NELSON MENDES MARRA

INFECÇÃO EXPERIMENTAL DE RATOS (*Rattus norvegicus*) DA LINHAGEM LEWIS POR *Strongyloides venezuelensis*: DINÂMICA DA INFECÇÃO, USO DE PCR PARA DETECÇÃO DO PARASITA E CARACTERIZAÇÃO DA RESPOSTA IMUNE HUMORAL

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração Genética do Instituto de Biociências, Universidade Estadual Paulista – UNESP, para obtencão do título de Doutor.

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RESUMO

No presente estudo foram analisadas a dinâmica da infecção primária por *Strongyloides venezuelensis* em ratos Lewis, a influência do sexo do hospedeiro e sua resposta imune. Também foi comparada a sensibilidade da PCR com as técnicas histológica e parasitológica na caracterização desta infecção utilizando amostras de tecidos e fezes, respectivamente. No primeiro artigo, o número de ovos por grama de fezes (OPG) foi determinado pela técnica de McMaster modificada e o DNA foi extraído para análise por PCR. Comparou-se a sensibilidade de ambos os métodos para o diagnóstico do parasita em fezes de ratos inoculados com 40, 400 e 4000 larvas infectantes (L3) e essas infecções foram consideradas leve, moderada e grave, respectivamente. Na PCR dois pares de *primers* foram empregados, um foi desenhado a partir da seqüência parcial de rDNA de *S. venezuelensis* e o outro, amplifica o rDNA de diversas espécies deste gênero. Nas amostras oriundas dos animais com infecção leve o *primer* específico não detectou DNA, já o *primer* gênero apresentou maior sensibilidade que a quantificação de OPG. No segundo artigo foi analisada a influência do sexo dos hospedeiros na suscetibilidade às infecções leve, moderada e pesada. Machos com infecção moderada e pesada apresentaram maior média de OPG e de número de parasitas recuperados do intestino delgado em comparação com as fêmeas, porém esse fenômeno não foi observado nas infecções leves. No terceiro artigo, os animais foram inoculados com 4000 L3 para a avaliação da dinâmica da infecção e caracterização da resposta imune durante as fases aguda e de recuperação. A dinâmica da infecção foi monitorada, diariamente, por OPG durante 32 dias. A infecção apresentou período pré-patente de seis dias e picos de eliminação de ovos no oitavo e no 11º dias. Ambos os anticorpos específicos IgG1 e IgG2b apresentaram nível elevado na fase aguda e houve um aumento significativo na concentração de IgG1 na fase de recuperação. IgE e IL-10 também apresentaram alta concentração na fase de recuperação, o que caracteriza o padrão típico de resposta Th2. No quarto artigo, a sensibilidade da PCR, com a utilização de dois pares de *primers,* foi comparada com a quantificação de OPG durante 32 dias de infecção, a partir das mesmas amostras apresentadas no terceiro artigo. Ambas as técnicas detectaram a presença do helminto a partir do 6° dia. No entanto, a PCR com o *primer* gênero detectou a presença de DNA em todas as amostras, enquanto a contagem de ovos variou entre 0 e 100 OPG em sete das 32 amostras, a partir do 23º dia. A PCR com o *primer* específico não detectou DNA do parasita a partir do 20º dia de infecção. Adicionalmente, análise histológica e PCR foram avaliadas, de acordo com sua sensibilidade, para definir a rota migratória do parasita. Dois grupos diferentes de ratos foram

inoculados com 4000 larvas L3. O segundo grupo de animais foi sacrificado em diferentes tempos após a infecção: 30 minutos, 24h, 48h, 72h e 120h. Amostras de tecidos foram coletadas e analisadas com o uso do *primer* gênero. Tanto a PCR quanto a análise histológica detectaram a presença do parasita na pele e na camada muscular 30 minutos após a infecção, no pulmão entre 48 e 72 horas de infecção, bem como no intestino delgado 120 horas após a infecção, mas não houve diferença estatística significativa na sensibilidade entre as duas técnicas.

Palavras-chave: *Strongyloides venezuelensis*, rato Lewis, PCR, infecção, resposta Th2.

ABSTRACT

In the present study the dynamics of *Strongyloides venezuelensis* primary infection in Lewis rats, the host sex influence and its immune response were analyzed. We also compared the sensitivity between PCR, parasitological and histological techniques to characterize this infection using feces and tissue samples. In the first paper the number of eggs per gram of feces (EPG) using a McMaster modified technique was enumerated and DNA was extracted to do PCR analysis. Sensitivity of both methods to diagnosis the parasite in feces of rats inoculated with 40, 400 or 4000 infective larvae (L3) was compared. These infections were considered light, moderate and heavy, respectively. Two PCR primer pairs were employed, a specific one was designed from a *S. venezuelensis* rDNA partial sequence and the other one amplifies several species within this genus. In light infection, the specific primer did not detect DNA in any sample, in the other hand the genus primer presented higher sensitivity than EPG. The host sex influence in susceptibility to light, moderate and heavy infection was evaluated in the second experiment. The FEC means and the mean parasite infection intensity were higher in males than in females in animals with moderate and heavy infection. But this phenomenon was not observed in light infection. In the third paper, animals were inoculated with 4000 L3 to determine the kinetics infection and to characterize the immune response during acute and recovery phases. The kinetics of infection was daily measured by FEC during 32 days after infection. Parasite eggs were detected in the feces for the first time at day 6 post-infection, but the maximal egg number was observed at days 8 and 11 post-infection. Both IgG1 and IgG2b specific antibodies were elevated at the acute phase and there was a significant increase of IgG1 concentration in the recovery one. IgE and IL-10 also presented a high concentration in the recovery phase which denotes a typical Th2 response pattern. In the forth paper, it was compared PCR sensitivity with two primer pairs and EPG enumeration during 32 days of infection with the same samples presented in the third paper. Both methods detected parasite presence at day 6. PCR with genus primer detected the parasite DNA in all samples and fecal egg count showed 0 to 100 EPG in seven out of 32 samples starting from day 23. PCR with specific primer did not detect the parasite DNA from day 20. Additionally, PCR and histological examinations were evaluated, according to their sensitivity, to define the parasite migration route. The other group of animals was sacrificed at different periods after infection: 30 minutes, 24h, 48h, 72h and 120h. Tissue samples were collected and analyzed by PCR only with the genus primer. PCR and histopathological analysis showed larvae presence below the muscular fibers of skin and at the muscular layer 30 minutes post-

infection. The pulmonar stage occurred between 48 and 72h and larvae appearance was detected after 120h in small intestine vilosities. There was no statistical difference between these two methodologies.

keywords: *Strongyloides venezuelensis*, Lewis rats, PCR, infection, Th2 response.

Lista de abreviaturas, unidades e símbolos

CPH: complexo principal de histocompatibilidade

DNA: ácido desoxirribonucléico

dNTP: Trifosfatos de desoxirribonucleosídeos

ELISA: ensaio imunoenzimático

EPG: eggs per gram of feces

FcεRI: receptor de alta afinidade para Fc de IgE

FEC: fecal egg counts

g: grama

GLM: general linear model

IFN-γ: interferon-gama

IgE, G1, G2b: imunoglobulinas E, G1, G2b

IL-3, 2, 4, 5, 9, 10, 13, 18: interleucinas 3, 2, 4, 5, 9, 10, 13, 18

KCl: cloreto de potássio

L: litro

L3: larva de terceiro estádio; larva infectante

L4: larva de quarto estádio

L5: larva de quinto estádio

log: logaritmo

M: molar

mg: miligrama

$MgCl_2$: cloreto de magnésio

ml: mililitro

mM: milimolar

ng: nanograma

^{o}C: graus Celsius

OPG: ovos por grama de fezes

pb: pares de base

PBS: salina tamponada com fosfato

PCR: reação em cadeia de polimerização

pg: picograma

ph: potencial hidrogeniônico

rDNA: DNA ribossômico

RPMI: Roswell Park Memorial Institute

spp. : espécies

TAE: tampão tris-acetato

$TCD4^{+}$: célula T cd4

Th1: célula T helper 1

Th2: célula T helper 2

U: unidade

UV: ultravioleta

β: beta

% : porcentagem

®: marca registrada

µg: micrograma

µl: microlitro

cm: centímetro

SUMÁRIO

1. INTRODUÇÃO

1. INTRODUÇÃO

1.1 Gênero *Strongyloides* e a Estrongiloidíase

Os nematódeos do gênero *Strongyloides* pertencem à família Strongyloididae (REY, 2001). Este gênero compreende mais de 52 espécies de nematódeos parasitas de vertebrados (COSTA-CRUZ, 2005). Diversas espécies são consideradas importantes parasitas gastrintestinais de humanos e animais domésticos (DAWKINS, 1989). Em mamíferos são descritas as espécies *S. chapini*, *S. fereirai*, *S. fuelleborni*, *S. myopotami*, *S. papillosus*, *S. ransomi*, *S. stercoralis*, *S. venezuelensis*, *S. ratti* e *S. westeri* (VICENTE et al., 1997).

As espécies que parasitam roedores são freqüentemente utilizadas como modelos experimentais para o estudo da estrongiloidíase (SATO e TOMA, 1990). *Strongyloides venezuelensis* é um parasita de roedores, naturalmente encontrado em ratos (*Rattus norvegicus*) (MARUYAMA et al., 2006). Este parasita tem sido utilizado como modelo experimental para estudos de biologia (NAKAI e AMARANTE, 2001; OLIVEIRA-SEQUEIRA e AMARANTE, 2001; MATSUDA et al., 2003; MARUYAMA et al., 2006), imunologia (KIMURA et al., 2006; NEGRÃO-CORRÊA et al., 2006), mecanismos de expulsão de parasitas (BAEK et al., 1999; EL-MALKY et al., 2003; NEGRÃO-CORRÊA et al., 2006), características bioquímicas (MARUYAMA e NAWA, 1997; MARUYAMA et al. 2006) e atividade anti-helmíntica (SATOU et al., 2001; SATOU et al., 2002).

As características clínicas da estrongiloidíase estão ligadas a diversos fatores, como grau de infecção parasitária, estado nutricional do hospedeiro, ausência de outras parasitoses gastrintestinais, migração das larvas no ciclo pulmonar, permanência e multiplicação da fêmea partenogenética na mucosa intestinal e nos locais ectópicos, e estado imunitário do hospedeiro (MACHADO, 2003). Esta enfermidade é uma das mais importantes infecções helmínticas que afetam a espécie humana e é causada por *S. stercoralis* (SIDDIQUI e BERK, 2001). As causas disso são sobretudo, a capacidade de auto-infecção por parte do agente causal, a resistência ocasional à erradicação terapêutica, a dificuldade para eliminar as formas larvárias e o risco de hiperinfecção em indivíduos imunodeprimidos (AMATO NETO et al., 1997). É uma parasitose pouco estudada que infecta por volta de 100 milhões de pessoas em todo o mundo, sendo geralmente encontrada em regiões tropicais e subtropicais (MATSUDA et al., 2003). Nos países desenvolvidos, a infecção acomete principalmente agricultores, hortigranjeiros, trabalhadores rurais, imigrantes e os viajantes que visitaram áreas endêmicas enquanto, nos países em desenvolvimento, que coincidem com essas áreas, a doença atinge

principalmente crianças, pela freqüente permanência em solos contaminados. No Brasil, a estrongiloidíase é uma doença de grande importância em saúde pública cujas taxas de infecção variam de acordo com a região estudada e métodos de escolha para o diagnóstico parasitológico (COSTA-CRUZ, 2005). A infecção causada por *S. stercoralis* pode se apresentar sob três formas distintas: aguda, crônica e disseminada (LY et al., 2003). Os pacientes sintomáticos apresentam alteração pulmonar e gastrintestinal, sendo que a maioria destes descobre que tem a estrongiloidíase após o exame laboratorial revelar a presença de eosinofilia (VADLAMUDI et al., 2006).

1.2 Período pré-patente, rota migratória e ciclo biológico de *Strongyloides venezuelensis*

Estudos mostram que o período pré-patente na infecção por *S. venezuelensis* em roedores (camundongos e ratos) varia entre o 5º e o 8º dia após a infecção com o pico de eliminação de ovos nas fezes ocorrendo entre o 6º e o 18º dias (TAIRA et al., 1995; NAKAI e AMARANTE, 2001; OLIVEIRA-SEQUEIRA e AMARANTE, 2001). As diferenças observadas nestes estudos podem ser atribuídas a fatores relacionados ao hospedeiro (espécie, linhagem, idade, sexo e resistência) e ao parasita (linhagem, dose do inóculo e cultura de larvas infectantes) (SOLOMON e HALEY, 1966; CARTER e WILSON, 1989; SATO e TOMA, 1990; KHAN et al., 1993).

Na infecção por *S. venezuelensis*, larvas infectantes de terceiro estádio (L3) penetram através da pele e migram para o pulmão onde ocorre muda para L4. Ao atingirem o intestino delgado se transformam em L5 e em fêmeas partenogenéticas, que apresentam de 8 a 12 ovos no útero (LITTLE, 1966; CARTER e WILSON, 1989).

Durante o processo de migração, esses parasitas secretam, por via oral, substâncias adesivas de origem glicoprotéica que auxiliam na invasão e no estabelecimento em tecidos do hospedeiro (MARUYAMA e NAWA, 1997). A composição dessas substâncias varia de acordo com o estádio de desenvolvimento da larva no hospedeiro (MARUYAMA et al., 2006).

Estudos sobre o ciclo biológico de *S. venezuelensis* em hospedeiros roedores relatam diferenças no tempo de migração e distribuição das larvas. Takamure (1995) inoculou larvas L3 em ratos da linhagem Wistar e em camundongos da linhagem ddy (*Mus musculus*) por via subcutânea. Em ratos, a migração até os pulmões levou 45 horas e em camundongos, 42. As larvas foram verificadas no intestino delgado de ambos, 60 horas após a infecção. Portanto, foi demonstrado que *S. venezuelensis* segue a mesma rota migratória através da via pulmonar

(pele, músculo, pulmão, traquéia e intestino delgado) nessas espécies. Matsuda et al. (2003) inocularam oralmente 1000 L3 em ratos da linhagem Sprague-Dawley e recuperaram larvas migratórias no sangue após 20 minutos e no fígado e pulmão após 45 minutos de inoculação. Os autores também recuperaram parasitas adultos do intestino delgado 120 horas depois da inoculação. Larvas migratórias foram detectadas em cortes histológicos realizados a partir de amostras de pulmão, fígado e intestino delgado. Focos de lesões necróticas e de hiperemia foram observados no fígado e no pulmão, juntamente com infiltrados de eosinófilos que também foram vistos no estômago. Sato e Toma (1990) recuperaram grande número de larvas da pele e músculo esquelético nas primeiras 42 horas, no pulmão e na traquéia entre 48 e 60 horas. Durante o curso da infecção poucas larvas foram encontradas na corrente sanguínea e nenhuma no cérebro ou em outros órgãos de camundongos.

Assim como *S. venezuelensis, S. ratti* também é parasita natural do rato e é utilizado como modelo experimental da estrongiloidíase (GEMMILL e WEST, 1998). Todavia, no ciclo biológico de *S. ratti*, L3 infectam o hospedeiro através da pele e migram para a cabeça através da região naso-frontal (TINDALL e WILSON, 1988; KOGA et al. 1998), sendo posteriormente deglutidas. Durante este processo, sofrem muda (L4) e passam para o estágio parasitário. Assim como em *S. venezuelensis* as fêmeas de *S. ratti* se fixam na parede do intestino delgado do hospedeiro, se reproduzem por partenogênese e realizam a postura de ovos que são eliminados juntamente com as fezes (WILKES et al., 2007).

A dinâmica da infecção por *S. venezuelensis* é conhecida em diversas linhagens de camundongos e ratos, mas não na cepa isogênica de ratos Lewis.

1.3 Utilização de *Rattus norvegicus* como modelo experimental

O rato de laboratório, *R. norvegicus*, é um roedor da família Muridae (HARKNESS, 1993). Os ratos são amplamente utilizados e particularmente vantajosos na pesquisa biomédica que abrange as áreas de toxicologia, gerontologia, cardiologia, endocrinologia, odontologia, imunologia, reprodução, neurociência e parasitologia (ANDERSEN, 2004). Os motivos para a escolha de ratos como modelo experimental são a facilidade de cuidado e de manejo, o tamanho e o custo reduzido, a alta capacidade reprodutiva, gerações de curta duração, fácil adaptação a ambientes variados, sociabilidade e a existência de linhagens geneticamente definidas (ANDERSEN, 2004), bem como, a possibilidade de efetuar infecções e acompanhá-las, o que possibilita a realização de análises variadas. Amato Neto et

al. (1997) postularam ainda que os resultados obtidos podem ser comparados com os aspectos da doença na espécie humana e em outras espécies animais.

1.4 Influência do sexo do hospedeiro em infecções helmínticas

A prevalência (proporção de indivíduos infectados) e a gravidade das infecções causadas por protozoários, nematódeos, trematódeos, cestódeos e artrópodes são mais elevadas em machos do que nas fêmeas (KLEIN, 2004). A influência do sexo em infecções helmínticas parece ser largamente hormonal, provavelmente em função do efeito dos hormônios sexuais na resposta imunológica do hospedeiro (ZUK e MCKEAN, 1996). Estudos em roedores confirmam que a influência do sexo pode ser mediada por interações entre os sistemas imunológico e endócrino (MORALES-MONTOR et al., 2004). A presença de receptores de hormônios sexuais em células do sistema imunológico, incluindo linfócitos, macrófagos, granulócitos e mastócitos, indica uma correlação direta entre os sistemas endócrino e imune. O sistema imunológico é influenciado por hormônios sexuais como testosterona, 17β-estradiol e progesterona (KLEIN, 2004).

De acordo com Wunderlich et al. (2002), a testosterona possui propriedades imunossupressoras. A resposta humoral e celular é maior nas fêmeas do que em machos (GROSSMAN, 1989). Nakanishi (1987) estudou a resposta celular de camundongos machos e fêmeas da linhagem BALB/c infectados por *Brugia pahangi* e verificou que as fêmeas apresentaram uma resposta mediada por macrófagos e eosinófilos mais intensa e se mostraram mais resistentes do que camundongos machos. Esse autor postulou que a influência do sexo na suscetibilidade ao parasita e na resposta celular foi devida ao hormônio sexual do macho, a testosterona. Nakanishi et al. (1989) também observaram a diminuição da resistência e a resposta de linfócitos, macrófagos e eosinófilos na cavidade peritoneal de camundongos machos C57BL/6 infectados por *B. pahangi*.

Estudos utilizando animais castrados comprovam a influência da testosterona na suscetibilidade a parasitas. Kamis et al. (1992) ao estudarem ratos castrados, não tratados com testosterona e infectados com o nematódeo *Angiostrongylus malaysiensis*, verificaram uma redução no número de parasitas recuperados das artérias pulmonar e cerebral e aumento do número de leucócitos circulantes em relação aos ratos castrados que receberam doses diárias de testosterona. Rivero et al. (2002) também observaram que ratos Wistar castrados foram menos suscetíveis à infecção por *S. venezuelensis* em relação aos não castrados. Este resultado foi atribuído à redução de testosterona plasmática nos animais castrados, pois ratas Wistar

tratadas com testosterona foram mais suscetíveis à infecção por *S. venezuelensis*. Além disso, os autores também verificaram que o número de parasitas recuperados no pulmão foi maior nos machos mais velhos, nos quais o nível de testosterona se mostrou mais elevado e menor nas fêmeas mais velhas, que apresentaram maiores níveis de estrogênio.

Estudos sobre a influência do sexo em infecções helmínticas têm sido realizados também em animais de produção. Ao infectarem cordeiros machos e fêmeas com 5000 L3 de *Haemonchus contortus* por via oral, Gauly et al. (2006) observaram que a contagem de ovos por grama de fezes (OPG) e a carga parasitária foram mais elevadas nos machos que nas fêmeas. Os autores também concluíram que as fêmeas foram mais resistentes à infecção experimental por *H. contortus*.

1.5 Resposta imunológica aos helmintos

A imunidade contra os nematódeos gastrintestinais se manifesta pela expulsão dos parasitas adultos, redução do tamanho do verme, diminuição da fecundidade da fêmea parasita, falha no estabelecimento bem como impedimento no desenvolvimento da larva no hospedeiro (ONAH e NAWA, 2000) e proteção contra re-infecção (DAWKINS, 1989).

As infecções helmínticas são caracterizadas pela sua capacidade de indução de uma resposta de padrão Th2 que geralmente resulta em eosinofilia, aumento no número de mastócitos na mucosa e aumento na produção de anticorpos não fixadores de complemento. As células Th2 produzem IL-4, IL-5, IL-9, IL-10 e IL-13, resultando na produção dos isotipos IgG1 e IgE (LAWRENCE, 2003). Os mastócitos são células efetoras importantes na infecção por nematódeos, embora sua necessidade absoluta na expulsão dos parasitas pareça também variar entre as espécies de nematódeos. O aumento do número de mastócitos é sempre observado em tecidos afetados durante a infecção causada por helmintos e esse aumento é dependente de citocinas tipo Th2, que são primariamente derivadas de células T $CD4^+$ (ANTHONY et al., 2007). Os mastócitos possuem muitos grânulos contendo histamina, heparina e proteases e podem secretar citocinas como IL-4 e IL-5, bem como leucotrienos e quimiocinas. A ativação clássica resultante da degranulação de mastócitos envolve a ligação da imunoglobulina E e a ligação cruzada do receptor FcεRI. A contribuição da IgE como mecanismo de proteção contra nematódeos gastrintestinais que penetram na mucosa do duodeno foi demonstrada em camundongos infectados por *S. venezuelensis* (NEGRÃO-CORRÊA, 2001). Silveira et al. (2002) também verificaram um aumento na concentração de

IgE em lavado broncoalveolar no processo inflamatório observado em pulmões de ratos infectados por *S. venezuelensis*.

Os eosinófilos são as células mais comuns no local da infecção por nematódeos. Eles contêm grânulos com proteínas catiônicas e podem liberar citocinas pró-inflamatórias, quimiocinas e mediadores lipídicos constituindo células efetoras potentes (DE VEER et al., 2007). A movimentação de eosinófilos dentro de sítios inflamatórios do trato gastrintestinal depende de ações coordenadas de algumas citocinas (IL-4, IL-5 e IL-13), moléculas de adesão e quimiocinas (LÖSCHER e SAATHOFF, 2008). A eosinofilia pode ser uma característica importante no diagnóstico de infecções helmínticas. No entanto, a incidência e a extensão da infecção relacionada com a eosinofilia não depende somente do tipo, intensidade e estágio da infecção; outros fatores como a variação nas respostas imunológicas inata e adaptativa, aspectos epidemiológicos e idade da primeira exposição, também são fatores importantes (LÖSCHER e SAATHOFF, 2008).

Os neutrófilos também são reconhecidos como componentes importantes da resposta Th2 durante a infecção causada por helmintos. Após a infecção os neutrófilos são rapidamente recrutados para os sítios de invasão de helmintos. Essas células trabalham em coordenação com outras populações de células, incluindo eosinófilos e macrófagos (ANTHONY et al., 2007).

Apesar desses padrões gerais comuns, a resposta imunológica difere consideravelmente entre as diferentes espécies de helmintos (GAUSE et al., 2003). De acordo com De Veer et al. (2007) as diferentes famílias de parasitas helmintos possuem padrões específicos de estruturas que influenciam sua interação com o hospedeiro.

A contribuição de eosinófilos, mastócitos, células Th2 e citocinas como IL-3 (interleucina 3), IL-4 (interleucina 4), IL-5 (interleucina 5) e IL-10 (interleucina 10) como mediadores da imunidade contra *S. venezuelensis* tem sido demonstrada em várias publicações (MARUYAMA et al., 2000; ONAH et al., 2000; SILVEIRA et al., 2002; EL-MALKY et al., 2003; NEGRÃO-CORRÊA, et al., 2004). Mais recentemente, Sasaki et al. (2005), avaliando camundongos C57Bl/6 STAT6(-/-) pré-tratados com IL-18 (interleucina 18) e IL-2 (interleucina 2), observaram um aumento no número de mastócitos intestinais e uma rápida expulsão de vermes adultos de *S. venezuelensis*. Por essa razão esses autores sugeriram que a ativação de mastócitos da mucosa intestinal depende da IL-18 para expulsão de parasitas.

Fatores relacionados ao hospedeiro como idade, sexo e base genética influenciam diretamente a carga parasitária (RIVERO et al., 2002). Estudos realizados com nematódeos

intestinais utilizando linhagens isogênicas de roedores demonstram a influência de fatores genéticos associados à resistência e a suscetibilidade à infecção (BEHNKE et al., 2000). O tipo de reposta imunológica apresentada pelo hospedeiro (rápida e aguda ou fraca e lenta) é regulada por diversos genes. A diferença observada entre os hospedeiros também pode ser explicada pela variação de alelos associados com a resposta imune. Estudos demonstram a importância de genes pertencentes ao complexo principal de histocompatibilidade (CPH) e de genes não relacionados ao CPH no controle da imunidade (BEHNKE et al., 2003).

1.6 Diagnóstico da estrongiloidíase

Os métodos parasitológicos são descritos como qualitativo e quantitativo. Enquanto o método qualitativo fornece informações sobre as espécies de parasitas presentes, o quantitativo fornece indicação sobre os níveis de infecção (PERECKIENÉ et al., 2007).

O diagnóstico da estrongiloidíase humana é usualmente realizado com base na detecção de larvas nas fezes (SIDDIQUI e BERK, 2001). Larvas de *Strongyloides* já foram encontradas no escarro, líquido ascítico, líquido pleural, urina e cistos cutâneos (FERREIRA, 1991). Exames como endoscopia digestiva, biópsia intestinal (realizada no duodeno, jejuno e íleo), necropsia de órgãos e esfregaços citológicos também foram descritos como métodos de detecção de larvas de *S. stercoralis* (COSTA-CRUZ, 2005).

Observações das características morfológicas das larvas rabditóides e filarióides de *S. stercoralis* e a adoção de métodos parasitológicos específicos possibilitam o diagnóstico correto. Porém, a presença de larvas de ancilostomídeos e de nematódeos de vida livre do gênero *Rhabditis* pode gerar confusão no diagnóstico microscópico (BEAVER e JUNG, 1985 apud PEDRAZA, 2004).

Para recuperar larvas de amostras fecais são empregados os métodos de Baermann (BAERMANN, 1917) ou de Rugai (RUGAI et al., 1954). Estes métodos caracterizam-se pela simplicidade e rapidez de execução (REY, 2001). Apresentam como desvantagens a necessidade de fezes frescas e a possibilidade de contaminação do manipulador devido a motilidade das larvas (COSTA-CRUZ, 2005).

O diagnóstico parasitológico da estrongiloidíase causada por *S. venezuelensis* pode ser realizado através da contagem de ovos por gramas de fezes (OPG) e da detecção de larvas realizando migração nos tecidos até o estabelecimento dos adultos no intestino.

As técnicas de flutuação separam os ovos de detritos fecais utilizando uma variedade de soluções com densidades específicas que fazem os ovos flutuarem para a superfície da

suspensão. A técnica quantitativa mais utilizada é a de Gordon e Whitlock (1939) (PERECKIENÉ et al., 2007). A técnica de Gordon e Whitlock (1939) modificada é um método parasitológico que se fundamenta no encontro de ovos de nematódeos gastrintestinais em fezes e requer uma lâmina especial denominada de "Câmara de McMaster". A determinação das espécies de parasitas é realizada através da observação do estado de desenvolvimento celular e da forma dos ovos recém excretados (UENO e GUTIERREZ, 1983). Conhecendo-se o OPG, pode-se estimar o número de vermes adultos, bem como a eficácia dos anti-helmínticos após o tratamento dos animais (DE CARLI, 2001). Entretanto pode haver variações da técnica de McMaster de laboratório para laboratório. Elas estão relacionadas à solução de flutuação, à diluição da amostra, ao tempo de flutuação e à escolha da área da lâmina analisada e isto pode comprometer a padronização da técnica (CRINGOLI, et al., 2004). Existem ainda outras técnicas que consistem na estimativa da contagem de ovos nas fezes, como a de Kato-Katz (KATZ et al., 1972), utilizada em alguns trabalhos para monitoramento de infecções.

Entretanto, os métodos parasitológicos para o diagnóstico da estrongiloidíase demonstram baixa sensibilidade (MACHADO et al., 2003). Ruano et al. (2005) observaram que a sensibilidade do método parasitológico foi de 30%, e chegou a 50% quando foram efetuadas repetições dos exames. Isto se dá, pois na maioria dos casos de estrongiloidíase crônica a eliminação das larvas ocorre de modo intermitente (PEDRAZA, 2004).

Diversas metodologias são descritas para detectar a resposta imune humoral em indivíduos infectados por *S. stercoralis*. Os métodos mais utilizados são imunofluorescência indireta e ELISA (SIDDIQUI e BERK, 2001). Entretanto, os testes sorológicos possuem algumas limitações, como a obtenção de quantidades suficientes de antígenos para fracionamento e análise, bem como a reatividade cruzada, devida à similaridade antigênica entre estes parasitas, bactérias e protozoários (ROSSI et al., 1993). Infecções com filárias e *Ascaris* spp. podem induzir resultados falso-positivos (KEISER e NUTMAN, 2004).

S. ratti e *S. venezuelensis* mantidas em laboratório têm sido usadas, com êxito, como fontes de larvas filarióides para preparar extratos antigênicos que permitem detectar anticorpos séricos específicos em pacientes com estrongiloidíase (MACHADO et al., 2003; RODRIGUES et al., 2007).

A detecção de antígenos nas fezes por diversos ensaios de captura de antígenos tem sido bastante utilizada (BOWMAN, 2006). A técnica de coproantígeno é baseada na detecção de antígeno, obtido a partir de produtos excretados e secretados por parasitas nas fezes do hospedeiro através do ELISA. Nageswaran et al. (1994) verificaram antígenos específicos de

S. ratti em ratos da linhagem Sprague-Dawley experimentalmente infectados. No entanto, a técnica apresentou reatividade cruzada com os nematódeos *Necator americanus* e *Syphacia muris*.

É portanto imprescindível dispor de melhores métodos diagnósticos a serem associados aos métodos convencionais a fim de propiciar maior sensibilidade e especificidade.

1.7 Uso de OPG e PCR para detecção de parasitas

A diferenciação e o diagnóstico das infecções parasitárias têm sido realizados por diversos métodos, desde os mais simples como técnicas biológicas e morfológicas até os testes mais complexos como bioquímicos, imunológicos e moleculares. Os métodos moleculares além de aumentarem a sensibilidade e a especificidade das técnicas, reduzem a subjetividade inerente à interpretação de resultados morfológicos e biológicos (ZARLENGA e HIGGINS, 2001).

O diagnóstico parasitológico de *S. venezuelensis* pode ser realizado através da OPG. Alguns trabalhos demonstram sua utilização no monitoramento da infecção em roedores (NAKAI e AMARANTE, 2001; OLIVEIRA-SEQUEIRA e AMARANTE, 2001). Entretanto essa técnica se mostra pouco sensível quando a carga parasitária é leve.

A análise de ácidos nucléicos na detecção ou caracterização de parasitos tem apresentado resultados promissores. As técnicas apresentam-se reprodutíveis, sensíveis e de alta especificidade, pois as seqüências alvo no DNA são conservadas durante as distintas fases do ciclo desses parasitas (DE CARLI, 2001).

A PCR (Reação em Cadeia de Polimerização) apresenta-se como um método de extrema sensibilidade, permitindo a geração exponencial de cópias de seqüências específicas a partir de um DNA molde, por meio de uma síntese enzimática *in vitro* (DE CARLI, 2001), podendo desempenhar, dessa forma, um importante papel na epidemiologia, prevenção e tratamento de doenças parasitárias. Por ser um método direto não apresenta as ambigüidades características dos métodos considerados indiretos (ZARLENGA e HIGGINS, 2001).

Alguns autores já utilizaram a PCR para detecção de outras espécies de parasitas em amostras de fezes e obtiveram bons resultados. A alta sensibilidade para detectar patógenos e a velocidade com que essa técnica pode identificá-los no organismo com uma baixa carga parasitária são vantagens dessa técnica (WEISS, 1995). Wongratanacheewin et al. (2001) desenvolveram um método baseado na PCR para detecção de ovos de *Opisthorchis viverrini*

nas fezes de hamsters experimentalmente infectados. Os autores compararam as técnicas de OPG e PCR e verificaram que a PCR apresentou uma sensibilidade de 100% principalmente nos grupos com infecções leves. Sandoval et al. (2006) concluíram que a PCR com DNA extraído de fezes de camundongos da linhagem BALB/c foi mais sensível e mais precoce para detecção de *Schistosoma mansoni* que a técnica parasitológica de Kato-Katz nos dois tipos de inóculos testados (com 50 ou com 200 cercárias). Em amostras de animais inoculados com 200 cercárias, a PCR mostrou 100% de sensibilidade contra cerca de 60% da técnica de Kato-Katz. Pontes et al. (2003) também relataram maior sensibilidade da técnica de PCR em relação à de Kato-Katz para detecção de ovos da mesma espécie nas fezes de humanos residentes de região endemica para *S. mansoni*. A técnica molecular apresentou 97,4% de sensiblidade, enquanto a parasitológica mostrou 78,9%.

Estudos empregando a técnica de PCR para detecção de DNA de parasitas em tecidos e músculos também têm sido realizados. Toe et al. (1998) detectaram a presença de DNA do parasita *Onchocerca volvulus* através da PCR em raspados de pele de humanos e verificaram que essa técnica foi mais sensível e menos invasiva em relação ao exame de biópsia. Arriaga et al. (1995) detectaram a presença de larvas do parasita do gênero *Trichinella* em 80 amostras de músculo do diafragma de cavalos abatidos. Quatro dessas amostras foram caracterizadas como *Trichinella* sp. através de critérios morfológicos e como *Trichinella spiralis* através de PCR.

1.8 A relevância deste estudo

Tendo em vista que *S. venezuelensis* é um parasita de roedores, e que não existem descrições sobre a infecção experimental deste helminto em ratos da linhagem Lewis, a proposta deste trabalho foi investigar, através de diferentes metodologias, os aspectos biológicos e immunológicos envolvidos na relação parasita-hospedeiro.

Este estudo incluiu o acompanhamento da dinâmica da infecção para determinar não só a rota inicial percorrida pelo parasita até seu estabelecimento no intestino, como também a caracterização das fases aguda e de recuperação. Neste sentido, a PCR foi comparada com a metodologia clássica, a OPG, para detectar ovos do parasita nas fezes, bem como com a análise histológica visando detectar larvas de *S. venezuelensis* em tecidos do hospedeiro infectado. Além disto, foram empregadas técnicas imunológicas para caracterizar a resposta imune no decurso da infecção.

Acredita-se que esta pesquisa possa esclarecer controvérsias sobre a biologia deste parasita e, ao demonstrar a rota migratória de *S. venezuelensis,* possibilite a distinção entre a migração de *S. ratti* e *S. venezuelensis.*

2. REFERÊNCIAS BIBLIOGRÁFICAS

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AMATO NETO, V. et al. Tratamento de ratos, experimentalmente infectados por *Strongyloides venezuelensis*, através da ivermectina administrada por via oral. *Rev. Soc. Bras. Med. Trop.*, v.30, n.6, p.481-484, 1997.

ANDERSEN, M.L. *Princípios éticos e práticos do uso de animais de experimentação.* São Paulo: UNIFESP, 2004. p.81-119.

ANTHONY, R.M. et al. Protective immune mechanism in helminth infection. *Nature*, v.7, n.12, p.975-987, 2007.

ARRIAGA, C. et al. Detection of *Trichinella spiralis* muscle larvae in naturally infected horses. *J. Parasitol*., v.81, n.5, p.781-783, 1995.

BAEK, B.K. et al. Partial cross-resistance between *Strongyloides venezuelensis* and *Nippostrongylus brasiliensis* in rats. *Korean J. Parasitol*., v.37, n.2, p.101-107, 1999.

BAERMANN, G. Eine einfache methode zur auffindun von Ankylostomun (nematoden) larven in erdproben mededeel. *Mit H Geneesk Lab Weltvreden Feestbundel Batavia*, p.41-47, 1917.

BEAVER, P.C.; JUNG, R.C. *Animals agents and vectors of human disease*. 5.ed. Philadelphia: Lea & Febiger, 1985.p.155-159.

BEHNKE, J.M. et al. Mapping the genes for resistance to gastrointestinal nematodes. *Acta Parasitol.*, v.45, n.1, p.1-13, 2000.

BEHNKE, J.M. et al. Chasing the genes that control resistance to gastrointestinal nematodes. *J. Helmintol*., v.77, n.2, p.99-109, 2003.

BOWMAN, D.D. (Ed). *Parasitologia Veterinária de Georgis*. 8.ed. São Paulo: Manole, 2006. p.197-201.

*ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. *NBR 6023*: informação e documentação - Referências – Elaboração. Rio de Janeiro, 2002. 22p.
BIOSIS. **Serial sources for the BIOSIS preview database**. Philadelphia, 1996. 468p.

CARTER, K.C.; WILSON, P.A.G. The course of infection in rats given small primary doses of *Strongyloides ratti* and *Strongyloides venezuelensis*. *J. Helminthol*., v.63, n.2, p.107-114, 1989.

COSTA-CRUZ, J.M. *Strongyloides stercoralis*. In: NEVES, D.P. (Ed). *Parasitologia Humana*. 11.ed. Rio de Janeiro: Atheneu, 2005. p.275-284.

CRINGOLI, G. et al. The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium dendriticum* in sheep. *Vet. Parasitol*., v.123, n.2, p.121-131, 2004.

DAWKINS, H.J.S. *Strongyloides ratti* infections in rodents: value and limitations as a model for human strongyloidiasis. In: GROVED, D.I. (Eds.) Strongyloidiasis: a major roundworm infection of man. London: Taylor & Francis, 1989. p.287-332.

DE CARLI, G.A. *Seleção de método e técnicas de laboratório para o diagnóstico das parasitoses humanas*. São Paulo: Ed. Atheneu, 2001. 810p.

DE VEER, M.J. et al. The innate host defence against nematode parasites. *Parasite Immunol*., v.29, n.1, p.1-9, 2007.

EL-MALKY, M. et al. Intraepithelial infiltration of eosinophils and their contribution to the elimination of adult intestinal nematode, *Strongyloides venezuelensis* in mice. *Parasitol. Int*., v.52, n.1, p.71-79, 2003.

FERREIRA, M.S. Estrongiloidíase. In: VERONESI, R. (Ed). *Doenças infecciosas e parasitárias*. 8.ed. Rio de Janeiro: Guanabara, 1991. p.856-865.

GAULY, M. et al. Influence of sex on the resistance of sheep lambs to an experimental *Haemonchus contortus* infection. *Dtsch. Tierarztl. Wochenschr*., v.113, n.5, p.178-181, 2006.

GAUSE, W.C. et al. The immune response to parasitic helminthes: insights from murine models. *Trends Immunol*., v.24, n.5, p.269-277, 2003.

GEMMILL, A.W.; WEST, S.A. Influence of rat strain on larval production by the parasitic nematode *Strongyloides ratti*. *J.Parasitol*., v.84, n.6, p.1289-1291, 1998.

GORDON, H.M.; WHITLOCK, H.V. A new technique for couting nematode egs in sheep faeces. *J. Council Sci. Ind. Res*., v.12, n.1, p.50-52, 1939.

GROSMANN, C.J. Possible underlying mechanism of sexual dimorphism in the immune response, fact and hypothesis. *J. Steroid. Biochem*., v.34, n.6, p. 241-251, 1989.

HARKNESS, J.E. *Biologia e clínica de coelhos e roedores*. 3.ed. São Paulo: Roca, 1993. p.238.

KAMIS, A.B.; AHMAD, R.A.; BADRUL-MUNIR, M.Z. Worm burden and leukocyte response in *Angiostrongylus malaysiensis*-infected rats: the influence of testosterone. *Parasitol Res*., v.78, n.5, p.388-391, 1992.

KATZ, N.; CHAVES, A.; PELLEGRINO, J. A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. *Rev. Inst. Med. Trop. Sao Paulo*, v.14, n.6, p.397-400, 1972.

KEISER, P.B.; NUTMAN, T.B. *Strongyloides stercoralis* in the Immunocompromised Population. *Clin. Microbiol. Rev*., v.17, n.1, p.208-217, 2004.

KHAN, A.I. et al. Mucosal mast cells and the expulsive mechanism of mice against *Strongyloides venezuelensis*. *J. Helminthol*., v.23, n.5, p.551-555, 1993.

KIMURA, K. et al. Interleukin-3 and Kit/stem cell factor are required for normal eosinophil responses in mice infected with *Strongyloides venezuelensis*. *Lab. Invest*., v.86, n.10, p.987-996, 2006.

KLEIN, S.L. Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunol*., v.26, n.6-7, p.247-264, 2004.

KOGA, M. et al. *Strongyloides ratti*: histological study of the descending route of larvae from the head in experimentally infected rats. *Parasitol. Int*., v.47, p.219-224, 1998.

LAWRENCE, C.E. Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunol*., v.25, n.5, p.271-281, 2003.

LITTLE, M.D. Comparative morphology of six species of Strongyloides (Nematoda) and redefinition of the genus. *J. Parasitol*., v.52, n.1, p.69-84, 1966.

LÖSCHER, T.; SAATHOFF, E. Eosinophilia during intestinal infection. *Best Pract. Res. Clin. Gastroenterol*., v.22, n.3, p.511-536, 2008.

LY, M.N. et al. Cutaneous *Strongyloides stercoralis* infection: An unusual presentation. *J. Am. Acad. Dermatol*., v.49, n.2, p.157-160, 2003.

MACHADO, E.R. *Estudo dos fatores determinantes do grau de infectividade de linhagens de Strongyloides venezuelensis e análise de antígenos heterólogos no imunodiagnóstico da estraongiloidíase humana*. 2003. Tese (Doutorado). Instituto de Biologia, Universidade Estadual de Campinas, Campinas.

MACHADO E. R. et al. *Strongyloides venezuelensis* Alkaline Extract for the Diagnosis of Human Strongyloidiasisby Enzyme-linked Immunosorbent Assay. *Mem. Inst. Oswaldo Cruz,* v.98, n.6, p.849-851, 2003.

MARUYAMA, H.; NAWA, Y. *Strongyloides venezuelensis*: adhesion of adult worms to culture vessels by orally secreted mucosubstances. *Exp. Parasitol*., v.85, n.1, p.10-15, 1997.

MARUYAMA, H. et al. Protective mechanisms against the intestinal nematode *Strongyloides venezuelensis* in *Schistosoma japonicum*-infected mice. *Parasite Immunol*., v.22, n.6, p.279-286, 2000.

MARUYAMA, H. et al. Successive changes in tissue migration capacity of developing larvae of an intestinal nematode, *Strongyloides venezuelensis*. *Parasitology*, v.132, n.13, p.1-8, 2006.

MATSUDA, K. et al. Migration of *Strongyloides venezuelensis* in rats after oral inoculation of free-living infective larvae. *J. Vet. Med. Sci*., v.65, n.9, p.971-975, 2003.

MORALES-MONTOR, J. et al. Host gender in parasitic infections of mammals: an evaluation of the female host supremacy paradigm. *J. Parasitol*., v.90, n.3, p.531-546, 2004.

NAGESWARAN, C.; CRAIG, P.S.; DEVANEY, E. Cropoantigen detection in rats experimentally infected with *Strongyloides ratti*. *Parasitology*, v.108, n.3, p.335-342, 1994.

NAKAI, E.S.; AMARANTE, A.F.T. Experimental infection in mice *(Mus musculus)* and *(Rattus norvegicus)* by *Strongyloides venezuelensis*. *Braz. J. Vet. Parasitol*., v.10, n.1, p.1-6, 2001.

NAKANISHI, H. Difference in the susceptibility to *Brugia pahangi* infection between male and female BALB/c mice: Differences of effector cell responses between sex. *Trop. Med*., v.29, p.153-163, 1987.

NAKANISHI, H. et al. Effect of testosterone on the susceptibility of C57BL/6 mice to infection with *Brugia pahangi* with reference to inflammatory cell response. *J. Parasitol*., v.75, n.3, p.455-460, 1989.

NEGRÃO-CORRÊA, D. Importance of immunoglobulin E (IgE) in the protective mechanism against gastrointestinal nematode infection: looking at the intestinal mucosae. *Rev. Inst. Med. Trop. São Paulo*, v.43, n.5, p.291-299, 2001.

NEGRÃO-CORRÊA, D. et al. Platelet-activating factor receptor deficiency delays elimination of adult worms but reduces fecundity in *Strongyloides venezuelensis* infected mice. *Infect. Immunol*., v.72, n.2, p.1135-1142, 2004.

NEGRÃO-CORRÊA, D. et al. Expression of IL-4 receptor on boné marrow-derived cells is necessary for the timely elimination of *Strongyloides venezuelensis* in mice, but not for intestinal IL-4 production. *Int. J. Parasitol*., v.36, n.10-11, p.1185-1195, 2006.

OLIVEIRA-SEQUEIRA, T.C.G.; AMARANTE, A.F.T. Dynamics of *Strongyloides venezuelensis* infection and relationship between fecal egg counts and parasite burden in swiss mice. *Braz. J. Vet. Parasitol*., v.23, n.3, p.99-102, 2001.

ONAH, D.N.; NAWA, Y. Mucosal immunity against parasitic gastrointestinal nematodes. *Korean J. Parasitol*., v.38, n.4, p.209-236, 2000.

ONAH, D.N. et al. Mucosal defense against gastrointestinal nematodes: responses of mucosal mast cells and mouse mast cell protease 1 during primary *Strongyloides venezuelensis* infection in FcRγ-Knockout mice. *Infect. Immun*., v.68, n.9, p.4968-4971, 2000.

PEDRAZA, R.A.M. *Freqüência de infecção por Strongyloides stercoralis no Chile: detecção da parasitose mediante métodos parasitológicos e soroepidemiológicos*. 2004. Tese (Doutorado). Instituto de Biologia, Universidade Estadual de Campinas, Campinas.

PERECKIENÉ, A. et al. A comparison of modifications of the McMaster method for the enumeration of Ascaris suum eggs in pig faecal samples. *Vet. Parasitol*., v.149, n.1-2, p.111-116, 2007.

PONTES, L.A.; OLIVEIRA, M.C.; KATZ, N.; DIAS-NETO, E.; RABELLO, A. Comparison of a polymerase chain reaction and the Kato-Katz technique for diagnosing infection with *Schistosoma mansoni*. *Am. J. Trop. Med. Hyg*., v.68, n.6, p.652-656, 2003.

REY, L. *Parasitologia*. 3ª.ed. Rio de Janeiro: Guanabara Koogan, 2001. p.856.

RIVERO, J.C. et al. Androgen- and estrogen-dependent sex differences in host resistance to *Strongyloides venezuelensis* infection in Wistar rats. *J. Vet. Med. Sci*., v.64, n.6, p.457-461, 2002.

RODRIGUES, R.M. et al. IgG1, IgG4, and IgE antibody responses in human strongyloidiasis by ELISA using *Strongyloides ratti* saline extract as heterologous antigen. *Parasitol. Res*., v.101, n.5, p.1209-1214, 2007.

ROSSI, C.L. et al. Total serum IgE and parasite-specific IgG and IgA antibodies in human strongyloidiasis. *Rev. Inst. Med. Trop. Sao Paulo*., v.35, n.4, p.361-365, 1993.

RUANO, A.L. et al. Avances en el estudio sobre la estrongiloidosis. *Enf. Emerg*., v.7, n.2, p.102-109, 2005.

RUGAI, E.; MATTOS, T.; BRISOLA, A.P. Nova técnica para isolar larvas de nematódeos das fezes. Modificação do método de Baermann. *Rev. Inst. Adolfo Lutz*, v.14, n.1, p. 5-8, 1954.

SANDOVAL, N. et al. Schistosoma mansoni: A diagnostic approach to detect acute schistosomiasis infection in a murine model by PCR. *Exp. Parasitol*, v.114, n.2, p.84-88, 2006.

SASAKI, Y. et al. IL-18 with IL-2 protects against *Strongyloides venezuelensis* infection by activating mucosal mast cell-dependent type 2 innate immunity. *J. Exp. Med*., v.202, n.5, p.607-616, 2005.

SATO, Y.; TOMA, H. *Strongyloides venezuelensis* infection in mice. *Int. J. Parasitol*., v.20, n.1, p.57-62, 1990.

SATOU, T. et al. Nematocidal activities of thiabendazole and ivermectin against the larvae of *Strongyloides ratti* and *S. venezuelensis*. *Vet. Parasitol*., v.99, n.4, p.311-322, 2001.

SATOU, T. et al. Assay of nematocidal activity of isoquinoline alkaloids using third-stage larvae of *Strongyloides ratti* and *S. venezuelensis*. *Vet. Parasitol*., v.104, p.131-138, 2002.

SIDDIQUI, A.A.; BERK, S.L. Diagnosis of *Strongyloides stercoralis* infection. *Clin. Infect. Dis*., v.33, n.7, p.1040-1047, 2001.

SILVEIRA, M.R. et al. Infection with *Strongyloides venezuelensis* induces transient airway eosinophilic inflammation, an increase in immunoglobulin E, and hyperresponsiveness in rats. *Infect. Immunol*., v.70, n.11, p.6263-6272, 2002.

SOLOMON, M.S.; HALEY, A.J. Biology of the rat nematode *Nippostrongylus brasiliensis* (Travassos, 1914). Characteristics of *N. brasiliensis* after serial passage in the laboratory mouse. *J. Parasitol*., v.52, p. 237-241, 1966.

TAIRA, N. et al. Massive experimental infection with *Strongyloides venezuelensis* in rats and absence of sudden death. *J. Vet. Med. Sci*., v. 57, n.5, p.855-858, 1995.

TAKAMURE, A. Migration route of *Strongyloides venezuelensis* in rodents. *Int. J. Parasitol*., v.25, n.8, p.907-911, 1995.

TINDALL, N.R.; WILSON, P.A. criteria for a proof of migration routes of immature parasites inside hosts exemplified by studies of *Strongyloides ratti* in the rat. *Parasitology*, v.96, n.3, p.551-563, 1988.

TOÉ, L. et al. Detection of *Onchocerca volvulus* infection by O-150 polymerase chain reaction analysis of skin scratches. *J. Infect. Dis*., v.178, n.1, p.282-285, 1998.

UENO, H.; GUTIERRES, V.C. *Manual para diagnóstico das helmintoses de ruminantes*. Tóquio: Japan International Cooperation Agency, 1983. 176p.

VADLAMUDI, R.S.; CHI, D.S.; KRISHNASWAMY, G. Intestinal strongyloidiasis and hyperinfection syndrome. *Clin. Mol. Allergy*, v.4, n.8, p.1-13, 2006.

VICENTE, J.J. et al. Nematódeos do Brasil. Parte V: Nematódeos de mamíferos. *Rev. Bras. Zool*., v.14, n.1, p.1-452, 1997.

WEISS, J.B. DNA probes and PCR for diagnosis of parasitic infections. *Clin. Microbiol. Rev*., v.8, n.1, p.113-130, 1995.

WILKES, C.P.; BLEAY, C.; PATERSON, S.; VINEY, M.E. The immune response during a *Strongyloides ratti* infection of rats. *Parasite Immunol.*, v.29, n.29, p.339-346, 2007.

WONGRATANACHEEWIN, S. et al. Develoment of a PCR-based method for detection of *Opisthorchis viverrini* in experimentally infected hamsters. *Parasitology*, v.122, n.122, p.175-180, 2001.

WUNDERLICH, F. et al. Testosterone signaling in T cells and macrophages. *Steroids*, v. 67, n.11, p. 535-538, 2002.

ZARLENGA, D.S.; HIGGINS, J. PCR as a diagnostic and quantitative technique in veterinary parasitology. *Vet. Parasitol*., v.101, n.3-4, p.215-230, 2001.

ZUCK, M.; MCKEAN, K. A. Sex differences in parasite infections: patterns and processes. *Int. J. Parasitol*., v.26, n.10, p.1009-1024, 1996.

3. OBJETIVOS

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3.1 Objetivo Geral

Determinar a dinâmica da infecção primária por *Strongyloides venezuelensis* em ratos da linhagem Lewis.

3.2 Objetivos Específicos

3.2.1 Comparar a sensibilidade das técnicas de McMaster modificada e PCR em ratos Lewis infectados com diferentes inóculos.

3.2.2 Acompanhar a dinâmica da infecção em ratos Lewis para determinar as fases aguda e de recuperação da infecção e avaliar os padrões de resposta imune nestas duas fases.

3.2.3 Determinar a rota migratória de *S. venezuelensis* em ratos Lewis sacrificados em diferentes períodos através das técnicas de histologia e PCR.

4. RESULTADOS E DISCUSSÃO

4. RESULTADOS E DISCUSSÃO

Os resultados e a discussão dos dados obtidos encontram-se apresentados na forma de Capítulos que se referem aos trabalhos científicos. Visando facilitar a apresentação e compreensão de cada um, as figuras e tabelas foram numeradas em relação a cada trabalho, bem como as referências bibliográficas mencionadas.

Capítulo I: Improving strongiloidiasis diagnosis by PCR;

Capítulo II: Sex differences in host resistance to *Strongyloides venezuelensis* infection in Lewis rats;

Capítulo III: Recovery from *Strongyloides venezuelensis* infection in Lewis rats is associated with a strong Th2 response;

Capítulo IV: Migratory route of *Strongyloides venezuelensis* in Lewis rats: comparison of histological analysis and PCR.

5. CAPÍTULO I

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Diagnosis of *Strongyloides* by PCR

Improving strongiloidiasis diagnosis by PCR

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Abstract

Fecal examination is the traditional method to diagnosis *Strongyloides* spp. eggs or larvae in mammals including humans. However, its sensitivity is low especially in light infections. There is a need of validation of more sensitive tests like those using the Polymerase Chain Reaction (PCR). This study compared the sensitivity of fecal examination and PCR to diagnosis *Strongyloides venezuelensis* in feces of Lewis rats (*Rattus norvegicus*) with a high, moderate or low rate of experimental infection. Animals were infected with 4000, 400 or 40 infective larvae (L3) and seven days later, were sacrificed. Additionally, adult nematodes were recovered from intestine and enumerated. DNA was extracted and the parasite fecal egg counts (FEC) were carried out. Two PCR primer pairs were employed, one was designed

from *S. venezuelensis* rDNA sequence (specific) and the other one amplifies several species within this genus. Regarding the rats inoculated with 400 or 4000 L3, PCR with both primers and also fecal examination detected the parasite presence. The PCR with genus primer pair presented better results than FEC in light infection, while the specific one did not detect the parasite DNA. The genus primer pair presented 90% sensitivity in the diagnosis in animals with low worm burden.

Keywords: *Strongyloides venezuelensis*, sensitivity, fecal examination, PCR, rats.

Introduction

Usually, the diagnosis of *Strongyloides* infection is based on fecal examination. However, the techniques employed are known to be unresponsive to low intensity infections. This problem can compromise the diagnosis leading to an underestimation of the infection rates in epidemiological studies. Therefore, it is necessary the development and validation of more sensitive assays to detect light helminth infections.

In some situations, the Polymerase Chain Reaction (PCR) has been a highly sensitive method to detect pathogens in light infections (Weiss 1995). Pontes et al. (2003) demonstrated that PCR presented 97.4% sensitivity when compared to Kato-Katz technique (78.9%) to detect *Schistosoma mansoni* in human feces. Analyses performed with infected mice revealed that PCR presented 100% sensitivity while Kato-Katz presented 60% to detect *S. mansoni* (Sandoval et al. 2006). Wongratanacheewin et al. (2001) by infecting hamsters with *Opisthorchis viverrini* confirmed that PCR showed 30% sensitivity whereas the fecal egg count (FEC) presented 10% in groups with low infection. Using human feces to detect the same helminth, Duenngai et al. (2008) also had positive results in 28.6% of the samples with PCR, whereas the same samples were negative by the parasitological analysis. The authors

suggested that PCR is the most sensitive method to analyze samples in groups with low intensity infection.

Species of *Strongyloides* are important intestinal parasites of humans and domestic animals (Dawkins 1989). *Strongyloides venezuelensis* is a rodent parasite, usually found in rats (*Rattus norvegicus*) and it is very useful as a model to study nematode infections (Maruyama et al. 2006). Infective larvae of *S. venezuelensis* penetrate into the skin and migrate to the lung where they become the fourth-stage larvae. Then, these larvae reach the small intestine where they finally become adult parasites (Tindall & Wilson 1988).

At the present study, we induced a high, a moderate and a low infection intensity by *S. venezuelensis* in Lewis rats to compare the sensitivity of fecal examination and PCR techniques to diagnosis this intestinal nematode species.

Material and Methods

Animals and experimental infection - Female and male Lewis rats (6-weeks-old) were obtained from a colony maintained in the Animal Facility from the Department of Microbiology and Immunology at Sao Paulo State University (UNESP) Botucatu, SP. Animals were maintained in plastic boxes with water and food *ad libitum*. The experiment was performed according to the Animal Ethics Committee from the Institute of Biosciences – UNESP – Botucatu (N° 45/07).

Strongyloides venezuelensis was isolated at the beginning of the 1980s and has been kept in Wistar rats at the Department of Parasitology- Institute of Biosciences – UNESP – Botucatu. The production of infective *S. venezuelensis* larvae (L3) was performed according to Amarante and Oliveira-Sequeira (2002). The infection intensity by *S. venezuelensis* was monitored by FEC using a McMaster modified technique (Ueno & Gonçalves 1998) in which each egg counted represented 100 eggs per gram of feces (EPG).

Three groups with 10 animals (5 males and 5 females) each were used. These groups were inoculated subcutaneously with 4000, 400 or 40 infective *S. venezuelensis* larvae (L3). Animals were sacrificed 7 days after the infection and fecal samples were collected from each animal to further FEC and PCR analyses. The parasitic females recovered from the first third of the small intestine were enumerated (Nakai & Amarante 2001).

DNA extraction, primer design and genotyping by PCR - DNA samples from fecal samples were extracted with QIAamp DNA stool mini kit and DNA samples from *S. venezuelensis* L3, spleen of a non infected rat and an adult specimen of *Syphacia muris*, nematode recovered from rats, were extracted with QIAamp DNA mini kit (QIAGEN, Hilden, Germany).

The specific oligonucleotide primer pair (Forward 5'-TTAGTGGTGCGTGGATTTGA-3' and Reverse 5'-ATGTGGAGCCGTTTATCAGG-3') was designed by the software program Primer3 (Rozen & Skaletsky 2000) from a *S. venezuelensis* rDNA partial sequence obtained at the GenBank (Accession number: AJ417026). A primer pair described to amplify species within the genus *Strongyloides* (Dorris et al. 2002) was also employed.

PCR reactions were performed in the My cycler (Bio-Rad, Hercules, CA, USA) and Gene Amp PCR System 9700® (Applied Biosystems, Foster City, CA, USA), thermocyclers. PCR mixtures included 10 mM dNTPs, 0.4 mM each primer, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, dNTP, 0.5 U of *Taq* polymerase® (GE Healthcare, Bucks, U.K.) and 100 ng of genomic DNA in a 10 µl reaction. The PCR conditions included initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds (denaturation) 60°C at 30 seconds (annealing for both primer pairs), extension at 72°C for 30 seconds and a final extension at 72°C for 7 min.

The PCR products were electrophoresed through 2% agarose gels in 1X TAE buffer containing ethidium bromide, photographed under UV light and analysed by the Infinity-Capt® software version 15.01 and the Infinity 3000 WL/LC/26M® image capture system (Vilber Lourmat, Marne-la-Valée, France).

Both primer pairs were typed twice with all DNA samples and scored independently in order to increase the accuracy of the results. Ambiguous results were typed a third time.

Statistical analysis - Pearson's correlation coefficient was determined between worm burden and FEC (log transformed data). The comparison between the techniques sensitivity used to detect *S. venezuelensis* was evaluated by the Fisher Exact Test.

Results

The mean parasite infection intensity (± standard deviation) in groups infected with 40, 400 and 4000 L3 were, respectively, 7.3 ± 5.6, 216.4 ± 81.2 and 2813.0 ± 668.0 *S. venezuelensis* specimens, with a mean FEC of 60.0 ± 51.6, 3533 ± 2648 and 24690 ± 24170 EPG. The correlation coefficient between worm burden and FEC was high (r = 0.893; P< 0.001).

Table I shows the comparison between the sensitivity of FEC and PCR techniques with specific and genus primer pair in each group (4000, 400 or 40 L3).

A PCR test with the specific oligonucleotide primer pair using different concentrations of DNA samples from *S. venezuelensis* was performed and a 198 bp amplification band was obtained (Figure 2). The genus primer pair amplified a 340 bp band from *Strongyloides venezuelensis* DNA (Lane 2), a 380 bp band from *Syphacia muris* DNA (Lane 3) and a 420 bp band from spleen DNA of a non-infected rat (Lane 4). These DNA samples were used as positive controls to contribute with the parasite DNA detection in fecal samples of rats infected with 4000, 4000 and 40 L3 infective larvae (Figures 4, 6 and 8). An unspecific 300

bp band was also amplified in fecal samples. Although, it could be easily distinguished from positive controls it was not possible to associate that to a specific helminth.

Both primer pairs were capable to detect DNA from *S. venezuelensis* in all fecal samples from animals infected with 4000 L3. Fecal examination also detected parasite eggs in all of those samples (Figures 3 and 4; Table I).

Fecal examination allowed detection of parasite eggs in all nine samples from animals infected with 400 L3. In this group there were only nine rats because one died (animal 16) during experiment. The specific primer pair detected the parasite DNA in seven out of nine samples. The absence of an amplification band in Lane 3 (animal 12) contrasts with its high FEC value (9100 EPG). It occurred because during DNA extraction procedure part of the fecal sample was lost. There was also absence of amplification band in Lane 10 (animal 20) and it could be explained because the FEC value was only 100 EPG, which means that there were only approximately 20 eggs within the analyzed sample. In both cases the DNA concentration was low (Figure 5). Otherwise, the genus primer amplified a 340 bp band (*S. venezuelensis*) in all nine samples, including animals 12 and 20, which corroborates presence of DNA in both samples. This primer pair also allowed the amplification of a band with 420 bp (rat DNA) in five out of the ten samples (Lanes 9, 10, 11 12 and 13) and the 300 bp (unspecific band) in Lanes 7, 10 and 11 (Figure 6).

The specific primer pair did not detect DNA parasite in none of the 10 samples in animals infected with 40 L3 (Figure 7), but concurrently the genus primer amplified bands in all fecal samples of this group (Lanes 5-14). A 340 bp band (*S. venezuelensis* DNA) was amplified in nine out of ten samples (Lanes 5 and 7 to 14). Simultaneously, a 380 bp band (*S. muris* DNA, Lanes 5, 6, 7, 10, 13, and 14), a 420 bp band (rat DNA, Lanes 5 to 14) and the unspecific 300 bp band (in all samples) were amplified (Figure 8).

Discussion

The sensitivity between PCR and parasitological technique to characterize this infection was compared in order to diagnosis the parasite in feces of rats inoculated with 40, 400 or 4000 infective larvae (L3). These infections were considered light, moderate and heavy, respectively.

FEC and PCR, with both primer pairs, presented 100% of sensitivity to detect *S. venezuelensis* in animals that received the highest inoculum (4000 L3), while in animals infected with 400 L3 the genus primer presented 100% sensitivity and the specific one just 77.7 %.

According with results presented, fecal examination showed positive results in 60% of samples in animals infected with 40 L3, and the specific primer pair, 0%. Hence, this primer pair was incapable to detect parasite presence when animals show 100 or zero EPG. Then, PCR with specific primer pair and FEC presented statistically significant differences in sensitivity ($P<0.05$). In contrast, the genus primer pair detected the parasite in 90% of the samples, and the difference in sensitivity revealed between the two primer pairs was highly significant ($P<0.001$). Considering animal 22 (Lane 6), the genus primer pair did not amplify a 340 pb band as expected. It is necessary to consider that only one parasitic female was recovered from its intestine. Then, it is possible that there were no available eggs in the fecal sample to be amplified by PCR. Although PCR with genus primer pair showed higher sensitivity (90%) when compared to FEC (60%), there was no significant difference, what could be influenced by the small number of animals used ($n=10$) in this experiment.

Wongratanacheewin et al. (2001), Pontes et al. (2003) and Sandoval et al. (2006) compared FEC and PCR sensitivity to detect the parasite in feces samples and observed 87.5%, 78.9% and 60% sensitivity for FEC and 100%, 97.4% and 100% sensitivity for PCR, respectively. PCR results with genus primer pair showed 100, 100 and 90% sensitivity for

heavy, moderate and light infection, respectively. Then, our results for moderate and heavy infection are in agreement with the ones presented by those authors.

It was observed that the higher sensitivity of the genus primer pair was also associated with the appearance of several bands. One of them was unspecific and another one was identified as *Syphacia muris* (380 bp). The presence of this band in fecal samples from the group infected with 40 L3 could be explained by the fact that the animals were kept in a conventional animal facility where the sanitary conditions are less rigorous. Pinto et al. (2001) described *S. muris* as a nematode parasite of rats, and Gilioli et al. (2000) observed a prevalence of 80% of this parasite in a study performed to verify the sanitary conditions within Brazilian bioterium. Therefore, it is likely that during the experiments the animals were infected by other parasites, based on the detection of 380 bp bands, identified as belonging to *S. muris*, and unspecific bands of 300 bp, related to the presence of other contaminants within the feces, once the genus primer pair is not specie-specific.

A high correlation coefficient between worm burden and FEC was observed in this study in agreement with Tamura (1993), Taira et al. (1995), and Nakai and Amarante (2001), who performed analyses with the same parasite, although with different rat strains and inoculum doses.

This was the first study dedicated to detect experimental infection by *S. venezuelensis* in Lewis rats using fecal examination, parasite counting and PCR. The results presented here indicated that PCR technique employing the genus primer pair (Dorris et al. 2002), was more sensitive than FEC mainly in light infection. This methodology could be applied in association with fecal examination in epidemiological studies in order to improve the diagnosis of strongiloidiasis, especially in hosts with small worm burden, i.e., with undetectable number of eggs or larvae in feces and without clinical symptoms of infection.

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References

Amarante AFT, Oliveira-Sequeira TCG 2002. *Strongyloides venezuelensis* infection susceptibility of seven inbred strains of mice. Arq Bras Med Vet Zootec 54: 273-278.

Dawkins HJS 1989. *Strongyloides ratti* infections in rodents: Value and limitations as a model for human strongyloidiasis. In Groved DI, Strongyloidiasis: a major roundworm infection of man, Taylor and Francis, London, p. 287-332.

Dorris M, Viney ME, Blaxter ML 2002. Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. Int J Parasitol 32: 1507-1517.

Duenngai K, Sithithaworn P, Rudrappa UK, Iddya K, Laha T, Stensvold CR, Strandgaard H, Johansen MV 2008. Improvement of PCR for Detection of *Opisthorchis viverrini* DNA in Human Stool Samples. J Clin Microbiol 46: 366-368.

Gilioli R, Andrade LAG, Passos LAC, Silva FA, Rodrigues DM, Guaraldo AMA 2000. Parasite survey in mouse and rat colonies of Brazilian laboratory animal houses kept under different sanitary barrier conditions. Arq Bras Med Vet Zootec 52: 60-69.

Maruyama H, Nishimaki A, Takuma Y, Kurimoto M, Suzuki T, Sakatoku Y, Ishikawa M, Ohta N 2006. Successive changes in tissue migration capacity of developing larvae of an intestinal nematode, *Strongyloides venezuelensis*. Parasitology 132: 1-8.

Nakai ES, Amarante AFT 2001. Experimental infection in mice *(Mus musculus)* and *(Rattus norvegicus)* by *Strongyloides venezuelensis*. Braz J Vet Parasitol 10: 1-6.

Pinto RM, Gonçalves L, Noronha D, Gomes DC 2001. Worm burdens in outbred and inbred laboratory rats with morphometric data on *Syphacia muris* (Yamaguti, 1935) Yamaguti, 1941 (Nematoda, Oxyuroidea). Mem Inst Oswaldo Cruz 96: 133-136.

Pontes LA, Oliveira MC, Katz N, Dias-Neto E, Rabello A 2003. Comparison of a polymerase chain reaction and the Kato-Katz technique for diagnosing infection with *Schistosoma mansoni*. Am J Trop Med Hyg 68: 652-656.

Rozen S, Skaletsky HJ 2000. Primer3 on the WWW for general users and for biologist programmers. In Krawetz S, Misener S, Bioinformatics methods and protocols: methods in molecular biology, Totowa, Humana Press, NJ, p. 365-386.

Sandoval N, Siles-Lucas M, Alban JL, Perez-Arellano JL, Garate T, Muro A 2006. *Schistosoma mansoni*: A diagnostic approach to detect acute schistosomiasis infection in a murine model by PCR. Exp Parasitol 114: 84-88.

Taira N, Nakamura Y, Almeida MAO, Saeki H 1995. Massive experimental infection with *Strongyloides venezuelensis* in rats and absence of sudden death. J Vet Med Sci 57: 855-858.

Tamura N 1993. Studies on availability of *Strongyloides venezuelensis* for experimental model of strongyloidosis. Bul Nip Vet A Sci 59: 94.96.

Tindall NR, Wilson PA 1988. Criteria for a proof of migration routes of immature parasites inside hosts exemplified by studies of *Strongyloides ratti* in the rat. Parasitology 96: 551-563.

Ueno H, Gonçalves PC 1998. Manual para diagnóstico das helmintoses de ruminantes, 4nd ed., Japan International Cooperation Agency, Tokyo, 143 pp.

Weiss JB 1995. DNA probes and PCR for diagnosis of parasitic infections. Clin Microbiol Rev 8: 113-130.

Wongratanacheewin S, Pumidonming W, Sermswan RW, Maleewong W 2001. Development of a PCR-based method for detection of *Opisthorchis viverrini* in experimentally infected hamsters. Parasitology 122: 175-180.

Table I - Fecal examination and PCR sensitivity, using the specific and the genus oligonucleotide primers and DNA from fecal samples of Lewis rats infected with 4000, 400 or 40 L3 of *Strongyloides venezuelensis.*

L3 infection	Number of samples	EPG		PCR (specific primer)		PCR (genus primer)	
		Positive	Sensitivity (%)	Positive	Sensitivity (%)	Positive	Sensitivity (%)
4000	10	10	100	10	100	10	100
400	9	9	100	7	77.7	9	100
40	10	6	60[a]	0	0[b]	9	90[a]

Percentages followed by different superscripts in the same line are significantly different by the Exact Fisher Test ($P<0.05$).

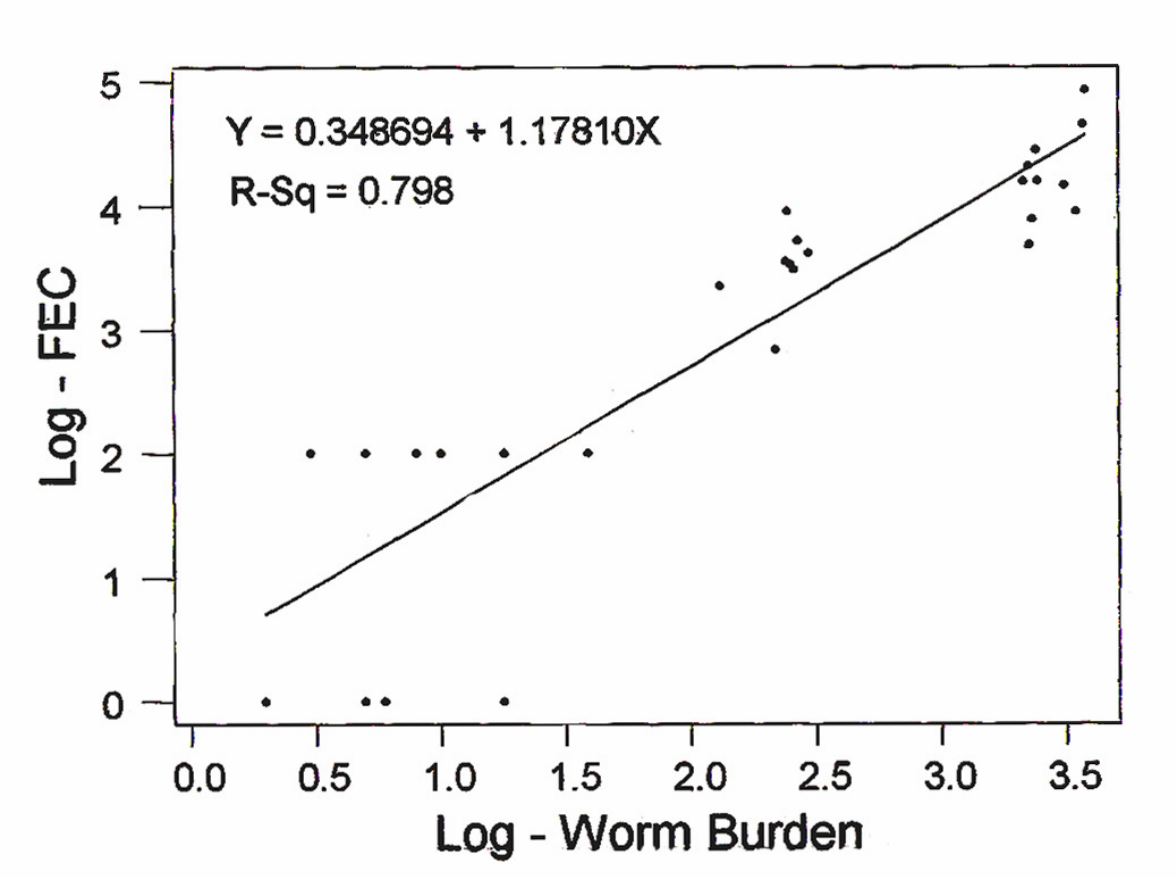

Figure 1 - The relationship between fecal egg counts (FEC) and the number of parasites recovered from the small intestine in Lewis rats infected with 4000, 400 or 40 L3 of *S. venezuelensis.*

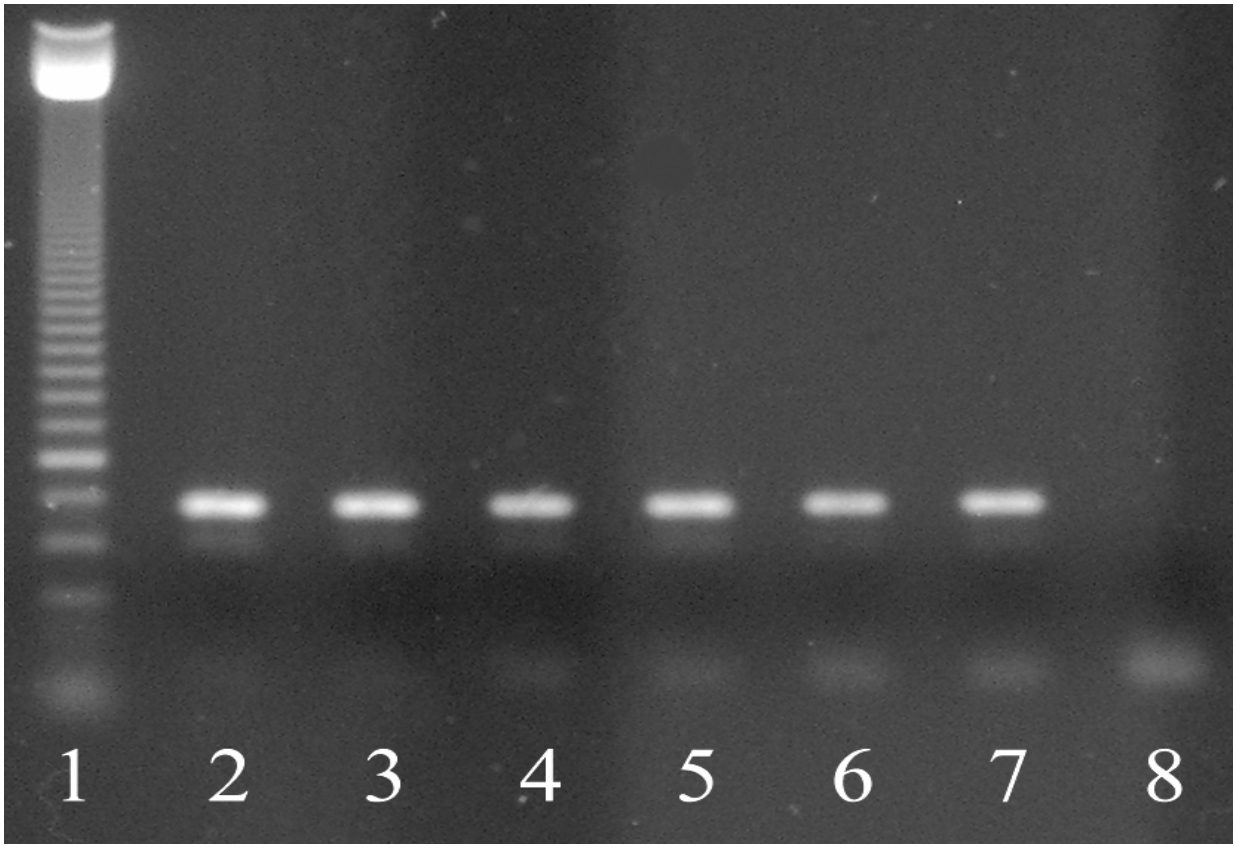

Figure 2 - Agarose gel electrophoresis (2%) showing a PCR test with the specific oligonucleotide primer pair using different concentrations of DNA samples from *S. venezuelensis* and amplification bands of 198 bp. Lane 1 – 50 bp ladder (GE Healthcare). Lanes 2 and 3 – 50ng/µl DNA concentration. Lanes 4 and 5 – 25ng/µl DNA concentration. Lanes 6 and 7 – 12.5ng/µl DNA concentration. Lane 8 - without DNA.

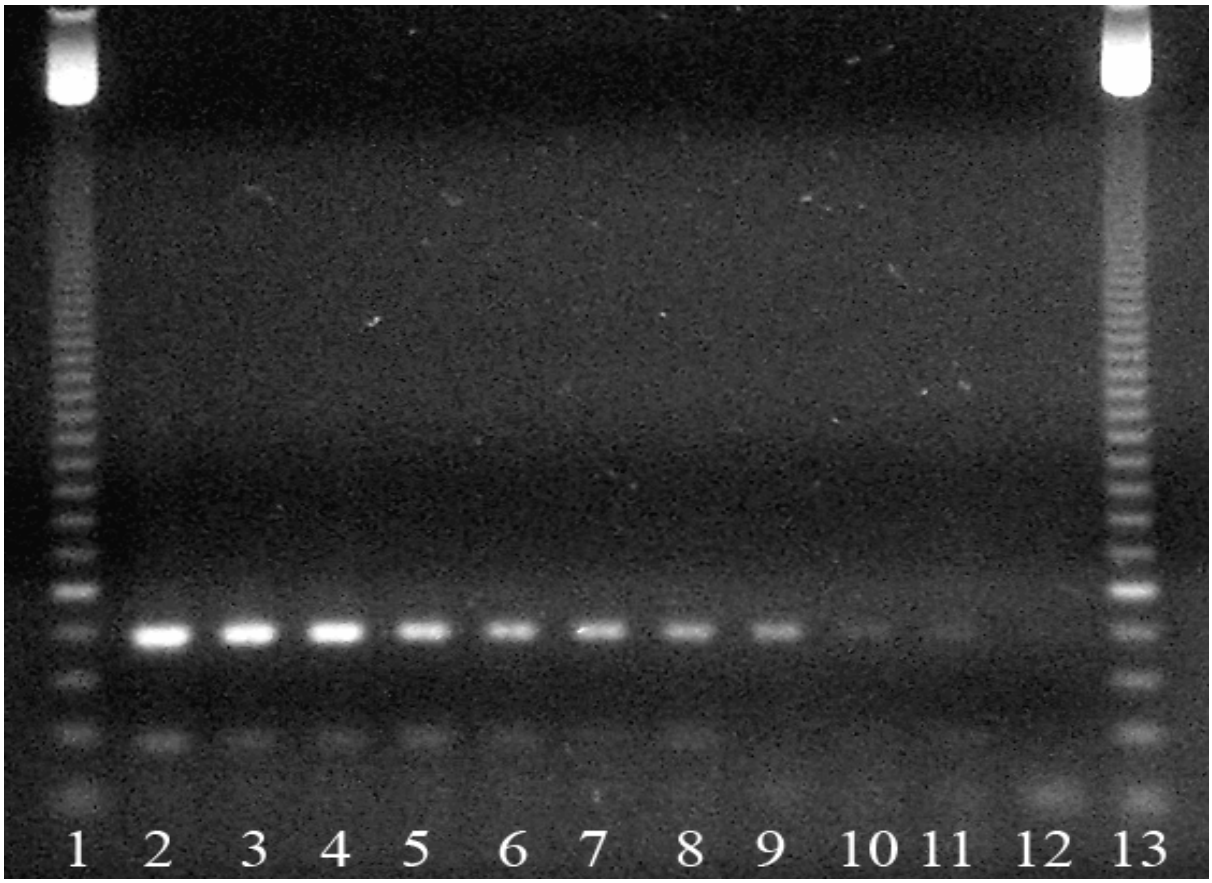

Figure 3 - Agarose gel electrophoresis (2%) showing PCR amplification with the specific oligonucleotide primer pair using DNA extracted from fecal samples of Lewis rats infected with 4000 L3. Lanes 1 and 13 – 50 bp ladder (GE Healthcare). Lanes 2 to 11 fecal samples. Lanes 2 to 10 - 198 bp – *Strongyloides venezuelensis* DNA. Lane 12 – without DNA.

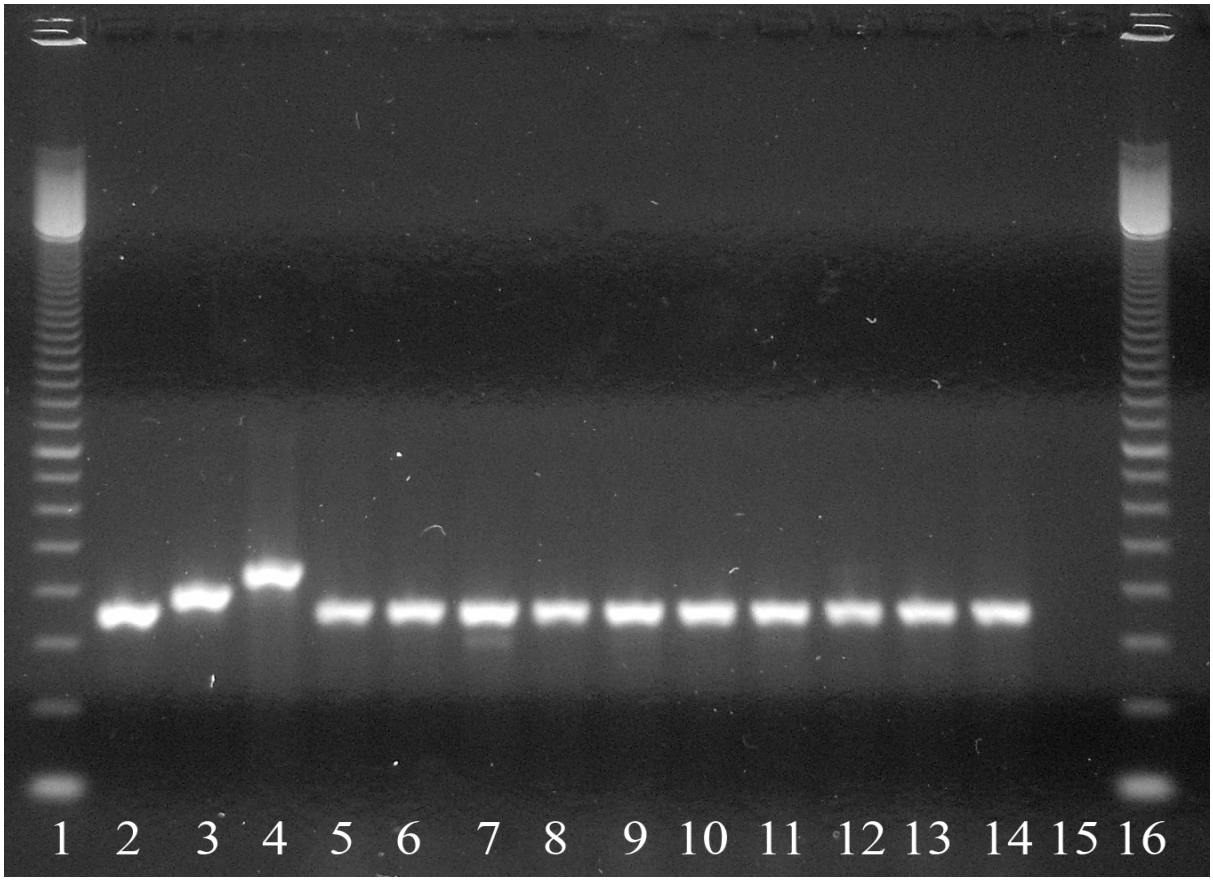

Figure 4 - Agarose gel electrophoresis (2%) showing PCR amplification with the genus oligonucleotide primer pair using DNA extracted from fecal samples of Lewis rats infected with 4000 L3. Lanes 1 and 16 – 100 bp ladder (GE Healthcare). Lane 2 – 340 bp (*Strongyloides venezuelensis* DNA). Lane 3 – a 380 bp (*Syphacia muris* DNA). Lane 4 – 420 bp (*Rattus norvegicus* DNA). Lanes 5 to 14 – DNA from fecal samples - 340 bp *S. venezuelensis* DNA. Lane 15 - without DNA.

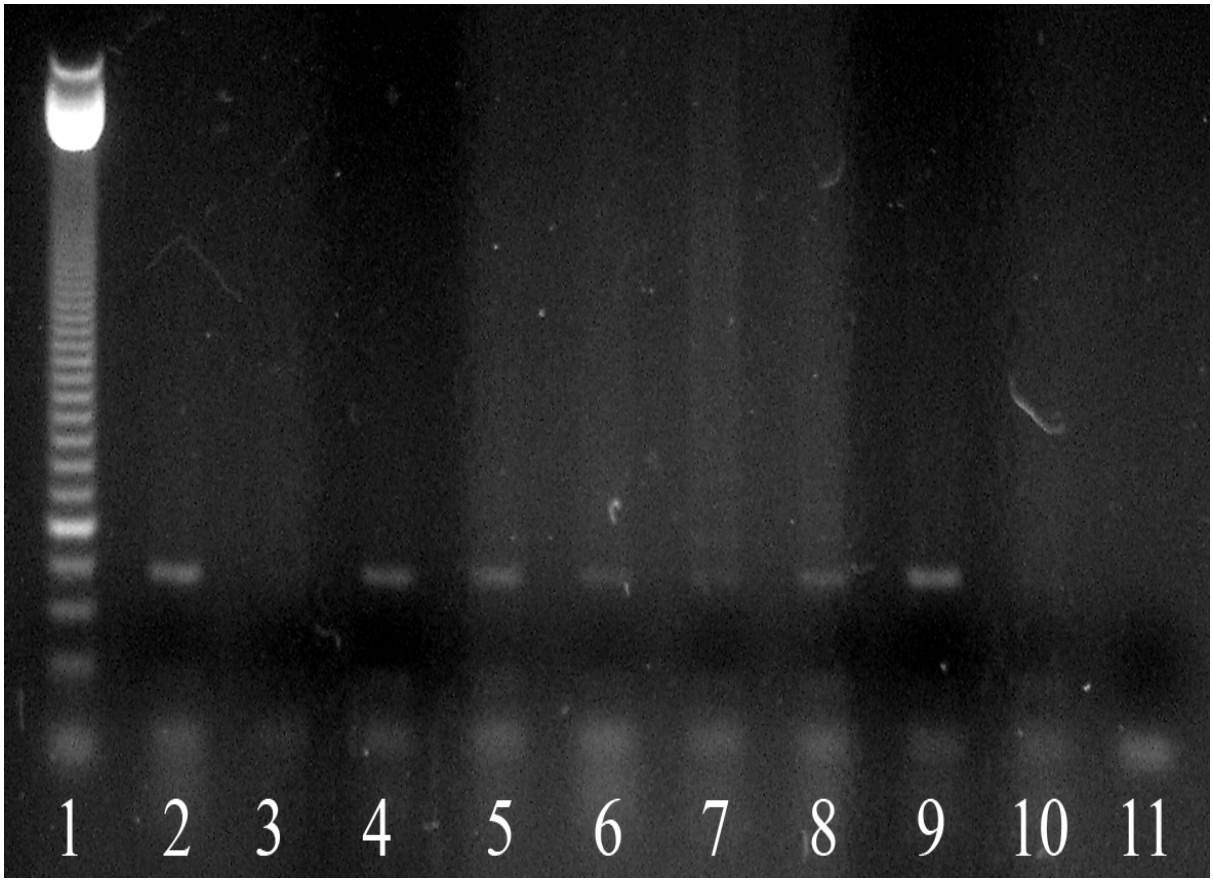

Figure 5 - Agarose gel electrophoresis (2%) showing PCR amplification with the specific oligonucleotide primer pair using DNA extracted from fecal samples of Lewis rats infected with 400 L3. Lane 1 – 50 bp ladder (GE Healthcare). Lanes 2- 10 fecal samples. Lanes 2, 4, 5, 6, 7, 8 and 9 – 198 bp - *Strongyloides venezuelensis* DNA. Lanes 3 and 10 – no amplification bands. Lane 11 - without DNA.

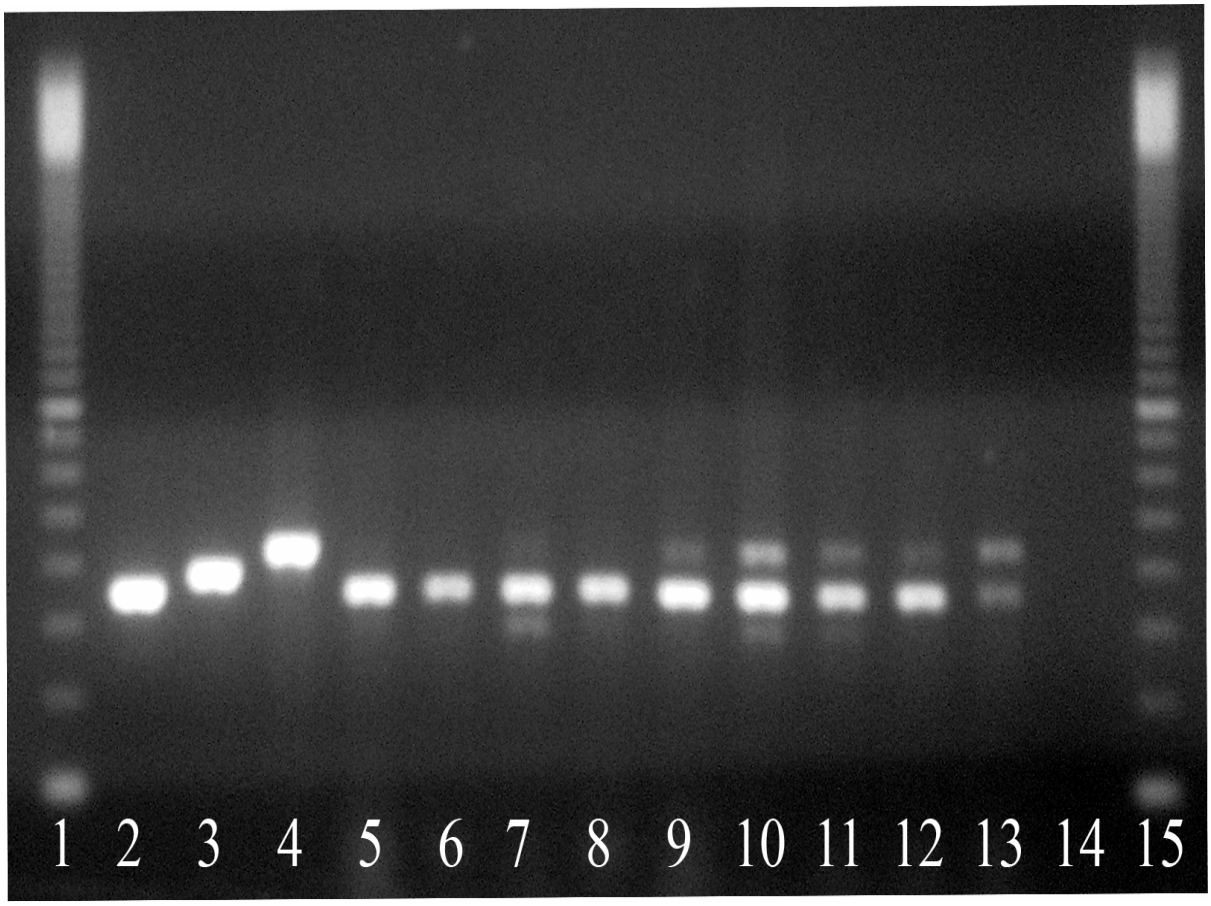

Figure 6 - Agarose gel electrophoresis (2%) showing PCR amplification bands with the genus oligonucleotide primer pair using DNA extracted from fecal samples of Lewis rats infected with 400 L3. Lanes 1 and 15 – 100 bp ladder (GE Healthcare). Lane 2 – 340 bp (*S. venezuelensis* DNA). Lane 3 – 380 bp (*Syphacia muris* DNA). Lane 4 – 420 bp (*Rattus norvegicus* DNA). Lanes 5 to 13 – DNA from fecal samples - 340 bp *S. venezuelensis* DNA. Lanes 9 to 13 – 420 bp *R. norvegicus* DNA. Lanes 7, 10 and 11 – 300 bp -unspecific band. Lane 14 - without DNA.

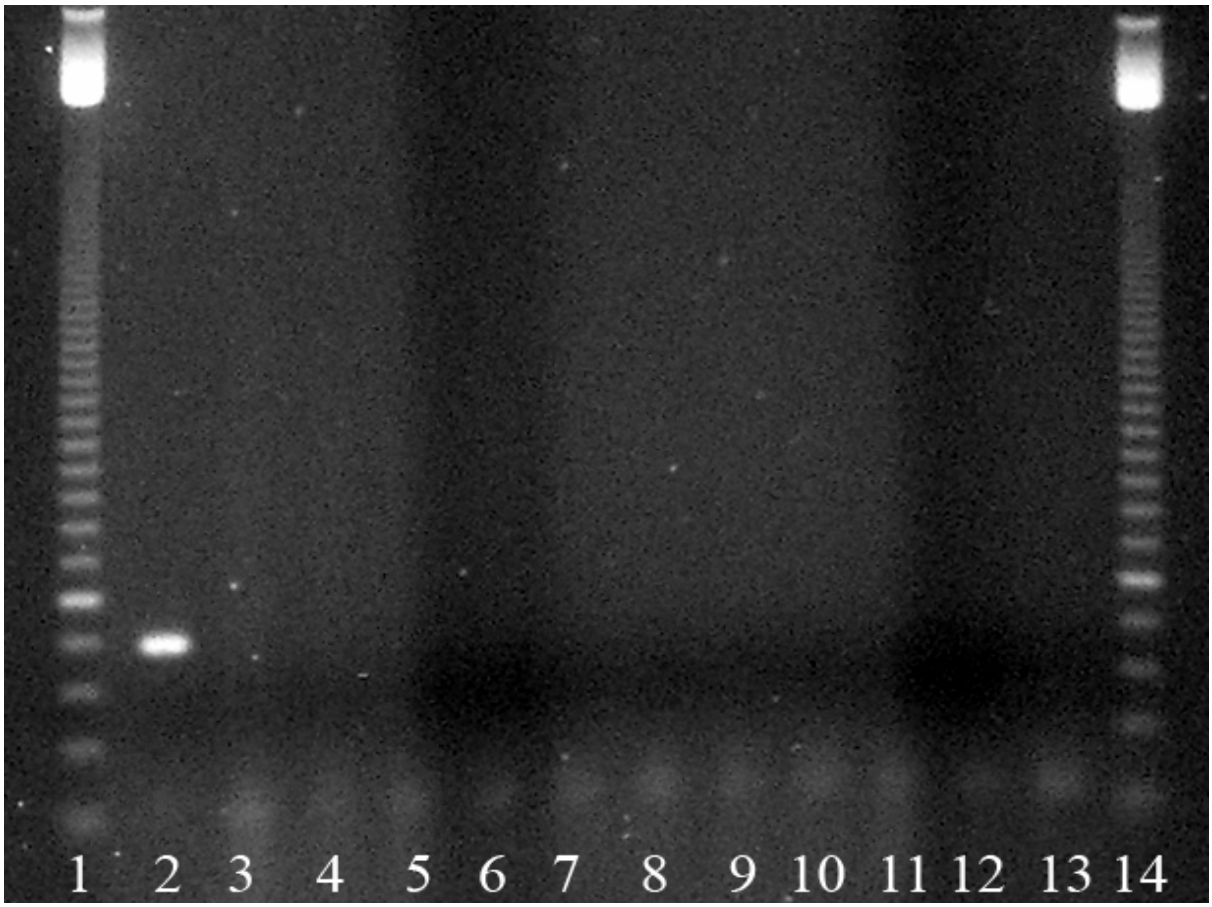

Figure 7 - Agarose gel electrophoresis (2%) showing PCR amplification with the specific oligonucleotide primer pair using DNA extracted from fecal samples of Lewis rats infected with 40 L3. Lanes 1 and 14 - 50 bp ladder (GE Healthcare). Lane 2 – 198 bp - *Strongyloides venezuelensis* DNA. Lanes 3 to 12 fecal samples. Lanes 3 to 12 – no amplification. Lane 13 - without DNA.

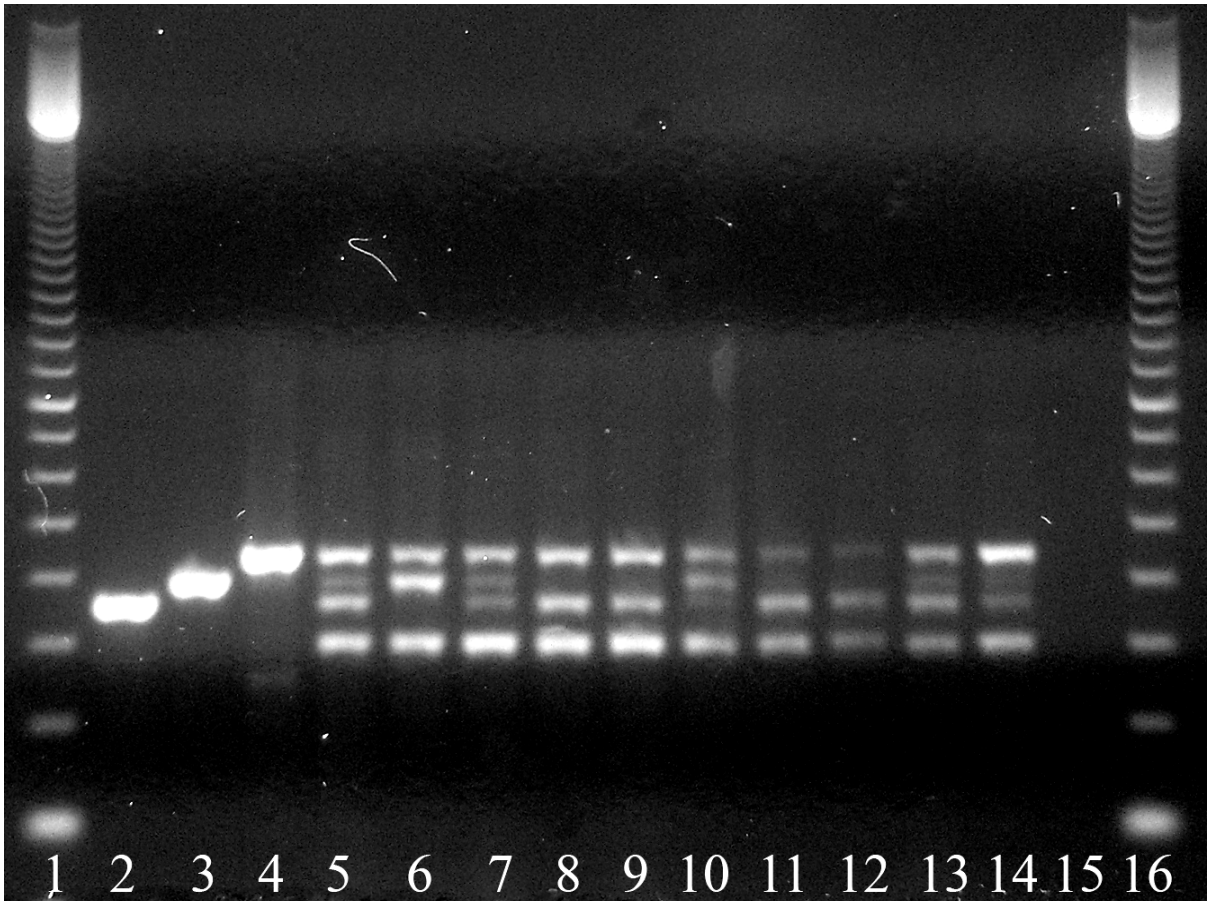

Figure 8 - Agarose gel electrophoresis (2%) showing PCR amplification with the genus oligonucleotide primer pair using DNA extracted from fecal samples of Lewis rats infected with 40 L3. Lanes 1 and 16 - 100 bp ladder (GE Healthcare). Lane 2 – 340 bp (*S. venezuelensis* DNA). Lane 3 – 380 bp (*Syphacia muris* DNA). Lane 4 – 420 bp (*Rattus norvegicus* DNA). Lanes 5 to 14 – DNA from fecal samples – Lanes 5 and 7 to 14- 340 bp *S. venezuelensis*. Lanes 5, 6, 7, 10, 13, and 14 – 380 bp *Syphacia muris* DNA. Lanes 5 to 14 – 420 bp *Rattus norvegicus* DNA. Lanes 5 to 14 – 300 bp unspecific band. Lane 15 - without DNA.

6. CAPÍTULO II

(Artigo enviado à Revista Brasileira de Parasitologia Veterinária ISSN: 0103-846X)

SHORT COMMUNICATION

Sex differences in host resistance to *Strongyloides venezuelensis* infection in Lewis rats

Influência do sexo de ratos Lewis na resistência às infecções por *Strongyloides venezuelensis*

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Abstract

The objective of this study was evaluate the host sex influence in the number of parasites recovered from the small intestine and in the fecal egg couts (FEC) in Lewis rats infected with 4000, 400 or 40 L3 of *Strongyloides venezuelensis*. Males inoculated with 400 or 4000 L3 showed higher parasite worm burden and FEC than females. Similar worm burden and FEC were found in males and females infected with 40 L3.

Key-Words: *Strongyloides venezuelensis*, sex, resistance, Lewis rats.

Resumo

O objetivo deste estudo foi avaliar a influência do sexo dos animais no número de parasitas recuperados do intestino delgado e na contagem de ovos nas fezes em ratos Lewis infectados com 4000, 400 e 40 L3 de *Strongyloides venezuelensis*. Nos animais inoculados com 400 e 4000 L3, a carga parasitária e a contagem de ovos nas fezes foram mais elevadas nos machos que nas fêmeas. A carga parasitária e a contagem de ovos nas fezes foram similares em machos e fêmeas inoculados com 40 L3.

Palavras-chave: *Strongyloides venezuelensis*, sexo, resistência, ratos Lewis

Studies with experimental rodent models confirmed that the sex influence is determined by interactions between immunological and endocrine systems (MORALES-MONTOR et al., 2004). Several reports have been showed the immunosuppressive effects of testosterone on the immune system (NAKANISHI et al., 1989; WATANABE et al., 1999; RIVERO et al., 2002). The presence of sexual hormone receptors in cells from the immunological system, including lymphocytes, macrophages, granulocytes and mast cells, indicates a direct correlation between the endocrine and the immune systems (KLEIN, 2004).

Female and male Lewis rats (6-weeks-old) were experimentally infected with *Strongyloides venezuelensis* in order to compare the host sex influence in the degree of infection. The experiment was performed according to the Animal Ethics Committee from the Institute of Biosciences – UNESP – Botucatu (N° 45/07).

Three groups with 10 animals (5 males and 5 females) each were used. These groups were inoculated subcutaneously with 4000, 400 or 40 infective *S. venezuelensis* larvae (L3). The production of infective *S. venezuelensis* larvae (L3) was performed according to Amarante and Oliveira-Sequeira (2002). Animals were sacrificed seven days after the infection and fecal samples were collected from each animal to enumerate eggs per gram of feces (EPG) using a McMaster modified technique (UENO and GONÇALVES, 1983) in which each counted egg represents 100 eggs. The parthenogenetic females recovered from the first third of the small intestine were also enumerated (NAKAI and AMARANTE, 2001).

Results were analyzed under logarithmic transformation (Log (x+1)). Data were analyzed using GLM of SAS program (1990). Sex (two) and inoculum (three) were included in the model. The interaction between sex and inoculum was evaluated. Means were compared by the Tukey's test, at 5% significance level. Pearson's correlation coefficient was determined between worm burden and FEC log transformed data.

Regarding worm burden and fecal egg count (FEC), the overall least Squares Means for Log transformed data are presented in Table 1. There were no significant interaction between inoculum and animal sex regarding those variables. The worm burden and FEC were proportional to the number of infective larvae administered to the animals with significant differences between inoculums used ($P<0.05$). Males showed higher worm burden and FEC than females, however there was a significant sex influence only on the number of nematodes recovered ($P<0.05$) (Table 1).

The highest mean parasite infection intensity was recorded in the male group infected with 4000 L3 (3276.2 ± 676.6 worms), which corresponded to a larval establishment rate of 81.9%, while in females receiving the same inoculum, 58.7% of L3 gave origin to adult

worms (2349.2 ± 95.3) (Figure 1). Conversely, the mean of parasites recovered from males was only 10.2 ± 6.9 and from females was 4.4 ± 1.8 in animals infected with 40 L3, while the FEC average was 60 ± 48 EPG in both males and females. The FEC means were higher in males than in females in animals infected with 4000 or 400 L3 (Figure 1).

It is likely that the higher susceptibility showed by males in this study is due to the influence of immunosuppressive properties from testosterone (WUNDERLICH et al., 2002). These results are in agreement with Rivero et al. (2002), who observed that castrated Wistar rats were less susceptible to infections by *S. venezuelensis* in comparison with non- castrated rats and also found a significant increase in the susceptibility to *S. venezuelensis* in castrated Wistar females treated with testosterone.

References

AMARANTE, A.F.T.; OLIVEIRA-SEQUEIRA, T.C.G. *Strongyloides venezuelensis* infection susceptibility of seven inbred strains of mice. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v. 54, n.3, p. 273-278, 2002.

KLEIN, S.L. Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunology*, v. 26, n.6-7, p. 247-264, 2004.

MORALES-MONTOR, J.; CHAVARRIA, J.; DE LEON, M.A.; DEL CASTILLO, L.I.; ESCOBEDO, E.G.; SÁNCHEZ, E.N.; VARGAS, J.A.; HERNÁNDEZ-FLORES, M.; ROMO-GONZÁLEZ, T.; LARRALDE, C. Host gender in parasitic infections of mammals: an evaluation of the female host supremacy paradigm. *Journal of Parasitology*, v. 90, n.3, p. 531-546, 2004.

NAKAI, E.S.; AMARANTE, A.F.T. Experimental infection in mice *(Mus musculus)* and *(Rattus norvegicus)* by *Strongyloides venezuelensis*. *Brazilian Journal of Veterinary Parasitology*, v.10, n.1, p.1-6, 2001.

NAKANISHI, H.; HORII, Y.; TERASHIMA, K.; FUJITA, K. Effect of testosterone on the susceptibility of C57BL/6 mice to infection with *Brugia pahangi* with reference to inflammatory cell response. *Journal of Parasitology*, v. 75, n. 3, p. 455-460, 1989.

RIVERO, J.C.; INOUE, Y.; MURAKAMI, N.; HORII, Y. Androgen- and estrogen-dependent sex differences in host resistance to *Strongyloides venezuelensis* infection in Wistar rats. *The Journal of Veterinary Medical Science*, v. 64, n. 6, p. 457-461, 2002.

SAS Institute (Ed.). Doing more with SAS/ASSIST software: version 6. Cary, United States, 1990. 789 pp.

UENO, H.; GUTIERRES, V.C. Manual para diagnóstico das helmintoses de ruminantes. Tóquio: Japan International Cooperation Agency, 1983. 176p.

WATANABE, K.; HAMANO, S.; NODA, K.; KOGA, M.; TADA, I. *Strongyloides ratti*: additive effect of testosterone implantation and carbon injection on the susceptibility of female mice. *Parasitology Research*, v. 85, n. 7, p. 522-526, 1999.

WUNDERLICH, F.; BENTEN, W.P.M.; LIEBERHERR, M.; GUO, Z.; STAMM, O.; WREHLKE, C.; SEKERIS, C.E.; MOSSMANN, H. Testosterone signaling in T cells and macrophages. *Steroids*, v. 67, n. 6, p. 535-538, 2002.

Table 1. Least squares means (log transformed data) of parasites recovered from the small intestine (worm burden) and fecal egg counts (FEC) of males and females Lewis rats seven days after infection with 40, 400 or 4000 infective larvae (Inoculum) of *Strongyloides venezuelensis.*

Variable			Mean	Standard error
worm burden	inoculum	40	0.8271	0.07113
		400	2.2660	0.07545
		4000	3.4388	0.07113
	sex	male	2.2895	0.05808
		female	2.0651	0.06045
FEC	inoculum	40	1.203	0.2304
		400	3.303	0.2444
		4000	4.245	0.2304
	sex	male	3.085 a	0.1882
		female	2.750 a	0.1958

All worm burden or FEC least squares means regarding the inoculum or the sex were significantly different by the Tukey test ($P<0.05$), except the means followed by equal letters.

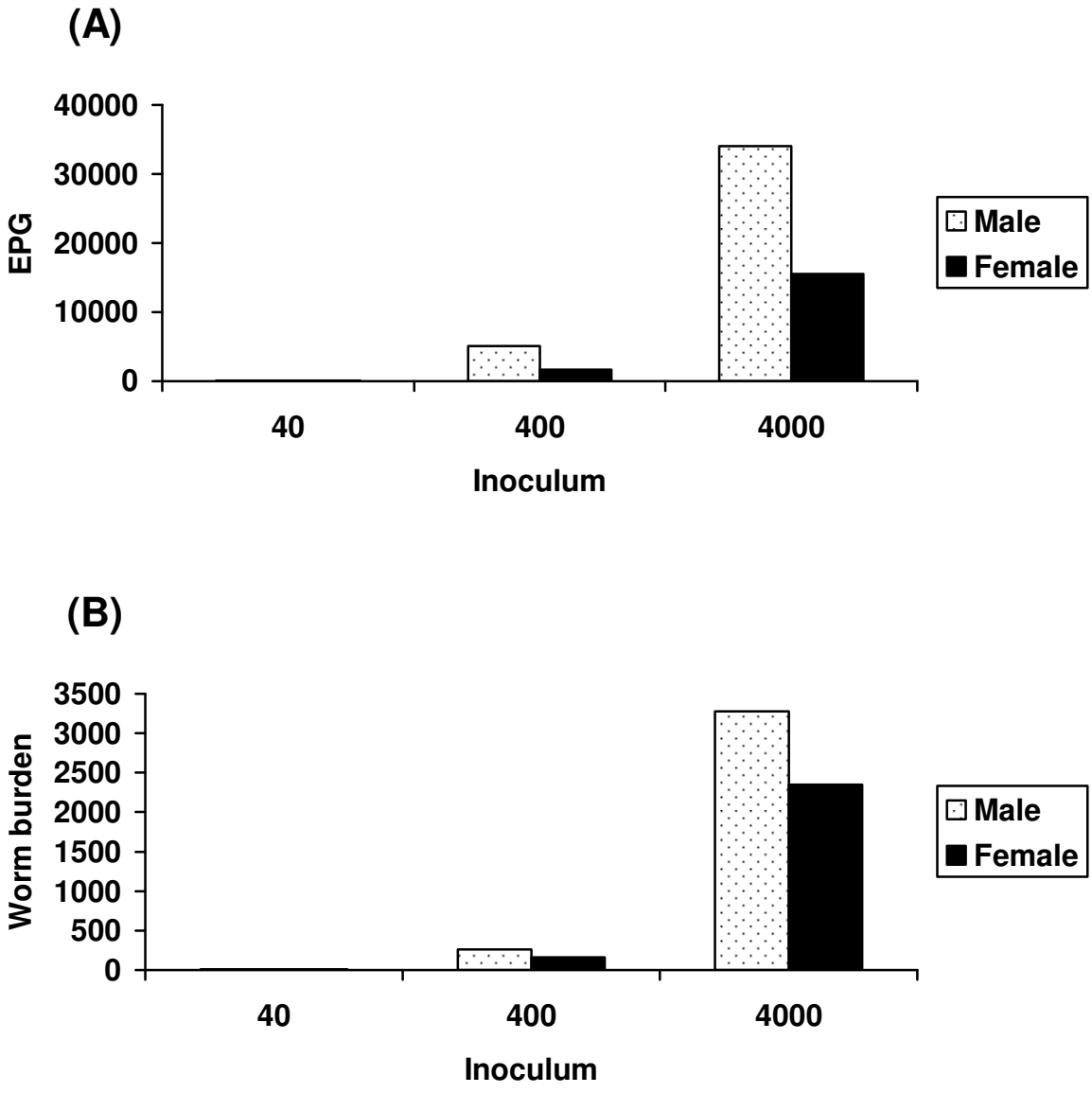

Figure 1. (A) Mean number of eggs per gram (EPG) and (B) number of parasites recovered from the small intestine (Worm burden) of male and female Lewis rats seven days after infection with 40, 400 or 4000 L3 of *Strongyloides venezuelensis*.

7. CAPÍTULO III

(Artigo enviado à revista Parasite Immunology ISSN: 0141-9838)

Recovery from *Strongyloides venezuelensis* infection in Lewis rats is associated with a strong Th2 response

***Strongyloides venezuelensis* infection is associated with a Th2 response**

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SUMMARY

In this study we investigated the characteristics of the infection and subsequent immunity induced by *Strongyloides venezuelensis* in Lewis rats. Animals were infected with 4000 L3 of *S. venezuelensis* and number of eggs per gram of faeces indicated an acute phase around day 8 and a recovery one around day 32 after infection. A strong Th2 polarization during recovery phase was ascertained by a significant increase in IgG1 and IgE comparing to the acute period. A shift in the cytokine profile confirmed these findings. A predominant production of IFN-γ during the acute phase was followed by IL-10 production during

recovery. Together these findings show that experimental infection of Lewis rats with *S. venezuelensis* presents a kinetics of parasite establishment and immunity similar to what is described in other models of helminthic infection.

Keywords *Strongyloides venezuelensis*, Lewis rats, Th2 cells

INTRODUCTION

Strongyloidiais is a parasitosis caused by *Strongyloides stercoralis.* Infection of rodents with *Strongyloides venezuelensis*, a gastrointestinal nematode that naturally infects wild rats, is an experimental model to study Strongyloidiais. The immune response to *Strongyloides* spp. is characterized by production of Th2-type cytokines such as IL-3, IL-4, IL-5 and IL-10 (1-3), increased levels of serum IgE (4) and IgG1 (3,5), tissue and blood eosinophilia (6) and intestinal mastocytosis (7). However, different kinds of immune response can be observed with different strains of *Strongyloides* spp. Recently, a study comparing two heterologous strains of *S. venezuelensis* showed that there was difference in the stimulation of humoral immune response between them (3). The dynamics of *S. venezuelensis* infection, especially concerning the kinetics of egg elimination, the induced immunity and the tissue migration route are already known in Wistar rats (8-9) and in several mice strains (10) but not in Lewis rats. Thus, the aim of this study was to determine the kinetics of *S. venezuelensis* infection and to characterize the immune specific response during acute and recovery phases in Lewis rats.

MATERIAL AND METHODS

Adult female Lewis rats were allocated into four experimental groups containing five animals each. Two groups were used as controls and the others were infected with 4000 *S. venezuelensis* infective filiform larvae by subcutaneous route. At the 8th day after infection

(acute phase), one control group and one infected group were euthanized. The other groups were euthanized at the 32nd day after infection (recovery phase). Larvae were obtained as previously described (9). Infection intensity was determined by daily counting the number of eggs per gram of faeces (EPG) by a modified Cornell McMaster method (11) and by couting the number of parthenogenetic female worms found in the first third portion of the small intestine. Leucogram, specific antibody levels, total IgE and cytokine production were evaluated at the 8th and 32nd day after infection. Parasite-specific IgG1 and IgG2b were estimated by ELISA. Parasite antigen preparation and ELISA methodology were done according to Negrão-Corrêa et al., 2004 (12). Total IgE was determined in blood samples diluted 1:10 also by ELISA according to manufacturer instructions (Immunology Consultants Laboratory, Inc, Newberg, OR, USA). The sensitivity of this assay was 0.5 ng/ mL. Spleen and lymph node (popliteal + inguinal) cells were collected and adjusted to $5x10^6$ cells/mL and $2.5x10^6$ cells/ mL, respectively. Cells were cultured in RPMI supplemented with 10% FCS, 2 mM L-glutamine and 40 mg/L of gentamicin, in the presence of 100 μg/mL of *S. venezuelensis* L3 antigen or 5 μg/mL of concanavalin A (ConA, Sigma, St. Louis, MO, USA). Cytokine levels were evaluated in culture supernatants collected 72h later by ELISA according to manufacturer instructions (R & D Systems, Minneapolis, MN, USA). ELISA sensitivity for IFN-γ and IL-10 were 19 and 31 pg/mL, respectively. Data were expressed as mean ± SD. Comparisons between groups were made by Student`s *t* test for parameters with normal distribution and by Mann-Whitney U test for parameters with non-normal distribution. Significance level was $p < 0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

RESULTS

Parasite eggs were detected in the faeces for the first time at day 6 of infection. The maximal egg number (42300 EPG) was observed at day 8 post-infection and this period was refered as acute phase. A second peak (21300 EPG) was also observed at 11 days post-infection. From this period on the egg number decreased steadly until day 21 when EPG varied from 0 to 100 (figure 1a). This very low level of infection was detected until day 32 that was considered the recovery phase. As expected, a significantly higher number of parthenogenetic females was recovered at the acute phase in comparison to the recovery period (figure 1b). Differences in antibody specific levels, eosinophil counts and cytokine production were observed by comparing these two phases. IgG1 (figure 1c) and IgG2b (figure 1d) specific levels were significantly higher in the acute phase comparing to non-infected control group. Production of specific IgG1 significantly increased during recovery phase whereas IgG2b levels remained similar to levels reached during acute phase. Total IgE was significantly more elevated in infected animals in comparison to control ones in both, acute and recovery phases (figure 1e). However, a significantly increase was observed in the recovery period comparing to the acute phase. Acute phase was also characterized by a significant increase in blood eosinophils (control = 0,02 (± 0,04), infected = 0,24 (± 0,16), $p<0.05$). IFN-γ induced by Con A or *S. venezuelensis* L3 antigen stimulation was evaluated in spleen cell cultures. IFN-γ levels stimulated by Con A were lower in infected animals, in both, acute and recovery phases (figure 2b and 2f). However, a significant decrease was observed in splenic cell cultures during recovery phase (figure 2f). Specific stimulation with *S. venezuelensis* L3 antigen did not induce IFN-γ production by lymph node cells from acute and recovery phases (data not shown). However, significantly higher levels of this cytokine were detected in splenic cell cultures during acute phase (figure 2a). Interestingly, IFN-γ concentration went down to basal levels during recovery phase (data not shown). Only

cultures from lymph node cells showed differences in IL-10 production between infected and normal rats. No IL-10 was detected in cultures stimulated with specific antigen during acute phase (not shown). However, there was IL-10 induction by specific stimulation during recovery (figure 2d). On the other hand, IL-10 levels induced by Con A were reduced in both phases, being statistically significant only in the acute period of infection (figure 2c).

DISCUSSION

This investigation was carried out to establish if inoculation of *S. venezuelensis* in Lewis rats triggers an infection and a subsequent immunity similar to what is described in other rodents and also in human infections by *Strongyloides stercoralis*. In Lewis rats subcutaneously infected with 4000 L3, parasite eggs were detected in the faeces for the first time at day 6 post-infection, but the maximal egg number was observed at day 8 post-infection. A second peak in the egg number was observed at 11 days post-infection decreasing steadly thereafter. This kinetics in egg number coincided with the amount of parthenogenetic females recovered from the small intestine. The highest amount was detected during the acute phase whereas a very low number was found at the recovery one. Considering these findings, the acute phase occurred around the 8^{th} day and the recovery phase around the 32^{nd} day of infection. This infection kinetics indicates a profile that is similar to infections caused by *S. venezuelensis* (8) and also by *S. ratti* in Wistar rats (13). Immunity against *Strongyloides* spp. is characterized by a typical Th2 pattern with a predominant production of IL-3, IL-4, IL-5 and IL-10 (1,3). Elevated levels of IgG1, IgE, eosinophils and intestinal mastocytosis has been abundantly described (3-7). In the present study, both IgG1 and IgG2b specific antibodies were significantly elevated at the acute phase. However, a much higher increase in IgG1 concentration already suggested a stronger Th2 polarization at this period. This tendency became evident at the recovery phase when IgG1 but not IgG2b presented a

significant increase comparing to the acute infection. These results are similar to the ones described in mice infected with *S. venezuelensis* (3) and Wistar rats infected with *S. ratti* (5). Wilkes et al., 2007 (5), even called attention for a finding that was very similar to our results, i.e., that there was a significant elevation of IgG1 specific levels during recovery phase comparing to the acute phase. They also stressed the fact that IgG1 higher levels coincided with worm elimination. Total IgE was significantly elevated in both, acute and recovery phases. Interestingly, IgE levels were significantly higher in recovery phase comparing to the acute period of infection. Even though IgE levels have been a hallmark in helminthic infections, its contribution to control these parasites has been, at least, controversial (14). Elevated IgE levels have been reported in both *S. venezuelensis* and *S. ratti* experimental infections (5,15). A significant raise in eosinophil`s number was detected in Lewis rats during the acute phase of *S. venezuelensis* infection. These counts returned to basal levels during recovery phase. These findings are in accordance with literature reports that showed increased number of blood eosinophils following helminthic infections (16). Their subsequent disappearance from the blood has been attributed to migration to the site of the infection where they degranulate, releasing eosinophil secondary granule proteins (17). Production of cytokines by secondary lymphoid organ cultures stimulated with specific antigens and Con A were used to characterize cellular immunity. Considering IFN-γ induction by specific stimuli, a significant production was detected during acute phase but not at the recovery one. The opposite happened with IL-10 production, i.e., absence of this cytokine at the acute period and detectable levels during recovery phase. Analysing this data together with antibody levels (IgG subclasses and IgE), we could suggest that an initial mixed pattern (Th1/ Th2) at the acute phase was followed by a predominantly Th2 polarization during recovery phase.

Production of IFN-γ and IL-10 stimulated by polyclonal activation with Con A showed a similar pattern, i.e., a general decreased production of these mediators by cultures of

spleen and lymph nodes. A theoretical explanation for this finding is that T lymphocytes able to produce these cytokines are migrating from lymphoid organs to the places of temporary (lungs) or final (intestine) establishment of the worm. This possibility is supported by recent literature reports (3,8,12). Together these results show that experimental inoculation of Lewis rats with *S. venezuelensis* triggers an infection that is similar in terms of kinetics of parasite establishment and immunity to experimental strongyloidiasis in other rodents and also in human *S. stercoralis* infection.

REFERENCES

1 Kobayashi T, Tsuchiya K, Hara T, *et al*. Intestinal mast cell response and mucosal defence against Strongyloides venezuelensis in interleukin-3 hyporesponsive mice. *Parasite Immunology* 1998; **20**: 279-284.

2 Machado ER, Ueta MT, Lourenço EV, *et al*. Leukotrienes play a role in the control of parasite burden in murine Strongyloidiasis. *J Immunol* 2005; **175**: 3892-3899.

3 Machado ER, Ueta MT, Lourenço EV, Anibal FF, Roque-Barreira MC & Faccioli LH. Comparison of immune response in mice infected with different strains of *Strongyloides venezuelensis*. *Parasite Immunol* 2007; **29**: 549-557.

4 Korenaga M, Nawa Y & Tada I. IgE response in Strongyloides ratti- infected rats with special reference to the life cycle of the parasite. *Z Parasitenk* 1986; **72**: 213-220.

5 Wilkes CP, Bleak C, Paterson S & Viney ME. The immune response during a *Strongyloides ratti* infection of rats. *Parasite Immunol* 2007, **29**: 339-346.

6 Ovington KS, Mckie K, Matthaei KI, Young IG & Behm CA. Regulation of primary *Strongyloides ratti* infections in mice: a role for interleukin-5. *Immunology* 1998; **95**: 488-493.

7 Tegoshi T, Okada M, Nishida M & Arizono N. Early increase of gut intraepithelial mast cell precursors following *Strongyloides venezuelensis* infection in mice. *Parasitology* 1997; **114**: 181-187.

8 Silveira MR, Nunes KP, Cara DC, *et al*. Infection with *Strongyloides venezuelensis* induces transient airway eosinophilic inflammation, an increase in immunoglobulin E, and hyperresponsiveness in rats. *Infect Immunol* 2002; **70**: 6263-6272.

9 Nakai ES, Amarante AFT. Infecção experimental de camundongos (*Mus musculus*) e ratos (*Rattus norvegicus*) com *Strongyloides venezuelensis*. *Rev Bras Parasitol Vet* 2001; **10**: 1-6.

10 Amarante AFT & Oliveira-Sequeira TCG. *Strongyloides venezuelensis* infection susceptibility of seven inbred strains of mice. *Arq Bras Med Vet Zootec* 2002; **54**: 273-278.

11 Gordon HM & WhitlocK HV. A new technique for couting nematode eggs in sheep faeces. *J Coun Scient Ind Res* 1939; **12**: 50-52.

12 Negrão-Corrêa D, Souza DG, Pinho V, Barsante MM, Souza ALS & Teixeira MM. Platelet-activating factor receptor deficiency delays elimination of adult worms but reduces fecundity in *Strongyloides venezuelensis*- infected mice. *Infect Immunol* 2004; **72**: 1135-1142.

13 Kimura E, Shintoku Y, Kadosaka T, Fujiwara M, Kondo S & Itoh M. A second peak of egg excretion in *Strongyloides ratti*-infected rats: its origin and biological meaning. *Parasitology* 1999; **119**: 221-226.

14 Cooper PJ, Ayre G, Martin C, Rizzo JA, Ponte EV & Cruz AA. Geohelminth infections: a review of the role of IgE and assessment of potential risks of anti-IgE treatment. *Allergy* 2008; **63**: 409-417.

15 Fernandes A, Pereira ATM, Eschenazi PD, *et al*. Evaluation of the immune response against *Strongyloides venezuelensis* in antigen-immunized or previously infected mice. *Parasite Immunol* 2008, **30**: 139-149.

16 Ganley-Leal LM, Mwinzi PN & Cetre-Sossah. Correlation between eosinophils and protection against reinfection with *Schistosoma mansoni* and the effect of human immunodeficiency virus type 1 coinfection in humans. *Infect Immunol* 2006; **74**: 2169-2176.

17 Antony RM, Rutitzky LI, Urban Jr JF, Stadecker MJ & Gause WC. Protective immune mechanisms in helminth infection. *Nature Rev Immunol* 2007; **7**: 975-987.

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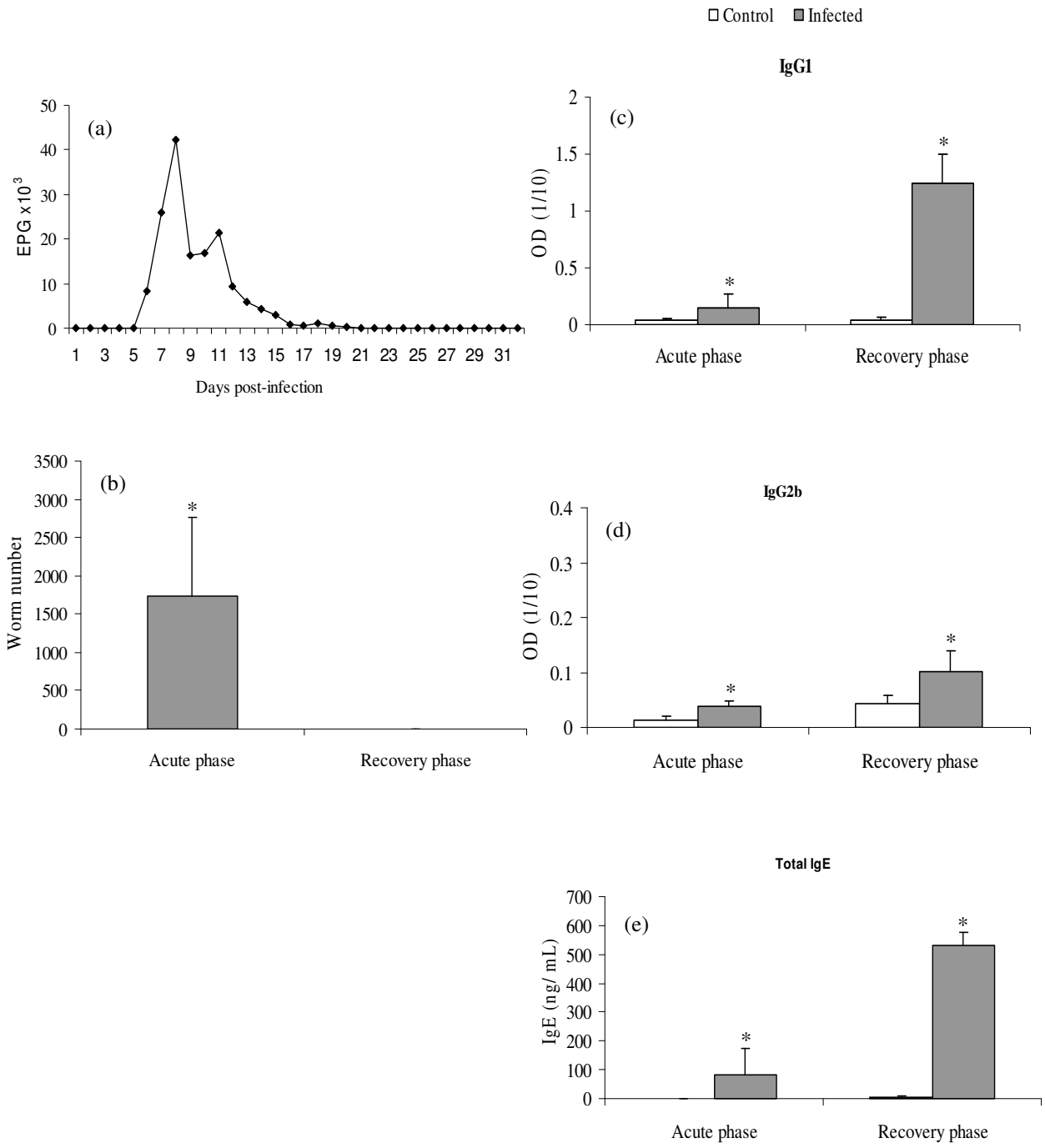

Figure 1 Kinetics of *S. venezuelensis* infection and induced humoral immunity. Eggs per gram of faeces (a) and number of female adult worms recovered from the small intestine (b). In the acute phase, the number of female adult worms varied from 97 to 2979. Seric levels of specific IgG1(c), IgG2b (d) and total IgE (e) in infected Lewis rats. The number of eggs per gram of faeces was determined in a faecal pool. Results are expressed as mean ± SD of 5 animals per group. * $p<0.05$ in comparison to recovery phase.

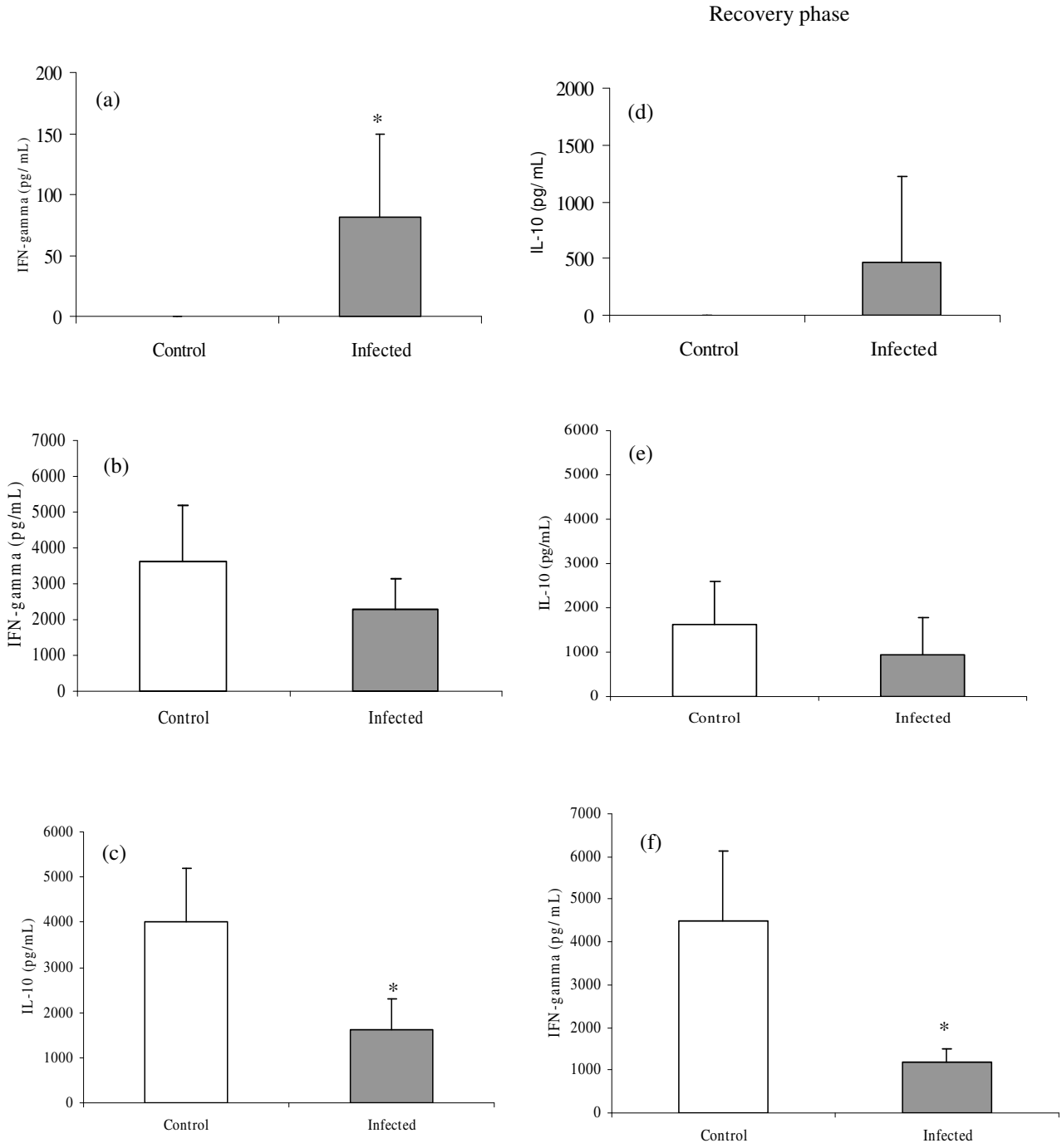

Figure 2 IFN-γ and IL-10 production in infected Lewis rats: acute phase (a,b and c) and recovery phase (d,e and f). Spleen (a,b and f) and lymph node (c,d and e) cells were stimulated with Con A (b,c,e and f) or *S. venezuelensis* L3 antigen (a and d). Results are expressed as mean ± SD of 5 animals per group. * $p<0.05$ in comparison to control group.

8. CAPÍTULO IV

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Migratory route of *Strongyloides venezuelensis* in Lewis rats: comparison of histological analyses and PCR

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Abstract

Strongyloides venezuelensis is a parasitic nematode that has been used as a model to study the relationship of human and/or animal strongyloidiasis. In the present study we compared the sensitivity between traditional methodologies and PCR assay to characterize the dynamics of *S. venezuelensis* infection and its migration route in Lewis rats subcutaneously infected with 4000 L3. The dynamics of the infection was determined by counting the number of eggs and by detecting parasite DNA in faeces samples. Both techniques similarly detected the

beginning of the infection 6 day after inoculation. However, PCR showed higher sensitivity during the recovery phase. Histological analysis and PCR assay were then used to follow parasite tissue migration. We observed that *S. venezuelensis* migration included the muscular fibers below the skin, the pulmonary alveoli and the small intestine vilosities. No parasite was detected in many others organs and tissues analyzed, including the brain. The sensitivity of these two techniques to detect parasite presence in these tissues was statistically similar.

Keywords: *Strongyloides venezuelensis*, migratory route, Lewis rats, histological, PCR.

Introduction

Strongyloidiasis is a common, usually asymptomatic helminthic infection caused by *Strongyloides stercoralis* that is an intestinal nematode which is mainly endemic in tropical and subtropical regions (Ly et al. 2003). It infects around 30 million people in 70 countries and usually results in asymptomatic chronic disease with characteristic features of autoinfection, hyperinfection involving pulmonary and gastrointestinal systems, and disseminated infection involving others organs (Siddiqui and Berk 2001).

Strongyloides venezuelensis is a parasitic nematode of rats that has been used as a model to study the host-parasite relationship of human and/or animal strongyloidiasis (Sato and Toma 1990; Taira et al. 1995). This parasite enters into the host through skin and them migrates to lungs, trachea, and then reaches the small intestine where it moults to become a sexually mature adult female (Baek et al. 1999). The infection of rodents with *S. venezuelensis* has been carried out in various strains of rats and mice (Takamure 1995; Silveira et al. 2002; Matsuda et al. 2003). However, the kinetic time-lines and migratory route were not yet established in the Lewis rat strain.

Histological methodology is also an important tool that has been used to determine parasite migration route and lesions caused by *S. venezuelensis* and *S. ratti* in rodents (Genta and Ward 1980; Koga et al. 1998; Matsuda et al. 2003).

More recently, DNA technology has had a major impact in many areas of parasitology, including the diagnosis of infection. Polymerase chain reaction (PCR) has found broad applicability mainly because its sensitivity allows the amplification of DNA fragments from minute amounts of parasite material (Gasser 1999). Several reports have showed a high PCR sensitivity for detection of parasite DNA in various biological samples (i.e., feces, tissues) from different hosts (Esteban-Redondo et al. 1999; Wongratanacheewin et al. 2001; Sandoval et al. 2006; Duenngai et al. 2008).

In the present study we compared the sensitivity between PCR and traditional methodologies to characterize *S. venezuelensis* infection in Lewis rats. This was done in two steps. We initially established the dynamics of infection by faecal examination and PCR analysis of faecal samples. We then evaluated the parasite migration route by PCR and histological analysis.

Material and Methods

Animals

Male and female Lewis rats weighing 120-150g (4-6 weeks old) were obtained from the CEMIB (UNICAMP, Campinas, SP, Brazil). Animals received sterilized food and water *ad libitum* and were manipulated in compliance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation, being the experimental protocol approved by the local Ethics Committee (protocol 45/07).

Parasite and infection

The *S. venezuelensis* strain was isolated from wild rats in 1980 and has been maintained in Wistar rats, routinely infected in the Parasitology Laboratory of the São Paulo State University (UNESP). Infective third-stage larvae (L3) of *S. venezuelensis* obtained from faecal cultures using sterilized horse manure as substrate were employed for experimental infection. The cultures were incubated at 25°C for 72h and the infective larvae were collected and concentrated by using a Baermann apparatus. Recovered larvae were washed in phosphate-buffered saline (PBS), the number of viable infective larvae was determined and 4000 L3 were subcutaneously inoculated at the animal's abdominal region.

Experimental design

In the first experiment it was determined the dynamics of *S. venezuelensis* infection by daily counting the number off eggs per gram of faeces (EPG) by modified Cornell McMaster (Gordon and Whitlock 1939), during 32 days. PCR analysis with both, a specific primer pair (designed from a *S. venezuelensis* rDNA partial sequence) and a non specific primer pair described by Dorris et al. (2002) was also performed in pooled faeces samples collected every other day until the 32nd day of infection. Five female rats were used in experimental group.

In the second experiment we compared sensitivity of PCR and histological examinations to define the parasite migration route. Male rats were allocated into six experimental groups. Five groups were infected and sacrificed at different periods after infection: 30 minutes, 24h, 48h, 72h and 120h. The non-infected group was used as a normal control. Tissue samples were collected and used to perform histological and PCR analysis.

Histological analysis

Histological analysis was performed in samples of brain, lung, spleen, first 14 cm of the small intestine, liver, lymph node (popliteal and inguinal), heart, vastus lateralis muscle,

skin and muscular layer (abdominal region). These samples were fixed in 10% formalin and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin and eosin and analyzed in a computerized system of image analysis (QWin Lite 2.5, Leica®).

PCR assay

PCR assays were employed to detect *S. venezuelensis* DNA in both, faeces and the same tissue samples employed in histological analysis. DNA from faecal samples was extracted with QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) and DNA from tissues, from *S. venezuelensis* L3, from adult *Syphacia muris* and from spleen of a non infected rat were all extracted with a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). The specific oligonucleotide primer pair (Forward 5'-TTAGTGGTGCGTGGATTTGA-3' and Reverse 5'-ATGTGGAGCCGTTTATCAGG-3') was designed by the software program Primer3 (Rozen and Skaletsky 2000) from a *S. venezuelensis* rDNA partial sequence obtained at the GenBank (Accession number: AJ417026). A primer pair described to amplify species within the genus *Strongyloides* (Dorris et al. 2002) was also employed. Both primer pairs were used to perform PCR in faecal samples and only the genus primer was used from tissue samples evaluation. DNA samples from *S. venezuelensis, Syphacia muris* and spleen of a non-infected rat were employed as positive controls, in all PCR reactions with both primer pairs.

PCR reactions were performed in the My cycler (Bio-Rad, Hercules, CA, USA) and Gene Amp PCR System 9700® (Applied Biosystems, Foster City, CA, USA), thermocyclers. PCR mixtures included 10 mM dNTPs, 0.4 mM each primer, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, dNTP, 0.5 U of *Taq* polymerase (GE Healthcare, Bucks, U.K.) and 100 ng of genomic DNA in a 10 µl reaction. The PCR conditions included initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds (denaturation) 60°C at 30 seconds (annealing for both primer pairs), extension at 72°C for 30 seconds and a final extension at

72°C for 7 min. PCR products were electrophoresed through 2% agarose gels in 1X TAE buffer containing ethidium bromide, photographed under UV light and analysed by the Infinity-Capt® software version 15.01 and the Infinity 3000 WL/LC/26M® image capture system (Vilber Lourmat, Marne-la-Valée, France). Both primer pairs were typed twice with all DNA samples and scored independently in order to increase the accuracy of the results. Ambiguous results were typed a third time.

Statistical analysis

The comparison between histological and PCR techniques sensitivity used to detect parasite larvae in tissue samples was evaluated by the Chi-Square Test and the agreement between techniques by the McNemar Test.

Results

Dynamics of infection by EPG and PCR

Infection outcome was initially determined by counting the number of eggs that were daily shed in the faeces (EPG) during 32 days. Parasite eggs were detected for the first time in the faeces at the 6th day of infection. The maximal faecal egg count (FEC) was 42300 EPG observed at day 8 pos-infection. A second peak (21300) was also observed at 11 days post-infection. From the 12th day on the amount of eggs steadly decreased until day 21 when FEC varied from 0 to 100 EPG (Figure 1). Fecal egg count showed 0 to 100 EPG in seven out of 32 samples starting from day 23.

Determination of infection by PCR depended upon the nucleotide sequence used as primers. The specific primer pair was effective to detect parasite DNA in the faeces from day 6 until day 18, being negative from this period on (Figure 2A). On the other hand, PCR

executed with the genus primer was able to detect *S. venezuelensis* DNA during a larger time interval, including all periods between day 6 and 32 that was the last evaluation day. Interestingly, the more intense parasite DNA amplification bands coincided with the highest amount of parasite eggs (Figure 2B).

Migration route characterization by histological analysis

This analysis begun 30 minutes after infection and at this time parasites were located below the muscular fibers of skin in 4 rats and at the muscular layer in one in a group with 5 animals. The presence of a typical larvae is documented at figure 3A. No histological alterations were observed in any of the organs examined at this early period. The parasite was not found during the analysis performed in the different tissues 24h after infection. However, tissue lesions associated with an inflammatory infiltrate were clearly observed at the most deeper skin layers and also among the muscle fibers. Appearance of larvae in the lungs was clear at 48h. At this period 3 of 5 animals already showed larvae located inside the alveoli. This was associated with inflammation, local edema and hyperemia (Figure 4A). The analysis carried out 72h post-infection revealed that the percentage of animals with larvae at the lungs increased to 100% and that all lung samples presented inflammatory foci. At this period no larvae were found in any of the organs that were analyzed.

At 120 hours the larvae disappeared from the lungs even thought the histopathological alterations described at 48 and 72 h were still present in this organ. Interestingly, at this time period, all samples from 5 infected animals presented larvae inserted at the small intestine vilosities (Figure 5A). A clear infiltration of eosinophils was found around the larvae's location.

Tissue parasite detection by PCR

Samples from all tissues submitted to histological analysis were also used to make PCR with the genus primer that generated a specific 340 bp amplification band from *Strongyloides venezuelensis* DNA. This band was detected in DNA samples obtained from the skin of 4 rats in a group of five animals that were euthanized 30 minutes after infection (Figure 3B). This specific band was also amplified in a DNA samples from the muscular layer (one animal), from the vastus lateralis muscle (one animal) and from muscular layer (2 animals) at 24h, 48h and 72h, respectively.

This 340 bp specific amplification band was present in lung DNA samples obtained from 3 rats at 48h (Figure 4B) and from all rats at 72 hours evaluation. All DNA samples from the small intestine got at 120 hours post-infection (Figure 5B), but not any one of the other organs analyzed, presented this specific band. As expected, this specific band was not detected in any of the samples obtained from the control experimental group.

Unspecific bands were also amplified by this technique in many of the samples. However, they could be identified by using positive controls or by their very distinctive molecular weight. Among the 4 unspecific bands two were identified as being derived from rat DNA (420 bp) or from *Syphacia muris* (380 bp). Two others unspecific bands of 260 and 300 bp were also detected.

The statistical comparison of these two methodologies shows that from the 261 samples analyzed by both techniques, 18 (6.7%) were positive by histological analysis and 21 (8.06%) were positive by PCR. These percentages were statistically similar ($P<0.05$). On the other hand, divergent results were observed in only 7 samples and this was not statistically relevant ($P<0.05$).

Discussion

In the first part of this investigation, by counting the number of eggs per gram of faeces (EPG), we demonstrated that strongyloidiasis caused by *Strongyloides venezuelensis* in Lewis rats presents a dynamics that is very similar to the infection by this helminth or by *Strongyloides ratti* in Wistar rats (Kimura et al. 1999). In Lewis rats subcutaneously infected with 4000 L3, parasite eggs were detected for the first time in the faeces at day 6 and reached a maximum value at day 8. From the 12th day on the amount of eggs steadily decreased until day 21 when EPG varied from 0 to 100. The presence of eggs in the faeces was continuously decreased until 32nd day post-infection and values remained very low (0 to 100). By using this criterion we could say that the acute phase was around day 8 and that recovery one was established between days 21 and 32 after infection.

One of your main goals was to compare the sensitivity of the traditional EPG technique with the PCR method that was performed with two kinds of primer pairs. The specific one was directly designed by us from the Gene Bank (Accession number: AJ417026). This primer pair was able to detect parasite DNA in the faeces the day 6 until 18, being negative thereafter. Comparison of EPG results with the PCR ones done with this primer pair indicated that PCR methodology was more sensitive as was expected. However, the substitution of this primer pair by the genus one was able to increase PCR's predictable higher sensitivity. In this case, *S. venezuelensis* DNA was detected for the first time also at day 6 but was continuously positive until day 32 that was the last time period of evaluation. It is important to stress that higher sensitivity of this primer pair was associated with the appearance of unspecific bands that were, however, easily identified by their distinct molecular weights. Calls attention the fact that even this more sensitive PCR was not able to detect the infection more precociously as was described by Wongratanacheewin et al. (2001),

in hamsters experimentally infected with the trematode *Opisthorchis viverrini*. We believe that this could be due to the fact that the experimental infection employed by us was initiated with a very high infective larvae inoculum. This probably triggered a simultaneous high production of eggs that achieved levels detected by the EPG methodology. The fact that PCR was positive throughout all the recovery period when EPG was only eventually positive suggests that PCR would be a better choice to make an early diagnosis avoiding evolution to a more severe on chronic disease. Interestingly, the more intense parasite DNA amplification bands coincided with the highest amount of parasite eggs. These findings shows that is viable in future studies setting up a quantitative PCR analysis, such as real time PCR, in order to estimate the worm burden.

In the second part of this investigation we asked if the tissue migration route of this parasite in Lewis rats was similar to the one observed in other rodents. We also evaluated if PCR was more sensitive than traditional histological technique to detect migration of the parasite throughout the different tissues. This histopathological analysis showed that from the subcutaneous infection site the larvae penetrated among the muscular fibers and then reached the lungs. This pulmonary stage occurred between 48 and 72 hours post-infection and has been described as the most relevant step in the strongyloidiasis cycle. According to Tindall and Wilson (1988) during this stage in the lungs the larvae molt to the 4^{th} stage.

At 120h the larvae were found inserted at the small intestine vilosities and this coincided with their disappearance from the lungs. In a general way these findings demonstrated that the intracorporeal migration route of *S. venezuelensis* in the Lewis rat was similar to the one observed in other rodents (Sato and Toma 1990; Takamure 1995; Matsuda et al. 2003). An interesting aspect revealed by this study was the absence of brain involvement during the parasite migration throughout the tissues in the Lewis rat. This possibility was investigated based on the work of Koga et al. (1998) that detected parasites in the brain,

cerebellum, olfactory bulbs and nasal mucosa 40 hours after infection of Wistar rats with 5000 L3 of *S. ratti* by subcutaneous route, at the abdominal region. The comparison of the results obtained by histological analysis and PCR technique lead us to conclude that both techniques were efficient to detected *S. venezuelensis* in tissues and organs from Lewis rats.

Acknowledgments

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References

Baek BK, Islam MK, Kim JH, Lee JW (1999) Partial cross-resistance *Strongyloides venezuelensis* and *Nippostrongylus brasiliensis* in rats. Korean J Parasitol 37:101-107.

Dorris M, Viney ME, Blaxter ML (2002) Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. Int J Parasitol 32:1507-1517.

Duenngai K, Sithithaworn P, Rudrappa UK, Iddya K, Laha T, Stensvold CR, Strandgaard H, Johansen MV (2008) Improvement of PCR for Detection of *Opisthorchis viverrini* DNA in Human Stool Samples. J Clin Microbiol 46:366-368.

Esteban-Redondo I, Maley SW, Thomson K, Nicoll S, Wright S, Buxton D, Innes EA (1999) Detection of *T. gondii* in tissues of sheep and cattle following oral infection. Vet Parasitol 86:155-171.

Gasser RB (1999) PCR-based technology in veterinary parasitology. Vet Parasitol 84:229-258.

Genta RM, Ward PA (1980) The histopathology of experimental strongyloidiasis. Am J Patol 99:207-220.

Gordon H.M, Whitlock HV (1939) A new technique for counting nematode eggs in sheep faeces. J Council Sci Ind Res 12:50-52.

Kimura K, Shintoku Y, Kadosaka T, Fujiwara M, Kondo S, Itoh M (1999) A second peak of egg excretion in *Strongyloides ratti*-infected rats: its origin and biological meaning. Parasitology 119:221-226.

Koga M, Huang X, Noda K, Tada I (1998) *Strongyloides ratti*: histological study of the descending route of larvae from the head in experimentally infected rats. Parasitol Int 47:219-224.

Ly MN, Bethel SL, Usmani AS, Lambert DR (2003) Cutaneous *Strongyloides stercoralis* infection: An unusual presentation. J Am Acad Dermatol 49:157-160.

Matsuda K, Kim BS, Whang IS, Lim CW, Baek BK (2003) Migration of *Strongyloides venezuelensis* in rats after oral inoculation of free-living infective larvae. J Vet Med Sci 65:971-975.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (ed) Bioinformatics methods and protocols: methods in molecular biology, Totowa, Humana Press, NJ, pp 365-386

Sandoval N, Siles-Lucas M, Alban JL, Perez-Arellano JL, Garate T, Muro A (2006) *Schistosoma mansoni*: A diagnostic approach to detect acute schistosomiasis infection in a murine model by PCR. Exp Parasitol 114:84-88.

Sato Y, Toma H (1990) *Strongyloides venezuelensis* infection in mice. Int J Parasitol 20:57-62.

Siddiqui AA, Berk SL (2001) Diagnosis of *Strongyloides stercoralis* infection. Clin Infect Dis 33:1040-1047.

Silveira MR, Nunes KP, Cara DC, Souza DG, Corrêa AJr, Teixeira MM, Negrão-Corrêa D (2002) Infection with *Strongyloides venezuelensis* induces transient airway eosinophilic

inflammation, an increase in immunoglobulin E, and hyperrresponsiveness in rats. Infect Immunol 70:6263-6272.

Taira N, Nakamura Y, Almeida MAO, Saeki H (1995) Massive experimental infection with *Strongyloides venezuelensis* in rats and absence of sudden death. J Vet Med Sci 57:855-858.

Takamure A (1995) Migration route of *Strongyloides venezuelensis* in rodents. Int J Parasitol 25:907-911.

Tindall NR, Wilson PA (1988) Criteria for a proof of migration routes of immature parasites inside hosts exemplified by studies of *Strongyloides ratti* in the rat. Parasitology 96:551-563.

Wongratanacheewin S, Pumidonming W, Sermswan RW, Maleewong W (2001) Development of a PCR-based method for detection of *Opisthorchis viverrini* in experimentally infected hamsters. Parasitology 122:175-180.

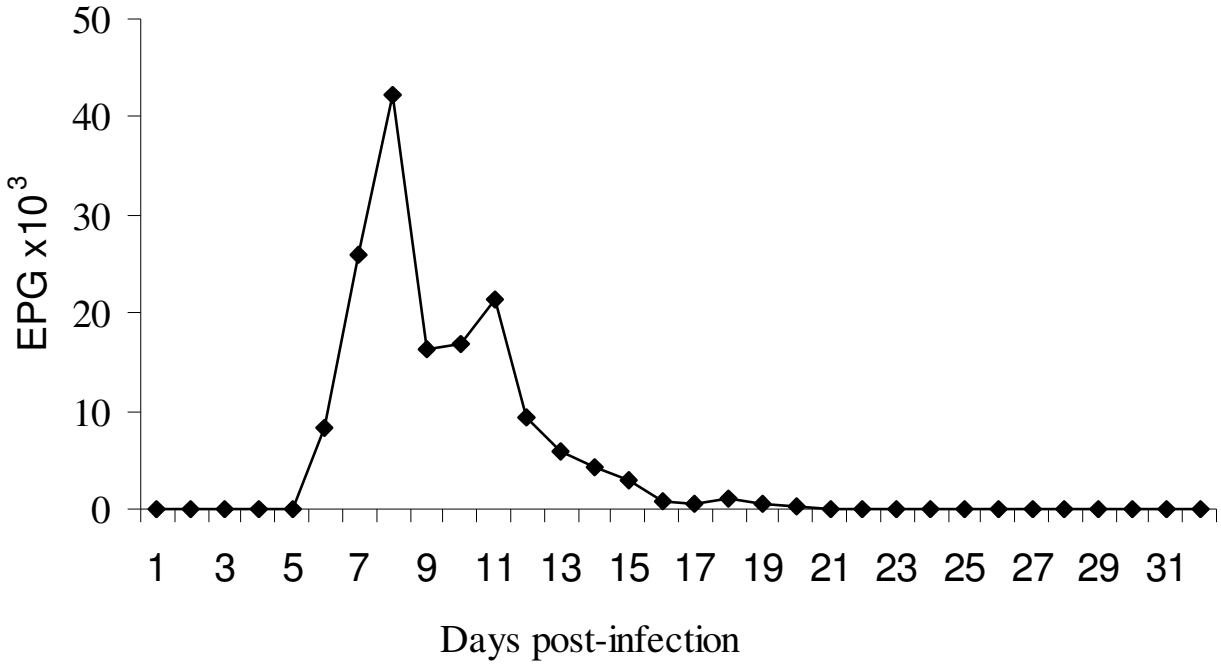

Figure 1- Eggs per gram of feces (EPG) of Lewis rats subcutaneously inoculated with 4000 infective larvae of *Strongyloides venezuelensis*.

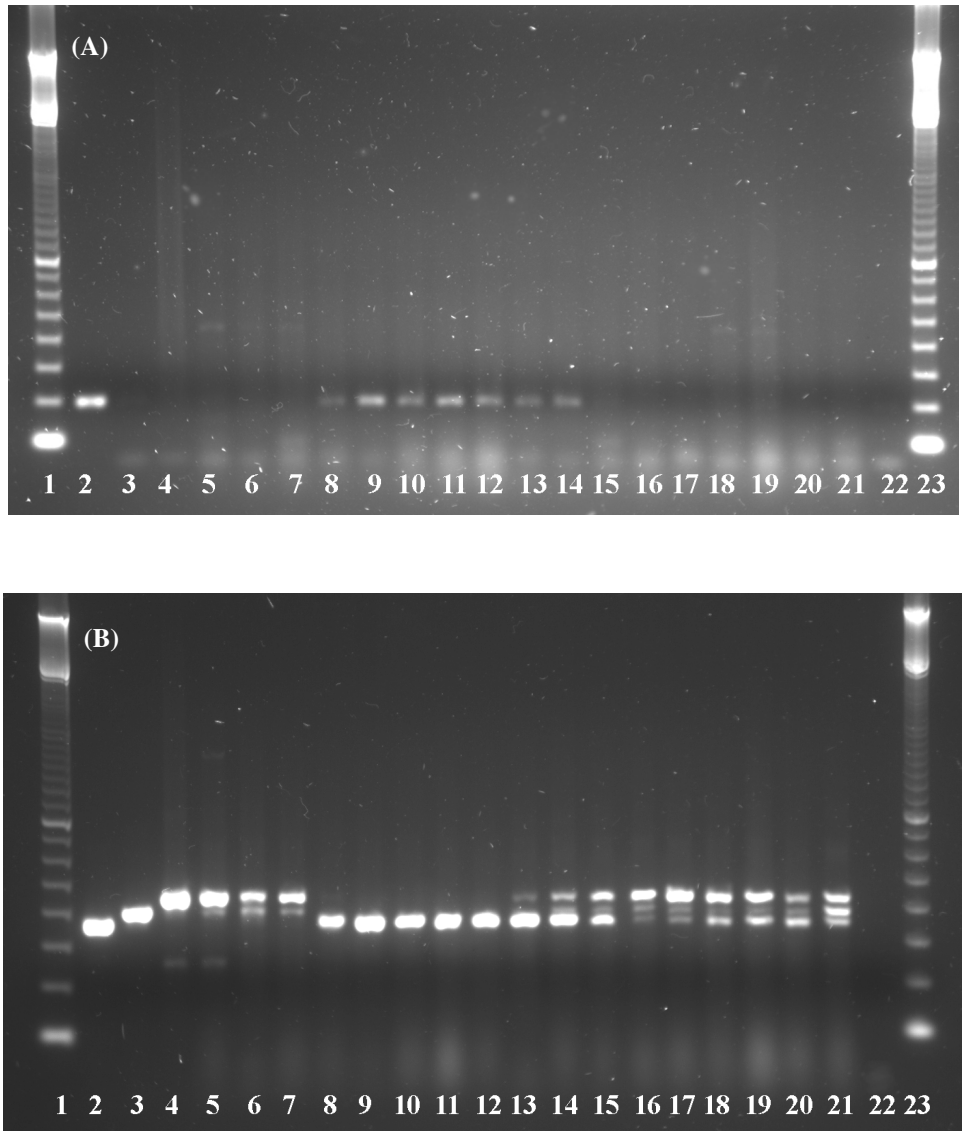

Figure 2- PCR performed with the specific primer pair (**A**). Lanes 1 and 23- 100 bp- ladder (GE Healthcare); Lane 2 – 198 bp (*S. venezuelensis* DNA); Lane 3 – no amplification (*Syphacia muris* DNA); Lane 4 – no amplification (spleen rat DNA); Lanes 5 to 7 - days 0, 2 and 4 - no amplification; Lanes 8 to 14 - days 6 to 18- 198 bp (*S. venezuelensis* DNA); Lane 22 (without DNA). PCR performed with genus primer pair (**B**). Lanes 1 and 23 - 100 bp-ladder (GE Healthcare); Lane 2 – 340 bp (*S. venezuelensis* DNA); Lane 3 - 380 bp (*Syphacia muris* DNA); Lane 4 – 420 bp (spleen rat DNA); Lanes 5 to 7 -days 0, 2 and 4 – 380 bp (*S. muris* DNA) and 420 bp (rat DNA); Lanes 8 to 12- days 6 to 14 - 340 bp (*S. venezuelensis* DNA); Lane 13 - day 16- 340 bp (*S. venezuelensis* DNA) and 420 bp (rat DNA); Lanes 14 to 21 – days 18 to 32 - 340 bp (*S. venezuelensis* DNA), 380 bp (*S. muris* DNA) and 420 bp (rat DNA); Lane 22 (without DNA).

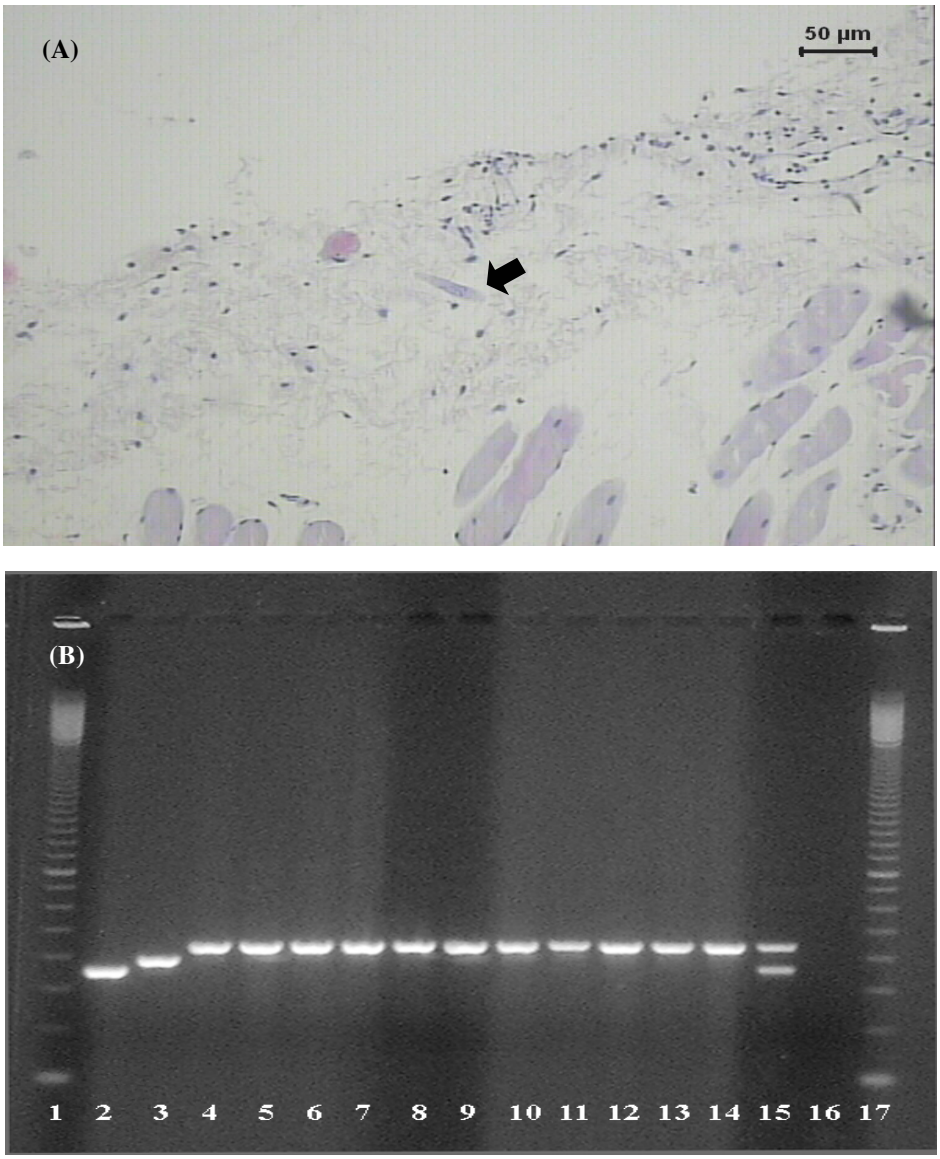

Figure 3- Histological analysis of skin section (30 min group) stained with H&E showing parasite larvae (**A**). PCR products using genus primer pair in tissue samples (**B**). Lanes 1 and 17 - 100 bp- ladder (GE Healthcare); Lane 2 - 340 bp (*S. venezuelensis* DNA); Lane 3 - 380 bp (*Syphacia muris* DNA); Lane 4 - 420 bp (spleen rat DNA); Lanes 5 to 14 - 420 bp - DNA from spleen (5), brain (6), lung (7), muscular layer (8), liver (9), lymph node popliteal (10), small intestine (11), vastus lateralis muscle (12), lymph node inguinal (13), heart (14); Lane 15 - 340 bp (*S. venezuelensis* DNA) and 420 bp - DNA from skin; Lane 16 (without DNA).

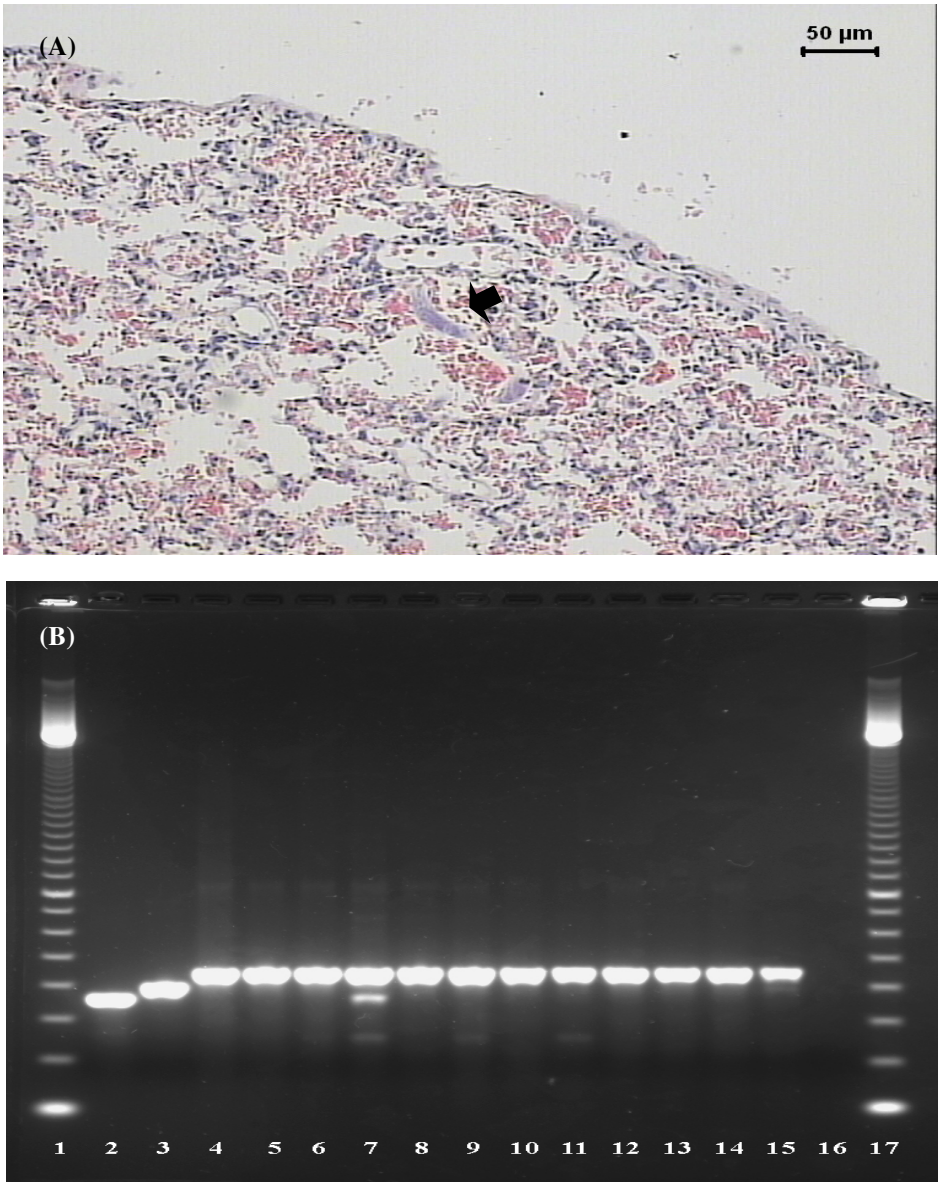

Figure 4- Histological analysis of lung section (48h group) stained with H&E showing the presence of the migrating larvae (**A**). PCR products using genus primer pair in tissue samples (**B**). Lanes 1 and 17 - 100 bp- ladder (GE Healthcare); Lane 2 - 340 bp (*S. venezuelensis* DNA); Lane 3 - 380 bp (*Syphacia muris* DNA); Lane 4 - 420 bp (spleen rat DNA); Lanes 5, 6 and 8 to 15 - 420 bp - DNA from spleen (5), brain (6), muscular layer (8), liver (9), lymph node popliteal (10), small intestine (11), vastus lateralis muscle (12), lymph node inguinal (13), heart (14), skin (15); Lane 7 – 260 bp (unspecific band), 340 bp (*S. venezuelensis* DNA) and 420 bp - DNA from lung; Lane 16 (without DNA).

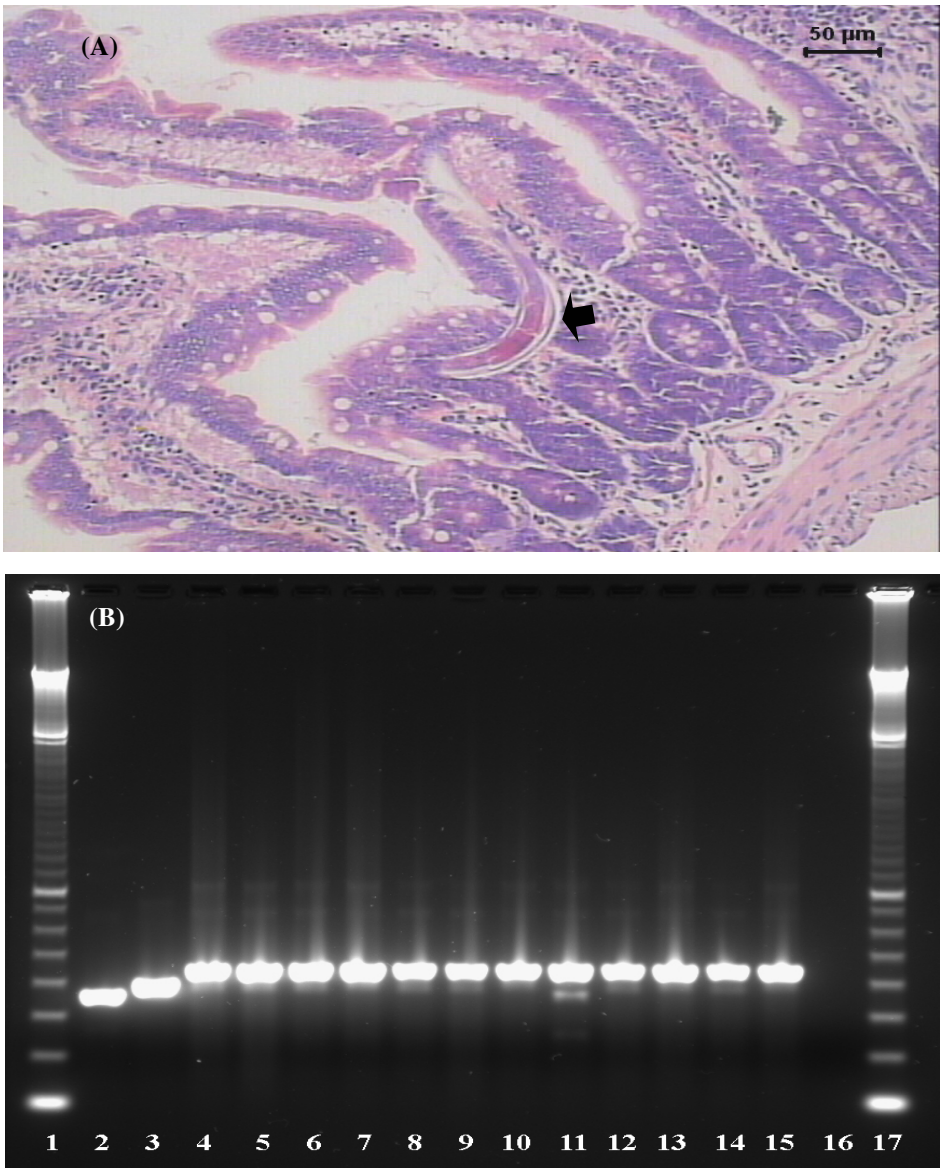

Figure 5- Histological analysis of small intestine section (120h group) stained with H&E showing the presence parasite larvae (**A**). PCR products using genus primer pair in tissue samples (**B**). Lanes 1 and 17 - 100 bp- ladder (GE Healthcare); Lane 2 - 340 bp (*S. venezuelensis* DNA); Lane 3 - 380 bp (*Syphacia muris* DNA); Lane 4 - 420 bp (spleen rat DNA); Lanes 5 to 10 and 12 to 15 - 420 bp - DNA from spleen (5), brain (6), lung (7), muscular layer (8), liver (9), lymph node popliteal (10), vastus lateralis muscle (12), lymph node inguinal (13), heart (14), skin (15); Lane 11 - 340 bp (*S. venezuelensis* DNA) and 420 bp – DNA from small intestine; Lane 16 (without DNA).

9. CONCLUSÕES

9. CONCLUSÕES

- A técnica de PCR com a utilização do par de *primers* gênero (DORRIS et al., 2002) demonstrou maior sensibilidade que a técnica de McMaster (modificada), principalmente nos animais com infecção leve (40 L3);

- A PCR constitui-se em importante ferramenta para monitorar e diagnosticar animais infectados experimentalmente com diferentes cargas parasitárias;

- Ratos machos da linhagem Lewis infectados com 4000 e 400 L3 demonstraram maior suscetibilidade à infecção por *Strongyloides venezuelensis* em relação às fêmeas;

- A dinâmica da infecção em ratos apresentou período pré-patente de seis dias com dois picos de elimanação de ovos (8° e 11° dias) e perdurou até o 32º dia após a infecção (com a detecção de fêmeas no intestino delgado e de ovos nas fezes).

- Ambos os anticorpos específicos IgG1 e IgG2b apresentaram nível elevado na fase aguda.

- A predominância de IgG1 em relação a IgG2b e a produção de IgE e IL-10 na fase de recuperação na infecção em ratos demonstra um padrão de resposta Th2;

- A resposta humoral e celular da linhagem Lewis apresentou um comportamento similar ao descrito para outras linhagens de ratos, podendo portanto, ser também um modelo experimental para o estudo da estrongiloidíase em humanos.

- Após a infecção subcutânea com 4000 L3, a rota das larvas teve início na pele e na camada muscular (30 minutos), seguindo para o pulmão (48 e 72 horas) e para o intestino delgado (120 horas), onde o parasita se estabeleceu posteriormente;

- As técnicas de Histologia e PCR foram igualmente eficientes na detecção de larvas nas amostras de órgãos e tecidos dos ratos analisados.

- A PCR demonstrou maior sensibilidade que a técnica de McMaster (modificada) na detecção de *S. venezuelensis* em amostras fecais durante os 32 dias de infecção;