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In Situ Hybridization Assay for the Diagnosis of Chagas Myocarditis and Orchitis in a Rhesus Macaque (*Macaca mulatta*): A case report

Megan DeLorenzo^{1,2}, Elvira Carias^{1,3}, Allison Mustonen¹, Olga Gonzalez¹, Edward J. Dick Jr¹, Shyamesh Kumar^{1,*}

¹Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX 78245

²College of Agriculture Forestry and Life Sciences, Clemson University, Clemson, SC

³Microbiology and Immunology Program, University of Texas at San Antonio, San Antonio, TX

Abstract

We present the first documented case of *Trypanosoma cruzi* induced orchitis in a rhesus macaque. Additionally, we describe an In Situ Hybridization based assay to confirm *Trypanosoma cruzi* infection in formalin fixed tissues.

Keywords

Trypanosome; Kinetoplast; RNAscope; PCR

1. INTRODUCTION

We present the first case of *Trypanosoma cruzi* (*T. cruzi*) induced orchitis in a rhesus macaque (*Macaca mulatta*), and an In Situ Hybridization (ISH) based sensitive and specific diagnostic method [1] to identify the parasite in formalin fixed tissues.

2. CASE REPORT

A five year old, male rhesus macaque was presented for lethargy, diarrhea, and weight loss. The animal had a 13% body weight loss during the three weeks he was treated with metronidazole (Oakdell Pharmacy, San Antonio, TX, 50mg/Kg, BID), enrofloxacin (Bayer Animal Health, 100mg/Kg SID), and supportive fluid therapy. Due to poor prognosis, the animal was euthanized. All animal care and procedures were approved by the Texas Biomedical Research Institute Institutional Animal Care and Use Committee.

At necropsy, the animal had diffuse subcutaneous edema, more severe around the scrotum and prepuce; the mucous membranes were pale. The thorax contained 20 ml of modified

*Corresponding author: Shyamesh Kumar, DVM, PhD, Diplomate ACVP, Texas Biomedical Research Institute, PO Box 760549, San Antonio, TX 78245-0549, USA., Tel.: (210) 258 9704; Fax: (210) 670 3305; shykumar@txbiomed.org.

transudate, edematous lungs, and an enlarged heart with a rounded apex. The left testicle had a focal area of dark red parenchyma.

Serial sections prepared from formalin fixed, paraffin embedded heart and testicle were processed for H&E staining or underwent ISH using RNAscope 2.5 HD Assay [1] (Advanced Cell Diagnostics, Newark, CA) as per the manufacturer's instructions [2], and a novel probe (Catalog # 300031, www.acdbio.com) specific for the *Trypanosoma cruzi* kinetoplast minicircle repeat DNA sequences (kDNA, Genbank ID [U07846.1](https://www.ncbi.nlm.nih.gov/nuclot/U07846.1)). Tissues from a published case of *Trypanosoma cruzi* induced myocarditis containing visible protozoan cysts served as a positive control [3]; an identical assay using a probe for bacterial DapB gene (Catalog # 300031, www.acdbio.com) served as a negative control. Positive signals were detected by FAST RED dye, and images were captured using Olympus BX46 microscope with Olympus DP27 camera (Olympus America, Center Valley, PA).

Histopathological examination identified multifocal accumulations of lymphocytes and plasma cells within the myocardium (Figure 1A). Scattered foci of myocardial fibers were shrunken, pale-staining, had ragged cell borders, and contained *Trypanosoma cruzi* cysts (Figure 1B). Testes were bilaterally composed of seminiferous tubules that lacked spermatogenesis and contained variably degenerate cells. Multifocally, in both testes, the interstitium was expanded by mononuclear infiltrates (lymphocytes, plasma cells, and macrophages) (Figure 1C). Tubules in these areas often contained areas of necrosis and multifocal protozoal cysts with multiple amastigotes (Figure 1D). There were scattered lymphocyte infiltrates around Auerbach's plexus, expanding the endomysium, and associated with skeletal myofiber degeneration in the stomach. There was multifocal acute centrilobular hepatic necrosis. The renal tubules were variably dilated and lined by attenuated epithelium.

ISH showed intense positive signals in the heart, testicle, and rare foci in stomach. In the heart, ISH labeled intracellular cysts (Figure 2A and B) and isolated foci within the areas of inflammation with no visible cysts (Figure 2C). Additionally, ISH labeled several cardiac myocyte nuclei and perinuclear cytoplasm (Figure 2D). In the testes, ISH labeled the intraepithelial cysts as well as individualized amastigotes in the lumen of the seminiferous tubules (Figure 2E and F). Rare smooth muscle cell nuclei in the outer or inner muscular layers of the stomach also were labelled by ISH.

Biannual serologic testing, including five months before presentation, was negative for *T. cruzi* antibody using Luminex bead assay coated with four *Trypanosoma cruzi* specific peptides supplied by Charles River Laboratories (www.crl.com). Serum collected at necropsy was strongly positive for *T. cruzi* antibody by Luminex assay, and by a commercial diagnostic laboratory using indirect ELISA, and QPCR (www.vrlsat.com/).

3. DISCUSSION

We present the first case of *T. cruzi* induced orchitis in a rhesus macaque with concurrent chagasic cardiomyopathy resulting in cardiovascular insufficiency and dependent edema.

Additionally, we present a tissue based diagnostic method for the detection of cysts and individual amastigotes in fixed tissues.

In addition to identifying cysts and amastigotes within the heart and testes, ISH detected positive signals within the nucleus and perinuclear cytoplasm of cardiac and gastric myocytes, which lacked visible cysts or amastigotes; no such staining was observed in serial sections stained with negative control probe. This indicates the possibility of a ruptured cyst with individual amastigotes or residual kDNA fragments being present, which are impossible to detect by routine H&E staining. At the SNPRC, there have been numerous cases with lymphoplasmacytic myocarditis and/or ganglioneuritis (colon, esophagus, and stomach) without visible cysts, suspected to be associated with *T. cruzi* infection [4, 5]. Due to the unavailability of a commercial antibody, and the loss of histopathological context when using PCR on formalin fixed tissues, these cases were difficult to confirm. ISH assay using this novel probe provides an innovative method to confirm *T. cruzi* infections in fixed tissues with or without visible cysts.

The potential for sexual transmission of *T. cruzi* has been suggested since the early 1900's [6–8] and there is supporting evidence in mouse models [9]. Araujo *et al* reported *T. cruzi* DNA in semen of chronically infected humans [10], and Hecht *et al* showed familial transmission of *T. cruzi* DNA over multiple generations in humans [11]. However, there is no report of nonhuman primates or humans transmitting *T. cruzi* through copulation. We identified intraepithelial amastigotes and cysts within the germ cells of the seminiferous tubules and also extracellular amastigotes in the lumen, further supporting the hypothesis of sexual transmission.

The subcutaneous and scrotal edema, centrilobular hepatic necrosis, and renal tubular epithelial degeneration in this macaque were attributed to cardiac insufficiency due to myocarditis and myocardial necrosis which is frequently observed in NHPs with *T. cruzi* infection [4]. The lack of seropositivity five months prior to necropsy, suggests an acute infection. The incidence of *T. cruzi* infections at the Southwest National Primate Research Center has been estimated at between 2–3% [12]. Because a majority of these primates are captive-bred (including the present case), it is likely that transmission occurred through either ingestion of triatomine insects or infection of bite wounds or mucous membranes [3, 13, 14]. This case report highlights the risks associated with *T. cruzi* infection, potential for sexual transmission, and a diagnostic assay which can be used to confirm *T. cruzi* infections irrespective of host species.

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Figure 1.

T. cruzi myocarditis and orchitis in a rhesus macaque. (A) Heart. Infiltrates of lymphocytes and plasma cells expand the interstitium and replace myocardial fibers. H&E. (B) Heart. Higher magnification of myocardium showing intracellular protozoal cyst containing multiple amastigotes. H&E. (C) Testicle. Interstitial mononuclear infiltrates and atrophy of seminiferous tubules. H&E. (D) Testicle. Higher magnification of seminiferous tubules showing intraepithelial amastigotes (black arrow). H&E.

Figure 2.

RNAscope based In-Situ Hybridization (ISH) assay to confirm *T. cruzi* myocarditis and orchitis in a rhesus macaque. (A) Heart. Location of a cyst is highlighted by the black box. Inset. Higher magnification of the cyst. H&E. (B) Adjacent serial section of heart showing the same protozoal cyst. Inset. Cyst and amastigotes show strong labelling for probe targeting the kinetoplast minicircle repeat DNA sequences. RNAscope based ISH. (C) Heart. ISH labelling for kinetoplast minicircle repeat DNA within areas of inflammation with no visible cyst. RNAscope based ISH. (D) Heart. ISH labelling for kinetoplast minicircle repeat DNA in cardiac myocyte nuclei and perinuclear cytoplasm. RNAscope based ISH. (E) Testicle. Note the heavy infiltration of inflammatory cells on the left half of the image; right half of image is relatively unaffected. Inset. Higher magnification of intraepithelial cyst with amastigotes. H&E (F) Adjacent serial section of testicle showing multiple intraepithelial and

intraluminal amastigotes. Note the distribution of the cysts is confined to the left half of image. Inset. Cyst and amastigotes show strong labelling for probe targeting the kinetoplast minicircle repeat DNA sequences. RNAscope based ISH.

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