ANDERSON SILVA DIAS

Pochonia chlamydosporia NO CONTROLE BIOLÓGICO DE *Fasciola hepatica* EM BOVINOS E COMPARAÇÃO DE TÉCNICAS DE SEDIMENTAÇÃO DE OVOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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BIOGRAFIA

Anderson Silva Dias, nascido em 10 de abril de 1979 em Coronel Fabriciano, filho de José Marcelino Dias e Maria Lúcia da Silva Dias.

Em 29 de Janeiro de 2004, graduou-se em Medicina Veterinária na Universidade Federal de Viçosa.

Em março de 2004, ingressou no mestrado no Programa de Pós Graduação da Universidade Federal de Viçosa. Em 23 de março de 2006, realizou a defesa da tese de Mestrado.

Em agosto de 2010, ingressou no Doutorado no Programa de Pós Graduação da Universidade Federal de Viçosa. Em 31 de março de 2014, realizou a defesa da tese de Doutorado.

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RESUMO

DIAS, Anderson Silva, D.Sc., Universidade Federal de Viçosa, março de 2014. ***Pochonia chlamydosporia* no controle biológico de *Fasciola hepatica* em bovinos e comparação de técnicas de sedimentação de ovos.** Orientador: Jackson Victor de Araújo. Co-orientador: Fábio Ribeiro Braga.

A presença de helmintos causando prejuízos na pecuária é significativa no Brasil e no mundo. A *Fasciola hepatica* figura dentre os principais agentes responsáveis por essas perdas. Devido ao aumento de ocorrências de problemas de resistência anti-helmínticas e falhas no controle desses helmintos, o emprego de agentes de controle biológico tem sido estudado e tem apresentado resultados promissores. O presente trabalho tem como objetivos: avaliar a ação do fungo *Pochonia chlamydosporia in vitro* e *in vivo* sobre ovos de *F. hepatica*, após a passagem através do trato gastrintestinal de bovinos em formulações peletizadas; avaliar as condições climáticas sobre a produção de ovos de *F. hepatica* e avaliar três técnicas de sedimentação para quantificar os ovos de *F. hepatica* em fezes de bovinos. Para isso, amostras de fezes foram coletadas nos bovinos do grupo tratado com péletes contendo o fungo *P. chlamydosporia* e grupo controle com péletes se fungo nos tempos de 12, 18, 24, 48, 72 e 96 h após a administração dos péletes. O efeito ovicida foi observado após sete dias da interação. O fungo apresentou atividade ovicida sobre ovos de *F. hepatica* nas amostras (grupo tratado) em todos os tempos a partir de 12 h. Diferenças significativas (p<0,01) na destruição de ovos dos animais do grupo tratado em comparação com o controle foi verificada. Esses resultados sugerem o emprego desse fungo de forma eficaz para o controle de ovos de *F. hepatica*. Trinta dias após a vermifugação, os animais foram separados em dois piquetes semelhantes e administrados via oral péletes contendo 25% de massa micelial de *P. chlamydosporia* (grupo B) e sem fungo (grupo A), a uma dose de 100

gramas, duas vezes por semana, durante 18 meses. A média de ovos de *F. hepatica* por grama de fezes foi maior (p<0,01) no grupo A (1,19) em comparação com o B (0,82). Após 18 meses, os animais do grupo B ganharam a mais 42,33 kg (17,82%) comparados aos do grupo A (p<0,01). Durante os 18 meses, mensalmente, as amostras de fezes de bovinos foram recolhidas nas pastagens dos grupos A e B e foram incubadas para observar estruturas referentes ao fungo *P. chlamydosporia* e foram identificados apenas nas amostras coletadas no piquete do grupo B. A média de ovos por grama de fezes das amostras coletadas entre os períodos de chuva (outubro a março) e seca (abril a setembro) não foram diferentes (P>0,05) e, dessa forma, é possível que os animais possam ser infectados por *F. hepatica* durante todo o ano. As técnicas de sedimentação modificadas de Dennis, Stone & Swanson (DSS), Girão e Ueno (*quatro tamises*) e Foreyt foram comparadas. A técnica de DSS modificada foi a mais sensível (p<0,01) (48,60%). As três técnicas apresentaram especificidade de 100%. Não houve correlação na contagem de ovos obtidos pelas três técnicas e coeficientes significativos não foram observados por análise de regressão. A técnica DSS modificada foi a mais eficaz para o diagnóstico de *F. hepatica* em bovinos (p <0,01). A aplicação de formulação fúngica com *P. chlamydosporia* (25%) foi eficaz na redução da disponibilidade de ovos no ambiente e, por conseguinte, nas reinfecções em bovinos.

ABSTRACT

DIAS, Anderson Silva, D.Sc., Universidade Federal de Viçosa, March, 2014. ***Pochonia chlamydosporia*** **in the biological control of** ***Fasciola hepatica*** **and comparison of techniques of eggs sedimentation.** Adviser: Jackson Victor de Araújo. Co-Adviser: Fábio Ribeiro Braga.

The presence of helminths causing losses in livestock in the Brazil and in the world is significant. The *Fasciola hepatica* is among the main agents responsible for these losses. Due to increased occurrences of problems anthelmintic resistance and failures in the control of this helminths, the use of agent of biological control have been studied and they have showed promising results. This study aims to evaluate: the action of the fungus *Pochonia chlamydosporia in vitro* and *in vivo* on eggs of *F. hepatica*, after passage through of gastrointestinal tract of cattle in formulations of pellets; assess the weather conditions on *F. hepatica* egg production and evaluate three techniques of sedimentation to quantify *F. hepatica* eggs in cattle faeces. Thus, stool samples were collected in cattle of treated group with pellets contained the fungus *P. chlamydosporia* and control group with pellets without fungus in times 12, 18, 24, 48, 72 and 96 h after administration of pellets. The ovicidal effect was observed after seven days of interaction. The fungus showed ovicidal activities on eggs *F. hepatica* in the samples (treated group) in all times from 12 h. Significant differences ($p < 0.01$) in the destruction of the eggs in animals of the treated group compared to those of the control was verified. This resuts suggest employment this fungus of effectively to the control of eggs of *F. hepatica*. Thirty days after deworming, the animals were divided into two similar paddocks e animals received *per os* pellets containing 25% mycelial mass of *P. chlamydosporia* (group B) and pellets without fungus (group A), at a dose of 100 g, twice a week over 18 months.

The mean count of *F. hepatica* eggs per gram of faeces was higher ($p<0.01$) in animals of group A (1.19) compared with those from group B (0.82). After 18 months, the animals from group B gained 42.33 kg above (17.82%) compared to those of control group (A) ($p<0.01$). During 18 months, monthly, cattle faecal samples were collected from the pastures of the paddocks of A and B groups and they were incubated to observe structures related at *P. chlamydosporia* fungus e they was identified only in sample collected in the paddock of B group. Mean number of eggs per gram of faeces of the samples collected between periods of rain (October-March) and dry (April-September) were not different ($p>0.05$) and, thus, it is possible that the animals can be infected by *F. hepatica* during all the year. The modified sedimentation techniques of Dennis, Stone & Swanson (DSS), Girão and Ueno (*quatro tamises*) and Foreyt were compared. The modified DSS technique was the most sensitivity ($p<0.01$) (48.60%). The three techniques showed a specificity of 100%. There was no correlation among the values of egg obtained by the three techniques, and significant coefficients were not observed by regression analysis. The modified DSS technique was the most effective for *F. hepatica* diagnosis in cattle ($p<0.01$). The application of this fungical formulation with *P. chlamydosporia* (25 %) mycelial mass was effective in reducing the availability of eggs in the environment, and consecutive, in the reinfections in calves.

1. INTRODUÇÃO

A criação de bovinos no Brasil ocupa grande importância socioeconômica (Honer, 1979) e os helmintos são um dos principais agentes responsáveis por perdas nessa atividade (Araújo et al., 2004). A *Fasciola hepatica*, um helminto trematóide, de distribuição cosmopolita, é responsável por perdas, principalmente na criação de ovinos e bovinos. Essas perdas se estendem a gastos com tratamentos e condenações de fígado no abate dos animais (Anderson et al., 1977; Ross et al., 1997; Acha & Szyfres, 2003), além das casuísticas humanas (Mas-Coma et al., 2005).

Há dificuldades no controle eficaz de *F. hepatica*, muitas vezes realizado apenas com o uso de antihelmínticos, que consiste de um método oneroso para a produção. Além disso, esse helminto tem apresentado resistência cada vez maior a essas drogas (Winkelhagen et al., 2012; Brockwell et al., 2014). Diante desse quadro, pesquisas para o controle desses agentes têm sido desenvolvida, dentre elas, destacam-se a investigação para o desenvolvimento de uma vacina eficaz, o que não tem obtido êxito, e o estudo do controle biológico, que tem apresentado resultados animadores *in vitro* e *in vivo*. Os fungos helmintófagos tem se destacado como controladores biológicos, e esses agentes atuam nas formas de vida livre dos helmintos, onde se alimentam das formas jovens de ovos e larvas dos helmintos e atuam de forma a prevenir as reinfecções dos animais (Stirling, 1991; Braga & Araújo, 2014).

Três grupos de fungos atuam de diferentes formas sobre helmintos: os endoparasitos, que se alimentam das porções internas dos helmintos e apresentam a desvantagem de depender dos helmintos no meio para se propagarem. os fungos predadores predam as larvas de helmintos no meio e os capturam através de formação de diversas armadilhas e; os fungos ovicidas ou oportunistas que apresentam atividade sobre ovos de helmintos parasitos de animais. Os dois últimos grupos de fungos são constituídos por representantes que produzem formas de propagação resistentes: os clamidósporos (Stirling, 1991; Braga & Araújo, 2014).

Para o controle da *F. hepatica*, na qual a fase de vida que está presente no meio por um período considerável é o ovo, um fungo ovicida seria o mais indicado para o seu controle. E fungos ovicidas *Pochonia chlamydosporia* tem se destacado (Braga et al., 2007, 2008; Araújo et al. 2008). Esse fungo apresenta eficácia satisfatória *in vitro* no controle de ovos de *F. hepatica*, o que não foi verificado ao observar a ação de fungos predadores como *Duddingtonia flagrans* e *Monacrosporium sinense*, que são eficazes em predar helmintos em sua forma larvária de vida livre e são usados nesses ensaios como controle negativo (Braga et al., 2008).

Resultados promissores têm sido obtidos em ensaios realizados com o emprego desses fungos sobre ovos e larvas de diversos helmintos, *in vivo* e *in vitro*, no sentido de se investigar a eficácia da ação desses fungos helmintófagos (Waller et al. 1994; Araujo et al., 2008; Braga et al., 2007; 2008; 2011; Braga & Araújo, 2014).

2. REVISÃO DE LITERATURA

A *Fasciola hepatica* é um helminto de distribuição mundial pertence ao filo Platyhelminthes, classe Trematoda, ordem Digenea e possui como hospedeiros intermediários moluscos dulcícolas da família Lymnaeidae. Morfologicamente ela é constituída por tegumento, musculatura, sistema nervoso, citoesqueleto, intestino e sistemas excretor e reprodutivo (Dalton, 1999).

No Brasil, a prevalência desse agente é registrada, principalmente, nas regiões Sul (regiões de fronteiras do Rio Grande do Sul, Vale do Itajaí, Santa Catarina e parte do estado do Paraná) e no Sudeste (no Vale do Rio Paraíba do Sul em partes do sul de Minas Gerais, Nordeste do estado de São Paulo e oeste e noroeste do estado do Rio de Janeiro e sul do Espírito Santo) (Queiroz et al., 2002). Nessas regiões, esse agente é responsável por uma parte considerável das perdas produtivas em criações de bovinos e ovinos. Dentre os prejuízos mais frequentes causados pela *F. hepatica* podem ser incluídos: a diminuição na produtividade, gastos com tratamento, mortalidade, descarte de fígados no abate, além de ser um risco à saúde pública, causando a mesma sintomatologia no homem (Oliveira, 2008; Lima et al., 2009). A destruição hepática causada pelas larvas de *F. hepatica* leva à hepatomegalia, à diminuição das proteínas plasmáticas e acúmulo de líquido nas cavidades (Honer, 1979; Anderson et al., 1999).

O ciclo de *Fasciola*, no hospedeiro definitivo, inicia-se com a deposição de ovos pelos adultos no ducto biliar. Os ovos, uma vez liberados no meio e sob condições climáticas favoráveis (temperatura e umidade altas), liberam o miracídio que procuram e penetram nos hospedeiros intermediários – moluscos aquáticos Lymnaeidae. No Brasil, os limnídeos mais importantes na transmissão de fasciolose são: *Lymnaea columela*, *L. viatrix*, *L. cubensis* (Oliveira, 2008). Nesses moluscos, ocorre a multiplicação e desenvolvimento do miracídio, que resulta na formação do esporocisto, um saco alongado formado por células germinativas, que dão origem às rédias e que se diferenciam até a fase de cercária. As cercárias saem do molusco e encistam em vegetações, passando a metacercária. Geralmente, ruminantes e outros animais silvestres ingerem as metacercárias que penetram no trato digestório e se alojam nos hepatócitos, onde se desenvolvem e, após isso, maturam e atingem os ductos biliares (Dalton, 1999).

A *F. hepatica* possui grande capacidade de dispersão, pois é capaz de se adaptar a novos hospedeiros e ao meio. Além disso, cada espécime presente no ducto biliar é capaz de ovipor até 20 mil ovos por dia (Mas-Coma et al., 2005). Embora a *F. hepatica* possa ser capaz de infectar os mamíferos terrestres em geral, ovinos e bovinos são as espécies mais susceptíveis à infecção causada por esse agente, onde as taxas de morbidade e mortalidade nessas espécies variam de região a região, e em áreas endêmicas não é raro encontrar taxas de infecção superiores a 50% (Ross et al., 1997). A helmintose causada por esse agente apresenta duas fases, a primeira, aguda, caracteriza-se por destruição dos hepatócitos quando as formas jovens do parasito invadem e migram pelo parênquima causando hemorragias, hematomas, rupturas e inflamação com posterior necrose e destruição do tecido hepático. Nessa fase, podem ocorrer debilidades repentinas, inapetência, dor à palpação na região hepática, perda

de peso, ascite e mortes e hipoproteinemia, essa fase é mais marcante entre ovinos, e branda entre bovinos (Anderson et al., 1977). E fase crônica, quando os helmintos estão nos ductos biliares e se caracteriza por perda de peso, emaciação, edema submandibular, anemia, debilidade, diarréia e ascite (Dalton, 1999; Acha & Szyfres, 2003).

A *F. hepatica* penetra em caramujos do gênero *Lymnaea* spp., e os tem como seus hospedeiros intermediários (Honer, 1979) e a sua permanência numa região está determinada pela presença desses caramujos nesse local (Pile et al., 2001), além de ser importante também à presença de hospedeiros definitivos apropriados, em geral, bons propagadores de ovos para o meio, como os ruminantes e alguns herbívoros silvestres (Honer, 1979). De acordo com Coelho & Lima (2003), esses caramujos dulcícolas são influenciados por duas fases climáticas: uma estação seca e menos úmida, em que esses se encontram em estarvação; e a outra, chuvosa, que se forem intensas, e houver a presença de áreas não drenadas com ocorrência de inundações, contribuem para a rápida multiplicação e rápida dispersão desses agentes. Ainda, segundo os mesmos autores, o aumento da densidade populacional contribui para o aumento da taxa de infecção dos moluscos pelos miracídios.

Considerando que a *F. hepatica* é responsável por perdas significativas na pecuária e que as perdas estão associadas à carga parasitária, a comparação e o aperfeiçoamento de técnicas de diagnóstico de fasciolose devem ser realizado com a finalidade de se indicar a técnica mais sensível é essencial, uma vez que, essa técnica poderia representar de forma mais confiável a carga parasitária nos animais e indicar se o manejo está ou deverá ser realizado de forma eficaz. Dentre aquelas mais empregadas destacam-se a de sedimentação simples, a de *flukefinder*, a de Hofmann et al., a de quatro tamises e a de Dennis et al. (Ueno & Gonçalves, 1998; Anderson et

al., 1999; Kleimann et al., 2005). As técnicas sorológicas apresentam a vantagem de detectar prévias infecções nos animais (Duménigo et al., 2000). Porém, de acordo com Kleimann et al. (2005), os testes imunológicos não são capazes de indicar a carga parasitária, nem o número de ovos que saem nas fezes que são os responsáveis pela dispersão do agente.

É imprescindível que se conheça as interações que determinam o parasitismo por helmintos para se estabelecer um sistema de controle. Por isso, conhecer as condições climáticas e as possibilidades associadas à transmissão de *F. hepatica* representa uma ferramenta auxiliar para aperfeiçoar o controle e profilaxia desse helminto (Araújo et al., 2004). Acha & Szyfres, (2003), por exemplo, relatam que as condições climáticas são essenciais no mecanismo de contaminação dos caramujos por larvas e de animais por metacercárias. Malek (1980) observou que em algumas regiões ocorrem surtos graves de fasciolose depois que as chuvas de fins de verão ocorrem.

Alguns meios de controles parasitários alternativos têm sido apresentados pela comunidade científica e dentre elas destacam-se o controle biológico e fitoterápicos (Waller & Faedo, 1996). As medidas que adotam apenas o uso de vermífugos são responsáveis pela rápida instalação de resistência pelos parasitos, e isto tem contribuído para que as pastagens estejam na maioria das vezes contaminadas por esses agentes (Waller & Faedo 1996; Sangster, 1999). Em relação à *F. hepatica*, tem sido registrado, em algumas regiões do mundo, resistência contra anti-helmínticos mais eficazes no mercado como triclabendanzole, tanto em bovinos e ovinos (Brockwell et al., 2014) quanto em humanos (Winkelhagen et al., 2012). Além disso, ocorrem problemas com o alto custo de aquisição desses fármacos e a possibilidade de ocorrência de resíduos químicos no ambiente e nos produtos de origem animal,

além das dificuldades técnicas e econômicas para o desenvolvimento de novas moléculas (Waller et al., 1994).

Os fungos nematófagos, atualmente chamados de helmintófagos, uma vez que os mesmos são também eficazes em predar formas de vida livre de outros grupos de helmintos além dos nematóides, compreendem diferentes tipos de fungos, que podem ser caracterizados como predadores, endoparasitas de nematóides e oportunistas ou parasitos de ovos (Braga & Araújo, 2014). Eles são cosmopolitas, ocorrendo em solos naturais, solos agricultáveis e em todos os tipos de matéria orgânica em decomposição (Kerry et al., 2008).

O fungo *Pochonia chlamydosporia* é oportunista, ovicida, classificado como pertencente ao reino Fungi, filo Ascomycota, subdivisão Pezizomycotina, classe Sordariomycetes, ordem Hypocreales e família Clavicipitaceae. Possui como características morfológicas: as hifas que constituem seu micélio de forma septada e produzem esporos em um saco fechado, denominado ascósporo (Gams & Zare, 2001). Por ser um fungo ovicida e saprófito, não depende da presença de ovos de helmintos no meio constantemente para se manter (Kerry et al., 2008). Esse fungo é capaz de parasitar ovos de uma grande variedade de espécies de helmintos e de moluscos, sua variabilidade genética é considerada ampla. E esse fato poderia estar ligado à interação com seus diversos hospedeiros e estar associada à sua eficácia no controle biológico desses agentes (Kerry et al., 2008). Stirling (1991) e Braga et al. (2011) relatam que esse fungo é capaz de produzir uma grande quantidade de clamidósporos (esporos de resistência) e possui ampla capacidade de colonizar o ambiente onde está presente. A produção dessas estruturas foi verificada por Ferreira et al. (2011) *in vitro*, após a passagem desse fungo através do trato gastrintestinal de suínos. Braga et al. (2011) conduziram um ensaio experimental *in vitro* e observaram

que a partir do emprego de concentrações maiores de clamidósporos desse fungo sobre ovos de *Taenia taeniformis* houve maior eficácia.

O fungo *P. chlamydosporia* é adaptado a diversas condições climáticas (Santiago et al., 2006). Ele cresce bem em meios de cultura e em temperaturas médias de 24 e 32°C (Lysek et al., 1982). Assim, estes poderiam ser utilizados em regiões de diferentes condições climáticas sem comprometimento para seu crescimento (Santiago et al., 2006). De acordo com Lysek et al. (1982), esses fungos crescem bem em condições climáticas diversas e em condições tropicais e as atividades ovicidas deles são elevadas.

P. chlamydosporia produz uma estrutura de predação denominada apressório, o qual é capaz de penetrar em ovos de helmintos por pressão mecânica e ação enzimática de proteases (Segers et al., 1996). Fungos ovicidas podem apresentar três formas de interação com o ovo. A saber, o efeito tipo 1, na qual o fungo não penetra o ovo, mas pode parar o desenvolvimento do embrião ou permitir a produção de uma larva defectiva; o efeito tipo 2, na qual o fungo não penetra o ovo, mas a casca e o embrião são enzimaticamente danificados; e o efeito tipo 3, na qual o fungo (as hifas) penetram a casca, colonizam, crescem (ramificam) e destroem o ovo (Lysek et al., 1982). Além disso, Lysek & Sterba (1991) e Irving & Kerry (1986) relatam que esse fungo é capaz de produzir efeito ovicida satisfatório em todas as fases de desenvolvimento embrionário de ovos de helmintos.

O fungo *P. chlamydosporia* é promissor *in vitro* para o controle de helmintos com ovos como forma de vida livre predominante (Lysek et al., 1982; Lysek & Sterba, 1991; Braga et al., 2008). esse fungo apresenta atividade ovicida sobre os ovos de *F. hepatica* (Braga et al., 2008), mas, testes *in vivo* com esse fungo sobre ovos desse trematóide ainda não haviam sido testados até o presente momento.

A forma mais prática de se fornecer fungos para o controle biológico é pela administração oral aos animais (Araújo et al., 2004). Após a passagem pelo trato gastrintestinal e ser eliminado junto às fezes no meio ambiente, o fungo coloniza o bolo fecal, estabelece contato com os ovos e alimenta-se dos mesmos (Stirling, 1991). A avaliação desse evento é realizada por execução de testes com alguns isolados fúngicos, o que tem sido o foco de diversos estudos (Araújo et al., 2004; Braga et al., 2007; 2008; 2011).

Medidas alternativas como o emprego de agentes de controle biológico tem se destacado como uma ferramenta auxiliar que visa dar suporte para que se faça o controle de parasitos, quando o mesmo está presente no ambiente como forma de ovos, larvas ou no hospedeiro intermediário (Stirling, 1991). Os agentes de controle biológico atuam sobre os ovos e larvas, atenuando as reinfecções de forma a minimizar as perdas econômicas, sem impactos ambientais (Garcia et al., 2008).

2. OBJETIVOS

2.1 Objetivo Geral

Testar o fungo *Pochonia chlamydosporia* no controle de *Fasciola hepatica in vitro* e *in vivo*, comparar a sensibilidade de técnicas de sedimentação e avaliar a produção de ovos de *F. hepatica* em bovinos no sul do Espírito Santo.

2.2 Objetivos Específicos

Avaliar a viabilidade do fungo *P. chlamydosporia* em predar ovos de *F. hepatica* após a passagem pelo trato gastrintestinal de bovinos.

Testar a eficácia do fungo *P. chlamydosporia* em bovinos a campo no sul do Espírito Santo.

Comparar a sensibilidade de três técnicas de sedimentação para o diagnóstico de ovos de *F. hepatica* em amostras fecais de bovinos.

Avaliar a produção de ovos de *F. hepatica* em fezes de bovinos sob condições tropicais no sul do Espírito Santo em época de chuvas e de estio.

4. HIPÓTESES

O fungo ovicida *Pochonia chlamydosporia* preda ovos de *Fasciola hepatica* após a passagem pelo trato intestinal de bovinos.

O fungo ovicida *P. chlamydosporia*, administrado em formulação peletizada para bovinos, reduz a infestação nas pastagens das fases de vida livre de *F. hepatica*.

As técnicas modificadas de sedimentação de Dennis, Stone e Swanson, Foreyt e Quatro tamises de Girão e Ueno apresentaram diferentes sensibilidades nas pesquisas por ovos de *F. hepatica* em fezes de bovinos.

Fatores climáticos interferem na produção de ovos de *F. hepatica* em bovinos no sul do Espírito Santo comparando as estações seca e chuvosa.

COMISSÃO DE ÉTICA

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CAPÍTULO 1

Biological control of *Fasciola hepatica* eggs with the *Pochonia chlamydosporia* fungus after passing through the cattle gastrointestinal tract

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Abstract

Fasciolosis is a disease caused by *Fasciola hepatica* responsible for causing significative losses in livestock. This study aimed to evaluate the *Pochonia chlamydosporia* fungus (isolate VC1) on *F. hepatica* eggs after passing through the cattle gastrointestinal tract. For this evaluation, 1 g pellet was given in sodium alginate matrix per kilogram live weight containing 25% of fungal mycelium from isolate VC1 per animal. Twelve animals were used, six treated and six untreated (control). Some stool samples were collected from the groups of treated and control animals, at the times of 12, 18, 24, 48, 72, and 96 h after the pellets' administration. Then, from each stool sample of treated and control groups, 2 g was placed in a Petri dish of 9 cm in diameter, containing 2% water–agar and 1,000 eggs of *F. hepatica*. The fungus effectively preyed the eggs present in the samples starting at 12 h. Furthermore, differences were observed ($p<0.01$) in the destruction of eggs in the Petri dishes in the treated group compared to the control group. The ovicidal effect was observed after 7 days of interaction. The ovicidal *P. chlamydosporia* fungus was effective in destroying *F. hepatica* eggs; therefore, this fungus could be used in the biological control of the helminthes eggs.

1. Introduction

Fasciola hepatica has a cosmopolitan distribution, infecting all land mammals, especially domestic ruminants (Lima et al. 2009). Thus, fascioliasis is the major cause of losses mainly in some Brazilian coming weight gain redution, hypoproteinemia, anemia, and liver condemnation at slaughter (Ross et al. 1997; Dalton 1999; Echevarria 2004). One *F. hepatica* adult produces ten to 20,000 eggs per day expelled with the stool. A right humidity, temperature, and light conditions in the pasture, the miracidia hatch, as the initial larval forms that will infect the *Lymnaea* spp. freshwater mollusk (Dalton 1999). Humans and domestic ruminants maybe contaminate by water consumption. But, most infections in ruminants occur during the dry season, grazing in these areas, usually not anymore flooded, and become infected orally by ingestion of metacercaria - the infective form (Pile et al. 2001).

To decrease the *Fasciola* incidence, chemical control is one of the most-used measures; however, resistance and the cost of animal treatment, especially small ruminants, show the necessary some control alternative, especially of the present eggs in the environment (Echevarria 2004; Braga et al. 2008). On the other hand, mentioned that biological control appears as a viable tool in an economic and environmental form, presenting itself as an additional tool for the control of losses by the responsible agents, among them *F. hepatica* (Larsen 1999; Singh et al. 2010).

Biological control with ovicidal nematophagous fungi especially *Pochonia chlamydosporia* fungus has shown promising results in the control of eggs of various helminth genera in laboratory and natural conditions (Stirling 1991; Dias et al. 2007; De et al. 2008; Braga et al. 2010). *P. chlamydosporia* destroys helminth eggs through structures known as appressoria (Kerry and Hidalgo 2004). However, there are no

reports that mention this fungus's predatory ability on *F. hepatica* eggs after passing through the cattle gastrointestinal tract.

This study aimed to evaluate the *P. chlamydosporia* fungus effectiveness in preying on *F. hepatica* eggs after passing through the cattle gastrointestinal tract.

2. Materials and methods

2.1. Organisms

An isolate of *P. chlamydosporia* fungus (isolate CV1) from the mycology collection of the Laboratory of Parasitology, from the Veterinary Hospital of Federal University of Viçosa, was used. This isolate was maintained in test tubes at 4°C, the culture medium containing corn meal agar (2%).

2.2. Obtainment of *F. hepatica* eggs

F. hepatica eggs were obtained by dissecting of ten adult samples coming from slaughtered cattle, and their morphology was analyzed by light microscopy at ×10 objective, according to Dalton (1999). Then, the eggs were washed ten times in distilled water and centrifuged at 1,000×g for 5 min.

2.3. Animals

Twelve crossbred cattle with a mean age of seven months, weighing 110±7.63 kg, lairaged and previously dewormed orally with the abamectin drug 200 µg/kg and triclabendazole 10 mg/kg was used.

2.4. Experimental assay

Fifteen days after deworming, the animals were divided into two groups, one group treated with the fungus and one control group. Each group was composed of six animals. These animals received water *ad libitum* and commercial feed for cattle, consisting of 18% soybean meal, 71% corn bran, 8% sodium chloride, and 3% mineral salt. Hereafter, each animal in the treated group received orally, mixed in commercial feed, 1 g of pellets per kilogram of weight body containing 25% mycelial mass of the *P. chlamydosporia* fungus (VC1). The control group animals received the pellets without the fungus with the commercial feed.

Fecal samples (about 20 g) were collected during the periods of 12, 18, 24, 48, 72, and 96 h after the oral administration of the fungus in the animals from treated with the fungus and control (without the fungus) groups. After then, two grams of feces from each sample was removed, homogenized, and placed in a Petri dish 9 cm in diameter, containing 2% water–agar. One thousand *F. hepatica* eggs were poured on Petri dishes of the treated and control groups and were incubated at 26°C for a period of 35 days, according to the methodology by Braga et al. (2010). Six replications were performed in the treated and control groups for each studied period.

Everyday, the Petri dishes of both groups were analyzed and it was evaluated for identify structures research (typical conidia and chlamydospores) for the *P. chlamydosporia* fungus in accordance with the ID key Gams and Zare (2001). At the end of 35 days, approximately 100 eggs were removed from each Petri dish of treated and control groups (Araújo et al. 1995) and were later analyzed for the ovicidal activity in accordance with the guidelines established by Lysek et al. (1982), as described below: type 1 effect, in which one can observe the fungus adherence to

the eggshell, but without morphological damage; type 2 effect, in which morphological damage occurs in the eggshell and to the embryo without hyphal penetration; and type 3 effect, in which lytic effect occurs on the egg and to the embryo with hyphal penetration into the egg.

2.5. Statistical analysis

The data obtained from the experimental assay were subjected to the Friedman nonparametric test, at 1% probability level. For the study, it was used the Biostat 5.0 software (Ayres et al. 2007).

3. Results

The *P. chlamydosporia* fungus passed through the cattle gastrointestinal tract, with viability and ovicidal activity on the *F. hepatica* eggs. The results for the ovicidal activity (type 3 effect) (Table 1) showed the following percentages for ovicidal activity (type 3 effect): "12 (47.2%), 18 (31.6%), 24 (25.1%), 48 (36%), 72 (23.4%), and 96 h (23.2%)." *P. chlamydosporia* fungus (VC1) showed activity on *F. hepatica* eggs, after incubation for 35 days in the collected faecal samples from cattle at 12 h interval after administration of the pellets. Furthermore, typical structures of this fungus were observed (conidia and chlamydospores) in the Petri dishes from the treated group. Moreover, the nematophagous fungi growth was not observed in the Petri dishes from the control group. The eggs' destruction showed the highest percentage for the type 3 effect was found on the plates containing the animal feces samples from the treated at 12 h interval (47.2%). However, fecal pellets were recovered from the treated animals until 96 h of.

A difference in destructed eggs ($p<0.01$) was observed at the end of 35 days on Petri dishes in the group treated with the fungus (VC1) compared with the dishes in the control group. Colonized eggs were found and later destroyed by the *P. chlamydosporia* fungus (VC1) as showed by light microscopy, x40 objective.

A comparison between the times of 12 until 96 h was also performed for the type 1, 2 and 3 effect. It was found that the samples collected during the period of 12 h showed difference in the type 3 effect compared to samples collected at 24, 72 and 96 h ($P<0.01$). Comparisons between type 1 and 2 effects in the times 12 h until 96 hours was not showed difference ($P> 0.01$).

4. Discussion

Infective forms (eggs and/or larvae) of gastrointestinal parasites, helminths, are present in the contaminated environment, causing recurrence infection in domestic animals, especially in domestic ruminants (Waller et al. 1994; Dias et al. 2007; Tavela et al. 2011). After passing through the cattle gastrointestinal tract, the *P. chlamydosporia* fungus grew, germinated, and destroyed the eggs of *F. hepatica* in vitro. To authors suggest the applicability of the *P. chlamydosporia* fungus as an alternative control to the *F. hepatica* eggs in the infected cattle feces under natural conditions. This population control would the helminth cycle, reducing reinfection of the snails and animals.

The ovicidal activity (type 3 effect) was registered and the *P. chlamydosporia* maintained its viability until the end (35 days). The formulation efficacy after an interval of 96 h suggests that this fungus, can be applied at intervals of twice a week to the animals. However, there is a lack of studies that aim to identify what would be

the best interval for the ovicidal fungi to make use in biological control of gastrointestinal helminthiasis, and this is the first report.

P. chlamydosporia fungus can be used in biological pest control (García et al. 2004). Moreover, chlamydospore production by fungi is essential to its spread after stressful conditions (Terrill et al. 2004). This fungus was effective in going through horses' gastrointestinal tract, keeping its ovicidal activity (Braga et al. 2010). Comparing our work with the records of the above work, it is noted that the results were similar, allowing to evaluate some points: "(1) in the two studies, *P. chlamydosporia* structures (chlamydospores) were observed present in stool samples on Petri dishes for the treated group animals; (2) both results mentioned the ovicidal activity from the interval of 12 h; (3) pellets were recovered in stool samples of the treated group animals until the last studied interval." These comparisons are justified because the transit time physiologies among cattle and horses are different, suggesting that the *P. chlamydosporia* fungus can be a potential biological control of gastrointestinal helminthiasis of domestic animals.

It can be observed that the samples collected at 12 h showed major percentual of the type 3 effect than those collected at 72 and 96 h, and this tendency can to be confirmed by fact that samples collected at 24h also have a lower percentage of type 3 effect on the eggs of *F. hepatica* compared to those samples collected at 12 h. It is noteworthy that the type 3 effect is what is considered by Lysek et al. (1982) as the true ovicidal effect.

Also, work, it was observed that at the end of 72 h, 28.4% of *Oxyuris equi* eggs (equine nematodes) were destroyed (Braga et al. 2010). Moreover, in the same interval (72 h), the *P. chlamydosporia* fungus preyed 23.4% of the *F. hepatica* eggs. This information is relevant, since there are morphological differences between the

helminthes eggs of the domestic animals that justify the difference in the action of this fungus's ovicidal activity (Braga et al. 2008, 2011; Araujo et al. 2009).

The period required for egg hatching and subsequent miracidium release from *F. hepatica* is around 2 weeks with favorable weather conditions (Dalton1999). In this study, the *P. chlamydosporia* ovicidal activity on eggs occurred after 12 h, so it is suggested that the fungus can be an effective biological control, contributing to the reduction of environmental contamination by this trematode eggs, since it is supposed that some of these eggs will be in the stool.

Moreover, it is suggested the stress from the passage through the animals' gastrointestinal tract could be suggested as a stimulating factor for the chlamydospore production by the fungus. This event did increase the required time for the fungal growth (Braga et al. 2008).

The fungus's presence in the environment would reduce the freshwater mollusk infection, what is an additional of helminth control. Furthermore, the treatment with benzimidazoles, which has a micostatic effect on fungi, did not inhibit the proliferation of the *P. chlamydosporia* fungus after interaction with the anthelmintic (Singh et al. 2010). These facts are favorable when considering the *P. chlamydosporia* fungus introduction in a program of helminth strategic control.

Effective ovicidal action of the *P. chlamydosporia* fungus on trematode eggs, namely *F. gigantica* and *Gigantocotyle explanatum* under *in vitro* conditions (De et al. 2008). ovicidal action on *F. hepatica* eggs *in vitro* also obtained by Braga et al. (2008). The aforementioned authors did not realize the fungus's passage through the gastrointestinal tract of the domestic animals. However, the fungus made the passage through the cattle intestinal tract and presented some ovicidal effect.

P. chlamydosporia did not show any adverse effect on animals and, compared to some anthelmintics, also has the advantage of not having any residual effect on the animals' fauna and flora. The possibility that these agents act only on the parasites is an attractive and clean alternative.

This was the first report about the feasibility and ability of the nematophagous *P. chlamydosporia* fungus after passage through the cattle intestinal tract without loss of the ovicidal activity on the *F. hepatica* eggs. The percentage of the fungus ovicidal effect on the eggs in feces suggests for us to use this agent as a supplement form for the control of *F. hepatica* eggs.

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Table 1 Mean values and standard deviation of the ovicidal activity of the nematophagous *P. chlamydosporia* fungus (VC1) on the *F. hepatica* eggs in fecal samples collected at 12, 18, 24, 48, 72, and 96 h after the pellets' passage containing 25% of the *P. chlamydosporia* fungus mycelium for the treated and control groups (without fungal treatment) and recovered on Petri dishes on 2% water–agar with six replications for each sample, after storage for 35 days.

GROUPS			
Effect at 12 h	Effect type 1*	Effect type 2**	Effect type 3***
VC1	$15.6^{A} \pm 7,5$	$37.2^{A} \pm 5.7$	$47.2^{A,C} \pm 8.8$
control	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Effect at 18 h			
VC1	$31.5^{A} \pm 11.8$	$36.9^{A} \pm 8.2$	$31.6^{A,C} \pm 7.9$
control	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Effect at 24 h			
VC1	$33.\ 6^{A} \pm 4.5$	$41^{A} \pm 6.5$	$25.1^{A,D} \pm 6.3$
control	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Effect at 48 h			
VC1	$29.6^{A} \pm 6.6$	34.5 ± 5.8	$36^{A,C} \pm 6.9$
control	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Effect at 72 h			
VC1	$42.1^{A} \pm 9.6$	$34.4^{A} \pm 6.5$	$23.4^{A,D} \pm 7.4$
control	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Effect at 96 h			
VC1	$42.2^{A} \pm 9.4$	$34.6^{A} \pm 6.6$	$23.2^{A,D} \pm 7.4$
control	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$

Percentages followed by the same letter (A, B) in the same column are not different ($p<0.01$) by the Friedman test. The ovicidal fungus activity on the eggs was evaluated on Petri dishes on 2% water–agar, after cattle fecal samples plating, when the cattle were fed with the *P. chlamydosporia* fungus

[a] Lytic effect without morphological damage to the eggshell with the hyphal adherence in the shell

[b] Lytic effect with morphological alteration of the embryo and the eggshell without the hyphal penetration through the eggshell

[c] Lytic effect with morphological alteration of the embryo and the eggshell and the hyphal penetration in the internal colonization

CAPÍTULO 2

***Pochonia chlamydosporia* in the biological control of *Fasciola hepatica* in cattle in Southeastern Brazil**

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Abstract

Biological control with nematophagous fungi has been described as a complementary control method, for free-living forms of helminths. The efficacy of the fungus *Pochonia chlamydosporia* against *Fasciola hepatica* eggs in faeces was evaluated in the field. Two bovine groups (six animals each) were used: A (control) and B (treated with fungus). Thirty days after deworming, the animals were separated into two similar paddocks with flooded areas and were used pellets with 25% mycelial mass (group B) or no fungus (group A) at a dose of 1 g/10 kg body weight, twice a week, during 18 months. Faecal samples were collected fortnightly from the animals of groups A and B and they were submitted at examination of quantitative sedimentation. The mean count of *F. hepatica* eggs per gram of faeces was higher in group A (1.19) compared with those from group B (0.82) ($P<0.01$). After 18 months, animals from group B had gained 42.33 kg above (17.82% more by weight) ($P<0.01$), than those of the control group (A). Every month, faecal samples from paddocks A and B were collected and they were incubated. *P. chlamydosporia* was identified only in sample source of the paddock B. The application of this fungical formulation with *P. chlamydosporia* at 25% mycelial mass reduced the availability of eggs in the environment and reinfections in calves in natural conditions.

1. Introduction

The overuse of chemotherapy in helminthic control has resulted in the rapid development of resistance (Brennan et al. 2007). There are several reports of *Fasciola hepatica* isolate resistance in the field, including active ingredients considered among the most efficient for the control of this agent, such as triclabendazole (Fairweather 2005; Oliveira et al. 2008; McKinstry et al. 2009). On the other hand, many attempts have been made to control the presence of this agent, especially among ruminants. Also, some preventive measures have been taken that may include reuse of pastures by other cultures in flooded areas, monitoring slaughterhouses for determining dissemination focus and treatment with chemotherapy, attempts to introduce competitor mollusk or predators in wetland environments and attempts to introduce plants that secrete harmful substances to mollusks (Araújo et al. 2002). An attempt to introduce a vaccine for the control of this agent has also been the subject of study (Smith and Zarlenga 2006).

Due to the increased incidence of *F. hepatica* in some Brazilian regions, the first reports in areas in which previously it had not been found in ruminants (Echevarria 2004), and the zoonotic potential, concern about this agent has increased. According to the World Health Organization, this zoonosis is considered emergent, and, according to Mas-Coma et al. (2005), Latin America is the region where the largest number of human cases in the world is observed.

Thus, alternative control measures have been the subject of study by several research groups. Biological control with the use of nematophagous fungi has been described very successfully by several authors (Waller et al. 2004; Araújo et al. 2006; Braga et al. 2008) and presents itself as a complementary control method, acting on the free-living forms of helminths. The use of ovicidal fungi, i.e. those able

to prey on helminth eggs, for example, *F. hepatica*, have shown satisfactory results *in vitro* (Braga et al. 2008; Dias et al. 2012), and this fungus is a good candidate for controlling *Ascaris suum* eggs *in vitro* (Araújo et al. 2008); however, there are no reports of the action of this agent under natural conditions. Thus, the fungus *Pochonia chlamydosporia*, that is capable of passing through the gastrointestinal tract and has ovicidal effect, was tested by this study in the field aiming to assess its ability to reduce the reinfection level of cattle raised in an environment with natural infection by *F. hepatica*.

2. Materials and methods

2.1. Organisms

A VC1 strain of *P. chlamydosporia* from the mycology collection of the Laboratory of Parasitology, Department of Veterinary Medicine, Federal University of Viçosa, Minas Gerais State, Brazil. This fungus was isolated from the soil of pasture in the municipality of Viçosa. This strain has been maintained through continuous transfer in test tubes at 4 °C, containing the medium culture 2% corn meal agar.

2.2. Animals

Twelve crossbred calves with a mean age of 7 months and an average weight of 110.10±7.63 kg were used. They were dewormed orally with a formulation based on abamectin (200 µg/kg body weight) and triclabendazole (10 mg/kg body weight) (Avotan Fasciola, Intervet- Schering Plough®), thirty days before the experimental assay was to be carried out.

The project was reviewed and approved by the Commission on Animal Experimentation of the Federal University of Viçosa (no. 052). About the number of calves involved, according to the guidelines proposed by the World Association for the Advancement of Veterinary Parasitology (Wood et al. 1995), as well as other requirements, the minimum number is six animals per group to test a drug's effect and its effectiveness on parasites of domestic animals.

2.3. Experimental assay in pasture

At 30 days after deworming, the animals were separated and isolated into two groups (the criteria of division was body weight), each one with six animals, a control group (group A) and a group treated with fungus (group B). The paddocks of the animals in groups A and B were similar in size (47,500 m2) and topographic character and shaped by *Brachiaria decumbens* pasture. The paddocks were partially flooded (about 10 % of area, 7 % of area during rainfall season and 15–20%, during dry season). In these paddocks before the trial period, cattle had been kept and they had been infected by *F. hepatica*, as confirmed by the presence of adults of *F. hepatica* in animals slaughtered with a parasite burden similar to animals from paddocks A and B. In both paddocks, there was a presence of lymnaeids in the pastures. These animals were kept in extensive farming system, received water *ad libitum* and were supplemented with commercial diets. Groups A and B were kept isolated from each other throughout the experimental period. The animals consumed commercial feed pellets for cattle, consisting of 18% of soybean meal, 71 % of corn bran, 8 % of sodium chloride and 3% of mineral salt formed by calcium, phosphorus and other micro- and macrominerals (Guabiphos 80 Corte, Guabi®). The animals in group B received a 1 g dose of pellets containing 25% of *P. chlamydosporia*

mycelium (VC1 strain)/10 kg body weight two times per week for a period of 18 months, according to procedures carried out by Dias et al. (2007). Pellets were made according to Walker and Connick (1983), as modified by Lackey et al. (1993). The same procedure was performed on animals of group A, but the pellets did not contain fungal mycelia. Pellets were previously tested to verify the growth of *P. chlamydosporia*. The treatment was offered for 18 months starting from July 2009.

Faecal samples were collected directly from the rectum of these animals, every 2 weeks, in amounts sufficient to perform stool examinations using the modified qualitative–quantitative sedimentation technique of Dennis, Stone and Swanson described by Ueno and Gonçalves (1998). Samples were analysed to quantify the eggs of *F. hepatica*.

The animals were weighed monthly and their pellet dosage containing fungus (group B) or no fungus (group A) was corrected according to their weight gain.

Monthly mean, minimum and maximum temperatures, rainfall and relative humidity were collected at a specialized weather station in the municipality of the Cachoeiro de Itapemirim, Espírito Santo State, Southeastern Brazil (20°40′60.00″ S, 41°12′0.00″ W) and were obtained according to the Food and Agriculture Organization-recommended method of Penman-Monteith (Allen et al. 1998).

2.4 Collection of faecal samples from the paddock pastures

Eight faecal samples were collected from paddocks where animals from groups A and B remained. These samples were about 20 g and they were collected monthly and randomly, for 18 months. They were sent to the laboratory, they were homogenized and they were lightly moistened. From them, 2 g was removed and plated on 2% water–agar and incubated at 26°C for 15 days. These procedures were

conducted according to Larsen et al. (1994). Pellets were also sought in these faecal samples and, when they were found, they were processed in a Petri dish containing 2% water–agar and were also incubated. Faeces were collected from pastures and incubated with the aim only to observe whether there was the presence of structures of *P. chlamydosporia* identified in the dishes and to count the number of dishes in which it was possible to observe the recovery of the structures of this fungus.

2.5. Fungus identification in incubated faecal samples and quantification

After the incubation of faecal samples from the animals of groups A and B, fungus identification was performed according to the classification of Gams and Zare (2001), using a light microscope (×10 objective). The number of the dishes with structures of *P. chlamydosporia* recovered after incubation and the amount of these structures present in the dishes were considered and analysed.

2.6 Statistical analysis

Egg count per gram of faeces and the weight gain of animals from groups B and A were statistically analysed using a completely randomized design. Results were interpreted by variance analysis (F test) at $P<0.01$. Averages of egg count per grams of faeces and weight gain of animals were compared by Tukey's test at $P<0.01$. To carry out statistical analysis on the data, Biostat 5.0 software was used (Ayres et al. 2007).

3. Results

The average monthly temperatures ranged from 18 in July 2010 to 30°C IN January and February 2010, with a mean of 25.05±3.46°C. The minimum temperature was 11°C in June 2010 and the maximum, 37°C in February 2010. The mean monthly relative humidity was 67% in June 2010 to 82% in December 2010 with a mean of 71.11±4.31%. The monthly rainfall ranged from 12 mm^3 in June 2010 to 580 mm^3 in December 2010, with a mean of 174.78±156.21 mm^3.

3.1. Egg count per gram of faeces

Mean number of *F. hepatica* eggs per gram of faeces showed after 18 months was in the Figure 1. Faecal sample collections from animals were every 15 days and eggs in the faecal sample was not observed in the first 2 months. The egg number per gram of faeces of group A (1.19±0.94) was higher ($P<0.01$) than that of group B (0.82±0.87). In the final of experimental assay (last 4 months), mean count of *F. hepatica* eggs in treated group (B) was 67% lower than those of control group (A). There were difference among the mean count of eggs per gram of faeces in the treated group (B) compared to the control group (A) with a reduction In July, 47.26; August, 50.19; September, 75.41; October, 100.00; November, 43.07; and December (2010), 61.35%.

3.2. Monthly mean of animal weight gain

The monthly mean weight of the animals (Fig. 2) is showed in both groups (A and B), of animals with weight gain after 18 months of the experimental period. Animals from group A had a mean weight gain of 194.87±63.49 kg (154.60% in weight gain) and animals from group B had 237.17±82.62 kg (190.50% in weight gain). The

major differences of weight gain were in July (7.32%), August (7.61%), September (8.02%), October (9.10%), November (10.71%) and December in 2010 (12.60%); with differences between months. Animal weight gain was 42.33 kg (17.32% weight gain) major in animals from group B compared to ones from group A ($P<0.01$).

3.3 Faecal samples collected from the paddocks

Fungus was observed in faecal samples collected from paddock B at 15 days after the first application. The mean number of Petri dishes with *P. chlamydosporia* was 6.50 of eight dishes from the treated group (B). The presence of *P. chlamydosporia* was not observed in any samples from group A.

The fungus was recovered in all months from the paddock B. Some pellets administered to animals were recovered from groups A and B. After 15 days of incubating in an oven at 26°C, pellets recovered from faecal samples collected from paddocks of the group B showed a positive result for *P. chlamydosporia*. However, this fungus did not grown in Petri dishes from the pellets recovered in samples collected from the paddocks of group A.

Larger amounts of fungal structures of *P. chlamydosporia* were recovered in Petri dishes incubated at 26°C containing 2% water agar and 2 g of stool and they were observed in microscopy (objective 10x). From the 7th to 15th day, the structures of *P. chlamydosporia* were identified in the dishes of the samples cultivated and derived from the paddock of the group B.

The mean number of Petri dishes in group B with the fungus increased throughout the period. At first, the mean was two dishes positive (33.33% of samples) to the specific presence of structures of *P. chlamydosporia*, and at the end of the test, *P. chlamydosporia* was recovered from all eight dishes (100%).

4. Discussion

In the present work, the efficacy of *P. chlamydosporia* on *F. hepatica* eggs after passaging through the gastrointestinal tract of cattle under field conditions was evaluated in faecal dung. There was difference of mean number of eggs per gram of faeces in the treated group (B) compared to the control group (A) ($P<0.01$). It was more pronounced at the end of experimental period. The nematophagous fungus reduced *F. hepatica* eggs in the dung, and consequently, decreased eggs survived. The fungus can reduce number of *F. hepatica* eggs in faecal sample of cattle after close to one year, i.e. in the last 6 months of experimental period.

P. chlamydosporia parasite helminth eggs of domestic animals, with effective control. This fungus showed satisfactory percentage of *F. hepatica* egg destruction in Petri dishes with 2% water–agar (Braga et al. 2008). *P. chlamydosporia*, *Duddingtonia flagrans* and *Monacrosporium thaumasium* fungi were tested on *Dipylidium caninum* eggs and only the first fungus showed good ovicidal effect (Araujo et al. 2009). As obtained with *P. chlamydosporia* employed on *A. suum* eggs (Araújo et al. 2008). Then results are promising because a decrease in the mean number of *F. hepatica* eggs per gram of faeces was observed ($P<0.01$). The evaluation of ovicidal activity of *P. chlamydosporia* fungus after passage through the gastrointestinal tract of cattle on helminth eggs was observed on *F. hepatica* eggs (Dias et al. 2012). this fungus was effective after passaging through the horse gastrointestinal tract, keeping their ovicidal activity against eggs of *Oxyuris equi* (Braga et al. 2010).

In addition, the animals showed difference in weight gain ($P<0.01$) and, in the last 6 months, showed high values for those treated with the fungus (group B). In the same period, a difference among number of *F. hepatica* eggs per grams of faeces

compared the treated group (B) and control group (A). Dias et al. (2007) obtained differences in weight gain, with formulations of *D. flagrans* on nematode larvae under field conditions. Similar results were obtained by Fontenot et al. (2003) and Waller et al. (2004). However, no work using *P. chlamydosporia* ovicidal fungus had been made under field conditions. Thus, the present results are summarized as unprecedented and show the ability to effectively use this agent under field conditions.

The occurrence of animal's infection by *F. hepatica* is higher during winter in southeastern Brazil, when flooded pastures from the rainy season (summer) dry and become of good quality, although leaving the pasture contaminated by metacercariae (Amato et al. 1986). The experimental period consisted of two drought periods and a prolonged rainy period, what say made possible to evaluate the effect of seasonal variation on this agent. Thus, the monthly rainfall index is a major differential to infection occurrence (Dalton, 1999). And that minimal rainfall per month to maintain the *F. hepatica* life cycle (Fox et al. 2011). The monthly rainfall ranged from 12 mm^3 in June 2010 to 580 mm^3 in December 2010, wich flooded some of the pastures, allowing the natural infection by *F. hepatica*. This climatic factor favoured pasture contamination by *F. hepatica* larvae (Dalton 1999; Fox et al. 2011). Amato et al. (1986) mention that regular rainfall and an average temperature around 25°C are required in order for *F. hepatica* eggs to develop feasibly in faeces.

In the initial months of this trial, the absence of *F. hepatica* eggs is justified by the following: first, the animals introduced in the area were free from this trematode; second, the lifetime required for it to become a parasitic form of helminth is longer than 45 days; and third, this experiment began at the time in which the animals usually become infected in pastures (dry season).

It was verified that from the 7th until the 15th day, *P. chlamydosporia* were identified in the dishes of the samples cultivated and derived from the dung in pasture with animals of group B. This condition indicates that the same animals decreased the release of *F. hepatica* eggs in their faeces, and this fungus remained in organic matter.

After incubation of stool samples, there were a greater number of Petri dishes with *P. chlamydosporia* on plates containing fungical structures after the first months of the application. In the beginning, the mean was only two dishes positive to the specific presence of structures of *P. chlamydosporia*, and at the end of the test, it was possible to recover this fungus on all dishes. Moreover, it was verified an increased amount of number of fungal structures on the dishes. This observation supports the premise that the presence of helminth eggs is not necessary for the fungus to develop in the environment.

This was first report of *P. chlamydosporia* fungus application *in vivo* conditions and with effectively reducing cattle infection. Thus, the pellet formulations applied to cattle twice a week at a dose of 1 g/10 kg body weight can effectively reduce the bioavailability of *F. hepatica* eggs in the environment.

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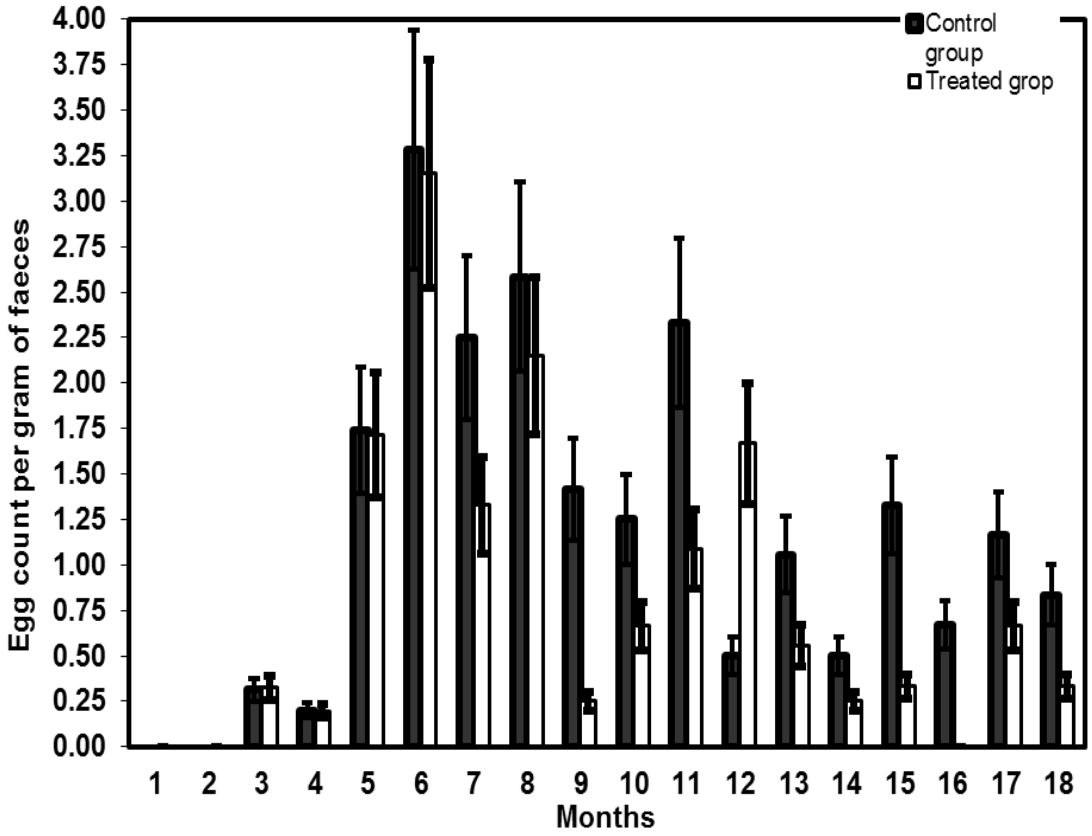

Fig. 1. Monthly mean number of *F. hepatica e*ggs and standard error per gram of faeces. Of the egg number of *F. hepatica* per gram of faeces from the animals in the control group (A) and treated group (B) with the *P. chlamydosporia* ovicidal fungus, collected from July, August, September, October, November and December (2009) and January, February, March, April, May, June, July, August, September, October, November and December (2010), in the municipality of Cachoeiro de Itapemirim, Espírito Santo State, Brazil.

Significant difference ($P<0.01$) between the treated group (A) and the control group (B).

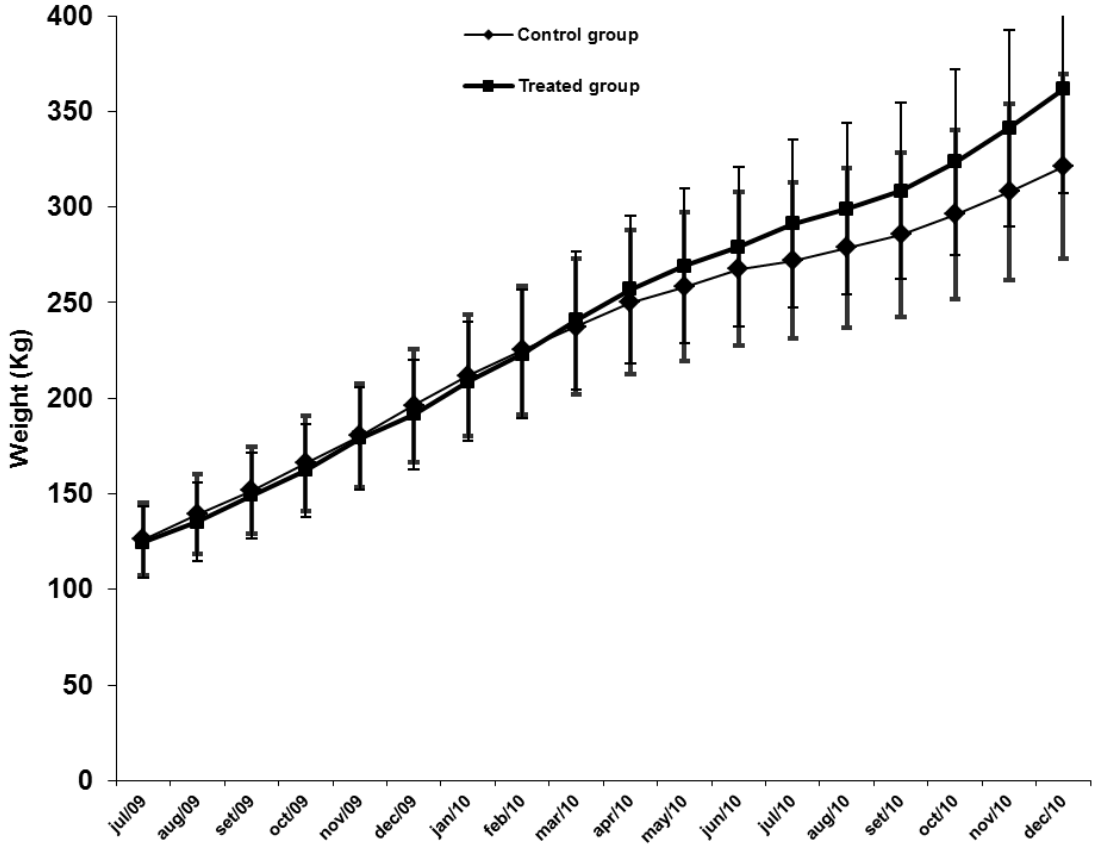

Fig. 2. Monthly mean weight and standard error (in kilograms) of the animals in the control group (A) and the group treated with the *P. chlamydosporia* ovicidal fungus (group B), from July, August, September, October, November and December (2009) and January, February, March, April, May, June, July, August, September, October, November and December (2010), in Cachoeiro de Itapemirim municipality, Espírito Santo State, Brazil.

Significant difference ($P<0.01$) between the treated (A) and the control groups (B).

CAPÍTULO 3

Comparison among three techniques in the parasitological diagnosis of *Fasciola hepatica* in cattle

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Abstract

Helminthiases are responsible by substantial economic losses. Many techniques have been developed to facilitate parasitological diagnosis and comparisons among these techniques are essential. The fluke worm *Fasciola hepatica* causes losses around the world in rearing of ruminants and it is zoonosis. The parasitological examinations through faecal samples showed low sensibility, for this, it is necessary to develop more sensitivities technique to diagnostic *F. hepatica*. The present work aimed to compare three techniques of quantitative sedimentation for parasitological diagnosis of *F. hepatica* in cattle. Faecal sample was collected from the rectum of 12 cattle fortnightly, during June to December, 2010. Faecal sample were submitted to three technique of parasitological sedimentation diagnostic of *F. hepatica* eggs: the modified sedimentation techniques of Dennis, Stone and Swanson (DSS), Girão and Ueno (*quatro tamises* - QT; four sieves) and Foreyt were compared using analysis of variance, linear regression and correlation tests. Evaluations of sensitivity, specificity and agreement were performed using the kappa test. The modified DSS technique had a high mean egg count and high sensitivity (p<0.01) (48.60%). The three techniques showed a specificity of 100%. There was no correlation among the values of egg count obtained by the three techniques, and significant coefficients were not observed by regression analysis. The egg number of *F. hepatica* with the techniques of *quatro tamises* and Foreyt showed excellent concordance by the kappa test. The modified DSS technique appeared to be the most effective for *F. hepatica* diagnosis in cattle (p<0.01).

Keywords: sedimentation quantitative techniques, diagnostic, eggs, trematode, ruminants.

1. Introduction

Fasciolosis caused by the trematode *F. hepatica* is a common zoonotic disease that affects cattle and sheep in endemic areas (Echevarria 2004, Faria et al. 2005) and is considered by the World Health Organization as an emerging disease (Mas-Coma et al. 1999). The economic losses by *F. hepatica* in cattle and sheep a are meaningful (Cringoli et al. 2002; Faria et al. 2005). Previous studies of prevalence indicate an increase in dispersion and incidence of this agent in endemic areas (Mas-Coma et al, 1999, Echevarria 2004, Dorchies 2007, Lima et al. 2009). These events probably are due to the dispersion of the intermediate hosts (lymnaeids aquatic mollusks) of this agent in some regions (Coelho, Lima 2003).

Due to chemical treatment to reduce the frequency of these agents among animals, the identification of this agent's presence in herds by parasitological examination of the animals' faeces is often masked, and early diagnosis is essential so that production losses are reduced. Despite, diagnosis often occurs the animal's slaughter; however, parasitological techniques of counting eggs in faeces are the most widely used, because it is practical and inexpensive. However, the sensitivity of the most commonly used parasitological techniques, such as the Dennis, Stone and Swanson (DSS) method (Dennis et al. 1954) *quatro tamises* Girão and Ueno (1985) (QT) (four sieves in portuguese), is around 30% (Reichel 2002; Dorchies (2007). Therefore, attempts to improve existing techniques or their implementation and studies to review them would be very valuable.

Many efforts have been made to increase the sensitivity of these parasitological diagnostic techniques, since they would be easier to perform and they would be low cost, are carried out effectively and do not require expensive reagents,

that increasing production costs. The present study aimed to compare the sensitivity of three sedimentation techniques for *F. hepatica* diagnosis in cattle faecal samples.

2. Materials and Methods (in annex)

2.1 Collection of samples from animals

A total of 12 animals crossbred with an average age of 2 year 3 months were reared in a paddock. These animals had been initially free of *F. hepatica*, they were previous tested through parasitological technique to detect *F. hepatica* eggs and, they were purchased of a region free of *F. hepatica*. For 16 replications, fortnightly, during June to December 2010, faecal samples were collected directly from the animals' rectum with a plastic bag, chilled and then sent to the laboratory of Parasitology, Federal University of Viçosa, totalling 192 samples e 576 examinations. The samples were collected from cattle reared in a farm in Cachoeiro de Itapemirim municipality (Espírito Santo State), 20° 40' 60.00"S, 41° 12' 0.00"W. The samples were subjected to three diagnostic techniques for *F. hepatica*, as described the following. To each technique were used 2 grams of faeces.

2.2. Techniques used in parasitological diagnosis

2.2.1 Modified technique of Dennis, Swanson & Stone by Belém et al. (1992)

Samples were subjected to a sedimentation technique modified by Belém et al. (1992) that was first described by Dennis, Stone and Swanson (Dennis et al. 1954), which aims to determine and to quantify eggs of *F. hepatica* through observation, of the entire content filtered, in 10x objective (100x increase). **(in annex)**

2.2.2 Girão–Ueno filtration and sieving technique

The samples were also evaluated by the QT modified technique as described by Girão and Ueno (1985), in which methylene blue is added to the pellet in a Petri dish, and then the reading is performed in a stereomicroscope increased 50 times (in annex).

2.2.3. Modified sedimentation technique

This technique was described by Foreyt (2001), in which the sediment is poured into a Petri dish and examined under a light microscope with 5x objective (in annex).

2.3. Obtaiment of *Fasciola hepatica* adults

After period experimental, cattle were slaughtered and the liver were separated specifically, the trematodes in biliar duct were collected and identified according to Foreyt (2001) (in annex).

2.4. Statistical analysis

Data were compared for egg counts of *F. hepatica* per grams of faeces obtained from the modified techniques of DSS, QT and Foreyt. Statistical analysis was performed by variance analysis (*F* test) at the 1% and 5% levels of probability. Mean of quantitative factors were compared by the Tukey test at the levels of 1% and 5%. Regression analysis was performed to identify the correlation among the results obtained by the three techniques. A test of sensitivity and specificity of the three techniques was performed. The kappa index was calculated to assess the degree of agreement of the presence of *F. hepatica* eggs in faecal samples through the

modified techniques of DSS, QT, and the modified sedimentation of Foreyt. All above statistical analyses were performed in the BioEstat 5.0 program (Ayres et al. 2007).

3. Results

Mean and standard errors of egg count per grams of faeces through three techniques are showed in Figure 1. In the table 1 is showed the number of adults of *F. hepatica* recovered in the bile duct of the animals after slaughter. In the table 2 displays the results of sensitivity testing of the three techniques for parasitological diagnosis. The three techniques showed 100% of specificity.

In the 16 different collections (192 samples) and examinations (576 through three techniques) of faecal samples performed, fortnightly, all animals showed to be positive count for *F. hepatica*. The modified technique of DSS had a mean of 0.82 *F. hepatica* eggs per grams of faeces and it ranged of 1 to 14; QT gave a mean of 0.04 and it ranged of 1 at 12, and the modified sedimentation method of Foreyt showed a mean of 0.028 and it ranged of 1 at 16. All techniques showed mode and median with value 0. A different number of eggs ($p<0.01$) was observed in the egg count obtained by the modified technique of DSS compared with counts obtained by the other two techniques. There were no differences between the number eggs verified through QT and modified Foreyt techniques ($p>0.05$).

The mean number of *F. hepatica* adults verified in biliar duct of slaughtered cattle was 1.75. In three animals, *Fasciola* adult in biliar duct was absence, and in positive animals, the presence of this agent ranged of 1 to 4 adults in biliar duct of the animals.

The correlation coefficients obtained in the linear regression showed no significance (p>0.05); thus the count eggs obtained by the three techniques of quantitative sedimentation did not follow a competitor standard of distribution. A weak linear correlation was observed.

A higher sensitivity of the DSS technique (48.60%) was verified, whereas the sensitivity obtained by the technique of QT was 15.30% and the modified sedimentation method of Foreyt was 20.80% (Table 2).

In the analysis of agreement, there was a proportion value of 0.7865 among the modified sedimentation techniques of DSS and QT, a value of 0.7448 among the modified sedimentation techniques of DSS and Foreyt, and a value of 0.9583 among QT and Foreyt. The expected concordance values were 0.6009, 0.6215 and 0.7656. The kappa index obtained to DSS and QT was 0.4549 (good replicability), to DSS and Foreyt was 0.3257 (weak) and to QT and Foreyt was 0.8222 (excellent).

4. Discussion

When using the three techniques for counting *F. hepatica* eggs, low values of eggs count per grams of faeces were found, and the technique of DSS gave the highest average (0.82). In this work, a low number of adult parasites in the bile duct of animals and low egg count in faecal samples were verified, i.e. the results were as expected. After the slaughter of these animals, an average of 1.75 adults of *F. hepatica* per animal was observed. The low parasite load in the animals in this experimental assay may account for the low sensibility of the techniques to detect *F. hepatica* eggs by parasitological examination of faeces (Acha, Szyfres 2003).

The technique of DSS possibly allowed the permanence of much debris at the bottom of the pellet, because the solution of aluminium potassium sulfate

[$KAl(SO_4)_2$] allows the sedimentation of less dense particles settling to the bottom of the cup. Moreover, this method has the advantage of reduced losses of egg losses per faecal sample.

Technique of Dennis et al. (1954) with the (unmodified) technique of QT (Girão and Ueno 1985) was compared in an assay by Mattos et al. (2009). They observed that the (unmodified) QT technique showed higher sensitivity than the (unmodified) DSS technique, but using faecal samples from sheep. Fasciolosis in cattle presents a profile of egg release different from that found in sheep. Cattle infected by *F. hepatica* had lower egg release than sheep and rodents (Cunha et al. 2007). Thus, under the conditions of the present study (for low parasite load), possibly the modified technique of DSS showed more reliable results.

Confirming the proposed by authors above cited, Reichel (2002) states that in regions where the incidence of *F. hepatica* is low, the sensitivity of diagnostic faecal tests is low (around 30%) in animals with low egg counts per grams of faeces. Anderson et al. (1999) reported a sensitivity of 66.70% for an egg-counting method, while the specificity was 100%. However, this assay was carried out in a region where the animals were found to be infested with about 100 *Fasciola* adults in the bile ducts after slaughter, whereas in the present study the average adult parasite number was 1.75, which probably contributed to the higher sensitivity than those obtained through the current work.

Therefore, although modified QT and Foreyt techniques showed a close mean, the regression between the two mean presented a very small correlation coefficient, which actually demonstrates that these two techniques showed a concordance that was not significant.

The kappa index between DSS and QT is classified as good replicability; the kappa value of 0.8222 was considered excellent, when studying the comparison between the QT and Foreyt techniques. On the other hand, between DSS and Foreyt the concordance is weak.

At least two replicates are carried out, as more reliable results can then be reported. The modified technique of DSS showed much greater sensitivity than the other two studied here, although it is low if compared with diagnosis by necropsy or even by slaughter; however, it allows early results and the indication of use of interventional measures.

Finally, when it is possible to compare the diagnostic techniques of *F. hepatica* in a quantitative manner in work in which QT and flukefinder techniques are compared, no difference in the results is obtained quantitatively (Faria et al. 2005; Kleiman et al. 2005). Furthermore, Duménigo et al. (2000) report that the immunological techniques present more sensitivity; however, they fail to detect active infections and do not report the number of eggs or predict the number of adult parasites that produce them.

5. Conclusion

The modified technique of DSS show greater sensitivity to identify cattle with *F. hepatica* infection than QT and Foreyt modified techniques.

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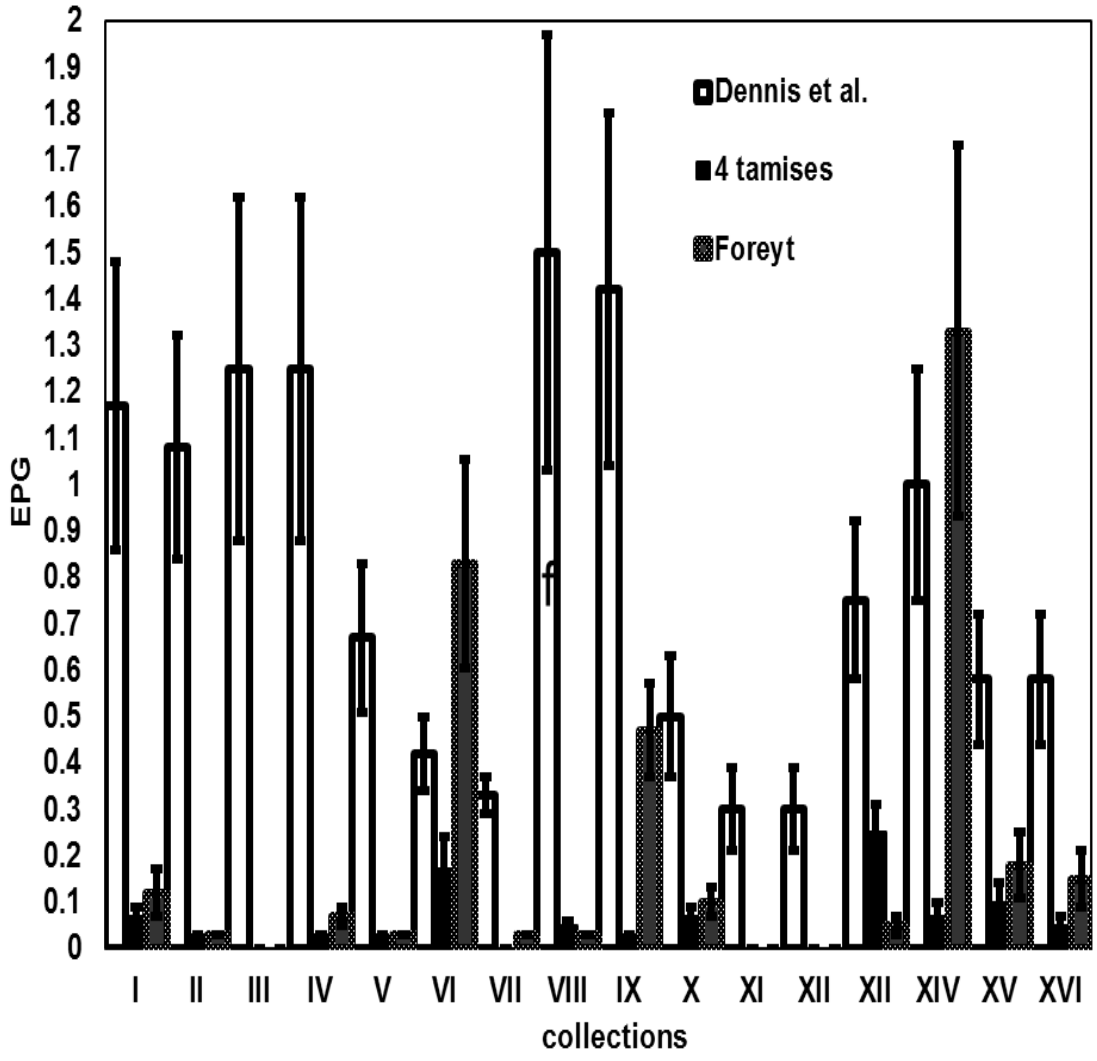

Figure 1 - Means and standard error of number *Fasciola hepatica* eggs per gram of faeces obtained by techniques of Dennis, Stone & Swanson modified (Belém et al. 1992), *quatro tamises* (four sieves) (Ueno and Girão, 1982), and sedimentation of Foreyt (2001). Samples were collected from 12 bovines with 16 repetitions, fortnightly. These samples were collected from these animals from June to December 2010. The experimental farm is at the municipality of Cachoeiro de Itapemirim, Espírito Santo State, southeastern Brazil.

Table 1 Number of *Fasciola hepatica* adults recovered from the bile ducts of cattle slaughtered after parasitological testing.

Animal	01	02	03	04	05	06	07	08	09	10	11	12
adult	2	1	2	4	0	3	2	0	3	2	2	0

Table 2 Sensitivity of parasitological examination of *Fasciola hepatica* eggs obtained through the modified techniques of Dennis, Stone & Swanson, *quatro tamises*, and sedimentation of Foreyt

	Samples		
	positive	**negative**	**total**
DSS	70 (48,6%)	74	144
4 tamises	22 (15,3%)	122	144
Foreyt	30 (20,8%)	114	144

CAPÍTULO 4

A experimental coprological survey of *Fasciola hepatica* in cattle in South of Espírito Santo State, Brazil

Abstract

This coprological survey aimed to evaluate the presence of *F. hepatica* eggs in faeces

of cattle under tropical climatic in the south of Espírito Santo State. Experimental coprological survey of *Fasciola hepatica* was carried out with cattle in a farm, during 12 months. Twelve cattle were stored in an area with natural transmission of *F. hepatica*. Coprological surveys in faecal sample of animals were carried out during October 2009 to September 2010. Fortnightly, faecal samples of these animals were collected and subjected to modified parasitological technique of Dennis et al. to count of *F. hepatica* eggs per grams of faeces. Climatic data were collected in a weather station in the region of work. No difference of mean number of eggs per gram of faeces of the samples collected between periods of rain (October-March) and dry (April-September). The weather conditions found and the experimental coprological surveys throughout the year made possible that the animals to be infected by *F. hepatica*.

Keywords: fluke, count eggs, climatic conditions, parasitological diagnosis, ruminants.

1. Introduction

Fascioliasis is a disease caused by the liver fluke *Fasciola hepatica* that parasite mainly cattle and sheep, possesses a cosmopolitan distribution and is zoonotic (Cringoli et al. 2002; Lima et al. 2009). The epidemiology of this disease is closely associated with environmental factors, such as temperature, rainfall and relative humidity (Garg et al. 2009; Dutra et al. 2010), and with ecology of freshwater lymnaeids (the intermediate host of *F. hepatica*). (Acha, Szyfres, 2003). There are several cross-sectional studies in Brazil are based mainly on parasitological diagnostic of eggs in faeces of animals and recovery of helminths from slaughtered animals. On the other land, efforts to understanding the epidemiological pattern and distribution of fascioliasis for a prolonged time needs to be better studied.

In Brazil, in endemic areas, the contamination of pastures by metacercariae occur almost all the year (Amato et al. 1986). However, temperate regions with there a variation of the temperature, humidity and rainfall without a rainy season, in wich occurs in the contamination of pasture and freshwater lymnaeids by eggs of *F. hepatica* and, a dry season, in which freshwater lymnaeids do not multiply, but the animals, when still are young, they can become infected after grazing in areas previously flooded (Acha, Szyfres, 2003). Epidemiological dynamics of *F. hepatica* in temperate climates was evaluated in southern Brazil by Dutra et al. (2010) and Silva et al. (2011). Knowledge of the seasonal distribution of the agent is essential for prophylaxis (Cruz-Mendoza et al. 2005). However, few studies have been conducted in tropical regions in Brazil for the knowledge of epidemiological dynamics of this agent.

The objective of this work was to evaluate the production of *F. hepatica* eggs in faeces of cattle under tropical weather conditions in the south of Espírito Santo State.

2. Materials and methods

2.1. Animals

Twelve animals were stabled on a farm located in rural area in the municipality of the Cachoeiro de Itapemirim, Espírito Santo state, Southeastern Brazil (S 20º 40' 60.00''S, W 41º 12' 0.00''W). The paddock where animals remained had 95,000 m^2. This area is characterized by predominance of plains surrounded by small mountains and the elevation is 50 meters. The predominant vegetation is pastures surrounded by a few of degraded areas in mountains and in smaller areas with vegetation typical like Atlantic forest. The climatic classification in the local is Aw, i.e., tropical climate with a dry season according to Koppen-Geiger. The period comprehended to these observations occurred during October 2009 to September 2010.

Animals were maintained under grazing conditions. Cattle were crossbred Holstein x Zebu, and at the beginning of the period showed about 10 months and weighted 163.17±10.53 kg. They were previously (3 months before) dewormed orally with a formulation based on abamectin (200 μg/kg body weight) and triclabendazole (10 mg/kg body weight) (Avotan Fasciola, Intervet- Schering Plough®).

2.2. Coprological examination

Faecal samples were collected from the rectum of the animals (about 20 grams), forthnightly, during October 2009 and September 2010, and they were

subjected to sedimentation technique initially described by Dennis, Stone and Swanson (Dennis et al. 1954), modified by Belém et al. (1992). This quantitative technique consists in; to weigh one gram of feces and places it in a Becker and adds 15 ml of double sulfate of potassium and aluminium [$KAl(SO_4)_2$] and homogenizes it slightly to avoid the formation of bubbles. Then, the content is filtered through a metal sieve (150 meshes/inch) for a sedimentation cup and then added 50 ml of detergent solution through the sieve and allowed to settle for 10 minutes. The precipitate collected with the aid of a pipette and placed a drop in a lamina, was covered with cover slip and taken under the microscope in a 10x objective and the eggs of *F. hepatica* are counted.

2.3. Weather conditions

The climatic classification in the local is Aw, i.e., tropical climate with a dry season according to Koppen-Geiger. The monthly means of minimum, medium, and maximum temperatures, rainfall and relative humidity were collected at a weather station, near the experimental local. Data were obtained according to the method recommended by the Penman-Monteith (FAO Standard, 1998).

2.4. Statistical Analysis

Number of *F. hepatica* eggs per gram of feces was compared among the rainy (October-March) and dry (April-September) seasons. Number eggs were statistically analyzed by analysis of variance (F test) at 5% probability. Mean the number eggs in two stations were compared by Tukey test at level 5% probability employing program BioEstat 5.0 program (Ayres et al. 2007). Correlation test between monthly

mean number of eggs per gram of *F. hepatica* and monthly climatic variables were conducted.

3. Results

Mean number of *F. hepatica* eggs per gram of feces of the experimental period was 1.08, with standard deviation was 0.52 (Figure 1). The largest number of egg per gram of faeces was observed during January 2010 (1.79) and the lower at the beginning, October 2009 (0.20).

The monthly minimum, medium and maximum temperatures, relative humidity and rainfall (Figure 2) showed that April-September, the counts of eggs of *F. hepatica* per gram was 26% lower, although with difference ($p > 0.05$) in egg counts per gram of feces in animals during periods of rainy and drought.

The lowest minimum temperature was in Juny 2010 (11°C), the highest, in January and February 2010 (37°C). The monthly mean relative humidity ranged between 67 (June and August 2010) and 80% (December 2009). Rainfall ranged between 12 mm^3 (Juny 2010) and 394 mm^3 (December 2009).

The correlation coefficient between the number of eggs of *F. hepatica* and the average temperature was 0.2416 and the ratio between the number of eggs and rainfall was 0.1739 and, was 0.1472, for number of eggs and relative humidity. The correlation between climate variables and the number of eggs were very low.

4. Discussion

Animals were dewormed 3 months before these surveys. And they were *F. hepatica* free. The region of this experiment assay had probability of infection of animals during each month and manly due to at previous observations by introduction of tracers animals in different season.

The highest count of eggs per gram was obtained during January 2010, 1.42 eggs per grams of faeces which agree with number of *F. hepatica* eggs per gram of faeces observed in cattle are low (Boray et al. 1969). The faecal samples of cattle had percentage of infected animals of 21.3, and 14.1%, respectively, in coprological examinations (Martins et al. 2008; Alves et al. 2011). However, count of eggs per gram of faeces is not considered in the work of these authors.

Weather factors such as temperature can influence the development of *F. hepatica* in intermediates stages, for example, miracidia emerge from the eggs in about 10 days when the average temperature presents around 26°C, which facilitates the occurrence of infection of freshwater lymnaeids (Nahm, 1997). During the entire rainy period, temperature was favorable to lymnaeids infection. The contamination of the animal in areas of primary sites is permanent (Acha, Szyfres 2003). These areas are those with permanent wet conditions for constant multiplication of lymnaeids, but, often with low and constant number of lymnaeids (Amato et al. 1986).

The paddock animals was partially flooded and wet during all experimental period, and the presence of lymnaeids was verified on site during dry and rainy seasons. High rates of rainfall and low altitudes influence positively the contamination of the environment and animals by *F. hepatica* (Dutra et al. 2010, Silva et al. 2011).

Temperatures above 10°C allow the development of larval stages of *F. hepatica* in the parenchyma of lymnaeids and that medium temperatures (10-20°C) are ideal for the development of cercariae in lymnaeids (Acha and Szyfres 2003). Throughout the trial period, the temperature showed values from 12 to 37°C (Fig. 2), this temperatures supported the infection of the lymnaeids by miracidia of *F. hepatica*.

Rate of humidity may be contributed positively to mitigate the drying of the soil, and thus, the multiplication of lymnaeids to be supported and to viability to hatching of *F. hepatica* eggs in faeces (Rangel, 1999).

Increase of population of lymnaeids depends on the amount of rainfall and not so high temperatures, because this contributes to reduce drying of the soil (Amato et al. 1986). The infection rate by *F. hepatica* depends on the precipitation rate, which allows the multiplication of lymnaeids (Rangel, 1999).

The weather conditions found made possible that the animals can be infected by *F. hepatica* in this region considering throughout the all year.

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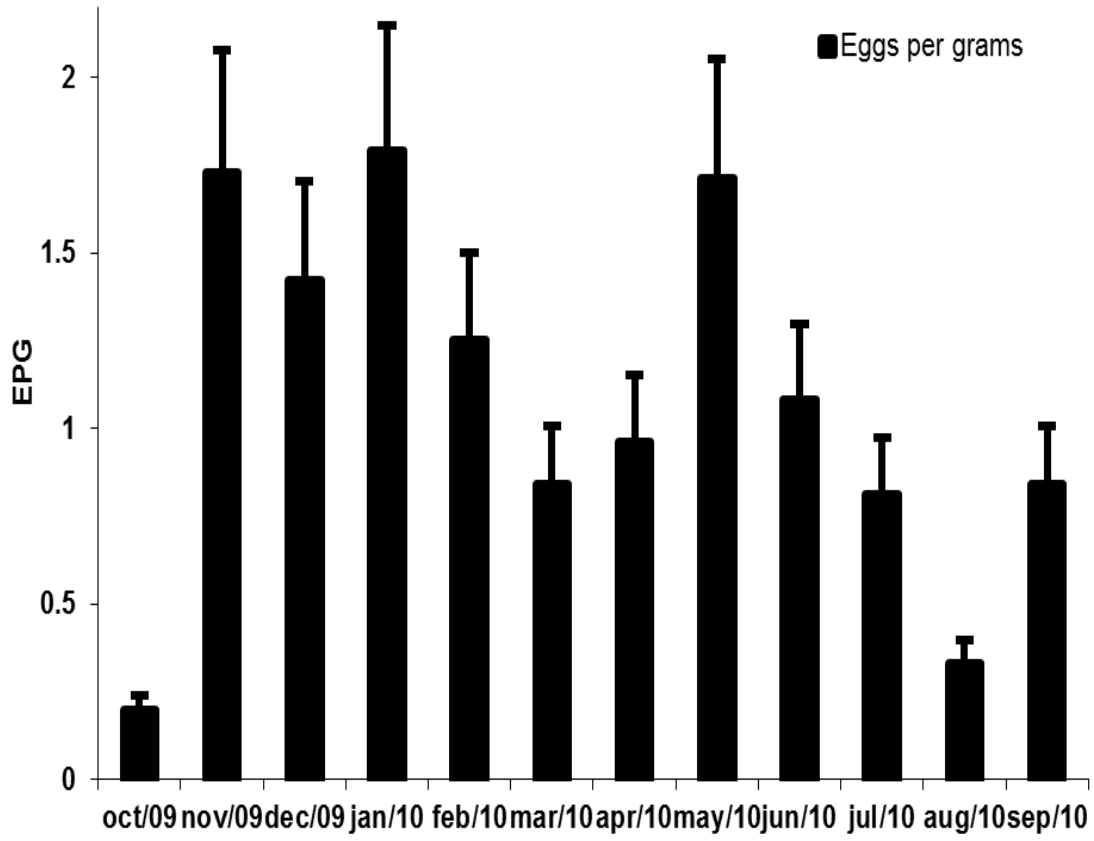

Figure 1 - Mean monthly number of egg per gram of *Fasciola hepatica* in cattle faeces. Faecal samples were collected from the rectum of 12 bovines, October 2009 to September 2010, forthnightly, and the sample were submitted at technique modified of Dennis, Stone and Swanson. The animals were reared at a farm at the municipality of Cachoeiro de Itapemirim, Espírito Santo State, Brazil.

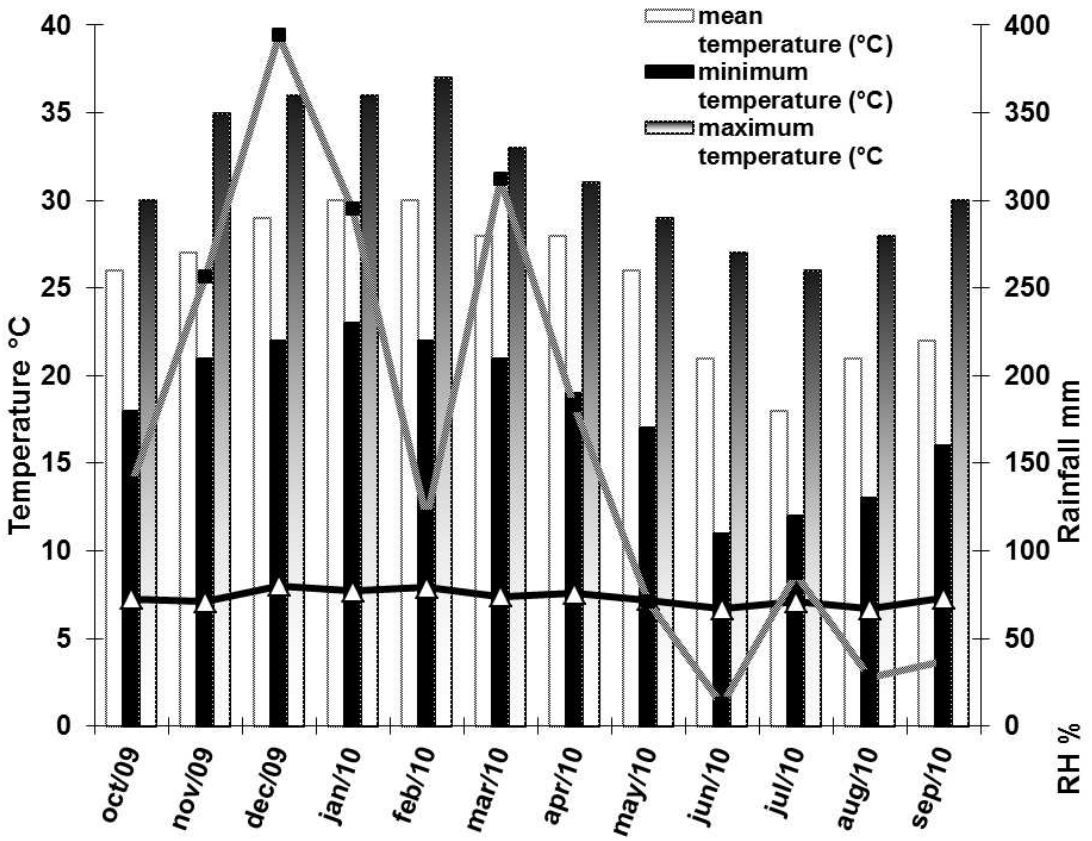

Figure 2 - Mean monthly minimum temperature, medium and maximum rainfall and relative humidity obtained from at a weather station of the region under study, in the municipality of Cachoeiro de Itapemirim, Espírito Santo state, Brazil. The data comprehend the period from October 2009 and September 2010.

5. CONCLUSÕES GERAIS

O fungo (*Pochonia chlamydosporia*) após atravessar o trato gastrintestinal de bovinos foi eficaz em predar ovos de *Fasciola hepatica*.

A aplicação de péletes contendo micélio fungico de *Pochonia chlamydosporia* reduziu as reinfecções por *F. hepatica* a bovinos criados em sistema extensivo, onde houve possibilidades de infecção de forma natural por esse agente.

A técnica de Denis, Stone e Swanson modificada é mais sensível no diagnóstico de ovos de *F. hepatica* em amostras de fezes de bovinos.

Não ocorre diferenças ($p>0,05$) na produção de ovos de *F. hepatica* em bovinos entre as épocas de chuva e seca no sul do Espírito Santo em populações de bovinos criados sob condições de clima tropical.

6. ANEXOS

6.1 Técnicas de diagnóstico parasitológico em fezes

6.1.1Técnica de sedimentação de Denis, Stone & Swanson modificada (DSS)

Essa técnica de sedimentação e tamisação foi descrita por Belém com modificações da técnica de Denis, Stone & Swanson (1954) e é assim descrita com as devidas modificações para diagnóstico de *F. hepatica*:

a) Coletar do reto de bovinos fezes e homogeneizá-las;

b) tomar 1 gramas dessa amostra de fezes e colocá-lo em um béquer e acrescentar 15 mL de solução detergente e homogeneizar levemente para evitar a formação de bolhas;

c) A seguir, filtrar através de um tamis com filtro metálico (100 malhas/ polegada, 174 μm de abertura) para um cálice de sedimentação e em seguida adicionar 50 mL da solução detergente através do tamis e deixar sedimentar por 10 minutos,

d) sifonar o sobrenadante deixando 1 a 2 ml no fundo e novamente ressuspender o sedimento com 50 ml de solução detergente; repetindo o processo por duas vezes;

d) e por fim colher o sedimento com auxílio de uma pipeta, colocar uma gota na lâmina, cobrir com lamínula e levar ao microscópio;

e) Após isso, observar toda a lâmina em objetiva de 10x (aumento de 100x, se possível) e contar todos os ovos de *F. hepatica*.

6.1.2 Técnica de filtração e tamisação de Girão–Ueno (QT)

Essa técnica de filtração e tamisação modificada foi descrita por Girão & Ueno (1985) e consiste no seguinte protocolo:

a) retirar fezes diretamente do reto de bovinos e homogeneizá-las;

b) retirar dessa amostra e pesar 1 grama dessa amostra de fezes e colocá-lo em um frasco. Diluir em 30 ml de torneira, com 5 gotas de solução detergente;

c) homogeneizar o conteúdo, agitando-o vigorosamente por 1 a 2 minutos. Passar a mistura lentamente no conjunto de tamises dispostos uns sobre os outros (os tamises são de 100, 180, 200 e 250 malhas por polegadas com abertura de 174, 96, 87 e 65 μm respectivamente);

d) lavar em água corrente lentamente, descartando-se, um por um, dos três primeiros tamises, reconhendo o material retido no último tamis (250 malhas/ polegadas) em uma placa de Petri riscada, utilizando-se um fino jato d´água no sentido inverso desse tamis;

e) esperar dois minutos e retirar, sem agitar o sedimento, o excesso de água da placa com uma pipeta de Pasteur;

f) adicionar 1 a 2 gotas de verde de metila ou azul de metileno a 0,5%;

g) examinar em estereomicroscópio com com objetiva em aumento de 10x;

6.1.3 Técnica de sedimentação modificada de Foreyt

Essa técnica é descrita por Foreyt (2001) com modificações da técnica de sedimentação comum de Hofmann, e consiste no seguintes procedimentos descritos abaixo:

a) a) retirar fezes diretamente do reto de bovinos e homogeneizá-las;

b) retirar dessa amostra e pesar 1 grama dessa amostra de fezes e colocá-lo em um frasco;

c) misturar a amostra à água com um bastão de vidro e após esse procedimento passa-la em um tamis e deixar sedimentar por 10 minutos em um cálice de sedimentação;

d) sifonar o sedimento 1 a 3 ml em uma placa de Petri e examinar em microscopia de luz com objetiva de 5 x.

6.2 Certificado de Aprovação da Comissão de Ética

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA EM PESQUISA NO USO DE ANIMAIS
Campus Universitário - Viçosa, MG - 36570-000 - Telefone: (31) 3899-3783

CERTIFICADO

A Comissão de Ética para Uso de Animais (CEUA) / UFV certifica que o processo n.º 52/2011, intitulado "Controle biológico de *Fasciola hepática* com o fungo ovicida *Pochonia chlamydosporia* (VC1) no sudeste do Brasil" coordenado pelo Dr. Jackson Victor de Araújo, do Departamento de Veterinária, está de acordo com o Código de Ética Profissional do Médico Veterinário, com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA) e com a legislação vigente, tendo sido aprovado por esta Comissão em 22/ 12 /2011.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 52/2011, named "Controle biológico de *Fasciola hepática* com o fungo ovicida *Pochonia chlamydosporia* (VC1) no sudeste do Brasil" is in agreement with the Medical Veterinary Professional Ethics Code, with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA) and with actual Brazilian legislation. This Institutional Commission on December 22, 2011 approved this process.

Viçosa, 22 de dezembro de 2011.

Professor Cláudio César Fonseca
Comissão de Ética para o Uso de Animais da UFV - CEUA
Coordenador