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Outcome of oral infection in mice inoculated with *Trypanosoma cruzi* IV of the Western Brazilian Amazon

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ABSTRACT

A new epidemiological view of American trypanosomiasis or Chagas disease has been formulated in recent decades. Oral transmission of the etiological agent of Chagas disease, *Trypanosoma cruzi*, has been the most common form of transmission. The *T. cruzi* discrete typing units TcI and TcIV have been involved in tens outbreaks of acute cases of Chagas disease in the Brazilian Amazon region. We investigated the intensity of infection in mice that were orally inoculated (OR group) with four strains of TcIV that were isolated from two outbreaks of acute Chagas disease that was orally acquired in the state of Amazonas, Brazil. We compared the OR group with mice that were intraperitoneally inoculated (IP group). Blood samples were analyzed by fresh blood examination, hemoculture, and conventional and qualitative real-time polymerase chain reaction (PCR). Samples of different tissues were analyzed by quantitative real-time PCR. The OR group exhibited a higher maximum peak of parasitemia, greater rates of positivity, and higher parasite loads in different tissues during acute infection compared with the IP group, indicating a greater intensity of orally acquired infection. Mice that were orally inoculated with TcIV strains that were obtained from two outbreaks of orally acquired Chagas disease in Amazonas, Brazil, exhibited a more intense course of infection compared with intraperitoneally inoculated mice, reflected by higher levels of parasitemia and parasite loads.

1. Introduction

Trypanosoma cruzi is the protozoan hemoflagellate causative agent of American trypanosomiasis or Chagas disease (Chagas, 1909). More than a century after its discovery by Carlos Chagas, despite advances in disease control in endemic countries, Chagas disease still has high morbidity and remains a major public health concern, especially because of its globalization. According to the World Health Organization (2015), more than 5 million people are infected in endemic countries in Latin America. In Brazil, epidemiological findings indicate the occurrence of cases and outbreaks that are related to ingesting foods that are contaminated with *T. cruzi*, especially in areas that were previously considered non-endemic, such as the Amazon region (Brasil, 2013). In 2000–2011, more than 1200 cases of acute infection were reported, and most of these (71%) were orally acquired (Brasil, 2013).

T. cruzi has high genetic variability, and the species is divided into six discrete typing units (DTUs): TcI to TcVI (Zingales et al., 2009). In the Brazilian Amazon, the presence of TcI and TcIV has been reported in humans with orally acquired Chagas disease (Marcili et al., 2009; Monteiro et al., 2012).

Patients with acute Chagas disease in the Amazon region have characteristic clinical signs of this phase, including fever, headache, myalgia, dyspnea, paleness, and abdominal pain (Pinto et al., 2009). Some patients also present patent parasitemia and are positive for IgM in serological tests (Valente et al., 2009). Electrocardiographic changes have also been reported, with severe involvement of the heart, characterized by myopericarditis that evolves to a cardiac form with chronic cardiopathy lesions (Pinto et al., 2010). These signs and symptoms, together with facial edema and exanthema, characterize a more severe acute phase compared with classic endemic areas (Pinto et al., 2008).

The few studies that have investigated oral and intraperitoneal routes of infection have mostly been conducted in mice. Camandaroba et al. (2002) found that the biodeme of *T. cruzi* influences murine infection by these routes. Dias et al. (2013) found that both the infective form of the parasite and volume of the inocu-

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lum differentially influence the rate of infectivity based on different routes of inoculation. Barreto-de-Albuquerque et al. (2015) showed that the initial site of parasite entrance critically affects the host immune response and disease outcome. The present study investigated the evolution of infection in mice that were either orally or intraperitoneally inoculated with TcIV strains from two outbreaks of orally acquired acute Chagas disease in the western Brazilian Amazon.

2. Materials and methods

2.1. Ethical aspects

The Ethics Committee of the Dr. Heitor Vieira Dourado Tropical Medicine Foundation approved the use of *T. cruzi* strains that were obtained from humans in the Amazon region (protocol no. 1940/08). Written consent was obtained from all of the patients in the study. The use, maintenance, and care of the experimental animals were in accordance with the guidelines of the National Council for Animal Experiments Control (CONCEA) and approved by the Ethics in Research on Animals Committee of the State University of Maringá (protocol no. 023/2014).

2.2. Parasites, animals, and infection

We used four *T. cruzi* IV strains from two outbreaks of orally acquired acute Chagas disease that occurred in the municipalities of Coari (AM14 and AM16 strains) and Santa Isabel do Rio Negro (AM64 and AM69 strains), located in the state of Amazonas in the western Brazilian Amazon. These strains were deposited in the *T. cruzi* strain collection of the Chagas Disease Laboratory of the State University of Maringá. The strains were thawed and cultured in liver infusion tryptose (LIT) medium in a Biochemical Oxygen Demand apparatus at 28 °C and pricked out weekly.

For each strain, 26 female Swiss mice were used (21 days old, weighing 18–20 g). The mice were housed in polypropylene cages with a 12 h/12 h light/dark cycle and food and water available *ad libitum*. The mice were equally divided into two groups such that the average weight of the groups was equal. Each animal was inoculated with 2×10^6 metacyclic trypomastigotes that were obtained from acellular cultivation. One group orally received 1.0 ml of inoculum by gavage (OR group; Dias et al., 2013). A cannula was introduced into the stomach, which was carefully and gradually removed so that the parasite suspension reached the oral cavity. The other group was intraperitoneally inoculated with 0.2 ml of inoculum with the same number of parasites (IP group; Camandaroba et al., 2002).

2.3. Parasitological parameters

Parasitemia was checked daily in blood that was collected from the tail vein beginning 3 days after inoculation (d.a.i.) until a negative result was obtained for at least 3 consecutive days through fresh blood examination (FBE) according to Brener (1962). The FBE was performed to confirm the presence of infection, obtain parasitemia curves, and determine the percentage of animals with a positive FBE (FBE+). This examination was also performed on blood samples that were collected for other tests. The following parameters were derived from the parasitemia curves: mean prepatent period (PPP; average period during which each mice remained FBE−), mean patent period (PP; average period during which each mice was FBE+), maximum peak of parasitemia (Pmax; average peak of parasitemia for each mice), and day of maximum peak of parasitemia (Dpmax; average number of days of peak parasitemia for each mice).

Hemoculture (HC) was performed 60 days after inoculation with blood that was collected from the retro-orbital venous sinus of each animal according to Filardi and Brener (1987) to obtain the percentage of animals with positive HC (HC+).

Conventional polymerase chain reaction (cPCR) was performed according to Miyamoto et al. (2006). Blood samples (200 μl) were collected on the same day as the HC and were added to double the volume of 6.0 M/0.2 M guanidine/ethylenediaminetetraacetic acid (EDTA). This technique was used to determine the percentage of mice that were cPCR+. Primers 121 (5′-AAATAATGTACGGG[T/G]GAGATGCATGA-3′) and 122 (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′) were used to amplify a 330-base-pair fragment that was specific to the DNA minicircle of the kinetoplast (*k*-DNA) of *T. cruzi*. The products were observed after 4.5% polyacrylamide gel electrophoresis and stained with silver.

DNA was extracted from part of the same aliquot of blood that was used for cPCR and subjected to qualitative real-time PCR (qlPCR) according to Caldas et al. (2012) using 5 μl of SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and 0.35 μM of primers specific to the genomic DNA of *T. cruzi* (5′-GCTCTTGCCCACAMGGGTGC-3′ and 5′-CCAAGCAGCGGATAGTTCAGG-3′; Cummings and Tarleton, 2003) in a final volume of 10 μl. Based on the results of these analyses, we obtained the percentage of mice that were qlPCR+.

Quantitative real time PCR (qPCR) was performed in blood and other tissues that were collected from mice in the different experimental groups. We collected 200 μl blood samples from two animals in each group before they were euthanized on 30 d.a.i. (acute phase) and 100 d.a.i. (chronic phase). The samples were added to Eppendorf tubes that contained 35 μl of 129 nM sodium citrate solution. After euthanasia by an overdose of ketamine + xylazine, the heart, esophagus, stomach, and abdominal wall were removed *in totum* from each animal and fragmented into 30 mg portions for DNA extraction. DNA extraction from blood and other tissues was performed according to Caldas et al. (2012) with modifications according to Gruendling et al. (2015). The amplification and quantification of DNA samples were performed using 5 μl of DNA, 75 μM primers (5′-AC/GTCGGCTGATCGTTTTCGA-3′ and 5′-AATTCCTCCAAGCAGCGGATA-3′), a TaqMan probe (5′-Fam-CACACACTGGACACCAA-NFQ-MGB-3′), and TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μl as previously described by Duffy et al. (2013). The amplifications were performed using StepOne equipment (Applied Biosystems, Foster City, CA, USA). Standard curves were constructed with serial dilutions of 1:10 DNA from 10^5 parasites/ml of blood or 30 mg tissue to determine the amplification efficiency. Based on the qPCR results, we obtained the percentage of animals that were qPCR+.

2.4. Infectivity and mortality

The percentage of infectivity (%INF) of the four TcIV strains in mice that were orally and intraperitoneally inoculated (OR and IP groups, respectively) were calculated based on positive results from at least one of the detection methods (FBE, HC, cPCR, and qlPCR for blood and qPCR for tissue). Mice survival was monitored daily throughout the experiment, which lasted for approximately 100 days. The percentage of mortality (%MOR) was recorded for the different experimental groups.

2.5. Statistical analysis

The data were analyzed using Biostat 5.3 software (Belém, Pará, Brazil). Frequency tables with percentages were generated. The χ^2 test and Fisher's exact test or G test were used to compare propor-