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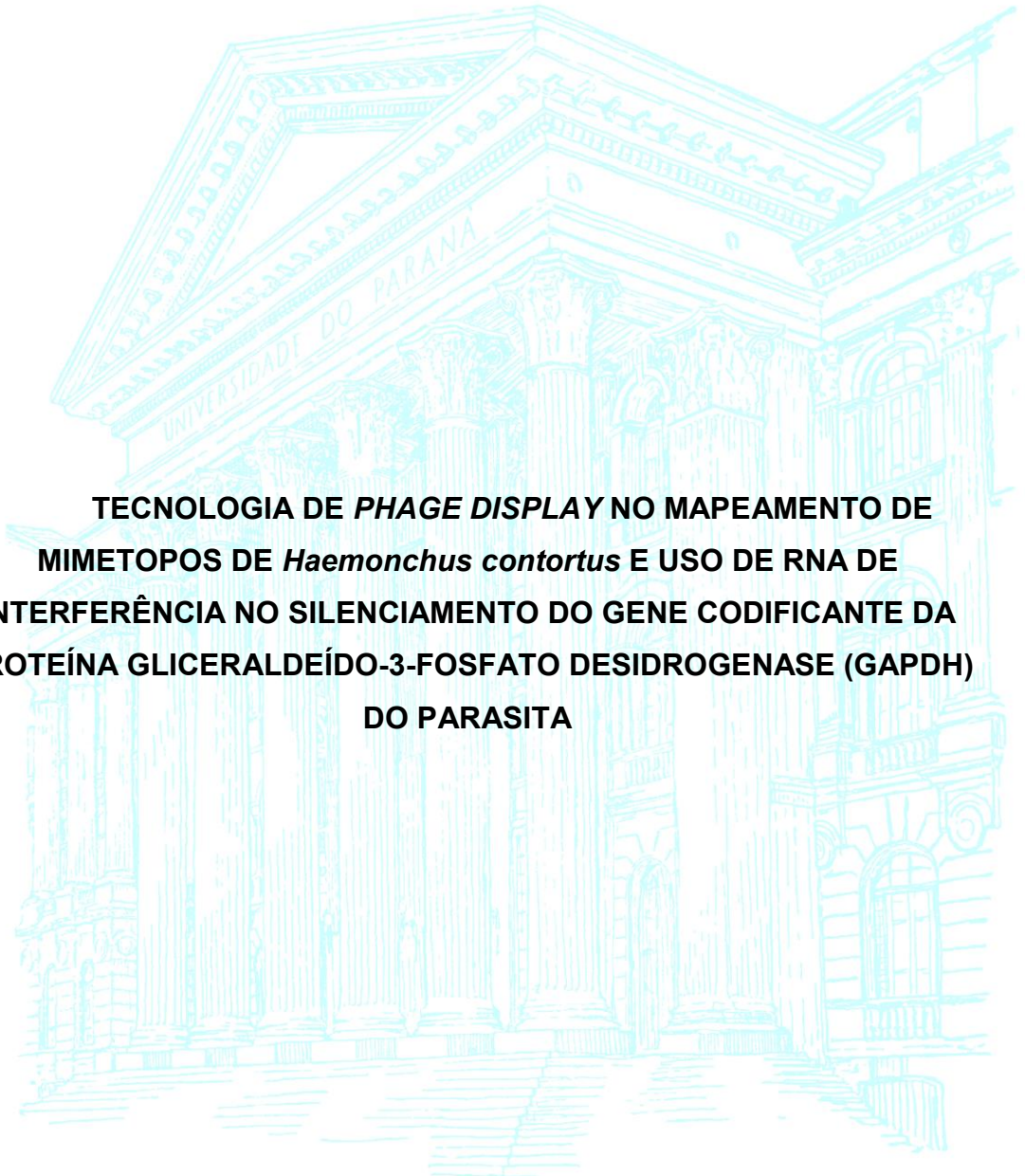
ANDRÉIA BUZATTI



TECNOLOGIA DE *PHAGE DISPLAY* NO MAPEAMENTO DE MIMETOPOS DE *Haemonchus contortus* E USO DE RNA DE INTERFERÊNCIA NO SILENCIAMENTO DO GENE CODIFICANTE DA PROTEÍNA GLICERALDEÍDO-3-FOSFATO DESIDROGENASE (GAPDH) DO PARASITA

CURITIBA, 2018

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Orientador: Prof. Dr. Marcelo Beltrão Molento
Co-orientador: Prof. Dr. Amilcar Arenal Cruz

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ANDRÉIA BUZATTI** intitulada: **Tecnologia de Phage Display no mapeamento de Mimetopos de *Haemonchus contortus* e uso de RNA de interferência no silenciamento do Gene codificante da protéina Gliceraldeído-3-fosfato desidrogenase (GAPDH) do parasita**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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CURITIBA, 12 de Abril de 2018.

MARCELO BELTRÃO MOLENTO
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Avaliador Externo

Aos animais,
sem eles nada seria possível!

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Das utopias
Se as coisas são inatingíveis… ora!
Não é motivo para não querê-las…
Que tristes os caminhos, se não fora
A presença distante das estrelas!

(MARIO QUINTANA)

RESUMO

Haemonchus contortus é um nematoda hematófago de pequenos ruminantes, extremamente importante em escala mundial, sendo responsável por muitos danos à saúde dos animais, além de perdas produtivas e econômicas aos criadores. O panorama atual é de resistência do parasita à maioria das classes de anti-helmínticos disponíveis no mercado, o que demonstra necessidade urgente de métodos alternativos de controle. Portanto, os objetivos deste estudo foram selecionar mimetopos de *H. contortus* e avaliar o uso de RNA de interferência no silenciamento do gene codificante da proteína gliceraldeído-3-fosfato desidrogenase (GAPDH) do parasita. A presente tese está dividida em 3 capítulos apresentados na forma de introdução geral e 2 manuscritos. O manuscrito 1 consistiu no uso de anticorpo policlonal de ovinos para mapear mimetopos de *H. contortus* usando a biblioteca de *phage display*. O clone 6 – mimetopo de GAPDH e clone 17 - mimetopo da família do músculo desorganizado (Dim 1) foram selecionados para ensaio de imunização de ovinos. Peptídeos mimetopos (sintéticos) foram avaliados como moléculas antigênicas por meio de ensaio imunoenzimático (ELISA). No ensaio de imunização constatou-se aumento dos títulos de IgG anti-fago M13, mas não ocorreu aumento de IgG anti-peptídeos mimetopos. Os peptídeos mimetopos sintéticos foram reconhecidos por IgG de ovinos naturalmente infectados por *H. contortus*. Este é o primeiro relato de uso bem sucedido da biblioteca de *phage display* para a identificação de mimetopos de *H. contortus*. O manuscrito 2 teve o objetivo de avaliar o uso de RNA de interferência (RNAi) para silenciamento do gene codificante da proteína GAPDH de *H. contortus.* Larvas infectantes foram incubadas com RNAi por 3; 6; 24 e 48h. Os resultados revelaram ausência de transcrição gênica em todas os períodos avaliados. Este estudo relata pela primeira vez o silenciamento de GAPDH em *H. contortus*, confirmando o gene como passível ao silenciamento por RNAi. Os dados expostos nesta tese apontam resultados promissores para uso em novas pesquisas voltadas ao desenvolvimento de terapias para o controle de *H. contortus.*

Palavras-chave: Pequenos ruminantes; ovinos; resistência parasitária; RNA de interferência.

ABSTRACT

Haemonchus contortus is a hematophagous nematoda of small ruminants, extremely important on a worldwide scale, being responsible for severe impact to the animal's health, besides productive and economic losses to the breeders. The current panorama is parasite resistance to the most anthelminthics classes commercially available for its control. This demonstrates the urgent need of alternative control methods. Therefore, the goals of this study were to select mimotopes from *H. contortus* and to evaluate the use of RNA interference (RNAi) to silence the gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the parasite. This thesis is divided into 3 chapters presented as 1 general introduction and 2 manuscripts. The goal of the first manuscript was to evaluate polyclonal antibody to map *H. contortus* mimetopos using the phage display library. Clone 6 - GAPDH mimetope and clone 17 - mimetope from the disorganized muscle family (Dim 1) were selected for sheep an immunization assay. Peptide mimotopes (synthetic) were evaluated as antigenic molecules by enzyme-linked immunosorbent assay (ELISA). In the immunization assay, IgG titers anti-phage M13 showed increase, but IgG anti-peptide mimotopes did not increased. Peptide mimotopes (synthetic) were recognized by IgG from sheep naturally infected by *H. contortus*. This is the first report of successful use of the phage display library for identification of *H. contortus* mimotopes. The aim of the manuscript 2 was to evaluate the RNAi to silence the GAPDH gene of *H. contortus*. Infective larvae were incubated with RNAi for 3; 6; 24 and 48h. The results showed absence of gene transcription in all evaluated periods. This study reports for the first time the silencing of GAPDH gene in *H. contortus*, confirming the gene as susceptible to silencing by RNAi. The data presented in this thesis point out promising results for use in new researches to development *H. contortus* control therapies.

Key-words: Small ruminants; sheep; parasite resistance; RNA interference.

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1 INTRODUÇÃO

Ovinos criados sob sistemas extensivos estão continuamente expostos à infecção por nematoides gastrointestinais, principalmente em regiões temperadas, tropicais e subtropicais. Geralmente os animais apresentam infecções múltiplas, sendo parasitados por diversas espécies ao mesmo tempo. Contudo o parasitismo por *Haemonchus contortus,* nematoide hematófago, destaca-se em escala mundial como o mais patogênico e prevalente em ovinos e caprinos (FORTES e MOLENTO, 2013; KNOX, 2013). A importância de *H. contortus* está relacionada a sua elevada prevalência e ao seu hábito alimentar hematófago, podendo ocasionar perda de peso, anorexia, severa anemia e até morte em infecções hiperagudas. Além dos danos diretos à saúde dos animais, o parasitismo também promove elevadas perdas econômicas em consequências da redução da produtividade, custos com mão de obra e tratamentos (SIMPSON, 2000).

Atualmente, o controle das endoparasitases de pequenos ruminantes constitui um grande entrave aos sistemas produtivos. O panorama mundial é de resistência dos parasitas à maioria das classes de anti-helmínticos comercialmente disponíveis (ROOS et al., 1990; VERÍSSIMO et al., 2012; FORTES e MOLENTO, 2013; BASSETTO; AMARANTE, 2015). Além disso, relatos mundias apontam *H. contortus* com um dos parasitas mais resistentes aos antiparasitários (revisado por MUCHIUT et al., 2018). Dessa forma, torna-se evidente a importância de pesquisas voltadas ao desenvolvimento de métodos alternativos de controle parasitário.

Os avanços biotecnológicos têm permitido o progresso de pesquisas nas áreas de vacinologia, imunoproteômica e genética de parasitas. Muitos estudos foram realizados com o objetivo de identificar proteínas imunogênicas de *H. contortus* e analisar seu potencial para induzir uma imunidade protetora (REDMOND e KNOX, 2006; YAN et al., 2010; KNOX, 2013). A tecnologia de *phage display*, biblioteca de expressão de peptídeos em fagos, é uma alternativa promissora para a seleção de moléculas com potencial imunogênico e/ou antigênico. A aplicação da biblioteca permite a seleção de sequências peptídicas que mimetizam as estruturas de epítopos naturais. Essa área de pesquisa ainda é recente com relação aos parasitas de animais (ELLIS et al., 2012). Já foram realizados estudos com *Trichinella spiralis* (GU et al., 2008), *Fasciola hepatica* (VILLA-MANCERA et al., 2008), *Rhipicephalus microplus* (PRUDENCIO et al., 2010), *Taenia solium* (ASSANA et al., 2010). Até o

momento, não há relatos da aplicação dessa tecnologia para pesquisa de mimetopos de *H. contortus*.

Mimetopos mapeados por *phage display* podem apresentar aplicação como moléculas imunogênicas com potencial vacinal e/ou moléculas antigênicas para uso em técnicas de diagnóstico. Resultados obtidos por *phage display* também podem direcionar o emprego de RNA de interferência (RNAi) para o silenciamento de genes específicos e verificação da importância da expressão desses genes para a sobrevivência do parasita. A tecnologia de RNAi foi descrita primeiramente com o nematoda *Caenorhabditis elegans* (FIRE et al., 1998) e desde então usada extensivamente para examinar a função de genes. A mistura de RNA mensageiro de fita dupla (dsRNA) pode causar uma interferência potente e especifica e inibir a expressão do gene alvo. O processo ocorre devido a mediação da degradação do transcrito correspondente do dsRNA (FIRE et al., 1998).

A aplicabilidade do RNAi foi demonstrada em vários sistemas e organismos, incluindo protozoários (McROBERT e McCONKEY, 2002), planárias (PINEDA et al., 2002), nematoideos parasitas (HUSSEIN et al., 2002), *Drosophila melanogaste*r (CAPLEN et al., 2000), e mamíferos (ELBASHIR et al., 2001). Em parasitas nematoides, estudos têm relatado efeitos após incubação, alimentação ou eletroporação com dsRNA correspondente (ABOOBAKER e BLAXTER, 2003; ISSA et al., 2005; LUSTIGMAN et al., 2004). O *knock-out* genético por estes métodos tem sido muito eficiente *em C. elegans,* porém a sua aplicação prática em parasitas nematodas não tem proporcionado resultados semelhantes.

Em *H. contortus* o RNAi já foi avaliado para alguns genes (GELDHOF et al., 2006; SAMARASINGHE et al., 2011; ZAWADZKI et al., 2012) e no geral os resultados observados foram variáveis, com redução da transcrição de alguns e efeito nulo ou até mesmo aumento na transcrição de outros. Os achados sugerem que determinados genes podem ser efetivamente silenciados, enquanto outros são refratários ao RNAi. Segundo Selkirk et al. (2012) o êxito ou falha do silenciamento podem estar associados, principalmente, à escolha do gene alvo a ser silenciado e a metodologia empregada nos ensaios. Os autores supracitados afirmam ainda que se necessita de uma padronização metodológica que considere a forma de uso do dsRNA (incubação, alimentação ou eletroporação) conforme a localização de cada gene alvo.

A presente tese está dividida em 3 capítulos. O capítulo 1 corresponde a introdução geral, a qual aborda a problemática do parasitismo por *H. contortus* em

ovinos e ressalta alguns avanços biotecnológicos na área de pesquisa voltada à novos métodos de controle. O capítulo 2 (artigo aceito para publicação) consiste na aplicação da tecnologia de *phage display* para a seleção de peptídeos mimetopos de *H. contortus* e avaliação do potencial imunogênico e antigênico das moléculas selecionadas. O capítulo 3 corresponde ao estudo sobre a aplicabilidade do RNA de interferência no silenciamento do gene codificante da proteína gliceraldeído-3-fosfato desidrogenase (GAPDH) de *H. contortus in vitro*.

.

1.1 OBJETIVO GERAL

Selecionar mimetopos de *Haemonchus contortus* por meio da tecnologia de *phage display* e avaliar o uso de RNA de interferência no silenciamento do gene codificante da proteína GAPDH de *H. contortus.*

1.2 OBJETIVOS ESPECÍFICOS

a) Avaliar o uso de anticorpo policlonal de ovinos anti-*H. contortus* para seleção de peptídeos mimetopos do parasita.
b) Avaliar o potencial imunogênico e antigênico dos mimetopos selecionados por *phage display.*
c) Avaliar o resultado da vacinação de ovinos com fagos expressando mimetopos de *Haemonchus contortus* sobre a carga parasitária e a contagem de ovos por grama de fezes.
d) Avaliar genotipicamente e fenotipicamente a aplicabilidade do RNA de interferência no silenciamento do gene codificante da proteína GAPDH em larvas infectantes de *H. contortus in vitro*.

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2 ANTICORPO POLICLONAL DE OVINOS PARA MAPEAR MIMETOPOS DE *Haemonchus contortus* USANDO A BIBLIOTECA DE *PHAGE DISPLAY*

RESUMO

O objetivo deste estudo foi avaliar a tecnologia de *phage display* no mapeamento de mimetopos de *Haemonchus contortus.* Anticorpo policlonal de ovinos anti-*H. contortus* foi usado para seleção de peptídeos mimetopos a partir da biblioteca *PhD-7 Phage Display Peptide Library Kit.* Após quatro rodadas, 50 clones de fagos expressando peptídeos foram selecionados e sequenciados. Dois clones que exibiram mimetopos de *H. contortus* foram escolhidos para imunização de ovinos: clone 6 – mimetopo de gliceraldeído-3-fosfato desidrogenase (GAPDH) e clone 17 - mimetopo da família do músculo desorganizado (Dim 1). Doze ovinos foram alocados em 3 grupos de 4 animais, da seguinte forma: G1: grupo controle, G2/GAPDH: imunizado com o clone 6 e G3/Dim1: imunizado com o clone 17. Quatro imunizações foram realizadas (0, 7, 14 e 21 dias). No dia 28 após a primeira imunização, todos os grupos foram desafiados oralmente com 2500 larvas infectantes de *H. contortus*. Os peptídeos mimetopos selecionados foram reconhecidos por IgG de ovinos naturalmente infectados por *H. contortus*. O ensaio de imunização revelou um aumento dos títulos de IgG anti-fago M13, mas não ocorreu aumento de IgG anti-peptídeos mimetopos. Este é o primeiro relato de uso bem sucedido da biblioteca de *phage display* para a identificação de mimetopos de *H. contortus*.

Palavras-chave: Mimetopos; biopanning; elisa; ruminantes; imunogênicos.

2 SHEEP POLYCLONAL ANTIBODY TO MAP *Haemonchus contortus* MIMOTOPES USING PHAGE DISPLAY LIBRARY

ABSTRACT

The aim of this study was to evaluate phage display technology for mapping *Haemonchus contortus* mimotopes. We screened the PhD-7 Phage Display Peptide Library Kit with a sheep polyclonal antibody against *H. contortus*. After four rounds of selection, 50 phage peptide clones were selected by biopanning and sequenced. Two clones displaying peptide mimotopes of *H. contortus* proteins were chosen for sheep immunization: clone 6 - mimotope of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and clone 17 - mimotope of a disorganized muscle family member (Dim 1). Twelve sheep were allocated into 3 groups of 4 animals as follow: G1: control group; G2/GAPDH: immunized with clone 6; and G3/Dim1: immunized with clone 17. Four immunizations were performed at intervals of seven days (0, 7, 14, and 21 days). On day 28 post initial vaccination, all groups were orally challenged with 2500 *H. contortus* infective larvae. The mimotope peptides (synthetic) selected by phage display were recognized by IgG from sheep naturaly infected with *H. contortus*. The immunization protocol showed an increase in IgG anti-M13 phage titers, but no effect was observed in IgG-specific for the anti-mimotope peptides. This is the first report of successful use of a phage display library for the identification of mimotopes of *H. contortus* proteins.

Keywords: Mimotopes, biopanning, ELISA, ruminants, immunogenic.

2.1 INTRODUCTION

Haemonchus contortus is a hematophagous gastrointestinal parasite of ruminants that is extremely important on a global scale. It is one of the most prevalent parasites, causing considerable blood loss, thereby having a substantial impact on farm production. The parasite has been reported to outlive the use of different anthelmintic products (FORTES and MOLENTO, 2013)

Phage display is a low-cost and fast method for mapping the epitope of an antigen that is involved in a specific protein interaction with the antibody. The identification of epitopes is essential in diagnostics, immunotherapy, drug discovery and vaccine development to combat diseases (PANDE et al., 2010). This application, enables selection of mimotopes, peptides mimicking natural epitopes of a pathogen, even without prior knowledge of the natural ligand area (ELLIS et al., 2012).

Concerning parasite control, phage display library technology is still a relatively novel research area (ELLIS et al., 2012) and it has not been applied previously in the screening of *H. contortus* mimotopes. Several reports have demonstrated the use of phage display libraries for targeting and diagnosis of parasites (HELL et al., 2009; GAZARIAN et al., 2012; CHEONG et al., 2016; RHAIEM & HOUIMEL, 2016), and promising studies on protective mimotopes have been reported on *Plasmodium falciparum* (ADDA et al., 1999), *Schistosoma japonicum* (TANG et al., 2004), *Trichinella spiralis* (GU et al., 2008), *Fasciola hepatica* (VILLA-MANCERA et al., 2008), *Rhipicephalus microplus* (PRUDENCIO et al., 2010), and *Taenia solium* (ASSANA et al., 2010). The objective of this study was to evaluate for the first time, the use of phage display technology for mapping *H. contortus* mimotopes.

2.2 Materials and methods

2.2.1 *Haemonchus contortus* polyclonal antibodies

Forty-eight adult sheep from a herd naturally infected with *H. contortus* and exposed to repeated infections, were used to obtain serum. We selected serum only from the animals with high IgG titter and zero parasite fecal egg count (FEC).

Blood samples (10 ml) were collected from the jugular vein of all animals. The serum was separated by centrifugation at 3000 x g for 10 min and stored at -20ºC until its use for the ELISA and biopanning assays.

2.2.2 Extraction of *Haemonchus contortus* proteins

Fifty worms were crushed in liquid nitrogen using a mortar and pestle. Protein extraction was performed using chilled 0.1% [v/v] PBS and Tween 20, with shaking for 1 h at 4°C. The samples were centrifuged at 10,000 x g for 20 min at 4ºC. The supernatant was filtered and aliquots were kept at -20°C until use. The protein concentration was determined by Qubit protein assay (Invitrogen, USA).

2.2.3 Enzyme-linked immunosorbent assay (ELISA)

The 96-well ELISA plates (PolySorp, Nunc) were coated with 5 µg ml^{-1} (100 µl $well^{-1}$) of *H. contortus* total proteins (DÍAZ et al., 2015) (5 µg ml^{-1} of chemically-synthesized peptides or 10^{11} plaque-forming units or 5 µg ml^{-1} according to the assay) diluted in coating buffer (0.1 mM sodium carbonate-bicarbonate, pH 9.6) and incubated at 4°C overnight. The plates were washed four times with 0.05% [v/v] Tween 20 solution (Merck, USA). The blocking of the reaction was performed with 3% [v/v] PBS for 1 h at 20°C. The plates were washed four times with 0.05% [v/v] Tween 20 solution. Sheep sera were used at 1:100, 1:300, 1:600, 1:1200, 1:2400, 1:4800, 1:9600 and 1:19200, depending on the assay, in PBS 1 x 0.2% [v/v] Tween 20 and incubated for 1 h at room temperature. After incubation, the plates were washed four times with Tween 20 solution 0.05% [v/v] and the anti-sheep IgG HRP 1:10.000 (Sigma A3415) was added. The plates were washed four times with 0.05% [v/v] Tween 20 solution and ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] buffer was added. The plates were then incubated in the dark at 20°C for 30 min. The optical density was determined using a microplate reader (BioTek, USA) at 405 nm. The cut-off optical density (OD) was determined using the mean OD + 3xSD (standard deviation) of the negative control (serum of sheep not infected with H. contortus).

2.2.4 Titration, purification and amplification of the phage

The PhD-7 Phage Display Peptide Library Kit (New England BioLabs, USA) was used according to methodology previously described by Wu et al. (2006). The technology consists of a randomized linear 7-mer peptides fused to a minor coat protein (pIII) of M13 phage.

2.2.5 Peptide selection from Phage-Display Library

A 96-well plate (PolySorp, Nunc) was coated with IgG protein-G purified from sheep polyclonal sera that recognized *H. contortus* (1:100), in 100 µl of binding buffer (0.1M NaHCO3, pH 9.6), overnight at 4°C. The panning process was performed according to Wu et al. (2006).

Four rounds of selection were performed, after which an individual plaque was picked up at random and subjected to analysis by ELISA and DNA sequencing, following amplification in *Escherichia coli* ER2537.

2.2.6 Analysis of the selected clones

After the four selection rounds, each clone obtained had its DNA purified and sequenced using a capillary electrophoresis apparatus (ABI3130) using BigDye® terminator v 3.1 and POP 7 polymer. The sequence analyses were carried out using the software Vector NTI (version 6.0) and Molecular Evolutionary Genetics Analysis (version 6.06). We evaluated the identity among the clones and also with *H. contortus* protein sequences available in GenBank.

2.2.7 Evaluation of peptides as mimotopes

Serum samples of 25 sheep naturally infected with *H*. contortus were subjected to ELISA testing (section 2.3) to evaluate the peptides' abilty as mimotopes. The plates were coated with two chemically-synthesized peptides, which had the same sequences and confomation (7 amino acids - aa) displayed on two clones selected by the biopanning, and used in the immunization assay.

2.2.8 Immunization protocol

To evaluate the potential of the selected mimotopes as experimental vaccine candidates, two clones displaying the peptides were selected to perform the *in vivo* study. Clone 6 - similar to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and clone 17 - similar to a disorganized muscle family member (Dim 1) were used. Twelve sheep, 8-10 months old, raised indoors under nematode-free conditions, were allocated into 3 groups of 4 animals as follows: G1: control group - infected but not immunized; G2: GAPDH - immunized with clone 6; and G3: Dim 1 - immunized with clone 17. Vaccinated animals were immunized with $5x10^{11}$ pfu with the adjuvant Montanide ISA 25 VG according to the manufacturer's recommendations. G1 was

unvaccinated but received 1 ml of the same adjuvant. Four immunizations were performed at seven-day intervals (0, 7, 14 and 21 days). On day 28 post-vaccination, all groups were orally challenged with 2500 infective *H. contortus* larvae (L3). All sheep were humanely slaughtered on day 63.

2.2.9 Serological analysis

Sheep serum samples, corresponding to days 0; 14; 21; 28; 44; 51; and 63 were used for testing antibody response (ELISA section 2.3). All samples were examined against five antigens: 1: M13 phage, 2: Clone 6, 3: Clone 17, 4: chemically-synthesized GAPDH (7 aa), and 5: chemically-synthesized Dim 1 (7 aa).

2.2.10 Haematological analysis

Blood samples were collected on day 0; 14; 28; 44 and 50 following the first vaccination, by jugular venipuncture into vaccum glass tubes containing EDTA as anti-coagulant. The samples were used for leukogram and haemoglobin determination. Differential cell counts were also carried out.

2.2.11 Fecal egg count (FEC)

Fecal samples were collected from each sheep at day 0; 28; 44; 47; 51 and 55 of the experiment. FEC was determined by a modified Gordon and Whitlock (1939) method, with a sensivity of 25 eggs per gram of feces. Previously the experiment start (day 0), we analyzed fecal samples of all animals for 4 consecutive days (day -4; day -3; day -2 and day -1) to confirm the absence of infection by gastrointestinal parasites.

2.2.12 Abomasum worm counts

The number of worms in the abomasum from all slaughtered animals was determined on day 63. The abomasal content was collected and the mucosa was scraped and washed with warm 0.9% sodium chloride to detach the worms. All *H. contortus* were picked up, collected, counted and sorted according to their sex.

All protocols were approved by the Animal Care and Ethics Committee of Camaguey University, Cuba (protocol No. 2013001).

2.2.13 Statistical analyses

The data were analyzed using the Shapiro-Wilk normality test. The Kruskal-Wallis test was used to evaluate differences between groups ($P<0.05$).

2.3 Results

2.3.1 Phage display library biopanning

To map the epitopes of *H. contortus*, a random heptapeptide phage display library (PhD-7) comprised of 1.0×10^{12} independent phage clones was screened with the coated sheep polyclonal antibodies (*H. contortus*-IgG). After four rounds of biopanning, the selected phage bound to *H. contortus*-IgG were well enriched (2100X), as indicated by the increased recovery. The number of phage peptides bound to *H. contortus*-IgG increased from 2.2×10^{4} pfu in the first round to 4.6×10^{7} pfu in the fourth round.

After four rounds of selection, 50 peptide phage clones were selected by biopanning and sequenced by capillary electrophoresis. The analysis of the sequences (Figure 2.1) revealed degrees of identity among some clones and also to proteins of *H. contortus*, namely, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a Disorganized Muscle Family Member (Dim 1).

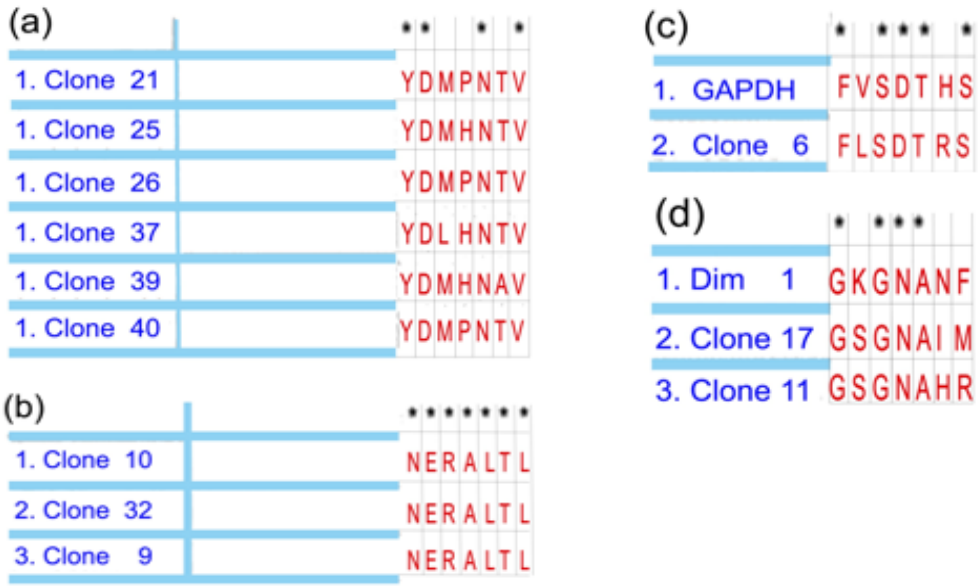
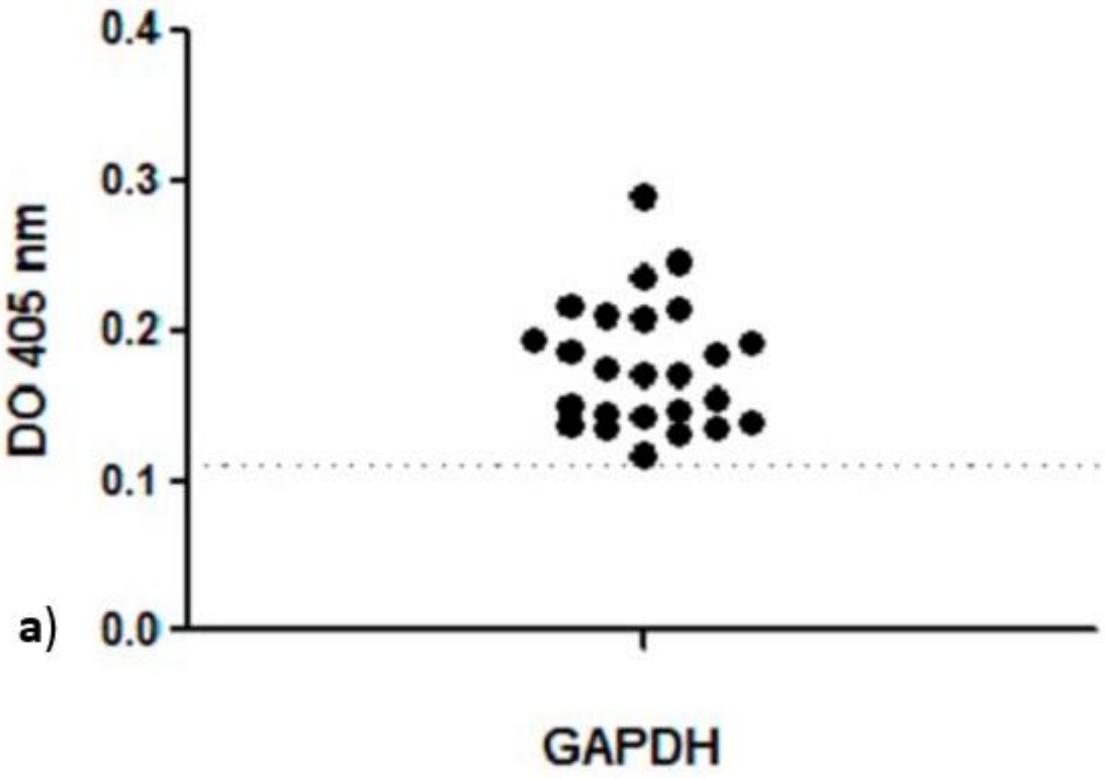


FIGURE 2.1- Phage display technology was employed to select mimotopes of *Haemonchus contortus* peptides. Analyses of the phage cloned sequences were performed with MEGA software Version 6.06. Consensus sequence (*) was observed among amino acids of some clones (a, b) and also to two *H. contortus* proteins, Glyceraldehyde-3-phosphate dehydrogenase (c) and a Disorganized muscle family member – Dim 1 (d). SOURCE: The author (2018).

The identity among clones 9; 10 and 32 was 100%; and among the clones 21; 25; 26; 37; 39 and 40 was 57.14%. The identity between GAPDH and clone 6 was 71.42%; the identity among Dim1, clone 11 and clone 17 was 57.14.

The evaluation of the chemically-synthesized peptides as mimotopes is demonstrated in Figure 2.2 (a and b). The serum samples analyzed against the peptide GAPDH (Figure 2.2 a) revealed 24 samples with IgG higher than the cut-off and just one sample below the cut-off. When used against Dim 1 (Figure 2.2 b), 12 samples demonstrated IgG means above and 12 samples below the cut-off value.



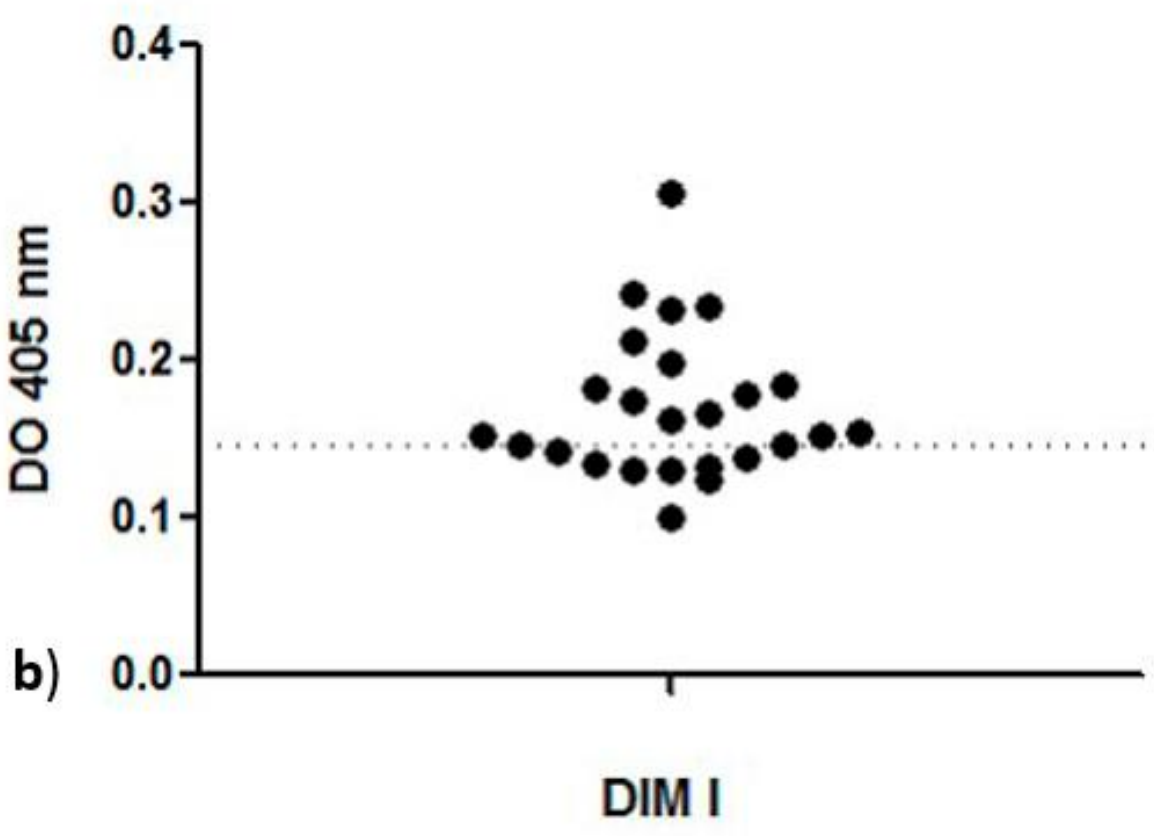
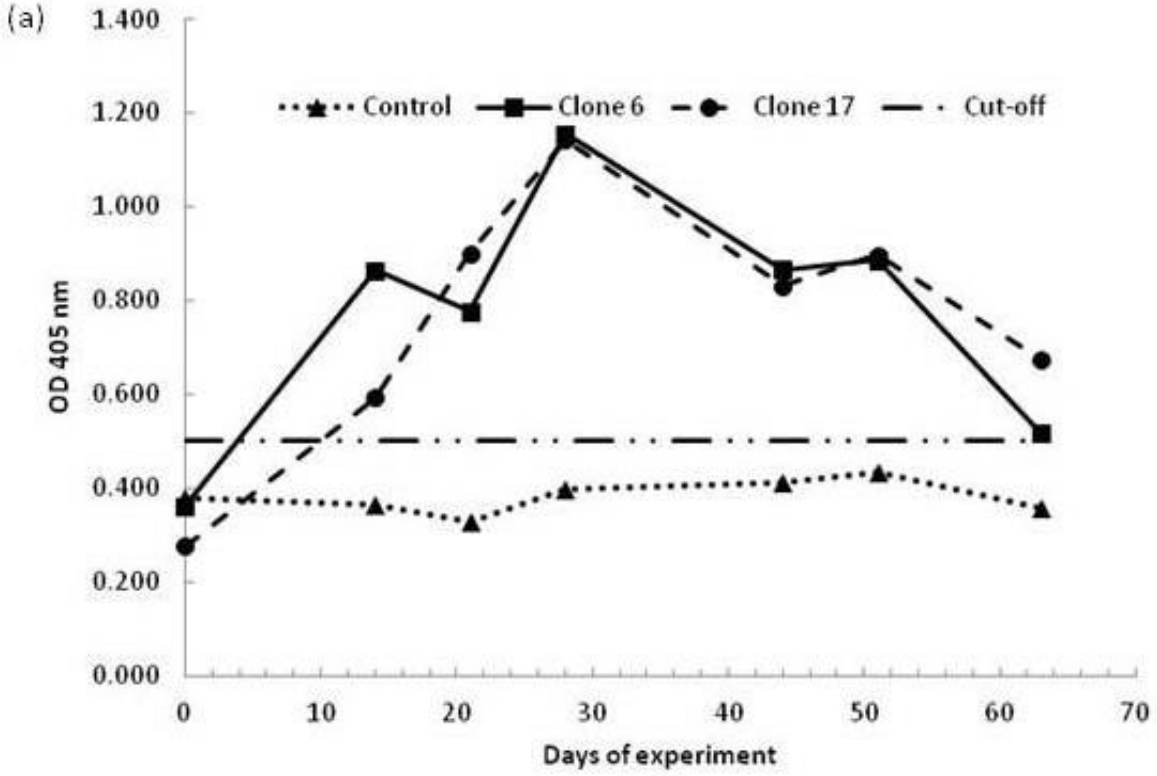
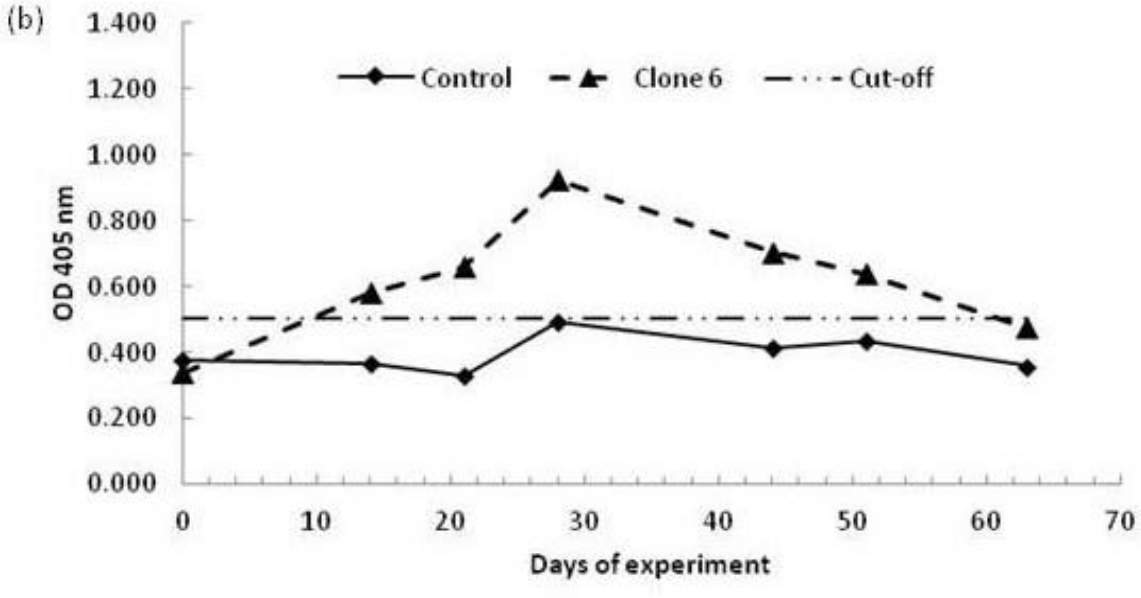


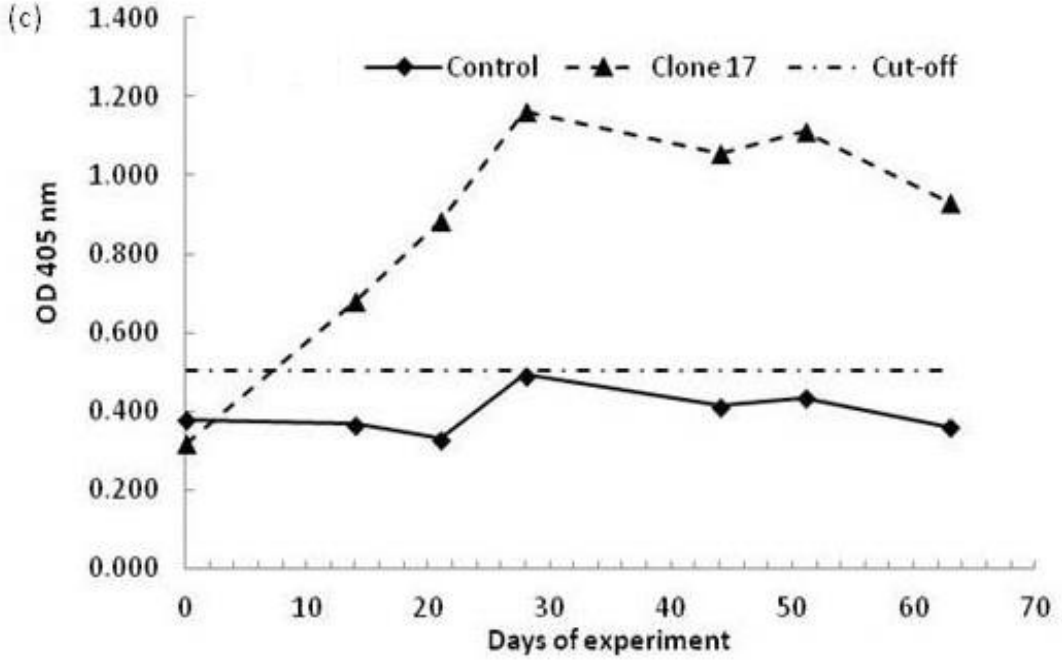
FIGURE 2.2 - Mean levels of plasma IgG of 25 adult sheep naturally infected with *Haemonchus contortus*. The serum samples were analyzed against two chemically-synthesized peptides: (a) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and (b) Disorganized muscle family member (Dim 1). These synthetic peptides have the same sequences (7 amino acids) displayed on two clones (6 and 17, respectively) selected by biopanning. SOURCE: The author (2018).

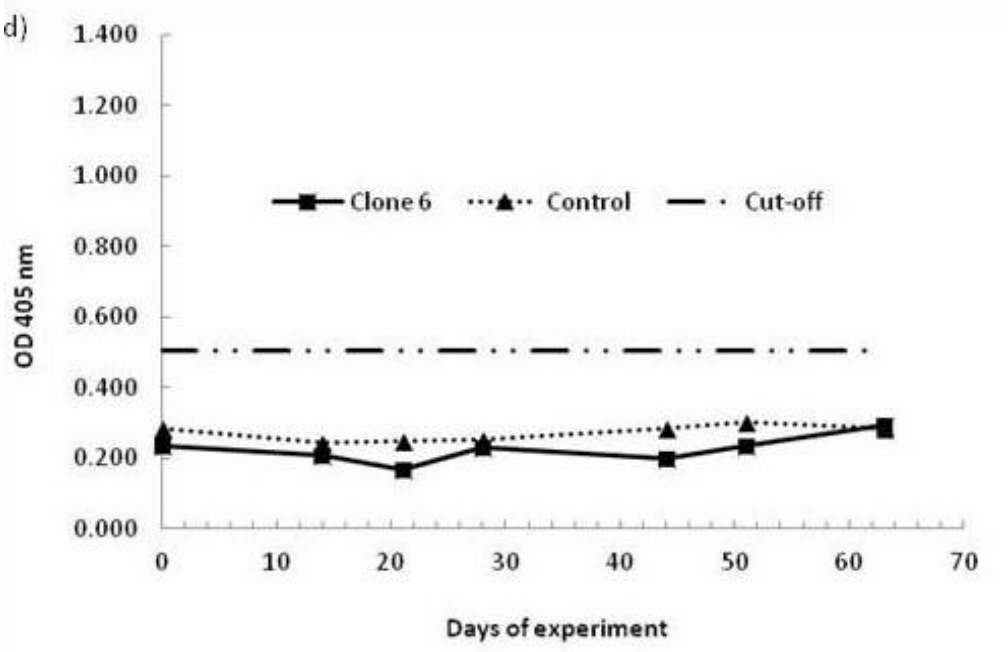
2.3.2 Immunization trial

Circulating antibody responses were observed in both vaccinated groups from day 14 onwards (Figure 2.3b and 3c). The same animals also revealed IgG against the M13 phage (Figure 2.3a). The IgG titers of the vaccinated groups were similar and followed the same pattern, rising after the first immunization (Day 0) and reaching a peak on day 28. The animals did not show an IgG response specifically against the GAPDH (Figure 2.3d) or Dim 1 (Figure 2.3e) chemically-synthesized peptides.


(a)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
OD 405 nm
Control
Clone 6
Clone 17
Cut-off
0
10
20
30
40
50
60
70
Days of experiment


(b)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
OD 405 nm
Control
Clone 6
Cut-off
0
10
20
30
40
50
60
70
Days of experiment


(c)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
OD 405 nm
Control
Clone 17
Cut-off
0
10
20
30
40
50
60
70
Days of experiment


d)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
OD 405 nm
Clone 6
Control
Cut-off
0
10
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40
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60
70
Days of experiment

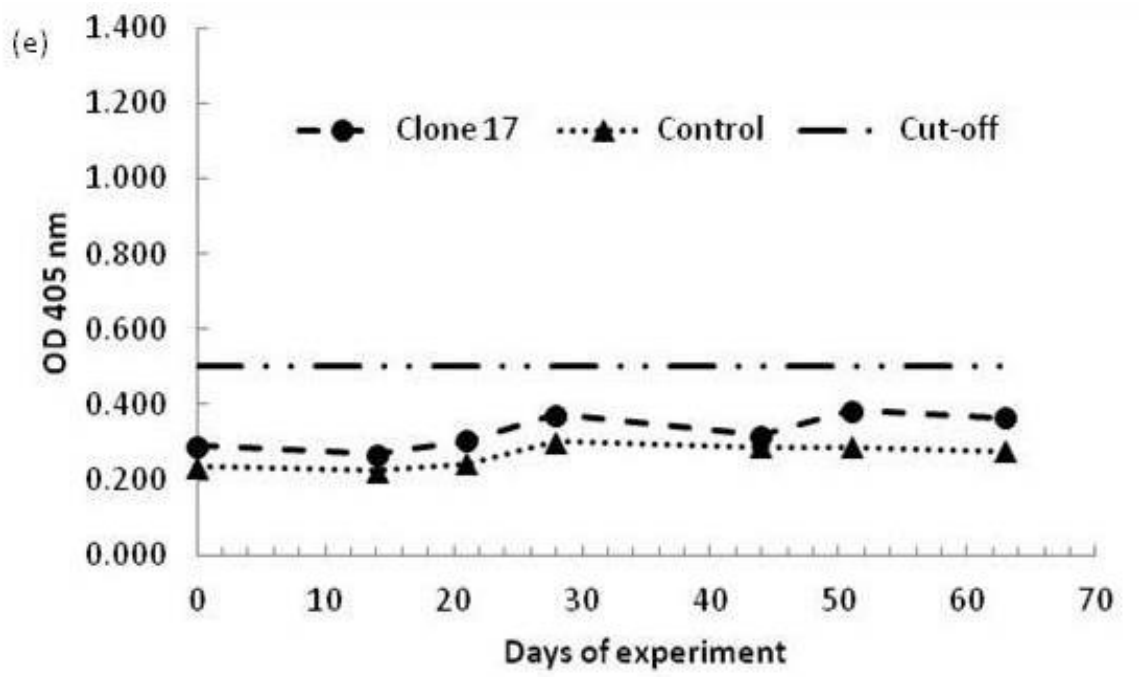

FIGURE 2.3 - Mean levels of plasma IgG of sheep against M13 phage (a), Clone 6 (b), Clone17 (c), Chemically-synthesized GAPDH mimotope (d) and Chemically-synthesized Dim 1 mimotope (e). Twelve 8-10 month-old Suffolk sheep were allocated into 3 groups of 4 animals as follows: Control: infected but not immunized; GAPDH: immunized with clone 6; and Dim 1: immunized with clone 17. Four immunizations were performed (0, 7, 14 and 21 days) with $5x10^{11}$ phage particles displaying mimotopes of *H. contortus* peptides - Clone 6 displayed a mimotope of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the clone 17 a mimotope of a Disorganized muscle family member (Dim 1). SOURCE: The author (2018).

TABLE 2.1 - Blood cell counts of the experimental groups.

Day of experiment	GAPDH	Dim 1	Control
Hemoglobin (g/dl)			
Day 0	10.64 ± 2.90	11.90 ± 2.92	11.03 ± 4.05
Day 14	14.50 ± 2.23	11.66 ± 3.82	13.37 ± 3.03
Day 28	11.75 ± 2.42	13.52 ± 3.97	13.20 ± 2.02
Day 44	12.56 ± 2.68	14.73 ± 1.68	14.28 ± 3.05
Day 50	13.89 ± 1.96	14.41 ± 2.06	13.10 ± 0.81
Total leukocytes (x $10^3/\mu L$)			
Day 0	11.85 ± 2.06	12.00 ± 1.48	12.12 ± 1.42
Day 14	12.20 ± 2.14	10.97 ± 0.28	12.12 ± 1.38
Day 28	11.07 ± 1.96	10.70 ± 2.09	12.50 ± 1.58
Day 44	11.37 ± 1.15	12.47 ± 0.75	12.27 ± 1.61
Day 50	12.05 ± 2.04	10.92 ± 1.90	12.57 ± 1.19
Lymphocytes (%)			
Day 0	58.75 ± 3.86	59.50 ± 4.72	57.50 ± 2.38
Day 14	68.00 ± 8.16	64.50 ± 6.35	66.00 ± 6.22
Day 28	61.00 ± 4.08	65.00 ± 0.82	61.50 ± 1.91
Day 44	59.25 ± 3.30	53.75 ± 0.50	56.75 ± 6.95
Day 50	64.25 ± 3.77	62.00 ± 6.22	60.50 ± 2.65
Neutrophil (%)			
Day 0	36.75 ± 3.30	36.75 ± 3.77	39.00 ± 2.44
Day 14	26.75 ± 7.80	31.00 ± 5.35	30.00 ± 6.48
Day 28	34.25 ± 3.59	31.00 ± 1.56	32.75 ± 1.72
Day 44	36.00 ± 3.37	41.25 ± 1.73	37.00 ± 5.71
Day 50	30.75 ± 2.75	32.75 ± 5.44	34.50 ± 2.38
Eosinophil (%)			
Day 0	2.25 ± 0.50	1.25 ± 0.50	1.50 ± 0.58
Day 14	2.25 ± 0.50	2.25 ± 0.50	1.75 ± 0.96
Day 28	1.75 ± 0.50	1.75 ± 0.96	2.75 ± 1.26
Day 44	1.75 ± 0.50	1.50 ± 0.58	2.25 ± 0.50
Day 50	2.50 ± 0.58	1.75 ± 0.96	1.75 ± 0.50
Monocytes (%)			
Day 0	1.50 ± 1.00	1.75 ± 0.50	1.50 ± 0.58
Day 14	1.50 ± 0.58	1.25 ± 0.50	1.75 ± 0.50
Day 28	2.00 ± 0.82	1.50 ± 0.58	2.00 ± 0.82
Day 44	2.00 ± 0.82	1.75 ± 0.50	1.75 ± 0.96
Day 50	1.75 ± 0.50	1.50 ± 0.58	1.25 ± 0.50
Basophils (%)			
Day 0	0.25 ± 0.50	0.50 ± 0.58	0.50 ± 0.58
Day 14	1.25 ± 0.96	0.75 ± 0.96	0.50 ± 0.58
Day 28	0.75 ± 0.50	0.50 ± 0.58	0.00 ± 0.00
Day 44	0.50 ± 0.58	0.75 ± 0.96	1.50 ± 0.58
Day 50	0.25 ± 0.50	1.50 ± 0.58	1.25 ± 0.96

Hemoglobin, total leukocyte number and percentage of lymphocyte, neutrophil and eosinophil cells in blood are shown as means ± SD. Twelve 8-10 month-old Suffolk sheep were alocated into 3 groups of 4 animals as follows: Control: infected but not immunized; GAPDH: immunized with clone 6; and Dim 1: immunized with clone 17. Four immunizations were performed (0, 7, 14 and 21 days) with $5x10^{11}$ phage particles displaying mimotopes of *H. contortus* peptides - On day 28 all groups were orally challenged with 2500 infective *H. contortus* infective larvae. All groups demonstrated similar results ($P>0.05$) by the Kruskall-Wallis test.

The results from leukogram and haemoglobin analyses are shown in Table 1, with a lack of significant variations during the experimental period. The infection with *H. contortus* was confirmed on day 44, but we did not detect any significant FEC reduction within the experimental groups (Apêndice 7.1); nor for the worm counts ($P>0.05$) after vaccination (data not shown).

2.4 Discussion

This is the first study using phage display to select *H. contortus* peptide mimotopes. This technology allowed mimotope selection for displayed peptides, using polyclonal serum of sheep naturally infected with *H. contortus*. The molecules bound to pathogen antibody are antigenic mimotopes of the pathogen (GAZARIAN et al., 2011). The ability of selected peptides to act as antigenic mimotopes was confirmed by serum analyzes against chemical peptides (GAPDH and/or Dim 1). We observed that IgG of animals naturally infected were able to recognize and bind to these peptides. The best results were for GAPDH, where only one sample had an IgG titre less than the cut-off.

In this study, after four selection rounds of biopanning, the bioinformatic analysis revealed two phage clones containing peptides with homology to GAPDH (clone 6) and Dim 1 (clone 17) proteins of *H. contortus*. Researchers have analyzed the immunogenic potential of many *H. contortus* molecules and immunoproteomic studies have already demonstrated that 23 immunogenic proteins are shared between males and females of *H. contortus*. These molecules include homologous of Dim 1 and GAPDH, among others (YAN et al., 2010).

GAPDH and Dim 1 are important vaccine candidates. In addition to their immunogenic potential, they perform important functions at cellular level. GAPDH is an enzyme crucial for energy production, in glycolysis and glyconeogenesis (NICHOLLS et al., 2012). It has also been related to other intracellular (SIROVER, 2011) and extracellular (GÓMEZ-ARREAZA et al., 2014) functions. According to Han et al. (2012), this molecule is a major therapeutic candidate for vaccines and a target for chemotherapy treatment against several parasites. Dim 1 is a structural protein located in the muscle cell membrane region, around and between dense bodies. It performs a key role in stabilizing the thin filament components of the sarcomere (YAN et al., 2013). Thus, an immunological response generated against these molecules might protect the animals against *H. contortus* infection.

In the *in vivo* assay, animals of both immunized groups GAPDH and Dim 1 presented IgG titers against M13 phage. The IgG increase followed the same pattern in both GAPDH and Dim 1 groups, with a peak after the last immunization. However, despite the sequence similarities to the immunogenic molecules of *H. contortus* (YAN et al., 2010), the peptides displayed by the phage did not promote specific IgG titers. It is important to note that the sequence displayed on the phage surface consisted of only 7 amino acids in linear conformation, being a small peptide compared to the full phage surface. Cui et al. (2013) observed significant immune responses and protection against *T. spiralis* infection in mice immunized with phage. However, they used a recombinant phage system, which displayed a 202-aa polypeptide.

To date, sheep immunization with phage displaying mimotopes has been reported only for *F. hepatica*. For this, animals were immunized at week 0 and 2 with $1x10^{14}$ phage particles expressing 7-aa mimotopes and challenged with 300 metacercariae, 4 weeks after immunization. The mean worm burden was reduced by 47.61% and the egg viability from 58.92 to 82.11%. The animals produced specific antibodies after the metacercariae challenge (VILLA-MANCERA et al., 2008).

Another study with a similar design had sheep receiving $1x10^{13}$ phage particles (12-aa mimotopes) and challenged with 300 metacercariae 4 weeks later. The worm burdens and FEC were reduced by 51.7 and 45.7%, respectively (VILLA-MANCERA & MÉNDEZ-MENDOZA, 2012). In our work, the animals were immunized with phage expressing mimotopes of GAPDH or Dim 1 (7aa) but there was no reduction in FEC or worm counts, indicating the absence of a protective effect. A DNA vaccine encoding Dim 1 antigen against *H. contortus* reduced the FEC and worm counts of goats by 47.5 and 51.1% respectively (YAN et al., 2013). Similarly, a GAPDH DNA vaccine reduced FEC and worm count by 34.9 and 37.7%, respectively (HAN et al., 2012). These studies support the immunogenic potential of these molecules.

Eosinophils also have an important function in immune responses against helminth infections, and are often associated with resistance to parasite infections in sheep (PFEFFER et al., 1996; PATNODE et al., 2014). In our work, eosinophils and the other leukogram parameters did not reveal any difference throughout the experimental period. Yan et al. (2014) evaluating a *H. contortus* actin DNA vaccine in goats failed to detect differences in the eosinophil, neutrophil, basophil and monocyte counts before L3 challenge. However, after the challenge of 5000 L3 infective *H. contortus*, only eosinophil counts were significantly increased.

Santos et al. (2014) tested a single infection with 4000 L3, and one week later the challenged groups presented significantly higher eosinophil numbers. However, there was a gradual decline in the average values, until the end of the trial. In the same experiment, they observed an increase in the eosinophil average in serially infected groups (3 times a week, from day 0 – 25 of the trial with 500 L3). Values significantly higher than the control group were observed on days 14, 21, 25, 28, and 32 with the peak of eosinophil counts on day 25. They concluded that the animals developed this high protection after being consecutively challenged with *H. contortus*. The authors reported that immunity against the parasite was not easily elicited other than after the prolonged infection. In our study, we used a single challenge infection with 2500 L3, and that may have been reflected in the failure to see an increase in eosinophil counts.

It is important to emphasize that the development of immunity to *H. contortus* antigens is complex and highly variable. It depends on the host-parasite interaction, which is influenced by many factors, following an intrinsic and extrinsic relationship (TAK et al., 2015; NISBET et al., 2016). In our trial, the two mimotopes selected by phage display failed to protect, and neither induced a distinct immune response in the animals. The peptides reacted with polyclonal antibodies in serum from sheep naturally infected with *H. contortus* but were not immunogenic when carried by the M13 phage, even though the two selected clones had high sequence similarity to Dim 1 and GAPDH of *H. contortus*. Our data highlight the importance of new studies to evaluate specific peptides from parasites.

2.5 Conclusion

The phage-display library was successfully used to identify *H. contortus* mimotopes. The selected clones revealed relatedness to Dim 1 and GAPDH of *H. contortus* proteins. The peptides were recognized by several sera from sheep naturally infected with *H. contortus*, indicating their potential use as a diagnostic. The sheep immunized with phage displaying the mimotopes did not develop protection against the parasite.

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3 SILENCIAMENTO DO GENE GLICERALDEÍDO-3-FOSFATO DESIDROGENASE EM *Haemonchus contortus*

RESUMO

A tecnologia de RNA de interferência (RNAi) apresenta um mecanismo funcional em *Haemonchus contortus*. Alguns estudos já foram desenvolvidos com este parasita e os resultados sugerem que determinados genes podem ser efetivamente silenciados, enquanto outros são refratários ao RNAi. Gliceraldeído-3-fosfato desidrogenase (GAPDH) é uma enzima chave na via glicolítica, além de outras importantes funções, o que a destaca como como um importante alvo terapêutico em doenças parasitárias. O objetivo deste estudo foi avaliar o uso de dsRNA no silenciamento do gene codificante da proteína GAPDH em larvas infectantes (L3) de *H. contortus.* Após os ensaios de incubação procedeu-se a avaliação do silenciamento por meio de Reação em cadeia da polimerase via transcriptase reversa (RT-PCR). O efeito fenotípico foi avaliado pelo Teste de Migração Larval (TML). Os resultados de RT-PCR revelaram ausência de transcrição após 3; 6; 24 e 48h de incubação de L3 com dsRNA do gene alvo, confirmando o efetivo silenciamento de GAPDH. Os resultados do TML foram semelhantes (P>0,05) entre os grupos tratado e controle e não foi observado efeito na motilidade de L3 em nenhuma das avaliações realizadas. Este estudo relata pela primeira vez o silenciamento do gene GAPDH em L3 de *H. contortus*, confirmando-o como passível ao silenciamento por RNAi. Os resultados constatados e as funções já relatadas à GAPDH destacam essa molécula como alvo potencial para novos estudos com enfoque na tecnologia RNAi para o desenvolvimento de novas terapias para o controle de *H. contortus*.

Palavras-chave: Inibição de genes; RNA de interferência; biotecnologia; controle de nematoides; ruminantes.

3 GENE SILENCING OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN *Haemonchus contortus*

ABSTRACT

Genetic knockdown by RNA interference (RNAi) technology have a functional RNAi pathway in *Haemonchus contortus.* Its use already has been evaluated in some genes and the results suggest that genes can be silenced or be seemingly refractory. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway, beside other functions. GAPDH is also been suggested as a major therapeutic target in several parasitic diseases. The aim of this study was to evaluate double-strand DNA (dsRNA) of GAPDH by soaking in third stage (L3) *H. contortus* larvae to silence the target gene. The evaluation of the gene knockdown was made by reverse transcription polymerase chain reaction (RT-PCR). Phenotypic effect was assessed by larval migration test (LMT). We observed an absence of transcript levels after 3; 6; 24 and 48h of soaking L3 with the dsRNA, confirming an effective GAPDH silencing. The results of LMT were similar ($P>0.05$) among all groups and we did not observe any effect of L3 motility. This is the first time *H. contortus* GAPDH gene was silenced, confirming it as a responsive RNAi pathway. GAPDH stands out as a potential target, employing RNAi technology for developing a novel treatment against *H. contortus*.

Key words: Gene knockdown; RNA interference; biotechnology; nematode control; ruminants.

3.1 INTRODUCTION

Haemonchus contortus is the most important endoparasite of small ruminants worldwide, mainly in tropical and subtropical areas and a major constraint on ruminant health and production (NISBET et al., 2016). The parasite is one of the most prevalent parasite in sheep and goats and the loss of blood caused by heavy infections can result in profound anemia, anorexia, body weight reduction, poor wool quality and death of animals (FORTES and MOLENTO, 2013; FORTES et al., 2013). Currently, the control of small ruminant endoparasites is a major obstacle to production systems. Worldwide, the heavy anthelmintic use has caused significant reduction of efficacy and the selection for resistant parasites, to all chemical options (KOTZE and PRICHARD, 2016). This problem had highlighted the need for new control strategies, particularly against *H. contortus*.

RNA interference (RNAi) technology was first described with the nematode *Caenorhabditis elegans* and since then it has been extensively used to examine the function of genes. The use of double-stranded messenger RNA (dsRNA) can cause potent and specific interference and inhibit target gene expression. The process occurs due to mediation of the degradation of the corresponding dsRNA transcript (FIRE et al., 1998). Following *C. elegans,* the application of RNAi has been demonstrated in several systems and organisms, including *Drosophila melanogaster* (CAPLEN et al., 2000), protozoan (MCROBERT and MCCONKEY, 2002), planarian (PINEDA et al., 2002), mammalian cells (ELBASHIR et al., 2001) and the nematode parasite *Nippostrongylus brasiliensis* (HUSSEIN et al., 2002).

According to Britton et al. (2016), genetic knockdown by RNAi has been very efficient in *C. elegans*, but its practical application in parasitic nematodes has not provided similar results. The *H. contortus* have a functional RNAi pathway and its use has been evaluated for some genes (KOTZE and BAGNALl, 2006; GELDHOF et al., 2006a; SAMARASINGHE et al., 2011; ZAWADZKI et al., 2012; ARENAL et al., 2017). In general, the results observed were variable, with complete silencing of some genes, transcription knockdown, no effect or even the increase in the transcription of others. These findings suggest that specific genes could be silenced, while others were seemingly refractory. This variability may be associated with the target to be silenced, its expression level, the site of expression, methodology used in the studies, the route of delivery the dsRNA, among others. These authors also affirm that a methodological standardization is necessary that considers the form of use of the dsRNA (soaking,

feeding or electroporation) according to the location of each target gene (SAMARASINGHE et al., 2011).

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), is a key enzyme in the glycolytic pathway, and it catalyzes the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (ZINSSER et al., 2014). The enzyme is important to energy production, and it has been suggested that it is also a major therapeutic target in several parasite diseases (i.e. target for chemotherapeutic treatment, vaccine candidate) (HAN et al., 2012). GAPDH from *S. mansoni* (GOUDOT-CROZEL et al., 1989), *S. bovis* (PÉREZ-SÁNCHEZ et al., 2006) and *H. contortus* (YAN et al., 2010) are bound by antibodies in the serum of immune hosts, proving the antigenic property of the enzyme and supporting to protein secretion by the these parasites.

The enzyme is also one of the components of *H. contortus* excretory/secretory (ES) products (SAHOO et al., 2013) and of others helminths (UMAIR et al., 2017). Besides ES, the protein is present in the infective stage larvae (L3) and adult parasites and may be present in other developmental stages of *H. contortus,* considering its function as a glycolytic enzyme (VEDAMURTHY et al., 2015). According to Vedamurthy et al. (2015), the GAPDH present into ES products acts as a key enzyme in the modulation of the immune response to parasite infections. The protein inhibits the membrane attack complex (MAC) formation, it may bound to C3 protein and impedes the complement-mediated lysis of sensitized erythrocytes. In *Fasciola hepatica,* this enzyme has other functions, such as gene regulation, vesicle transport and the prevention of telomere shortening(ZINSSER et al., 2014). Base on all important functions previously reported to GAPDH, the aim of this study was to evaluate dsRNA of GAPDH by soaking in *H. contortus* L3 to silence the target gene.

3.2 Materials and methods

3.2.1 Collection of adult *Haemonchus contortus*

This study was approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of Parana (n° 080/2015). Adult *H. contortus* worms were collected from a sheep slaughtered in a commercial slaughterhouse, located in Curitiba, PR for the extraction of total RNA.

The abomasum was excised and placed in a container with water at 37ºC. Adult female *H. contortus* were removed of the abomasum contend and placed in petri dishes containing PBS 1X at 37ºC. The worms were washed in PBS 1X until

completely remove the debris and after that they were placed in supplemented RPMI-1640 medium at 37ºC with pH 6.8, and 1% glucose, 0.25µg/ml amphotericin B, 10 units/ml penicillin, 10µg/ml streptomycin e 10mM HEPES buffer. The parasites identification (Apêndice 7.3) was proceded according to Ueno and Golçalves (1998).

3.2.2 Total RNA Extraction of *Haemonchus contortus*

Total RNA extraction from adults *H. contortus* was performed with the PureLink RNA Mini Kit (Ambion) according to the manufacturer's instructions. The extraction product was aliquoted and kept frozen at -80°C until use for cDNA synthesis (complementary DNA).

3.2.3 Oligonucleotides

TABLE 3.1 - Oligonucleotides used in PCR and/or RT-PCR reactions.

Primers	Sequence 5' – 3'	Amplified product (bp)
1A: GAPDH- gf	ACGGATGTGGCCGTATTGGTCGCCT	998
1B: GAPDH- gr	GGCCTTGCTTGCAATGTAGGCTAAC	998
2A: GAPDH -4f	CCACGAGACCTACAATGCAGCCAAC	400
2B: GAPDH -4r	AACAGACACATCAGGCGTGGGTACA	400
3A: GAPDH-4f/promotor T7	GGATCCTAATACGACTCACTATAGGN CCACGAGACCTACAATGCAGCCAAC	419
3B: GAPDH-4r/ promotor T7	GGATCCTAATACGACTCACTATAGGN AACAGACACATCAGGCGTGGGTACA	419
4A: GAPDH-3f	GGCTTTCCGTGTACCCACGC	321
4B: GAPDH-3r	ATGTAGGCTAACAGATCGAC	321

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; bp: base pairs; gf: complete gene forward; gr: complete gene reverse; 4f: 400 base pairs forward; 4r: 400 base pairs reverse; 4f/T7 promoter: 419 base pairs with T7 promoter forward; 4r / T7 promoter: 419 base pairs with T7 promoter reverse; 3f: 321 base pairs forward; 3r: 321 base pairs reverse. SOURCE: The author (2018).

The oligonucleotides (primers) used in this study are shown in Table 1. The primers were designed based on the sequence of the gene encoding Glyceraldehyde 3-Phosphate Dehydrogenase protein (GAPDH) of *H. contortus* (GenBank - Accession Number: ADI46817.1).

The primers (GAPDH-3f/3r) for RT-PCR amplification, to verify the gene expression after incubation with dsRNA, were designed to a region of the gene outside of that used as a template to dsRNA production. This was done to eliminate the possibility of RT-PCR amplification, from the applied dsRNA treatment, interfering in the detection of mRNA levels (KOTZE and BAGNALL, 2006).

3.2.4 Synthesis of complementary DNA (cDNA)

Reverse transcription polymerase chain reaction (RT-PCR) was performed to the synthesis of complementary DNA (cDNA). Prior the cDNA synthesis, the quality and quantity of RNA samples were evaluated by gel electrophoresis and spectrophotometer (Qubit 2.0, Thermo Fisher Scientific), respectively. To degrade any genomic DNA contamination, each total RNA sample was treated with RQ1 RNase-Free DNase (Promega), according to the manufacturer's protocol.

The DNase-treated total RNA was used as template for the RT-PCR. The integrity of each RNA preparation was verified using the 18S rRNA as an internal control. For this, each reaction mixture was split into two tubes, in which either control 18S primers (non-target gene) or RNAi target primers were added. Negative (no template) controls were also included in each assay.

The reactions were performed with the One-Step RT-PCR Kit (Qiagen), employing specific primers (Table 1) and cycling conditions as following: 45ºC x 60 min (1cycle); 94°C x 15 min (1cycle); 94°C x 1 min, 55°C x 1 min, 72°C x 1:30 sec (45 cycles); 72°C x 10 min and after the samples were kept at 10 ° C. Amplification products were separated and visualized on 1% agarose gel containing 0.001% SYBR safe (Invitrogen, USA).

3.2.5 In vitro DNA amplification

Complementary DNA (cDNA) obtained from the RT-PCR reactions was amplified by nucleotide polymerization reaction (PCR) to serve as a template to dsRNA target GAPDH. Proportions of reagents were optimized and reactions were standardized. Each PCR reaction consisted of PCR buffer (1X); 0.6 mM of each primer

(forward and reverse); 0.2 mM of each dNTP; 1.5 mM $Mg2^{+}$; 0,04 U of Taq DNA polymerase and 4 µl of cDNA template, to volume of 50 µl/reaction. For the amplification of the complete gene, 1.0 mM of each specific primer was used (Table 1). After each PCR, all amplification products were separated and visualized on 1% agarose gels containing 0.001% SYBR safe (Invitrogen, USA). After amplification, the products of 998 and 400 bp were sequenced (Myleus Biotechnology, Brazil) and the gene identity was verified using BLAST with GenBank association (accession number: HM145749.1).

The thermocycling was performed under variable conditions according to each objective, as described below:

a) Amplification of the complete GAPDH gene: 94°C x 5 min (1 cycle); 94°C x 30 sec, 45°C x 30 sec, 72°C x 30 sec (45 cycles); and 72°C x 10 min (1 cycle).

b) Amplification of the 400 base pairs fragment of GAPDH: 94º C x 5 min (1 cycle); 94ºC x 30 sec, 58ºC x 30 sec, 72ºC x 30 sec (30 cycles); and 72ºC x 10 min (1 cycle).

c) Amplification of the 400 base pairs fragment of GAPDH with T7 promoter: 94ºC x 5 min (1 cycle); 94ºC x 1 min, 58ºC x 30 sec, 72ºC x 30 sec (10 cycles); 94ºC x 1 min, 62ºC x 1 min, 72ºC x 30 sec (35 cycles); and 72ºC x 10 min (1 cycle).

After the end of the thermocycling (a, b or c), the samples were kept at 10°C until they were removed from the thermal cycler.

3.2.6 Synthesis of double-stranded RNA (dsRNA)

The templates for synthesis of dsRNA were generated by PCR from either, cDNA prepared from adult *H. contortus* (GAPDH) or *Arabidopsis thaliana*. We used *A. thaliana* photosystem II light harvesting complex (Photosystem II), as a control treatment (non-target gene) for RNAi experiments. The sequence of the photosystem II fragment (accession number NM 0012027 90.1) did not have homology with the *H. contortus* gene in public access databases (Arenal et al., 2017).

The primers 3A and 3B (Table 3.1) were used to produce *H. contortus* DNA template (419 bp) to dsRNA synthesis. To produce *A. thaliana* DNA template, we used the primers according to Arenal et al. (2017). After that, the samples were purified with the Wizard Genomic DNA Purification Kit (Promega, USA). The dsRNA was synthesized with the T7 Ribomax RNAi Express System kit (Promega, USA) according to the manufacturer's protocol. The quality and integrity of the dsRNA was confirmed by electrophoresis in 1% gel, stained with 0.001% of SYBR safe (Invitrogen, USA).

The sample concentration was determined on Qubit fluorimeter 2.0 (Thermo Fisher Scientific) and conditioned at -80 °C for later use.

3.2.7 Treatment of *Haemonchus contortus* infective larvae

Infective larvae (L3) of *H. contortus* were obtained from coproculture (Roberts and O'sullivan, 1950) of naturally infected sheep. The feces were maintained in a biological oxygen demand (BOD)-type incubator at 25-28°C, for 10 days. The culture analysis showed the presence of *H. contortus* L3 as the abundant (90%) nematode species.

The L3 were washed in distilled water and placed into a solution of 0.3% (v/v) sodium hypochlorite for approximately 15 min, at room temperature, to remove the cuticle. After, the contend was washed 3 times with PBS 1X (140mM NaCl, 5mM KCl, 20mM Na2HPO4, 5mM NaH2PO4), the L3 were counted (in triplicate) under optical microscopy and aliquoted for the assays. Each aliquot corresponded to approximately 8000 (for genotypic assessment) or 200 (for phenotypic evaluation) exsheathed L3, which were placed into Eppendorf tubes for RNAi treatment and centrifuged to obtain a final volume of 10 µl. The dsRNA treatments were prepared as follows*:* dsRNA of *H. contortus* or *A. thaliana*, were pre-incubated for 10 min at room temperature with 1 ml Lipofectin reagent (Invitrogen, USA) and RNasin (0.4 U/ml.). The treatments were added to each L3 tube, previously prepared, and mixed. Control incubations with no dsRNA were also set up. The volume of each reaction was 35 µl and the final concentration of dsRNA was 440 ng/µl.

All assays were performed with three experimental groups, as follow: Negative control (no dsRNA) - in duplicate; Positive control: Photosystem II - in duplicate; dsRNA of GAPDH – in quadruplicate. The total of 29 assays were performed for the evaluation of transcript levels (genotypic assessment) and 27 assays for phenotypic evaluation (in triplicates). The incubation was set at 37ºC for 3; 6; 24 and 48h. To each time point, the respective aliquots were removed and washed extensively in PBS 1X (140 mM NaCl, 5mM KCl, 20mM Na2HPO4, 5mM NaH2PO4), and used to evaluated de effect of the dsRNA treatment and larval migration test.

3.2.8 Evaluation of transcript levels

After each incubation time point we evaluated the transcript levels (genotypic assessment). The total RNA was extracted according to section 3.3.2 RT-PCR was

carried out according to section 3.2.4 After each RT-PCR, all amplification products were separated and visualized on 1% agarose gels containing 0.001% SYBR safe (Invitrogen, USA).

3.2.9 Larval migration test (LMT)

The LMT (DEMELER et al., 2010) were performed as a phenotypic evaluation, to determine the effect of dsRNA incubation on the mobility in of *H. contortus* L3. After each incubation time point (3; 6; 24 and 48h) the correspondent aliquots of treated and control groups were transferred into 24-multiwell plates with apparatus containing a 22 µm-mesh nylon membrane sieve. Each well was completed with PBS 1X to a final volume of 1 mL. The plates were incubated in a BOD incubator at 25-28°C, for 12h. A source of light (150 W) was positioned below the plates to stimulate their movement. After the incubation, the apparatus was removed and the number of L3 that migrated through the sieve were counted under optical microscopy.

3.2.10 Statistical analysis

The data that did not pass the normality tests made by the Shapiro-Wilk; D'Agostino-Pearson omnibus, and Kolmogorov-Smirnov test. The statistical analysis was performed using Kruskal–Wallis at 5% probability ($P<0.05$). We tested differences among treatment groups and time of incubation differences in the LMT.

3.3 Results and discussion

The internal control 18S confirmed the integrity of all RNA preparation used to verify the gene transcript levels by RT-PCR. In this study, *H. contortus* GAPDH gene was effective silenced after 3; 6; 24 and 48h of soaking exsheathed L3 with dsRNA. The control Photosystem II dsRNA did not affect GAPDH expression (Fig. 3.1). According to Geldhof et al. (2006b), the use of a dsRNA control (non-target gene) is indispensable to confirm the success of silencing assays because, sometimes, the dsRNA itself can have effect on the parasites. This way, the silencing of GAPDH may be confirmed by the absence of transcription of the target gene, compared to the control and Photosystem II dsRNA in the same period and experimental conditions. Our results support the presence of a functional RNAi pathway in *H. contortus*.

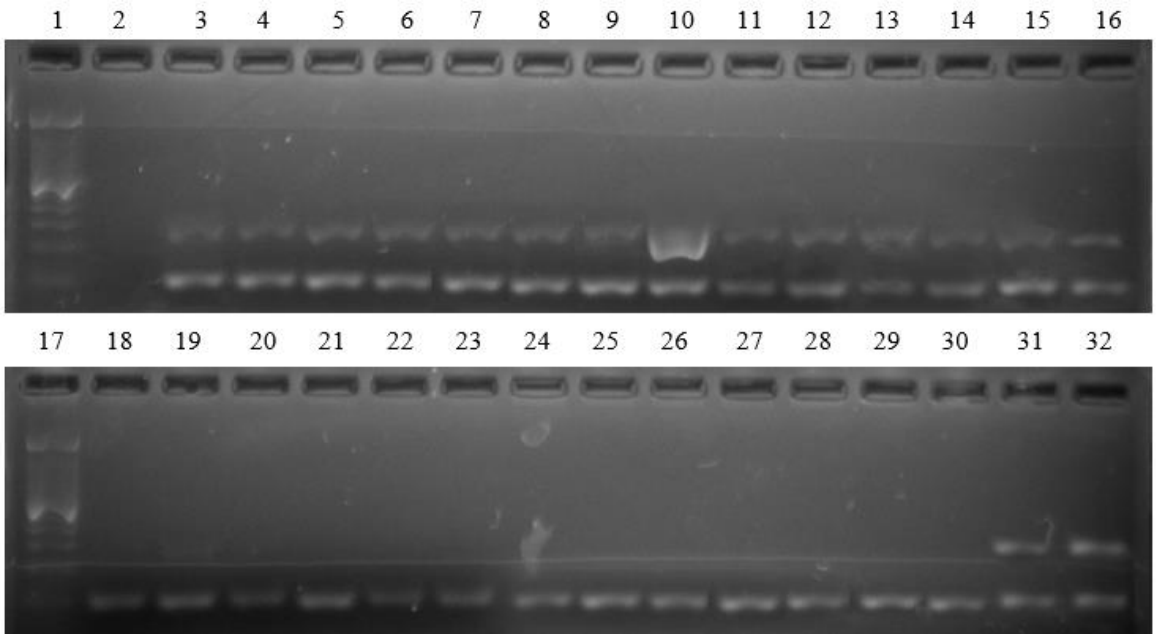

FIGURE 3.1 - Transcript levels of RNAi target genes following soaking of *Haemonchus contortus* exsheathed L3 larvae in a negative control (no dsRNA), positive control: dsRNA Photosystem II (a non-target gene) and dsRNA *H. contortus* GAPDH. Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect mRNA levels as follow: Negative control at 3 h (line 3 and 4), 6 h (line 5 and 6), 24 h (line 7 and 8) and 48h (line 9 and 10) of soaking; Photosystem II (a non-target gene) at 3 h (line 11 and 12), 6 h (line 13 and 14), 24 h (line 15 and 16) and 48h (line 31 and 32); GAPDH at 3 h (line 18, 19, 20 and 21), 6 h (line 22, 23 and 24), 24 h (line 25, 26 and 27) and 48h (line 28, 29 and 30). The 100 base pairs (bp) DNA Ladder is shown in lines 1 and 17 (100 ng/μL). The integrity of each RNA sample used for RT-PCR was verified using the 18S rRNA (non-target gene) as an internal control. The 18S primers were applied to amplify a 100 bp fragment of all samples. The GAPDH primers were used to amplify a 321 bp fragment.

The RNAi pathway is present in *H. contortus* but to achieve success in silencing is a challenge, in this parasite and also in other nematodes. Studies have already related that a few genes may be silenced, while others were seemingly refractory (GELDHOF et al., 2006b). Thus, to apply RNAi to examine gene functions in *H. contortus* is still an obstacle and there are still a few studies that successfully silenced its genes (SAMARASINGHE et al., 2011). To our knowledge, we have demonstrated for the first time, that GAPDH is amenable to RNAi by soaking exsheathed L3 and may be added to the list of genes already silenced in *H. contortus*. In *Schistosoma mansoni*, successful down-regulation of GAPDH have already been reported in sporocysts, where miracidia were allowed to undergo to sporocysts in the presence of dsRNA and incubated for 6 days. The effect of dsRNA treatment on transcript levels

(70-80%) was evident since 10 min until 28 days after the initial dsRNA treatment (BOYLE et al., 2003).

The majority of the studies that tested RNAi in soaked worms for 24 to 72h (BRITTON and MURRAY, 2006; GELDHOF et al., 2006b, 2007; VISSER et al., 2006; SAMARASINGHE et al., 2011). In our case, we obtained success using RNAi in all evaluations performed, after 3, 6, 24, and 48h of soaking exsheathed L3. Tzelos et al. (2015) evaluated the dsRNA to silence the Tci-asp-1 gene of *Teladorsagia circumcincta*, a member of the ASP (Activation-associated Secreted Proteins) gene family. The authors observed the absence of gene expression after 1 h of L3 incubation. However, transcript abundance increased markedly with the *in vitro* maintenance of L3 (24; 48; 72 and 96h). The authors related that successful RNAi may be achieved in *T. circumcincta* but was inconsistent. According to Britton et al. (2016), in some cases, prolonged soaking periods may allow the recovery of normal transcript levels and promote inconsistent results. However, we confirmed that this does not apply to *H. contortus* GAPDH, where the silencing was effective and stable until 48h of soaking.

However, despite the absence GAPDH transcription, we did not observe the effect of L3 motility in any of the evaluations. This phenotype was evaluated by LMT (Fig. 3.2), in which the results were similar ($P>0.5$) among the experimental groups. Our results are consistent with previous finding that identifying RNAi-mediated phenotypes in vitro is a major hurdle, when working with parasitic nematodes (BRITTON et al., 2016).

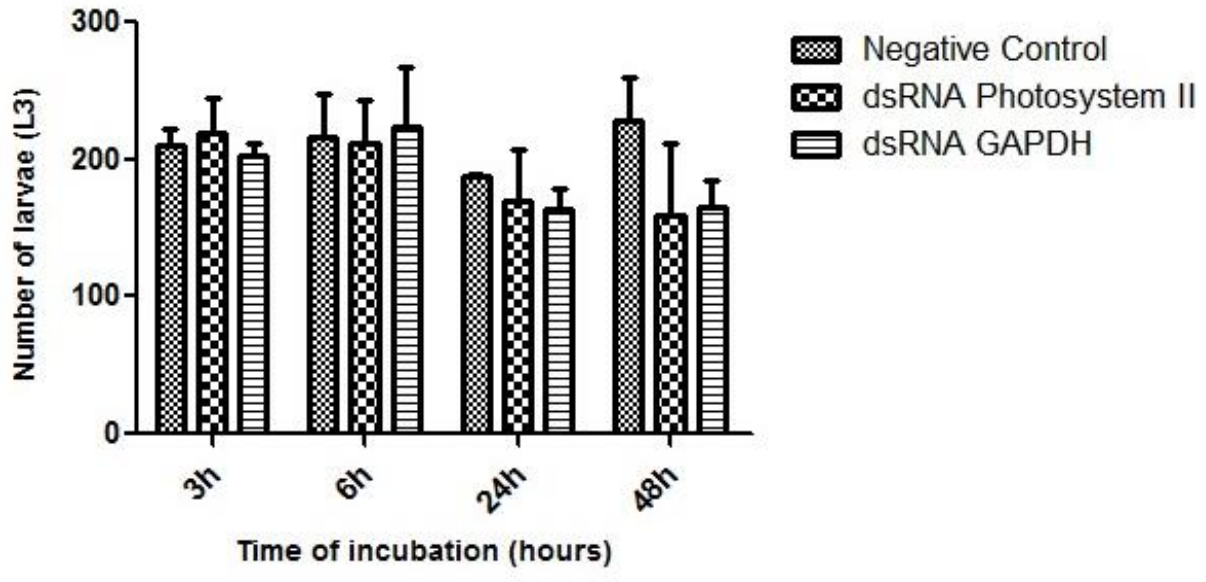

FIGURE 3.2 - Larval migration test (LMT) after dsRNA treatment (GAPDH or Photosystem II). A negative control (no dsRNA, RNasin, and lipofectamine) was tested. The ability of the larvae to migrate after the exposure to dsRNA was evaluated after 3; 6; 24 and 48h after dsRNA treatment. Each column represents the mean (± standard deviation) of three measurements in separate filter assays. Kruskal–Wallis test did not reveal statistical significantly differences (P>0.05) among the experimental groups.

In *H. contortus,* RNAi-mediated phenotypes have already been related to only a few genes, in specific parasite stages. In a study performed by Kotze and Bagnall (2006) two beta-tubulin genes were targeted in exsheathed L3, L4 and adult parasites by soaking dsRNA. Transcript levels of both genes dropped in all three parasite stages after 24 h of incubation. Phenotypic effects were observed after 6 days of treatment, where L3 worms showed a decreased motility compared to control worms and less worms developed to the L4 stage. The adult worms showed a marked gene suppression but did not show any phenotypic effects. Geldhof et al. (2006a), evaluated L1 and exsheathed L3 for 24 h of soaking in dsRNA on 11 target genes. Among these genes, a specific reduction in mRNA levels occurred in only two: Hc-ben-1, the beta tubulin gene, in agreement with the results of Kotze and Bagnall (2006) and the Hc-sec-23, a gene involved in vesicle transport. For all the evaluated genes, the authors did not observe any phenotypic effects of larval survival or integrity.

Samarasinghe et al. (2011) have succeeded using RNAi in *H. contortus* L3 by soaking with target genes putatively expressed in the intestine, excretory cell or amphids. The genes silenced included the gut aminopeptidase H11, secretory protein Hc-ASP-1, b-tubulin, homologues of aquaporin and RNA helicase. Their results suggest that genes that are expressed in sites accessible to the environment are more likely to be responsive to dsRNA by soaking. For all the genes effectively silenced, was not observed phenotypic effect *in vitro*. However, they also performed an *in vivo* assay using L3 pre-treated with *H. contortus* H11 dsRNA to infect sheep and observed a small phenotypic effect. The animals showed a reduction of 57% and 40% in faecal egg count and worm burden, respectively. These findings are in agreement with our results in relation to the absence of RNAi-mediated phenotypes after gene silencing. Although, they have demonstrated that the lack of phenotype results *in vitro* is not a confirmation of the failure to induce RNAi response.

According to Zawadzki et al. (2012), the silencing of phenotype effect in parasitic nematodes is at an early stage of development in comparison with protocols available to *C. elegans.* The ability of the parasite to survive, grow and develop in *in vitro* culture conditions is an importante factor to evaluate the susceptibility to RNAi. For example, adult *H. contortus* can survive for only 2-3 days *in vitro*, which may limit the identification of gene function by phenotypic analysis. L4, collected 7 days post-infection remained viable for up to a week (BRITTON et al., 2016). This time-frame can be a problem to identify gene function, mainly if compared to a study with *Schistosoma mansoni,* which took 2-3 weeks to confirm the knockdown of some phenotypic effects (GUIDI et al., 2015). Thus, the development of *in vitro* cultures and the progress in maintaining parasite stages in more physiological conditions and for longer periods, would promote progress in identifying phenotypic effects that could be mediated by RNAi.

3.4 Conclusion

The present study reports for the first time the silencing of H. contortus GAPDH gene, confirming it as responsive to RNAi pathway. Our findings and the functions already related to GAPDH link this molecule as a potential target to new studies employing RNAi technology for developing a treatment against *H. contortus*.

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4 CONSIDERAÇÕES FINAIS

Pequenos ruminantes criados sob sistema extensivo estão continuamente expostos a infecções por endoparasitas. Geralmente os animais apresentam infecções mistas, podendo ser parasitados por várias espécies ao mesmo tempo. Contudo, tanto em infecções mistas ou parasitismo isolado, *H. contortus,* destaca-se como o mais patogênico e prevalente em ovinos e caprinos, principalmente em regiões de clima temperado, tropical e subtropical. Considerando inúmeros relatos mundias, a espécie também se destaca como altamente resistente aos antiparasitários.

A fim de garantir a viabilidade dos sistemas produtivos é imprescindível o contole parasitário. Dessa forma, torna-se fundamental a realização de pesquisas voltadas ao desenvolvimento de novas terapias. Nos últimos anos, avanços biotecnológicos têm permitido o progresso de pesquisas na área de saúde humana e animal. Nesse sentido, a tecnologia de *phage display* e o RNA de interferência destacam-se como ferramentas importantes na busca de moléculas terapêuticas.

Esta tese demonstrou, pela primeira vez a aplicação da tecnologia de *phage display (PhD-7 Phage Display Peptide Library Kit)* para a seleção de mimetopos de *H. contortus.* O uso de anticorpo policlonal de ovinos infectados por *H. contortus* permitiu a seleção de clones expressando mimetopos de proteínas anteriormente relatadas como imunogênica: gliceraldeído-3-fosfato desidrogenase (GAPDH) e família do músculo desorganizado (Dim 1). A imunização de ovinos com *phagos* expressando os mimetopos em sua superfície não induziu resposta imune específica em ovinos imunizados. Contudo, os peptídeos mimetopos (sintéticos) foram reconhecidos por IgG de ovinos naturalmente infectados pelo parasita, confirmando as moléculas como mimetopos antigênicos, as quais apresentam potencial para uso como antígenos em técnicas de diagnóstico imunológico do *H. contortus.*

Pesquisas já desenvolvidas com RNA de interferência (RNAi) em *H. contortus* indicam que, determinados genes podem ser efetivamente silenciados, enquanto outros são refratários ao RNAi. GAPDH é uma enzima chave na via glicolítica, além de outras importantes funções, o que a destaca como um importante alvo para o silenciamento. Os resultados desta tese demonstraram, pela primeira vez, efetivo silenciamento de GAPDH, confirmando-o como passível ao silenciamento por RNAi. Os resultados constatados e as funções já relatadas à GAPDH destacam essa

molécula como alvo potencial para novos estudos com enfoque na tecnologia RNAi para o desenvolvimento de novas terapias para o controle de *H. contortus*.

Esta tese confirmou o potencial de desenvolvimento e aplicabilidade de estratégias biotecnológicas para a busca de moléculas direcionadas ao controle de *H. contortus.* Com base nos resultados, sugere-se a avaliação dos peptídeos mimetopos (sintéticos) diretamente na imunização de ovinos. Além disso, recomenda-se também a combinação dos 2 mimetopos (GAPDH e Dim1) sintetizados em conjunto, a fim de se aumentar o tamanho da molécula a ser empregado como antígeno imunizante. Os mimetopos apresentam sequência de 7 aminoácidos e se administrados isolados da sequência do *phago* M13 podem ser reconhecidos diretamente como moléculas antigênicas e possivelmente também como imunogênicas.

O gene de GAPDH demostrou ser passível ao silenciamento por incubação de larvas infectantes com RNAI, dessa forma recomenda-se estudos que envolvam outros métodos de produção e aplicação da dupla fita de RNA como tratamento. Por exemplo, o uso de bactérias recombinantes expressando dupla fita de RNA (dsRA) pode servir como uma alternativa interessante do ponto de vista econômico e com possível capacidade de produção de dsRNA em larga escala e seu uso em futuros testes *in vivo.*

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5 ANEXOS

5. 1 Aprovação pela Comissão de Ética no Uso de Animais (CEUA) do Setor de Ciências Agrárias da Universidade Federal do Paraná.

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 080/2015, referente ao projeto "**Biotecnologias aplicadas ao controle parasitário em Ruminantes**", sob a responsabilidade de **Marcelo Beltrão Molento** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro, de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 3 de invasividade, em reunião de 17/12/2015

Vigência do projeto	02/2016 a 10/2017
Espécie/Linhagem	Ovinos
Número de animais	12
Peso/Idade	5-12 meses
Sexo	Machos e fêmeas
Origem	Criatórios comerciais

CERTIFICATE

We certify that the protocol number 080/2015, regarding the project "**Biotechnologies applied to parasitary control in ruminants**" under **Marcelo Beltrão Molento** supervision – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October, 2008, of Decree nº 6.899, of 15 July, 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of the State of Paraná, Brazil), with degree 3 of invasiveness, in session of 12/17/2015.

Duration of the project	February 2016 to October 2017
Specie/Line	Sheep
Number of animals	12
Wheight/Age	5-12 months
Sex	Females and males
Origin	Comercial

Curitiba, 17 de Dezembro de 2015.

5.2 Aprovação pela Comision de ética para el uso de animales de la Facultad de Ciências Agropecuarias de la Universidade de Camaguey, Cuba.

CERTIFICADO

Certificamos que el protocolo número 001/2013, referente al proyecto **"Biotecnologías aplicadas al control parasitario en Rumiantes"**, bajo la responsabilidad de **Amilcar Arenal Cruz** – que incluye la producción, manejo, alimentación y utilización de animales pertenecientes al *filum* Chordata, *subfilum* Vertebrata (excepto el hombre), para fines de investigación científica o la enseñanza – está de acuerdo con los preceptos de la Ley nº No 180/07 (Gaceta Oficial 084, 19/12/2007), y fue aprobada por la COMISION DE ÉTICA PATRA EL USO DE ANIMALES DE LA FAULTAD DE CIENCIAS AGROPECUARIAS DE LA UNIVERSIDAD DE CAMAGÜEY, CUBA, con grado 3 de invasividad, en reunión realizada el 07/01/2013.

Vigencia del proyecto	2013 a 2017
Especie	Ovinos
Número de animales	40
Peso/Edad	5-12 meses
Sexo	Machos y hembras
Origen	Bioterio Facultad de Ciencias Agropecuarias

Presidenta del Comité de 'ética

Dra Teresa González Compte

5.3 Artigo publicado – Molecular & Biochemical Parasitology

Molecular & Biochemical Parasitology 211 (2017) 71–74

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Molecular & Biochemical Parasitology

Short communication

Gene silencing of Dim-1, a member of the disorganized muscle family, in *Haemonchus contortus*

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ABSTRACT

RNA interference has been widely used in parasites. In *Haemonchus contortus*, reproducible silencing has been reported; however, in this species an altered phenotype has been observed for only a few genes silenced by RNA interference. The aim of this work was to evaluate the effect of silencing Dim-1, a member of the disorganized muscle family on third stage larvae (L3) of *H. contortus*. Effective silencing of Dim-1 in L3 led to reduced L3 migration and slowed larval development from L3 to early L4. To our knowledge, this is the first report to demonstrate Dim-1 silencing affecting phenotypic characteristics of this parasite.

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1. Introduction

Intestinal nematodes are a major health problem for ruminant production and *Haemonchus contortus* is the most important such parasite worldwide. Anthelmintic control with medication is including parasitic nematodes [6], *Fasciola hepatica* [7], *Schistosoma mansoni* [8] protozoans [9], and trypanosomes [10].

In general, the reports have shown phenotypic effects following soaking, feeding or electroporation of nematode parasites with dsRNA [11–13]. However, their direct application to *H. contortus* has

5.4 Artigo aceito para publicação

Decision Letter (RBPV-2017-0164.R2)

From: zacariascbpv@fcav.unesp.br

To: deiabuzatti@gmail.com

CC:

Subject: Revista Brasileira de Parasitologia Veterinária - Decision on Manuscript ID RBPV-2017-0164.R2

Body: 09-Mar-2018

Dear Dr. Buzatti:

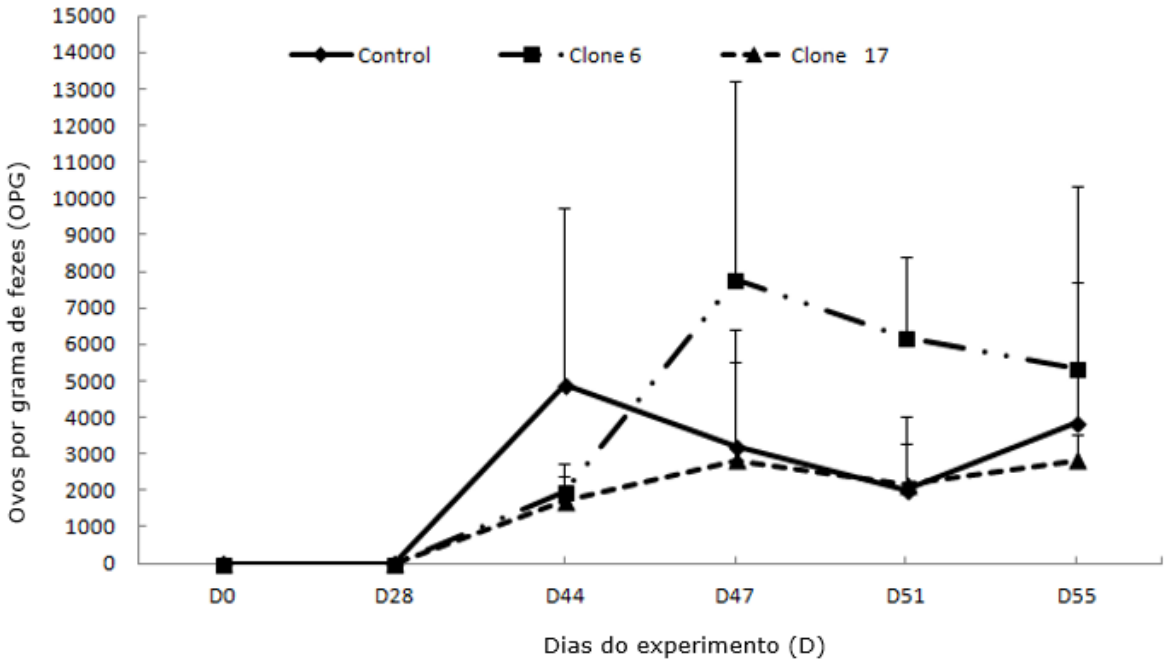
It is a pleasure to accept your manuscript entitled "Sheep polyclonal antibody to map Haemonchus contortus mimotopes using phage display library" in its current form for publication in the Revista Brasileira de Parasitologia Veterinária. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Revista Brasileira de Parasitologia Veterinária, we look forward to your continued contributions to the Journal.

Sincerely,
Prof. Rosangela Machado
Editor-in-Chief, Revista Brasileira de Parasitologia Veterinária
zacariascbpv@fcav.unesp.br

6 APÊNDICES

6.1 Média (+desvio padrão) da contagem de ovos por grama de fezes (OPG) proveniente do ensaio de imunização de ovinos demonstrado no capítulo 2.

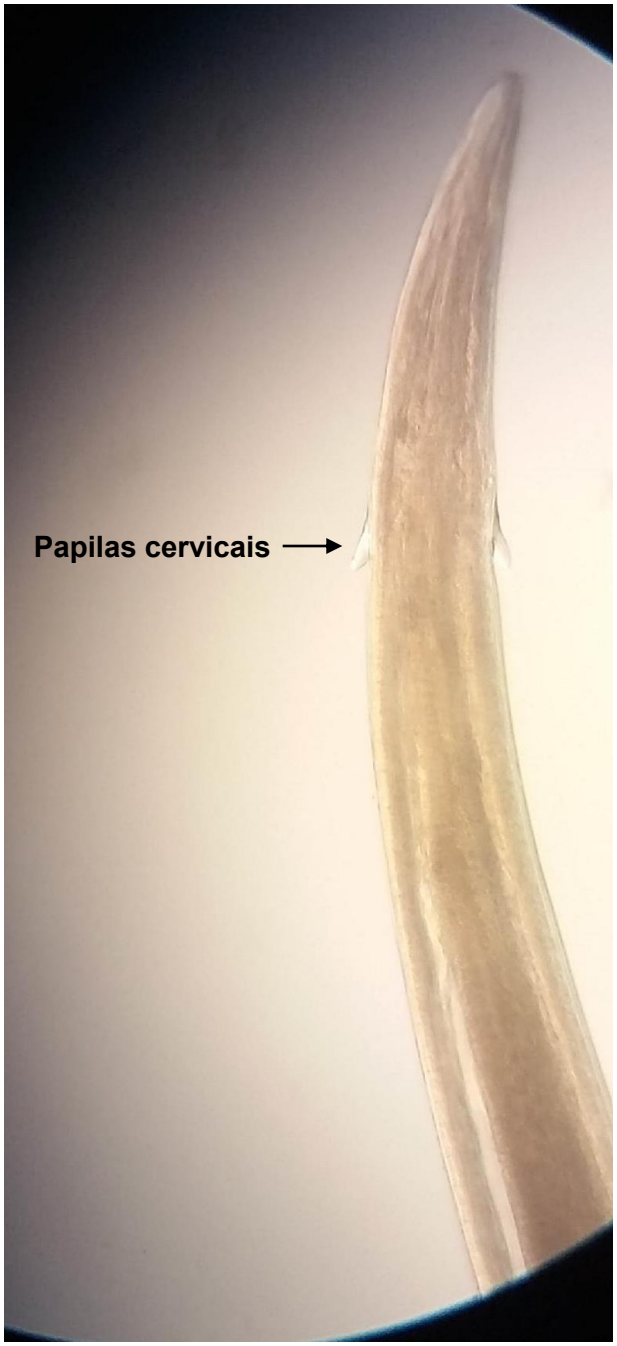


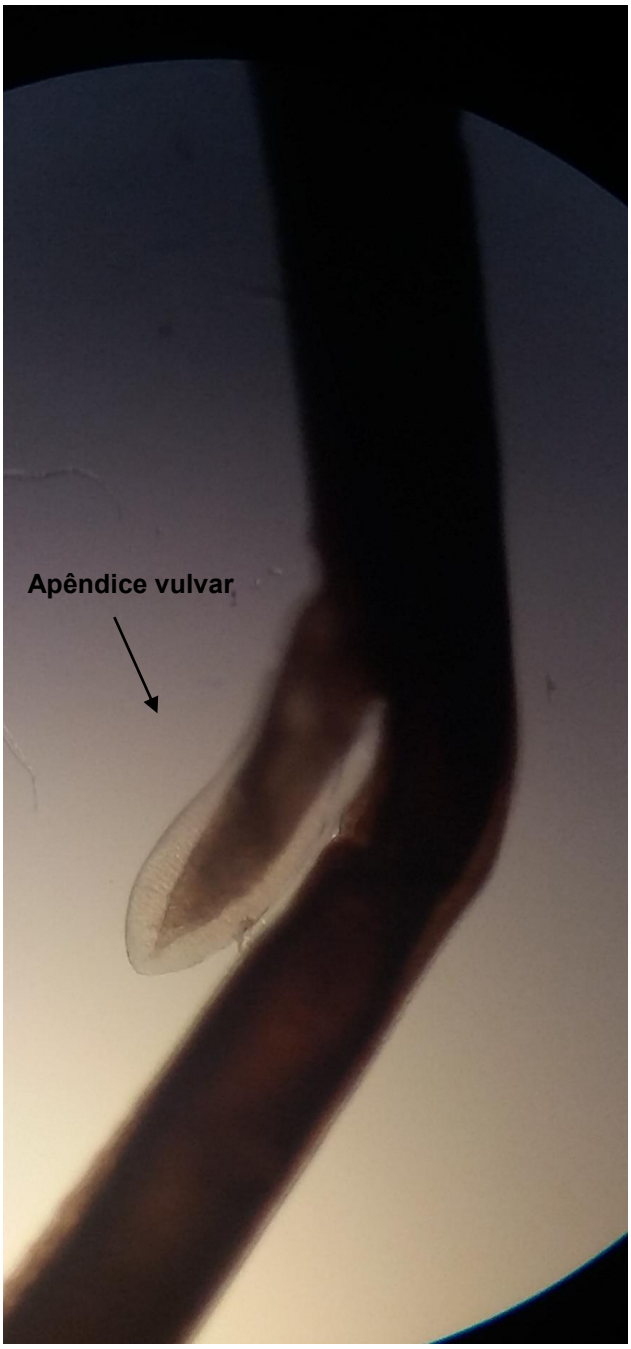
Média (+desvio padrão) da Contagem de ovos por grama de fezes (OPG) proveniente do ensaio de imunização de ovinos com fagos expressando mimetopos de glyceraldehyde-3-phosphate dehydrogenase (GAPDH) e disorganized muscle family member (Dim 1) de *Haemonchus contortus*. No estudo foram utilizados 12 ovinos adultos distribuídos em 3 grupos de 4 animais cada: Controle - infectado, mas não imunizado (recebeu 1 mL do adjuvante); Clone 6 – imunizado com mimetopo de GAPDH; Clone 17 – imunizado com mimetopo de Dim 1. Foram realizadas 4 imunizações com $5x10^{11}$ ufc com adjuvante Montanide ISA 25 VG nos dias (D) 0; 7; 14 e 21 do estudo. No dia 28 (D28) todos os animais foram infectados via oral com 2500 larvas infectantes de *H. contortus*. Contagem de OPG foi realizada nos dias (D) 0; 28; 44; 47; 51 e 55. Fonte: O autor (2018).

6.2 Exemplares adultos de *Haemonchus contortus.*

Exemplares adultos de *Haemonchus contortus* coletados do abomaso de um ovino proveniente do Laboratório de Produção de ovinos e caprinos (LAPOC) da Universidade Federal do Paraná. Fonte: O autor (2018).

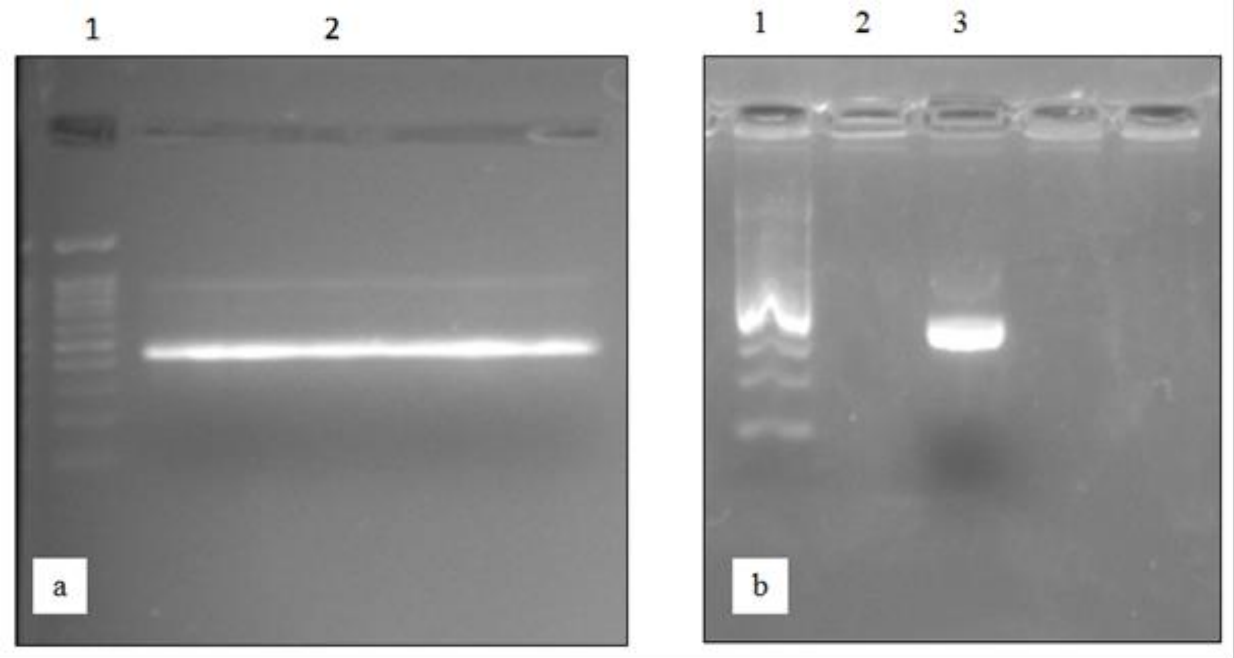
6.3 Identificação morfológica de fêmea de *Haemonchus contortus.*





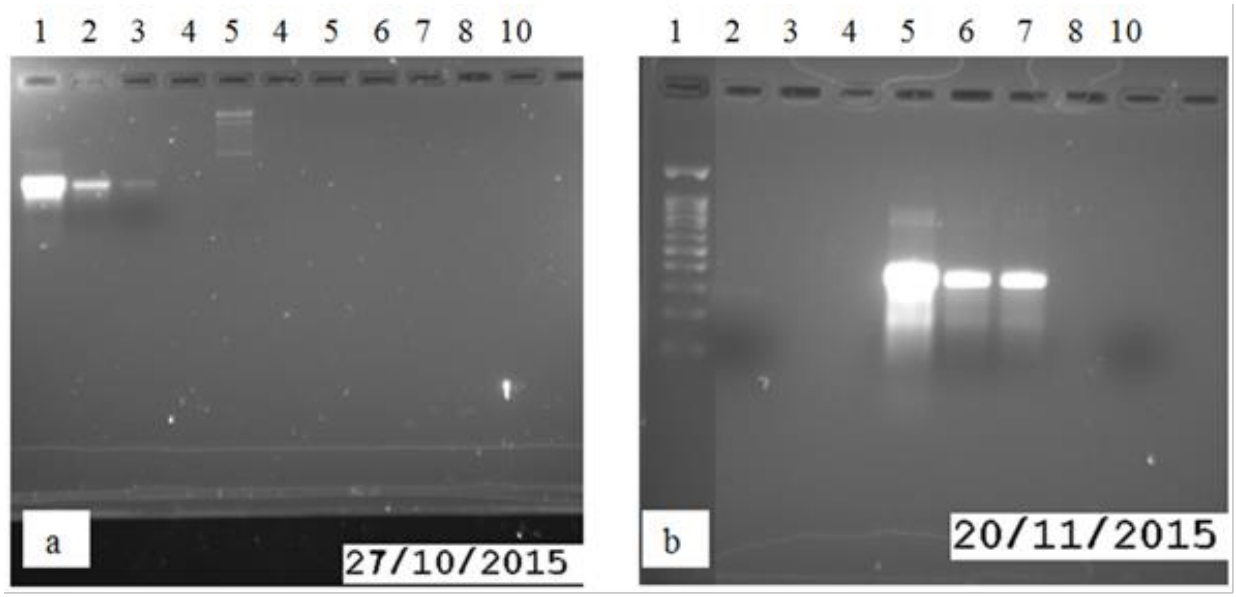
A identificação morfológica foi procedida conforme Ueno e Gonçalves (1998).

6.4 Eletroforese em gel de agarose com amostras de DNA do gene que codifica a proteína Gliceraldeído-3-fosfato desidrogenase de *Haemonchus contortus.*



Eletroforese em gel de agarose 1%. (a) - poço 1: Marcador de peso molecular 100 pb DNA ladder: 3 µL (100 ng/µL); poço 2: 25 µL da amostra; (b) – poço 1: Marcador de peso molécula 100 pb DNA ladder: 3µL (100 ng/µL); poço 2: controle negativo da reação (ausência de amostra); poço 3: 4 µL de amostra após processo de purificação. A amostra consistiu em fragmento de DNA (419 pares de bases) do gene que codifica a proteína Gliceraldeído-3-fosfato desidrogenase de *Haemonchus contortus* juntamente com promotor T7. A amostra está representada antes (a) e após o processo de purificação (b). Fonte: O autor (2018).

6.5 Eletrofrorese em gel de agarose com amostras de RNA dupla fita (RNA de interferência) do gene que codifica a proteína Gliceraldeído-3-fosfato desidrogenase de *Haemonchus contortus*.



Eletroforese em gel de agarose 1%. (a) – poço 5: Marcador de peso molecular 100 pb DNA ladder: 3µL (100 ng/µL); poço 1: 5 µL de amostra; poço 2: 5 µL de amostra diluída 1:10 (água ultrapura); poço 3: 5 µL de amostra diluída 1:100 (água ultrapura). (b) - poço 1: Marcador de peso molecular 100 pb DNA ladder: 3µL (100 ng/µL); poço 5: 5 µL de amostra; poço 6: 5 µL de amostra diluída 1:10 (água ultrapura); poço 7: 5 µL de amostra diluída 1:100 (água ultrapura). As amostras consistiram em RNA dupla fita (RNA de interferência) do gene que codifica a proteína Gliceraldeído-3-fosfato desidrogenase de *Haemonchus contortus*. A síntese do RNA de interferência foi realizada por meio de transcrição *in vitro*. Fonte: O autor (2018).

VITA

Andréia Buzatti é Médica Veterinária graduada pela Universidade Federal de Santa Maria (UFSM/2010). Mestre em Ciências Veterinárias, pela Universidade Federal do Paraná (2014). Em 2014 ingressou no doutorado, no Programa de Pós-Graduação em Ciências Veterinárias da UFPR e desenvolveu os seguintes projetos: - Biotecnologia aplicada ao controle parasitário em ruminates; - Avaliação de genes funcionais do parasita *Haemonchus contortus* usando RNAi para desenvolvimento de novas terapias. Durante esse período publicou (autoria e co-autoria) 16 artigos científicos em periódicos e teve 1 artigos aceitos para publicação. Publicou 1 artigo técnico e 1 capítulo de livro (co-autoria), participou de 1 banca de concurso público, ministrou palestra e atuou como Professora Visitante na Universidade do Oeste de Santa Catarina. Além disso, participou de palestras e congressos e publicou resumos em anais de eventos.

1. Artigos científicos publicados em periódicos:

1.1 SPRENGER, L.K.; YOSHITANI, U.Y.; BUZATTI, A.; MOLENTO, M.B. Occurrence of gastrointestinal parasites in wild animals in State of Paraná, Brazil. ANAIS DA ACADEMIA BRASILEIRA DE CIÊNCIAS. An. Ahead of print Epub Jan 11, 2018.

1.2. BUZATTI, A.; DE PAULA SANTOS, C.; VIEIRA, D. L.; MOLENTO, M. B. (2017). Nematoides gastrintestinais de equinos com ênfase no biocontrole por *Duddingtonia flagrans*. Archives of Veterinary Science, v. 22, n.4, 2017.

1.3. BUZATTI, A.; SANTOS, C.P.; FERNANDES, M.A.M.; YOSHITANI, U.Y.; SPRENGER, L.K.; MOLENTO, M.B. Control of free living gastrointestinal nematodes of horses using *Duddingtonia flagrans*. ARQUIVO BRASILEIRO DE MEDICINA VETERINARIA E ZOOTECNIA, v. 69, p. 364-370, 2017.

1.4. DIAS DE CASTRO, L.L.; ABRAHÃO, C.L.H.; BUZATTI, A.; MOLENTO, M.B.; BASTIANETTO, E. RODRIGUES, D.S.; LOPES, L.B.; SILVA, M.X.; DE FREITAS, M. G.; CONDE, M. H.; BORGES, F.A. Comparison of McMaster and Mini-FLOTAC fecal

egg counting techniques in cattle and horses. VETERINARY PARASITALOGY: Regional Studies and Reports, v. 10, p. 132-135, 2017.

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1.9. GARCIA, Y.C.; SANCHEZ, J.C.; DIAZ, A.F.; ARENAL, A.C.; BUZATTI, A.; MOLENTO, M. B. Albendazol and Triclabendazol as Treatment for Fasciolosis in Sheep. REVISTA DE PRODUCCIÓN ANIMAL, v. 28, p. 29-32, 2016.

1.10. SANTOS, F.C.; BUZATTI, A.; SCHEUERMANN, L.M.; VOGEL, F.S.F. Head position and administration of levamisol disofenol in sheep. AGRARIAN, v. 8, p. 111-114, 2015.

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2. Artigo científico aceito para publicação

2.1. BUZATTI, A.; FERNANDEZ, A.D.; ARENAL, A.; PEREIRA, E.; MONTEIRO, A.L; MOLENTO, M.B. Sheep polyclonal antibody to map *Haemonchus contortus* mimotopes using phage display library. Aceito para publicação na Revista Brasileira de Parasitalogia Veterinária.

3. Capítulos de livros publicados

3.1. MOLENTO, M.B.; FORTES, F.S.; BUZATTI, A.; KLOSTER, F.S.; SPRENGER, L.K.; SOARES, L.D. Tratamento parcial seletivo do *Rhipicephalus (B.) microplus* e variações raciais de resistência em vacas de corte no Rio Grande do Sul, Brasil: uma experiência em larga escala. In: Cecília José verissímo. (Org.). Resistência e Controle do Carrapato do boi. 1ed. Nova Odessa: 2015, v.1, p. 57-76.

4. Textos em jornais de notícias/revistas

4.1. BUZATTI, A.; FERNANDES, M. A. M.; MOLENTO, M. B. Verminoses - Por que os medicamentos às vezes não funcionam? Revista Leite Integral, Piracicaba - SP, p. 18 - 22, 01 out. 2014.

5. Concurso público

5.1. BUZATTI, A. Banca de Concurso Público para Provimento de Cargo de Professor de Anatomia Veterinária do Instituto Federal de Educação, Ciência e Tecnologia Baiano. 2016. Instituto Federal de Educação, Ciência e Tecnologia Baiano.

6. Docência

6.1. Professora visitante - Docência do componente curricular Parasitalogia Clínica (30 horas) no curso de Pós-Graduação - Especialização em Análises Clínicas promovido pela Universidade do Oeste de Santa Catarina - UNOESC, Campus de Videira (2017).

6.2. Controle Parasitário em Ovinos. Palestra proferida no Dia de Campo e Leilão de Ovinos em Pedro Canário, ES. Evento promovido pela Associação dos Criadores de Ovinos e Caprinos do Espirito Santo (2014).

7. Participação em eventos durante o doutorado

7.1 Curso: Nanotecnologia - Aplicação Prática em Saúde Animal. (Carga horária: 40h). Setor de Ciências Agrárias, PPG em Ciências Veterinárias, UFPR. 19 a 23 de outubro de 2015.

7.2 Curso: A evolução da vacina em um mundo vacinado (Carga horária: 40h). Setor de Ciências Biológicas, UFPR.

7.3 Curso: “Análise de dados usando o R em ciências agrárias”. Ministrado por André L. Giron, consultor técnico Progene. Universidade Federal do Paraná. 15 a 17 de setembro de 2014.

7.4 Palestra: “Desvendando o PCR em Tempo Real: princípios da técnica e conceitos sobre análise de resultados”. Ministrada por Dr. Renan Alves, analista de marketing da Promega Biotecnologia do Brasil. Setor de Ciências da Saúde, PPG em Ciências Farmacêuticas, UFPR.

27 de maio de 2014.

7.5 Palestra: “RNAi: entregando silenciamento à resistência antihelmíntica em *Haemonchus contortus*”, promovida pelo Laboratório de Produção e Pesquisa em Ovinos e Caprinos da Universidade Federal do Paraná (LAPOC-UFPR) realizado no dia 11 de setembro de 2017, com duração de 3 horas.