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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 human and animal strongyloidiasis. In this 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 deoxyribonucleic acid in faeces samples. Both techniques similarly detected the infection at day 6 after larvae inoculation. However, PCR performed with the genus primer showed higher sensitivity during the recovery phase. Histological analysis and PCR assay were then used to follow parasite tissue migration. *S. venezuelensis* migration route included the muscular fibers below the skin, the pulmonary alveoli and the small intestine vilosities. The sensitivity of these two techniques to detect parasite's presence in these tissues was statistically similar.

1. Introduction

Strongyloidiasis is a common, usually asymptomatic helminthic infection caused by *Strongyloides stercoralis*, an intestinal nematode which is mainly endemic in tropical and subtropical regions (Ly et al., 2003), and affects an estimated 30–100 million people in the world (Siddiqui and Berk, 2001). Strongyloidiasis encompasses five clinical syndromes: acute infection with Loeffler's syndrome, chronic intestinal infection, asymptomatic autoinfection, symptomatic autoinfection and hyperinfection syndrome with dissemination. Presentation and outcome of infection are determined by interaction between the host and the parasite (Marcos et al., 2008).*Strongyloides venezuelensis* is a parasitic nematode of rats that has been used as a model to study the host–parasite relationship in human and/or animal strongyloidiasis (Sato and Toma, 1990; Taira et al., 1995). This parasite enters the host through the skin, then migrates to lungs and trachea, and finally reaches the small intestine where it molts to become a sexually mature adult female (Baek et al., 1999). Infection of rodents with *S. venezuelensis* has been carried out in various strains of rats and mice (Takamure, 1995; Silveira et al., 2002; Baek et al., 2003; Matsuda et al., 2003; Chiuso-Minicucci et al., 2010). However, the kinetic time-lines and migratory route have not been established in the Lewis rat strain. Histological methodology is a classical but still important tool to determine parasite migration routes and lesions caused by *S. venezuelensis* and *Strongyloides ratti* in rodents (Genta and Ward, 1980; Koga et al., 1998; Matsuda et al., 2003). More recently, deoxyribonucleic acid (DNA) technology has had a major impact in many areas of parasitology, including infection diagnosis. 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 shown a high PCR sensitivity for detection of parasite DNA in various biological samples (i.e., faeces, 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, the sensitivity between PCR and traditional methodologies was compared to characterize *S. venezuelensis* infection in Lewis rats. This comparison was carried out 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.

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2. Materials and methods

2.1. Animals

Male and female Lewis rats, weighing 120–150 g (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, the experimental protocol being approved by the local Ethics Committee (protocol 45/07).

2.2. 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 72 h 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 injected into the animal's abdominal region.

2.3. Experimental design

In the first experiment, the dynamics of *S. venezuelensis* infection was determined by daily counting the number of eggs per gram of faeces (EPG) using the modified McMaster technique (Gordon and Whitlock, 1939) for 32 days. The EPG counting was performed in a pool of faecal samples obtained from the five infected animals. PCR analysis with both a species 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 on pooled faeces samples collected every other day until the 32nd day of infection.

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

2.4. Histological analysis

Histological analysis was performed in samples from the brain, lungs, spleen, the first 14 cm of the small intestine, liver, lymph nodes (popliteal and inguinal), heart, vastus lateralis muscle, skin and muscular layer (abdominal region). These samples were fixed in 10% formalin and embedded in paraffin wax. Five micrometer sections were cut and stained with hematoxylin and eosin and analyzed on a computerized image analysis system (QWin Lite 2.5, Leica®).

2.5. PCR assay

PCR assays were employed to detect *S. venezuelensis* DNA in both faeces and the same tissue samples as used in the histological analysis. DNA from faecal samples was extracted with QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany). DNA from tissues, *S. venezuelensis* L3, adult *Syphacia muris* and spleen of a non-infected rat were all extracted with a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). Integrity of DNA samples obtained from different tissues was ascertained by electrophoresis and showed no degradation. The species oligonucleotide primer pair (forward 5′-TTAGTGGTGCGTGGATTTGA-3′ and reverse 5′-ATGTGGAGCCGT TTATCAGG-3′) was designed using the Primer-BLAST program and a *S. venezuelensis* partial 18S rDNA sequence (GenBank Accession No. AJ417026). Primer3 software program (Rozen and Skaletsky, 2000) was employed to design this primer pair; the expected product being 198 bp. This oligonucleotide was submitted to BLAST and searched against *Homo sapiens, Rattus norvegicus, S. venezuelensis*, *Strongyloides* spp. and *S. muris* genome sequences. The genus primer pair, SSUA and SSU22R amplifies the small subunit ribosomal RNA gene and was described by Dorris et al. (2002) to analyse several species within the genus *Strongyloides*. This primer pair allows the amplification of a 380 bp band. The characterization of the molecular weight of this band was previously determined by using purified L3 larvae.

Both primer pairs were used to perform PCR in faecal samples and only the genus primer was used for tissue samples evaluation. DNA samples from *S. venezuelensis*, *S. muris* and spleen of a non-infected rat were employed as positive controls in all PCRs with both primer pairs. *S. muris* was also evaluated because it is frequently found in rats raised in the laboratory.

PCRs 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, UK) and 100 ng of genomic DNA in a 10 μl reaction. The PCR conditions included initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s (denaturation), 60 °C at 30 s (annealing for both primer pairs), extension at 72 °C for 30 s and a final extension at 72 °C for 7 min. PCR products were electrophoresed through 2% agarose gels in 1× 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/26 M® 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.

2.6. 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.

3. Results

3.1. 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 a 32-day period. Parasite eggs were detected for the first time in the faeces at the 6th day of infection. The maximal faecal egg count was 42,300 EPG observed at day 8 post-infection. A second peak (21,300) was also observed at day 11 post-infection. From the 12th day on, the amount of eggs steadily decreased until day 21. After that, EPG was 0–100 until day 32. During this period, no eggs were detected in seven out of 10 samples (Fig. 1).

Determination of infection by PCR depended upon the nucleotide sequence used as primers. The species primer pair detected parasite DNA in the faeces from days 6 to 18, being negative thereafter (Fig. 2a). On the other hand, PCR using the genus primer was