Video Article

Sexual Transmission of American Trypanosomes from Males and Females to Naive Mates

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Abstract

American trypanosomiasis is transmitted to humans by triatomine bugs through the ingestion of contaminated food, by blood transfusions or accidently in hospitals and research laboratories. In addition, the *Trypanosoma cruzi* infection is transmitted congenitally from a chagasic mother to her offspring, but the male partner's contribution to in utero contamination is unknown. The findings of nests and clumps of amastigotes and of trypomastigotes in the theca cells of the ovary, in the goniablasts and in the lumen of seminiferous tubules suggest that *T. cruzi* infections are sexually transmitted. The research protocol herein presents the results of a family study population showing parasite nuclear DNA in the diploid blood mononuclear cells and in the haploid gametes of human subjects. Thus, three independent biological samples collected one year apart confirmed that *T. cruzi* infections were sexually transmitted to progeny. Interestingly, the specific *T. cruzi* antibody was absent in the majority of family progeny that bore immune tolerance to the parasite antigen. Immune tolerance was demonstrated in chicken refractory to *T. cruzi* after the first week of embryonic growth, and chicks hatched from the flagellate-inoculated eggs were unable to produce the specific antibody. Moreover, the instillation of the human semen ejaculates intraperitoneally or into the vagina of naive mice yielded *T. cruzi* amastigotes in the epididymis, seminiferous tubule, *vas deferens* and uterine tube with an absence of inflammatory reactions in the immune privileged organs of reproduction. The breeding of *T. cruzi*-infected male and female mice with naive mates resulted in acquisition of the infections, which were later transmitted to the progeny. Therefore, a robust education, information and communication program that involves the population and social organizations is deemed necessary to prevent Chagas disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/57985/

Introduction

The protozoan parasite *Trypanosoma cruzi* belonging to the family Trypanosomatidae undergoes trypomastigote and amastigote life cycle stages in mammalian hosts and exists as epimastigotes in the insect-vector's (Reduviid: Triatominae) gut and in axenic culture. In recent decades, several studies have shown the presence of Chagas disease in countries on four continents considered triatomine bug free[1,2,3,4,5,6,7,8,9,10,11,12,13]; the dispersion of American trypanosomes was initially attributed to Latin American immigrants to the Northern Hemisphere, but the possibility that some are autochthonous cases of Chagas disease can no longer be denied[3,4,5,6,7,8,9,10,11,12,13,14]. The only recognizable endogenous source of *T. cruzi* transmission has been ascribed to the chagasic mother's transfer of the parasite to the offspring in approximately 10% of pregnancies[15]; the male partner's contribution to in utero infections through semen ejaculates has remained unrecognized.

Over one century ago, investigators[16,17] observed intracellular *T. cruzi* amastigotes in the theca cells of the ovary and in the germ line cells of the testicles of acute cases of Chagas disease. The nests and clumps of *T. cruzi* trypomastigotes and amastigotes in theca cells of the ovary, in goniablasts and in the lumen of seminiferous tubules (**Figure 1**) of fatal acute Chagas disease cases develop immune privilege in the organs of reproduction in the absence of inflammatory infiltrates[18,19]. In recent decades, a few experimental studies have shown nests of the round amastigote forms of *T. cruzi* in the seminiferous tubule, epididymis, and *vas deferens* as well as in the uterus, tubes and ovary theca cells of acutely infected mice[1,20,21,22]. Furthermore, in the course of family studies to document the transfer of protozoan mitochondrial DNA from parental Chagas patients to their descendants, *T. cruzi* nuclear DNA (nDNA) was verified in human haploid germ line cells[23], and parasite life cycle stages were observed in the ejaculates of chagasic mice[24]. These findings are in agreement with reports on the immune tolerance attained by the progeny of *T. cruzi*-infected hosts in the absence of the specific antibody[1,25,26]. Additionally, epidemiological reports that suggested the spread of endemic Chagas disease to the other continents[3,4,5,6,7,8,9,10,11,12,13] are now supported by experimental studies showing that Chagas disease can be transmitted sexually[1]. The present investigation presents an epidemiologic family study protocol and shows that *T. cruzi* infection propagates by sexual intercourse.

Protocol

The Human and the Animal Research Committees of the Faculty of Medicine of the University of Brasilia approved all the procedures with human subjects and laboratory animals, respectively, in research protocols 2500.167567 and 10411/2011. The Ethics Committee of the Public Foundation Hospital Gaspar Vianna (protocol nº 054/2009 and CONEP 11163/2009) approved the free consent forms for the field study, with extension to the Ministry of Health National Commission on Human Research (CONEP 2585/04). The protocol was adjusted to assess *T. cruzi* DNA in diploid blood mononuclear cells and in haploid gametes of semen ejaculates. The laboratory animals received humane care; the mice, subjected to heart puncture before sacrifice, were under anesthesia.

1. Recruitment of human participants

1. Ensure that the research team participates in a Health System Chagas Disease Program to deliver health care to Chagas patients.
2. Recruit human participants from families enrolled in the program, showing at least one case with fever, malaise, headache, tachycardia, and edema, the main clinical symptoms of the acute Chagas disease[14].
3. Deliver health care to the people in the study families for a period of five years.
4. Obtain 15 mL of venous blood from the study participants on three occasions one year apart, divide the sample into three 5 mL aliquots, and store them in the refrigerator at 4 °C.
5. Collect 2 mL of the semen ejaculates from adult volunteer family members and proceed as described in step 3.3.

2. Growth of parasites

1. Using the aliquot from step 1.4, mix the blood with 5 units of anticlotting sodium heparin; make a slope culture by the inoculation of blood from family participants with the diagnosis of acute Chagas disease.
    1. Inoculate 5 mL of the unclotted human blood into a 50 mL screw cap tube blood-agar slant plus 5 mL of liver infusion-tryptose medium (LIT), and incubate the sample in a shaker at 27 °C for 3 months.
    2. Place 100 µL of the supernatant medium on top of glass slides, cover with slips and search for blood *T. cruzi* epimastigotes under the microscope, every four weeks.
    3. Harvest the epimastigote isolates in the supernatant of axenic LIT medium at 27 °C; wash the cells in PBS pH 7.4, centrifuge at 1,000 x *g* for 10 min; dilute the epimastigotes in the pellet in 5 mL of Dulbecco's-modified essential medium (DMEM).
    4. Inoculate the confluent L6 muscle cell culture flasks with 1 x 10^6 ECI1-to- ECI21 *T. cruzi* epimastigote isolates.
    5. Grow the *T. cruzi* ECI1-to-ECI21 and the Berenice archetype trypomastigotes in L6 muscle cell cultures in 75 mL culture flasks.
    6. Feed cells with 15 mL of DMEM at pH 7.4 supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 250 nM L-glutamine, and 5% CO_2 at 37 °C[1].
    7. Use the positive control *T. cruzi* Berenice trypomastigotes to phenotype the ECI1-to-ECI21 isolates from tissue culture, as described in step 5.2.
    8. Grow the negative control *Leishmania braziliensis*[27] in DMEM supplemented with 20% fetal bovine serum only, and use the parasite promastigotes as a negative control.
    9. Use 1 x 10^6 *T. cruzi* ECI1-to-ECI21 trypomastigotes in the supernatant from the cell culture to infect mice.
    10. Search for *T. cruzi* trypomastigotes in the tail blood after the first week of the infection.
    11. Search the nests of amastigotes in hematoxylin-eosin stained sections of the heart, skeletal muscle, and reproductive organs of the infected mice, one month thereafter.

3. DNA extraction and PCR analyses

1. Place 5 mL of the blood (step 1.4) aliquot in a sterile EDTA tube, and perform density gradient centrifugation for 45 min at 3,000 x *g*. Use a pipette to harvest the mononuclear cells from the whitish phase above the red cells, wash the white cells twice in 5 mL of PBS, pH 7.4, by centrifugation for 10 min at 1,500 x *g* in a different 15 mL tube, and use the cells for the DNA extraction.
2. Extract the DNA from ECI-1 to ECI-21 *T. cruzi*, from the positive control Berenice *T. cruzi*, from the negative control *L. braziliensis* (step 2.1.8), and from the test blood mononuclear cells of 109 human family study subjects[1,27,28].
3. Dilute 2 mL of the sperm samples obtained in step 1.5 in DMEM (1:4, v/v); incubate for 45 min at 5% CO_2 and 37 °C, recover spermatozoa from the supernatant after centrifugation for 5 min at 13,000 x *g*, and extract the haploid DNA[23].
4. Place 1 mL of the cells in extraction buffer (10 µM NaCl, 20 µM EDTA, 1% SDS, 0.04% proteinase-K, and 1% dithiothreitol), and mix the solutions by inversion and shaking.
    1. Centrifuge the solutions for 10 min at 13,000 x *g* and 25 °C, and transfer the viscous supernatant to a spin column.
    2. Centrifuge the spin column for 1 min at 10,000 x *g* and discard the eluate; add 500 µL binding buffer to the spin column (**Table of Materials**), centrifuge it for 1 min at 12,000 x *g*, and discard the eluate.
    3. Add 600 µL washing buffer to the spin column, centrifuge it for 1 min at 12,000 x *g*, and discard the eluate.
    4. Repeat this step twice, and transfer the spin column to a sterile 1.5 mL micro centrifuge tube.
    5. Add 100 µL of TE buffer, incubate the tube at room temperature for 2 min, and then centrifuge the tube for 1 min at 12,000 x *g*. The buffer in the microcentrifuge tube contains the DNA.
    6. Measure DNA concentrations by running aliquots on a 0.8% agarose gel and by reading the absorbance at 260 nm with a spectrophotometer. Store the DNA samples at -20 °C until use in the PCR analysis.
5. Use *T. cruzi* Tcz1/2 primers annealed to the specific 188-nt specific telomere sequence probe[29] and run the PCR with DNA from the family study subjects' blood and sperm, from the Berenice *T. cruzi* positive control and from the *L. braziliensis* negative control.

1. Prepare the PCR mixture with 10 ng template DNA, 0.4 µM of each pair of primers, 2 U of Taq DNA polymerase, 0.2 µM dNTPs, and 15 µM $MgCl_2$ in a 25 µL final volume.
2. Initiate the DNA amplification program at 94 °C for 30 s to denature the template, and cool the samples to 55 °C for 30 s. Then, incubate the samples at 94 °C for 90 s to extend the annealed primers. Return the temperature to 94 °C for 30 s to initiate the next cycle, and incubate the samples an additional 3 min at 72 °C. At the end of the 32nd cycle, cool the samples for 10 min at room temperature, and store them in the refrigerator at 4 °C[29].
3. Amplify a *T. cruzi* DNA telomere repeat sequence annealed to the Tcz1/2 primers at both extremities.
4. Analyze the amplification products on a 1.3% agarose gel and observe the 188-nt DNA bands on a UV-illuminator.

4. *Southern* hybridization

NOTE: *Southern* hybridization was used to discard most of the false positive PCR amplicons in the agarose gel.

1. Subject the PCR amplification products from uninfected controls, from Chagas case positive controls, from 109 test samples of diploid DNA and from haploid DNA of 21 study family participants to *Southern* hybridizations.
2. Employ the *T. cruzi* 188-nt DNA-specific telomere sequence probe annealed to the Tcz1/2 primers shown; label the probe with [α-^{32}P] 2'-deoxyadenosine triphosphate (dATP) using a random primer labeling kit, and analyze the amplification products on a 1.3% agarose gel at 60 V overnight at 4 °C.
3. Transfer the gel to a positively charged nylon membrane using the capillary method overnight.
4. Hybridize the DNA bands transferred to the nylon membrane with the radiolabeled 188-nt probe, which binds the *Eco*R1 digests of the genomic DNA in 25 µL of the enzyme-specific buffer for variable periods.
5. Wash the membrane twice for 15 min at 65 °C with 2x SSC and 0.1% SDS.
6. Expose the X-ray films to the nylon membrane and autoradiograph the bands on the membrane for one week.
7. Grow the clones selected from PCR and *Southern* hybridization with the *T. cruzi* PCR amplification products, which are hybridized with the specific radiolabeled DNA probe.
8. Commercially sequence the clones using the Tcz1/2 primers set annealed to the *T. cruzi*-specific telomere footprint[2,23].

5. Immunological assays

NOTE: The sensitivity and specificity of the indirect immunofluorescence (IIF) and of the enzyme-linked immunosorbent assay (ELISA) were assessed in the serum from six Chagas patients with demonstrable parasitemia and from six Chagas-free, deidentified serum bank samples. The assays conducted with the double serum dilutions in PBS, pH 7.4, revealed that the IIF at 1:100 dilutions and the ELISA optical densities (ODs) at 0.150 and above separated the positive from the negative results.

1. Use 5 mL of the unclotted blood aliquot (step 1.4) kept at room temperature for 1 h, and centrifuge it at 1,500 x *g* for 30 min to separate the serum in the supernatant.
2. Perform IIF and ELISA in triplicate 1:100 serum dilutions to detect the *T. cruzi* and *L. braziliensis* antigens as described previously[28].
3. IIF
    1. Conduct the IIF assay in triplicate serum dilutions of the 109 study subjects' samples obtained on three occasions one year apart.
    2. Place 5 µL suspensions of the formalin 1% -treated *T. cruzi* epimastigotes or *L. braziliensis* promastigotes onto glass slides; let the parasites dry overnight in a hood at room temperature and store the glass slides at -20 °C until use.
    3. Place 20 µL of the patients' serum dilutions on top of glass slides coated with 5 µL of the formalin-killed (10 parasites/µL) *T. cruzi* epimastigotes or *L. braziliensis* promastigotes.
    4. Incubate the glass slide covered with a slip for 1 h in a moist chamber at 37 °C and wash thrice with PBS.
    5. Incubate the air-dried glass slide with a 1:1,000 dilution of a fluorescein-labeled rabbit antibody (**Table of Materials**) to human IgG for 1 h at 37 °C; wash and dry the slide.
    6. Mount the slide with a cover slip and examine it under an UV light microscope.
    NOTE: A positive exam is an apple-green *T. cruzi* epimastigote silhouette, shown in the video.
4. ELISA
    1. Run an ELISA to detect the *T. cruzi* and the *L. braziliensis* soluble antigens (1 µg/100 µL in 0.1 M carbonate buffer, pH 9.6) on coated microplate wells.
    2. Incubate the 1:100 serum dilutions in triplicate coated wells for 2 h at room temperature.
    3. Wash the plates thrice with PBST (PBS containing 0.5% Tween-20), pH 7.4, solution before drying.
    4. Incubate the plates with 50 µL of a 1:1,000 dilution of rabbit anti-human IgG antibody for 90 min at 37 °C.
    5. Wash the plates thrice with PBST solution and allow them to dry at room temperature.
    6. Incubate the plates with 100 µL of 1:1,000 dilutions of alkaline phosphatase-conjugated goat anti-rabbit IgG (**Table of Materials**) for 90 min at 37 °C.
    7. Wash the plates thrice with PBST, add the substrate p-nitro phenyl phosphate, and wait for color development.
    8. Read the ODs at 630 nm in a multimode plate reader.
    9. Run the triplicate dilutions of the test serum from the study population and plot the ODs to identify the specific *T. cruzi* antibody titers.

6. Assessments of immune tolerance

NOTE: A chicken model system was used to test *T. cruzi* infections after the first week of embryo development.

1. Inoculate chicken fertile eggs with 100/10 µL *T. cruzi* trypomastigotes harvested from tissue culture medium; inoculate mock control eggs with 10 *T. cruzi* trypomastigotes per µL culture medium. Seal the hole with tape.
2. Incubate 20 *T. cruzi*-infected eggs and an equal number of mock control fertile eggs at 37 °C and 65% humidity for 21 days.
3. Keep the chicks that hatch in the incubator for 24 h and, thereafter, at 32 °C in hoods with temperature control for three weeks.
4. Grow the chicks hatched from *T. cruzi*-inoculated eggs and from mock control eggs to the adult stage in a positive air pressure room at 24 °C, in individual cages placed in separate aisles.
5. Challenge all the adult chicks thrice at six months of age with 10^7 formalin-killed trypomastigotes injected subcutaneously, weekly, according to the scheme in the video.
6. Draw blood from a wing vein of chickens hatched from the *T. cruzi*-inoculated eggs and from the mock controls four weeks after the last immunization to obtain the serum.
7. Use the chicken serum to detect the specific *T. cruzi* antibody by IIF and ELISA as described in step 5 of the protocol.

7. Infection of mice with *T. cruzi* from Chagas patients' semen ejaculates

1. Use the semen ejaculates from an adult PCR+ Chagas disease patient (step 1.5) and from an adult PCR- Chagas-free individual.
2. Use two groups of 12 one-month-old BALB/c naive mice kept in hoods under positive air pressure at 24 °C and fed food *ad libitum*.
3. Instill the human Chagas-positive semen aliquots (100 µL) into the peritoneum and equal amounts into the vagina of group-A mice.
4. Instill the control Chagas-free semen aliquots (100 µL) into the peritoneum and an equal amount into the vagina of group-B mice.
5. Sacrifice the experimental mice under anesthesia five weeks after the semen instillations and subject the tissue sections to staining with hematoxylin-eosin.
6. Use microscopy to search for *T. cruzi* trypomastigotes and amastigotes in the heart, skeletal muscle, and reproductive organs in groups of mice.

8. Transmission of *the T. cruzi* infection by intercourse

1. Use 10 male and female six-week-old BALB/c mice in the experiments.
2. Inoculate five male and five female mice intraperitoneally with 1 x 10^5 *T. cruzi* trypomastigotes from the tissue culture.
3. Breed the mice until three months of age: group I will be formed by five *T. cruzi*-infected female mice and five control noninfected male mates; group II will be formed by five *T. cruzi*-infected male and five control noninfected female mates. Group III will be formed by five male and five female control naive uninfected mates.
4. House each breeding pair in one cage placed inside a safe box with a 5 mm grid and lock-in door to prevent escape.
5. Feed the mice chow and water ad libitum. Raise a total of 70 weaning progenies in groups II and I for at least six weeks.
6. Draw blood by heart puncture from the parental (FO) and progeny (F1) mice under anesthesia, sacrifice the mice, and submit sections of the heart, skeletal muscle, and reproductive organs for pathological analysis.

9. Assessment of immune privilege

1. Obtain tissues from *T. cruzi*-infected and naive control mice (step 8.6), and cut the paraffin-embedded samples into 4 µm thick sections.
2. Remove the paraffin and dehydrate the sections onto glass slides with several changes of xylene and graded washes with 100% to 70% ethanol, for 1 min each.
3. Incubate the tissue sections with 0.05% saponin once, followed by three distilled water washes at room temperature.
4. Block the tissue sections with 5% nonfat powdered milk for 45 min. Wash the slides in 0.1 M PBST and incubate the sections with the Chagas mouse anti-*T. cruzi* serum or with the control uninfected mouse serum at a 1:20 dilution for 2 h.
5. Wash the slides thrice for 5 min in PBST and dry at room temperature before incubation with a 1:1,000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse IgG.
6. Rinse the slides in PBST and add 100 µL of 3,3' diaminobenzidine for a 5 min incubation, followed by three washes with PBST and counterstaining with Harris hematoxylin for 30 s.
7. Wash the slides in distilled water for 5 min, dehydrate them in 70%, 80%, 90%, and 100% ethanol for 1 min, and mount them in buffered glycerin.
8. Examine the slides with a bright-field light microscope, and capture photo images with a microcamera with software and an analyzer program.
9. Document the immune privilege of the parasite in the absence of inflammatory reactions in the reproductive organs.

10. Statistical analyses

1. Use Biomedical Edit for sequence analysis, perform alignments with BLAST, and determine the E-value statistical significance ($p < 0.05$).
2. Perform a one-way analysis of variance (ANOVA) and the Tukey test to compare the OD means plus or minus standard deviations.

Representative Results

This research, conducted according to the protocol, aimed to detect acute cases of Chagas disease by clinical and parasitological exams. Venous blood samples were subjected to direct microscopic examination and in vitro culture for parasite growth. Twenty-one acute cases of Chagas disease showed *T. cruzi* in blood. The research protocol secured the isolation of *T. cruzi* ECI1-to ECI21 from acute Chagas disease, and the DNA samples exhibited positive DNA footprints in the remainder of the study population: nDNA-PCR assays yielded the typical telomere repeat sequence with the 188-nt bands present as well as the *T. cruzi* Berenice archetype[1]. The Chagas cases and their family members who volunteered to participate in the study were grouped into four families[1].

In this family study, the *T. cruzi* nDNA was PCR amplified with primer sets[1,23] annealed to the specific telomere sequence from each of the 21 acute Chagas disease cases. These *T. cruzi* nDNA amplicons hybridized with the specific radiolabeled sequence probe (**Figure 2**); the cloning and sequencing revealed that the amplicons comprised the *T. cruzi* 188-nt telomere repeat motif. The specificity of these hybridization procedures was shown in the negative control performed with *L. braziliensis* promastigotes. The pathology analysis validated that the hemoflagellates in the acute Chagas disease patients were truly virulent *T. cruzi*. We conclude that the *T. cruzi* nDNA (188-bp) band found in the 21 acute Chagas cases (**Figure 2**) is a direct demonstration of persistent infections.

Sexual Transmission of *Trypanosoma cruzi* in Humans

To evaluate the ratios of the *T. cruzi* infections, we applied the nucleic acid test for high sensitivity detection of the parasite footprints in the family study population[1]. In these PCR assays, the amplification products that hybridized with the specific radiolabeled 188-nt probe formed nDNA bands in 76.1% (83/109) of the test samples; the results of *Southern* hybridization of the nDNA-PCR amplification products with the specific 188-nt radiolabeled probe are shown in **Figure 3**. Furthermore, the hybridizations showed the parasite DNA in the germ cell line of the volunteer family members (**Figure 4**).

IIF was employed to phenotype the ECI1 to ECI21 *T. cruzi* trypomastigotes with the human serum IgG from a Chagas disease serum bank sample with parasitological demonstration of the protozoan in the blood, and a fluorescein conjugated anti-human IgG was used. *T. cruzi* Berenice was a positive control for the Chagas antibody, and the negative control was the promastigote of its family relative *L. braziliensis*. **Figure 5** shows that the positive apple-green silhouette of the Berenice archetype correlates with the wild-type *T. cruzi* envelope shown in the video.

The ELISA and IIF revealed the specific *T. cruzi* antibodies[1,28] in 28.4% (31/109) of the test samples. The results of the ELISA and IIF, as well as those from the nDNA-PCR amplicon and *Southern* hybridizations, are plotted in **Figure 6**. The discrepancies among the results of the nDNA-PCR footprints and those from the specific antibody assays are depicted in the heredograms (**Figure 7**), Family A, four subjects had positive nDNA and the anti-*T. cruzi* antibody, and 11 had only the positive nDNA; five males had *T. cruzi* in the semen ejaculate. In family B with 44 people, 11 had the specific *T. cruzi* antibody, and 23 had both the specific antibody and the parasite nDNA; seven individuals had *T. cruzi* in the semen ejaculate. Family C with 29 members had the antibody and the *T. cruzi* nDNA in five individuals, and 17 had the parasite nDNA alone; four males had the nDNA-PCR positive in the semen ejaculate. In Family D, among 21 subjects, 11 had the specific anti-*T. cruzi* antibody and the nDNA footprint, and nine had positive nDNA-PCR alone. **Figure 3**, **Figure 6** and **Figure 7** depict the broad discrepancies among the results, consistently, in the biological samples obtained from family subjects in three independent experiments run one year apart.

Table 1 shows the quantitative differences between the IIF, the ELISA, and the nDNA-PCR assays in the samples from the human study families A-to-D. The discrepancies between the ratios of antibody assays (28.4%) and those of positive nucleic acid assays (76.1%) are statistically significant ($p < 0.005$). In these families, the differences among groups of *T. cruzi*-infected people (III and IV) accounted for 62.6% (52/83) of the population, showing a positive nucleic acid test alone. The broad discrepancies among the ratios of positive nDNA footprints and those of the specific *T. cruzi* antibody were explained by the experiments conducted in the chicken model system.

Immune Tolerance

The evaluation of the immune responses conducted in groups of chickens raised to the adult stage in individual cages in separate aisles containing naive control chickens (A); mock control chickens hatched from eggs inoculated with culture medium (B); and chickens hatched from the *T. cruzi* trypomastigotes-inoculated eggs (C)[26]. The adult chickens in groups B and C were challenged three times with the formalin-killed *T. cruzi* trypomastigote antigen, weekly, as shown in the video. The ELISA and the IIF assays were run with the serum collected from group A, B and C chickens one month after challenge. **Figure 8** shows the absence of the specific antibody in group A and C chickens, which contrasted sharply with the specific antibody production in group B immunized with the *T. cruzi* antigen. The results clearly showed the immune tolerance in group C hatched from the *T. cruzi*-inoculated eggs.

Sexual Transmission of *Trypanosoma cruzi* in a Mouse Model System

Moreover, the infectivity of *T. cruzi* from a Chagas patient's ejaculate, which tested positive in the PCR and lacked the specific antibody, was demonstrated through instillations of 100 µL of semen into the peritoneal cavity of male mice and through an equal amount of semen instilled into the vagina. Five weeks later, the *T. cruzi* amastigote nests were detected in the heart and skeletal muscles, and clumps of differentiating parasites were present in the lumen of the *vas deferens* and uterine tube. Interestingly, the destructive inflammatory reactions did not surround the nests and clumps of the *T. cruzi* amastigotes (**Figure 9**).

The assessment of the sexual transmission of *T. cruzi* infections was further conducted in two groups of mice inoculated intraperitoneally with 1 x 10^5 *T. cruzi* Berenice trypomastigotes forms[1,30,31]. In experimental group I, 10 *T. cruzi*-infected males mated with 10 naive control female mice. In experimental group II, 10 *T. cruzi*-infected females mated with 10 naive control males. **Figure 10** shows that the *T. cruzi*-infected male mice (A-to-E) and the *T. cruzi*-infected female mice (F-to G) yielded 188-bp nDNA bands (odd numbers). After breeding, the naive mates (even numbers) readily acquired *T. cruzi* following a unique sexual encounter with a chagasic mate. Similar repeat experiments performed under identical conditions confirmed that each naive female or male mouse that sexually mated with a *T. cruzi*-infected male or female acquired the flagellate infection. These nDNA-positive founders (F0) generated progeny that they raised until the age of six weeks. Then, the test and the control uninfected mice were bled *via* heart puncture to collect approximately 0.5 mL of blood. The nDNA-PCR assays showed that the founders' (F0) sexually acquired infections were transmitted to the F1 progeny, as shown by the 188-bp nDNA bands (**Figure 11**). The F1 progeny were nDNA-positive in 41 of the 70 (58.6%) samples examined. Of these mice with nDNA bands suggestive of vertically acquired infections, as few as 9 of 41 (22%) had *T. cruzi* antibodies.

The F1 progeny mice were sacrificed under anesthesia, and the body tissues were subjected to pathological and immune peroxidase-staining analyses. The results of these experiments are shown in **Figure 12**. The results for the *T. cruzi* amastigotes were documented in the interstitial cells of the epididymis and in goniablasts; amastigotes differentiating into trypomastigotes were present in the lumen of seminiferous tubules in the absence of inflammatory reactions.

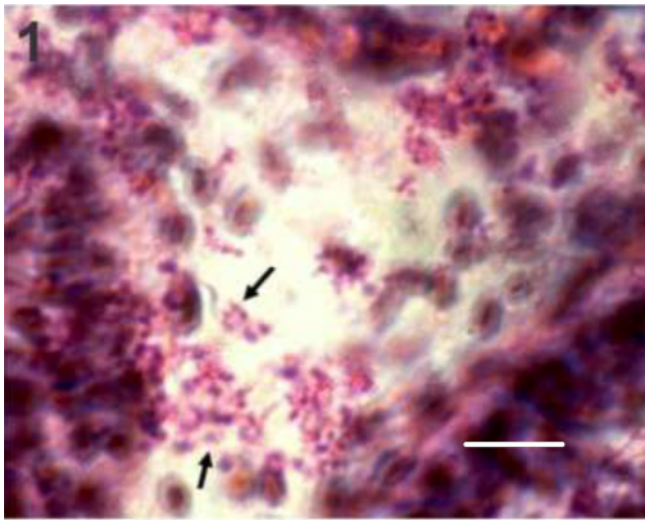

Figure 1. *Trypanosoma cruzi* infection in the seminiferous tubule of a boy who died of acute Chagas disease. Microphotograph from Doctor Teixeira's file, 1970[18]. The *T. cruzi* forms are in goniablasts, and clumps of amastigotes and free trypomastigotes (arrows) are present in the lumen of the seminiferous tubule in the absence of inflammatory infiltrates[1]. Hematoxylin-eosin stains. Bar, 20 *μ*m. Reprinted with permission from the publisher and the authors[1,19].

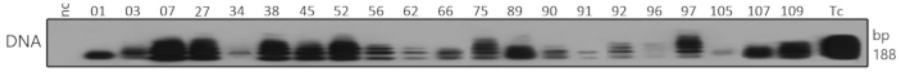

Figure 2. The footprint of *Trypanosoma cruzi* from acute Chagas disease. The *T. cruzi* nDNA-PCR amplification products formed 188-nt bands with a specific radiolabeled 188-nt probe. Tc, *T. cruzi*; nc, negative control. Reprinted with permission from the publisher and the author[1]. Please click here to view a larger version of this figure.

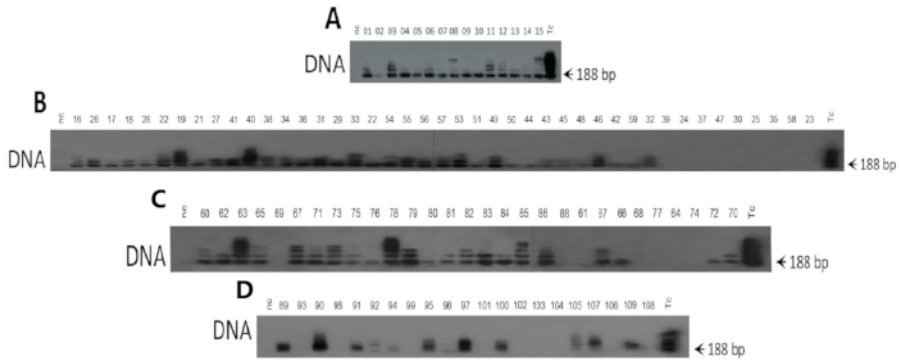

Figure 3. *Southern* blotting analysis of *Trypanosoma cruzi* infections in subjects of human study families. Family A - All 15 subjects showed the specific nDNA-PCR 188-nt bands. In Family B, a total of 35 of 43 subjects (81.4%) formed the specific nDNA bands. In Family C, among 29 members, 22 (75.8%) formed the nDNA bands. In Family D, 11 of 21 subjects (52.4%) had the nDNA bands. The *T. cruzi*-specific nDNA bands were confirmed by cloning and sequencing. Reprinted with permission from the publisher and the author[1]. Please click here to view a larger version of this figure.

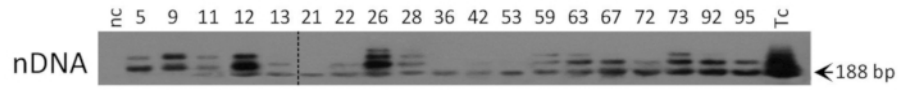

Figure 4. The active *Trypanosoma cruzi* infections in the semen ejaculate from study family volunteers. The infections in Chagas patients' ejaculates identified by the nDNA-PCR 188-bp bands. Tc, *T. cruzi* positive control. Nc, *L. braziliensis* negative control. Reprinted with permission from the publisher and the author[1]. Please click here to view a larger version of this figure.

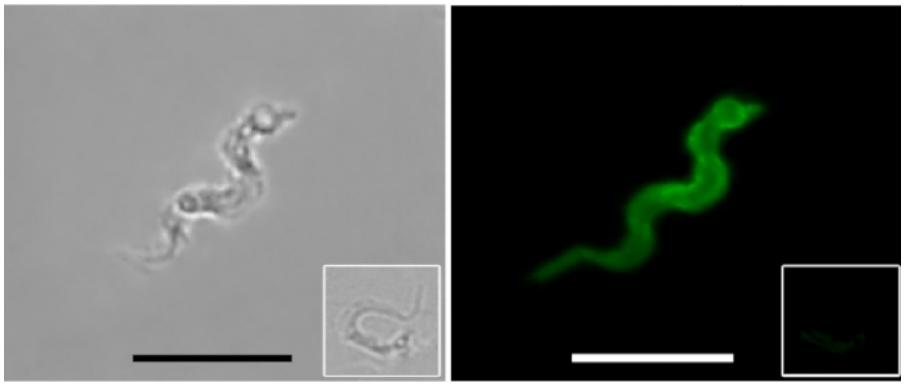

Figure 5. The phenotype of *Trypanosoma cruzi* with the Chagas disease patients' serum antibody. *T. cruzi* identified with the Chagas serum IgG antibody that recognizes the parasite trypomastigote treated with an FITC-labeled monoclonal Ab anti-human IgG. The anti-*T. cruzi* Ab does not recognize *Leishmania braziliensis* promastigotes. The insets show the negative controls. Bars, 20 µm. Reprinted with permission from the publisher and the author[1]. Please click here to view a larger version of this figure.

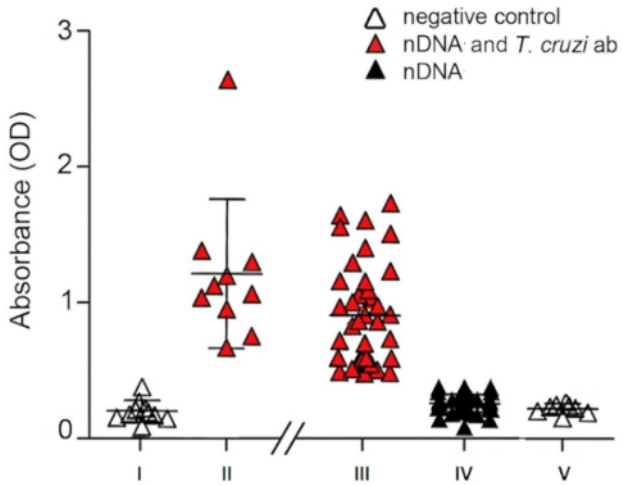

Figure 6. Graphic representation of the ELISAs and nDNA-PCR assays in the family study population. Group I (n=10) and group II (n=20) were the negative control and the positive control sera, respectively, from *T. cruzi* infections with parasitological demonstration. Group III (n=31) included samples from family subjects with the 188-bp nDNA bands and specific antibodies to *T. cruzi*. Group IV (n=52) comprised sample from subjects with *T. cruzi* infections detected by the nDNA-PCR 188-nt amplicons in the absence of the specific antibody. Group V (n=26) were negative test samples comprising the infection-free people in the family study. Reprinted with permission from the publisher and the author[1]. Please click here to view a larger version of this figure.

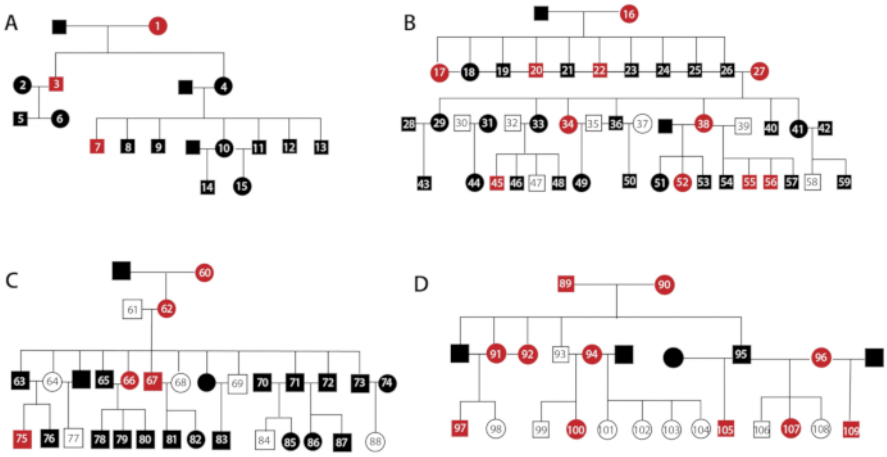

Figure 7. The heredograms and mapping of the *Trypanosoma cruzi*-infected family population. The figure shows the discrepancies among the ratios of the anti-*T. cruzi* antibody and those of the nDNA-PCR assays. Open square and circle, negative male and female. Red squares and circles, positive anti-*T. cruzi* antibody and nDNA-PCR. Black squares and circles, positive nDNA-PCR alone. Please click here to view a larger version of this figure.

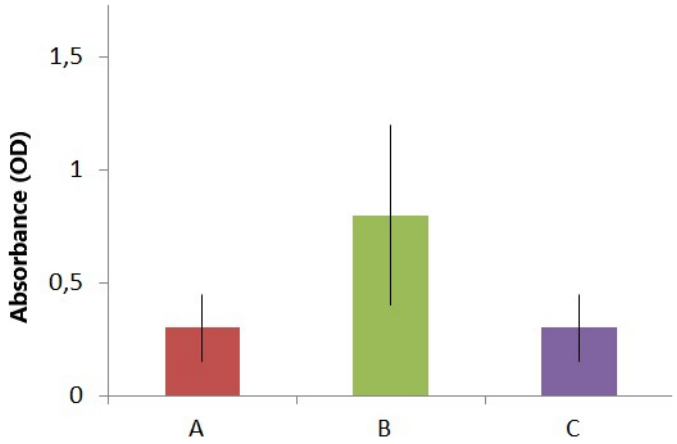

Figure 8. The immune tolerance in chickens hatched from *Trypanosoma cruzi*-inoculated eggs. A) Preimmune antibody profile in the mock control chickens (n = 10). B) The specific antibody response in the naive control chickens challenged with the *T. cruzi* antigen (n = 20). C) The absence of a specific immune response in chickens hatched from the *T. cruzi*-inoculated eggs after challenge with the *T. cruzi* antigen (n = 20). The optical density difference between A and C (024 ± 0.17) towards B (0.85 ± 0.6) is statistically significant ($p<0.05$). This figure has been modified from reference[26] and is reprinted with permission from the publisher and the author.

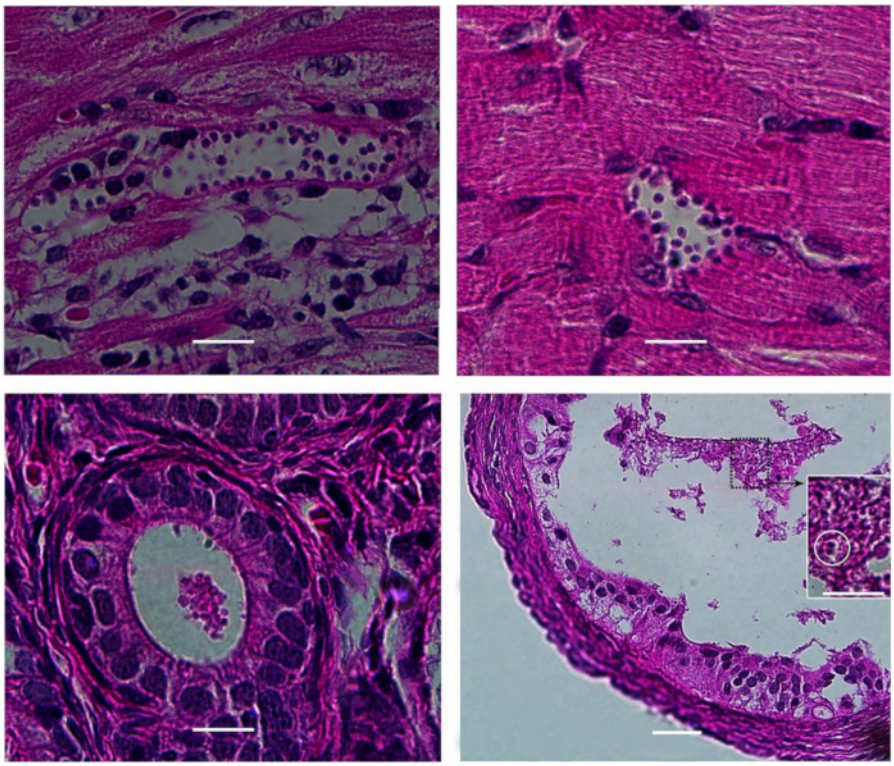

Figure 9. The infective *Trypanosoma cruzi* in human ejaculates translates into an active murine infection. Aliquots of Chagas patient ejaculates were instilled into the peritoneal cavity or into the vagina of mice. The mice were sacrificed three weeks after instillation. Top lane, *T. cruzi* amastigotes nests in the heart (left) and in the skeletal muscle (right). Bottom lane, *T. cruzi* amastigote nests in the *vas deferens* (left) and in the uterine tube (right). The insert shows a dividing amastigote (circle). Notice the absence of inflammatory infiltrates in the tissue sections. Hematoxylin-eosin stains. Bars: top and bottom left, 20 µm; bottom right, 10 µm. Reprinted with permission from the publisher and the author[1].
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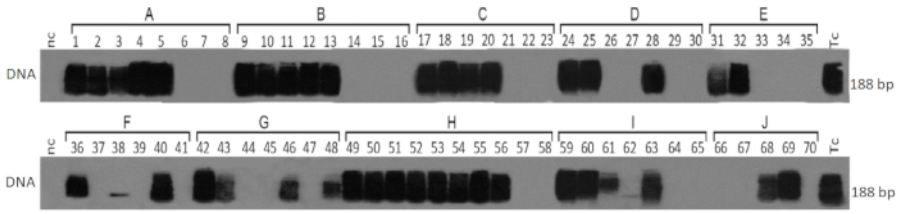

Figure 10. The sexual transmission of *Trypanosoma cruzi* infections in the mouse model system by intercourse. The transmission of the *T. cruzi* infections from chagasic to naive mates demonstrated by the specific nDNA 188-bp bands revealed in the *Southern* hybridizations. Top lane) Prebreeding profiles of the PCR amplification products of the *T. cruzi*-infected mice and of the naive mice. The odd numbers indicate the *T. cruzi*-infected male A-to-E and female F-to-I mouse samples. The even numbers are naive female (2-to-10) and male (12-to-20) mice. Bottom lane, after breeding, the profiles show that even mice 2-to-20 acquired *T. cruzi* infections. Reprinted with permission from the publisher and the author[1,30]. Please click here to view a larger version of this figure.

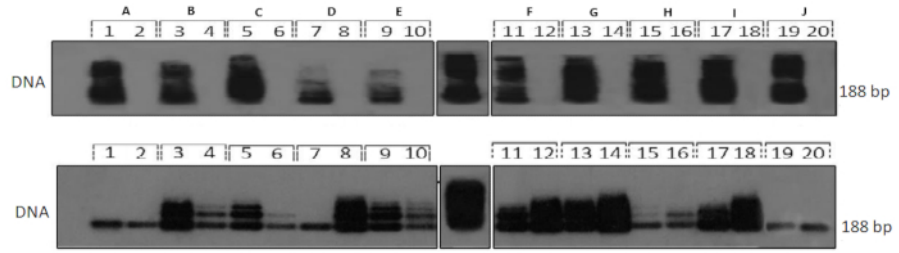

Figure 11. The *Trypanosoma cruzi* infection is vertically transferred from the F0 chagasic parental to the F1 progeny mice. The chagasic parent transmitted the *T. cruzi* infections by a single breeding encounter. The *T. cruzi*-infected females were mated to naive males A-E, and the naive females were mated to the *T. cruzi*-infected males F-J. After breeding, all the founders (F0) showed the positive protozoan nDNA-PCR 188-bp band. *Southern* blotting revealed the specific nDNA band after hybridization with the radiolabeled 188-nt probe in a majority of the F1 litters. Nc, *L. braziliensis* negative control; Tc, *T. cruzi* positive control. Reprinted with permission from the publisher and the author[1,31]. Please click here to view a larger version of this figure.

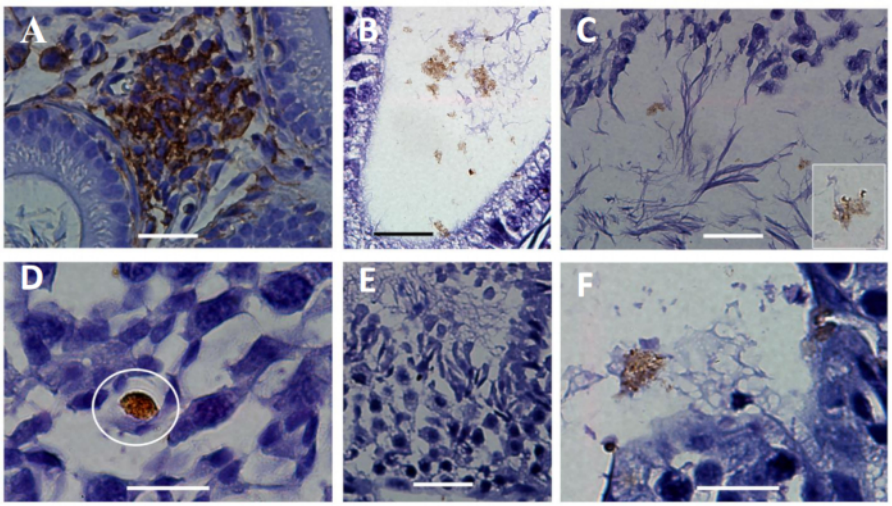

Figure 12. The histopathology documented *Trypanosoma cruzi* sexually transmitted from F0 to F1 progeny and immune tolerance in the absence of an inflammatory reaction. The sections show growth of the *T. cruzi* forms in the epididymis, goniablasts and the seminiferous tubules of the F1 mice. The mice were sacrificed under anesthesia, and the immune peroxidase-stained sections were examined under a microscope. The photomicrographs show brownish immune peroxidase-stained *T. cruzi* amastigotes in the interstitial of the epididymis *(***A**) and clumps of amastigotes differentiating into trypomastigotes shed into the lumen of the seminiferous tubule (**B, C,** and **F**). The amastigote nest seen in a goniablast (**D**). The positive control mouse's seminiferous tubule normal histology (**E**). Notice the absence of inflammatory infiltrates in the testes of the F1 mice showing loads of the Chagas parasites. Giemsa's stain. Bars: A, B, C and E, 20 µm; D and F, 10 µm. Please click here to view a larger version of this figure.

Table 1. The discrepancies between the ratios of positive IIF and ELISA exams and those of nDNA-PCR assays in the samples collected from the human study families A-to-D*

Groups**	ELISA: serum anti-*T. cruzi* Ab (%)		*T. cruzi* nDNA- PCR (%)	
I- Control Ab- PCR- (n=10)	10/10	100	10/10	100
II- Control Ab+ PCR+ (n = 20)	20/20	100	20/20	100
III- Chagas Ab+ PCR+ δ (n = 31)	31/109	28.4	31/109	28.4
IV- Chagas Ab- PCR+ δ (n= 52)	-	-	83/109	76.1
V- Chagas-free Ab- PCR- (n = 26)	26/109	23.9	26/109	23.9

* Results of three independent ELISA and nDNA-PCR assays run in samples collected in three different occasions at years 1, 2, and 3. [1]. The amplification of the 188-nt *T. cruzi* DNA repeat confirmed by cloning and sequencing.
**The differences among negative (groups I and V) and the positive subjects (groups III and IV) are statistically significant ($p < 0.05$).
δ The discrepancies between groups III and IV explained by the immune tolerance in the absence of the *T. cruzi* antibody attained in 62.6% (52/83) of the PCR positive subjects.

Discussion

Herein, we discuss a family-based research protocol that answered the question of whether human Chagas disease stems from sexually transmitted intraspecies *T. cruzi* infections. Early studies could not provide evidence of the sexual transmission of *T. cruzi* infections, probably because the available data and information on Chagas disease were obtained separately from the individual[3,4,5,6,7,8,9,10,11,12,13]. The finding of *T. cruzi* in the seminiferous tubule of a boy (**Figure 1**) was the spark that spurred clinical and epidemiological investigation. After several decades, conceivably when family study approaches and the technologies described in this research protocol were available, *T. cruzi* life cycle stages appeared in the human ejaculate[1,19].

The direct parasitological demonstration of the protozoan in 21 acute Chagas disease cases was crucial to validate the nDNA-PCR amplification products, which formed specific bands in samples from all the subjects acutely infected with *T. cruzi*. This point-of-care laboratory marker evaluated the results of the immunological and nucleic acid assays. The fundamental long-run Chagas disease family study, therefore, combines the findings in humans with those obtained in groups of laboratory animals. The research conducted according to the protocol revealed for the first time that *T. cruzi* infections are sexually transmitted in humans[1].

The broad difference between the ratios from the parasite-specific antibody assays and those from the nucleic acid tests indicates that the majority of the nDNA footprints resulted from sexually transmitted cases in the absence of specific anti *T. cruzi* antibodies. Thus, the sexual transmission of *T. cruzi* in the family members exhibiting positive nDNA in the absence of the specific IgG antibody was due to immune tolerance.

Immune tolerance was demonstrated in a chicken model system refractory to *T. cruzi* infections after the first week of embryo growth[1,25,26,32,33,34]. Thereafter, the immature immune system's inability to recognize the parasite as a foreign component of the body indicated the chicken's late mature immune system tolerance towards *T cruzi.* In view of these results, tolerance is a natural phenomenon[1] resulting from the immune system's self-recognition and maintenance of its own body components under physiological conditions[25,26,32,33,34]. The shift from the state of immune tolerance to autoimmune Chagas heart disease can therefore be associated with effector cell modifications resulting from *T. cruzi* kinetoplast (kDNA) mutations in the host's genome[1,2,14,23,25,26].

The critical steps in the research protocol describe the main technique modification and troubleshooting in order to disclose the sexually transmitted Chagas parasites[1,14,23,25,26]: *i)* selecting study families with cases of acute Chagas disease[35,36]; *ii*) isolating of wild-type *T. cruzi* from the blood of the acute cases; *iii*) obtaining DNA samples from the families' participants blood flagellates, from the Berenice *T. cruzi* archetype, from positive deidentified bank DNA samples, and from the negative control *L. braziliensis*; *iv*) performing tidy technical procedures to demonstrate that the participants' flagellates nDNA footprint is identical to that of the Berenice *T. cruzi* archetype and of those positive bank DNA samples; *v*) running independent triplicate nDNA footprinting to demonstrate the *T. cruzi* infections in the family members on three occasions one year apart; *vi*) ensuring that the nDNA-PCR technique conducted at the point of care yields results confirmed by cloning and sequencing all the amplicons annealed to the specific primer sets, thus consistently showing the *T. cruzi* 188-nt sequence[1,25,28,29,30]; *vii*) using high-quality trademark reagents to reproduce the antibody titers still in serum samples collected at three different time points; *viii*) the family study protocol revealed the existing live infection in the germ line cells upon the demonstration of the *T. cruzi* nDNA in the absence of specific serum antibodies in semen ejaculates collected from Chagas parasite-infected individuals[1]; *ix*) the perspective is that the research protocol designed to unravel the sexually transmitted *T. cruzi* infections should disclose the autochthonous Chagas disease on five continents; *x*) the nDNA and the kDNA footprints secure the diagnosis of chronic asymptomatic Chagas disease in humans[1,2]; *xi*) in the absence of the nDNA, the mutation*of the T. cruzi* kDNA sequence[1,2,23,25,26] alone is a laboratory marker for achieving the differential diagnosis from the idiopathic dilated cardiomyopathies[23,25,37,38,39].

Additionally, the virulent *T. cruzi* documented in Chagas patient ejaculates were capable of initiating widespread infections upon instillation into the mouse vagina and into its peritoneal cavity. The pathology study showed *T. cruzi* amastigote nests in the heart and skeletal muscles as well as in the *vas deferens* and uterine tube. Interestingly, the parasite nests did not provoke inflammatory reactions that would hamper vital

reproductive functions. The absence of inflammatory reactions renders the immune privilege in vital functional body structures[40,41,42,43,44,45] and therefore explains the uncurbed growth of *T. cruzi* in the reproductive organs.

Furthermore, the experimental studies in chagasic mice that bred with naive mates further explained the sexual transmission of *T. cruzi* infections in humans. The infected females and males transmitted the *T. cruzi* infections to the uninfected naive mates during intercourse, and the majority of their litters acquired the *T. cruzi* vertically transferred from parent to progeny. In these experiments, the initial phase referred to the growth of *T. cruzi* in the tube and in the uterus, as well as in the seminiferous tubule and *vas deferens*, where the immune privilege took place. Then, sexual transmission occurred through the parasitic stages in the semen or in the uterine secretions into the vagina. Immune privilege[40,41,42,43,44,45] is a phenomenon that allows some organs (reproductive system, eyes, and brain) to downregulate inflammatory reactions and avoid damage to important, sensitive and specific functions[40]. Hormones[41] and several immune factors downregulate macrophages[41,42,43], natural killer cells[41], T-lymphocytes, and T-regulatory (Treg) cells, thus orchestrating the inhibition of a number of proinflammatory cytokines and immune-privilege triggers[40,41,42,43,44,45].

The sexual transmission of *T. cruzi* infections from males and females to naive partners indicates that the control of Chagas disease requires international solidarity. The results discussed herein suggest that more creative research is needed. The following immediate goals are achievable: *i)* to develop high-throughput platforms for specific and highly sensitive nucleic acid testing to reach an accurate diagnosis, aiming for the prevention of infections transmitted by sexual intercourse, blood transfusions and organ transplantation as well as facilitating clinical and epidemiologic enquiries to determine the diagnosis and the prevalence of Chagas disease; *ii*) to promote a multicenter drug development program to obtain new drugs for the eradication of *T. cruzi* infections; and *iii)* to implement a suitable education, information and communication program that includes the participation of schools, churches, social organizations, and health institutions to prevent the spread of Chagas disease.

Disclosures

The authors declare that they have no competing financial interests.

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