of different protocols of metaphylactic treatment with 5% toltrazuril (15mg/kg) in naturally infected, extensively reared beef calves from birth to one year of age. We used 92 calves aged up to two weeks of life, initially divided into two experimental groups: treated and control. The animals were evaluated fortnightly with individual count of oocysts per gram of feces (OPG) tests until weaning; thereafter, they were again divided into four subgroups: I — treated at both birth and weaning; II — treated at birth but not treated at weaning; III — not treated at birth but treated at weaning; and IV — neither treated at birth nor at weaning (control). During the experimental period, the animals were evaluated for weight gain, excretion of oocysts, and presence of pathogenic species of *Eimeria* spp. In the first stage of the study, less oocysts were excreted in the treated group for up to 75 days and at 150 days of age ($p<0.05$). *Eimeria bovis* was the most frequently identified species during the experimental period. Moreover, there was a positive correlation between the presence of diarrhea in the calves and the number of oocysts excreted ($p<0.05$). There was no significant difference in weight gain of animals in any of the study periods ($p>0.05$).

Keywords: coccidiosis, eimeriosis, calves, toltrazuril, *Eimeria* spp.

INTRODUCTION

Bovine coccidiosis is a worldwide disease caused by protozoa of the genus *Eimeria* that cause intestinal disorders and, in some cases, death. Although mixed infections are commonly observed under natural conditions, only two highly pathogenic species *E. zuernii* and *E. bovis* are generally involved in the outbreaks of clinical disease in calves, and can cause moderate to severe enteritis, sometimes leading to death (Daugschies & Najdrowski, 2005). The other species are considered less pathogenic (e.g. *E. alabamensis*) or are rarely associated with the presence of clinical signs (Jolley & Bardsley, 2006).

The prevalence of *Eimeria* infection in cattle is highly age dependent (Cornelissen et al. 1995). Young animals up to one year of life are usually the most susceptible. As a result, when they grow into adults, they usually becom

e asymptomatic hosts after recurrent reinfections, serving as a source of infection for younger animals (Matjila & Penzhorn, 2002).. Interest in forage-based meat production systems has increased in recent years. For grass-based systems with continuous use of pastures, internal parasitism by coccidia may be a limiting factor of production in young animals (Lucas et al. 2014). In southern Brazil, eimeriosis is a disease that frequently affects young calves, in different management systems (Oliveira et al. 2017).

Clinical coccidiosis is more common in intensive systems than other systems due to epidemiological issues, which provided a more viable environment for oocyst sporulation (Veronesi et al. 2011). However, extensively reared animals can also be affected (Daugschies & Najdrowski, 2005). The greatest susceptibility of young animals is primarily explained by the fact that there is a lack of adaptive immunity in that stage and passive immunity may not be sufficiently protective against this infection. As the animal age and exposure progresses, it develops an adequate immune response capable of preventing the development of clinical coccidiosis (Sánchez et al. 2008).

Toltrazuril is a triazone compound that acts in all intracellular stages of the parasite, mainly by interfering with cellular respiration and pyrimidine synthesis (Harder & Haberkorn, 1989).

Furthermore, the prophylactical use of toltrazuril is highly recommended because it does not interfere with the development of adaptive immunity, and an increase in antibody titers can be observed after treatment. In general, the therapeutic treatment of clinical coccidiosis is not advantageous, as lesions of the mucosa are already present in the intestine (Greif, 2000). Knowing the life cycle of protozoa and the pharmacology of anticoccidials is important and this knowledge should be considered while planning the treatment (Veronesi et al. 2011; de Souza Rodrigues et al. 2017).

This study was aimed at evaluating the efficacy of the metaphylactic use of toltrazuril 5% in different conditions, in extensively reared beef calves that were naturally infected with *Eimeria* spp., in addition to comparing weight gains and the dynamics of excretion of oocysts between treated and untreated animals.

MATERIALS AND METHODS

Property and animals

This study was conducted in a cattle farm located in the municipality of São Francisco de Assis (29°19 35.76” S 54°58.01.36”), state of Rio Grande do Sul, Brazil. We selected 92 nelore and hereford crossbred calves of both sexes, aged from one to two weeks, randomly from the same herd. The animals were kept in native pasture together with their mothers until weaning, which occurred between five and nine months of age. After this period, they were transferred to a winter pasture (*Lolium multiflorum*), where they remained for approximately five months. All animals had free access to water and mineral salt. The project was approved by the Animal Ethics Committee of Federal University of Santa Maria under CEUA under no 7304121218.

Experimental groups

The calves aged from one to two weeks (n=92), during the birth season, which comprised the months between August and November, and were divided into two experimental groups: treated with toltrazuril 5% (15 mg/kg) (n=46) and control group without treatment (n=46). The animals in this first stage of the study (from birth to weaning) were divided into two blocked randomized groups, according

to birth and sex data, every fifteen days, as far as births occurred. After weaning and before being transferred to winter pasture, the calves were finally redistributed into four blocked randomized groups, according to sex, OPG and weight: I — treated at both birth and weaning (n= 23); II — treated at birth but not treated at weaning (n= 23); III — not treated at birth but treated at weaning (n= 23); and IV — neither treated at birth nor at weaning (control group, n= 23) (fig 1).

The toltrazuril was applied orally using a specific dosing gun for cattle with drench type applicator 60 ml, Nasco Farm & Ranch. None of the animals on the property received anticoccidial treatment prior to the experimental period. All calves were treated with anthelmintic at birth (ivermectin) and weaning (moxidectin), according to efficacy tests previously performed.

Clinical, parasitological, and weight evaluation

We calculate OPG as well as clinical evaluation of the animals for presence or absence of diarrhea, were performed fortnightly, from the birth of the animals to weaning, which occurred at 165 to 285 days of age. After weaning, the animals were relocated to a winter pasture, where they remained for approximately five months. During this period, clinical evaluations and feces were collected in three weeks, and then at intervals of 60 days. The animals were weighed at 45 days of age, at weaning, and at the end of the experimental period.

The samples were first evaluated to quantify OPG using the McMaster technique with a sensitivity of 50 with 4 grams of feces from each animal that was collected directly from the rectal ampoule. Fecal samples with OPG count above 500 were separated to identify the species of *Eimeria*. For this purpose, a feces pool was formed at each day of collection. For this, the feces were homogenized, dissolved in water, and strained through sieves with 60 μm meshes. Several washes were performed using the technique proposed (Hoffman et al., 1934). After this, potassium dichromate was added in sufficient quantity to obtain a 2% solution. This solution was oxygenated using an air pump, kept at room temperature (±23 °C) for one week so that the oocysts could sporulate.

After this process, the centrifugal flotation technique was performed in saturated sugar solution for separating oocysts (Ueno & Gonçalvez, 1988). Morphological and morphometric

characteristics, such as shape, color, presence/absence of micropyle, and length and width of oocysts and sporocysts were evaluated to identify the species of *Eimeria* collected from the feces of animals (Daugschies & Najdrowski, 2005; Florião et al. 2016).

Statistical analysis

OPG and weight data were tested for normality using the Kolmogorov-Smirnov test. The results of OPG in the first part of the study, from birth to weaning, were evaluated by the Mann-Whitney Test. In the second stage of the experiment, the OPG values of the same group over the experimental period (paired samples) were evaluated by the non-parametric Kruskal-Wallis multiple comparisons test with *post-hoc* Wilcoxon rank sum. Live weight gain among the animals were compared using the Kruskal Wallis test. The Spearman correlation test was used to identify correlations between the presence or absence of diarrhea and OPG in animals of different groups. These statistical tests were performed with a 95% confidence interval ($p<0.05$) in the R software (R Core Team 2017).

RESULTS

Feces analysis

In the first stage of the experiment, comprising the period from birth to weaning till up to 75 days of life, animals receiving treatment with toltrazuril 5% had lower mean excretion of oocysts in feces compared to the control group ($p<0.05$). The highest OPG count in the treated group occurred at 105 days of life with a mean OPG of 4098, while peak excretion in the non-treatment group occurred at 75 days of life, with a mean OPG of 6560. Moreover, oocysts were excreted later in the treated group when compared to the control group (Fig 2).

When analyzing the excretion data by collection dates, we observed that from September to May, the highest number of OPG in the control group occurred in the 13th collection (second half of February), while the highest number of OPG in the treated group occurred in the 8th collection (first half of December) (Fig 3). With regard to the frequency of detection of oocysts of *Eimeria* spp. in the feces, oocysts were identified in 60.8% (443 out of 728) of all samples collected from the groups

receiving treatment, and oocysts were identified in 70.1% (522 out of 744) of the samples analyzed in the group control.

In the second stage of the experiment, when the animals remained for 150 days in winter pasture, the two groups (I and III) that received anticoccidial treatment after weaning showed a decrease in the excretion of oocysts, reaching almost zero in three weeks (Fig 4). In this period, there were significant differences only in the second collection, and these differences occurred between groups I and II; I and IV; II and III; III and IV ($p<0.05$).

The animals were also analyzed for the presence or absence of diarrhea during feces collection, in the first stage of the study, from birth to weaning. The animals in the toltrazuril 5% group had diarrheal feces in lower degree during collections, compared to the control group, for up to 105 days of age (Fig 5). Moreover, treated animals had a higher number of diarrhea cases compared to the control group. during the first experimental period, 23 cases in the treated animals and 38 cases in the control group. Furthermore we observed a positive correlation between cases of diarrhea and excretion of oocysts in the control group ($p<0.05$).

Of the 46 animals treated at birth, 16 (34.8%) had diarrhea in at least one stool collection. The same was observed in 24 (52.1%) of the 46 animals that received no treatment at birth (groups III and IV). No animals with bloody diarrhea were observed during the study period and no calf from the control group needed anticoccidial treatment.

***Eimeria* spp. species**

Throughout the experimental period, *E. bovis* was the most frequently identified species in the samples (Fig 6). However, oocysts of other species such as *E. zuernii*, *E. ellipsoidalis*/*cylindrica*, *E. alabamensis*, *E. subspherical* and *E.* auburmensis were present in animal feces. Since the oocysts of *E. cylindrica* and *E. ellipsoidalis* cannot be reliably morphologically distinguished, all small and cylindrical shaped oocysts of *Eimeria* spp. measuring 19 to 36 μmm by 8 to 18 μmm were designated as *E. ellipsoidalis*/ *cylindrical* (Levine & Ivens, 1986).

Weight gain

In the first stage of the study (birth upon weaning), the animals without treatment gained an average weight of 100.7 kg, while the animals treated with toltrazuril 5% gained an average of 88.4 kg (Fig 7). In the winter pasture, group I animals were the ones that had the highest mean gain (84.5 kg), followed by groups II (79.6 kg), III (79.1 kg) and IV (78.8 kg) (Fig 8). However, there were no significant differences for mean weight gains between the treated and control groups in the first and second stages of the study ($p>0.05$).

DISCUSSION

Studies involving toltrazuril treatment in extensively raised beef calves are still scarce. This study showed that 5% toltrazuril can be used to control infection with *Eimeria* spp. in calves naturally exposed to oocysts in a grazing system. Positive effects of metaphylactic treatment with toltrazuril on the degree and duration of oocysts excretion, and in the presence or absence of diarrhea have been demonstrated in field animals (Enemak at al., 2015).

The excretion of oocysts was significantly lower in the treated groups than in the control groups in terms of duration of excretion per animal, in days and rate of excretion of oocysts. The primary criterion for evaluating efficacy was oocyst excretion, since this is the most sensitive parameter for evaluating coccid infection (Mundt et al. 2005b). The effect of a sharp decrease in the excretion of oocysts observed in the present study corresponds to the observations previously described for *E. bovis* and *E. zuernii* (Jonsson et al. 2011). It is known that infection with *Eimeria spp.* may be the primary or secondary cause of diarrhea in calves (Mundt et al. 2005b; Mundt et al. 2003). Consequently, a decrease in the rate of infection with *Eimeria* spp. and endothelial injury reduces the rate of neonatal diarrhea caused by *Eimeria* and agents that have infections aggravated by lesions caused by *Eimeria* (Keeton & Navarre, 2018). The invasion of endothelial cells occurs with the formation of first generation macrogamontes, with a morphological alteration of the parasitized cells (Lopez-Osorio et al. 2020)

Diarrhea in calves has a multifactorial etiology, in which both infectious (viruses, bacteria, and protozoa) and non-infectious (facilities, feeding, hygiene) agents can be directly involved in the development of this disease (Bartels et al. 2010). Coinfection is often observed in diarrheal calves, although a single primary pathogen may be causing the disease (Cho & Yoon, 2014). Previous studies have shown that animals infected with *Eimeria* spp. are more susceptible to infection by other viral, bacterial, or parasitic enteropathogens. This occurs because coccids cause damage to enterocytes in their stage of intracellular multiplication in the host (Keeton & Navarre, 2018). Although the area where this study was conducted had a history of coccidiosis in young animals, we could not say that this was the only causal factor for cases of diarrhea during the study period, since no differential diagnosis was made in the affected animals. Despite this, there was a positive correlation between excretion of oocysts with data on diarrhea in control animals ($p<0.05$), indicating that coccidiosis is likely to be the primary cause of diarrhea that occurred during the experimental period. In order to be considered a potential cause of clinical coccidiosis in herds, a threshold of 500 OPG of pathogenic species of *Eimeria* spp. in cattle is needed (Mundt et al. 2005a; Bangoura et al. 2012). In this study, we observed that most animals with diarrhea had a high excretion of oocysts associated with a predominant presence of pathogenic species of *Eimeria* spp.

Four main criteria were required for comparing the results of studies with toltrazuril (Mundt et al. 2003; Daugschies & Najdrowski, 2005; Epe et al. 2005; Veronesi et al. 2011): (1) study duration, (2) excretion of oocysts (pathogenic species), (3) diarrhea index, and (4) body weight gain or/and mean daily gain. In this study, indicators such as excretion of oocysts and presence of diarrhea were shown to be good criteria to evaluate the efficacy of toltrazuril in this exploration model, being evaluated in a long-term study. The number of animals with diarrhea during the experimental period was higher in the control group than in the treated group, from birth to weaning. However, no statistical significance was observed ($p>0.05$). In a study conducted by Epe et al. (2005), metaphylactic treatment with toltrazuril significantly decreased the number of days on which animals had diarrhea in a grazing system. More remarkable results can be expected in areas with high incidence of neonatal diarrhea and death of animals.

Toltrazuril can affect all phases of the life cycle of *Eimeria* spp., which results in the maintenance of intestinal integrity and nutrient absorbing function, besides having a considerably lasting effect compared to other anticoccidials used for cattle (Balicka-Ramisz A, 1999). The application of metaphylactic treatment in beef calves covers the period between the decline of maternal antibodies and immunity acquired with age. The results of this study indicate that a metaphylactic approach through the treatment of exposed animals is a beneficial tool for efficient control of the disease when the outbreak of coccidiosis is expected, as the objective is to avoid economic losses associated with clinical disease and those associated with subclinical coccidiosis (Bosco et al. 2015).

In the present study, animals began to excrete oocysts at 30 days of life, which is close to the pre-patent period of most species of *Eimeria* spp., from 2 to 3 weeks (Daugschies & Najdrowski, 2005). In the control group, the first peak occurred at 45 days of life and the highest mean excretion occurred at 75 days of life. In naturally infected and stable calves, the first peak of excretion of oocysts can be observed at about three months of age, involving *E. ellipsoidalis, E. bovis.,* and *E. zuernii* (Parker & Jones, 1987). The fact that feces were collected only every two weeks may have increased the differences in OPG results among calves, as the excretion of oocysts of these animals can last for more or less days, so that the maximum and minimum excretion of each animal may have occurred on a day without sampling (Zechner et al. 2015). Lucas et al. (2014) observed higher excretion of oocysts near the age of six months in extensively reared beef calves.

Different results can be attributed to differences in breeding systems and the species involved in infection. Throughout the experimental period, the main species identified in the animals of this study was *E. bovis*, while in other studies involving bovine coccidiosis under field conditions, the prevalent species that has usually been reported is *E. alabamensis* (Svensson et al. 1994; Svensson, 2000).

It is known that subclinical coccidiosis also has economic importance mainly related to the decrease in weight gain and the worsening of diarrhea caused by other agents (Daugschies & Najdrowski, 2005). In this study, no significant difference was observed in the weight gain of animals

at weaning and leaving grazing. This observation can be attributed to low environmental contamination, and less challenging situation since the animals were kept in extensive grazing system throughout the experimental period. Most studies using metaphylactic treatment with toltrazuril that showed significant weight gains were conducted in intensive breeding systems, which provided a more viable environment for oocyst sporulation (Veronesi et al. 2011; Enemark et al. 2015). Although there were no differences in gains in the analyzed groups, the final effect was beneficial, since the reduction of oocyst excretion indicated the minimization of the infection pressure in calves subsequently kept in that area, thus controlling coccidiosis in the long term. In addition to the contamination of calves, cows in the peripartum period show an increase in the excretion of oocysts, which also contributes to greater environmental contamination (Faber et al. 2002; Mundt et al. 2005). In this context, a preventive program to control coccidiosis and reduce the environmental burden of oocytes can minimize the risk of serious infection in subsequent generations.

The treatment time in animals shortly after birth was chosen to optimize the management of calves, since in extensive livestock farming, many farmers treat myiasis and helminths in newborn animals. In order to benefit from metaphylactic treatment against eimeriosis, calves should receive treatment in the pre-patent period, i.e. one to two weeks after infection (Mundt et al. 2005b). In the present study, the greatest excretion of oocysts in calves occurred approximately at 75 days of life (fifth collection). Thus, the results could perhaps be enhanced if the treatment was given to older animals. However, this possibility should be evaluated by the farmer, since it involves an additional management procedure for young animals, which may be not possible in certain production systems.

During the experimental period, animals of different groups and different levels gained weight, as expected, but without any significant differences. However, these gains can be attributed to the peculiarities of the individual, as well as the age and sex of the animals. Although in the second stage of the experiment, group I had a final mean gain 5.7 kg higher than group IV, this difference was not statistically significant ($p>0.05$). This result was expected, as few animals showed severe clinical signs during the study. It has already been shown that the number of excreted oocysts is not necessarily associated with the severity of coccidiosis, which consequently can be applied to weight gain

(Daugschies & Najdrowski, 2005). In a study conducted by Veronesi et al. (2011), in which metaphylactic treatment with toltrazuril was evaluated, a greater daily weight gain was observed in the treated calves. However, there were no significant differences in the final weight of the animals in the long term. Although we did not evaluate the evolution of weight gain over the experimental period and only evaluated the initial and final weight gain, it is likely that this effect may have occurred in the present study, since it was also conducted with animals in the growth phase and during a similar period.

Toltrazuril 5% showed good efficacy against mixed infections of *Eimeria* spp. in extensively reared calves, either in the first weeks of life or at weaning. Moreover, treatment lasted a considerable amount of time, significantly decreasing the excretion of oocysts up to approximately 75 days of life. However, since there was no significant difference in animal weight gain during the experimental period, the treatment should consider the type of management used in the area and the presence of high rates of infection by pathogenic species, in order to avoid clinical cases resulting in animal death.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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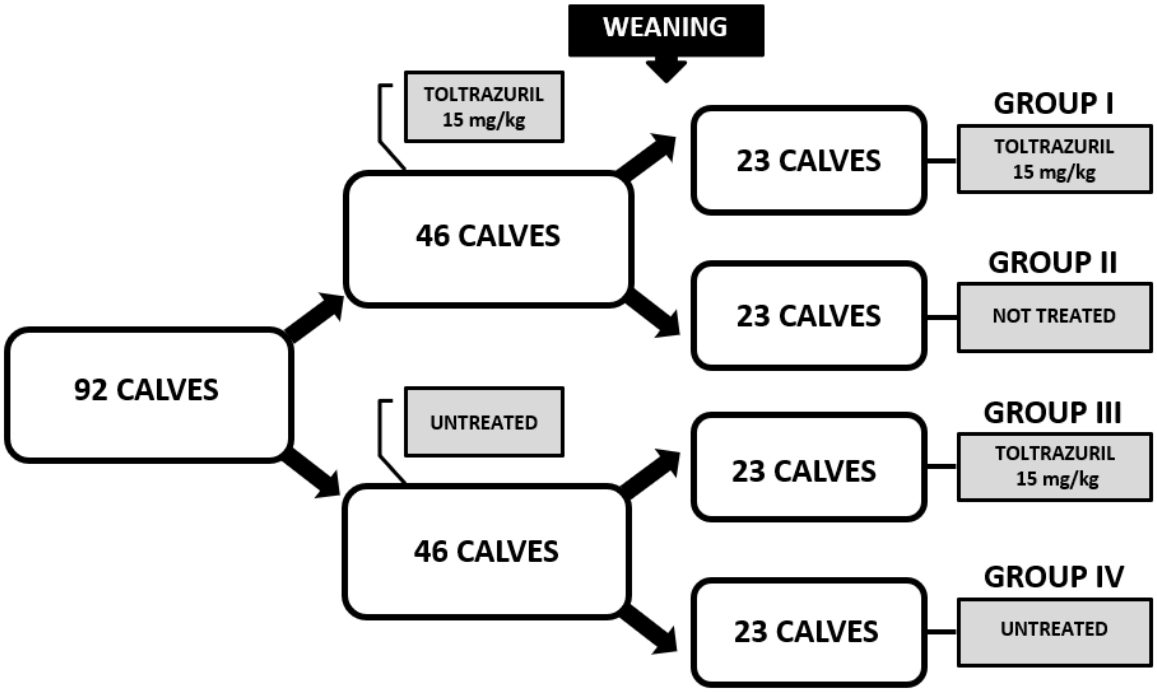


Figure 1. Treatment planning and division of experimental groups (group I — treated at both birth and weaning; group II — treated at birth but not treated at weaning; group III — not treated at birth but treated at weaning; group IV — neither treated at birth nor at weaning).

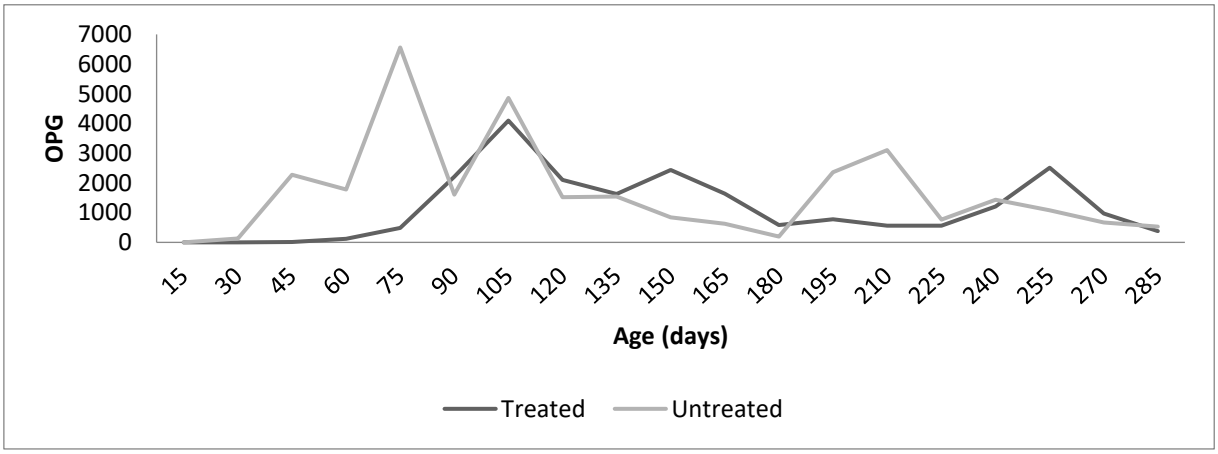


Figure 2. Dynamics of excretion of *Eimeria* spp., according to the age of the calves, in the first stage of the study: from birth to weaning.

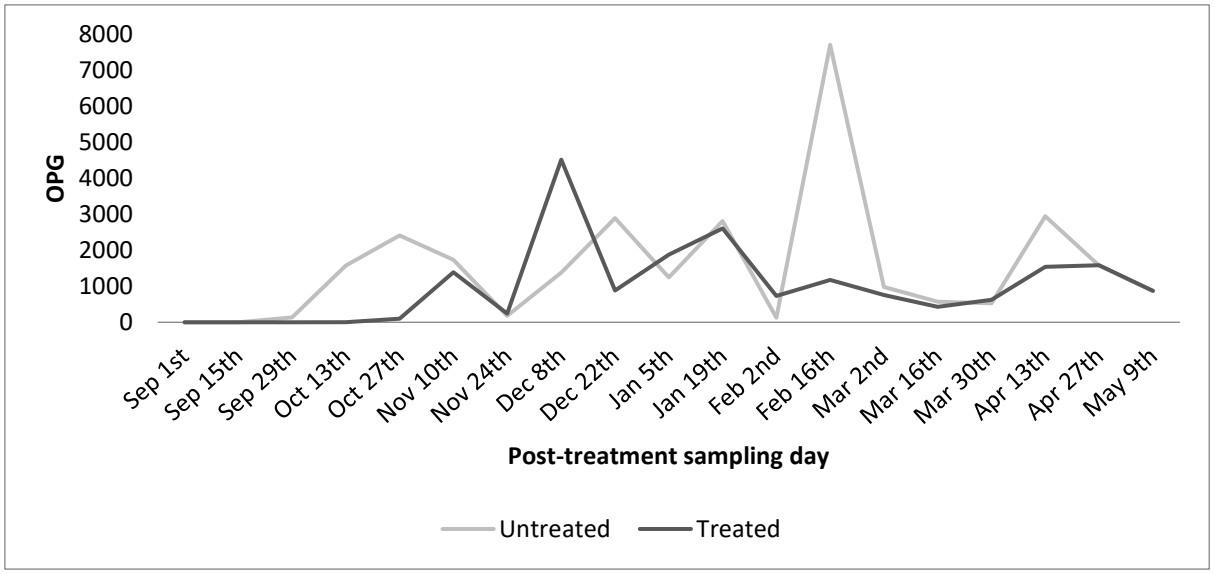


Figure 3. Excretion of oocysts per collection day, from september to may (from birth to weaning).

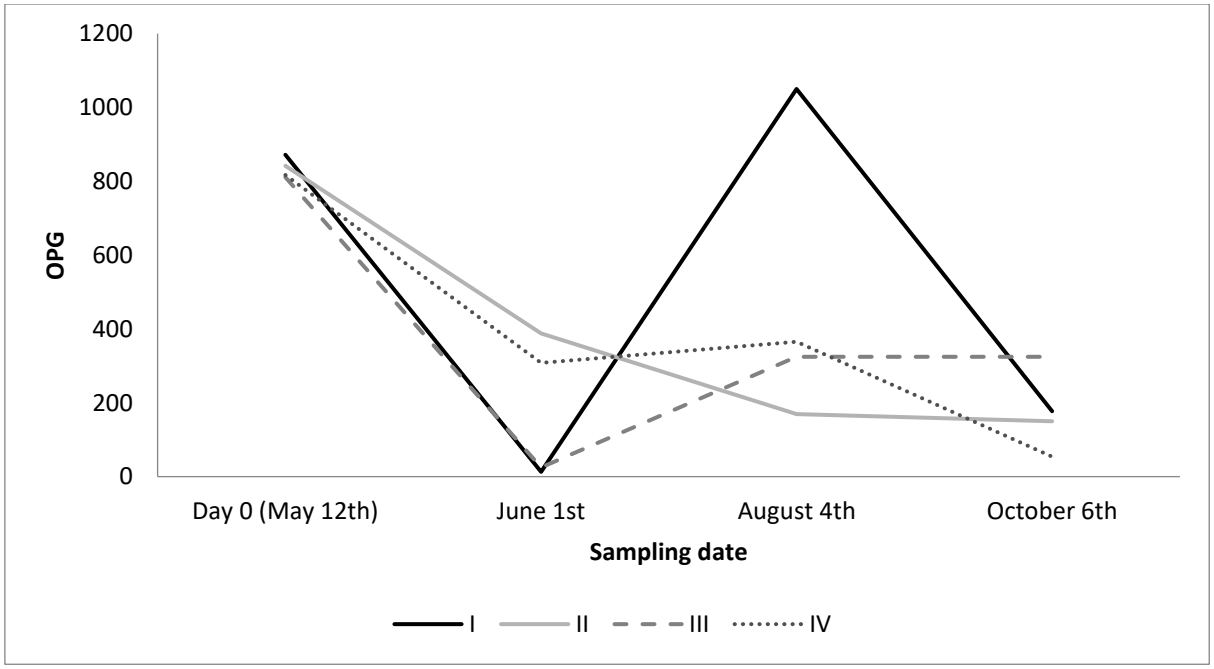
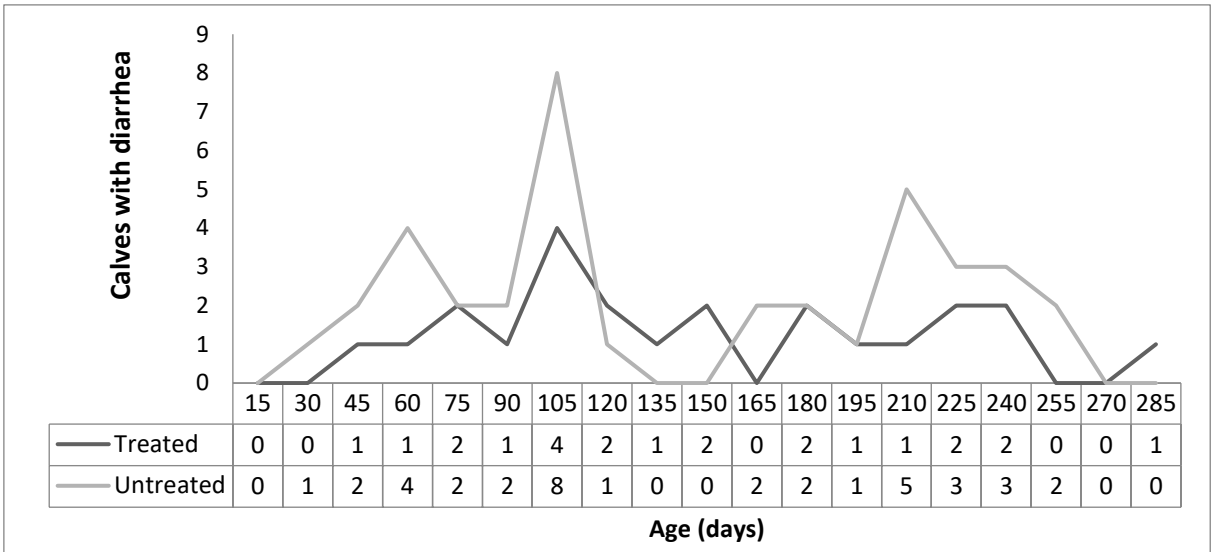


Figure 4. Eimeria spp. excretion dynamics from weaning to exit from winter pasture (group I — treated at both birth and weaning; group II — treated at birth but not treated at weaning; group III — not treated at birth but treated at weaning; group IV — neither treated at birth nor at weaning). Significant differences in the 2nd collection, between groups I and II; I and IV; II and III; III and IV ($p<0.05$).



	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285
Treated	0	0	1	1	2	1	4	2	1	2	0	2	1	1	2	2	0	0	1
Untreated	0	1	2	4	2	2	8	1	0	0	2	2	1	5	3	3	2	0	0

Figure 5. Number of animals with diarrhea in the treated and control groups, according to the age of the calves, in the first stage of the study: from birth to weaning. Positive correlation between cases of diarrhea and excretion of oocysts ($p<0.05$).

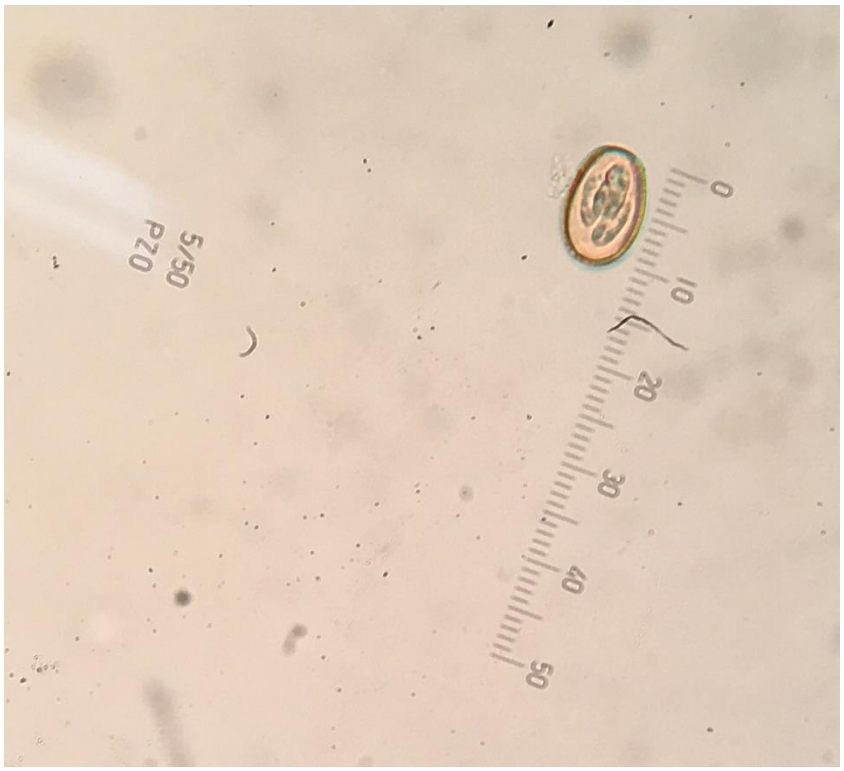


Figure 6. *Eimeria bovis*, optical microscope, 40-fold increase.

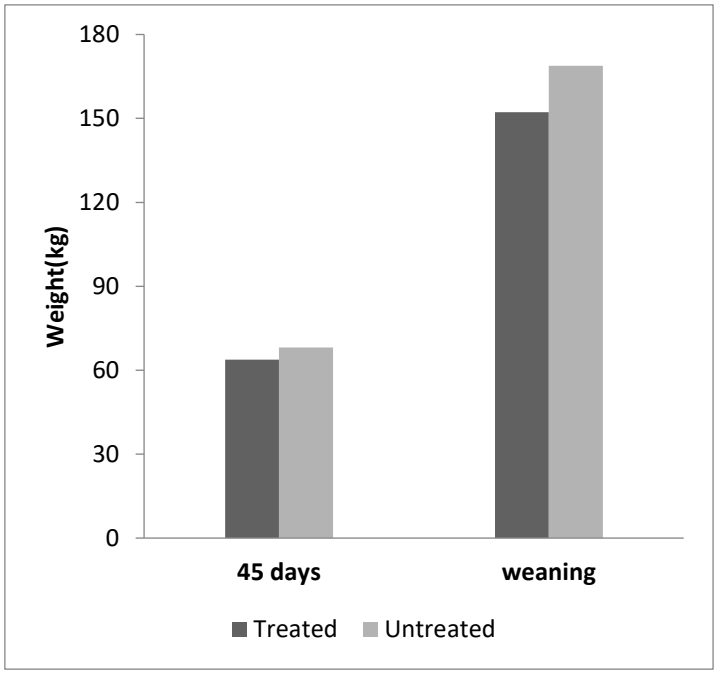


Figure 7. Weight of animals at 45 days of life and after weaning, in the first stage of the study. No significant difference ($p>0.05$).

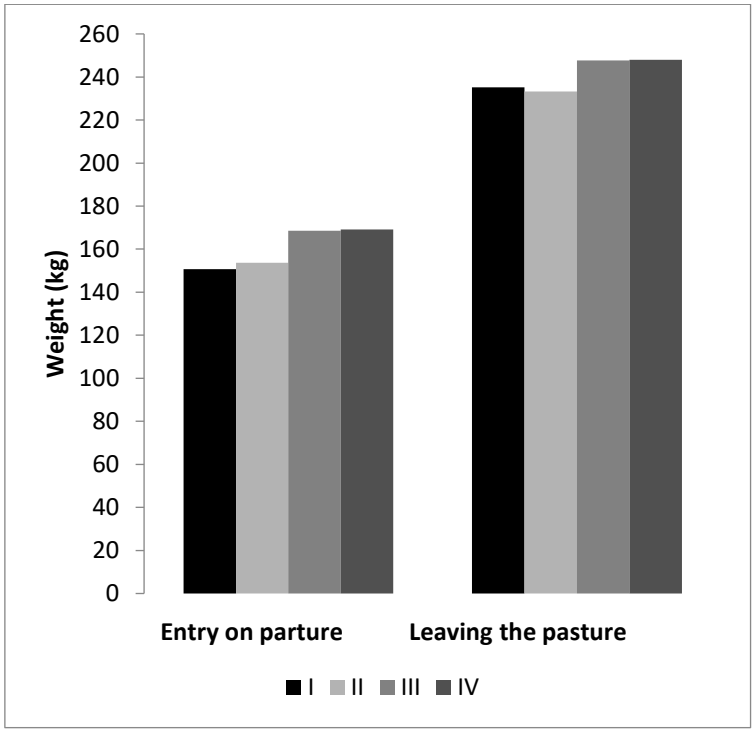


Figure 8. Weight of animals at weaning and 140 days after (leaving winter pasture), by treatment group: I — treated at both birth and weaning; II — treated at birth but not treated at weaning; III — not treated at birth but treated at weaning; IV — neither treated at birth nor at weaning. No significant difference ($p>0.05$).

4. CAPÍTULO 2 – Artigo científico

Este capítulo originou um artigo científico que foi publicado na revista Pesquisa Veterinária Brasileira, v. 40, n. 7, p. 514-518, 2020.

DNA extraction methods for molecular detection of *Eimeria* spp. in cattle and sheep

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ABSTRACT

Molecular detection of *Eimeria* species in fecal samples can be useful for experimental and diagnostic purposes. However, the parasite quantity presence in feces and the oocyst wall are an obstacle in DNA extraction protocols. Therefore, adequate sampling and effective disruption of the oocysts are essential to improve the accuracy of DNA detection by PCR. The aims of this study were to evaluate the suitability of six protocols for DNA extraction from *Eimeria* spp. present in bovine and sheep. Twenty pools of fecal samples from cattle (10 pools) and sheep (10 pools) were distributed to six DNA extraction protocols: commercial kit, commercial kit with modification, DNAzol, cetyl-trimethyl ammonium bromide (CTAB), glass beads and commercial kit for fecal samples. Fecal samples were submitted to DNA extraction and PCR. Among the protocols tested, CTAB was determined to be most suitable for DNA extraction from oocysts (90% of DNA detection by PCR); DNAzol and CTAB resulted in higher DNA detection from bovine samples (80%). CTAB and commercial kit with modification improved PCR detection of *Eimeria* spp. in sheep samples, with positive amplification of DNA in all tested samples.

INDEX TERMS: DNA extraction, molecular detection, *Eimeria* spp., cattle, sheep, PCR, fecal samples.

RESUMO [**Métodos de extração de DNA para detecção molecular de *Eimeria* spp. em bovinos e ovinos.**]

A detecção molecular de espécies de *Eimeria* em amostras fecais pode ser útil para fins experimentais e de diagnóstico. No entanto, a quantidade de parasitas nas fezes e a parede do oocisto são um obstáculo nos protocolos de extração de DNA. Portanto, uma amostragem adequada e a ruptura efetiva dos oocistos são essenciais para melhorar a precisão da detecção de DNA por PCR. Os objetivos deste estudo foram avaliar seis protocolos para extração de DNA de *Eimeria* spp. em amostras de bovinos e ovinos. Foram distribuídos 20 grupos de amostras fecais de bovinos (10 grupos) e ovinos (10 grupos) em seis protocolos de extração de DNA: kit comercial, kit comercial com modificação, DNAzol, brometo de cetil-trimetil amônio (CTAB), pérolas de vidro e kit comercial para amostras fecais. As amostras fecais foram submetidas à extração de DNA e PCR. Entre os protocolos testados, CTAB foi considerado o mais adequado para extração de DNA de oocistos (90% de detecção de DNA por PCR); DNAzol e CTAB resultaram em maior detecção de DNA em amostras de bovinos (80%). CTAB e kit comercial com modificação melhoraram a detecção por PCR de *Eimeria* spp. em amostras de ovinos, amplificação positiva de DNA em todas as amostras testadas.

TERMOS DE INDEXAÇÃO: Extração de DNA, detecção molecular, *Eimeria*, bovinos, ovinos, DNA, PCR, amostras fecais.

INTRODUCTION

Coccidiosis is an infection caused by protozoa of the genus *Eimeria* of great economic importance because of the losses due to clinical disease (diarrhea) and subclinical infections (poor weight gain) (Chartier & Paraud 2012). *Eimeria* spp. develop in the small and large intestines of the hosts, is specie specific and several species of *Eimeria* were identified infecting cattle and sheep affecting mainly young animals (Daugschies & Najdrowski 2005, Chartier & Paraud 2012).

Eimeria bovis and *Eimeria zuernii* are most commonly pathogenic species in calves worldwide causing morbidity and mortality by disturbing intestinal absorption and often associated with diarrheic feces (Daugschies & Najdrowski 2005). The most pathogenic *Eimeria* spp. in sheep are *Eimeria ovinoidalis* which develops in the large intestine and *Eimeria crandallis* (Chartier & Paraud 2012).

Subclinical form of coccidiosis is difficult to diagnose and low rates of fatality are associated with this disease, however *Eimeria* spp. infection negatively impact ruminants mainly on decreasing growth and increasing susceptibility to diseases (Vieira et al. 2005). Diagnosis of eimeriosis infections should be based on epidemiological aspects, clinical signs and laboratory test results, including: light microscopy traditional fecal examination and molecular techniques, especially polymerase chain reaction (PCR) (Carvalho et al. 2011, Chartier & Paraud 2012).

Molecular assays for the diagnosis of *Eimeria* spp. infection are known to have greatly improved through more effective DNA extraction techniques and more sensitive PCR techniques for identifying protozoa (Kawahara et al. 2010, Carvalho et al. 2011, Malek & Kuraa 2018). Previous studies have revealed that one of the steps that can influence PCR results is the DNA extraction methods used (Zhao et al. 2001, Kaya et al. 2007, Tang et al. 2018).

DNA extraction process is essential for the success of the PCR technique, favoring a more sensitive and specific diagnosis. Thus, the present work aimed to compare different DNA extraction methods of *Eimeria* spp. oocysts from sheep and cattle.

MATERIALS AND METHODS

Samples and parasitological examination. Cattle and sheep feces were collected directly from the rectum using sterile gloves and placed in clean plastic bottles. The fecal samples were transferred under refrigeration to the laboratory and kept at 4°C until processing.

Eggs and oocysts per gram of feces (EPG) counting was performed using a modified McMaster technique, with a sensitivity of 50 EPG. Samples that had an oocysts count ≥500 were selected for both cattle and sheep.

***Eimeria* spp. oocysts examination.** Positive fecal samples (oocysts count≥500) were separated according animal species (cattle or sheep) and 10 pools of feces from each experimental group was formed. Therefore, 2 to 4g from a pool of feces were homogenized, dissolved in distilled water and filtered through sieves (60μm). Several washes were performed according Hoffman et al. (1934) and when the supernatant became clear, flotation centrifugation technique (Ueno & Gonçalves 1998) was used to separate and concentrate the oocysts. Finally, the recovered oocysts were stored in 2% potassium dichromate solution, quantified in Newbauer chamber and stored at 4°C until DNA extraction.

DNA extraction protocols. Each sample was twice washed with distilled water, centrifuged for 10 min at 14,000 × g to remove the potassium dichromate solution. The pellet with a number of approximately 5000 oocysts/pool was subsequently submitted to DNA extraction protocols.

Protocol 1 (commercial kit). Genomic DNA was extracted using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) according to manufacturer´s instructions. Briefly, 600μL of Nuclei Lysis Solution was added to the pellet of oocysts, homogenized, and incubated at 65°C for 30 min. After incubation, 3μL of RNase Solution was added, incubated for 30 min at 37°C, followed by addition of 200μL of Protein Precipitation Solution, vortexed, chilled on ice for 5 min, and centrifuged at 13000 × g for 4 min. The supernatant was transferred to a fresh tube containing 600μL of isopropanol, mixed and centrifuged at 13000 × g for 1 min. After centrifugation, the supernatant was removed, and 600μL of 70% ethanol was added to the pellet and centrifuged at the conditions described above. The ethanol was aspirated, the pellet was air-dried, and the DNA rehydrated in 50μL of DNA Rehydration Solution for 1 hour at 65°C.

Protocol 2 (commercial kit with modification). Genomic DNA was extracted using the commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) with a modification: after RNase addition, the lysis step was carried out at 55°C and kept overnight (16h) (adapted from Moré et al. 2011).

Protocol 3 (DNAzol reagent). Genomic DNA was extracted using a specific reagent (DNAzol Reagent, Invitrogen, USA) according to manufacturer´s instructions. Briefly, 1ml DNAzol was added to the pellet of oocysts followed by homogenization and centrifugation (5 min at 10,000 × g at 4°C). Each resulting viscous supernatant was

transferred to a new tube. Genomic DNA precipitation was carried out by adding 0.5ml of cold 100% ethanol, homogenized, incubated for 3 min at room temperature and centrifuged at the conditions described above. Finally, genomic DNA was washed with 1ml of cold 75% ethanol and centrifuged (same conditions described above), after centrifugation, supernatant was discarded and the resulting pellet suspended in 50µL of MilliQ water.

Protocol 4 (CTAB). The pellets of oocysts were incubated for 10 min with 100µL of lysis buffer (lysozyme 10mg/ml, SDS 10% and proteinase K 10mg/ml) at 37°C. This was followed by addition of 100µL cetyl-trimethyl ammonium bromide (CTAB) and 100µL of 5M NaCl into the solution and incubation at 65°C for 10 min. Finally, genomic DNA was isolated from the lysate by the phenol-chloroform method (Sambrook & Russel 2001), precipitated by cold ethanol, and solubilized in 50µL MilliQ water.

Protocol 5 (glass beads). To perform the protocol with glass beads, in to the oocysts pellet was added 100µL of STES buffer (0.2 M Tris-HCl, 0.1% SDS (w/v), EDTA 0.01M, pH 7.6), 50µL of 0.5mm glass beads and 100µL phenol-chloroform, vortexed for 1 minute. After that, the solution was centrifuged at 13000g for 5 min, the supernatant was collected and transferred to a new, sterile tube and precipitated by adding cold 2V (volume) absolute ethanol and 0.1V 5M NaCl. After 30 minutes, the solution was centrifuged again at 1200g for 10 min. The pellet was washed twice with 70% ethanol and after that, DNA was eluted in 50µL MilliQ water.

Protocol 6 (commercial kit for fecal samples). A specific kit for extraction of genomic DNA from fecal samples was also used according to manufacturer's instructions (PureLink® Genomic DNA Mini Kits). Basically, kit consists in specific digestion buffers, purification column for DNA, washing buffers and an elution buffer, resulting in a final volume of 50µL.

The concentration and quality of DNA extracted from each sample were analyzed using spectrophotometer NanoDrop 1000 (absorbance of 260/280nm ratio for purity evaluation) (Thermo Scientific, USA). After that, the DNA samples were stored at -20°C until use in PCR.

PCR and electrophoretic analysis. DNA extracted by the different protocols described above was submitted to the polymerase chain reaction (PCR) under the same conditions, using a set of primers (F: 5´-GCA AAA GTC GTA ACA CGG TTT CCG -3' and R:

5´-CTG CAA TTC ACA ATG CGT ATC GC-3') (Malek & Kuraa 2018) for amplification of a 348-546 bp fragment from the internal transcribed spacer 1 (ITS-1). Each PCR was performed in a total volume of 25µL, containing 10X buffer (Promega, USA); 10mM dNTPs (Ludwig Biotec, Brazil); 50µm of each primer (Sigma-Aldrich, Brazil); 1U Taq DNA polymerase (Promega, USA); and 50ng of DNA as template. DNA extracted from a pool of sporulated oocysts using the commercial kit was used as positive control and MilliQ water was used as negative control. The PCR was carried out using a T100 thermal cycler (BioRad, USA) under the following conditions: 30 seconds at 94°C for the initial hot denaturation step, followed by 35 cycles of 10 seconds at 94°C, 20 seconds at 55°C, 20 seconds at 72°C, and a final extension step of 2 min at 72°C. The PCR products were visualized by UV illumination after electrophoresis at 1% agarose gel stained with Gel Red Nucleic Acid Stain (Biotium, USA).

Statistical analysis. Software IBM SPSS Statistics 23 was used to calculate the kappa coefficient to estimate the concordance rate among the extraction protocols. The frequencies of positive and negative samples obtained using each DNA extraction protocol were compared by the Fisher's exact test at a 99% confidence level.

RESULTS

All the extraction protocols have resulted in DNA amplification by PCR (Table 1). CTAB (Protocol 4) showed better results with regard to DNA extraction from pools of oocysts (Table 1), with the *Eimeria* spp DNA detected from 90% (18/20) of the samples. This frequency was higher than the 70% (14/20) detection obtained using either the commercial kit for fecal samples (Protocol 6) or DNAzol (Protocol 3). Commercial kit (Protocol 1) showed a lower detection frequency (45, 9/20). Kappa coefficient was obtained using Protocol 4 as standard therefore, Protocols 1 (commercial kit), 3 (DNAzol) and 6 (commercial kit for fecal samples) demonstrated slight concordance (kappa 0.167 and 0.118) with Protocol 4 (CTAB). However, Protocol 5 (glass beads) showed moderate (kappa 0.48) and Protocol 2 (commercial kit with modification) fair concordance (kappa 0.318) with CTAB (Protocol 4).

In cattle fecal samples, Protocols 3 (DNAzol) and 4 (CTAB) showed better results (80%), 8/10 samples were positive in PCR when these protocols were used for DNA

extraction. Interestingly, in fecal samples from sheep, Protocols 2 (commercial kit with modification) and 4 (CTAB) resulted in 100% (10/10) of DNA amplification.

DISCUSSION

Coccidiosis causes economic losses in livestock as a result of decrease in feed efficiency which leads to slow weight gain and increased predisposition to other diseases (Malek & Kuraa 2018). Therefore, accurate diagnosis of coccidiosis is important to promote animal health and consequently to reduce losses. There is different diagnosis of *Eimeria* spp. using different methods with different specificity and sensitivity (Carvalho et al. 2011) and PCR which is known as a specific and sensitive method of diagnosis showed a high frequency of *Eimeria* spp. detection in the present study, reinforcing that coccidia are widely distributed across bovine and ovine.

Haug et al. (2007) observed that there is a variation in the detection of each *Eimeria* spp., depending on the number of oocysts used and the technique adopted for DNA extraction. However, Fernandez et al. (2003) identified *Eimeria* spp. in samples containing from two to eight oocysts proving that PCR correctly detect and discriminate species from small quantities of oocysts. Therefore, PCR is much sensitive compared to oocyst-counting using McMaster's method that can quantify as much as 100 oocysts in 1g of faeces (Kawahara et al. 2010).

In field samples, many factors may interfere in the success of diagnosis by PCR, especially the presence of contamination (Carvalho et al. 2011). According to Haug et al. (2007) the DNA extraction process in stool samples is influenced by the formation of inhibitors of Taq DNA polymerase that affect the reaction. We compared results obtained from six DNA extraction methodologies and found that CTAB was more efficient. Glass bead grinding is based on frequent physical contact between oocysts to break the wall, but the frequency of contact decreases with the small number of oocysts, resulting in less oocyst breakage (Tang et al. 2018). In addition, the amount of DNA from ruptured sporozoites that remains adhered to the glass beads and/or the container wall also reduces the total yield of DNA (Zhao et al. 2001, Tang et al. 2018). In contrast, Protocol 3 (DNAzol) and 4 (CTAB) are performed in microtubes resulting in minimal loss of DNA. In addition, since the commercial DNA kit can adsorb substances that could degrade DNA

and inhibit downstream enzymatic reactions, it also dramatically reduced PCR sensitivity (Kumar et al. 2014, Tang et al. 2018).

Eimeria's oocyst walls contain two or more shell layers (Scholtyseck et al. 1971, Hammond & Long 1973, Duszynski et al. 1981, Long 1982). The thick and elastic outer skeletal layer is made of a chitin-like material (Wilson & Fairbairn 1961) and contains quinone-tanned proteins that can be dissolved in hypochlorite (Hammond & Long 1973). Some DNA extraction protocols use sodium hypochlorite to promote oocysts lysis (Carvalho et al. 2011, Tang et al. 2018) and although none of the six protocols tested used hypochlorite, they showed to be efficient in oocysts lysis resulting in positive PCRs.

Results demonstrated that the PCR assay targeting the ITS-1 region of *Eimeria* spp. in cattle and sheep can be used for detection of this parasite. The ITS-1 regions are flexible corresponding with species variation, as compared with whole rRNA genes, showing a pattern of low intra-specific and high inter-specific variations in the DNA sequence (Kawahara et al. 2010). Accurate identification of *Eimeria* spp. is important not only for the diagnosis of disease and management of subclinical infections but also for epidemiological studies (Sun et al. 2009, Lee et al. 2010). PCR facilitates the detection of parasites including at subclinical levels, at concentrations that may be missed by routine microscopy (Tang et al. 2018) and in this study we tested six DNA extraction protocols in which oocysts were ruptured, DNA released and extracted in concentration and purity appropriately for PCR detection.

CONCLUSIONS

All the extraction protocols tested have promoted DNA releasing from *Eimeria* spp. oocysts. CTAB protocol showed to be better in DNA extraction from pools of *Eimeria* oocysts. Therefore, CTAB should be considered as DNA extraction method in molecular studies involving *Eimeria* oocysts from sheep and cattle.

Conflict of interest - The authors declare that they have no conflict of interest.

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TABLE 1 – PCR results from DNA extracted by six extraction protocols from cattle and sheep isolated oocysts

Sample	PCR results from the six DNA extraction protocols tested					
	*P1	P2	P3	P4	P5	P6
	Kit	Kit with modification	DNAzol	CTAB	Glass beads	Kit for fecal samples
C1**	+	+	+	+	+	+
C2	+	+	+	+	+	+
C3	+	+	+	+	+	+
C4	-	+	+	+	+	+
C5	-	+	+	+	-	+
C6	-	+	+	+	-	+
C7	-	+	+	+	-	-
C8	-	-	+	+	-	-
C9	-	-	-	-	-	-
C10	-	-	-	-	-	-
Total bovine samples	3/10	7/10	8/10	8/10	4/10	6/10
S1***	+	+	+	+	+	+
S2	+	+	+	+	+	+
S3	+	+	+	+	+	+
S4	+	+	+	+	+	+
S5	+	+	+	+	+	+
S6	+	+	+	+	+	+
S7	-	+	-	+	+	+
S8	-	+	-	+	+	+
S9	-	+	-	+	-	-
S10	-	+	-	+	-	-
Total sheep samples	6/10	10/10	6/10	10/10	8/10	8/10
Total fecal samples	9/20	17/20	14/20	18/20	12/20	14/20

*P: protocol; **C: pool of cattle fecal samples; ***S: pool of sheep fecal samples

5. CONCLUSÃO

Em um primeiro momento, buscou-se compreender quais as formas de utilização de tratamentos metafiláticos com toltrazuril 5% apresentam os melhores resultados tanto em ganho de peso quanto na diminuição da exceção de oocistos. Como conclusões do estudo realizado no capítulo 1, o toltrazuril 5% mostrou-se efetivo na redução de OoPG para infecções mistas de *Eimeria* spp. de bezerros, seja nas primeiras semanas de vida ou no momento do desmame. Além disso, apresentou também um considerável período de duração, chegando a diminuir significativamente a excreção de oocistos até aproximadamente os 75 dias de vida.

O momento da administração do toltrazuril é a chave para alcançar bons resultados a nível de rebanho. O tratamento durante o período pré-patente garante que os animais não sofrerão com doenças clínicas. No entanto a utilização deste tipo de tratamento deve ser avaliada conforme características inerentes ao tipo de manejo da propriedade além da presença altas taxas contaminação ambiental por espécies patogênicas de *Eimeria* spp.

Entretanto, não houveram diferenças significativas no ganho de peso dos animais durante o período experimental e este fato pode ser atribuído a uma baixa contaminação ambiental na propriedade e consequentemente um menor desafio. Sendo assim, sugerimos que outros estudos sejam realizados, como continuidade a este, especialmente em propriedades com maiores taxas de contaminação ambiental, casos clínicos recorrentes de eimeriose bovina e ocorrência de óbitos.

No segundo capitulo, buscou-se identificar uma técnica de ideal para extração de DNA de *Eimeria* spp. em amostras fecais de bovinos e ovinos, através da avaliação de seis diferentes protocolos para a extração de DNA.

Todos os protocolos de extração testados resultaram na amplificação do DNA pela PCR sendo que o CTAB (Protocolo 4) apresentou melhores resultados no que diz respeito à extração de DNA de pools de oocistos, sendo detectado o DNA de *Eimeria* spp. em 90% (18/20) das amostras.

A escolha da técnica de extração de DNA é uma etapa crucial para diagnóstico molecular, especialmente em protozoários, devido a estrutura resistente da parede dos oocistos. O diagnóstico molecular de espécies de *Eimeria* tem sido cada vez mais empregado tanto em pesquisa quanto em diagnóstico. Desta forma, os ensaios moleculares para o diagnóstico da infecção por protozoários podem ser

substancialmente melhorados por meio de técnicas mais eficazes de extração de DNA, resultando assim em técnicas de PCR com maior sensibilidade para identificação de *Eimeria* spp.

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