Comparison of parasitological, immunological and molecular methods for evaluation of fecal samples of immunosuppressed rats experimentally infected with *Strongyloides venezuelensis*

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SUMMARY

Definitive diagnosis of strongyloidiasis in humans is typically achieved by detection of larvae in fecal samples. However, limitations on sensitivity of parasitological methods emphasize the need for more robust diagnostic methods. The aim of this study was to compare the diagnostic value of three methods: eggs per gram of feces (EPG), coproantigen detection by enzyme linked immunosorbent assay (ELISA), and DNA detection by conventional polymerase chain reaction (PCR). The assays were performed at 0 and 5, 8, 13, 21 and 39 days post-infection (dpi) using fecal samples from experimentally infected immunocompetent and immunosuppressed rats. In immunocompetent rats, eggs were detected in feces on days 5, 8 and 13 dpi; coproantigen detection and PCR amplification were successful at all post-infection time points (5, 8, 13, 21 and 39 dpi). In immunosuppressed rats, eggs were detected at 5, 8, 13 and 21; coproantigen detection and PCR amplification were successful at all post-infection time points. In conclusion, these results suggest that coproantigen detection and PCR may be more sensitive alternatives to traditional methods such as EPG for diagnosis of *Strongyloides venezuelensis* infection.

Key words: diagnosis, fecal antigen, immunosuppression, PCR, strongyloidiasis, *Strongyloides venezuelensis*.

INTRODUCTION

Strongyloidiasis is a widespread infection that affects 30–100 million people across 70 countries, mainly in tropical and subtropical regions of the world (Genta, 1989; Puthiyakunnon *et al*. 2014). *Strongyloides venezuelensis* naturally infects rodents, and has been used as an experimental intestinal parasite model (Rodrigues *et al*. 2013; Yasuda *et al*. 2014; Corral *et al*. 2015). In experimental infections, infective-stage of *S. venezuelensis* larvae migrates to the lungs before implanting in the duodenal mucosa. In this way, migration in the rodent host is similar to that of *Strongyloides stercoralis* in humans (Negrão-Corrêa *et al*. 2003; Ferreira *et al*. 2009). Thus parasite has been used as a convenient tool for development and validation of more sensitive procedures for human strongyloidiasis diagnosis (Gonçalves *et al*. 2008, 2010).

Immunological assays, such as enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test and western blotting are useful for evaluating the host immune response and diagnosing asymptomatic cases and for epidemiological surveys (Paula and Costa-Cruz, 2011; Levenhagen and Costa-Cruz, 2014; Gottardi *et al*. 2015). The principal limitation in establishing such specific methods is the difficulty of obtaining infective *S. stercoralis* larvae. Parasite antigen detection in fecal samples by immunological assays may contribute to earlier diagnosis, which is particularly an important consideration for patients with increased risk of strongyloidiasis, such as immunocompromised individuals (Bailey, 1989; Gonçalves *et al*. 2012*a*).

DNA technology has greatly impacted many areas of parasitology, including infection diagnosis (Wang and Gao, 2014). Polymerase chain reaction (PCR) has broad applicability, mainly because its high sensitivity allows the amplification of DNA fragments from minute amounts of parasite material (Gasser, 1999; Saugar *et al*. 2015). Several reports have shown high PCR sensitivity for detection of parasite DNA in various biological sample types (i.e. feces, tissues) and from different host species (Esteban-Redondo *et al*. 1999; Wongratanacheewin *et al*. 2001; Sandoval *et al*. 2006; Marra *et al*. 2010).

The aim of this study was to compare the diagnostic value of three diagnostic methods: the eggs per

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gram of feces (EPG) technique, the detection of coproantigens by ELISA and DNA amplification/detection by conventional PCR. We tested these methods on feces samples of both immunocompetent and immunosuppressed rats that were experimentally infected with *S. venezuelensis*.

MATERIALS AND METHODS

Animals

Male *Rattus norvegicus* (Wistar) weighing 100–120 g were maintained in the *Centro de Experimentação e Utilização de Animais, UFU* provisioned with laboratory chow and water available *ad libitum*.

Parasites

Strongyloides venezuelensis third-stage infective larvae (L3) were obtained from charcoal cultures of infected rat feces. The cultures were stored at 28 °C for 48 h, and the infective larvae were collected and concentrated using the Rugai method (Rugai *et al*. 1954). For infection, each rat was subcutaneously inoculated with 1500 *S. venezuelensis* L3 larvae.

Experimental groups

The rats were divided into four groups: immunocompetent infected rats ($n = 30$), immunosuppressed infected rats ($n = 30$), control immunocompetent ($n = 6$) and control immunosuppressed ($n = 6$). Prior to infection, immunosuppressed groups received 5 μg mL^{-1} of dexamethasone disodium phosphate in water for 5 days, as previously described by Romand *et al*. (1998). Animals from the infected groups were then inoculated subcutaneously with 1500 L3 *S. venezuelensis* larvae. Rats were examined in groups of 6 at 0 and 5, 8, 13, 21 and 39 days post-infection (dpi). Each fecal sample was divided into three subsamples. The first subsample was submitted to the Cornell-MacMaster quantitative method (Gordon and Whitlock, 1939) the others were stored at −70 °C for later coproantigen detection assay (ELISA) and PCR. Fecal samples used for the ELISA were prepared prior to assay by adding 0·15 M phosphate buffer pH 7·4 (PBS, containing 0·3% Tween 20) at a ratio of 1:1.

EPG

Experimentally infected rats were placed individually on clean absorbent paper moistened with distilled water, and allowed to defecate over an 18 h period at the following time points: 5, 8, 13, 21 and 39 dpi. The feces were then collected, diluted in saturated saline and homogenized. The supernatant was placed in a McMaster chamber, and the number of EPG was estimated using the Cornell-McMaster method (Gordon and Whitlock, 1939).

Production of immune serum and secondary antibodies

For the production of immune serum, two rabbits were immunized with crude larval somatic antigen as described by Gonçalves *et al*. (2008). Briefly, the rabbits were inoculated with 500 μg mL^{-1} of alkaline extract of *S. venezuelensis* L3 larvae emulsified in Freund's complete adjuvant in the first immunization. Inoculations were made at intervals of 2 weeks after the first immunization, using Freund's incomplete adjuvant. Blood samples were collected by auricular puncture, at intervals of 2 weeks, for evaluation of the antibody titers. Three months following immunization, when a high antibody titer (15 000) was detected, the animals were submitted to a cardiac puncture and blood was collected and an Immunoglobulin G (IgG) fraction was prepared using a Protein A Sepharose CL-4B column. After testing IgG activity against the specific antigen, a portion of the IgG fraction was stored at −20 °C to be used as capture antibody in ELISA. The other portion was conjugated with horseradish peroxidase (Sigma) according to Wilson and Nakane, (1978).

Coproantigen detection by ELISA

ELISA was performed according to Gonçalves *et al*. (2010). Briefly, high-binding microtiter plates (Corning-Costar; Laboratory Sciences Company, New York, NY) were coated with 40 μg mL^{-1} of anti-*S. venezuelensis* IgG in 0·06 M carbonate bicarbonate buffer (pH 9·6) and stored overnight at 4 °C. Plates were washed three times for 5 min each with PBS containing 0·05% Tween 20 (PBS-T). After washing, plates were incubated with fecal supernatant (1:8 dilution) for 45 min at 37 °C. The secondary antibody consisting of peroxidase-labelled rabbit anti-*S. venezuelensis* IgG at the optimal dilution of 1:40 was added, and the plates were incubated again for 45 min at 37 °C. The reaction was revealed by adding the enzyme substrate (0·03% H_2O_2 and o-phenylenediamine [OPD] in 0·1 M citrate–phosphate buffer, pH 5·0) with a final incubation for 15 min at room temperature. The reaction was stopped by adding 2 N H_2SO_4 and the optical density (OD) was determined at 492 nm using a plate reader (Titertek Multiskan; Flow Laboratories, McLean VA). Results were arbitrarily expressed as ELISA index (EI), according to the following formula: EI = OD sample/cut-off, where the cut-off was established as the mean OD of three negative control sera plus two standard deviations and EI > 1·0 was considered positive.

DNA extraction and PCR

Genus-specific primer pairs were utilized to amplify the small subunit ribosomal RNA gene, as described by Dorris *et al*. (2002) to analyze several species within the genus *Strongyloides* (Forward

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5′-AAAGATTAAGCCATGCATG-3′; Reverse 5′-GCCTGCTGCCTTCCTTGGA-3′). The QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) was used to extract DNA from feces pool samples following the manufacturer's instructions. DNA from *S. venezuelensis* L3 larvae was used as positive control in all PCR reactions. DNA from feces of non-infected rats and adult *Syphacia muris* specimens (nematodes commonly recovered from rat DNA) was used as controls to identify similar and dissimilar bands amplified by the primers. A blank tube (PCR mix without DNA) was used as negative control.

PCR was performed in a Master Cycler ep Gradient S thermocycler (Eppendorf, Hamburg, Germany). Samples containing 10 mM dNTPs, 0·4 mM each primer, 1·5 mM MgCl2, 50 mM KCl, pH 8·3, 0·1 U of Taq polymerase® (5 U μL^{-1}, Invitrogen, Life Technologies) and 100 ng of genomic DNA for a final reaction volume of 10 μL. PCR program (cycle specifications) were chosen according to Marra *et al.* (2010). PCR products were run on 5% polyacrylamide gels in 1× Tris-Borate-EDTA (TBE) buffer (Freitas *et al.* 2004). Direct sequencing of PCR products were performed using an automatic sequencer (ABI 3500 genetic analyzer DNA Sequencer, Applied Biosystems, Thermo Fisher Brand, Foster City, CA) and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Brand, Foster City, CA) according to the manufacturer's instructions. The sequences obtained were aligned using the BioEdit program (Biological Sequence Alignment Editor) (http://www.mbio.ncsu.edu/bioedit/page2.html) and compared with previously reported sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/tbl2asn2), using Basic Local Alignment Search Tool.

Statistical analysis

Eggs were counted and expressed as mean ± standard error of the mean (S.E.M.) percentages and were calculated using Excel. Each experiment was performed in duplicate. Data were analysed using one-way analysis of variance (ANOVA), followed by a Bonferroni test. The level of statistical significance was set at $P < 0{\cdot}05$.

RESULTS

The initial time point for egg shedding was 5 dpi for both immunocompetent and immunosuppressed rats, reaching a peak at 8 dpi and decreasing at 13 dpi in both groups. Immunosuppressed rats presented higher egg counts at day 8 ($P < 0{\cdot}001$) and 13 ($P < 0{\cdot}05$) compared with immunocompetent rats. *Strongyloides venezuelensis* eggs were not detected in the feces of immunocompetent rats beyond 21 dpi, or in immunosuppressed rats beyond 39 dpi (Fig. 1).

The antigen detection of immunocompetent and immunosuppressed rats was compared. In immunocompetent rats, coproantigen detection was positive during the entire kinetics (Fig. 2) and antigen was best detected on 13 dpi ($P < 0{\cdot}01$); at 21 and 39 dpi, a lower EI was observed probably due to the reduction of antigen in feces as a result of elimination of the infection. In immunosuppressed animals, similar results were observed on 8–21 dpi and a lower EI was observed on 39 dpi.

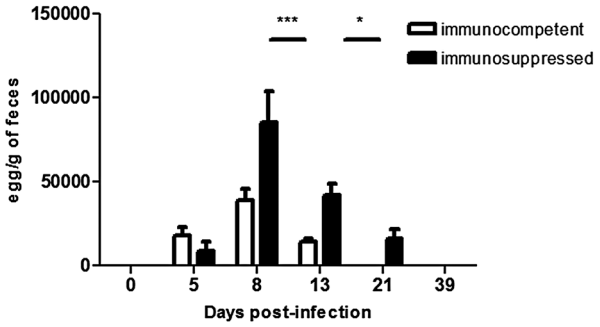

Fig. 1. Total number of eggs per gram in feces of immunocompetent and immunosuppressed rats infected with *Strongyloides venezuelensis* on 0 and 5, 8, 13, 21 and 39 dpi *$P < 0{\cdot}05$ and ***$P < 0{\cdot}001$. Abbreviation: dpi, days post-infection.

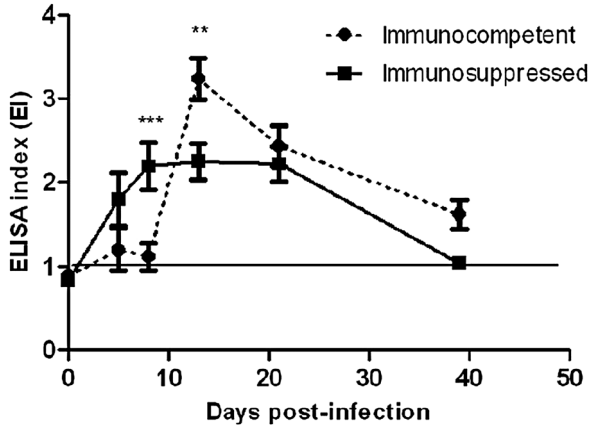

Fig. 2. Detection of coproantigen, with antigen levels expressed as ELISA-index in fecal samples from immunocompetent and immunosuppressed rats infected with *Strongyloides venezuelensis* on 0 and 5, 8, 13, 21 and 39 dpi **$P < 0{\cdot}01$ and ***$P < 0{\cdot}001$. Fecal supernatants were diluted 1:8 and incubated with anti-L3 polyclonal antibody. Abbreviations: dpi, days post-infection; ELISA, enzyme-linked immunosorbent assay.

In the PCR assay, a DNA fragment of approximately 405 base pairs corresponding to the positive control (L3 larvae of *S. venezuelensis*) was amplified. The primers amplified bands that were not associated with a specific parasite, although they were distinguishable from the positive control. PCR products (amplified bands from pooled fecal samples) from both immunocompetent and immunosuppressed rats are shown in Fig. 3. PCR amplification produced sufficient bands throughout the post-infection period in both groups.

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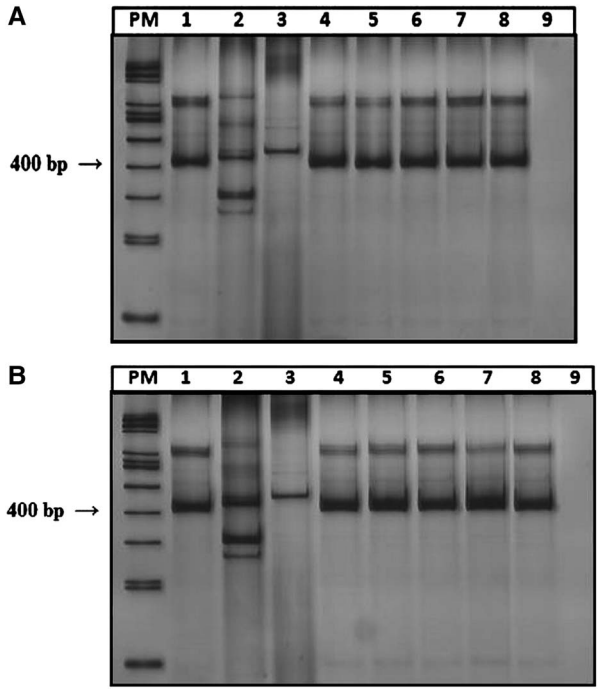

Fig. 3. Electrophoretic profiles of PCR products from fecal samples of rats infected with *Strongyloides venezuelensis*. (A) Immunocompetent and (B) immunosuppressed groups. MW lane: 100 base pairs ladder (Life Technologies); Lane 1: ~405 base pairs (positive control, *S. venezuelensis* L3 DNA); Lane 2: ~440 base pairs (corresponding to the *Syphacia muris*); Lane 3: ~480 base pairs (non-infected *Rattus norvegicus* fecal sample day 0); Lanes (4–8): ~405 base pairs. (pooled DNA from fecal samples collected 5, 8, 13, 21 and 39 dpi. Lane 9: negative control (without DNA). Abbreviations: dpi, days post-infection; PCR, polymerase chain reaction; MW, molecular weight.

DNA sequences obtained from filariform *S. venezuelensis* larvae and feces samples in both experimental groups showed 100% similarity with *S. venezuelensis* gene for 18S small subunit ribosomal RNA (AB923887.1) in GenBank (Fig. 4).

The comparison of the three diagnostic methods shows that for immunocompetent individuals, egg shedding was highest at 8 dpi, coproantigen detection by ELISA was highest at 13 dpi, and PCR amplification produced sufficient bands throughout the post-infection period. In the immunosuppressed group, egg shedding was also highest at 8 dpi, coproantigen detection by ELISA was highest at 8–21 dpi, and PCR amplification also produced sufficient bands throughout the post-infection period. Table 1 summarizes the comparison between parasitological, immunological and molecular methods for fecal samples from experimentally infected immunocompetent and immunosuppressed rats.

DISCUSSION

The detection of *S. venezuelensis* antigens in stool samples by ELISA may contribute to accuracy diagnostic, an important consideration for immunocompromised groups due to higher potential risk for severe forms of strongyloidiasis (Gonçalves *et al.* 2010). The coproantigen technique is more sensitive than direct observation of eggs because the antigen was detected in feces up to 39 dpi in both groups. In immunosuppressed rats, the timing of egg elimination was affected by dexamethasone disodium phosphate treatment, in which infection was more severe and long lasting. Peak egg shedding occurred at 8 dpi in both groups, with a decrease in egg counts at the end of the post-infection period. Similar results were shown by Nakai and Amarante (2001), Gonçalves *et al.* (2010) and Paula *et al.* (2013).

Coproantigen detection had higher positive detection of *S. venezuelensis* infection compared with the EPG method, particularly in immunosuppressed animals, in concordance with results obtained by Gonçalves *et al.* (2010). Diagnosis using polyclonal antibodies has also been investigated by different authors, and the results revealed potential for the development of a financially viable kit for rapid coproantigen detection (Nageswaran *et al.* 1994; Gonçalves *et al.* 2010; Sykes and McCarthy, 2011).

Molecular analysis of the subunit 18S ribosomal RNA gene region has been used as a target for phylogenetic analysis, especially for intestinal parasites (Dorris *et al.* 2002). In the present study, analysis revealed that the same region amplified a 405 base pairs fragment in fecal samples from both immunocompetent and immunosuppressed rats throughout the duration of infection. The fragment was larger than described by Marra *et al.* (2010) and Paula *et al.* (2013), despite using the same set of primers; this may be due to greater separation sensitivity of the polyacrylamide gel, which is usually 10 times higher than agarose gel method (Freitas *et al.* 2004). The amplification products from *S. venezuelensis* L3 larvae and feces samples from both experimental groups showed high sequence similarity to ribosomal RNA genes of *Strongyloides* species.

PCR using genus-specific primers may function as sensitive methods for detection of *S. venezuelensis* genetic material in feces of rats with low parasite load (Nakai and Amarante, 2001, Marra *et al.* 2010; Paula *et al.* 2013) and it was demonstrated that the detection of *S. venezuelensis* in fecal samples showed higher sensitivity in the PCR assay than the parasitological methods. In this study, specific amplification occurred until 39 dpi in both, immunocompetent and immunosuppressed rat groups.

Coproantigen detection and PCR amplification showed similar results in the immunosuppressed group, perhaps a reflection of greater susceptibility to *S. venezuelensis* infection. According to Wallen *et al.* (1991) and Gonçalves *et al.* (2012*b*) the glucocorticoids inhibit the activation, proliferation and survival of inflammatory cells such as eosinophils and mast cells, and block the release of IL-4, IL-5

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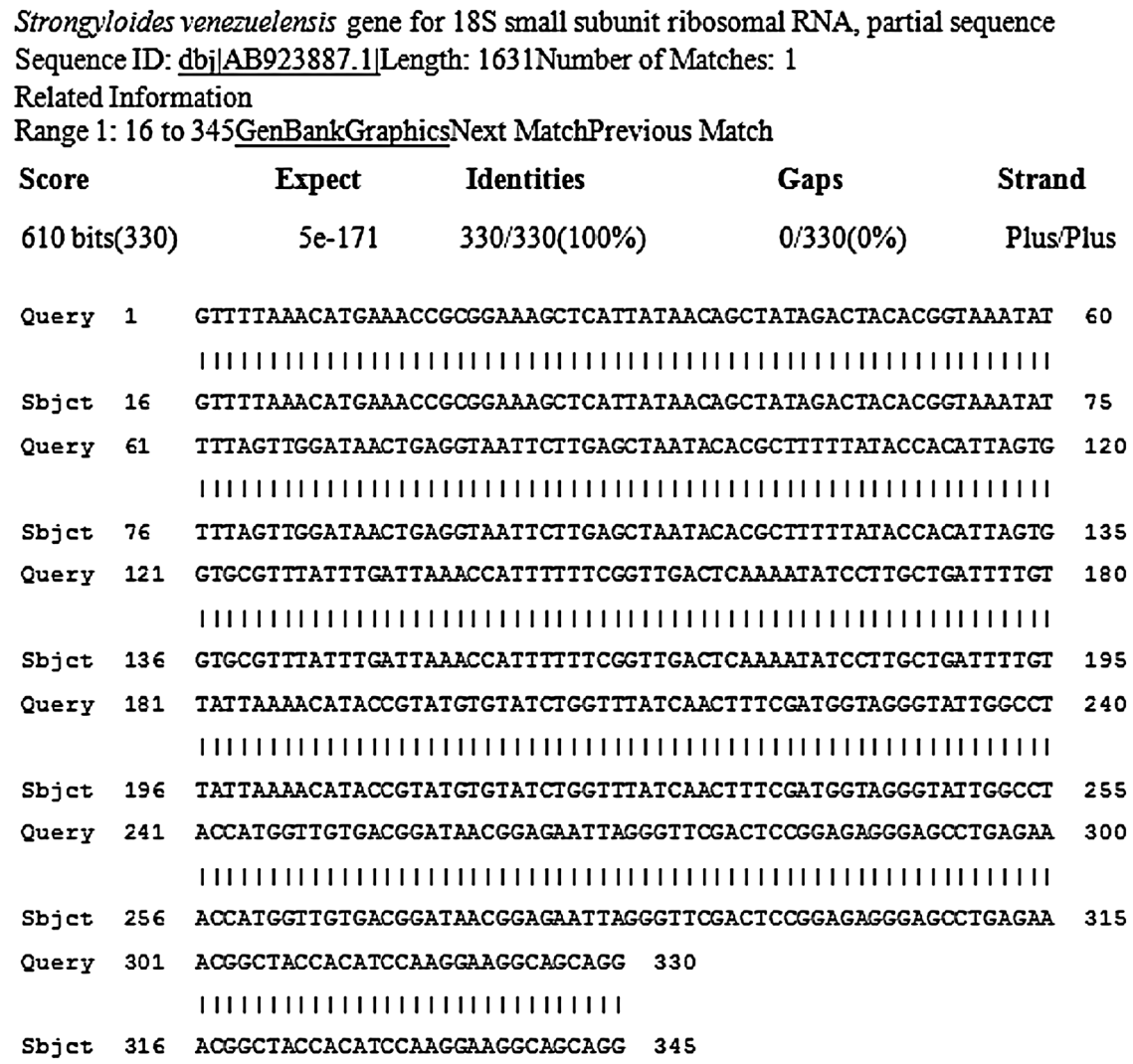

Fig. 4. Alignment of products amplification from *Strongyloides venezuelensis* L3 larvae and feces samples from experimental groups with gene sequence of the subunit 18S rRNA of *S. venezuelensis* deposited in NCBI database. Abbreviations: NCBI, National Center for Biotechnology Information.

Table 1. Comparison of eggs per gram of feces (EPG), coproantigens detected by enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) amplified bands using fecal samples from immunocompetent and immunosuppressed rats infected with *Strongyloides venezuelensis*

Immunocompetent				Immunosuppressed			
Post-infection days	EPG	Coproantigen	PCR	Post-infection days	EPG	Coproantigen	PCR
0	–	–	–	0	–	–	–
5	+	+	+	5	+	+	+
8	+	+	+	8	+	+	+
13	+	+	+	13	+	+	+
21	–	+	+	21	+	+	+
39	–	+	+	39	–	+	+

+, positive result; –, negative result.

and IL-13 cytokines involved in response to helminthic infections.

The comparative results of the EPG method and the coproantigen detection by ELISA showed to be similar to the results of Gonçalves *et al*. (2010), for diagnosis of *S. venezuelensis*, and also to the results of Gordon *et al*. (2012) and Brockwell *et al*. (2013), for diagnosis of *Fasciola hepatica*. The high

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sensitivity of PCR for diagnosis of *S. venezuelensis* infection, when compared with the EPG method, was previously demonstrated by Marra *et al*. (2010).

Results from the current study demonstrate the reliability and sensitivity of the coproantigen detection by ELISA, and of the PCR assay for diagnosis of experimental strongyloidiasis. These results could help to improve the diagnosis of human strongyloidiasis as alternatives for applying in epidemiological research, considering the importance of early diagnosis to prevent the possibility of chronicity and hyperinfection establishment in endemic areas.

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ETHICAL STANDARDS

Authors assert that all procedures contributing to this study comply with the ethical standards of laws of Brazil on the care and use of laboratory animals, in accordance with the ethical principles upon approval of the Comitê de Ética na Utilização de Animais of Universidade Federal de Uberlândia (CEUA/UFU no 004/08).

REFERENCES

Bailey, J. W. A. (1989). A serological test for the diagnosis of *Strongyloides* antibodies in ex Far East Prisoners of War. *Annals of Tropical Medicine and Parasitology* **83**, 241–247.

Brockwell, Y. M., Spithill, T. W., Anderson, G. R., Grillo, V. and Sangster, N. C. (2013). Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. *Veterinary Parasitology* **196**, 417–426.

Corral, M. A., Paula, F. M., Gottardi, M., Meisel, D. M., Chieffi, P. P. and Gryschek, R. C. (2015). Membrane fractions from *Strongyloides venezuelensis* in the immunodiagnosis of human strongyloidiasis. *Revista do Instituto de Medicina Tropical de São Paulo* **57**, 77–80.

Dorris, M., Viney, M. E. and Blaxter, M. L. (2002). Molecular phylogenetic analysis of the genus *Strongyloides* and related nematodes. *International Journal for Parasitology* **32**, 1507–1517.

Esteban-Redondo, I., Maley, S. W., Thomson, K., Nicoll, S., Wright, S., Buxton, D. and Innes, E. A. (1999). Detection of the *T. gondii* in tissues of sheep and cattle following oral infection. *Veterinary Parasitology* **86**, 155–171.

Ferreira, C. M., Pereira, A. T. M., Amaral, F. A., de Souza, R. S., Coelho, F. M., Souza, D. G., Negrão-Corrêa, D. and Teixeira, M. M. (2009). Mechanisms of the airway hyperresponsiveness induced by *Strongyloides venezuelensis* infection in rats: role of capsaicin-sensitive neurons. *Microbes and Infection* **11**, 315–320.

Freitas, M. A., Vianna, E. N., Martins, A. S., Silva, E. F., Pesquero, J. L. and Gomes, M. A. (2004). A single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba dispar*. *Parasitology* **128**, 625–628.

Gasser, R. B. (1999). PCR-based technology in veterinary parasitology. *Veterinary Parasitology* **84**, 229–258.

Genta, R. M. (1989). Global prevalence of strongyloidiasis: critical review with epidemiologic insights into the prevention of disseminated disease. *Journal of Infectious Diseases* **11**, 755–767.

Gonçalves, A. L. R., Rodrigues, R. M., Silva, N. M., Gonçalves, F. A., Cardoso, C. R., Beletti, M. E., Ueta, M. T., Silva, J. S. and Costa-Cruz, J. M. (2008). Immunolocalization and pathological alterations following *Strongyloides venezuelensis* infection in the lungs and the intestine of MHC class I or II deficient mice. *Veterinary Parasitology* **158**, 319–328.

Gonçalves, A. L. R., Silva, C. V., Ueta, M. T. and Costa-Cruz, J. M. (2010). A new faecal antigen detection system for *Strongyloides venezuelensis* diagnosis in immunosuppressed rats. *Experimental Parasitology* **125**, 338–341.

Gonçalves, A. L. R., Rocha, C. A., Gonzaga, H. T., Gonçalves-Pires, M. do R., Ueta, M. T. and Costa-Cruz, J. M. (2012*a*). Specific IgG and IgA to larvae, parthenogenetic females and eggs of *Strongyloides venezuelensis* in the immunodiagnosis of human strongyloidiasis. *Diagnostic Microbiology and Infectious Diseases* **72**, 79–84.

Gonçalves, A. L. R., Ribeiro, T. S., Silva, C. V., Ueta, M. T. and Costa-Cruz, J. M. (2012*b*). A novel approach based on antigen, antibody and immune complex detection in bronchoalveolar lavage fluid samples from rats experimentally infected with *Strongyloides venezuelensis*. *Acta Tropica* **124**, 166–169.

Gordon, D. K., Zadoks, R. N., Stevenson, H., Sargison, N. D. and Skuce, P. J. (2012). On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. *Veterinary Parasitology* **187**, 436–444.

Gordon, H. M. and Whitlock, H. V. A. (1939). New technique for counting nematode eggs in sheep faeces. *Journal of the Council for Scientific and industrial Research* **12**, 50–52.

Gottardi, M., Paula, F. M., Corral, M. A., Meisel, D. M., Costa, S. F., Abdala, E., Pierrotti, L. C., Yamashiro, J., Chieffi, P. P. and Gryschek, R. C. (2015). Immunofluorescence assay for diagnosis of strongyloidiasis in immunocompromised patients. *Infectious Diseases* (*London, England*) **2**, 1–5.

Levenhagen, M. A. and Costa-Cruz, J. M. (2014). Update on immunologic and molecular diagnosis of human strongyloidiasis. *Acta Tropica* **135**, 33–43.

Marra, N. M., Chiuso-Minicucci, F., Machado, G. C., Zorzella-Pezavento, S. F., França, T. G., Ishikawa, L. L., Amarante, A. F., Sartori, A. and Amarante, M. R. (2010). Faecal examination and PCR to detect *Strongyloides venezuelensis* in experimentally infected Lewis rats. *Memórias do Instituto Oswaldo Cruz* **105**, 57–61.

Negrão-Corrêa, D., Silveira, M. R., Borges, C. M., Souza, D. G. and Teixeira, M. M. (2003). Changes in pulmonary function and parasite burden in rats infected with *Strongyloides venezuelensis* concomitant with induction of allergic airway inflammation. *Infection and Immunity* **71**, 2607–2614.

Nageswaran, C., Craig, P. S. and Devaney, E. (1994). Coproantigen detection in rats experimentally infected with *Strongyloides ratti*. *Parasitology* **108**, 335–342.

Nakai, E. S. and Amarante, A. F. T. (2001). Experimental infection of mice (*Mus musculus*) and rats (*Rattus norvegicus*) with *Strongyloides venezuelensis*. *Revista Brasileira de Parasitologia Veterinária* **10**, 1–6.

Paula, F. M. and Costa-Cruz, J. M. (2011). Epidemiological aspects of strongyloidiasis in Brazil. *Parasitology* **138**, 1331–1340.

Paula, F. M., Sitta, R. B., Malta, F. M., Gottardi, M., Corral, M. A., Gryschek, R. C. and Chieffi, P. P. (2013). Parasitological and molecular diagnosis in experimental *Strongyloides venezuelensis* infection. *Revista do Instituto de Medicina Tropical de São Paulo* **55**, 141–143.

Puthiyakunnon, S., Boddu, S., Li, Y., Zhou, X., Wang, C., Li, J. and Chen, X. (2014). Strongyloidiasis – an insight into its global prevalence and management. *PLoS Neglected Tropical Diseases* **8**, e3018.

Rodrigues, R. M., Cardoso, C. R., Gonçalves, A. L., Silva, N. M., Massa, V., Alves, R., Ueta, M. T., Silva, J. S. and Costa-Cruz, J. M. (2013). Increased susceptibility to *Strongyloides venezuelensis* infection is related to the parasite load and absence of major histocompatibility complex (MHC) class II molecules. *Experimental Parasitology* **135**, 580–586.

Romand, S., Thulliez, P. and Dubey, J. P. (1998). Direct agglutination test for serologie diagnosis of *Neospora caninum* infection. *Parasitology Research* **84**, 50–53.

Rugai, E., Mattos, T. and Brisola, A. P. (1954). A new technique for the isolation of nematode larvae from faeces: modification of the Baermann method. *Revista do Instituto Adolfo Lutz* **14**, 5–8.

Sandoval, N., Siles-Lucas, M., Lopez Alban, J., Pérez-Arellano, J. L., Gárate, T. and Muro, A. (2006). *Schistosoma mansoni*: a diagnostic approach to detect acute schistosomiasis infection in a murine model by PCR. *Experimental Parasitology* **114**, 84–88.

Saugar, J. M., Merino, F. J., Martín-Rabadán, P., Fernández-Soto, P., Ortega, S., Gárate, T. and Rodríguez, E. (2015). Application of real-time PCR for the detection of *Strongyloides* spp. in clinical samples in a reference center in Spain. *Acta Tropica* **142**, 20–25.

Sykes, A. M. and McCarthy, J. S. (2011). A coproantigen diagnostic test for *Strongyloides* infection. *PLoS Neglected Tropical Diseases* **5**, e955.

Wallen, N., Kita, H., Weiler, D. and Gleich, G. J. (1991). Glucocorticoids inhibit cytokine-mediated eosinophil survival. *Journal of Immunology* **147**, 3490–3495.

Wang, D. and Gao, G. (2014). State-of-the-art human gene therapy: part II. Gene therapy strategies and clinical applications. *Discovery Medicine* **18**, 151–161.

Wilson, M. B. and Nakane, P. K. (1978). Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In *Immunofluorescence and Related Technique* (ed. Knapp, W., Holuban, K. and Wick, G.), p. 215. North Hol Biomedicine, Amsterdam, Vol. 1978.

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

Yasuda, K., Matsumoto, M. and Nakanishi, K. (2014). Importance of both innate immunity and acquired immunity for rapid expulsion of *S. venezuelensis*. *Frontiers in Immunology* **5**, 118.

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