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Lucas Trevisan Gressler

PRODUÇÃO DE IMUNOGLOBULINA Y ANTI-*Haemonchus contortus* E APLICAÇÃO IMUNOTERÁPICA EM *Meriones unguiculatus*

Santa Maria, RS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de Doutor em Medicina Veterinária

Orientador: Prof. Silvia Gonzalez Monteiro

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O cientista não é o mais honesto dos homens,
mas a ciência dá um grande prêmio à honestidade.

(Max Planck)

RESUMO

PRODUÇÃO DE IMUNOGLOBULINA Y (IgY) ANTI-*Haemonchus contortus* E APLICAÇÃO IMUNOTERÁPICA EM *Meriones unguiculatus*

Autor: Lucas Trevisan Gressler
Orientador: Silvia Gonzalez Monteiro

Na última década, dentre os rebanhos de médio porte (aves, suínos, ovinos), o que apresentou o maior índice de crescimento foi o efetivo ovino. Para a ovinocultura, as helmintoses gastrintestinais podem ser consideradas um dos principais entraves para criação atualmente. Dentre essas, destacamos a haemoncose, causada pela espécie *Haemonchus contortus*, parasito cosmopolita, altamente patogênico e prevalente. Esta enfermidade, além de causar a morte de animais e retardo no desenvolvimento, reduz a produção, gerando maiores custos e maior tempo para o retorno do capital empregado. Devido à importância do controle desta parasitose, o uso massivo e indiscriminado de anti-helmínticos culminou com seleção de nematódeos multirresistentes e por consequência, a deficiente ação dos produtos disponíveis no mercado. Assim, pesquisas por métodos de controle não químicos tornam-se indispensáveis para continuidade deste seguimento. Neste contexto, esta tese foi elaborada com os objetivos de avaliar diferentes protocolos experimentais para estabelecimento da infecção de *Meriones unguiculatus* por *H. contortus* com e sem imunossupressão (manuscrito 1); de produzir e caracterizar imunoglobulinas Y (IgY) anti *H. contortus* a partir de larvas de terceiro estágio (L3) (manuscrito 2) e estágios adultos desse helminto (manuscrito 3) e de avaliar a atividade imunoterapêutica da IgY anti-*H. contortus* em *Meriones unguiculatus* infectados experimentalmente. Concluímos assim, que é possível estabelecer a infecção de gerbils por *H. contortus*, sendo a imunossupressão com metilprednisolona a que obteve os melhores resultados comparado a dexametasona e com animais imunocompetentes. Galinhas imunizadas com larvas de terceiro estágio e adultos de *H. contortus* produziram imunoglobulinas específicas anti-*H. contortus* e com alta avidez. A imunoterapia interferiu no estabelecimento de *H. contortus* em gerbils infectados experimentalmente.

Palavras chave: *Haemonchus contortus*. IgY. Imunoterapia. *Meriones unguiculatus*.

ABSTRACT

PRODUCTION OF IMMUNOGLOBULIN Y (IgY) ANTI-*Haemonchus contortus* E IMMUNOTHERAPY APPLICATION IN *Meriones unguiculatus*

Author: Lucas Trevisan Gressler
Advisor: Silvia Gonzalez Monteiro

In the last decade, among the medium-sized herds (poultry, swine, sheep), the one with the highest growth rate was sheep. For the sheep farming, gastrointestinal helminths can be considered one of the main barriers for breeding today. Among these, we highlight the haemoncosis, caused by the *Haemonchus contortus* species, a cosmopolitan parasite, highly pathogenic and prevalent. This disease, in addition to causing the death and delayed development of animals, reduces production, resulting in increased breeding costs. Due to the importance of the control of this parasitosis, the massive and indiscriminate use of antihelmintics promoted the selection of multiresistant nematodes and, consequently, the deficient action of the products commercially available. Thus, to research this topic is essential for the prevention and control of infections caused by *H. contortus*. In this context, this thesis was elaborated with the aim of evaluate different experimental protocols for the establishment of *H. contortus* infection of *Meriones unguiculatus* with and without immunosuppression (manuscript 1); to produce and characterize anti-*H. contortus* immunoglobulins Y (IgY) from third instar larvae (L3) (manuscript 2) and adult stages of this helminth (manuscript 3), and to evaluate the IgY immunotherapeutic activity anti-*H. contortus* in *Meriones unguiculatus* experimentally infected. We observed that is possible to establish gerbils' infection by *H. contortus*, after immunosuppression with methylprednisolone compared to dexamethasone immunosuppression and immunocompetent animals. Chickens immunized with third-stage larvae and adults of *H. contortus* produced immunoglobulins anti-*H. contortus* displaying specificity and high avidity. Lastly, we verified that immunotherapy interfered in the establishment of *H. contortus* in experimentally infected gerbils.

Keywords: *Haemonchus contortus*. IgY. Immunotherapy. *Meriones unguiculatus*.

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1 INTRODUÇÃO

1.1. HAEMONCOSE: DESAFIOS PARA A OVINOCULTURA

De acordo com dados do IBGE (2015), o efetivo de ovinos foi de 18,41 milhões em 2015. A Região Nordeste se destaca na criação de ovinos concentrando aproximadamente 60% do rebanho nacional no último ano. A Região Sul figura em seguida, representando aproximadamente 26% do efetivo da espécie, seguida pelas Regiões Centro-Oeste, Sudeste e Norte. Rio Grande do Sul é o estado com o maior número de animais, representando aproximadamente 21% do total nacional. Para a ovinocultura, as helmintoses gastrintestinais podem ser consideradas um dos principais entraves para criação desses animais atualmente. Além de causar a morte de animais e retardo no desenvolvimento, reduz a produção, gerando maiores custos e maior tempo para o retorno do capital empregado. Devido à importância do controle desses parasitos, o uso massivo e desenfreado de anti-helmínticos culminou em elevada resistência parasitária. Atualmente, a ineficácia dos antiparasitários vem se tornando cada vez mais expressiva, dificultando o controle e em muitos casos levando os produtores ao abandono da atividade. A deficiente ação dos produtos disponíveis no mercado e a falta de novos princípios ativos torna-se um forte agravante para este seguimento. Além disso, a crescente exigência por alimentos saudáveis e provenientes de sistemas produtivos que não ofereçam risco ao meio ambiente vem instigando o desenvolvimento de métodos de controle alternativos, objeto do nosso estudo.

1.2. HAEMONCHUS CONTORTUS

Haemonchus contortus é um nematódeo gastrintestinal (NGI) altamente patogênico, principalmente em pequenos ruminantes. Devido ao seu comportamento hematófago e ao desenvolvimento rápido de grandes cargas de parasitos, é uma causa frequente de mortalidade em ovinos e caprinos, sendo o parasito mais importante em regiões de clima quentes (BESIER et al. 2016). Os NGIs em geral apresentam ciclo de vida direto, com um ciclo de vida livre e um ciclo de vida parasitário. Os ovos são eliminados nas fezes e após a eclosão da larva de primeiro estágio (L1) ocorrem quatro mudas. As duas primeiras mudas ocorrem no ambiente, levando à formação de uma larva infectante (L3). Após a ingestão e desembainhamento no

rúmen, as larvas sofrem duas mudas, passando L4 e finalmente L5, que representa o adulto imaturo. O período pré-patente é de duas a três semanas (TAYLOR et al., 2010).

A haemoncose se caracteriza por anemia hemorrágica aguda em virtude dos hábitos hematófagos do parasito. Exatamente antes da muda final elas desenvolvem a lanceta perfurante, com a qual são capazes de obter sangue dos vasos da mucosa do abomaso, local de fixação do parasito. Os adultos movem-se livremente na superfície da mucosa. Na haemoncose aguda, a anemia torna-se aparente cerca de duas semanas após a infecção sendo caracterizada por queda progressiva e perceptível no hematócrito. Menos comumente, em infecções mais intensas com mais de 30.000 parasitos, ovinos aparentemente saudáveis podem morrer repentinamente de gastrite hemorrágica grave, denominada neste caso de haemoncose hiperaguda (DOMINGUES et al., 2013).

Além de causar inapetência e diminuição significativa da ingestão de alimentos no hospedeiro, os parasitos podem lesionar a mucosa do abomaso devido ao seu habito hematófago e, para compensar a lesão, o organismo do animal utiliza as proteínas da dieta, que usualmente seriam destinadas para a sua manutenção, desenvolvimento e reprodução. Além desses fatores, a proteína da dieta pode ser desviada para contribuir com a resposta imune, pois muitos componentes do sistema imunológico como as imunoglobulinas, citocinas e proteases liberadas pelos mastócitos celulares são proteínas *in natura*. Sendo assim, a resposta imune eficiente contra infecções helmínticas, gera um custo ao metabolismo do animal. Estima-se que a manutenção da imunidade contra nematódeos gastrintestinais em ovinos implica em perdas de 15% na produtividade (SCHAFER, 2014). Além disso, a resposta imunológica contra a reinfecção se desenvolve de maneira lenta e incompleta, deixando os rebanhos sujeitos à reincidência das formas clínica e subclínica deste parasito (VANDAMME & ELLIS, 2004).

1.3. RESISTÊNCIA PARASITÁRIA

A resistência que os nematoides gastrintestinais, particularmente *Haemonchus contortus*, *Trichostrongylus* sp., *Oesophagostomum* sp. e *Cooperia* sp., vêm adquirindo aos anti-helmínticos tem sido uma grande limitação no controle da verminose. O fenômeno da resistência pode ser observado através da substituição acelerada do alelo SS, homozigoto susceptível, por alelo(s) RR, homozigoto resistente (MOLENTO et al., 2013).

As principais causas do surgimento de resistência estão relacionadas ao uso intensivo e inadequado de medicamentos antiparasitários, como o curto intervalo entre tratamentos, a

utilização de uma mesma classe de anti-helmíntico por longos períodos, a subdosagem, a rápida alternância de diferentes grupos de quimioterápicos, tratamentos não-seletivos, movimento frequente do rebanho para pastos limpos combinado com desverminação, o uso de medicamentos de longa persistência e a aquisição de animais infectados com cepas resistentes (CARVALHO, 2011).

A partir das evidências de resistência aos fármacos, os registros de problemas com a verminose começaram a ser mais comuns principalmente na região sul do Brasil, incluindo relatos de resistência múltipla (CEZAR et al., 2010). Em algumas propriedades, pode ser observada a ineficácia de associações de dois ou mais fármacos, o que agrava ainda mais o controle parasitário e em alguns casos, inviabiliza a atividade produtiva.

1.4. MODELO EXPERIMENTAL: *MERIONES UNGUICULATUS*

Inúmeras pesquisas *in vivo* são desenvolvidas em laboratório através da utilização de gerbils (*M. unguiculatus*). Este modelo experimental tem apresentado grande valor para a helmintologia e farmacologia, sendo utilizado em estudos de reversão da resistência (MOLENTO et al. 1999), para fins de avaliação da eficácia de anti-helmínticos (KATES & THOMPSON, 1967) e, principalmente, na pesquisa de novas moléculas com potencial antiparasitário (OSTLIND et al. 2006; ROJAS et al. 2006; KÖNIGOVÁ et al. 2008; SQUIRES et al. 2010; DE JESÚS-GABINO et al. 2010; SQUIRES et al. 2011; KÖNIGOVÁ et al. 2012; RIBEIRO et al. 2013; GRANDO et al. 2016), com destaque para o princípio ativo monepantel (KAMINSKY, R. et al. 2008).

M. unguiculatus é considerado o melhor modelo experimental devido, principalmente, as características fisiomorfológicas do seu estômago em comparação ao trato gástrico de ovinos. Segundo Conder et al. (1992) *M. unguiculatus* é proposto como um modelo experimental de laboratório adequado para o estudo das interações parasita-hospedeiro em infecções por *H. contortus*, pela semelhança histológica do abomaso de ovinos com a do estômago desses roedores. Sendo possível observar um grande número de protocolos para manutenção da infecção.

1.5. IMUNOGLOBULINA Y

A anticorpogênese é o principal mecanismo de defesa natural dos vertebrados, sendo o processo pelo qual ocorre a formação de anticorpos específicos sempre que o indivíduo se depara com agentes estranhos (SAMPAIO, 2014). De um modo geral, muitos tipos de antígenos podem ser utilizados para produzir IgY antígeno-específica, tais como: proteínas, bactérias, vírus, parasitos, fungos, polipeptídios, hormônios, toxinas, entre outros (SCHADE et al., 2005).

Há pouco mais de um século, Klemperer (1893) demonstrava pela primeira vez que a imunização de galinhas resultava na transferência de anticorpos para a gema do ovo. Esses anticorpos foram reconhecidos como imunoglobulina Y ou IgY. A denominação IgY refere-se as imunoglobulinas da gema do ovo (Y = yolk). Entre os três isotipos aviários conhecidos (IgY, IgM e IgA), a IgY é a imunoglobulina mais abundante no soro de aves, podendo ser isolada também de anfíbios e répteis (SOARES, 2013; SAMPAIO, 2014).

1.5.1 Características moleculares

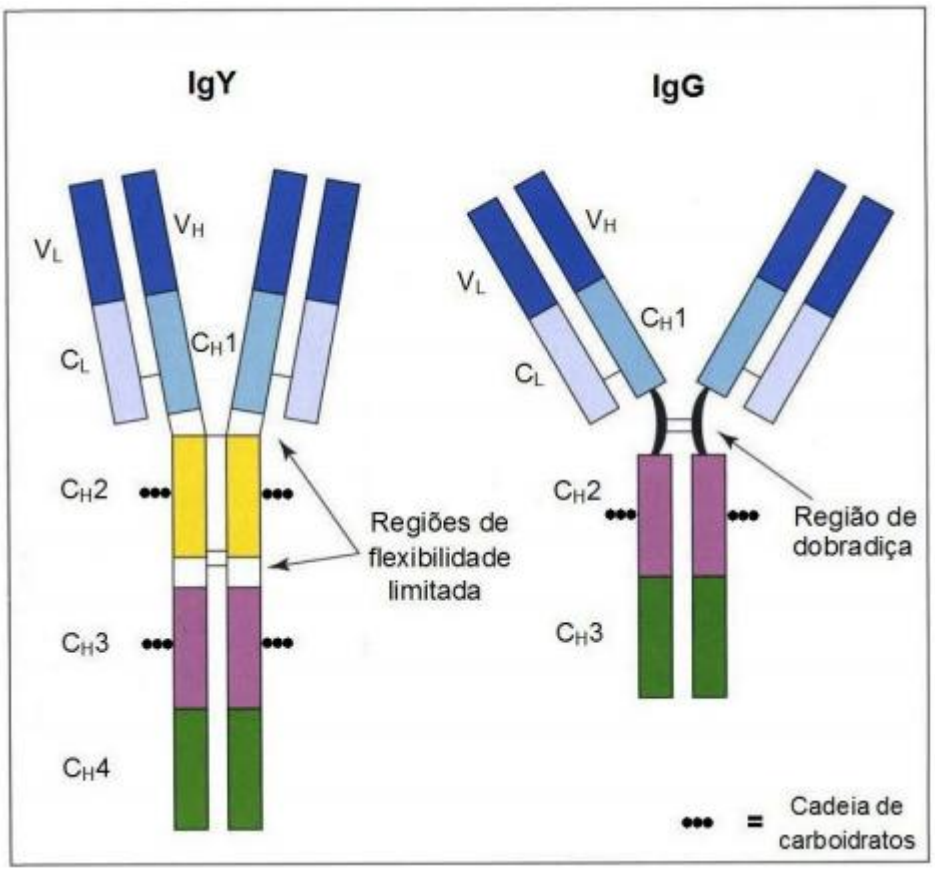
1.5.1.1 Estrutura

Historicamente, a IgY era chamada de IgG, no entanto, tornou-se claro que esta denominação é inadequada devido a diferenças estruturais entre as duas moléculas. Essas diferenças estruturais da molécula de IgY são refletidas nas diferentes interações moleculares e bioquímicas (SCHADE et al, 2005). Agora se sabe que esta imunoglobulina é evolutivamente a precursora das imunoglobulinas IgG e IgE de mamíferos (WARR et al., 1995).

Estruturalmente a IgY é composta por duas cadeias leves (L – “light”) idênticas e duas cadeias pesadas (H –“heavy”) também idênticas (Figura 2). Apresenta peso molecular em torno de 180 kDa, sendo mais pesada que a IgG de mamíferos (aproximadamente 150 kDa) (SHIMIZU et al., 1993). A cadeia leve pode pesar 18 kDa (SUN et al., 2001) até 21 kDa (HATTA et al., 1993) e seu fragmento Fab pesa em torno de 45 kDa (SUN et al., 2001). A cadeia pesada tem peso molecular entre 65-105 kDa e apresenta um domínio constante a mais do que a IgG dos mamíferos. Além disso, a região da dobradiça não é desenvolvida, sendo atribuída aos resíduos de prolina e glicina a flexibilidade limitada do fragmento Fab da IgY (NARAT, 2003; BIZANOV & JONAUSKIEN, 2003). O grande peso molecular da IgY se deve ao número aumentado de domínios constantes e cadeias de carboidratos da cadeia pesada

(WARR et al., 1995). Podendo apresentar característica hidrofóbica superior a molécula de IgG (DAVALOS-PANTOJA et al., 2000).

Figura 1 - Representação esquemática da IgY de aves e da IgG de mamíferos.



Fonte: (VASCONCELOS, 2010).

1.5.1.2 Estabilidade

Em temperatura ambiente a meia vida desta imunoglobulina é superior a seis meses. Em temperaturas superiores a 37 °C a meia vida pode ser igual ou inferior a 30 dias (SAMPAIO, 2014), porém, pode perder aproximadamente 20% da sua atividade a 60ºC por 10 min (SHIN et al., 2002). A 70ºC pode perder grande parte de sua atividade (SHIMIZU et al., 1988; 1992; HATTA et al., 1993), chegando a uma perda de mais de 90% quando aquecida a 80ºC (CHANG et al., 1999; SHIN et al., 2002).

O congelamento ou liofilização da IgY não interferem na sua atividade, a menos que esses processos sejam realizados repetidamente (SHIMIZU et al., 1988). O congelamento a -70°C, por 12 meses, provoca a perda de 50% de sua atividade, enquanto que a -20°C, pelo

mesmo período, a perda é mínima (STAAK et al., 2001; LEE et al., 2002). A IgY pode ser liofilizada, possibilitando assim, longa vida nas prateleiras. Os anticorpos são bastante estáveis frente às variações de temperatura. Podem ser armazenados em solução salina a 0,85% associada à azida sódica (0,02%) a 4ºC, por até 10 anos. Sua atividade no ovo *in natura* estocado a 4ºC se mantém por no mínimo seis meses (SAMPAIO, 2014).

A atividade da IgY pode sofrer influência da variação do pH, reduzindo sua ação em pH 3,5 e perdendo-a quase que por completo em pH 3. Em condições alcalinas, a atividade não se alterou até atingir pH 11, reduzindo significativamente em pH 12 ou mais (SHIMIZU et al., 1988, 1992, 1993). A IgY é relativamente resistente a tripsina e quimiotripsina, entretanto, apresenta elevada sensibilidade a pepsina. Sua estabilidade a pepsina parece ser altamente dependente do pH e da razão enzima/substrato (SHIMIZU et al., 1988).

1.5.2 Transferência passiva de imunidade em galinhas

A transferência de imunoglobulinas da mãe para o filho é um processo comum a todas as espécies e é denominada imunidade passiva. Enquanto nos mamíferos a IgG é transferida no ambiente uterino ou após o nascimento via colostro, nas aves ocorre a transferência de IgY do soro para a gema do ovo (BERNARDO, 2009).

A IgY é transferida para o ovo durante sua formação no ovário da ave, sendo transportada ativamente para a gema através do epitélio folicular. Este processo é mediado por receptores. Para que ocorra a transferência é necessário que a fração Fc da imunoglobulina esteja intacta, uma vez que é necessária a ligação desta fração com receptores específicos no epitélio do oócito (MORRISON et al., 2001; DE SOUSA, 2008).

Durante o desenvolvimento embrionário o pinto absorve a IgY da gema. As IgA e IgM da mãe se difundem através do líquido amniótico e são ingeridas pelo embrião. Ao nascer, os pintos já apresentam a IgY no soro e as demais na mucosa intestinal (BERNARDO, 2009).

A quantidade de imunoglobulina transferida depende da concentração sérica, sendo toda a concentração transferida (MOHAMMED et al., 1998). Já a IgM e a IgA são incorporadas juntamente com a albumina à clara do ovo durante a passagem do ovo pelo oviduto, em quantidades bastante inferiores quando comparada a IgY. A quantidade de IgY transferida independe do tamanho do ovo (PATTERSON et al., 1962). A passagem transovariana leva aproximadamente cinco dias (SCHADE et al., 2005). A meia vida circulante em aves adultas varia entre 36-65 horas (MORRISON et al., 2001).

Após a exposição a um antígeno, os anticorpos são detectados no soro e na gema após sete a dez dias respectivamente. No entanto, existem variações nestes períodos de acordo com o ensaio biológico realizado (SAMPAIO, 2014).

1.5.3 Anticorpos aviários versus anticorpos de mamíferos

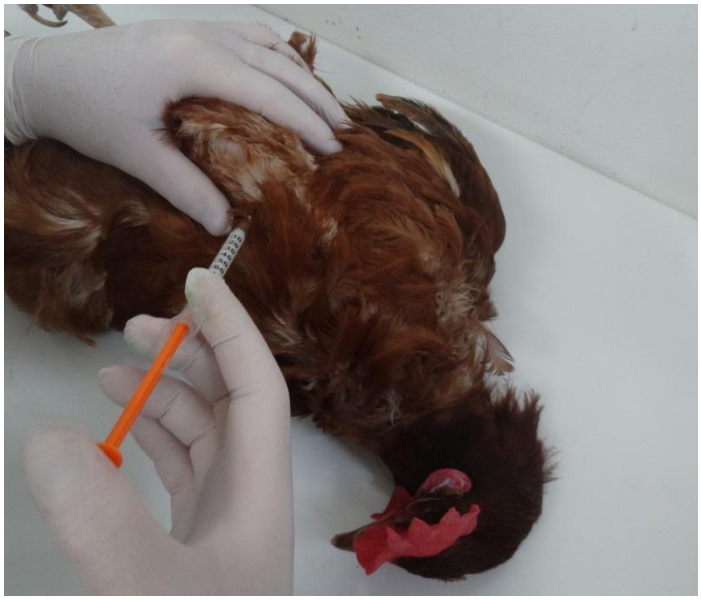
A IgY aviária combina as funções da IgG e IgE de mamíferos, promovendo proteção contra infecções, como também pode ser mediadora da anafilaxia. Nos mamíferos a IgG forma imunocomplexos e facilita a opsonização, ativa o sistema complemento (SC). A IgE não ativa o sistema complemente (SC) e nem atravessa a placenta, entretanto, pode sensibilizar células efetoras, principalmente mastócitos e basófilos e mediar reações anafiláticas. Assim como a IgE de mamíferos, IgY também apresenta a capacidade de sensibilizar mastócitos de galinhas, sendo responsáveis por reações anafiláticas locais no intestino, os quais desempenham um papel importante na defesa contra infecções protozoárias (TAYLOR et al., 2008).

Dentre as vantagens da produção de anticorpos IgY frente a anticorpos de mamíferos, destacamos algumas: O manejo das aves é simples e relativamente mais barato quando comparado a experimentos com mamíferos, produzindo anticorpos a baixo custo e em larga escala. Além de produzir IgY rapidamente e em grandes quantidades, as galinhas mantêm altos níveis de anticorpos específicos por um longo período. Permite a obtenção de maior quantidade de anticorpos devido à distância filogenética entre aves e mamíferos. O sistema imunitário das aves pode produzir anticorpos contra antígenos de mamíferos altamente complexos, podendo reconhecer partes de uma molécula que a IgG não reconheceria e os anticorpos produzidos possuem alta afinidade e avidez. Não é necessário sangrar a ave porque os anticorpos são extraídos da gema do ovo. A coleta de ovos é um método simples, não invasivo e reduz o número de animais utilizados na produção de anticorpos (CONTRERAS et al. 2005; SCHADE et al. 2005). Individualmente, uma galinha é capaz de produzir muito mais anticorpos que coelhos, cabras, equinos e roedores. Quando comparado a IgG dos mamíferos, o rendimento da IgY pode ser 5 a 10 vezes superior (GOTTSTEIN & HEMMELER, 1985), dependendo do adjuvante utilizado, protocolo de imunização e purificação (SVENDSEN et al.,1996).

1.5.4 Produção e purificação de IgY

As aves podem ser usadas para a produção de anticorpos a partir do momento em que iniciam a postura (SAMPAIO, 2014). O desenvolvimento e a produção de anticorpos IgY podem ser obtidos através da apresentação de antígenos complexos (vírus, bactérias e parasitos) ou simples (proteínas, polissacarídeos e toxinas) (SCHADE et al., 2005). Para assegurar um alto nível de produção de anticorpos, as aves devem ser imunizadas periodicamente. Estes anticorpos mostram grande avidez logo após a primeira imunização. Porém, este resultado depende de algumas variáveis: tipo, dose e peso molecular do antígeno, adjuvante, via de administração, genética do animal e tipo de criação. A concentração do antígeno pode variar de 0,1 a 1 mg, em casos especiais 10 µg, de acordo com o tipo de adjuvante escolhido (SAMPAIO, 2014). Atualmente, o adjuvante mais utilizado é o adjuvante incompleto de Freund (AIF) (VASCONCELOS, 2010). O volume das administrações varia entre 0,5 a 1 ml e a via de aplicação usual é a intramuscular, preferencialmente no musculo peitoral (SAMPAIO, 2014). De acordo com Chang et al. (1999) a imunização via intramuscular resulta em maior quantidade de anticorpos específicos quando comparada com a imunização subcutânea. O intervalo de aplicações pode ser de 2 a 8 semanas e o número de imunizações depende do interesse da produção de anticorpos (SAMPAIO, 2014)

Figura 2 - Inoculação de uma ave via intramuscular.



Fonte: arquivo pessoal.

Após a primeira inoculação as aves iniciam a produção de anticorpos, os quais após alguns dias são encontrados na gema dos ovos. O processo de extração sempre inicia pela separação da gema e da clara. Para separar a fase aquosa da gema utilizam-se solventes orgânicos, substâncias hidrófilas ou congelamento a –20ºC. Este processo é denominado deslipidação (STAAK et al., 2001).

O isolamento da imunoglobulina a partir da gema pode ser realizado por técnicas de precipitação com sais (DEIGNAN, 2000; CHACANA et al., 2003), técnicas cromatográficas (MEULENAER & HUYGHEBAERT, 2001) ou ultrafiltração (KIM & NAKAI, 1998). A escolha do método está na dependência da quantidade, pureza e atividade biológica desejada, assim como do custo da técnica.

Dentre os sais citados nas técnicas de precipitação destacam-se o sulfato de sódio, ácido caprílico, dextransulfato, sulfato de amônio e polietilenoglicol. Bernardo (2009), ao produzir anticorpos aviários contra *Leishmania amazonenses*, comparou as extrações realizadas com sulfato de amônio e polietilenoglicol 6000 (PEG 6000), e constatou que enquanto o primeiro apresentou maior rendimento, o segundo obteve maior pureza. O polietilenoglicol 6000 (PEG-6000) é um polímero de alto peso molecular formado a partir do etileno glicol. O uso desse polímero para extração de IgY da gema foi introduzido por Polson et al. (1980). O método tem como vantagem a possibilidade de manipulação em temperatura ambiente sem risco de desnaturação da imunoglobulina (AKITA & NAKAI, 1993).

A quantidade de IgY na gema é dependente da sua concentração no soro. Segundo Carlander et al. (2002) a concentração varia entre 10 e 20 mg/ml, podendo ser obtido entre 100 e 400 mg por ovo. Tini et al. (2002) encontraram valores entre 6 e 13 mg/ml, o que permite obter até 200 mg de anticorpo em uma única gema. Em estudo de Sampaio (2014), usando o método descrito por Polson et al. (1980), foram obtidos em média 9,46 mg/mL de proteína total e 2,87 mg/mL de imunoglobulina específica anti-*T.evansi*. A média de IgY obtida neste experimento, próxima de 3mg/mL, foi similar aos valores descritos por Contreras et al. (2005), Malekshahi et al. (2011) e Paula et al. (2011) usando PEG-6000, assim como Mendoza et al. (2012) e Vega et al. (2012) ao usarem a técnica de precipitação com sulfato de amônio. O rendimento proteico do purificado de IgY anti-*T. evansi* foi superior aos encontrados por Garcia et al. (2005) e Ferreira Júnior et al (2012) ao utilizarem sais de sódio e amônio para precipitação. O que leva a crer que o método descrito por Polson et al. (1980) tem rendimento proteico similar ou até mesmo superior aos métodos de precipitação com sulfato de sódio e sulfato de amônio.

1.5.5 Aplicação da tecnologia IgY

É importante ressaltar que, embora os conhecimentos sobre estes anticorpos datem desde o século XIX, sua aplicação no meio científico ressurgiu a partir do final da década de 1950 como técnica alternativa para minimizar o sofrimento de animais utilizados na pesquisa. A partir de 1980 houve um incremento na utilização de IgY nos ensaios laboratoriais, principalmente devido a disponibilidade de reagentes e novas tecnologias. Outros fatores que vem estimulando as pesquisas com estas imunoglobulinas referem-se ao custo de produção, facilidade de manejo dos animais e grande volume de anticorpo produzido quando comparado a outros modelos animais (SAMPAIO, 2014).

A tecnologia IgY é recomendada pelo Centro Europeu para Validação de Métodos Alternativos em substituição a IgG de mamíferos. Também foi aprovada como recurso alternativo a favor do bem-estar animal pelo *Office Vétérinaire Federal* da Suíça (CHACANA et al., 2004; SCHADE et al., 2005).

1.5.6 Imunoterapia IgY

A atividade terapêutica de imunoglobulinas aviárias é relatada por Mulvey e colaboradores (2011) frente à bactéria *Clostridium difficile*. Utilizando uma concentração de 45 mg de purificado de IgY, por 10 dias consecutivos, os autores obtiveram aumento da sobrevivência de hamsters em relação ao grupo controle.

Malekshahi et al. (2011) utilizando doses de 60 mg de IgY, via oral, em ratos, por um período de 28 dia obtiveram sucesso na inibição da bactéria *Helicobacter pylori* e considerável redução da inflamação nos tecidos estomacais, concluindo que a imunoterapia pode ser uma importante alternativa ao uso de terapias antimicrobianas.

Em geral, a administração de IgY por via oral destina-se a um local específico ao longo do trato digestório, é altamente específica e baseia-se na interação antígeno-anticorpo, em grande parte previsível e geralmente eficiente. Alguns mecanismos de ação são propostos na proteção do hospedeiro, com a inibição da adesão de microrganismos às superfícies celulares, supressão da colonização viral por prevenção da disseminação célula-a-célula, a aglutinação bacteriana, com a consequente imobilização microbiana e a morte ou a facilidade de serem eliminados do intestino, inibição da atividade de enzimas e a neutralização da atividade de toxinas (RAHMAN et al., 2013).

De acordo com Kovacs-Nolan et al. (2005) algumas proteínas presentes na clara e gema do ovo, como a ovomucóide e ovoinibidores apresentam atividade antiviral, já a ovotransferrina e cistatina apresentam ação antibacteriana. Estes resultados demonstram que outras proteínas podem estar presentes no purificado de IgY, podendo gerar efeitos adicionais além do efeito primário dos anticorpos.

Lee e colaboradores (2009a, 2009b) ao avaliarem IgYs produzidas a partir de diferentes espécies de *Eimeria* (*E. acervulina, E. tenella e E. maxima*), demonstraram que a imunização passiva de aves fornece significativa proteção frente ao parasito. Futuros estudos devem ser conduzidos para elucidação dos mecanismos mediados por anticorpos frente à coccidiose.

Sampaio et al. (2014) avaliaram a atividade *in vivo* do anticorpo aviário contra protozoários da espécie *T. evansi*. Utilizando uma concentração de 30mg/Kg, via intraperitoneal, cinco dias pré e dez dias pós-infecção, os autores relataram um aumento do período pré-patente, assim como, da longevidade dos animais tratados. Os mecanismos envolvidos no controle da infecção ainda são desconhecidos.

2 MANUSCRITO 1

(Artigo aceito para publicação – Journal of Helmintology)

***Meriones unguiculatus* infected by *Haemonchus contortus*: evaluation of different experimental protocols**

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Abstract

Many studies of great value on resistance reversion, anthelminthic efficacy and, especially, new molecules with antiparasitic effect are performed in laboratories using gerbils (*Meriones unguiculatus*) as the experimental model. This study aimed to evaluate the use of corticosteroids (dexamethasone and methylprednisolone acetate) in gerbils experimentally infected with different doses of infective larvae (sheathed or exsheathed) of *Haemonchus contortus*. For the first experiment, twenty-eight gerbils were divided into seven groups infected by 2 to 6 x 10^3 larvae, with or without immunosuppression using corticosteroids. In the second experiment, eight gerbils were divided into two groups of animals infected by 2 x 10^3 sheathed or exsheathed larvae. For the third assay, seven immunosuppressed gerbils were infected with

2 x 10^3 sheathed larvae and were killed 15 days post-infection (PI). The highest number of parasites was recovered from methylprednisolone-immunosuppressed animals. We observed red and white blood cell alterations and biochemical parameters in infected animals that had undergone immunosuppression with methylprednisolone. We highlight that in the first and second experiments a satisfactory number of worms was recovered using sheathed larvae and immunocompetent animals. When exsheathed larvae were used, the number of worms recovered was unsatisfactory. A considerable larval burden was recovered from immunosuppressed gerbils 15 days PI, and body weight did not influence establishment of larvae.

Keywords: Nematode, gerbil, biochemical, hematological parameters

Introduction

Gastrointestinal nematodes are responsible for health issues and economic losses. The parasite *Haemonchus contortus* is a nematode of small ruminants with high prevalence and pathogenicity (Arosemena et al., 1999). It causes severe clinical signs such as anemia, due to its hematophagous life, and it has become the most pathogenic parasite for these animals (Urquhart et al., 1990). The increasing prevalence of helminths presenting multi-drug resistance has increased the need to better understand parasite resistance mechanisms and life cycles. Thus, *in vitro* and *in vivo* laboratory experiments have been described recently (Grando *et al*., 2015, 2016).

Many studies have been conducted using gerbils (*Meriones unguiculatus*), and this experimental model has demonstrated much promise regarding reversion of resistance (Molento *et al*., 1999), anthelminthic evaluations (Kates & Thompson, 1967) and investigations on new antiparasitic compounds (Ribeiro *et al*., 2013). Gerbils are susceptible to infections

caused by several nematodes including: *Strongyloides stercoralis* (Nolan *et al*., 1993), *Strongyloides venezuelensis*, *Nippostrongylus brasiliensis* (Horii *et al*., 1993), *Trichostrongylus colubriformis* (Conder *et al*., 1991; Ziam *et al*., 1999), *H. contortus* (Conder *et al*., 1990) and *Ostertagia circumcincta (Teladorsagia circumcincta)* (Court *et al*., 1988).

Use of new experimental models and methodologies with high reliability and repeatability is important for comparing scientific results in an easier and faster manner. Moreover, use of alternative protocols that cause less discomfort to animals has been suggested in order to improve animal welfare. Currently, some aspects of optimal protocols for infection of gerbils by *H. contortus* remain unclear, such as doubts regarding the use of sheathed or exsheathed larvae and the employment of corticosteroids to induce animal immunosuppression. Therefore, this study aimed to compare the infectivity of sheathed and exsheathed larvae of *H. contortus* in gerbils that either were immunocompetent or had undergone immunosuppression through different immunosuppression protocols.

Materials and methods

Isolation of H. contortus

A multi-drug resistant *H. contortus* isolate (Almeida *et al.*, 2010) was used to infect a sheep. This animal was fed with hay and received water *ad libitum*. Initially, this animal was treated with the anthelminthic monepantel (Zolvix®). Five days later, fecal examination was performed using the zinc sulfate centrifugation-flotation technique and no parasite was detected. Later, the animal was infected orally with 1.0×10^4 third-stage larvae at three different times: firstly, a dose of 4.0×10^3 larvae and then another two doses of 3.0×10^3 larvae, at three-day intervals. The larvae that were used to infect the gerbils were recovered by means of coproculture, in accordance with the method described by Roberts & O'Sullivan (1950), as

modified by Ueno & Gonçalves (1994). The larvae for infecting the animals were previously stored at room temperature for seven days.

In vivo experiment number 1

Twenty-eight male and female five-week-old outbred gerbils (*M. unguiculatus*), of average body weight 37 g, which were visually in a healthy and parasite-free condition, were obtained from the Animal Care Center of the Federal University of Santa Maria (UFSM). The gerbils were kept in polypropylene boxes under controlled temperature and humidity (22 °C ± 2 ºC; 40% relative humidity, RH) under a 12/12 h dark/light cycle and were fed with commercial feed and water *ad libitum*. After a week of adaptation, the animals were randomly divided according to their body weight into seven groups (A to G) of four animals each. They were infected orally on day 0, except for group G (uninfected), which received a placebo solution (0.9% NaCl). The gerbils were subjected to a 24-hour fasting period in order to enhance the chances of larval infection. The groups were composed as follows:

Immunosuppression protocols:

Protocol 1: Immunosuppression applied to gerbils on days -2, -1, 0, 1 and 2 using 0.1 ml (0.2 mg) of dexamethasone (Azium®, Schering Plus; 2 mg/ml), intramuscularly (IM);

Protocol 2: Immunosuppression applied to gerbils on days -2, -1, 0, 2, 4, 6, 8 and 10 using 0.1 ml (0.2 mg) of dexamethasone (Azium®, Schering Plus; 2 mg/ml), IM;

Protocol 3: Immunosuppression applied to gerbils on days -2, -1, 0, 1 and 2 using 0.1 ml (4 mg) of methylprednisolone acetate (Depo-Medrol®, Pfizer; 40 mg/ml), IM.

Method for obtaining exsheathed larvae

Infective larvae were exsheathed in 0.9% sodium hypochlorite (NaClO), by adding 14 µl of NaClO per 1 ml of water. When 90% of the larvae had become exsheathed, they were washed with distilled water, followed by centrifugation for 3 min at 2000 rpm. This procedure was repeated three times. Later, the larvae were placed on a mesh (25 µm) and those with high motility were selected.

Groups formed

Group A - immunocompetent gerbils infected with 2 x 10^3 sheathed larvae;

Group B - immunosuppressed gerbils (protocol **1**) infected with 2 x 10^3 sheathed larvae;

Group C - immunosuppressed gerbils (protocol **1**) infected with 6 x 10^3 sheathed larvae;

Group D - immunosuppressed gerbils (protocol **2**) infected with 2 x 10^3 sheathed larvae;

Group E - immunosuppressed gerbils (protocol **3**) infected with 2 x 10^3 sheathed larvae;

Group F - immunosuppressed gerbils (protocol **1**) infected with 2 x 10^3 exsheathed larvae;

Group G - immunocompetent gerbils that remained uninfected.

Parasite recovery and sampling

Ten days post-infection (PI), the animals were anesthetized with isoflurane and total blood was collected for hematological and biochemical analyses. The stomach was removed, externally washed with 10 ml of warm distilled water (37 °C), opened longitudinally in a Petri dish and incubated with 20 ml of 0.9% NaCl at 37 °C in a biological oxygen demand (BOD) chamber at 37 °C for 5 hours, following the method of Conder *et al*. (1990). After this period, the stomachs were washed with 0.9% NaCl and the larvae were placed in falcon tubes with 50 ml of 4% buffered formaldehyde. The parasites were counted using an inverted optical microscope (40x).

Hematological analysis

Hematological parameters were assessed in whole blood that had been collected in tubes containing EDTA, using an automatic counter (Coulter T890®; Coulter Electronics, Inc., Hialeach, FL, USA). Total leukocytes (WBC), total erythrocytes (RBC), hematocrit (HCT), hemoglobin concentration (HGBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and platelets (PLT) were determined. Blood smears were fixed in methanol and were stained with Instant-Prov (NewProv®) stain for differential WBC counts; in these, at least 200 WBCs were counted.

Biochemical analysis

Blood was collected, and the serum was separated by means of centrifugation (3000 rpm for 15 min) and stored at -20 °C for biochemical analysis. Serum levels of albumin and total protein (TP) were analyzed using Labtest kits (Labtest Diagnóstica SA) through an automatic analyzer (CELM SBA 200®; CELM, Barueri, SP, Brazil). Globulin values were obtained by subtracting the albumin from the total protein. All tests were carried out in duplicate.

In vivo experiment number 2

A second experiment was performed using two groups: group A, infected with 2 x 10^3 sheathed larvae; and group B, infected with 2 x 10^3 exsheathed larvae. Eight male six-week-old gerbils, of body weight 44.5 g, were divided into two groups with four animals each. The animals were orally infected on day 0. Ten days later, the stomachs were removed for larval recovery.

In vivo experiment number 3

The correlation between establishment of third-stage larvae of *H. contortus* 15 days PI and body weight was investigated. Seven five week-old gerbils, males and females, of body weight 35 g, were subjected to immunosuppression using three doses of Depo-Medrol® (Pfizer) (2-4 mg IM, according to body weight) on days -2, -1 and 7 PI. The gerbils were subjected to fasting for 24 hours (18 h prior to infection and 6 hours PI), to enhance the chances of infection by 2 x 10^3 sheathed larvae on day 0. The animals were sacrificed on day 15 PI in an isoflurane chamber and their stomachs were removed and washed. The larval content of their stomachs was saved as described in the section *Parasite recovery and sampling*, with minor modifications.

Data analysis

For *in vivo* experiment 1, data were compared using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. For experiment 2, the Mann-Whitney test was used to compare means. In experiment 3, Pearson's correlation coefficient was used to determine the strength of the correlations among the variables: number of larvae recovered and body weight of the gerbils. All analyses were performed with the significance level taken to be $P < 0.05$.

Results

In vivo experiment number 1

The number of parasites recovered at the necropsy and the animals' body weights on days 0 and 10 are shown in Table 1. A higher number of parasites was quantified in group A than in group B. Groups B and F had significantly fewer parasites than groups A, C and D. The highest number of parasites was recovered in group E (using the immunosuppressive drug Depo-Medrol® and sheathed larvae). In group F, using exsheathed larvae and

immunosuppressed animals, the lowest number of parasites was found, in comparison with all the other groups. All the animals had higher body weight 10 days PI, except those in the group E, which was treated with methylprednisolone. This drug possibly reduced the mean body weight.

Table 2 shows the mean red and white blood counts and biochemical parameters of gerbils infected by *H. contortus* and the control group (uninfected). For group E, significant alterations to RBC, HCT, PLT, MCHC, WBC, lymphocyte and neutrophil counts can be observed. There were also significant differences in TP and total globulins in all groups, compared with the uninfected control group (group G), with highest alterations in group E (treated with methylprednisolone). Other parameters of the red blood series (HGBC) and white blood series (rod neutrophils, eosinophils and monocytes) were also measured (data not shown).

In vivo experiment number 2

A higher number of worms was recovered from gerbils infected by sheathed larvae (189.33 ± 126.26) than from those infected by exsheathed larvae (7 ± 2.64). However, this result was not statistically significant because of the high variability.

In vivo experiment number 3

In the third experiment, the mean number of larvae recovered 15 days PI was approximately 225. There was a weak correlation ($R = 0.36$) between the number of larvae recovered and animal body weight. However, no significant differences were found ($p = 0.05$) (Fig. 1).

Discussion

Over the last two decades, a large number of scientific studies on nematodes have been conducted using *M. unguiculatus* as the experimental model. Many protocols for infecting gerbils with *H. contortus* can be found in the literature, and some of them are compiled in Table 3. The high number of worms recovered in the present study shows that infection became established in immunocompetent animals, as also demonstrated by Ostlind *et al*. (2006), Rojas *et al*. (2006) and Squires *et al*. (2010, 2011). For unknown reasons, we found significantly more parasites in immunocompetent gerbils (group A) than in those subjected to immunosuppression using dexamethasone (group B). However, using the same *H. contortus* isolate as in the present study, Grando *et al*. (2016) recovered an average of 44.0 worms from gerbils 12 days PI while using the same protocol as used for group B in our study.

We found that the main glucocorticoids (GC) used in gerbils to improve *H. contortus* infection and, consequently, the number of larvae recovered, were dexamethasone and hydrocortisone (Table 4). Machado *et al*. (2006) evaluated gerbils infected by *H. contortus* and *Trichostrongylus colubriformis* using 4 mg of methylprednisolone per animal (Depo-Medrol®; Pharmacia) every 21 days. The gerbils were sacrificed 58 days PI for adult worm recovery, which encouraged us to compare the activity of methylprednisolone and dexamethasone. According to Machado *et al*. (2006), methylprednisolone reduced the bio-nutritional efficiency of the gerbils, such that treated animals showed significant lower performance than untreated animals. This finding corroborates the reduction in mean body weight seen among the animals treated with methylprednisolone in our study.

Larval exsheathment is a critical part of the process of experimental infection (Macedo *et al.*, 2015). However, only a few studies on establishment of *H. contortus* infection in gerbils by means of larvae exsheathed using sodium hypochlorite have been conducted (Conder *et al.*, 1990; De Jesús-Gabino *et al.*, 2010; Squires *et al.*, 2010; 2011; Ribeiro *et al.*, 2013, and Macedo

et al., 2015). Conder and Johnson (1996) reported that none of the *in vitro* exsheathing media, including sodium hypochlorite, was optimal for parasites and that they appeared to reduce larval viability. However, among the exsheathment media used, the best infection rate was achieved using carbon dioxide for exsheathment. To standardize a larval migration inhibition test, Demeler *et al*. (2010) used exsheathed *Cooperia oncophora* larvae and found significantly fewer viable larvae, and migration rates as low as 50%, compared with use of sheathed larvae. We observed that exsheathed larvae of *H. contortus* showed lower motility. Even though we selected the ones with highest motility to infect the gerbils, it was not possible to have a satisfactory rate of parasite recovery in both experiments. The use of exsheathed larvae resulted in decreased establishment of worms in experiment 2, thus supporting the results of the first experiment.

In relation to the immune response, GC caused lymphopenia affecting T lymphocytes through inhibiting the Th1 response and, especially, the Th2 recruits and activates cells responsible for IgE production (Lirini, 2008. Furthermore, GC induces neutrophilia and eosinopenia and reduces the number of macrophages (Pereira *et al*., 2007). Because of the inhibitory effect of GC, it facilitates dissemination and establishment of infectious agents, including parasites such as *H. contortus*. In our study, infected gerbils that had undergone immunosuppression using methylprednisolone showed higher numbers of leukocytes, despite also showing lymphocytosis and neutropenia. Concerning the hematological and biochemical parameters, strong thrombophilia was observed even with increased total globulins, due to increased synthesis of hepatic proteins as an adverse effect of GC administration (Freitas & Souza, 2007).

It is known that the effect of corticosteroids on mucosal mast cells, mast cell proteinases and eosinophils, and on the antibody response, is capable of influencing B cell and T-helper

cell responses (Ziam *et al*. 1999). According to Amorim *et al*. (2010), gerbils infected by *Giardia duodenalis* showed specific IgA fecal antibodies and serum levels of IgG_1 and IgM, 7 days PI. However, the antibody levels decreased as soon as immunosuppression induced by methylprednisolone acetate was started (Amorim *et al*., 2008).

In addition to IgE, other immunoglobulins may perform important functions towards protecting the host against larvae (Taylor, 2014), thereby hampering larval establishment in gerbils. Therefore, one explanation for the higher number of larvae recovered from methylprednisolone-immunosuppressed gerbils is that this occurred through reduction of the humoral response due to increased plasma levels after GC administration, as a result of high doses of methylprednisolone administered over a short period of time (Pereira *et al*., 2007).

Our study provided additional evidence that weaned gerbils at an age of approximately five weeks appear to be an acceptable alternative for use as an experimental model, since they showed body development, which contributes towards immunosuppression and/or infection. The decision regarding which methodology should be used might be influenced by other factors, such as the parameters that will be analyzed in the research and the influence of GC on them. We highlight that in the first and second experiments it was possible to recover a satisfactory number of worms using sheathed larvae and immunocompetent animals. Use of methylprednisolone increased the number of parasites recovered, compared with untreated gerbils or those receiving dexamethasone. Infected gerbils that had been subjected to immunosuppression using methylprednisolone showed alterations to hematological and biochemical parameters, along with poor performance. A considerable larval burden was recovered from the immunosuppressed gerbils 15 days PI, and body weight did not influence establishment of larval infection.

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Statement of interest

The authors declare that there were no conflicts of interest in conducting this study.

Ethical committee

This study was approved by the Ethics Committee for Animal Research (CARE) of the Federal University of Santa Maria (UFSM), under protocol numbers 3768260515/2015 and 3787160917/2017.

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Table 1. Mean numbers of *Haemonchus contortus* larvae recovered from the stomachs of gerbils on day 10 post-infection (PI) and mean body weights pre-infection (day 0) and 10 days

PI. Groups: **A** - infected with 2 x 10^3 larvae; **B** - infected with 2 x 10^3 larvae and immunosuppressed (protocol 1); **C** - infected with 6 x 10^3 larvae and immunosuppressed (protocol 1); **D** - infected with 2 x 10^3 larvae and immunosuppressed (protocol 2); **E** - infected with 2 x 10^3 larvae and immunosuppressed (protocol 3); **F** - infected with 2 x 10^3 larvae (exsheathed) and immunosuppressed (protocol 1); **G** - Uninfected. N/A - Not applied.

Groups (n = 4)	**Mean number of worms (± SD)**	**Mean weight (± SD)**	
		Day 0	**Day 10**
A	169.25 ± 40.63 [a]	39.5 ± 6.6	49.02 ± 6.63
B	43.24 ± 29.06 [b]	40.0 ± 5.41	51.02 ± 5.25
C	184.75 ± 56.94 [a]	35.5 ± 6.80	43.07 ± 3.98
D	247.0 ± 30.11 [a]	39.5 ± 8.69	49.1 ± 7.0
E	442.5 ± 57.81 [c]	39.5 ± 8.38	37.82 ± 8.72
F	0.75 ± 1.5 [b]	34.0 ± 8.48	42.1 ± 6.5
G	N/A	40.6 ± 7.19	49.75 ± 4.71

Data are expressed as mean ± SD and compared with each group. Different letters indicate significantly different mean values ($P < 0.05$).

Table 2. Red and white blood cell counts and biochemical indicators of gerbils with or without infection by *Haemonchus contortus* on day 10 PI.

	A	B	C	D	E	F	G
			Red blood cells				
RBC (x 10^6/µl)	6.6 ± 0.4 [a]	6.7 ± 0.8 [a]	6.8 ± 0.5 [a]	6.5 ± 0.7 [a]	4.2 ± 0.3 [b]	6.7 ± 0.4 [a]	6.39 ± 0.32 [a]
HCT (%)	36.2 ± 1.7 [a]	36.6 ± 4.1 [a]	37.7 ± 1.6 [a]	36.1 ± 2.8 [a]	23.2 ± 1.3 [b]	36.7 ± 1.2 [a]	38 ± 1.84 [a]
PLT (x 10^3/µl)	45.2 ± 5.1 [a]	47.2 ± 20.8 [a]	40.0 ± 8.1 [a]	64.0 ± 24.5 [a]	185.2 ± 25.5 [b]	48.25 ± 12.0 [a]	53.5 ± 14.84 [a]
MCV (fl)	54.3 ± 1.8 [a]	54.4 ± 1.3 [a]	54.9 ± 2.11 [a]	55.9 ± 2.4 [a]	54.8 ± 1.6 [a]	55.4 ± 1.5 [a]	55.4 ± 1.5 [a]
MCHC (%)	37.1 ± 0.9 [a]	35.9 ± 1.0 [a]	36.6 ± 1.7 [a]	36.9 ± 1.3 [a]	52.4 ± 2.8 [b]	35.5 ± 0.1 [a]	30.22 ± 0.29 [c]
			White blood cells				
WBC (x 10^3/µl)	4.6 ± 0.9 [a]	3.8 ± 1.9 [a]	3.3 ± 0.42 [a]	2.2 ± 1.2 [a]	17.2 ± 7.1 [b]	1.7 ± 0.3 [a]	3.7 ± 2.01 [a]
Lymphocytes (%)	71.5 ± 6.8 [a]	63.2 ± 5.9 [a]	68.5 ± 5.8 [a]	65.0 ± 6.4 [a]	91.7 ± 2.0 [b]	64.5 ± 4.4 [a]	72 ± 1.82 [a]
Neutrophils (%)	25.7 ± 6.6 [a]	34.0 ± 5.4 [a]	28.5 ± 6.5 [a]	32.7 ± 6.7 [a]	6.2 ± 1.9 [b]	35.2 ± 2.7 [a]	25 ± 1.41 [a]
			Biochemical indicators				
Total protein (mg/dl)	5.5 ± 0.4 [a]	5.2 ± 0.6 [a]	5.3 ± 2.1 [a]	6.1 ± 0.3 [a]	9.0 ± 2.2 [b]	5.5 ± 0.13 [a]	4.82 ± 0.26 [c]
Total globulins (mg/dl)	2.7 ± 0.2 [a]	2.5 ± 0.3 [a]	2.8 ± 0.8 [a]	2.9 ± 0.5 [a]	6.2 ± 2.9 [b]	2.7 ± 0.23 [a]	2.22 ± 0.09 [c]
Albumin (mg/dl)	2.8 ± 0.2 a	2.7 ± 0.3 a	2.4 ± 1.2 a	3.1 ± 0.2 a	2.8 ± 0.8 a	2.8 ± 0.2 a	2.6 ± 0.18 a

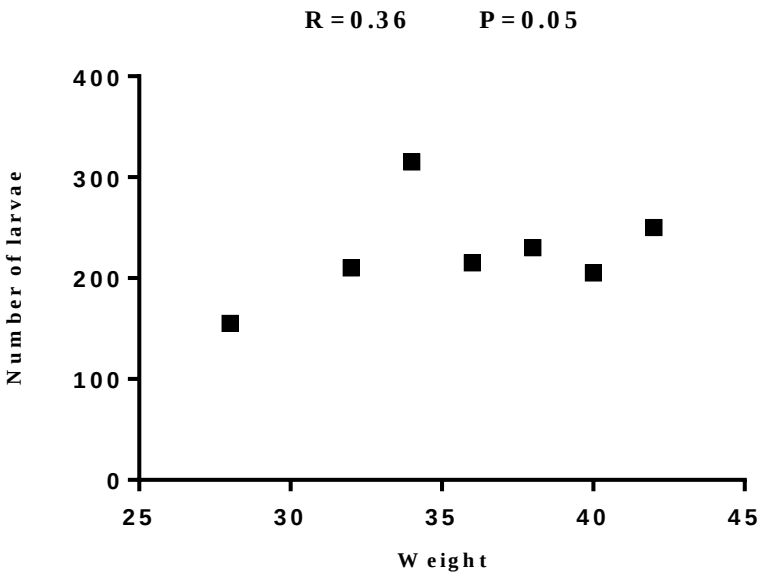
Data are expressed as mean ± SD and each treatment was compared with the control. The letters compare means in the columns. Different letters indicate significantly different mean values ($P < 0.05$). **A** - infected with 2 x 10^3 larvae; **B** - infected with 2 x 10^3 larvae and immunosuppressed (protocol 1); **C** - infected with 6 x 10^3 larvae and immunosuppressed (protocol 1); **D** - infected with 2 x 10^3 larvae and immunosuppressed (protocol 2); **E** - infected with 2 x 10^3 larvae and immunosuppressed (protocol 3); **F** - infected with 2 x 10^3 larvae (exsheathed) and immunosuppressed (protocol 1); **G** - Uninfected.

Table 3. Protocols used to infect gerbils with *Haemonchus contortus* over the last two decades.

Reference	Sex	Age	Weight	Immun.	Protocol	Number of larvae	Sheathed larvae	Mean number of worms
GRANDO et al., 2016	M/F	8-9 weeks	35-40 g	Yes	A	2000	Yes	44.0
MACEDO et al., 2015	M/F	5 weeks	25-35 g	Yes	B	5000	No	363.2
RIBEIRO et al., 2013	M/F	± 7 weeks	30-34 g	Yes	C	4500	No	171.8
KÖNIGOVÁ et al., 2012	NI	NI	60-65 g	Yes	D	1000	Yes	157.1
SQUIRES et al., 2011	Female	± 5 weeks	50 g	No	_	600	No	97.1
SQUIRES et al., 2010	Female	± 5 weeks	50 g	No	_	600	No	78.25
DE JESÚS-GABINO et al., 2010	M/F	± 5 weeks	20-25 g	Yes	F	40000	No	78.0
KÖNIGOVÁ et al., 2008	NI	6-8 weeks	60-68 g	Yes	E	1000	Yes	92.14
OSTLIND et al., 2006	M/F	± 5 weeks	30-34 g	No	_	500	Yes	135.08
ROJAS et al., 2006	Male	22 days	14 g	No	_	1000	Yes	145
MOLENTO et al., 1999	Female	NI	30-35 g	Yes	G	1000	No	109.66

Protocols for immunosuppression: A - Dexamethasone (Azium®, Coopers Animal Health), 0.2 mg per animal, 3 days before and 2 days after infection; B - Hydrocortisone (Azium®, Schering-Plough Labs), 0.2 mg per animal, 2 days before infection; C - Dexamethasone (Azium®, Coopers Animal Health) 0.2 mg per animal, 3 days before infection; D - Hydrocortisone, 6 mg per animal, 7 days before infection and every day after infection; E - Hydrocortisone, 6 mg/kg, 7 days before infection and every other day after infection; F - Hydrocortisone (Azium®, Shering-Plough Labs), 100 µl per animal, 2 days before infection; G - Hydrocortisone 0.02% in the feed, 5 days before infection and during maintenance of the infection. M/F = male/female; Immun. = immunosuppression; NI = not informed.

Figure 1. Relationship between the number of larvae recovered and host body weight (g).



3 MANUSCRITO 2

(Artigo submetido para publicação – Veterinary Parasitology)

Anti-*Haemonchus contortus* IgY produced against L3 hampers the larval establishment in experimentally infected *Meriones unguiculatus*

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Anti-*Haemonchus* IgY *contortus* L3 and therapeutic potential

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ABSTRACT

The aims of this study were to produce antibodies against third instar larvae of *Haemonchus contortus* and to evaluate their biological activity using *Meriones unguiculatus* as the experimental model. For the production of IgY, larvae antigens were inoculated into hens, the eggs were collected, and the antibody extracted. The characterization of the anti-*H. contortus*

IgY was performed using polyacrylamide gel electrophoresis, Western and Dot blotting and Enzyme-Linked Immunosorbent Assay (ELISA). The Dot Blotting and ELISA-avidity results showed the reaction of the antigen-antibody and a high bond strength, respectively. The biological activity was evaluated in steroid-treated and experimentally infected gerbils in three different assays. In our first assay no one difference in the parasite burden was observed, however, the death was prevented in gerbils treated with IgY orally but not on those treated intraperitoneally and the control group. In other assay was showed a reduction on the parasitic burden of orally IgY treated gerbils on day 10 post infection. The results showed that antigens of *H. contortus* larvae induce the production of immunoglobulins highly avidity and the immunotherapy hampered significantly the establishment of the parasitic infection in gerbils killed 10 days pi. Further studies should be performed to clarify the activity of these antibodies and mainly their mechanism of action.

Keywords**:** Gerbil; Gastrointestinal nematode; Haemonchus; Immunotherapy; Protective antibody; IgY

INTRODUCTION

Haemonchus contortus is a highly pathogenic helminth mainly due to its blood-feeding behavior and fast development of large burdens (Besier et al., 2016a). Currently, haemonchosis is one of the most important parasitic diseases of livestock worldwide affecting thousands of sheep and goats, causing substantial production losses estimated at tens of billions of dollars every year (Preston et al., 2015).

In conjunction with the increasing resistance to anthelmintic, the cost of livestock production is rising, as well as the need to develop new and sustainable preventative strategies. Alternative methods of control may reduce the frequency of chemical treatments, decreasing

the selection pressure for resistant nematodes as well as anthelmintic residues in animal products and the environment (Besier et al., 2016b).

Non-chemical strategies are necessary for better parasite control, and some methods, as biological control and alternative anthelmintic compounds are interesting but clear evidences of their effectiveness are still needed (Besier et al., 2016b). Perhaps, today the most efficient method available is the vaccine “Barbervax” that provides an interesting protection against *H. contortus* infection in sheep (Bassetto et al., 2014) and dairy goats (De Matos et al., 2017) been required a vaccination schedule with frequently applications, mainly in the periods with high challenge.

The oral administration of IgY has been proven successfully for the treatment of a variety of gastrointestinal pathogens such as *Helicobacter pylori* (Malekshahi et al., 2011), *Clostridium difficili* (Mulvey et al., 2011), canine parvovirus (Van Nguyen et al., 2006) and bovine rotavirus (Vega et al., 2011). Regarding the use of IgY for passive immunization against gastrointestinal parasites, Lee et al. (2009a; 2009b) evaluated IgY produced against different species of *Eimeria* and showed its immunotherapy activity. Kobayashi et al. (2004) showed *in vitro* and *in vivo* that specific IgY prepared against whole oocyst antigens has protective effects against *C. parvum*. However, the immunotherapy using IgY technology is restricted to protozoa. In relation to helminthes, the IgY is focused on the development of IgY-based to apply yolk antibodies in enzyme-linked immunosorbent assay (ELISA) for diagnosis of parasites such as *Trichinella spiralis* (Xu-xu et al., 2010) and *Schistosoma japonicum* (Cai et al., 2012).

Considering that the parasite *H. contortus* is able to stimulate the immune system of hens producing highly specific antibodies, the aims of this study were to produce specific avian

antibodies (IgY) against *H. contortus* L3 and evaluate their activity on *Meriones unguiculatus* experimentally infected by this parasite.

MATERIALS AND METHODS

Production of L3 antigens

The multiresistant *H. contortus* isolate (Almeida et al., 2010) was used to infect a sheep absence of parasitism. The animal was fed with hay and water *ad libitum*. The infection occurred orally with 10.000 *H. contortus* larvae at three different moments at three days intervals. For the first infection approximately 4000 larvae were used and the other two used 3000 larvae. The larvae used for the production of antigens were recovered from coprocultures, according to Roberts and O'Sullivan (1950) modified by Ueno (1998) and stocked in falcon tubes (50 mL) under refrigeration at 4°C.

The L3 were sonicated using the equipment Ultrasonic Processor (Sonics VibraCell T.M.) in 3 times (3 min each) with intervals of 3 min. The first time was divided in 2 amplitudes, the first 90 sec at 40 Hz and the other 90 sec at 80 Hz. The last two times were used one amplitude of 80Hz. The pulse was of 3 sec in all the times (Cai et al., 2012) with modifications.

Protein quantification

The protein concentration content of the antigen solution was measured by the method of Bradford (1976). Coomassie Blue (2.5 mL) was mixed with 50 µL of the antigen. After 10min, the sample was read by measuring the maximum absorbance of the solution at 595 nm,

using bovine serum albumin as the standard. For the calculation of protein concentration, the following formula was used:

Absorbance (Abs) x correction factor of Coomassie = mg/ mL protein

After this process, aliquots of antigen solution of 1 mL with approximately 1500 µg/mL were stored in 2 mL tubes in the freezer at -20 ºC.

Chicken immunization protocol

The chicken inoculation protocol was performed as described by Sampaio et al. (2014). Briefly, 1 mL of antigen solution with approximately 1500µg of protein concentration was added to incomplete Freund's adjuvant (Difco®) at a 1:1 ratio, with a final volume of 2 mL. A volume of 1 mL was injected (peer chicken) intramuscularly at five different sites of the pectoral muscle of two 22-week-old Leghorn red chickens. The interval between immunizations was set as two weeks with six inoculations total. Chicken eggs were collected every day from the first week post-immunization for 28 weeks and stored at fridge 4 °C up to analysis.

Extraction of anti-H. contortus IgY

Extraction was performed by the method of precipitation of polyethylene glycol 6000 (PEG-6000) (Pauly et al., 2011; Polson et al., 1980). The yolk was separated from the white, filtrated in paper filter and placed in a 250 mL tube. The fat content was removed using PBS (1:2 ratio) and PEG 6000 at a final concentration of 3.5%. This mixture was held in a roller mix for 30 min and centrifuged for 80 min at 4.000 rpm and 4 °C. The supernatant was filtered in paper filter (28 µm) and the final volume measured. PEG 6000 was once again added (8.5%),

and the solution kept in a roller mix for 10 min, and centrifuged for 30 min at 4000 rpm and 4 °C. At this stage, the supernatant was discarded, and the pellet was resuspended in 10 mL of PBS. A final step using PEG 6000 (12%) was performed as described previously. The final pellet was resuspended in 2.4 mL of PBS, and this solution was dialyzed overnight in a saline solution (0.1%) at 4 °C. A final dialyzation was performed for three more hours, the extract was collected, placed in microtubes, stored at −20 °C, and lyophilized until further use.

The polyacrylamide gel electrophoresis

The IgY sample were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% (Sampaio et al., 2014). For this, 20 µL of the IgY sample collected from chicken post-immunization were mixed with 20 µL of the buffer (Laemmli Sample Buffer – BioRad®), boiled in a water bath at 95 °C for 5 min, and applied to the stacking gel wells in a final volume of 5 µL. The electrophoresis was submitted to a current of 70 V for 30 min and 120 V for 120 min. Finally, the gel was colored with Coomassie Blue R (BioRad®) for at least 1 h and treated with a decolorizing solution (glacial acetic acid and methanol).

Western blot

Western blot was performed as described by Sampaio et al. (2014). Initially, electrophoresis was performed under the same conditions as described above. After protein separation in polyacrylamide gel, it was electrophoretically transferred to a nitrocellulose membrane of 0.45 µm (BioRad) using a current of 140 V for 120 min. Finally, the membrane was removed carefully, and blocked overnight in a PBS-Tween 20 solution with 5% skimmed milk powder. The membrane containing the IgY was washed three times with PBS-Tween and

incubated for 1 h with rabbit anti-chicken antibody peroxidase conjugate (Sigma-Aldrich®) diluted in PBS-Tween 20 (1:2000). The membrane was washed three times with PBS-Tween, and finally incubated with a developing solution (DAB–diaminobenzidine, Tris HCl, Nickel sulfate 0.3%, and H_2O_2) for the visualization of any reactive band.

Dot blot

Dot blot was performed through the application of the L3 antigen on nitrocellulose membrane (BioRad) in 24-well-plate. The nitrocellulose membrane containing *H. contortus* antigen was blocked for one hour in a PBS-Tween solution with 5% skimmed milk powder. It was washed three times with PBS-Tween 1% (1 min each) and incubated for one hour with anti-*H. contortus* IgY and the control IgY. The membrane was washed again three times with PBS-Tween, incubated for one hour with rabbit anti-chicken antibody peroxidase conjugated (Sigma-Aldrich®). The washing was repeated three more times with PBS-Tween, and finally incubated with a developing solution (DAB–diaminobenzidine, Tris HCl, Nickel sulfate 0.3%, and H_2O_2) for the visualization of any colorimetric reaction.

ELISA and ELISA avidity

Ninety-six-well ELISA plates (Corning Costar Corporation, Cambridge, MA, USA) were sensitized with soluble antigen (1 µg/well) diluted in 0.05 M of carbonate buffer (pH 9.6) and incubated overnight at 4 ºC. After incubation, the plates were washed four times with saline buffer solution (SBS) (pH 7.2). For testing, the plates were first blocked with 100 µL per well of fetal bovine serum (FBS) (Cripion®, SP, Brazil) (FBS + SBS) and incubated at 37 ºC. After this, the washing procedure was repeated using SBST (SBS pH 7.4 with 0.05% of Tween 20). The tested IgY were diluted at 1:10, 1:100, 1:500, 1:2000, 1:3000, and 1:5000 in FBS + SBS,

distributed on the plates (100 µL/well), and incubated for 1 h at 37 ◦C. Followed this last incubation, the washing procedure was repeated. Each tested sample was subjected to three replicates per plate and two inter-plate repetitions. After incubation with the primary antibody, the plates were washed four times with SBST and subjected to an incubation with the secondary antibody (IgY anti-peroxidase conjugate) (Sigma–Aldrich®, USA) at 1:3000 dilution and incubated for 1 h at 37 ºC. Finally, the plates were washed again and 100 µL of chromogenic substrate (orthophenylene diamine, OPD) was added. After 15 min, the reaction was blocked with 10 µL of H_2SO_4 (4 N), and reading was performed on a microplate spectrophotometer using a 490 nm filter.

To perform the ELISA avidity test, 96-well ELISA plates were sensitized with antigen and incubated with IgY (1:3000), as described above. Each well received 100 µL of 6 M urea in SST (pH 7.2) for five minutes. Plates were washed three times with SSTT and the secondary antibody was added. Revelation was performed as described earlier. The results were expressed as the avidity index (AI) determined by the ratio between optical density values of samples treated with urea (U+) and the optical density of untreated samples (U−) and expressed in percentage (AI = U+/U− × 100). The values (AI) <40% were considered of low avidity, AI between 41 and 70% of medium avidity and AI >70% of high avidity (Sampaio et al., 2014).

IgY concentration

The concentration of anti-*H. contortus* IgY in the protein extract (PE) dialyzed was measured according to the Lambert-Beer's law using an extinction coefficient of 1.33 for IgY (Polson et al., 1980). Two independent samples were prepared diluted 20mg of PE lyophilized to 1ml of distilled water in microtubes 1.5 mL. The protein content of the samples

was measured in duplicate with Coomassie blue using bovine serum albumin as standard, and monitored by measuring the maximum absorbance of the solution at 595 nm (Bradford, 1976).

In vivo assay 1 – IgY in two routes (oral and intraperitoneal)

Larvae ex-sheathed method - The larvae were ex-sheathed by incubation in sodium hypochlorite (NaClO) [0.9%], add 14 µL of NaClO per 1 mL. When 90% of the larvae were ex-sheathed, they were washed by adding distilled water and centrifugated for 3min at 300 *xg*. This procedure was repeated three times. After this process, the larvae were placed on a mesh of 25µm and those with high motility were selected.

To evaluate the immunotherapy activity of IgY two routes were used in twenty-four outbred gerbils (*M. unguiculatus*), parasite-free and clinically healthy, male or female, average weight 31g, five-week-old (34-36 days) obtained from the Animal Care Center. After a week of acclimatization, the animals were randomly divided into three groups of 8 animals each. The gerbils were kept in the polypropylene boxes under controlled temperature and humid (22 °C ± 2 ºC; 40% UR), a 12/12 h dark/light cycle and fed commercial feed and water *ad libitum*. The animals were previously immunosuppressed for better establishment of the infection with Methylprednisolone (Depo-Medrol®Pfizer), 4 mg/IM, three doses (days -2, -1, 0) according Gressler et al. (not published yet), infected orally (day 0) with $2x10^3$ larvae ex-sheathed (1 day) and treated daily for 10 days (3 days before infection and 7 dpi). The experimental groups were:

Group 1 - treated with NaCl 0.9% (control group);
Group 2 - treated orally with 25 mg/Kg IgY dissolved in NaCl 0.9%;
Group 3 - treated intraperitoneally with 25 mg/Kg IgY dissolved in NaCl 0.9%.

Parasite recovery and sample collection – Ten days after infection the animals were previously euthanized using an isoflurane chamber. The stomach was removed, externally washed with 10 mL of water pre-heated at 35-39°C (average 37°C), opened longitudinally on petri dish and incubated with 20 mL NaCl 0.9% at 37°C in an incubator at 37°C for 5 hours following the method by Conder et al. (1992) with modifications. After this period, the stomachs were washed with NaCl 0.9% and the larvae solution stored in falcon tubes of 50 mL with tamponed formaldehyde at 4%. The parasites were counted using inverted optical microscopic 40x.

In vivo assay 2 - Evaluation of the oral administration by IgY in gerbils immunosuppressed infected with L3 sheated

Twenty-eight gerbils, male or female, 35 days-old (32-38), average weight 35g, were immunosuppressed with Depo-Medrol®Pfiser, 2-4mg/IM according to body weight on days -2 and -1. The gerbils were kept fasted 18h for a better larvae establishment, infected on day 0 with $2x10^3$ of larvae sheathed (20 day). The groups 2 and 4 received an extra dose of corticoid 7 dpi.

Group 1 - control group treated 10 days (on days -2 − 7 pi) with 0.25 mL NaCl 0.9%;
Group 2 - control group treated 15 days (on days -2−12 pi) with 0.25 mL NaCl 0.9%;
Group 3 - treated 10 days later (on days -2 − 7 pi) with 25mg/Kg IgY;
Group 4 - treated 15 days later (on days -2 − 12 pi) with 25mg/Kg IgY.

Groups 1 and 3 were euthanized on day 10 pi and groups 2 and 4 on day 15 pi. The animals were euthanized using an isoflurane chamber and the stomachs were removed, washed and stocked as described above with minor modification.

Statistical analysis

Normality and homoscedasticity were analyzed through the Shapiro-Wilk and Levene test, respectively. For the *in vivo* experiment one and three, data were compared using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test, and levels of significant differences using $P<0.05$. For the experiment three, the reduction (%) of the parasite burden was estimated using the following formula: (mean parasite burden in the control group – mean parasite burden in the treated group)/mean parasite burden in the control group x 100.

RESULTS

Gel electrophoresis SDS–PAGE and Western blot assay

Fig. 1a shows SDS-PAGE bands of stained peptides with molecular weight between 75 to 50 kDa and 37 to 25 kDa. The recognition of IgY peptide bands was performed by Western blot, where anti-*H. contortus* IgY reacted with the secondary antibody peroxidase conjugated, showing specific peptide bands corresponding to the heavy and light chains of IgY (Fig. 1b).

Dot blot assay

Fig. 2 shows that IgY was able to recognize *H. contortus* antigens and the IgY control was negative, indicating IgY specificity to its target antigen.

ELISA and ELISA avidity assay

ELISA results demonstrated an IgY increase from yolk collected on the 3rd and 4th week after the first inoculation (day 0). After the last immunization (10th week) IgY concentration decreased in the 22th week, and IgY synthesis was noted until the 30th week despite its low concentration (Fig. 3).

The avidity index at 1:10 dilution after the 4th week was higher than 75% and kept this index until the 30th week. For dilutions 1:100 and 1:1000, it was observed an avidity between 50 to 75% until the 26th week. Already at 1:5000 dilution avidity index was lower than 50% in all observed weeks (Fig. 4).

IgY concentration

The dilution of 20mg/mL of the PE showed the average concentration of IgY of 5.06 ± 0.87mg/mL.

In vivo assay 1

The kinetics of larvae populations in the stomach of gerbils treated orally and intraperitoneally (IP) are summarized in Table 1. There was no significant difference (P=0.05) between experimental groups. However, during the *in vivo* assay two animals of the control group and three animals IP treated died or were euthanized when they deemed too sick.

In vivo assay 2

Fig. 5A shows a significant reduction (P<0.01) in the mean number of larvae recovered from stomach of gerbils orally treated with IgY 10 dpi compared to the control group. There

was no difference (p=0.05) between treated and the control group on day 15 pi (Fig. 5B). The ratio of larvae recovered of the control group was significantly different on days 10 and 15 p.i. (P<0.01) (Fig. 5C).

DISCUSSION

Due to the importance of *H. contortus* and the role of the immune response to control the infection, our study aimed to evaluate the ability of hens to produce specific antibodies against third instar *H. contortus* larvae and its immunotherapeutic activity in gerbils experimentally infected by this parasite. Our results demonstrated for the first time to produce IgY after the inoculation of *H. contortus* antigens into hens. There are several available IgY isolation methods, but mostly based on polyethylene glycol (PEG) precipitation of supernatants, which usually yiel also protein impurities (Amro et al., 2017). However, other methods can be performed by chromatographic techniques and ultrafiltration. In this study, the salt precipitation method using PEG–6000 as described by Polson et al. (1980) was used. This method has been explored by our research group and it proved to be effective (Sampaio et al., 2014). In addition, it is easy to perform, yields high quantities of antibody and it can be done at room temperature without immunoglobulin denaturation (Grando et al., 2017).

The characterization of anti-*H. contortus* IgY was performed by SDS-PAGE and Western blot. The IgY produced in our experiment showed 75-50 kDa for the heavy chain and 37-25 for the light chain, resulting in the molecular weight of approximately 105 kDa. The results found in our study were similar to those described for anti-*Schistosoma japonicum* IgY with light and heavy chain between 25 and 68 kDa, respectively (Cai et al., 2012) and IgY against excretory-secretory antigens of *T. spiralis* muscle larvae with molecular weight of 67 kDa and 23 kDa (Xu-xu et al., 2010), demonstrating that there is a certain variability in the molecular weight of IgY.

The active antibodies against *H. contortus* larvae were demonstrated by Dot-blot and ELISA. Our results showed that chickens may produce antibodies for long time after the last inoculation (± 20 weeks), and that they are quite responsive against parasitic antigens. The strength of the antigen-antibody was clearly demonstrated by ELISA-avidity and the result shows the immunogenic potential of larvae to induce the synthesis of antibodies with high avidity.

Specific chicken antibodies have been successfully used against a wide variety of parasites (Table 2), especially for diagnosis purposes. Among these experiments, we highlight the IgY activity against some species of *Eimeria* since their results provide clear evidences that passive immunization of chickens with egg yolk IgY provides significant protection against *E. tenella* or *E. maxima* (Lee et al., 2009b) and *E. acervulina* (Lee et al., 2009a). However, there are no reports on IgY therapy against helminthic infections.

Meriones unguiculatus is considered the best experimental model due to physiological and morphological characteristics of its stomach compared to sheep stomach (Conder et al., 1990; Conder et al., 1992). A large number of *in vivo* studies are conducted using gerbils as the experimental model. This model has shown considerable value in the knowledge of resistance reversion (Molento and Prichard, 1999), evaluation of drug efficacy (Kates and Thompson, 1967) and currently, on the research of new molecules with antiparasitic potential, such as monepantel (Kaminsky et al., 2008).

In our study only animals treated with IgY orally did not die and it has been reported that steroid-treated gerbils may die of severe hemorrhage associated with migrating autoinfective larvae after hyperinfection by *Strongyloides stercoralis* (Kerlin et al., 1995). Moreover, it has been suggested that paralytic ileus underlies the subsequent death observed in gerbils after implantation of adult *Strongyloides papillosus* (Kobayashi et al., 2009).

Among the mechanisms used by immunoglobulins to protect the animal against larval infections, it should be included the antibody-mediated neutralization of larval proteases, anal and oral pore blockade by immunocomplexes, mutation prevention and inhibition of larval development (Tizard, 2014). The lower number of larvae recovered on day 10 pi, approximately 50%, in gerbils treated for IgY suggest that the activity of the antibodies on the larvae would hamper the larvae establishment and consequently the parasite rejection would happen. According to Nisbet *et al.* (2016) an antigen Hc-sL3 recognized by the sheep immune system during a response against *H. contortus* shown to be expressed in a stage-specific manner on the surface of L3. In the present study, the antibody production was performed through L3 antigens, therefore is possible there are specific IgY antibodies against surface antigens. Recently, vaccination trials confirmed the protective properties of the larval surface antigen and level of reduction in worm burden approximately 60% in field sheep (Piedrafita et al., 2012).

On the contrast with the results found on day 10 pi, no differences were observed on day 15 pi. Our hypothesis is that the large reduction of the larvae recovered in the control group on day 15 may have influenced significantly and this should be better evaluated in upcoming studies. The ratio of larvae recovered in the control group was different on days 10 and 15 pi. In agreement with our finding, the reduction of parasite burden throughout infection was also observed in *M. unguiculatus* infected by *H. contortus* on days 4, 7, 10 and 14 by Königová et al. (2008).

It is clear that chickens immunized with *H. contortus* larvae are capable of producing specific immunoglobulins and these are highly avid against the parasite. Our result allows us to claim that the immunotherapy may interfere significantly with the establishment of the *H. contortus* infection in gerbils. Therefore, it is suggested new studies to clarify the activity of the antibodies, such as the mechanism of action.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STANDARDS

This study was approved by the Committee on Animal Research and Ethics (CARE) of the Universidade Federal de Santa Maria (UFSM), under protocol number 3216240915/2016 and 3787160917/2017.

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Figure 1. (A) IgY electrophoresis in polyacrylamide gel (12%), stained with Coomassie Blue R250 reagent (Bio-Rad). (B) Black arrows indicate the heavy and light chains IgY produced post-immunization. The red arrows indicate the bands 75, 50, 37 and 25 kDa in the protein marker. Intervals of the bands being, 75 and 50 kDa refers to the heavy chain and 37 and 25 kDa refers to light chain.

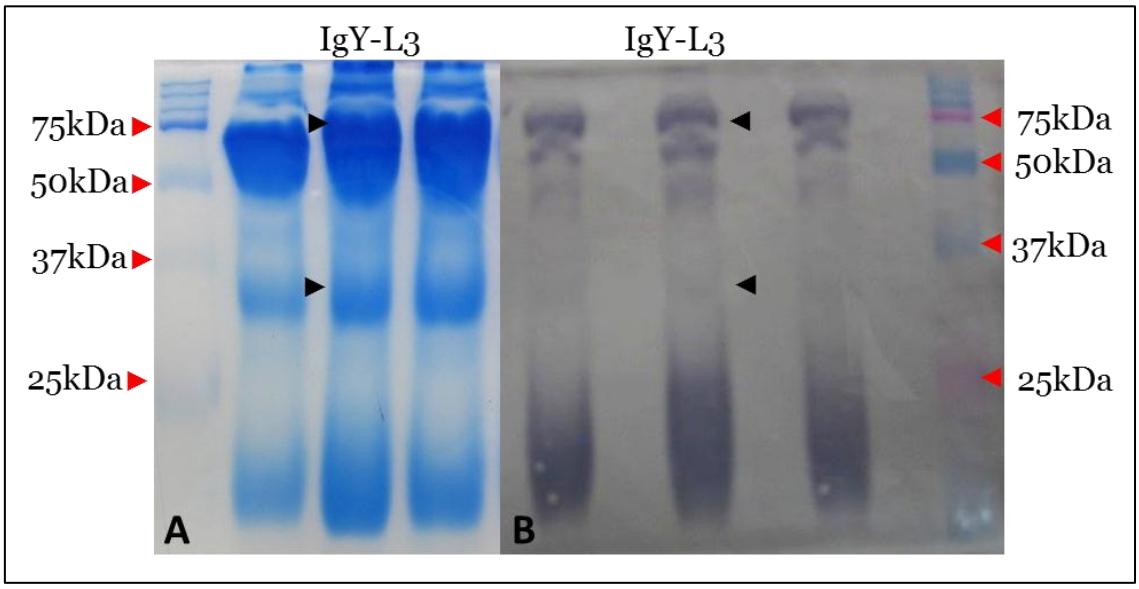


Figure 2. Dot-blot showing the specific recognition of IgY anti-*H. contortus* to the protein antigens from larvae (L3) of *H. contortus* after the addition of peroxidase-conjugated secondary antibody and incubated with a developing solution.

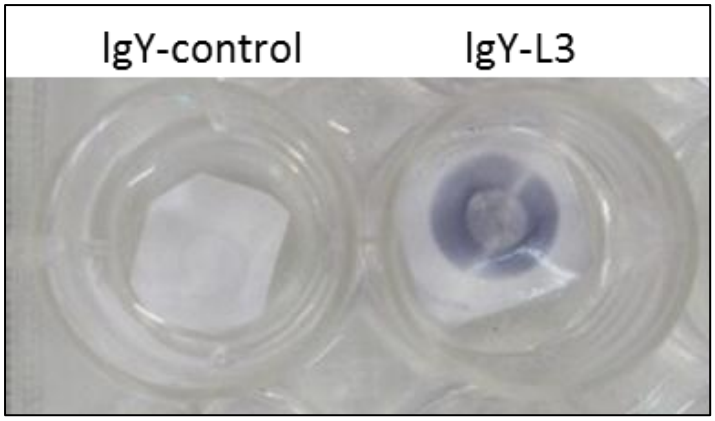


Figure 3. Optical density (O.D) values obtained in the ELISA using purified IgY from egg yolk of immunized chickens. The samples tested were IgY extracted each 2 weeks after immunization. The circle indicates the time of the last immunization.

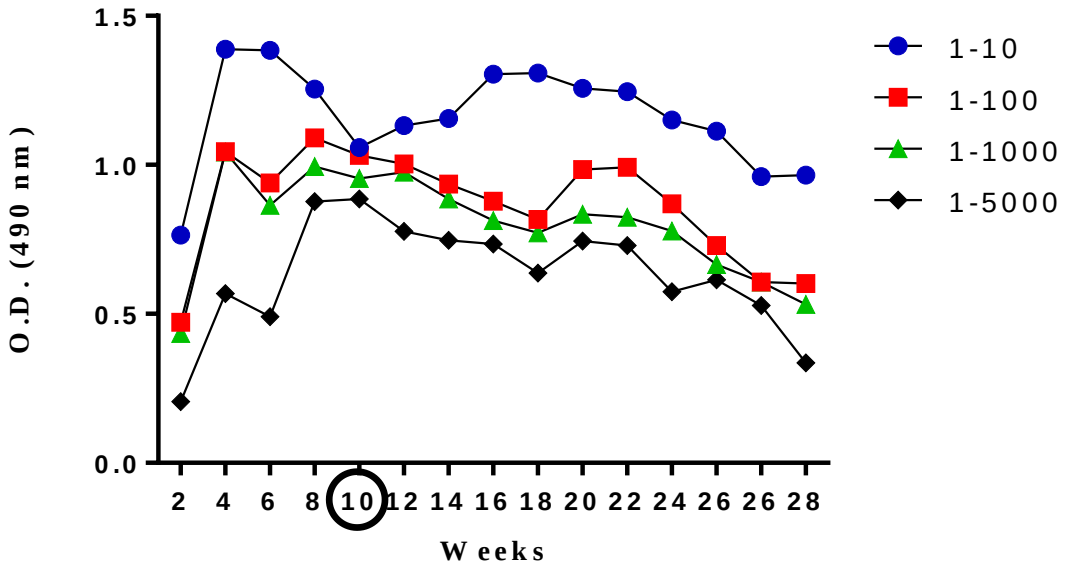


Figure 4. Avidity index (AI) of IgY antibodies produced during immunization of the hens with *H. conortus* antigens. AI < 40% were considered of low avidity; AI between 41 and 70% represented a medium avidity; and AI > 70% was considered of high avidity.

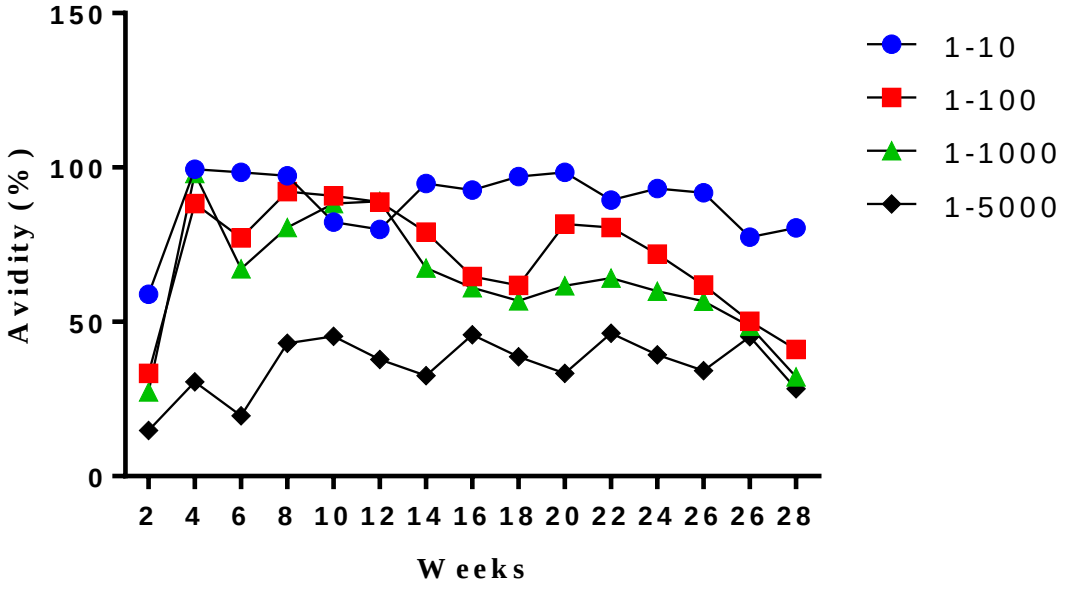
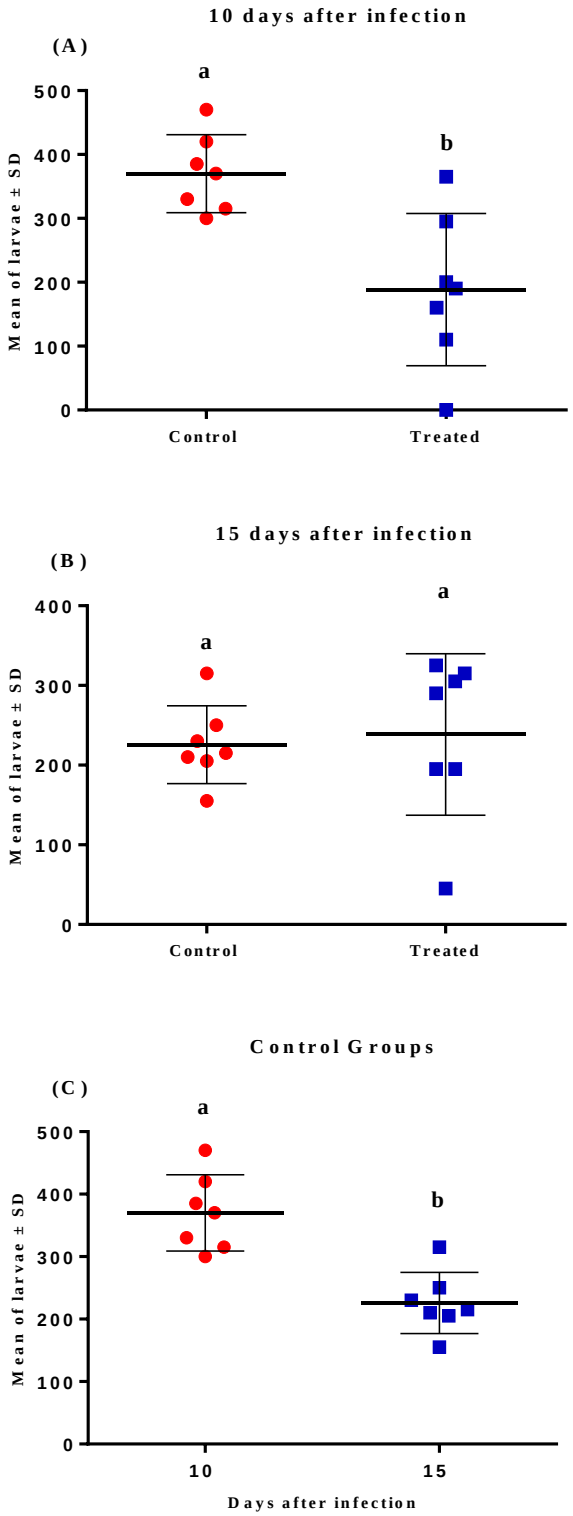


Table 1. Mean numbers of *H. contortus* larvae recovered at necropsy on days 10 post-infection (p.i.) from the stomach of gerbils.

Groups (n=8)	Mean worm (±SD)	Number of dead animals (Dead/Total)
Control	58.5±14.0 [b]	2/8
Treated IgY VO	74.71±32.7 [b]	0/8
Treated IgY IP	60.80±23.5 [b]	3/8

Data are expressed as mean ± SD and compared to each group. Upper case letters compare mean in the columns. Different letters indicate significant differences for P value < 0.05.

Figure 5. Mean number of larvae recovered from the stomach of gerbils treated orally with IgY anti-*H. contortus* on days 10 and 15 post infection.



Data are expressed as mean ± SD (standard deviation) and compared between groups. The letters compare mean in the columns. Different letters indicate significant differences for P value < 0.01.

Supplementary Table. Parasites used to perform the production of IgY.

Agent	Reference
Protozoa	
Cryptosporidium parvum	(Kobayashi *et al.*, 2004); (Omidian *et al.*, 2014)
Eimeria acervulina	(Lee et al., 2009)
E. tenella and E. maxima	(Lee et al., 2009)
Giardia duodenalis	(García *et al.*, 2005)
Toxoplasma gondii	(Hassl *et al.*, 1987); (Ferreira *et al.*, 2012)
Trypanosoma cruzi	(Contreras *et al.*, 2005); (Grando *et al.*, 2017)
Trypanosoma evansi	(Sampaio *et al.*, 2014)
Trematoda	
Schistosoma japonicum	(Cai et al., 2012)
Cestoda	
Echinococcus granulosus	(Gottstein and Hemmeler, 1985)
Nematoda	
Haemonchus contortus	Presente study
Opisthorchis viverrini	(Teimoori *et al.*, 2017)
Trichinella spiralis	(Xu-xu *et al.*, 2010)

4 MANUSCRITO 3

(Artigo em preparação para publicação – Parasitology)

Therapeutic activity of IgY antibodies anti-*Haemonchus contortus* producted from adult stages in *Meriones unguiculatus*

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Abstract

The use of chicken IgY is an attractive approach due to the fact that chicken egg yolk is an inexpensive and convenient source for mass production of specific antibodies. Therefore, the aims of this study were to produce antibodies against adult stages of *Haemonchus contortus*, to characterize and to evaluate their biological activity. For the production of IgY, adult antigens were inoculated into hens within two weeks for 3 months, the eggs were collected, and the antibody extracted. The characterization of the IgY anti-*H. contortus* was performed using polyacrylamide gel electrophoresis, Western and dot blotting and enzyme-linked immunosorbent assay (ELISA). The biological activity was evaluated in steroid-treated and experimentally infected gerbils. The characterization of the antibody reveled bands of stained peptides with molecular weight between 75-50 kDa and 37-25 kDa. The dot-blotting and ELISA-avidity results showed the reaction of the antigen-antibody and a high bond strength,

respectively. In gerbils treated with IgY were found a low parasitic burden 10 dpi., showing the activity of avian antibodies over the larvae establishment.

Keywords: immunotherapy, helminth, gerbil, protection

Introduction

Current state of anthelmintic resistance around the world is unsafe. In some studies conducted in the US, Brazil, Africa, Australia, New Zeland and Europe the results showed there is multiple resistance parasites and in some goat and sheep farms the resistance exist to all available anthelmintic drugs (Kaplan and Vidyashankar, 2011).

The main nematode of small ruminant is the blood sucking parasite *Haemonchus contortus*. Beyond the high prevalence and intensity of the infection, this specie has shown a high ability to develop resistance to all drug classes available. Hence, it is observe significant economic impact on a worldwide (Kotze and Prichard, 2016). The researches about alternative control methods objectifying to supplement the use of anthelmintic had been performed mainly for nematophagous fungi and bioactive compounds with anthelmintic potential (Besier *et al.*, 2016).

The therapeutic activities of avian antibodies (IgY) are reported against different microorganisms such as virus, bacteria, fungi and parasites. The use of chicken IgY as an attractive approach resulted from the fact that chicken egg yolk is an inexpensive and convenient source for mass production of specific antibodies (Omidian *et al.*, 2014).

Our recently study showed for the first time that the *H. contortus* L3 is able to enhance the immune system of hens producing specific antibodies and highly avidity. The therapeutic activity of IgY reduced approximately 50% of the parasitic burden in gerbils infected with *H. contortus* 10 days post infection (Gressler, unpublished yet). Due to the encouraging result, the

aims of this study were to produce specific avian antibodies against adults stages and evaluate their therapeutic potential on *Meriones unguiculatus* experimentally infected by *H. contortus*.

Materials and methods

Production of antigens and proteins quantification

One sheep infected with multirresistente *H. contortus* isolate (Almeida *et al.*, 2010) was euthanized and adults recovered and stocked in falcon tubes (50mL) under refrigeration at 4ºC. The adults were sonicated using the equipment Ultrasonic Processor Sonics VibraCell$^{T.M}$ (Gressler, unpublished yet) and stocked again. The protein concentration content of the antigen solution was measured by the method of Bradford (Bradford, 1976) and aliquots (1mL) with approximately1500 µg/ml were storage in 2ml tubes in the freezer at -20ºC.

Chicken immunization protocol

For the chicken immunization, 1 mL of antigen solution was added to incomplete Freund's adjuvant (Difco®) at a 1:1 ratio, with a final volume of 2 mL. A volume of 1 mL was injected intramuscularly at five different sites of the pectoral muscle of two 32-week-old Leghorn red chickens. The interval between immunizations was set as 14 days with six inoculations. Chicken eggs were collected from the first week post-immunization and stored at 4 °C up to analysis (Sampaio *et al.*, 2014).

Extraction of IgY anti-H. contortus

Extraction was performed by the method of precipitation of polyethylene glycol 6000 (PEG-6000) (Polson *et al.*, 1980; Pauly *et al.*, 2011). The yolk was separated of white,

transferred to a filter paper and after to a 250 mL tube and the proteins were extracted and purified by PEG 6000 at a final concentration of 3.5%, 8.5% and 12%. The solution was dialyzed and transferred into 2 mL microtubes, stored at −20 °C, and lyophilized until further use.

IgY Caracterization

The methods for characterization and evaluation of the specificity of IgY anti-*H. contortus* were performed according Sampaio et al. (2014).

Briefly, IgY sample were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After protein separation in polyacrylamide gel, the wester-blot was performed by electrophoretically transferring the gel to a nitrocellulose membrane. Finally, it was incubated with a developing solution for the visualization of any reactive band.

The dot-blot was performed to report the antigen antibody reaction. For this, the adult antigen was applied on nitrocellulose membrane in 24-well-plate, incubated with IgY anti-*H. contortus* and the control IgY and lastly with rabbit anti-chicken antibody peroxidase conjugated (Sigma-Aldrich®). After, adding the developing solution for the visualization of any colorimetric reaction.

The IgY produce over the weeks was performed by ELISA and the strength of binding of the antibody to the antigen was demonstrated by avidity-ELISA. For this, ninety-six-well ELISA plates (Corning Costar Corporation, Cambridge, MA, USA) were firstly sensitized with soluble antigen (1 µg/well). The tested IgY were diluted at 1:10, 1:100, 1:500, 1:2000, 1:3000, and 1:5000, distributed on the plates (100 µL/well), and incubated for 1 h at 37 ºC. After incubation with the primary antibody, the plates were washed and subjected to an incubation

with the secondary antibody (IgY anti-peroxidase conjugate) (Sigma–Aldrich®, USA) at 1:3000 dilution and incubated for 1 h at 37 ºC. The reading was held in microplate spectrophotometer with 490 nm filter.

To perform the ELISA avidity test, 96-well ELISA plates were sensitized with antigen and incubated with IgY (1:3000). Then, in each well, it was added 100 µL of 6 M urea in SST, pH 7.2 for five minutes. Plates were washed three times with SSTT and the secondary antibody added. Revelation was performed as described earlier. The results were expressed as the avidity index (AI) determined by the ratio between optical density values of samples treated with urea (U+) and the optical density of untreated samples (U−) and expressed in percentage (AI = U+/U− × 100). The values (AI) < 40% were considerate of low avidity, AI between 41 and 70% of medium avidity and AI > 70% of high avidity.

IgY specific concentration

The concentration of IgY anti-*H. contortus* was measured according to the Lambert-Beer's law using an extinction coefficient of 1.33 for IgY (Polson *et al.*, 1980). Two independent samples were prepared diluted 20mg of the lyophilized to 1ml of distilled water in microtubes 1.5 mL. The protein content of the samples was measured in duplicate with Coomassie blue using bovine serum albumin as standard, and monitored by measuring the maximum absorbance of the solution at 595 nm (Bradford, 1976).

In vivo assay

Twenty-eight gerbils, male or female, 35 days-old, average weight 35g, were immunosuppressed with Depo-Medrol®Pfiser, 2-4mg/IM according to body weight on days -2

and -1. The gerbils were kept fasted 18h for a better larvae establishment, infected on day 0 with $2x10^3$ of larvae sheathed (20 day). The groups 2 and 4 received an extra dose of corticoid on day 7 pi. All groups were treated orally during 10 or 15 days daily.

Group 1 - treated 10 days (on days -2 − 7 pi) with 0.25 mL of NaCl 0.9% (control group);
Group 2 - treated 15 days (on days -2 −12 pi) with 0.25 mL of NaCl 0.9% (control group);
Group 3 - treated 10 days (on days -2 − 7 pi) with 25 mg/kg of IgY;
Group 4 - treated 15 days (on days -2 − 12 pi) with 25 mg/Kg of IgY.

Parasite recovery and collect of samples

Groups 1 and 3 were euthanized on day 10 pi and groups 2 and 4 on day 15 pi. The animals were euthanized using an isoflurane chamber and the stomachs removed, externally washed with 10ml distillated water pre-heated at 35-39°C (average 37°C), opened longitudinally on petri dish and incubated with 30ml NaCl 0.9% at 37°C in an incubator at 37°C for 3-5 hours following the method by Conder *et al.* (1992) with modifications. After this period, the stomachs were washed with tap water and the larvae solution stored in falcon tubes 50ml with tamponed formoldeide 7%. The parasites were counted using inverted optical microscopic 40x

Statistics

Normality and homoscedasticity were analyzed through the Shapiro-Wilk and Levene test, respectively. For the *in vivo* experiment the mean were compared using Student's t-test and levels of significant differences using $P<0.05$. The reduction (%) of the parasite burden was estimated using the following formula: (mean parasite burden in the control group – mean parasite burden in the treated group)/mean parasite burden in the control group x 100.

Results

Characterization of IgY-H. contortus

IgY antibodies were successfully purified from egg yolk using PEG method. The samples were evaluated by SDS-PAGE (Fig. 1A) indicates the heavy and light chains between 75 to 50 kDa and 37 to 25 kDa, respectively. In addition, western blot analysis confirmed the presence of two heavy and light chains of IgY antibody in the sample (Fig. 1B). Dot blot assay was perfumed and IgY was able to recognize *H. contortus* antigens indicating IgY specificity to its target antigen (Fig. 2).

The level of IgY antibodies from chickens immunized with *H. contortus* were evaluated by the ELISA during all immunization period. Results showed a relatively strong immune response against *H. contortus* antigens. The level of IgY antibodies increased until on week 12 which remained relatively high 10 weeks after the last immunization (Fig. 3).

The avidity index at 1:10 dilution increased after four immunizations keeping a high index until on week 20. In the other dilutions was observed a low index during immunization period (Fig. 4).

IgY concentration

The average concentration of IgY purified after immunization was 4.87 ± 0.56mg/mL.

In vivo assay 3

Significant reduction ($P<0.01$) in the mean number of larvae recovered from stomach of gerbils treated with IgY 10 dpi compared to the control group is show in the Fig. 5A. There was no difference ($p=0.05$) between treated and the control group on day 15 pi (Fig. 5B).

Discussion

Haemonchus contortus has shown able to develop resistance to whatever chemicals are used intensively for its control, beyond is expected the increase in prevalence and severity of resistances in field isolates (Kotze and Prichard, 2016). On the other hand, the existence of microorganism resistant to the immunotherapy is unlikely, arising the interest for the production of antibodies against pathogens with medical and veterinary importance (Xu *et al.*, 2012; Tobias *et al*., 2012, Vega *et al*., 2011).

Our research group showed recently a lower number of larvae recovered in gerbils treated for IgY produced by L3 antigens, suggesting that IgY antibodies would hamper the larvae establishment and consequently the parasite rejection would happen (Gressler, unpublished yet). In current study we produced the IgY antibodies from adult stages and therapeutic effectiveness was evaluated. Our results show a potential cross-activity of antibodies produced from adult stages on the establishment of *H. contortus* -L3-L4 *in vivo*, since L4 and adult stages may present antigens in common, such as gut antigens (Nisbet *et al.*, 2016). Solhi *et al*. (2017) revealed that the IgY which is specific for one strain could inhibit the growth of the other strains of *H. pylori*. This useful cross inhibitory effect could be attributed to the interactions of IgY antibodies with similar antigens which are distributed among different strains of *H. pylori.*

The results demonstrate strain-specific and cross-strain inhibitory effects of the IgY polyclonal antibody on growth and urease activity of *H. pylori* (Solhi *et al.*, 2017). This results evidence that IgY polyclonal antibody can not only inhibit the growth of *Helicobacter pylori* but also may inhibit other specific antigens involved in different pathogenesis pathways of *H. pylori*. It is possible that IgY antibodies anti-*H. contortus* may act on enzyme-encoding antigens, such as proteases, interfering with the establishment of the parasite.

Further studies are planned to characterize the dose–response of IgY and elucidate its mechanism of action. More attempts to study *in vivo* effectiveness will must be performed.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Standards

This study was approved by the Committee on Animal Research and Ethics (CARE) of the Universidade Federal de Santa Maria (UFSM), under protocol number 3216240915/2016 and 3787160917/2017.

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Figure 1. (A) SDS-PAGE analysis of the purified IgY antibodies and (B) immunoblot showing the recognition of IgY, both under reducing conditions. The scale of molecular weight expressed in kDa, which the red arrows indicate the bands 75, 50, 37 and 25 in the protein marker. Intervals of the bands being, 75 and 50 kDa refers to heavy chain and 37 and 25 kDa refers to light chain. Black arrows indicate the heavy and light chains IgY produced post-immunization.

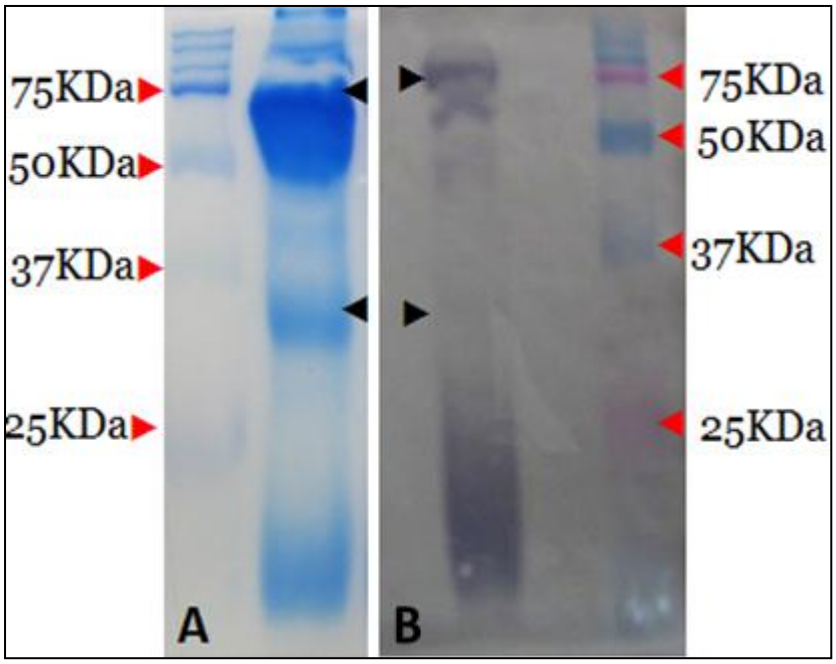


Figure 2. Dot-blot showing the specific recognition of IgY - anti-*H. contortus* to the protein antigens from adults of *H. contortus* after addition of the peroxidase-conjugated secondary antibody and incubated with a developing solution.

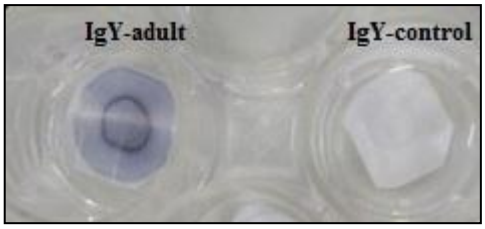


Figure 3. Optical density (O.D) values obtained in the ELISA using purified IgY from egg yolk of immunized chickens. The samples tested were IgY extracted each 2 weeks after immunization. The circle indicates the time of the last immunization.

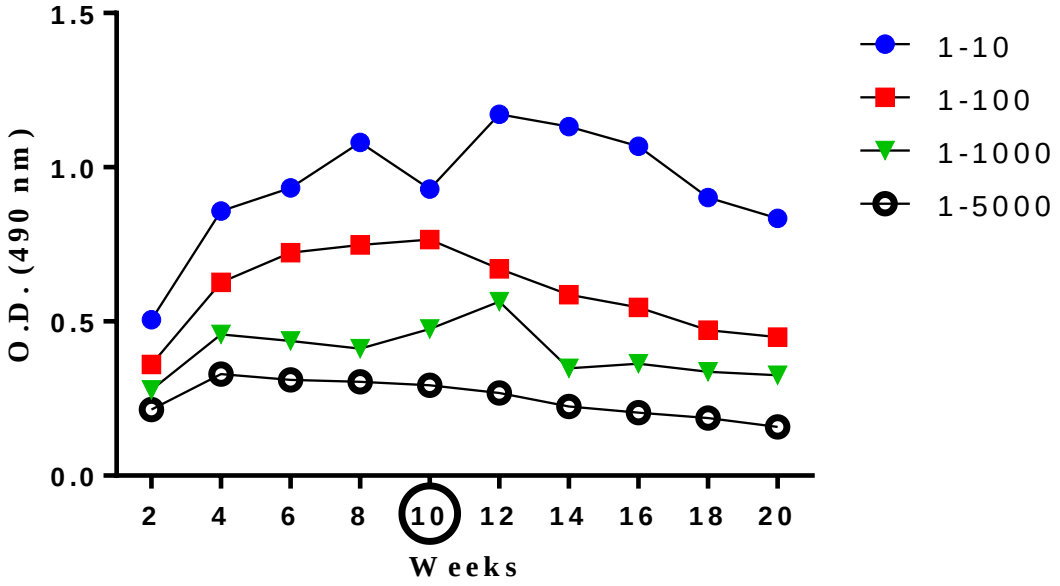


Figure 4. Avidity index (AI) of IgY antibodies produced during immunization of the hens with *H. conortus* antigens. AI < 40% were considerate of low avidity; AI between 41 and 70% represented a medium avidity; and AI > 70% was considered as high avidity.

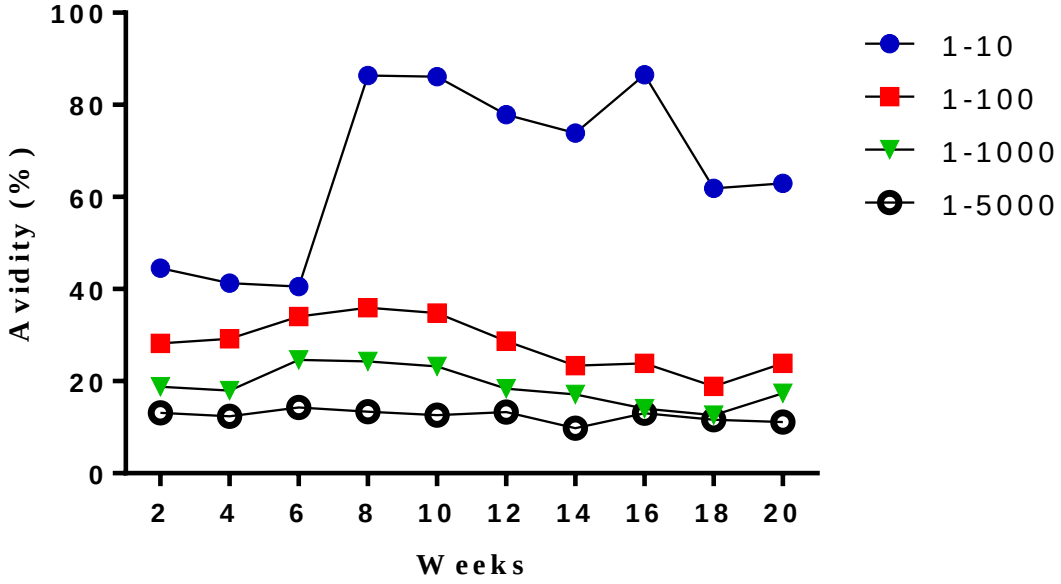
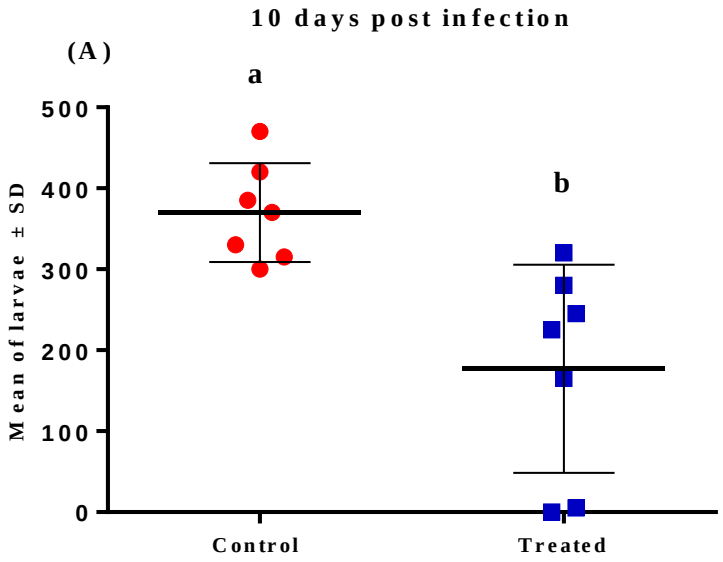
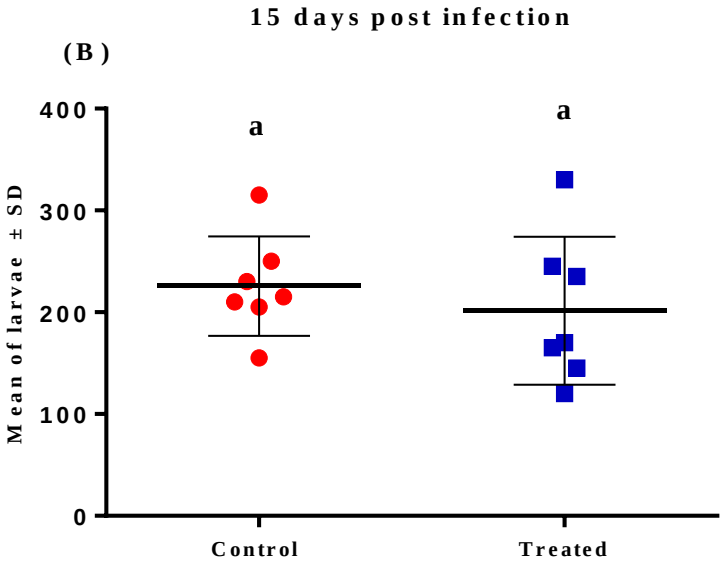


Figure 5. Mean number of larvae recovered at necropsy at 10 and 15 dpi from the stomach of gerbils treated orally with IgY anti-*H. contortus*.





Data are expressed as mean ± SD and compared with each group. The small letters compare mean in the columns. Different letters indicate significantly different values (P< 0.01).

5 CONSIDERAÇÕES FINAIS

Apesar dos esforços despendidos nas últimas décadas por maiores esclarecimentos acerca do controle da infecção por *H. contortus*, essa enfermidade ainda se apresenta como um dos principais desafios na criação de ovinos. No Brasil, a doença é responsável por significativas perdas econômicas e mortalidade de animais. Entre os principais obstáculos para o controle da infecção podemos destacar a alta prolificidade das fêmeas de *H. contortus*, gerando elevada taxa de contaminação ambiental e com isso, um grande desafio parasitário. Outro grande obstáculo está atrelado a intensidade da infecção e a capacidade hematófaga dos parasitos, estabelecendo assim um quadro de anemia e hipoproteinemia, característicos da doença.

Haemonchus contortus é um parasita versátil, o qual transita entre um estado de vida livre e um ciclo parasitário. Em seu ciclo de vida livre, ovos e larvas infectante (L3) apresentam tolerância a variações de temperatura, permanecendo viáveis por meses em condições ambientais favoráveis. Sendo assim, essas características contribuem para manutenção de parasitos no ambiente, dificultando muitas vezes práticas de controle integrado. Durante a fase infectante, larvas podem interromper seu ciclo patogênico, entrando em um estado de hipobiose, retornando ao seu estágio ativo, ocasionando surtos da infecção. Além de sua ampla capacidade de manutenção ambiental e parasitaria, atualmente existe uma grande preocupação em relação ao avanço da resistência parasitária.

Embora novos princípios ativos como monepantel e mais recentemente derquantel, tenham sido lançados na última década, relatos de ineficácia passaram a ser observados rapidamente, reiterando a necessidade de um programa de controle parasitário e principalmente, a aplicação de métodos de controle não-químicos. Atualmente, uma única vacina está disponível comercialmente, restrita a alguns países como Austrália e Nova Zelândia. A vacina “barbervax” pode apresentar interessantes resultados no controle de *H. contortus*, sendo necessário a realização de um cronograma de vacinação com aplicações frequentes, principalmente nos períodos do ano com maior desafio parasitário.

Diante desta situação, este estudo buscou produzir anticorpos a partir de antígenos larvais e de adultos de *H. contortus* e avaliar seu potencial imunoterápico *in vivo*, utilizando-se *M. unguiculatus* como modelo experimental. A partir dos nossos resultados, podemos observar

que gerbils podem servir como uma importante ferramenta para avaliação de complexos ativos sobre *H. contortus*, previamente a estudos em ovinos. Ainda, nossos resultados sugerem uma atividade de anticorpos IgY sobre o estabelecimento de *H. contortus* em *M. unguiculatus* atrelada a mecanismos ainda não esclarecidos.

6 CONCLUSÕES

- A infecção experimental de gerbils por larvas (L3) embainhadas resultou um maior número de parasitos recuperados 10 dias pós infecção.
- A infecção pode ser estabelecida satisfatoriamente em animais imunocompetentes, sendo uma alternativa para estudos nos quais as alterações fisiológicas causadas pelo uso de imunossupressores são indesejáveis.
- O uso de acetato de metilprednisolona resultou em um melhor estabelecimento da infecção de gerbils por *H. contortus*.
- O protocolo utilizando-se 2.000 larvas embainhadas e tratamento imunossupressor foi eficiente para recuperação de larvas 15 dias pós infecção.
- O peso dos gerbils não interferiu no número de parasitos recuperados 15 dias pós infecção.
- O protocolo imunogênico foi eficientemente para estimular o sistema imune de aves e essas foram capazes de produzir anticorpos IgY específicos e com alto índice de avidez.
- O tratamento com anticorpos IgY produzidos a partir de L3 e adultos interferiu no estabelecimento de *H. contortus* em gerbils infectados experimentalmente. O mecanismo de ação dos anticorpos devem ser alvo de novos estudos, fornecendo maiores esclarecimentos aos resultados apresentados nesta tese.

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