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Fernando de Souza Rodrigues

CONTROLE DA COCCIDIOSE EM OVINOS UTILIZANDO O TOLTRAZURIL

Santa Maria, RS
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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 título em **Doutor em Medicina Veterinária**

Orientador: Profª. Drª Sônia de Avila Botton

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RESUMO

CONTROLE DA COCCIDIOSE EM OVINOS UTILIZANDO O TOLTRAZURIL

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A coccidiose em pequenos ruminantes é uma infecção causada por protozoários do gênero *Eimeria.* Atualmente pelo menos onze espécies parasitam ovinos, sendo *E. ovinoidalis* e *E. crandallis* consideradas as mais patogênicas. A importância econômica da coccidiose nos rebanhos ovinos decorre principalmente da diminuição do ganho de peso relacionada à doença clínica e às infecções subclínicas, especialmente em criações intensivas e com alta densidade animal. O controle da coccidiose pode ser realizado com a utilização de fármacos que interrompam o ciclo do parasito e através de práticas sanitárias, ambas visando diminuir a contaminação ambiental. Atualmente, a coccidiose em ovinos é considerada uma doença negligenciada; a despeito das consideráveis perdas econômicas que causa à produção ovina. Diante do exposto acima, esta tese apresenta três capítulos, nos quais foram avaliados: (1) a eficácia, o custo/benefício e o ponto de equilíbrio econômico de dois diferentes regimes de tratamento utilizando o toltrazuril a 5% em cordeiros em lactação naturalmente expostos à reinfeção por *Eimeria* spp. em sistema de pasto; (2) o efeito do tratamento com toltrazuril a 5% quatro e duas semanas antes do parto na excreção de oocistos por ovelhas prenhas, bem como a influência na dinâmica de infecção pelo parasito nos seus cordeiros criados em sistema extensivo; e (3) a avaliação do efeito do tratamento com toltrazuril a 5% em cordeiros mantidos em condições naturais favoráveis ao desenvolvimento de coccidiose. Com base nos resultados obtidos, ressaltamos que o toltrazuril, na concentração avaliada, apresenta eficácia em cordeiros em lactação e mantidos em condições para o desenvolvimento de coccidiose. Utilizando o esquema de tratamento com intervalos a cada 14 dias os cordeiros permanecem protegidos da reinfecção por *Eimeria* spp. e uma dose única em cordeiros mantidos em condição de risco reduz a excreção de oocistos por até 35 dias. A utilização de toltrazuril a 5% quatro e duas semanas antes do parto em ovelhas reduziu significativamente a excreção de oocistos 21 dias antes do parto e 14 dias após o parto; entretanto, não influenciou na dinâmica de infecção dos cordeiros. *E. ovinoidalis*, *E. crandallis*, *E. parva* e *E. ahsata* foram as espécies mais frequentes causando principalmente infecção subclínica. Cordeiros em lactação e em condição de risco tratados com toltrazuril a 5% não apresentaram ganho de peso significativo em relação aos mantidos naturalmente infectados. O tratamento com toltrazuril na concentração testada apresenta alto custo quando os animais apresentam infecção subclínica, porém na ocorrência de casos clínicos e morte, a utilização deste medicamento pode ser economicamente viável. A viabilidade econômica do tratamento pode ser avaliada com a utilização do modelo econômico apresentado neste trabalho, modelo este que visa determinar o ponto de equilíbrio (*breakeven point)* a partir do qual o tratamento torna-se viável economicamente pela prevenção de perdas produtivas. Desta forma o controle da coccidiose em ovinos deve avaliar a situação específica de cada propriedade, bem como os riscos de desenvolvimento da doença e os potenciais prejuízos decorrentes dela e, assim, a necessidade de utilizar o toltrazuril.

Palavras-chave: Eimeriose. Coccidiose. Apicomplexa. *Eimeria ovinoidalis.* Ovino

ABSTRACT

CONTROL OF COCCIDIOSIS IN SHEEP USING TOLTRAZURIL

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Coccidiosis in small ruminants is an infection caused by protozoan of genus *Eimeria*. Currently, at least eleven species parasite sheep, being *E. ovinoidalis* and *E. crandallis* the most pathogenic. The economic importance of coccidiosis in sheep livestock, mainly in intensive system and with high stock density, is related with losses caused by clinical and subclinical infection, causing decrease of the weight gain. Coccidiosis control can be accomplished using treatment that interrupt the life cycle of the parasite and with sanitary practices, both aiming decrease the environment contamination. Currently, coccidiosis is considering a neglected disease, however coccidia infection cause economic losses to ovine production. In this context, this thesis presents three chapters, which evaluated: (1) the efficacy, cost-benefit ratio, and economic break-even point of two different toltrazuril treatment regimens for suckling lambs naturally exposed to *Eimeria* spp. re-infection in a grazing system; (2) evaluated the effect of treatment with toltrazuril 5%, four and two weeks before the parturition in pregnant ewes, as well as the influence of it in the dynamics of infection of lambs naturally infected by *Eimeria* spp. in an extensive breeding system; (3) and evaluated the effect of toltrazuril 5% in a single dose in lambs maintained in a natural condition favorable to the development coccidiosis. Based on results, we emphasize that toltrazuril at the concentration assessed, has efficacy in lactating lambs and kept in condition to developmente coccidiosis. Treatment with toltrazuril at 14-day intervals was effective in controlling re-infection of lambs and a single dose can reduce the excretion of oocysts in lambs kept in conditions favorable to development coccidiosis. The use of toltrazuril 5% four and two weeks before the parturition reduced significantly oocyst excretion 21 days before the parturition and 14 days postpartum but did not influence the dynamics of lamb infection. *E. ovinoidalis*, *E. crandallis*, *E. parva* and *E. ahsata* were the frequently species identified causing mainly subclinical infection. Lambs kept in conditions favorable to development coccidiosis and lactating lambs treated with toltrazuril did not have a significant weight gain ($p>0.05$) than animals maintained naturally infected. Treatment with toltrazuril at the concentration assessed may be costly when the animals have subclinical infection, but where there are clinical cases and death, the use of this medicine may be economically viable. Economic viability of the treatment can be evaluated with the use of the economic model that aims to determine the point where the treatment becomes economically feasible. To control coccidiosis in sheep, should evaluate the specific situation of each property, the risks of developing the disease and if is necessary to use toltrazuril.

Keywords: Eimeriosis. Coccidiosis. Apicomplexa. *Eimeria ovinoidalis*.Ovine

SUMÁRIO

1 INTRODUÇÃO

A coccidiose em pequenos ruminantes é uma infecção causada por protozoários do gênero *Eimeria*. Estes parasitos desenvolvem-se nos intestinos delgado e grosso dos hospedeiros, sendo que há várias espécies de *Eimeria* que infectam os ruminantes (bovinos, ovinos e caprinos), porém, sem haver relatos de infecção cruzada entre as espécies. Estes parasitos pertencem à família Eimeriidae, subordem Eimeriorina, ordem Eucoccidiorida, subclasse Coccidiasina, classe Conoidasida, filo Apicomplexa e Reino Protista (TENTER et al., 2002). Atualmente, pelo menos onze espécies parasitam ovinos, sendo *E. ovinoidalis* e *E. crandallis* consideradas as mais patogênicas (CHARTIER; PARAUD, 2012).

Em ovinos, após um período pré-patente de 12 a 20 dias, dependendo das espécies envolvidas e da sua respectiva patogenia, os animais excretam os oocistos através das fezes e, desta forma, contaminam o meio ambiente (ANDREWS, 2013). A importância econômica da coccidiose nos rebanhos ovinos, especialmente de criação intensiva e com alta densidade animal se deve às perdas relacionadas à doença clínica e às infecções subclínicas, principalmente ocasionando a diminuição do ganho de peso (CHARTIER; PARAUD, 2012; FOREYT, 1990).

A coccidiose ovina tem o seu maior impacto em cordeiros com menos de três meses de idade, causando lesões graves no intestino. Os sinais clínicos observados em pequenos ruminantes são: diarreia aquosa e profusa por 3 a 6 dias, que raramente pode ser sanguinolenta, inapetência, tenesmo, desidratação, perda de apetite e relutância em caminhar (ANDREWS, 2013; CHARTIER; PARAUD, 2012).

O diagnóstico da enfermidade deve ser baseado em: aspectos epidemiológicos, sinais clínicos, resultado da contagem de oocistos por grama de fezes (OoPG), achados patológicos e espécies de *Eimeria* envolvidas. Podem ser utilizados exames laboratoriais, como exame de fezes, histopatológico e reação em cadeia da polimerase (PCR) para confirmação diagnóstica (ANDREWS, 2013).

O controle da coccidiose pode ser realizado com a utilização de fármacos que interrompam o ciclo do parasito visando diminuir a carga parasitária e através de práticas sanitárias com diminuição da contaminação ambiental. Um dos fármacos que apresenta alta eficácia é o toltrazuril, onde recomenda-se tratar os ovinos e mudar para um locar limpo ou com baixa contaminação (JALILA et al., 1998; SARATSIS et al., 2011).

As estratégias de controle utilizando toltrazuril nas infecções por *Eimeria* spp. em ovinos mantidos em criação extensiva e os benefícios econômicos da sua utilização ainda

precisam ser esclarecidos. Diante do exposto acima, esta tese apresenta o compilado dos estudos realizados na forma de três capítulos, os quais visam apresentar diferentes regimes de tratamento para o controle da coccidiose em ovinos utilizando o toltrazuril a 5%, avaliar sua eficácia antiparasitária e seus efeitos produtivos e econômicos. Os estudos foram relacionados (1) ao controle da reinfecção e o custo benefício de dois regimes de tratamento; (2) o efeito do tratamento em ovelhas no terço final da gestação e a influência na dinâmica da infecção dos cordeiros; e, (3) a eficácia em cordeiros em condições de adquirir a infecção e desenvolver a doença.

A tese está composta por uma revisão de literatura sobre a coccidiose em ovinos abordando os principais aspectos de epidemiologia, imunologia, diagnóstico e controle. Na sequência são apresentados três artigos científicos com os objetivos de i. avaliar a eficácia, custo benefício e o ponto de equilíbrio econômico de dois diferentes regimes de tratamento utilizando toltrazuril a 5% em cordeiros lactentes naturalmente expostos a reinfeção por *Eimeria* spp. em sistema de pasto; ii. verificar o efeito do tratamento com toltrazuril 5% quatro e duas semanas antes do parto em ovelhas prenhas, bem como a influência na dinâmica de infeção em cordeiros em sistema extensivo naturalmente expostos à infecção por *Eimeria* spp.; e, iii. avaliar o efeito do tratamento com toltrazuril a 5% em cordeiros em condições naturais favoráveis ao desenvolvimento de coccidiose. A discussão e conclusões encontram-se no final desta tese, e apresentam comentários gerais sobre os estudos realizados.

2 REVISÃO DE LITERATURA

2.1 CICLO DE VIDA DE *Eimeria* spp.

O ciclo de vida de *Eimeria* spp. é divido em três etapas: merogonia ou esquizogonia (reprodução assexuada), gametogonia (reprodução sexuada) e esporogonia (esporulação). O animal se infecta quando ingere o oocisto esporulado junto ao alimento ou água. No intestino, os esporozoítos penetram nas células da mucosa intestinal e passam a ser denominados trofozoítos, que começam a sofrer divisão binária, formando merozoítos que darão origem ao esquizonte. À medida que o esquizonte se torna maior, a célula intestinal rompe-se, liberando os merozoítos que penetram em células adjacentes. Cada merozoíto se diferencia em macrogametócito ou microgametócito, que após a fusão completam a gametogonia, dando origem ao zigoto ou oocisto não esporulado. A fase de merogonia normalmente ocorre no final do duodeno e jejuno, e a gametogonia pode ocorrer no final do íleo, ceco e cólon; dependendo da espécie envolvida. A fase de esquizogonia e gametogonia pode durar de 12-20 dias (GREGORY et al., 1989; JOLEY; BARDSLEY, 2006).

No meio ambiente, em condições ideais de temperatura, umidade e oxigênio, o oocisto torna-se infectante, através da esporulação, contendo em seu interior quatro esporocistos com dois esporozoítos cada. Esse processo pode variar de 48 horas até sete dias dependendo da espécie de *Eimeria* (CHARTIER; PARAUD, 2012).

2.2 EPIDEMIOLOGIA E RESPOSTA IMUNE À INFECÇÃO POR *Eimeria* spp. EM OVINOS

A coccidiose em ruminantes tem distribuição mundial e acomete ovinos submetidos aos diferentes sistemas de criação. No entanto, os animais mantidos nos sistemas intensivos apresentam maiores chances de infecção pelo protozoário. No Brasil, estudos indicam que o parasito está amplamente difundido em todas as regiões onde foi pesquisado (AMARANTE; BARBOSA, 1992; BRESCIANI et al., 2002; REBOUÇAS et al., 1997; RODRIGUES et al., 2016; SILVA et al., 2007).

Os oocistos não esporulados são mais susceptíveis às alterações climáticas extremas do que os esporulados. Desta forma, os oocistos esporulados são capazes de resistir às temperaturas de -5ºC a -9ºC durante vários meses (FOREYT, 1986).

Após a ingestão de oocistos esporulados, durante os primeiros dias de vida, os cordeiros podem iniciar a excreção de oocistos entre a 2ª a 3ª semana de idade. O número de animais que excretam oocistos e a intensidade da excreção no ambiente têm um aumento progressivo até a 6ª semana de vida e pode permanecer elevado até o período do desmame. Posteriormente, a intensidade de excreção diminui; entretanto, em animais de maior idade os oocistos são eliminados continuamente, em níveis mais baixos, não havendo a presença de imunidade etária (CHARTIER; PARAUD, 2012).

Pelo menos onze espécies podem parasitar ovinos; todavia, *E. ovinoidalis* e *E. crandallis* são consideradas as mais patogênicas e responsáveis por doença clínica. O período pré-patente da infecção por *E. ovinoidalis* situa-se entre 12 a 15 dias (ANDREWS, 2013). A coccidiose clínica é uma doença autolimitante e está geralmente relacionada à ingestão de elevada quantidade de oocistos esporulados e à multiplicação assexuada exacerbada no hospedeiro, associadas a uma baixa resistência do animal. Estas situações podem coexistir em condições naturais (CHARTIER; PARAUD, 2012).

Algumas condições de habitação ou de pastoreio dos animais, incluindo superlotação e a presença de áreas úmidas, predispõem a contaminação massiva do ambiente e aumento da taxa de translação do parasito ocasionando uma alta infecção do hospedeiro (CAI; BAI, 2009; JALILA et al., 1998). Além disso, todas as causas de estresse, tais como: frio ou calor, mudança alimentar brusca, subnutrição, desmame, doenças concomitantes e transporte são fatores que afetam a resposta imunológica dos animais e favorecem o surgimento de casos de coccidiose (LIMA, 2004).

O contato do hospedeiro com estágios infectantes do parasito, determina o desenvolvimento de uma resposta imunológica espécie-específica (ROSE, 1987; 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). A imunidade específica para as principais espécies patogênicas de *Eimeria* ocorre de maneira precoce, enquanto que para as espécies menos patogênicas é estabelecida tardiamente (REEG et al., 2005).

A resposta imune de ovinos infectados por *Eimeria* spp. não foi totalmente caracterizada, sendo que a maioria das informações disponíveis para coccídeos são descritas para bovinos. Durante uma infecção primária por *Eimeria* spp. em ruminantes, geralmente ocorre um aumento de linfócitos T $CD4^+$ e $CD8^+$ (HERMOSILLA et al., 1999; SUHWOLD et al., 2010). No período pré-patente as respostas imunológicas celulares são do tipo Th1, sendo caracterizadas por produção de IFN-γ (TAUBERT et al., 2008). Em bovinos, nas infecções por

E. bovis há um aumento de fenótipos celulares, expressando os marcadores CD4+, CD8+ e CD2+ e há uma reatividade prolongada da população de linfócitos T específica para o estímulo antigênico (HERMOSILLA et al., 1999; HUW et al., 1989).

Embora estas populações de células T ativadas não sejam capazes de interromper o ciclo de vida do parasito em infecções primárias, a resposta de células T pode interferir com o nível e a duração da excreção de oocistos, bem como pode estar relacionada ao controle imunológico de novas infecções (HERMOSILLA et al., 1999). Todavia, o papel preciso das populações de células T com diferentes fenótipos ainda não foi estabelecido; entretanto, Hermosilla et al. (1999) sugeriram que as células T CD4+ podem participar da resolução de uma infecção primária.

O nível sanguíneo de fator de necrose tumoral alfa (TNF-α) é reduzido durante a coccidiose, sendo considerado um fator a favor do parasito, uma vez que esta citocina pode ter efeito sobre a reprodução do parasito (HEATH et al., 1997). Os níveis de anticorpos (IgG) em cordeiros variam fortemente durante os primeiros 100 dias de vida. Alguns animais podem não apresentar anticorpos e outros podem apresentar altos títulos. Pondera-se que, em geral, os animais apresentam níveis elevados por volta de sete dias de vida, diminuem os valores até 40 dias e aumentam novamente até os 80 dias de vida (REEG et al., 2005).

2.3 DIAGNÓSTICO, TRATAMENTO E CONTROLE DE *Eimeria* spp. EM OVINOS

O diagnóstico das infecções por *Eimeria* spp. deve ser baseado nos aspectos epidemiológicos, sinais clínicos, resultado da contagem de oocistos por grama de fezes (OoPG), nos achados patológicos e nas espécies de *Eimeria* envolvidas. Para tanto, há necessidade de utilizarem-se os exames laboratoriais, incluindo: exame de fezes, histopatológico, técnicas de biologia molecular para a confirmação diagnóstica (ANDREWS, 2013). Oocistos são facilmente encontrados nas fezes com microscopia de luz, preferencialmente, após a concentração utilizando as técnicas de flutuação convencional (DAUGSCHIES; NAJDROWSKI, 2005).

Os métodos sorológicos empregando as técnicas de *Enzyme-Linked Immunosorbent Assay* (ELISA) e *Western blot* foram desenvolvidos para a detecção da infecção de *E. bovis* em bezerros. No entanto, estas metodologias apresentam vários entraves, destacando-se a reatividade com anticorpos adquiridos via colostro e a reação cruzada entre as espécies. Apesar de não serem os métodos mais adequados para a rotina de diagnóstico, os métodos sorológicos

são úteis para os estudos experimentais (FABER et al., 2002; FIEGE et al., 1992). Outras ferramentas para a diferenciação das espécies de *Eimeria*, incluem os métodos moleculares; entretanto, em ovinos há a necessidade de padronização destes testes. Desta forma, os ensaios moleculares ainda não estão disponíveis para fins de rotina laboratorial para a detecção da coccidiose ovina.

O controle da coccidiose está diretamente relacionado às informações concernentes às características e à evolução da infecção (CHARTIER; PARAUD, 2012). O controle pode ser realizado utilizando medidas de higiene e com utilização de anticoccidianos. Um grande número de fármacos tem sido recomendado para o tratamento da coccidiose em ruminantes, que podem ser os coccidiostáticos, os quais impedem o desenvolvimento do parasito, ou os coccidicidas que eliminam os parasitos. Estas drogas agem 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 controle da infecção por *Eimeria* spp. podem ser utilizados de forma metafilática, isto é, a aplicação do princípio ativo durante o período pré – patente da infecção ou quando os animais estão em risco; de forma terapêutica, com aplicação quando os animais estão excretando oocistos e apresentando os sinais clínicos da infecção (EPE et al., 2005); e o tratamento preventivo com uso de fármacos de forma continua a fim de evitar o estabelecimento das infecções (TAYLOR, 2000).

Entre os fármacos mais empregados para o controle de *Eimeria* spp. incluem-se as sulfonamidas (sulfanilamidas ou sulfas), os compostos ionóforos e os derivados do benzeno acetonitrila. Estes medicamentos podem ser utilizados de forma metafilática, terapêutica ou profilática (ALZIEU et al., 1999; DAUGSCHIES; NAJDROWSKI, 2005; LE SUER et al., 2009; TAYLOR et al., 2011).

As sulfonamidas constituem fármacos extensivamente utilizados para o tratamento de infecções por diversos micro-organismos e são classificadas como antimetabólitos, que são os fármacos que antagonizam um metabólito essencial ao organismo vivo. A estrutura química das sulfonamidas é muito semelhante à do ácido para-aminobenzóico (PABA). Os micro-organismos necessitam de PABA extracelular para a formação do ácido diidrofólico, um constituinte essencial na produção de purinas e na síntese de ácidos nucléicos. Desta forma, as sulfonamidas inibem competitivamente a diidropteroatosintetase e, consequentemente, inibirão o crescimento do micro-organismo ao bloquear reversivelmente a síntese de ácido fólico (CONNOR, 1998).

As sulfonamidas primariamente atuam na fase de reprodução assexuada de *Eimeria* spp., é empregado principalmente como medicamentos de ação terapêutica e são consideradas coccidiostáticos, pois não apresentam atividade suficiente contra gamontes (DAUGSCHIES; NAJDROWSKI, 2005; MUNDT et al., 2005). Gutierrez-Blanco et al. (2006) testaram a eficácia de sulfametazina em formulação *bolus* intra-ruminal, onde os animais tratados mostraram uma maior tendência ao ganho de peso acumulativo.

Os compostos ionóforos são utilizados principalmente como profiláticos e os principais princípios ativos deste grupo incluem amprólio, lasalocida sódica, salinomicina e monensina sódica. Os compostos ionóforos atuam nos trofozoítos e na primeira geração de merontes, o que impede a diferenciação em merozoítos, por isso, são considerados coccidiostáticos. A sua principal atuação está relacionada ao transporte de elétrons e no metabolismo mitocondrial, por consequência, isto resultará na diminuição na eliminação de oocistos (ASIM; ALI, 2008).

No final do tratamento com estes compostos, poderá haver interferência positiva no ganho de peso dos animais (STROMBERG et al., 1986; WAGGONER et al., 1994; YOUNG et al., 2011). Vieira et al. (2004) observaram que o tratamento preventivo de caprinos leiteiros com salinomicina é eficaz na fase de cria e recria, promovendo ganho de peso significativo.

Decoquinato é um coccidiostático que atua na fase assexuada do parasito principalmente no esporozoíto. O mecanismo de ação se dá através do bloqueio do transporte de elétrons no sistema citocromo da mitocôndria. É indicado como preventivo da coccidiose (PLUMB, 2011). Andrade Junior et al. (2012) verificaram a eficácia do tratamento com decoquinato em ovinos, sem observação de casos clínicos, com redução na quantidade de oocistos liberados, porém, sem diferença significativa no ganho de peso.

Formulações a base de benzeno acetonitrila são compostos que atuam contra todos os estágios endógenos do parasito e são utilizados particularmente como metafiláticos, mas servem também como medicamentos terapêuticos. Os princípios ativos deste grupo incluem o toltrazuril e o diclazuril, os quais apresentam alta eficácia contra eimerídeos (DAUGSCHIES; NAJDROWSKI, 2005). Ovinos e caprinos tratados com estes fármacos comumente apresentam maior ganho de peso (ALZIEU et al., 1999; DIAFERIA et al., 2013; LE SUER et al., 2009; RUIZ et al., 2012). O toltrazuril apresenta resultados superiores ao diclazuril devido a uma meia-vida mais prolongada e com uma ação terapêutica mais eficaz (SARATSIS et al., 2013).

A prevenção da coccidiose pode ser realizada através do emprego de práticas sanitárias visando diminuir a contaminação ambiental. Medidas incluindo a manutenção dos animais em locais limpos, secos e separados de acordo com a idade devem ser preconizadas. Estas práticas sanitárias apresentam uma boa eficácia e devem ser empregadas, sobretudo, em animais

mantidos em sistemas de criação intensivos e semi-intensivos (JALILA et al., 1998; SARATSIS et al., 2011).

Em relação ao controle e profilaxia utilizando tratamento, é recomendado que se estabeleça um tratamento metafilático, utilizando uma única dose de toltrazuril a 5% (20 mg/kg) no período pré–patente da coccidiose, desde que estes animais sejam, em seguida, realocados em áreas não contaminadas com oocistos. Este tipo de tratamento visa reduzir a contaminação ambiental por oocistos liberados nas fezes dos cordeiros e evitar que os animais se reinfectem (DIAFERIA et al., 2013).

O controle eficiente da coccidiose abrange, além do tratamento metafilático e profilático, o conhecimento das relações parasito-hospedeiro, destacando-se especialmente os fatores de patogenicidade do agente e os imunológicos relacionados ao hospedeiro (CHARTIER; PARAUD, 2012).

3 ARTIGO 1 - EFFICACY AND ECONOMIC ANALYSIS OF TWO TREATMENT REGIMENS USING TOLTRAZURIL IN LAMBS NATURALLY INFECTED WITH *Eimeria* spp. ON PASTURE

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Efficacy and economic analysis of two treatment regimens using toltrazuril in lambs naturally infected with *Eimeria* spp. on pasture

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Abstract

This study evaluated the efficacy and the economic viability of two anticoccidial treatment regimens tested in lambs naturally exposed to *Eimeria* spp. re-infections in a grazing system during a 140-day period. Twenty-four suckling lambs were distributed into three groups based on the individual count of oocysts per gram of feces (OPG) and body weight. Animals were treated with toltrazuril 5% (20 mg/kg) at 14 (GI) or 21-day (GII) intervals, and GIII was kept as untreated control. A cost-benefit analysis of each treatment regimen was calculated. Additionally, economic analysis was performed on four hypothetical scenarios, in which lambs could be having 10%, 25%, 50%, or 85% decrease in their expected body weight gain due to clinical eimeriosis. Efficacy of toltrazuril against *Eimeria* spp. was 96.9-99.9% (GI) and 74.2-99.9% (GII). *E. ovinoidalis* was most frequently identified, but no clinical signs of eimeriosis were observed in lambs. There were no differences in weight gain among the groups. The cost of treatment per lamb was $13.09 (GI) and $7.83 (GII). The estimation model showed that the cost-benefit ratio favored treatment with toltrazuril when lambs fail to gain weight. In the studied flock, the break-even point for toltrazuril administered at 14-day intervals was reached with 85% decrease in mean weight gain. In conclusion, toltrazuril can be used at 14-day intervals to control *Eimeria* spp. (re)-infection in lambs raised on pasture. This treatment regimen was not economically feasible for subclinical eimeriosis; however, it may be feasible when used to prevent weight loss caused by clinical eimeriosis.

Keywords eimeriosis, coccidiosis, sheep, anticoccidial, toltrazuril, Coccidia

Introduction

Eimeria spp. are coccidian protozoa that infect domestic and wild animals (Tenter et al. 2002). Although sheep can harbor at least eleven *Eimeria* species, *Eimeria ovinoidalis* and *Eimeria crandallis* are considered the most pathogenic (Gregory et al. 1989; Andrews 2013). Eimeriosis is generally subclinical in sheep, but chronic infection can decrease growth and weight gain (Diaferia et al. 2013). Clinical signs can be observed, especially in

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young and weaned lambs raised in intensive or semi-intensive systems (Chartier and Paraud 2012). Clinical disease is generally the result of multiple conditions, including decreased host immunity, high parasite load, and presence of pathogenic *Eimeria* species. Clinical eimeriosis in lambs can result in severe diarrhea, weight loss, and death (Chartier and Paraud 2012).

Epidemiological knowledge is essential to achieve effective *Eimeria* spp. control, using anticoccidial drugs together with hygiene and prophylactic measures. Thus, both the life cycle of the protozoa and the pharmacology of the anticoccidial drugs should be considered when planning management and treatment regimens. For example, the life cycle of *E. ovinoidalis* includes a prepatent period of 12-15 days; the first generation meronts appear in the ileum 10 days post-infection (dpi), and the second generation meronts reach the cecal crypts 12 dpi; gamonts further develop in the cecal crypts 15 dpi. Finally, oocysts discharged in host feces become infective after sporulation within 2-3 days in the environment, and may cause re-infection (Gregory and Catchpole 1987; Andrews 2013).

Toltrazuril is a triazone compound that acts against all intracellular stages of *Eimeria* spp. and is highly effective as metaphylactic treatment for ruminants under feedlot conditions (Le Suer et al. 2009). However, protocols using toltrazuril to control re-infection with *Eimeria* spp. in lambs raised on contaminated pasture have not been well established. Therefore, this study aimed to evaluate the efficacy, cost-benefit ratio, and economic break-even point of two different toltrazuril treatment regimens for suckling lambs naturally exposed to *Eimeria* spp. re-infection in a grazing system.

Material and Methods

All experimental practices involving animals were approved by the Ethics Committee for Animal Experimentation at Universidade Federal de Santa Maria (UFSM) (approval number 8088190815).

Farm and sheep flock

This study was carried out on a sheep farm located in the Central region of the Rio Grande do Sul State (29°41’14.88’’S – 54°26’34.06’’O), in a subtropical area in Southern Brazil. The average climate conditions during the experimental period (expressed as mean and confidence intervals [CI] or standard deviations) were: average rainfall 6.59 ± 12.41 mm; average temperature 19.74 ± 3.16 °C; and relative humidity 76.67 (CI: 68.52–84.82%) (Fig. 1). Texel x Ile de France crossbred ewes, and their suckling lambs, were kept in a natural pasture intercropped with *Panicum maximum* and *Pennisetum glaucum*. Ewes and lambs were grazed during daylight (10 to 11 hours) and were housed at night in a stable with rice straw bedding.

Previous anthelmintic treatment used in the flock consisted of a two drugs combination: ivermectin 1% (0.2 mg kg^{-1}, subcutaneously) plus levamisole 5% (5 mg kg^{-1}, orally). Sheep were selectively treated based on the FAMACHA© score (van Wyk and Bath 2002) assessed at weekly intervals. Additionally, all ewes were strategically treated during the last trimester of pregnancy and one month after parturition. Ewes and lambs did not receive any anticoccidial treatment prior to this study.

Experimental design

Two treatment regimens were designed considering the following epidemiological and pharmacological factors: (1) the *Eimeria* spp. life-cycle, using the 12-15 days prepatent period of *E. ovinoidalis* (Andrews 2013); (2) environmental conditions, since lambs could not be moved from oocyst contaminated pasture after treatment; and (3) toltrazuril (Baycox® 5% Bayer technical information, 2008) pharmacological features including the time to reach the peak plasma concentration (48 h) and half-life (9 days). The two treatment regimens were: toltrazuril 5% (20 mg/kg, orally) every 14 days (12 days of the parasite prepatent period [ppp] plus 48 h to reach the peak plasma concentration) or every 21 days (12 days of the ppp plus 9 days of drug half-life).

Twenty-four suckling lambs, approximately 25 days of age and of both genders, were distributed into three randomized blocks (n = 8), based on oocyst count (oocysts per gram of feces, OPG) and live weight. Each block was randomly assigned as a group. Group I (GI) was treated with toltrazuril at 14-day intervals; group II (GII) was treated with toltrazuril at 21-day intervals; and group III (GIII) was the untreated control. Lambs were individually numbered by painting on the wool. All groups were treated with monepantel (Zolvix® Novartis), before and during the experimental period, to avoid influence of spoliation by gastrointestinal nematodes. Lambs were weighed every 21 days, using a 100g digital scale.

Starting seven days prior (D -7) of the treatment until 98 days after (D98) the onset of anticoccidial treatment (D0), individual fecal samples were collected weekly and examined for OPG and eggs per gram of feces (EPG). An additional fecal collection on day -3 was used to obtaining OPG counts for randomized blocks distribution. The last toltrazuril treatment occurred at D84. A final fecal collection was performed on D140, when the lambs reached slaughter weight, which was 14 days after the required 42-day drug withdrawal period (Fig. 2).

Parasitological analysis

Fecal consistency was judged for each lamb at each collection according to a fecal score (FS) adapted by Le Suer et al. (2009): 0 (normal pellets), 1 (mild diarrhea), 2 (moderate diarrhea), 3 (severe diarrhea), and 4 (severe diarrhea with blood and/or tissues present). Each fecal sample was evaluated by the McMaster technique with a sensitivity of 50 OPG and 50 EPG. Briefly, 2 g of feces were mixed and diluted with 28 mL of saturated solution of sugar, re-suspended, sifted, and transferred to a McMaster chamber for microscopic identification of oocysts and eggs.

Eimeria species were identified from each experimental group at each sampling date. Positive samples were homogenized, dissolved in water, and sieved through a 60-µm steel mesh filter. Several washes were performed until the supernatant became transparent (Hoffman et al. 1934). After sedimentation, the liquid was removed and potassium dichromate ($K_2Cr_2O_7$) solution 2.0% (w/v) was added in equal proportion. The suspension was maintained in Erlenmeyer flasks at room temperature with forced aeration using an air pump. To recover oocysts and identify the species under microscopy, a sample from each preparation was processed by the flotation technique in a modified concentrated sugar solution (Ueno and Gonçalves 1998). *Eimeria* species identification included morphometric measures of the length and width of both oocysts and sporocysts, and wall thickness of oocysts. Morphological characteristics, including shape, color, and presence or absence of micropyle on the surface of oocysts, was noted (Levine 1970; Chartier and Paraud 2012). *E. crandallis* and *Eimeria weybridgensis* oocysts could not be differentiated due to their morphological similarity (O'Callaghan et al. 1987).

Statistical analysis

Efficacy of each treatment regimen was calculated using OPG reduction percentage at 95% confidence interval. Fecal oocysts count reduction test (FOCRT), comparing mean OPG of each treatment group with mean OPG of the untreated control group (unpaired samples) on each collection day, was used (available at http://www.math.uzh.ch/as/index.php?id=eggCounts). A hierarchical Bayesian method, described for the fecal egg count reduction test (FECRT; Torgerson et al. 2014), was adapted for the FOCRT estimation. A treatment could be classified as effective, when percentage reduction (PR%) ≥ 95% and lower 95% confidence limit ≥ 90%; ineffective, when PR% <95% and lower 95% confidence limit <90%; or inconclusive (when none of the criteria were fulfilled), e.g. PR <95% and lower 95% confidence limit ≥90%.

Pre-treatment and post-treatment EPG counts were used to calculate the efficacy of monepantel treatment based on the percentage reduction (PR%) in EPG, using the method of Torgerson et al. (2014). The same criteria described above were used to evaluate the efficacy of the treatments with monepantel.

The OPG was not normally distributed (Kolmogorov-Smirnov test, $p < 0.05$, data not shown). Therefore, the non-parametric Friedman test was used for comparison of the mean OPG among the groups at each time point. In addition, the mean OPG of the same group over time (paired samples) were compared using the non-parametric Dunn's Kruskal-Wallis multiple comparisons test. Weight gain was normally distributed (Kolmogorov-Smirnov test, $p>0.05$), and was compared by ANOVA and the post hoc Tukey test. The frequency of diarrhea/non-diarrhea (assessed by fecal scores) in each group was compared using Fisher's exact test. These statistical tests were performed with a 95% confidence interval and 5% significance level. The Kolmogorov-Smirnov and Fisher's exact tests were performed using R (R Core Team 2016) language. The ANOVA, post hoc Tukey, Dunn's Kruskal-Wallis, and Friedman tests were performed using SAS® software (SAS Institute 2011).

Economic analysis Cost-benefit ratio of each treatment regimen was calculated. The cost of treatment included the cost of the drug plus the cost of employee time to treat the lambs. The benefit of treatment was calculated as the additional weight gain when compared to the untreated control group. A model was developed to determine the break-even point when treatment with toltrazuril 5% (at 14- or 21-day intervals) was economically feasible for a particular sheep flock. Using this model, both the cost of the treatment and the potential revenue provided by the treatment can be estimated and compared for individual flock conditions. This can be used as a tool to guide the decision to treat or not to treat a flock. The model was constructed as:

(1) Cost of the drug per animal (*Cda*):

Where:

0.29 is the volume of toltrazuril 5% (mL kg^{-1}) used;

Pk is the sum of the expected mean weight of an animal in the flock at each treatment, since the weight of the animals will increase during the treatment period. Thus, *Pk* is the mean weight of the animal at each day *k* of the treatment, with: $k \in \{1,2,3,4,5,6,7\}$, considering seven treatments at 14-day intervals; and *price* is the price of each 1 mL of toltrazuril 5%.

(2) Cost of the work (*Cw*) to perform the treatments: $Cw = t.ph.nt$

The cost of the work was calculated considering the time (*t)* spent to treat each animal, the hourly wage of the worker (*ph*), and the number of treatments needed (*nt*). The time spent to treat each animal was estimated as 15 min (0.25 hour).

Thus:

(2.1) $Cw = 0.25.ph.nt$

Therefore, the total cost of all the treatments (*Ct*) per animal was calculated as:

(3) $Ct = Cda + Cw$

In addition, this model was used to evaluate four hypothetical scenarios, in which untreated lambs could be having a 10%, 25%, 50%, or 85% decrease in weight gain due to clinical eimeriosis during a period of 140 days. Regarding these hypothetical scenarios, the break-even point, where treatment with toltrazuril 5% could become economically feasible in the flock studied, was estimated for treatment at 14- or 21-day intervals. For this purpose, decrease in weight gain (kg) was established as a fixed effect in four different degrees representing: 10%, 25%, 50%, or 85% decrease in the expected weight gain of the lambs. Expected weight gain was calculated as a mean of the weight gain of the untreated control lambs during the present study. The price of the live lambs sold to slaughterhouse (US$ per kg) was included as a variable. Thus, the break-even point from which the benefit (revenue) would be greater than the cost of treatment was determined using the price of the live lamb in the Brazilian market (US$ 1.30 per kg; following Emater quotation, 2015), and estimated for a range of prices from US$ 1.00 up to $5.00 per kg (Figure 5). The cost of the treatment and the kg price of the live lamb were converted from Brazilian monetary units (R$) to US dollar (US$) at a rate of R$ 3.85 to US$ 1.00.

Results

Efficacy of the treatments Efficacy of toltrazuril against *Eimeria* spp. (Table 1) ranged from 96.9 to 99.9% (GI) and from 74.2 to 99.9% (GII) between days 7 and 98 (two weeks after the last treatment).

Parasitological analysis As shown in Figure 3A, the prevalence of lambs excreting *Eimeria* spp. oocysts decreased to very low levels during the treatment period, but it increased levels again at 14 days after the last treatment. The prevalence of positive OPG in GII was reduced to zero at D35, but it was above 20% during most of the treatment period. Prevalence of OPG positive lambs remained at high levels in GIII (untreated control). Figure 3B describes the means of OPG excreted by the lambs during the treatments period (days 7 to 98). The mean OPG in GI was 22.8 (0-500); GII 186.5 (0-7600); and GIII 9887.9 (0-530,000).

E. ovinoidalis was the most frequently identified species. However, *E. crandallis/E. weybridgensis*, and other species (*E. ahsata*, *E. parva*, *E. intricata*, *E. faurei*, and *E. bakuensis*) were also identified (Fig. 4). Lambs presented with mixed infections of two to seven *Eimeria* species during the experimental period.

Climate conditions were favorable for the development of both *Eimeria* spp. and gastrointestinal nematodes (Fig. 1). Monepantel treatment was effective to avoid gastrointestinal nematodes spoliation in all experimental groups, with EPG reduction of ≥ 95%.

Clinical examination and weight gain No adverse drug reactions were observed in any treated lamb. Despite the high frequency of pathogenic *E. ovinoidalis*, no clinical signs of eimeriosis were observed. Fecal scores were all normal in the GI and GII. Four episodes of abnormal fecal consistency were observed in untreated lambs (GIII) during the experimental period (FS = 1 at three times, and FS = 2 at one time). Anyway, no significant differences were found comparing FS frequencies among the groups ($p > 0.05$). Therefore, subclinical eimeriosis was characterized in untreated lambs, while treated lambs were free of eimeriosis (GI) or episodically infected (GII).

In addition, despite the efficacy of the treatment, no significant differences were found comparing the mean weight gain of the lambs among the groups ($p > 0.05$) (Fig. 3C).

Economic analysis Total cost of treatment for GI was $104.81 and for GII was $62.65. The total and individual costs of the treatments with toltrazuril 5% in lambs are shown in Table 2. Therefore, despite its efficacy, treatment with toltrazuril at 14- or 21-day intervals was not economically feasible in the presence of subclinical eimeriosis in lambs in this study. However, our estimation model (Fig. 5) showed that the cost-benefit ratio of toltrazuril treatment could be positive in the presence of decreased weight gain due to clinical eimeriosis. Thus, the break-even point, where treatment is economically feasible, can be calculated for each scenario, showing the point from where the benefits (prevention of decreased weight gain) overcome the cost (drug plus work).

In the present study, the farmer was paid $1.30 per live lamb kg. The mean weight gain of the control group during the experimental period was of 12.08 kg, and the cost of treatment per animal was $13.09 for GI and $7.83 for GII (Table 2). Thus, in the flock studied, treatment at 14- or 21-day intervals could be economically feasible if the lambs were losing 85% or 50% of their expected mean weight gain, respectively. However, it is important to highlight that treatments at 21-day intervals were not completely effective to avoid re-infection of the lambs by *Eimeria* spp. Thus, despite its higher cost, efficacy analysis indicates that treatments at 14-day intervals should be recommended.

Discussion

In this study, toltrazuril 5% was effective against multiple species of *Eimeria* infecting lambs raised on pasture. This drug has been recommended for metaphylactic treatment in lambs raised in feedlot conditions (Gjerde and Helle 1991; Le Suer et al. 2009; Mundt et al. 2009; Saratsis et al. 2013; Scala et al. 2014), however, this approach is not feasible in grazing systems. The present study shows that toltrazuril 5% when given at 14-day intervals can be used to control re-infection with *Eimeria* spp. in suckling lambs naturally exposed to oocysts in a semi-intensive grazing system. Additionally, lambs were not fully protected against re-infection when toltrazuril was used at 21-day intervals. Therefore, *Eimeria* spp. completed their life cycle in less than 21 days in the studied conditions. Similarly, Le Suer et al. (2009) observed that housed lambs treated with toltrazuril 5% excreted oocysts 14 days after treatment, even they continued to excrete only low levels of oocysts up to 28 days after treatment.

The spectrum of action of toltrazuril covers all the stages of *Eimeria* spp. present into the host cells at the time of the treatment (Haberkorn and Stoltefuss 1987; Gjerde and Helle 1991). However, extracellular stages of the parasite present in the host gut can be not susceptible to toltrazuril (Jonsson et al. 2011). Thus, as shown in GI lambs, parasites that escape the first treatment can be destroyed 14-days later. Anyway, a lack of protection during at least four weeks before the slaughter of the lambs is obligatory, since the withdrawal period for toltrazuril covers 42 days, and re-infections by *Eimeria* spp. can appear since 14-days after the treatment.

Flock management characteristics, as semi-intensive or intensive raising systems, and high stocking rate, can predispose to massive environmental contamination by the oocysts. Housing can provide an environment that increases the viability and favors the rapid sporulation of the oocysts to become infective (Berriatua et al. 1994; Cai and Bai 2009). In the present study, untreated lambs had a high prevalence of positive OPG and higher levels

of OPG excretion (Fig. 3A and 3B). Otherwise, treated animals had negligible excretion of oocysts. Thus, the treatment regimens evaluated can decrease environmental oocysts contamination.

In the context of a preventive program to control eimeriosis, reduction of the environmental oocysts burden can minimize the risk of severe infection (Gjerde and Helle 1991; Le Suer et al. 2009). After the required slaughter withhold period of 42 days, lambs from all groups were excreting similar levels of OPG ($p > 0.05$) (Fig. 3B). Despite the selection pressure exerted by frequent treatment of lambs with toltrazuril, the presence of untreated ewes co-habiting in pasture can be useful to keep *Eimeria* spp. parasites in refugia, avoiding rapid development of parasite resistance.

As observed in this study, mixed infections with *E. ovinoidalis* and *E. crandallis/weybridgensis* are commonly reported in sheep (Vercruysse 1982; Platzer et al. 2005; Le Suer et al. 2009; Saratsis et al. 2011). The predominance of these *Eimeria* species could be related to their high reproductive efficiency, when compared to other sheep coccidian parasites (Catchpole et al. 1976). As described for *E. crandallis* (Gregory and Catchpole 1990), sheep eimeriosis can cause soft feces from 8 to 11 days post-infection and may cause diarrhea on the 15th day post-infection. However, most untreated lambs (GIII) had normal feces, excepting four lambs that had soft feces (FS = 1, three episodes and FS = 2, one episode). Thus, no evidence of clinical eimeriosis was observed, even in untreated lambs continuously excreting *Eimeria* spp. oocysts. As expected, lambs from both treated groups (GI and GII) had feces of normal consistency (FS = 0) during the experimental period.

No significant difference was found in weight gain among groups (Fig. 3C). This was not surprising since untreated lambs (GIII) had subclinical infection. No differences in weight gain were identified in other studies using toltrazuril on housed lambs or dairy sheep under intensive management systems (Mundt et al. 2009; Saratsis et al. 2013). However, Scala et al. (2014) observed that control of eimeriosis with toltrazuril resulted in increased weight gain in weaned lambs in a dairy husbandry system in Italy. Thus, based on the high costs of treatment, toltrazuril 5% is not economically feasible in sheep flocks with subclinical eimeriosis. However, this drug could be recommended to prevent production losses caused by clinical eimeriosis in lambs. Consequently, the economic analysis proposed in the present study may be useful to estimate the cost–benefit ratio, and to guide treatment decisions for each particular flock.

Although subclinical eimeriosis is most common, the risk of clinical surges in the presence of pathogenic *Eimeria* species cannot be ignored. Diarrhea, weight loss, and mortality can result from infection with pathogenic *Eimeria* species combined to decreased immune response or increased re-infection rate (Chartier and Paraud 2012). When individual clinical cases are observed, farmers can consider the use of toltrazuril 5% at 14-day intervals to prevent the emergence of clinical eimeriosis in the flock and also to decrease mortality of lambs.

Despite the efficacy of toltrazuril 5% against *Eimeria* spp. infection, the cost of the drug makes treatment economically feasible under a scenario of high production losses caused by clinical eimeriosis. As shown in Figure 5, treatment with toltrazuril at 14-day intervals could be recommended if an 85% decrease in mean weight gain is observed. It depends on the lamb meat price in each particular market and the cost of drug and labor in performing the treatments. Furthermore, in the presence of mortality caused by clinical eimeriosis, treatment becomes economically feasible. For instance, considering the price of the lambs at the slaughter point, in the present study economic loss caused by the death of one lamb would be enough to pay the treatment of 2.5 lambs of the flock at 14-day intervals during a 140-day period.

The economic model presented in this study is useful in evaluating the economic viability of treatment for individual flocks. Each farmer should consider factors such as: the risk of clinical eimeriosis, lamb mortality, costs of the treatment, expected mean weight gain, and the value of lamb in the market.

Conclusions

Toltrazuril 5% showed high efficacy against mixed *Eimeria* spp. infection in lambs on pasture. Treatment with toltrazuril at 14-day intervals was effective in controlling re-infection of lambs, raised in naturally oocysts contaminated pasture. However, the high cost of the treatment limits its use for subclinically infected lambs. Thus, the cost of treatment and the value of the lamb can influence the treatment cost-benefit ratio. Treatment can become a cost effective option when used to prevent weight loss and mortality caused by clinical eimeriosis in lambs.

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

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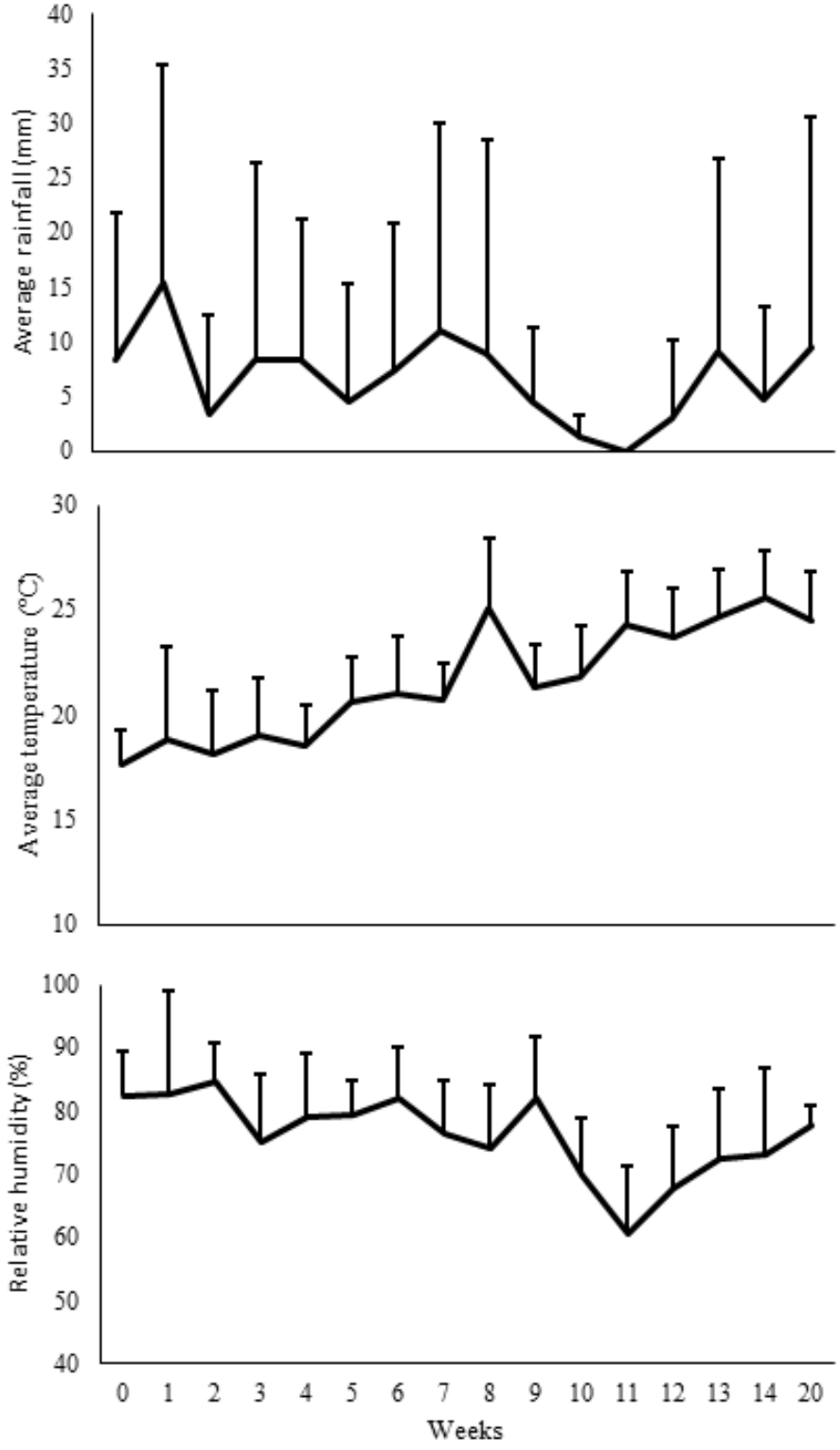

Fig. 1 Average climate conditions (rainfall, temperature and relative humidity) during the experimental period in the central region of Rio Grande do Sul

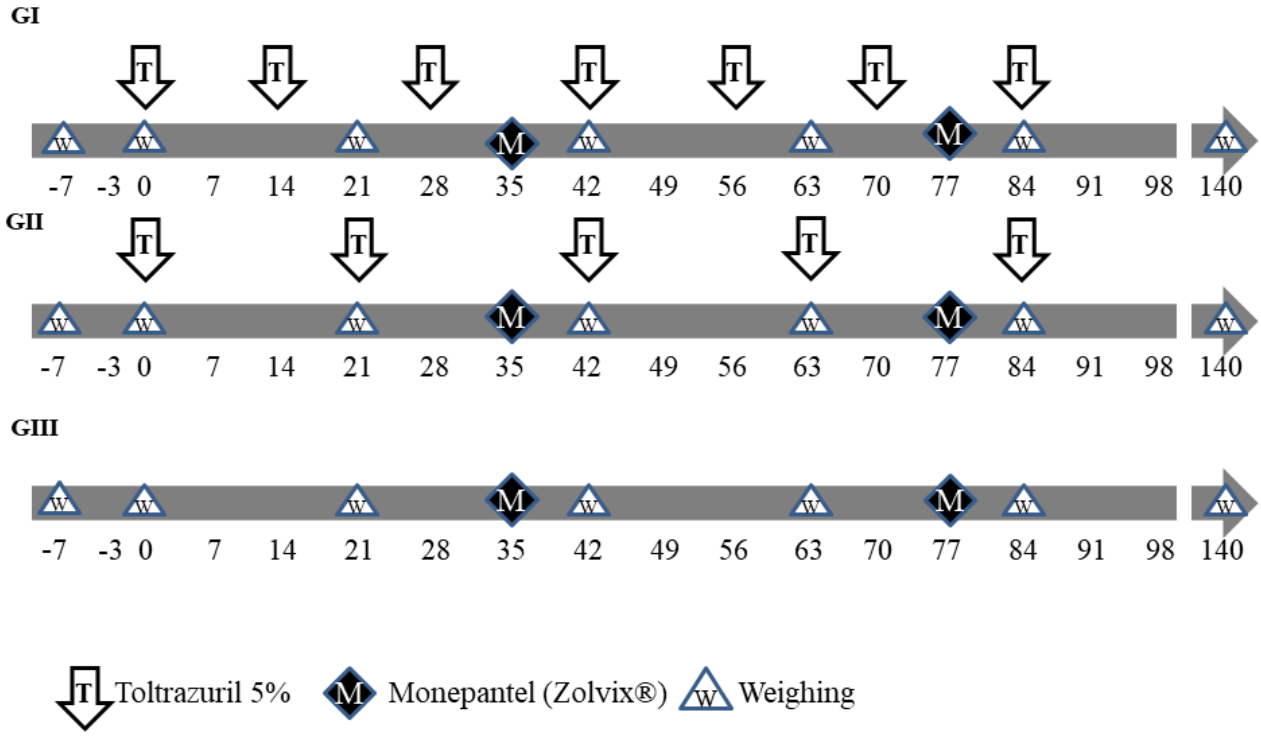

Fig. 2 Schematic of treatments and weighing in lambs during the experimental period. Group I - Treated every 14-day. Group II- Treated every 21-day. Group III – Control group. The numbers represent the days of individual fecal samples collection

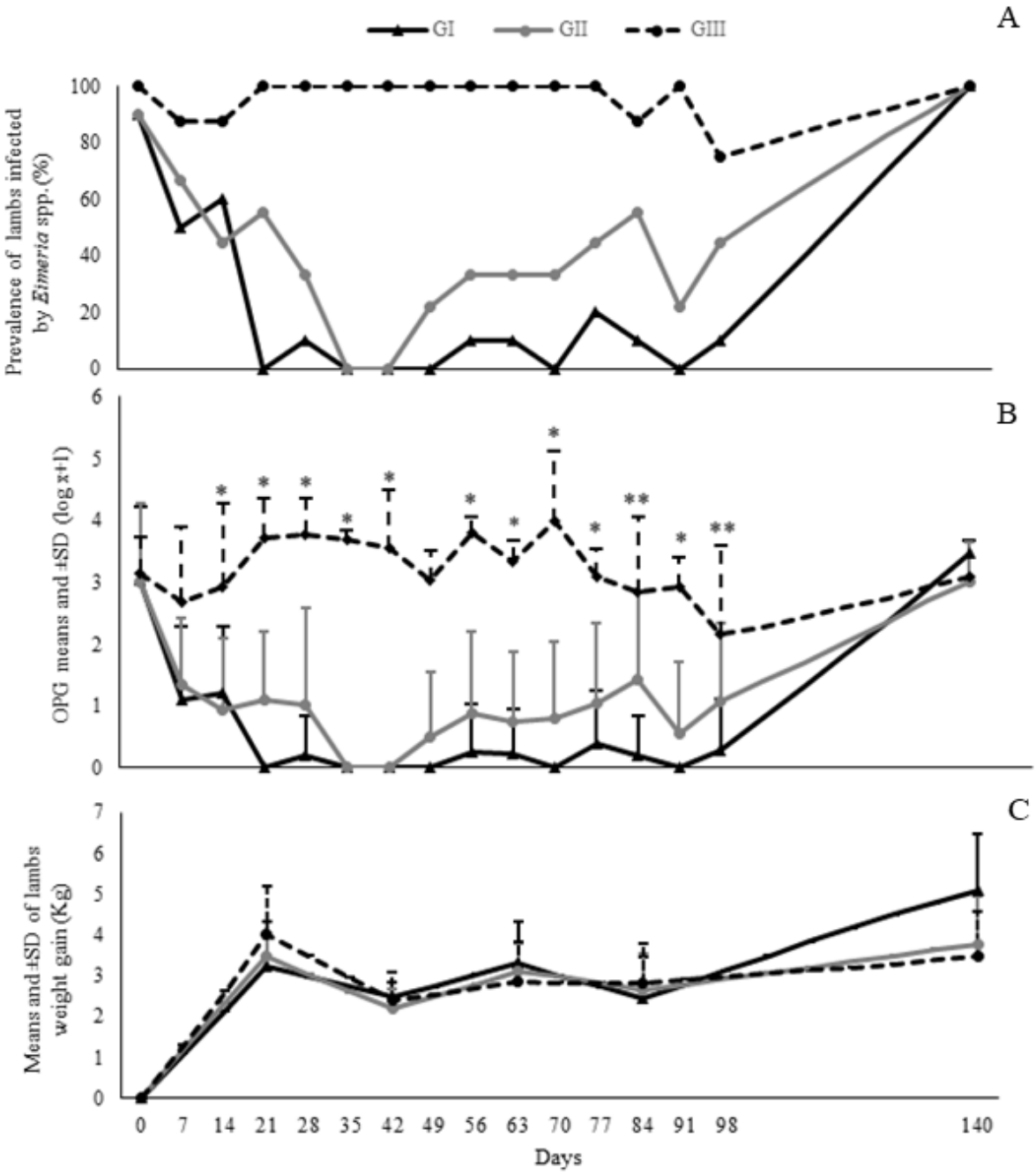

Fig. 3 A - Prevalence of lambs infected by *Eimeria* spp. during the experiment in treated group with toltrazuril in different periods (GI and GII) and control group (GIII). B - Means of OPG excreted by the lambs during the experiment in treated group with toltrazuril in different periods (GI and GII) and control group (GIII). C - Mean of weight gain in lambs during the experimental period. GI- Treated every 14-day. GII- Treated every 21-day. GIII – Control group. * Significant difference among GI and GII as compared to GIII ($p < 0.05$). ** Significant different ($p<0,05$) between GI and GIII

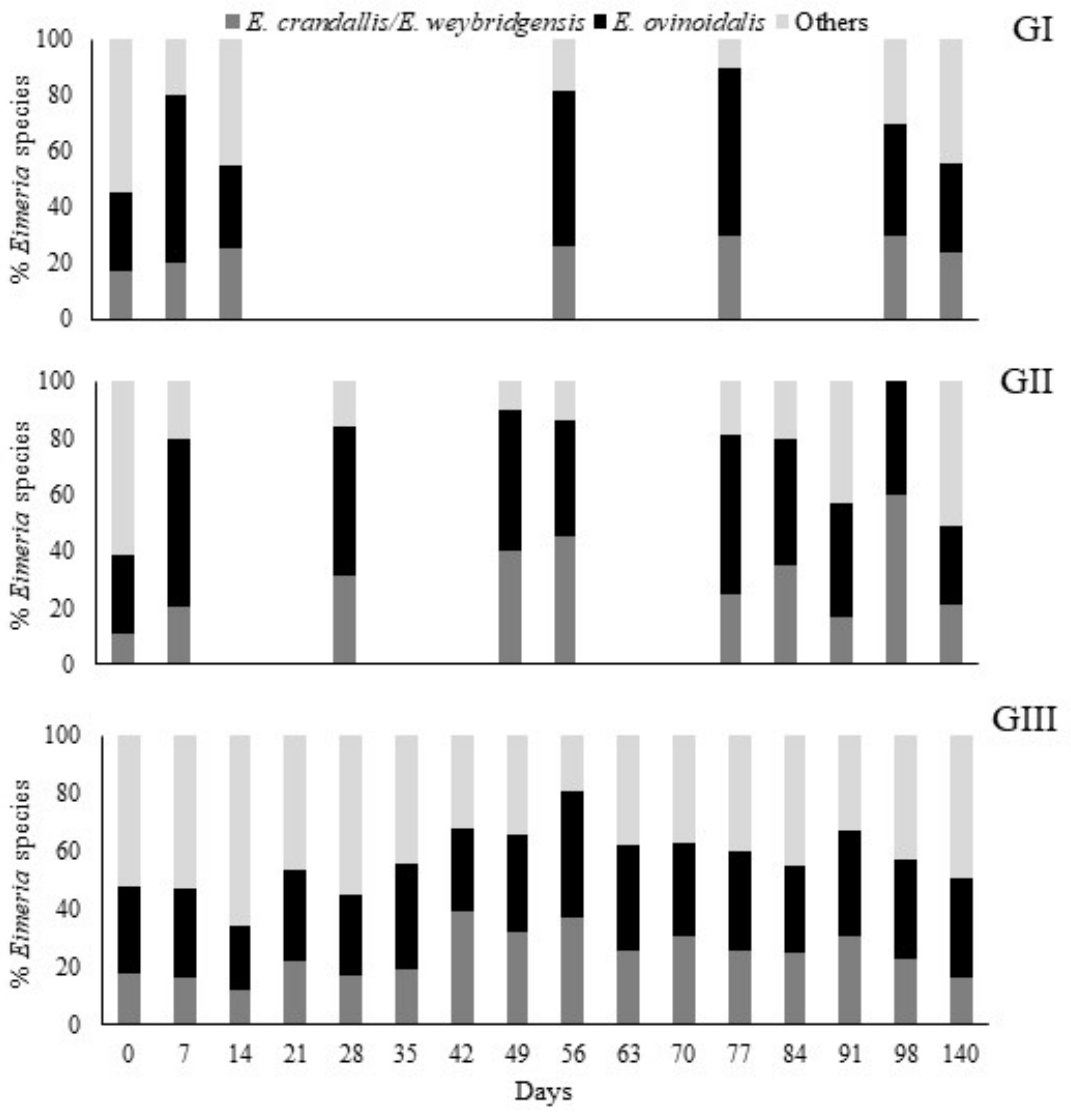

Fig. 4 Frequency (%) of species of *Eimeria* in lambs naturally infected in different regimens of treatment with 5 % toltrazuril and control group. A -Group I- Treated every 14-day. B- Group II- Treated every 21- day. C – Group III – control group

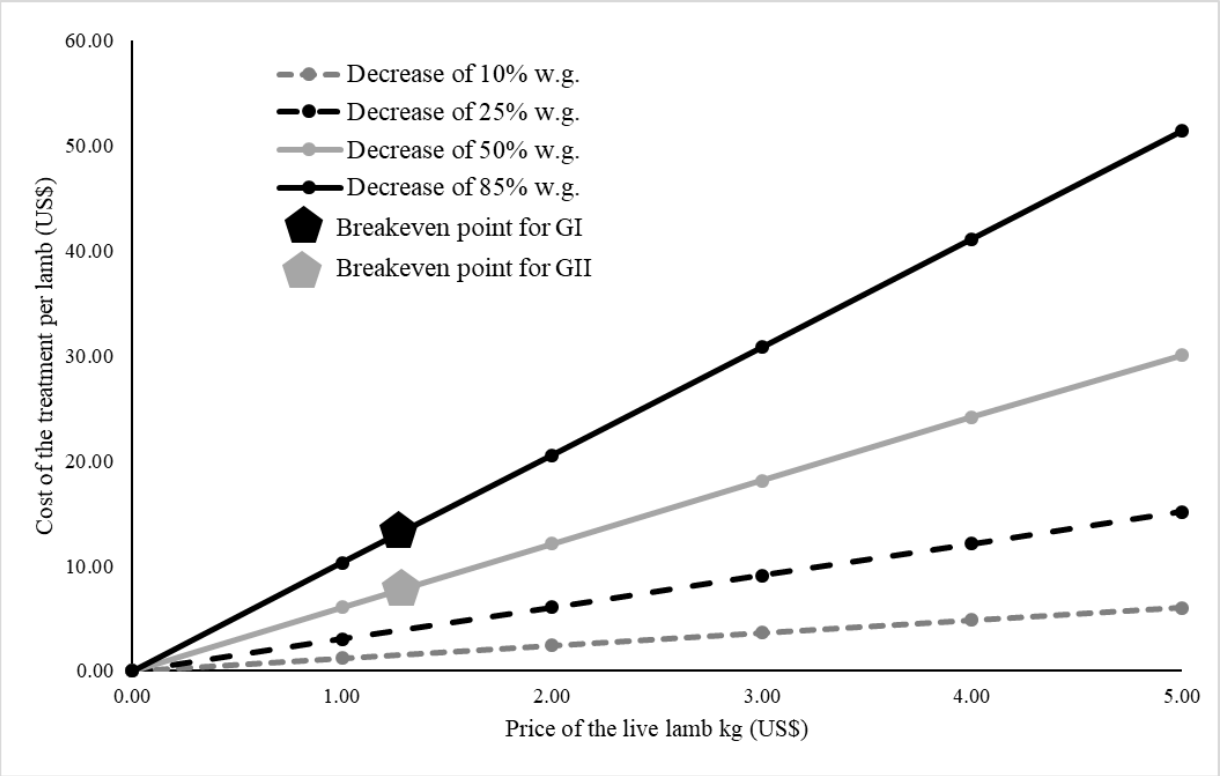

Fig. 5 Costs/benefits balance of the treatments with toltrazuril 5% in lambs under four hypothetical scenarios of production losses caused by clinical eimeriosis: 10%, 25%, 50%, or 85% decrease on the mean expected weight gain (w.g) of the lambs. Remuneration (benefits) calculated (y-axis) resulting from prevention of 10%, 25%, 50%, or 85% decreases on the w.g. were calculated for ranging values of live lamb kg prices (up to US$ 5.00) in the x-axis. The diamonds indicate the breakeven points from which the costs of the treatments at 14-day (black diamond) or 21-day (gray diamond) intervals become economically lower than the mean production losses. Where: the costs of the treatments were of US$ 13.10 (14-day intervals) or US$ 7,83 (21-day intervals) per animal; the mean expected weight gain was of 0.604 kg per lamb/week; and the price paid for the live lamb kg was of US$ 1.30

Table 1 Efficacies of two treatment regimens against *Eimeria* spp. in naturally infected lambs raised on pasture. The first treatment with toltrazuril 5% was given at day 0 and lambs were treated at 14-day (GI) and at 21-day (GII) intervals until the last treatment given at day 84 for both groups

Days	Treatments with toltrazuril		Efficacy of toltrazuril against *Eimeria* spp. in lambs	
	GI	GII	GI	GII
7	-	-	96.9 (95.6 – 98)	96.8 (95.2 – 97.9)
14	Second	-	97.3 (95.8 – 98.3)	97.6 (96.3 – 98.5)
21	-	Second	99.9 (99.5 – 100)	97.8 (96.5 – 98.8)
28	Third	-	99.9 (99.5 – 100)	74.2 (70.1 – 77.9)
35	-	-	99.9 (99.5 – 100)	99.9 (99.5 – 100)
42	Fourth	Third	99.9 (99.5 – 100)	99.9 (99.5 – 100)
49	-	-	99.9 (99.5 – 100)	98.4 (97 – 99.2)
56	Fifth	-	98.9 (97.5 – 99.6)	94.6 (92.7 – 96.2)
63	-	Fourth	99.2 (98.2 – 99.7)	98.1 (96.6 – 98.9)
70	Sixth	-	99.9 (99.5 – 100)	94.6 (92.7 – 96.3)
77	-	-	99.3 (98.4 – 99.8)	93 (90.1 – 94.7)
84	Seventh (last)	Fifth (last)	99.6 (98.9 – 99.9)	90.9 (88.1 – 93.2)
91	-	-	99.9 (99.5 – 100)	96.4 (94.7 – 97.7)
98	-	-	98.4 (97.3 – 99.2)	96.4 (94.7 – 97.7)

Table 2 Total and individual costs of the treatments with toltrazuril 5% in lambs, including the cost of the drug and the cost of the work to perform the treatments at 14- (GI) or 21-day (GII) intervals

Costs of the treatments [a, b]	Groups [c]	
	I	II
Total cost of the drug per group [d]	81.57	46.05
Total cost of the drug per animal	10.19	5.75
Total cost of work per group	23.24	16.6
Total cost of work per animal	2.90	2.08
Total cost of the treatment per animal	13.09	7.83
Total cost of treatment for the group	104.81	62.65

[a] Cost of toltrazuril 5% = U$ 0.38 per ml

[b] The mean time spent at each treatment of each animal was of 15 min., and the hourly wage of the worker was of $1.66 per hour of work of a Brazilian agricultural worker

[c] The number of treatments per animal was seven in the GI and five in the GII.

[d] Total volume of toltrazuril used during all the experimental period were of 314.05 ml (GI) and 177.31 ml (GII)

4 ARTIGO 2 – EFFECTIVE REDUCTION OF *Eimeria* spp. OOCYSTS EXCRETION IN THE PERIPARTUM PERIOD BY EWES TREATED WITH TOLTRAZURIL 5% BEFORE THE PARTURITION

Artigo submetido para Parasitology Research.

Effective reduction of *Eimeria* spp. oocysts excretion in the peripartum period by ewes treated with toltrazuril 5% before the parturition

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Abstract This study was performed to evaluate the effect of the treatment of pregnant ewes with toltrazuril 5% in the dynamic of *Eimeria* spp. oocysts excretion by the ewes and their offspring. The animals were kept under natural exposure of oocysts in an extensive breeding system. Twenty-three pregnant ewes infected by *Eimeria* spp. were distributed in two groups: GI. 11 untreated ewes; GII. 12 ewes treated with toltrazuril 5% (20 mg/kg) in two drenches at four and two weeks before the parturition. Twenty-six lambs born from these ewes formed other two experimental groups: GIII. 11 lambs born from the GI ewes; GIV. 15 lambs born from the GII ewes. Feces were weekly examined during the last trimester of gestation and up to nine weeks after parturition in sheep and from fourteen days up to nine weeks of age in the lambs. Efficacy of toltrazuril ranged from 26.2 to 91.8% in pregnant ewes. The OPG of ewes ranged from 0-1000 (GI) and 0-600 (GII) during the experiment. Lambs started oocyst shedding at 21 days of age and the OPG ranged from 0-18,900 (GIII) and 0-171,200 (GIV). *E. ovinoidalis* was the most frequent species identified in the flock. In conclusion, the treatment with toltrazuril 5% in pregnant ewes significantly reduced the excretion of *Eimeria* spp. oocysts in the peripartum period and had no influence in the dynamics of infection by the lambs exposed to *Eimeria* spp. (re)infection at pasture.

Keywords coccidiosis, eimeriosis, *E. ovinoidalis*, *E. crandallis*, anticoccidial

Introduction

Coccidiosis caused by *Eimeria* species is an important and prevalent disease in sheep (Fitzgerald 1980; Dittmar et al. 2010). Production losses can be due to clinical disease (diarrhea) or subclinical infection resulting in low weight gain. These losses negatively impact the different production systems (Chartier and Paraud 2012). Sheep can be affected by at least eleven *Eimeria* species, being *E. ovinoidalis* and *E. crandallis* considered the most pathogenic (Gregory et al. 1989; Andrews 2013). Infection with *Eimeria* spp. is generally subclinical in sheep

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however, in susceptible animals diarrhea, dehydration, weight loss, and death can result from heavy infection by pathogenic *Eimeria* spp. (Taylor and Catchpole 1994). Young sheep are particularly susceptible to coccidiosis (Taylor and Catchpole 1994). Suckling lambs can be infected due to the presence of the environmental oocysts. After infection, the excretion of oocysts occurs at 2–3 weeks of age, peaking at weaning (Chartier and Paraud 2012). Adult sheep do not excrete a large number of oocysts, but shed *Eimeria* spp. oocysts in low levels, contributing to environment contamination (Carrau et al. 2016).

The periparturient period is a critical regarding the health and production of the ewes (Raoofi et al. 2013). A periparturient rise in parasite shedding has been identified worldwide and is recognized in ewes infected with *Eimeria* spp. (Carrau et al. 2016). Control of coccidiosis can be achieved with hygienic measures and an understanding of the parasites life cycle, including pre-patent period and time to *Eimeria* spp oocyst sporulation (Chartier and Paraud 2012). Under certain conditions, for example in grazing systems where the hygienic measures may not be feasible, the use of anticoccidial drugs can aid in the control of infection (Rodrigues et al. 2017).

Toltrazuril is a triazone compound that acts against all intracellular stages of *Eimeria* spp. and is a highly effective treatment for lambs raised in feedlot and grazing system (Le Suer et al. 2009; Mundt et al. 2009; Scala et al. 2014; Rodrigues et al. 2016). However, the effect of toltrazuril treatment on pregnant ewes and the influence of this approach on the infection of their lambs have not been investigated. The aim of this study was to evaluate the effect of the treatment of pregnant ewes with toltrazuril 5% in the dynamic of *Eimeria* spp. oocysts excretion by the ewes and their offspring kept under natural exposure to environmental oocysts in an extensive breeding system.

Material and Methods

All experimental practices involving animals were approved by the Ethics Committee for Animal Experimentation at Universidade Federal de Santa Maria (UFSM).

Farm and sheep flock This study was carried out on a sheep farm located in the central region of the Rio Grande do Sul State (29°49'53.64" S – 53°42'46.72" O), in a subtropical area in Southern Brazil. The average climate conditions during the experimental period (expressed as mean and confidence intervals [CI] or standard deviations [SD]) were: average monthly rainfall 224.25 ± 126.68 mm; average temperature 17.63 ± 1.91 °C; and relative humidity 80.51 (CI: 73.2–88.4%) (Fig. 1). Corriedale ewes and their suckling lambs were kept in a natural pasture intercropped with oats *(Avena sativa)* and ryegrass *(Lolium* spp.*)*. Ewes and lambs grazed during the day (10 to 12 hours) and were housed at night in a stable with a soil floor and no bedding. Previous anthelmintic treatment was moxidectin (1%, 1 mg kg^{-1}, subcutaneously). During the experimental period, sheep were selectively treated based on the FAMACHA© score (van Wyk and Bath 2002) assessed at weekly intervals. Additionally, all ewes were strategically treated during the last trimester of pregnancy and one month after parturition. Ewes and lambs had not received anticoccidial treatment prior to this study.

Experimental design The ewes were kept with ram for natural reproduction, and the reproduction season was three months (February to April, 2015). Pregnancy status and estimated gestational stage were assessed by

ultrasonography, one month after the end of the reproduction season (June, 2015). A total of 23 pregnant ewes were selected for this study.

During the last trimester (corresponding to 35 days prior to the first parturition), fecal samples were collected weekly for oocysts per gram of feces count (OPG). According to OPG, the ewes were distributed into two groups: group 1 (GI; control group) included 11 pregnant ewes naturally infected with *Eimeria* spp. and group 2 (GII; treated group) included 12 pregnant ewes naturally infected by *Eimeria* spp. and treated with toltrazuril 5% (Baycox®; 20 mg/kg, orally). Ewes in the GII received two drenches, based on the predicted day of parturition, four and two weeks before the parturition. Fecal samples were collected from the rectum of the ewes weekly up to 63 days postpartum (D63).

Thirty lambs were born from the 23 ewes (GI and GII). Three ewes in GI and four in GII had twins. During the study, three lambs from GI and one from GII died, and the cause of death was not determined. Therefore, twenty-six lambs were evaluated. Lambs were identified after birth by numbered ear tags and distributed into two groups based on treatment of the ewe. Group 3 (GIII) had 11 lambs from GI dams (not treated with toltrazuril) and group 4 (GIV) had 15 lambs from GII dams (treated with toltrazuril 5%). Fecal samples were collected weekly after lambing from day 14 (D14) to D63. All the fecal samples were examined for OPG and eggs per gram (EPG) counts.

Parasitological analyses Fecal consistency was judged for each lamb at each collection according to a fecal score (FS) adapted by Le Suer et al. (2009): 0 (normal pellets), 1 (mild diarrhea), 2 (moderate diarrhea), 3 (severe diarrhea), and 4 (severe diarrhea with blood and/or tissues present). Each fecal sample was evaluated by a McMaster technique with a sensitivity of 50 OPG and 50 EPG. Briefly, 2 g of feces was mixed and diluted with 28 mL of saturated sugar solution, re-suspended, sifted, and transferred to a McMaster chamber for microscopic identification of oocysts and eggs.

Identification of species was possible when there were more than 200 OPG. *Eimeria* species were identified from each experimental group at each sampling period. Positive samples were homogenized, dissolved in water, sieved through a 60-μm steel mesh filter, and washed until the supernatant become transparent (Hoffman et al. 1934). After sedimentation, the liquid was removed and potassium dichromate ($K_2Cr_2O_7$) solution 2.0% (w/v) was added in equal proportion. The suspension was maintained in Erlenmeyer flasks at room temperature with forced aeration using an air pump. To recover oocysts and identify the species under microscopy, a sample from each preparation was processed by the flotation technique in a modified concentrated sugar solution (Ueno and Gonçalves 1998). *Eimeria* species identification included morphometric measures of the length and width of both oocysts and sporocysts, as well as the wall thickness of oocysts. Morphological characteristics evaluated included: shape, color, and presence or absence of micropyle on the surface of oocysts (Levine 1970; Chartier and Paraud 2012). *E. crandallis* and *E. weybridgensis* oocysts could not be differentiated due to their morphological similarity (O'Callaghan et al. 1987).

Statistical analysis Efficacy of treatment with toltrazuril was determined using percent OPG reduction at 95% CI. The fecal oocysts count reduction test (FOCRT), was used to compare mean OPG of treated and untreated groups (unpaired samples) on each collection day. A hierarchical Bayesian method, described for the fecal egg count

reduction test (FECRT; Torgerson et al. 2014), was adapted for the FOCRT estimation, included random sampling errors, degree of aggregation between individuals within each group, and the 95% CI.

The OPG was not normally distributed (Kolmogorov-Smirnov test, $p < 0.05$, data not shown). Therefore, the non-parametric Mann Whitney U test was used for comparison of the mean OPG between the groups. In addition, the mean OPG of the same group over time (paired samples) were compared using the non-parametric Wilcoxon signed sum test. The frequency of diarrhea/non-diarrhea (assessed by fecal scores) in each group was compared using Fisher's exact test. These statistical tests were performed with a 95% confidence interval and 5% significance level. All analyses were performed using R (R Core Team 2016) language.

Results

Efficacy of toltrazuril 5% against *Eimeria* spp. ranged from 26.2 to 91.8% in pregnant ewes (Table 1). The prevalence of ewes infected with *Eimeria* ranged from 0 – 36% in GI and 0 – 25% in GII. In GI, the highest prevalence was during the parturition (D0) and on D14. In GII, prevalence increased after parturition at D7, D21, and D28 (Fig. 2A). The OPG of the ewes ranged from 0-1000 in GI and 0-600 in GII. Differences between the oocysts excretion of the groups were observed at D-21, D-14, D-7, D0, D14 and D56, where the GI had higher OPG than GII ($p<0.05$; Fig.2B). Mixed infections, including *E. ovinoidalis* (58%), *E. crandallis* (19%), *E. ahsata* (14%), *E. granulosa* (7%), and *E. parva* (2%), were observed in the ewes. No clinical signs of coccidiosis or drug reaction was observed in any treated ewes. Moxidectin treatment was effective to avoid gastrointestinal nematodes in GI and GII (data not shown).

As shown in the Figure 3A, the prevalence of lambs shedding *Eimeria* spp. oocysts ranged from 0-100% in GIII and 0-93% in GIV. The prevalence was greater than 50% during most of the experimental period. The highest prevalence was recorded at D56 in both groups of lambs. In all groups of lambs, oocyst shedding began on D21 and peaked on D56 in GIII and D35, D49 and D63 in GIV. The OPG ranged from 0-18,900 in GIII and 0-171,200 in GIV. No statistical differences were found among groups. When analyzing if there was a significant difference in OPG in the same group over time (Wilcoxon signed sum test), the OPG results were significantly different in GIV between days 14 to 21, 35 to 42 and 42 to 49. The OPG values of the lambs of both groups are shown in Figure 3B.

Lambs presented mixed infections with two to seven *Eimeria* species. *E. ovinoidalis*, *E. crandallis* and *E. parva* were the most frequent species identified (Table 2). *E. ovinoidalis* was the predominant species in lambs from D21 to D63. The other species varied in frequency during the experimental period. One lamb in GIV had diarrhea (FS=3) on D32 with an OPG of 171,200.

Discussion

In the present study, treatment of the pregnant ewes with toltrazuril 5% before parturition significantly reduced the mean OPG excreted in the peripartum period (21 days before and 14 days after the parturition). An increase in OPG excretion is expected in the peripartum period under natural conditions, a phenomenon called periparturient rise (Carrau et al. 2016). Although the mechanisms responsible for periparturient rise remain unclear, activation of inhibited stages associated with stress during the parturition may be involved (Faber et al.

2002). This stressful period matches with the peak of oocysts excretion, indicating the relation between stress and immune status (Carrau et al. 2016). Due the duration of time from treatment, the difference ($p<0.05$) in oocyst shedding by ewes at D56 (Fig. 2B) was not directly related to treatment with toltrazuril 5%. In general, the prevalence and oocyst excretion of ewes was low. This was expected, because adult sheep continuously excrete small number of oocysts contributing to pasture contamination (Carrau et al. 2016). Thus, toltrazuril 5% was effective in reducing environmental contamination by decreasing fecal oocyst excretion by periparturient ewes.

In this research, the treatment of the ewes before the parturition had no influence on the dynamics of infection and excretion of oocysts by their lambs. The fact that all ewes and lambs were kept together in a naturally contaminated pasture may have influenced the infection and excretion of oocysts by lambs. Saratsis et al. (2011) showed that early excretion of oocysts occurred in lambs 13-15 days after birth. In the present study, oocyst shedding began on D21 after birth with a prevalence of 36% in GIII and 66% in GIV. This suggests that both groups of lambs acquired the infection in the first few days after birth. Considering that the prepatent period of *Eimeria* species is approximately 12 days (Taylor et al. 1995), the occurrence of this early infection was explained by the previous contamination of the environment and the absence of a protective immunity, independent of the treatment of their mothers.

Excretion of oocysts by lambs increased significantly during the experimental period, reaching the highest values on D56 in GIII and D35, D49 and D63 in GIV. Gregory et al. (1983) found British lambs had peak oocysts excretion at 6 weeks of age followed by a rapid decrease. In different breeding systems, the peak excretion of oocysts in lambs occurs between 4-7 weeks of age. During this period, the lambs are particularly susceptible to *Eimeria* infection (Catchpole et al. 1993, Gregory and Catchpole 1989; Platzer et al. 2005).

Seven species of *Eimeria* were found in lambs (Table 2). *E. ovinoidalis* was most frequently identified at D21 in both groups. *E. ovinoidalis* has been reported as the most frequent *Eimeria* spp. species in sheep in temperate (Reeg et al. 2005) and dry tropical areas (Vercruysse 1982). The predominance of this *Eimeria* species could be related to the high reproductive efficiency (Catchpole et al., 1976) and short pre-patent period of these protozoa (Andrews, 2013). Other *Eimeria* spp. identified in lambs showed variations throughout the experiment. The factors responsible for this variation are unclear. This may be due to the development of immunity against these parasites, with decreased of parasitic load after a period of infection (Reeg et al. 2005).

In sheep farms with confirmed cases of coccidiosis, the use of toltrazuril 5% at four to two weeks before the parturition can be recommended aiming to reduce the excretion of *Eimeria* spp. oocysts by the ewes in the peripartum period. However, these ewes should be moved to an oocyst-free area (or a low contaminated area) few days before or soon after the parturition with their lambs to decrease the risk of lambs coccidiosis. As showed in this study, untreated lambs (GI) excreted a great number of *Eimeria* spp. oocysts during the peripartum period, acting as an important source of environmental contamination for the lambs. Unfortunately, moving treated ewes with their newborn lambs to a new pasture area could be not advisable on regards to the parasite resistance selection pressure. Anyway, it should be considered that the other sheep in the flock as non-pregnant ewes, rams, and older lambs could be maintained as untreated sheep, acting as sources of toltrazuril sensitive *Eimeria* spp. oocysts for the parasite population in *refugia* in the farm.

Conclusions

In conclusion, the treatment with toltrazuril 5% in ewes at four and two weeks before parturition reduces the mean of excretion of *Eimeria* spp. oocysts in the peripartum period. However, this approach had no influence in the dynamics of *Eimeria* spp. infection by the lambs born from these ewes under natural exposure to (re)infection at pasture. This treatment management can be used in ewes in the peripartum period to reduce the environmental contamination with oocysts.

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

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Table 1 Efficacy of the treatment using toltrazuril 5% against *Eimeria* spp. in naturally infected ewes raised on pasture.

Treatments	First drench		Second drench					
Days (parturition as day 0)	-28	-21	-14	-7	0	+7	+14	+21
GI OPG mean (untreated)	22.9	56.9	33.8	61.2	105.3	51.9	68.5	183.5
GII OPG mean (treated)	13.5	8.3	3.5	5.2	40.9	35.7	5.6	94.5
OPG reduction percentage (% and CI)	-	85.8 (27.4-98.3)	89.7 (26.3-99.7)	91. 5(14.7-99.8)	69 (8.6-94.6)	26.2 (0.01-74.1)	91.8 (17.5-99.8)	41.1 (0.02-88)

Table 2 Frequency (%) of species of *Eimeria* spp. in lambs naturally infected kept in an extensive breeding system.

Species (%)	Days of experiment (D)							
	D14	D21	D28	D35	D42	D49	D56	D63
E. ahsata	0	0	0	16	33	16	0	0
E. crandallis/ E. weybridgensis	0	18	31	17	0	20	0	24
E. faurei	0	0	0	0	22	0	0	28
E. granulosa	0	0	0	0	0	13	0	0
E. intricata	0	0	0	0	0	2	0	5
E. ovinoidalis	0	46	63	34	34	47	100	38
E. parva	0	36	6	33	11	2	0	5

Fig. 1 Average climate conditions (rainfall, temperature and relative humidity) during the experimental period in the central region of Rio Grande do Sul

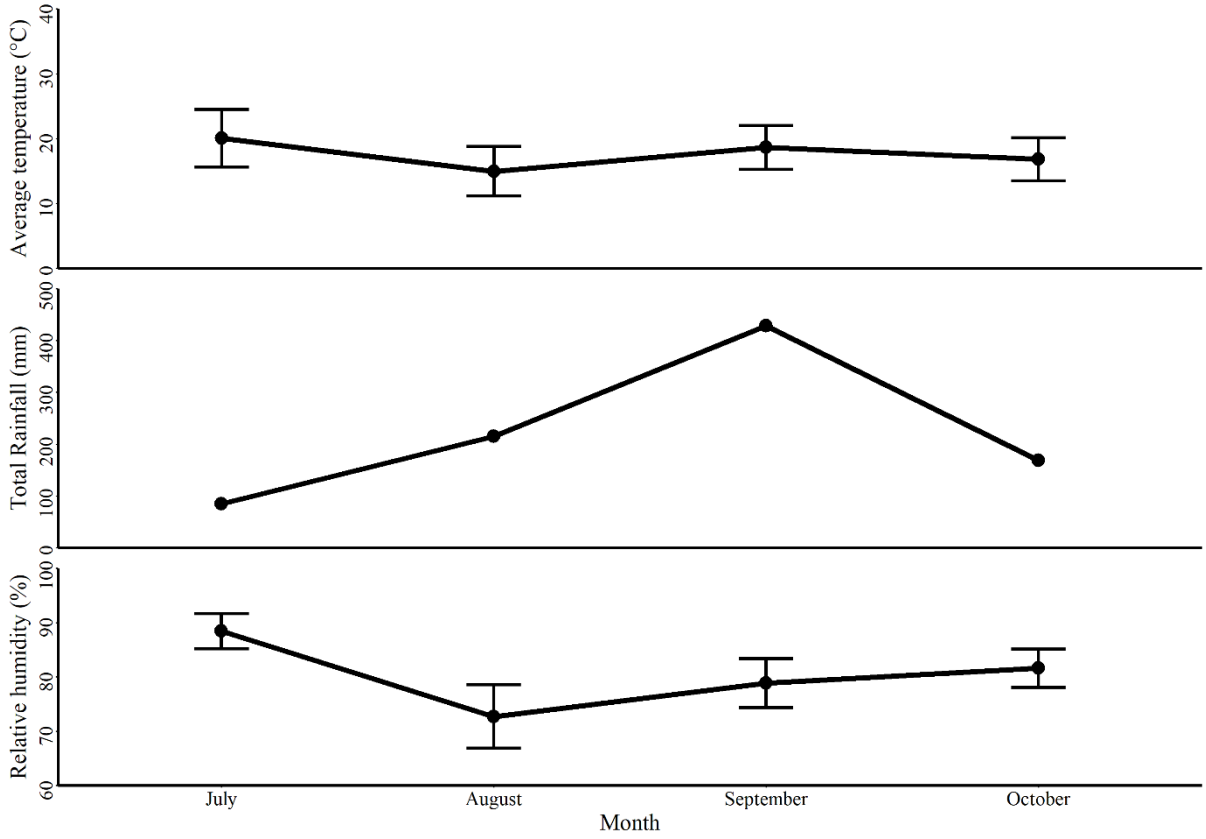


Fig. 2 A - Prevalence of *Eimeria* spp. infection in ewes during the experimental period. B - Means of OPG excreted by the ewes during the experimental period. Group 1 (GI; control group) pregnant ewes naturally infected with *Eimeria* spp. and group 2 (GII; treated group) pregnant ewes naturally infected by *Eimeria* spp. and treated with toltrazuril 5% (Baycox®; 20 mg/kg, orally). * Significant difference ($p<0.05$) between GI and GII.

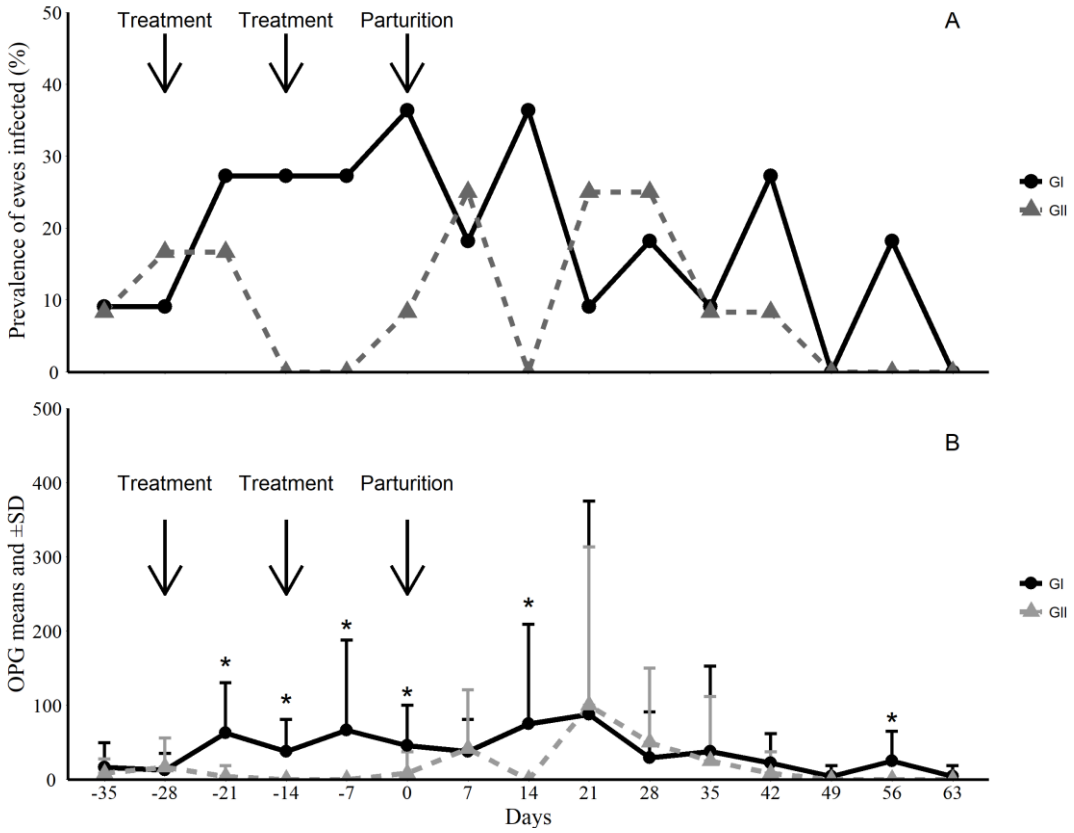
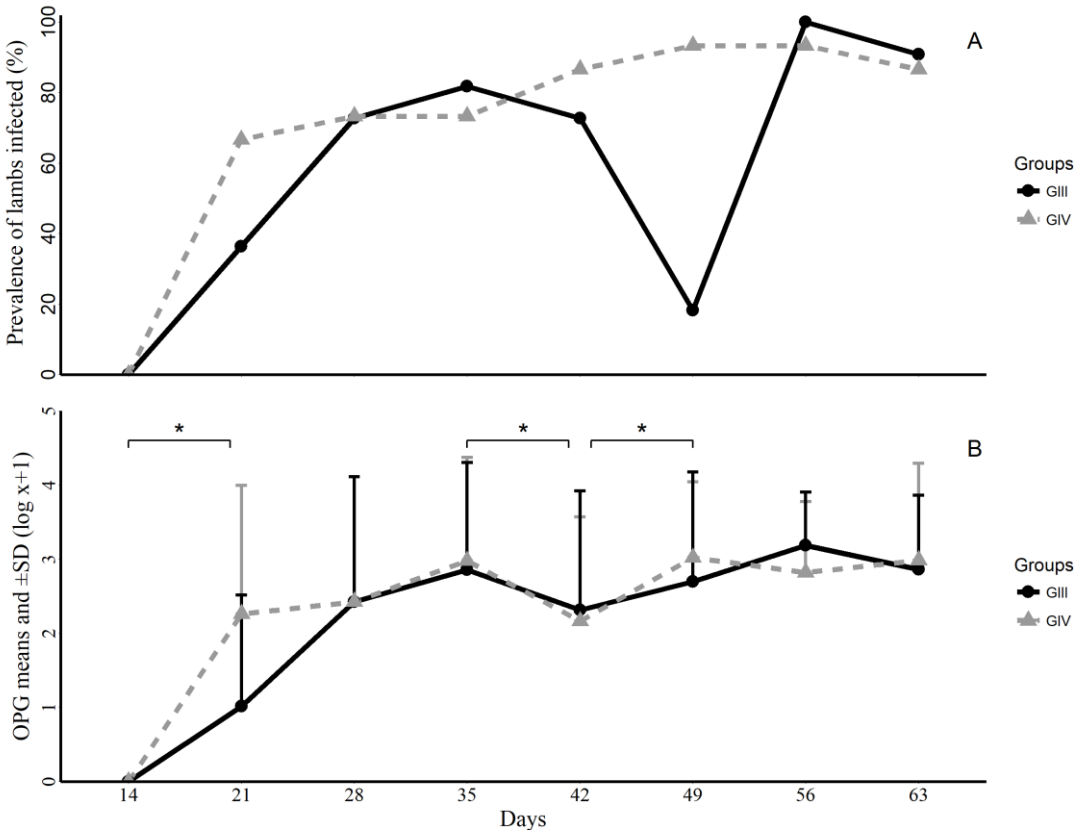


Fig. 3 A - Prevalence of *Eimeria* spp. infection in lambs during the experimental period. B - Means of OPG excreted by the lambs during the experimental period. Group 3 (GIII) lambs from GI dams (not treated with toltrazuril) and group 4 (GIV) lambs from GII dams (treated with toltrazuril 5%).



5 ARTIGO 3 - EFFICACY OF TREATMENT USING TOLTRAZURIL IN LAMBS MAINTAINED IN A NATURAL CONDITION FAVORABLE TO THE DEVELOPMENT COCCIDIOSIS

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Efficacy of treatment using toltrazuril in lambs maintained at pasture under natural condition favorable to the development coccidiosis

Eficácia do tratamento usando toltrazuril em cordeiros mantidos em condições naturais favoráveis ao desenvolvimento de coccidiose.

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Abstract

The objective of this study was to evaluate the efficacy of a single dose of toltrazuril 5% against *Eimeria* spp. infection in lambs raised at pasture in a natural condition favorable to the development of coccidiosis. The experiment was designed after the confirmation of death by clinical coccidiosis in a lamb from the studied flock. Thirty-two lambs, with approximately 6-7 months of age and both genders, were distributed into two randomized blocks, based on oocyst count and live weight. The group I was treated with toltrazuril 5% in a single dose and the group II was the control group. Individual feces were collected weekly from day 0 until day 84 after the onset of the treatment. Lambs were weighed at days 0, 84, and 126. Costs of the

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treatment regimen were calculated. Efficacy of the treatment using toltrazuril 5% was higher than 95% until day 7. Treated lambs become re-infected since day 28. *E. ahsata* was the most frequent species found in the lambs' feces. There was no significant difference in weight gain between the groups. The total cost of the treatment was U$2.32 per animal. The treatment with toltrazuril in subclinically infected lambs was not economically feasible, but prevention of clinical coccidiosis and deaths of lambs can become the treatment a cost-effective option to be used in lambs raised on pasture.
Keywords: Eimeriosis; *Eimeria* spp.; *E. ahsata; E. ovinoidalis;* Metaphylactic

Resumo

O objetivo do estudo foi avaliar o uso de toltrazuril 5% em dose única em cordeiros mantidos em condições naturais favoráveis para o desenvolvimento de coccidiose. O experimento foi desenhado após a confirmação da morte de um cordeiro por coccidiose. Trinta e dois cordeiros com aproximadamente 6-7 meses de vida de ambos os sexos foram distribuídos em dois blocos randomizados baseado em contagem de oocistos e peso vivo. O grupo I foi tratado com toltrazuril 5% em dose única e o grupo II foi o grupo controle. Fezes foram coletadas semanalmente do dia 0 até dia 84 após o início do tratamento. Cordeiros foram pesados nos dia 0, 84 e 126. Custo do tratamento foi calculado. Eficácia do tratamento com toltrazuril 5% foi maior que 95% até o dia 7. Cordeiros tratados tiveram reinfecção no dia 28. *Eimeria ahsata* foi a espécie mais frequente. Não houve diferença significativa no ganho de peso entre os grupos. O custo total de tratamento por animal foi de $2,32. Tratamento com toltrazuril em infecções subclínicas deve ser avaliado, mas pode se tornar uma opção com custo/benefício quando usado para prevenir mortalidade causado por coccidiose em cordeiros.
Palavras-Chave: Eimeriose; *Eimeria* spp.; *E. ahsata*; *E. ovinoidalis*; Metafilático

Introduction

Coccidiosis is a disease caused by protozoa of the genus *Eimeria* spp. with a high occurrence infecting the small and large intestine of sheep worldwide (Tenter et al., 2002; Raue et al., 2017). Eleven species are described infecting sheep, *E. ovinoidalis* and *E. crandallis* are the most pathogenic (Levine, 1985; Chartier & Paraud, 2012). The pre-patent period is variable among the *Eimeria* species, being approximately 12-20 days (Andrews, 2013). Subclinical infection is common in livestock and it can cause decreased weight gain. Clinical coccidiosis is less frequent, and it is characterized by diarrhea, dehydration, decreased appetite up to anorexia, weight loss, and death (Chartier & Paraud, 2012; Andrews, 2013).

Lambs are more susceptible to have higher parasitic loads and subclinical and clinical infection than adult sheep. Lambs can start excretion of oocysts in the feces after 3 weeks of age, tending to increase the intensity of oocysts excretion around 6 weeks (Reeg et al., 2005). Adult sheep use to excrete oocysts in low levels and with no clinical signs. However, clinical disease can occur under certain epidemiological conditions including stressing situations, high animal density, wet environment, and sub-nutrition (Jalila et al., 1998; Lima, 2004; Cai & Bai, 2009; Taylor, 2013). Control of coccidiosis is based on hygienic prophylaxis and the use of anticoccidial drugs (Lopes et al., 2014; Rodrigues et al., 2016). Many drugs have been recommended for the treatment of coccidiosis in ruminants, which prevent the development of the parasite (Lima, 2004; Andrews, 2013). Toltrazuril is a triazone compound that acts against all intracellular stages of *Eimeria* spp. lifecycle and it is highly effective for ruminants in intensive and semi-intensive systems (Balicka-Ramisz, 1999; Le Suer et al., 2009, Rodrigues et al., 2017).

The objective of the study was evaluated the use of toltrazuril 5% in a single dose to control the development of coccidiosis in lambs maintained in a natural condition favorable to the development of this infection.

Material and Methods

Farm and sheep flock

This study was carried out on a sheep farm located in the Central region of the Rio Grande do Sul State (29°41'14.88''S – 54°26'34.06''O), in a subtropical area in Southern Brazil. The average climate conditions during the experimental period (expressed as mean and confidence intervals [CI] or standard deviations [SD]) were: average temperature 14.28 ± 4.07 °C; average rainfall 67.78 ± 67.78 mm; and relative humidity 83.97% (CI: 80.28–87.34%) (Fig. 1). The sheep farm had between 100 to 110 Texel x Ile de France crossbreeding sheep in an area of four hectare (ha)[2] during the experimental period. Sheep were kept in a natural pasture intercropped with *Panicum maximum* and *Pennisetum glaucum*, grazed during daylight (10 to 11 hours) and they were housed at night in a stable with rice straw bedding. Sheep did not receive any anticoccidial treatment prior to this study.

Experimental design

The study was designed after a confirmation of coccidiosis in a lamb of the flock that died. Clinical signs and evolution of the disease, as the post-mortem observations by necropsy,

[2] 1 hectare correspond to 10000m[2]

intestinal tissue examination, and feces analysis confirmed the diagnostic of coccidiosis caused by *Eimeria* spp. Thirty-two lambs from the same flock with approximately 6-7 months of age and both genders, were distributed into two randomized blocks (n = 16), based on oocyst count (oocysts per gram of feces, OPG) and live weight. The group I (GI) was treated with toltrazuril 5% (20mg/kg, orally) at day 0 (D0) and the group II (GII) was the untreated (control group). Individual feces were weekly collected from each lamb rectum from D0 until D84 after the onset of the treatment and examined for OPG and *Eimeria* species identification. Sheep were weighed at D0, D84 and D126 using digital scale.

Parasitological analysis

Fecal consistency was evaluated for each lamb at each collection day according to a fecal score (FS) adapted by Le Suer et al. (2009): 0 (normal pellets), 1 (mild diarrhea), 2 (moderate diarrhea), 3 (severe diarrhea), and 4 (severe diarrhea with blood and/or tissues present). Each fecal sample was assessed by the McMaster technique with a sensitivity of 50 OPG. Briefly, 2 g of feces were mixed and diluted with 28 mL of saturated solution of sugar, re-suspended, sifted, and transferred to a McMaster chamber for microscopic identification of oocysts.

Eimeria species were identified from each experimental group at each sampling date. Positive samples were homogenized, dissolved in water, and sieved through a 60-μm steel mesh filter. Several washes were performed until the supernatant became transparent (Hoffman et al., 1934). After sedimentation, the liquid was removed and potassium dichromate ($K_2Cr_2O_7$) solution 2.0% (w/v) was added in equal proportion. The suspension was maintained in Erlenmeyer flasks at room temperature with forced aeration using an air pump. To recover oocysts and identify the species under microscopy, a sample from each preparation was processed by the flotation technique in a modified concentrated sugar solution (Ueno & Gonçalves, 1998). *Eimeria* species identification included morphometric measures of the length and width of both oocysts and sporocysts, and wall thickness of oocysts. Morphological characteristics, including shape, color, and presence or absence of micropyle on the surface of oocysts, was noted (Levine, 1970; Chartier & Paraud 2012). *Eimeria crandallis* and *E. weybridgensis* oocysts could not be differentiated due to their morphological similarity (O'Callaghan et al., 1987).

Economic analysis

Total cost of the treatment regimen per animal was calculated using the model proposed by Rodrigues et al. (2017). Briefly, the cost of the treatment included the cost of the drug plus the cost of employee time to treat the lamb. The price of the live lamb in the Brazilian market was US$ 1.59 per kg (Emater quotation, 2016). The cost of the dead lamb caused by coccidiosis was calculated based on the price of a kilogram of live lamb multiplied by the weight of the lamb. The cost of the treatment and the kg price of the live lamb were converted from Brazilian monetary units (R$) to US dollar (US$) at a rate of R$ 3.44 to US$ 1.00.

Statistical analysis

Efficacy of each treatment regimen was calculated using OPG reduction percentage at 95% confidence interval. For this purpose, fecal oocysts count reduction test (FOCRT) was used, comparing mean OPG of each treatment group with mean OPG of the untreated control group (unpaired samples) on each collection day. A hierarchical Bayesian method, described for the fecal egg count reduction test (FECRT; Torgerson et al. 2014), was adapted for the FOCRT estimation, which took into account the random sampling errors, the degree of aggregation between individuals within each group and the 95% confidence interval.

The OPG was not normally distributed (Kolmogorov-Smirnov test, $p < 0.05$, data not shown). Therefore, the non-parametric Mann-Whitney test was used for comparison of the mean OPG between the groups at each time point. Weight gain was normally distributed (Kolmogorov-Smirnov test, $p>0.05$), and it was compared by two samples Student's t-test. The frequency of diarrhea/non-diarrhea (assessed by fecal scores) in each group was compared using Fisher's exact test. These statistical tests were performed with a 95% confidence interval and 5% significance level. All the analysis was performed using R (R Core Team, 2016) language.

Results

Efficacy of the treatment using toltrazuril 5% was higher than 95% until D14 (Table 1). After the treatment the lambs in GI reduced the OPG to zero. Lambs from GII increased their OPG counts on day 7 (D7) and they have higher OPG means ($p<0.05$) compared to GI at D7, D14, D21, D28, and D35. After D35 all the lamb kept a low OPG without statistical difference ($p>0.05$) between the groups (Figure 2). Treated lambs (GI) had a re-infection on day 28 (D28) with two lambs excreting oocysts in feces. After this period, most of the lambs had low levels of oocysts excretion.

Three and four species of *Eimeria* were identified in GI and GII, respectively (Figure 3). *E. ahsata* was the most frequent species found in this study. Lambs presented infections with one to four *Eimeria* species. *E. ovinoidalis* or *E. ahsata* monoinfection was also identified. In both groups, subclinical infection remained in most of the cases (FS=0). One lamb of GII presented moderate diarrhea (FS=2) in the D14. No adverse drug reactions were observed in any treated lamb.

No significant differences were found comparing the mean weight gain of the lambs between the groups ($p > 0.05$). From D0 to D84 all lambs of both groups decreased the live body weight, however, after the D84 until D126 (end of the experiment) the lambs recovery the body weight (Figure 4). During the experimental period the lambs were kept togheter with other sheep in the flock in a higher stock rate (100 to 110 sheep heads kept in four Ha area). The climate conditions were favorable to sporulation of *Eimeria* spp. oocysts during the experimental period (Figure 1).

The total cost of the treatment was $2.32 per animal and $34.8 for all the lambs in GI, including the cost of the drug and the cost of the work to treat the lambs. The cost of the lamb dead by coccidiosis was $38.4 considering the mean weight of the lambs at the beginning of the experiment.

Discussion

This study evaluated the efficacy of toltrazuril in seven months old lambs kept in a high stock density area. Toltrazuril was effective to reduce the OPG under a condition favorable to the development coccidiosis, but there was no significant difference in weight gain at the end of the experiment.

The lambs treated with toltrazuril 5% (GI) had no diarrhea (FS=0), and the untreated control had one lamb with moderate diarrhea (FS=2). OPG of untreated lambs were significantly higher than treated lambs until the D35. After this period, lambs of both groups showed OPG in low levels with no significant difference ($p>0.05$). Coccidiosis is a disease self-limiting, and after the infection the animal can develop immune response against the parasite, however, in cases of poor nutrition, high density population, weaning, abrupt change of temperature and diet or stress situation the risk of clinical coccidiosis is increased (Jalila et al., 1998; Lima, 2004; Cai & Bai, 2009; Taylor, 2013). The use of toltrazuril can avoid the damage caused by *Eimeria* spp. in the intestine villus, preventing the losses of subclinical infection and the new cases of coccidiosis in the flock.

Treated lambs had re-infection showed by excretion of OPG at 28 days after the treatment. Toltrazuril has a pharmacological peak plasma concentration at 48 h and half-life on 9 days (Baycox® 5% Bayer technical information, 2008) and the lambs were not fully protected against re-infection when toltrazuril was used at 21-day intervals. Re-infection can occur 14 - 21 days post treatment and some species complete the life cycle in less than 21 days (Le Suer et al., 2009; Rodrigues et al., 2017).

In our study, mixed infections of *Eimeria* spp. occurred with four species, *E. ahsata*, *E. ovinoidalis*, *E. bakuensis,* and *E. parva*. These species are commonly reported in sheep (Vercruysse, 1982; Platzer et al., 2005; Le Sueur et al., 2009). *E. ovinoidalis,* considered the most pathogenic and responsible for the majority of clinical disease cases, has a high potential of multiplicity in relation to the other species (Catchpole et al., 1976; Chartier & Paraud, 2012). *Eimeria ahsata* is considered a pathogenic species and it is frequently identified in fecal exams in sheep (Levine, 1985; Amarante e Barbosa, 1992; Wang et al., 2010; Saratsis et al., 2011).

No significant difference was found in the weight gain between the groups, though toltrazuril showed efficacy and some pathogenic species have been identified. Little is known about the impact of subclinical coccidiosis on the performance of sheep (Gauly et al., 2004), but in our study even under high-density population and the poor nutrition, infection by *Eimeria* spp. had no influence on the lambs' weight gain. Other studies also reported no differences in weight gain using toltrazuril on housed lambs, dairy sheep, and suckling lambs naturally exposed to *Eimeria* spp. re-infection in grazing systems (Mundt et al., 2009; Saratsis et al., 2013; Rodrigues et al., 2017).

The cost of treatment was $2.4 per animal including the cost of the drug plus the cost of the work needed to treat each lamb. Economic benefits from the treatment with toltrazuril can be calculated using the weight gain of the treated lambs in comparison with the untreated lambs. In our study, no significant difference in the weight gain was found. However, before the initial of the experiment, one lamb died of coccidiosis and the cost of the loss of this lamb was U$38.4, being this value higher than the total cost to perform the treatment in all lambs in treated group. If, we consider the efficacy of toltrazuril and the benefits to prevent severe losses of coccidiosis in a herd, including the death of animals, the treatment could be economically feasible.

Conclusions

Treatment with toltrazuril 5% showed efficacy against mixed *Eimeria* spp. infection in lambs maintained in a natural condition favorable to the development of coccidiosis. There was no significant difference in weight gain between the toltrazuril-treated and untreated lambs.

Treatment in subclinical infection should be evaluated, but it can become a cost-effective option when used to prevent mortality caused by coccidiosis in lambs.

Compliance with ethical standards

All experimental practices involving animals were approved by the Ethics Committee for Animal Experimentation at Universidade Federal de Santa Maria (UFSM) (Protocol number 8088190815).

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends

Table 1. Efficacy of the treatment using toltrazuril 5% against *Eimeria* spp. in naturally infected lambs raised on pasture.

Fig 1. Average climate conditions (temperature [SD], total rainfall, and relative humidity [CI]) during the experimental period in the central region of Rio Grande do Sul

Fig 2. Means of OPG excreted by the lambs during the experimental period in treated group (GI) and untreated control group (GII). * Significant difference between GI and GII ($p < 0.05$).

Fig 3. Frequency (%) of species of *Eimeria* in lambs naturally infected. GI- Treated with toltrazuril 5%. GII- control group.

Fig 4. Mean of weight gain in lambs during the experimental period. GI- Treated with toltrazuril 5%. GII- control group.

Table 1.

Days	Efficacy of toltrazuril 5% against *Eimeria* spp. in lambs
7	96.1 (85.9 – 99.9)
14	94.8 (80.2 – 99.9)
21	82.4 (29.6 – 99.9)
28	48.8 (0 – 84.4)
35	75.8 (17.2 – 100)
42	25.3 (0 – 62.6)

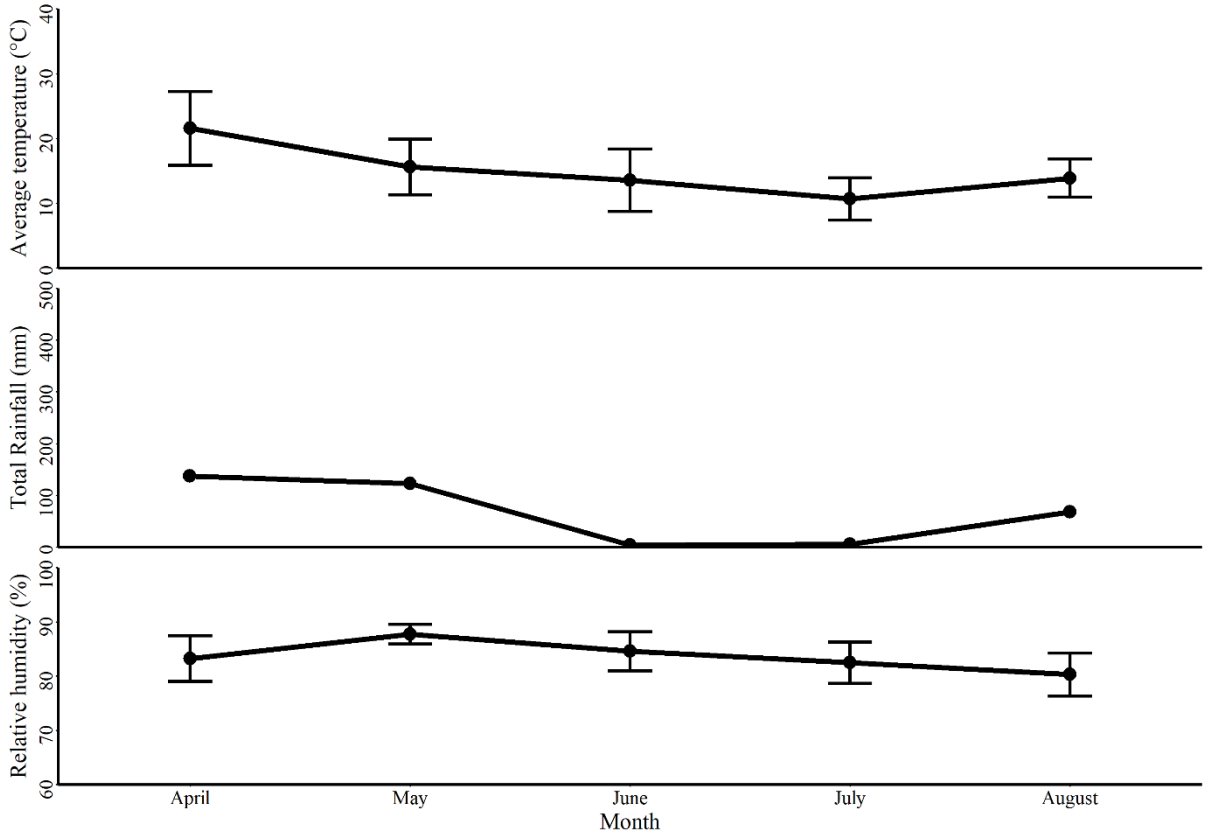
Average temperature (°C)
0
10
20
30
40
Total Rainfall (mm)
0
100
200
300
400
500
Relative humidity (%)
60
70
80
90
100
April
May
June
July
August
Month

Fig 1.

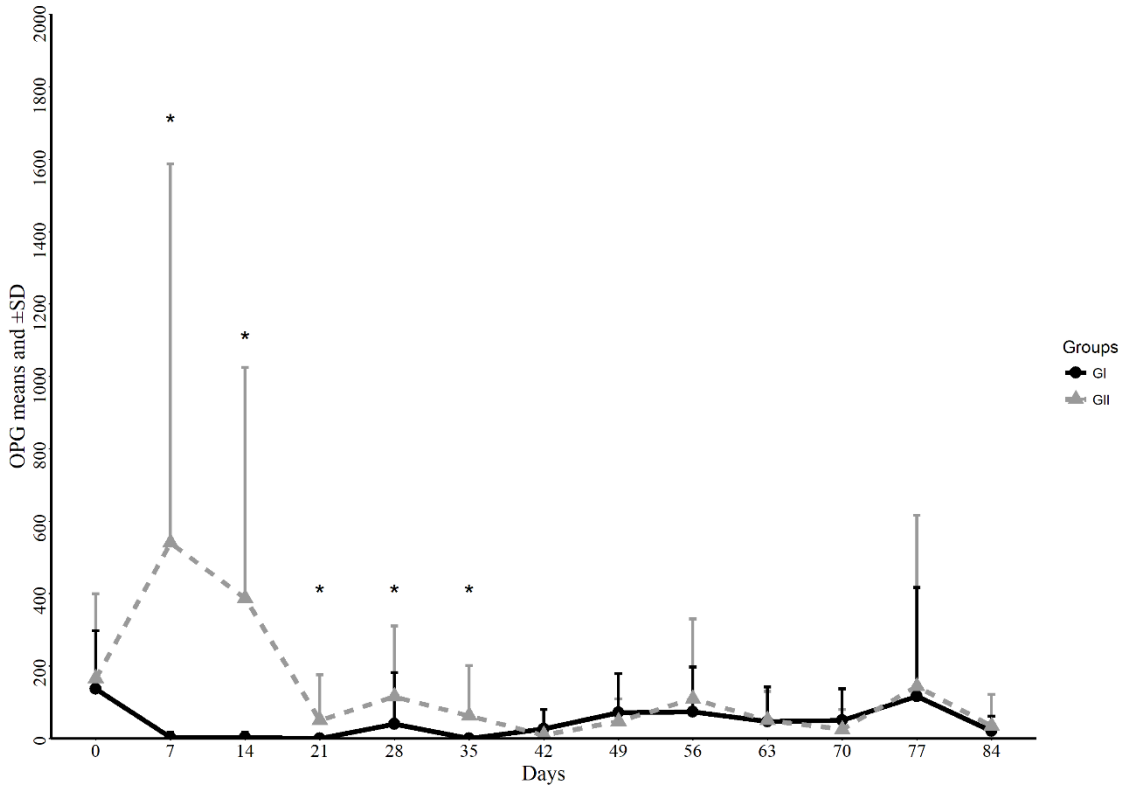
OPG means and ±SD
0
200
400
600
800
1000
1200
1400
1600
1800
2000
*
*
*
*
*
0
7
14
21
28
35
42
49
56
63
70
77
84
Days
Groups
GI
GII

Fig 2.

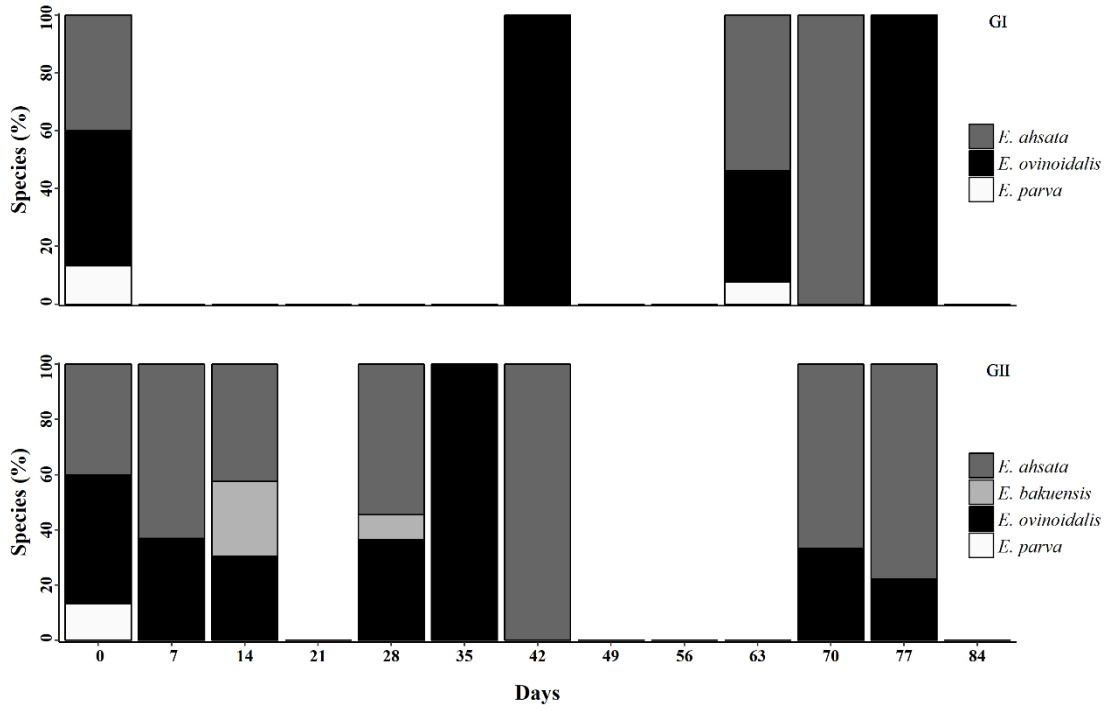
GI
GII
Species (%)
Days
E. ahsata
E. bakuensis
E. ovinoidalis
E. parva
0
20
40
60
80
100
0
7
14
21
28
35
42
49
56
63
70
77
84

Fig 3.

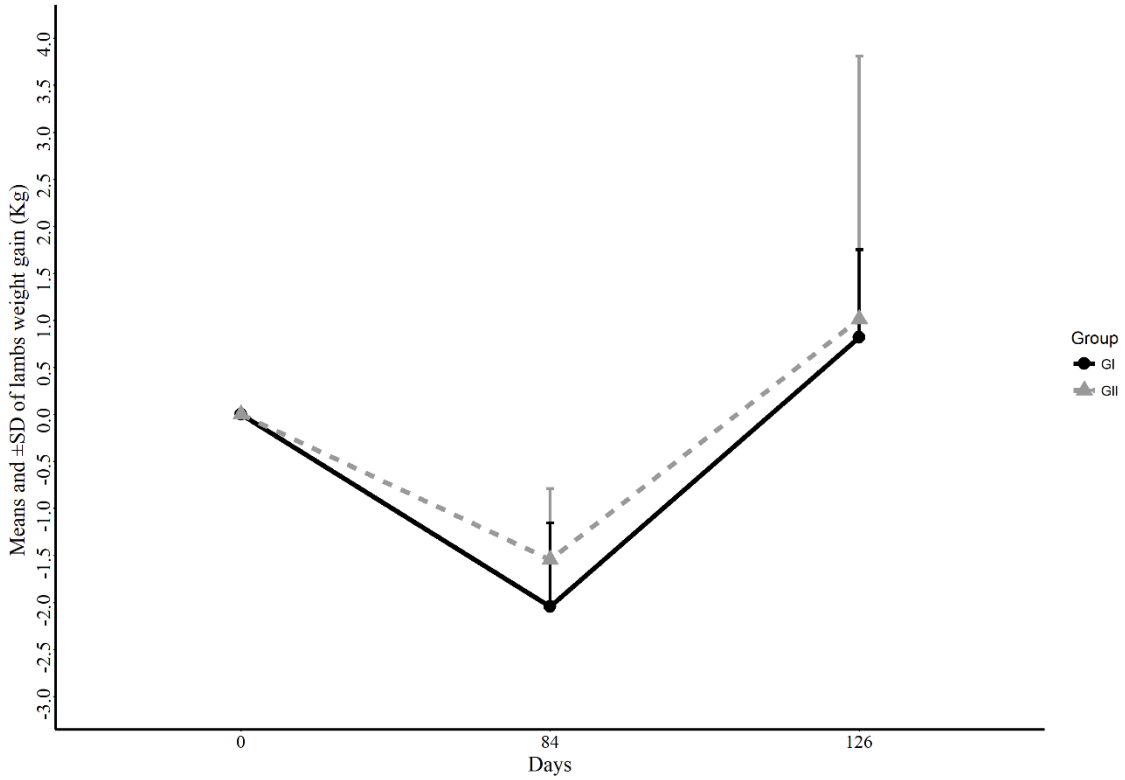
Means and ±SD of lambs weight gain (Kg)
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
84
126
Days
Group
GI
GII

Fig 4.

6 DISCUSSÃO

Nesta tese, o nosso grupo de estudo buscou entender quais são as melhores formas de utilizar o toltrazuril em cordeiros em lactação, em situação de risco para o desenvolvimento de coccidiose e em ovelhas prenhes, visando verificar a eficácia desta droga, bem como a maior produtividade e a viabilidade econômica para uso do medicamento na ovinocultura. O desenvolvimento desta tese permitiu a compilação de três artigos científicos.

No artigo 1, pode-se observar que toltrazuril a 5% aplicado a cada 14 dias pode ser usado para controlar a reinfecção contra *Eimeria* spp. em cordeiros em lactação naturalmente infectados em um sistema semi-intensivo. Contudo o tratamento a cada 21 dias não confere proteção completa contra reinfecção. *Eimeria* spp. consegue completar o ciclo de vida em um período menor que 21 dias nas condições estudadas. Le Suer et al. (2009) observaram que cordeiros em sistema intensivo tratados com toltrazuril a 5% excretam oocistos 14 dias depois do tratamento.

Infecções mistas com *E. ovinoidalis* e *E. crandallis/weybridgensis* foram observadas no estudo e são frequentemente relatadas em ovinos (LE SUER et al., 2009; PLATZER et al., 2005; SARATSIS et al., 2011; VERCRUYSSE, 1982). A predominância destas espécies pode ser relacionada com a alta eficiência reprodutiva quando comparada com as demais espécies deste parasito que acomete ovinos (CATCHPOLE et al., 1976). Nesta pesquisa, não foi encontrada uma diferença significativa no ganho de peso entre os animais tratados e os animais do grupo controle. Outros estudos em cordeiros em criação intensiva ou leiteira também não encontraram tal diferença (MUNDT et al., 2009; SARATSIS et al., 2013). Em determinadas situações específicas como em infecções subclínicas, deve-se verificar a necessidade de utilização do toltrazuril associado com os benefícios econômicos; entretanto, em casos onde há casos clínicos e até morte dos animais, a utilização do medicamento pode ser economicamente viável.

No artigo 2, pode-se constar que o tratamento com toltrazuril a 5% em ovelhas antes do parto reduziu significativamente ($p<0.05$) a média de excreção de oocistos 21 antes e 14 dias após o parto. O aumento da excreção de oocistos no período peri-parto foi previamente demostrado (CARRAU et al., 2016). O mecanismo responsável por este fenômeno permanece desconhecido. Uma possível explicação pode ser a ativação dos estágios inativos do parasito associados com fatores de estresse (FABER et al., 2002).

O tratamento com toltrazuril a 5% antes do parto não causou influência na dinâmica de excreção de oocistos nos cordeiros, embora tenha reduzido significativamente a excreção do

parasito pós-parto pelas ovelhas, Nestes animais, a excreção de oocistos iniciou na terceira semana após o nascimento, sugerindo que a infecção acontece nos primeiros dias de vida. A ocorrência dessa infecção ocorre devido a contaminação ambiental e ao curto período pré-patente de algumas espécies (TAYLOR et al., 1995). Por ocasião do estudo, sete espécies de *Eimeria* foram identificadas (*E. ahsata*, *E. crandallis*, *E. ovinoidalis*, *E. faurei*, *E. granulosa*, *E. intricata* e *E. parva*), sendo *E. ovinoidalis* a mais frequente. Esta espécie tem sido reportada como a de maior ocorrência em áreas de clima temperado (REEG et al., 2005) e áreas de clima seco (VERCRUYSSE, 1982).

No artigo 3, há o relato do estudo abrangendo os cordeiros que estavam mantidos em uma condição que favorecia a ocorrência da infecção por *Eimeria* spp. A utilização do toltrazuril foi efetiva em reduzir o OoPG nesta situação, porém, não se observou uma diferença significativa no ganho de peso ao final do experimento. É escasso o conhecimento sobre a influência da infecção subclínica desencadeada por *Eimeria* spp. na performance produtiva de ovinos (GAULY et al., 2004). Os cordeiros tratados com o fármaco apresentaram reinfecção 28 dias após o tratamento. Toltrazuril apresenta uma concentração plasmática máxima em 48 horas e meia vida de 9 dias (Baycox® 5% Bayer technical information, 2008) e os animais não estão completamente protegidos após o intervalo de 21 dias. A reinfecção pode acontecer 14 dias após o tratamento (LE SUER et al., 2009).

Neste trabalho foi possível identificar quatro espécies, incluindo *E. ahsata*, *E. ovinoidalis*, *E. parva* e *E. bakuensis*. No entanto, *E. ahsata* foi a mais frequente. Esta espécie é considerada patogênica e normalmente encontrada em exame de fezes (LEVINE, 1985; AMARANTE; BARBOSA, 1992; WANG et al., 2010; SARATSIS et al., 2011). O custo com o tratamento foi de R$ 8,25 por animal incluindo o preço do fármaco e custo da mão de obra. Levando em conta que não houve diferença entre o ganho de peso dos animais, o tratamento não foi financeiramente satisfatório. Todavia, considerando que o valor do cordeiro que morreu devido a coccidiose que foi de R$132,1, o tratamento poderia compensar se fosse realizado antes da morte. Portanto, o tratamento preventivo com toltrazuril pode ser recomendado em rebanhos nos quais haja episódios de coccidiose clínica nos ovinos.

Espera-se que os resultados aqui apresentados possam representar subsídios para o controle da coccidiose de pequenos ruminantes, especialmente em ovinos. Ressalta-se a importância de avaliar a situação específica de cada propriedade, bem como avaliar os riscos de desenvolvimento da doença e verificar a necessidade de utilizar o toltrazuril em rebanhos ovinos.

7 CONCLUSÕES

O tratamento com toltrazuril a 5% a cada 14 dias é efetivo em controlar a reinfecção por *Eimeria* spp. em cordeiros lactentes mantidos em ambiente naturalmente contaminado.

O tratamento com toltrazuril na concentração de 5% em ovelhas prenhes quatro e duas semanas antes do parto reduz significativamente a excreção de oocistos 21 dias antes e 14 dias após o parto e não influência na dinâmica de infecção por *Eimeria* spp. nos cordeiros.

Cordeiros em lactação e mantidos em uma condição favorável ao desenvolvimento de coccidiose tratados com toltrazuril não apresentaram um maior ganho de peso significativo em relação aos mantidos naturalmente com infecção por *Eimeria* spp.

E. ovinoidalis, *E. crandallis*, *E. parva* e *E. ahsata* foram as mais frequentes e causaram, principalmente, infecção subclínica.

A viabilidade econômica do tratamento com toltrazuril em diferentes sistemas de criação de ovinos pode ser avaliada com a utilização do modelo econômico que visa determinar o ponto onde o tratamento torna-se viável financeiramente.

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ANEXO A - A AUTORIZAÇÃO DA REVISTA PARASITOLOGY RESEARCH PARA INCLUIR O ARTIGO NA TESE

Santa Maria, Brazil 97110800
Attn: Dr. Fernando Rodrigues

Total 0.00 USD

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ANEXO B – COMPROVANTE DE SUBMISSÃO DO ARTIGO 2 PARA A REVISTA PARASITOLOGY RESEARCH

Parasitology Research - Submission Notification to co-author

PR Parasitology Research
11:59

Para: Fernando de Souza Rodrigues

Re: "Effective reduction of Eimeria spp. oocysts excretion in the peripartum period by ewes treated with toltrazuril 5% before the parturition"
Full author list: Fernando de Souza Rodrigues; Alfredo Skrebsky Cezar; Fernanda Rezer Menezes; Fernanda Ramos; Luiza Pires Portella; Daniela Isabel Brayer Pereira; Luis Antônio Sangioni; Fernanda Silveira Flores Vogel; Sonia Botton

Dear Dr Fernando Rodrigues,

We have received the submission entitled: "Effective reduction of Eimeria spp. oocysts excretion in the peripartum period by ewes treated with toltrazuril 5% before the parturition" for possible publication in Parasitology Research, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr Sonia Botton who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

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Parasitology Research