Journal of Visualized Experiments

Sexual transmission of the American trypanosomes from males and females to naive mates

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE57985R6
Full Title:	Sexual transmission of the American trypanosomes from males and females to naive mates
Keywords:	Family study; Trypanosoma cruzi; semen ejaculates; uterine tube secretion; mouse model system; vertical transmission; immune privilege; chicken model system; immune tolerance; Chagas disease
Corresponding Author:	Antonio R.L. Teixeira Universidade de Brasilia Brasílila, Federal District XX
Corresponding Author's Institution:	Universidade de Brasilia
Corresponding Author E-Mail:	antonioteixeirarl@gmail.com
Order of Authors:	Adriana Benevides Almeida
	Perla Fabiola Araujo
	Francisco Moreno Bernal
	Ana de Cassia Rosa
	Sebastião Aldo Valente
	Antonio R.L. Teixeira
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

TITLE:

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

AUTHORS AND AFFILIATIONS:

Adriana B. Almeida[1], Perla F. Araújo[1], Francisco M. Bernal[1], Ana de Cassia Rosa[1], Sebastião A. Valente[2], Antonio R. L. Teixeira[1]

1. Chagas Disease Multidisciplinary Research Laboratory, Faculty of Medicine of the University of Brasilia, Brazil
2. Instituto Evandro Chagas, Belém, PA, Brazil

KEYWORDS:

Family study; *Trypanosoma cruzi*; semen ejaculates; uterine secretion; mouse model system; vertical transmission; immune privilege; chicken model system; immune tolerance; Chagas disease.

SUMMARY:

The *Trypanosoma cruzi* agent of Chagas disease produces long-lasting asymptomatic infections that abruptly develop into clinically recognized pathology. The following research protocol describes a short-run family-based epidemiological study to unravel the *T. cruzi* infection transmitted sexually from parent to progeny.

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.

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[1-13]; 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[3-14]. 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[1,20-22]. 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[3-13] 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

1.1 Ensure that the research team participates in a Health System Chagas Disease Program to deliver health care to Chagas patients.

1.2 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].

1.3 Deliver health care to the people in the study families for a period of five years.

1.4 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.

1.5 Collect 2 mL of the semen ejaculates from adult volunteer family members and proceed as described in step 3.3.

2. Growth of parasites

2.1 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.

2.1.1 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.

2.1.2 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.

2.1.3 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).

2.1.4 Inoculate the confluent L6 muscle cell culture flasks with 1 x 10^6 ECI1-to- ECI21 *T. cruzi* epimastigote isolates.

2.1.5 Grow the *T. cruzi* ECI1-to-ECI21 and the Berenice archetype trypomastigotes in L6 muscle cell cultures in 75 mL culture flasks.

2.1.6 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].

2.1.7 Use the positive control *T. cruzi* Berenice trypomastigotes to phenotype the ECI1-to-ECI21 isolates from tissue culture, as described in step 5.2.

2.1.8 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.

2.1.9 Use 1 x 10^6 *T. cruzi* ECI1-to-ECI21 trypomastigotes in the supernatant from the cell culture to infect mice.

2.1.10 Search for *T. cruzi* trypomastigotes in the tail blood after the first week of the infection.

2.1.11 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

3.1 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.

3.2 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].

3.3. 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].

3.4. 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.

3.4.1 Centrifuge the solutions for 10 min at 13,000 x *g* and 25 °C, and transfer the viscous supernatant to a spin column.

3.4.2. 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.

3.4.3 Add 600 µL washing buffer to the spin column, centrifuge it for 1 min at 12,000 x *g*, and discard the eluate.

3.4.4 Repeat this step twice, and transfer the spin column to a sterile 1.5 mL micro centrifuge tube.

3.4.5 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.

3.4.6 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.

3.5. 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.

3.5.1 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.

3.5.2 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].

3.5.3 Amplify a *T. cruzi* DNA telomere repeat sequence annealed to the Tcz1/2 primers at both extremities.

3.4.4 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.

4.1 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.

4.2 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.

4.3 Transfer the gel to a positively charged nylon membrane using the capillary method overnight.

4.4 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.

4.5 Wash the membrane twice for 15 min at 65 °C with 2x SSC and 0.1% SDS.

4.6 Expose the X-ray films to the nylon membrane and autoradiograph the bands on the membrane for one week.

4.6 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.

4.7 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.

5.1 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.

5.2. Perform IIF and ELISA in triplicate 1:100 serum dilutions to detect the *T. cruzi* and *L. braziliensis* antigens as described previously[28].

5.3. IIF

5.3.1 Conduct the IIF assay in triplicate serum dilutions of the 109 study subjects' samples obtained on three occasions one year apart.

5.3.2 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.

5.3.3 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.

5.3.4 Incubate the glass slide covered with a slip for 1 h in a moist chamber at 37 °C and wash thrice with PBS.

5.3.5 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.

5.3.6 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.

5.4 ELISA

5.4.1 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.

5.4.2 Incubate the 1:100 serum dilutions in triplicate coated wells for 2 h at room temperature.

5.4.3 Wash the plates thrice with PBST (PBS containing 0.5% Tween-20), pH 7.4, solution before drying.

5.4.4 Incubate the plates with 50 µL of a 1:1,000 dilution of rabbit anti-human IgG antibody for 90 min at 37 °C.

5.4.5 Wash the plates thrice with PBST solution and allow them to dry at room temperature.

5.4.6 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.

5.4.7 Wash the plates thrice with PBST, add the substrate p-nitro phenyl phosphate, and wait for color development.

5.4.8 Read the ODs at 630 nm in a multimode plate reader.

5.4.9 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.

6.1 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.

6.2 Incubate 20 *T. cruzi*-infected eggs and an equal number of mock control fertile eggs at 37 °C and 65% humidity for 21 days.

6.3 Keep the chicks that hatch in the incubator for 24 h and, thereafter, at 32 °C in hoods with temperature control for three weeks.

6.4 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.

6.5 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.

6.6 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.

6.7 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

7.1 Use the semen ejaculates from an adult PCR+ Chagas disease patient (step 1.5) and from an adult PCR- Chagas-free individual.

7.2 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*.

7.3 Instill the human Chagas-positive semen aliquots (100 µL) into the peritoneum and equal amounts into the vagina of group-A mice.

7.4 Instill the control Chagas-free semen aliquots (100 µL) into the peritoneum and an equal amount into the vagina of group-B mice.

7.5 Sacrifice the experimental mice under anesthesia five weeks after the semen instillations and subject the tissue sections to staining with hematoxylin-eosin.

7.6 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

8.1 Use 10 male and female six-week-old BALB/c mice in the experiments.

8.2 Inoculate five male and five female mice intraperitoneally with 1 x 10^5 *T. cruzi* trypomastigotes from the tissue culture.

8.3 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.

8.4 House each breeding pair in one cage placed inside a safe box with a 5 mm grid and lock-in door to prevent escape.

8.5 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.

8.6 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

9.1 Obtain tissues from *T. cruzi*-infected and naive control mice (step 8.6), and cut the paraffin-embedded samples into 4 µm thick sections.

9.2 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.

9.3 Incubate the tissue sections with 0.05% saponin once, followed by three distilled water washes at room temperature.

9.4 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.

9.5 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.

9.6 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.

9.7 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.

9.8 Examine the slides with a bright-field light microscope, and capture photo images with a microcamera with software and an analyzer program.

9.9 Document the immune privilege of the parasite in the absence of inflammatory reactions in the reproductive organs.

10 Statistical analyses

10.1 Use Biomedical Edit for sequence analysis, perform alignments with BLAST, and determine the E-value statistical significance ($p < 0.05$).

10.2 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 μm. 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].

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].

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].

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].

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].

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.

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].

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].

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].

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.

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[3-13]. 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[1,25-26,32-34]. 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[25,26,32-34]. 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[1,25,28-30]; *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[23,25,37-39].

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[40-45] 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[40-45] 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[41-43], 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 [40-45].

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.

ACKNOWLEDGMENTS:
We acknowledge the laboratory facilities and the critical comments of Izabela Dourado, Carla Araujo, and Clever Gomes and the technical assistance of Bruno Dalago and Rafael Andrade. We are indebted to the Foundation for the Advancement of Science (FAPDF), The National Research Council, Ministry of Science and Technology (CNPq/MCT), and The Agency for Training Human Resources, Ministry of Education (CAPES/ME), Brazil, for supporting these investigations.

DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES

1. Araujo, P.F et al. Sexual transmission of American trypanosomiasis in humans: a new potential pandemic route for Chagas parasites. *Memórias do Instituto Oswaldo Cruz*. **112** (6), 437–446 (2017).
2. Teixeira, A.R.L., Nitz, N., Bernal, F.M., Hetch, M.M. Parasite Induced Genetically Driven Autoimmune Chagas Heart Disease in the Chicken Model. *Journal of Visualized Experiments (*65), 3716 (2012).
3. Kalil-Filho, R. Globalization of Chagas Disease Burden and New Treatment Perspectives. *Journal of the American College of Cardiology.* **66** (10), 1190-2 (2015).
4. Nunes, M.C.P., Dones, W., Morillo. A. Encina, J.J., Ribeiro, A.L. Chagas Disease: An Overview of Clinical and Epidemiological Aspects. *Journal of the American College of Cardiology*. **62** (9), 767-776 (2013).
5. Pinazo, M.J., Gascon J. Chagas disease: from Latin America to the world. *Reports in Parasitology.* **2015** (4), 7-14 (2015).
6. Kessler, D. A., Shi, P.A., Avecilla, S.T., Shaz, B. H. Results of lookback for Chagas disease since the inception of donor screening at New York Blood Center. *Transfusion*. **53** (5), 1083-1087 (2013).
7. Klein, N., Hurwitz, I., R. Globalization of Chagas Disease: A Growing Concern in Nonendemic Countries. *Epidemiology Research International* (2012).
8. Pérez-Molina, J.A., Norman, F., López-Vélez, R. Chagas disease in non-endemic countries: epidemiology, clinical presentation and treatment. *Current Infectious Diseases Report*. **14** (3), 263-74 (2012).
9. Hotez, P.J. et al. Chagas disease: "the new HIV/AIDS of the Americas". *PLoS Neglected Tropical Diseases*. 6, e1498 (2012).
10. Schmunis, G.A., Yadon, Z.E. Chagas disease: a Latin American health problem becoming a world health problem. *Acta Tropica.* **115** (1-2), 14-21 (2010).
11. Franco-Paredes, C., Bottazzi, M.E., Hotez. P.J. The Unfinished Public Health Agenda of Chagas Disease in the Era of Globalization. *PLoS Neglected Tropical Diseases.* **3** (7), e470 (2009).
12. Teixeira, A.R.L., Vinaud. M., Castro, A.M. Emerging Chagas Disease. *In:* Chagas Disease: - A Global Health Problem. Chapter 3, pgs. 18-39. Bentham Science Publishers, New York. 2009. https://books.google.com.br/books?isbn=1608050416.
13. Lee, B.Y., Bacon, K.M., Bottazzi, M.E., Hotez, P.J. Global economic burden of Chagas disease: a computational simulation model. *Lancet Infectious Diseases*. **13** (4), 342-348 (2013).
14. Teixeira, A.R.L., Hecht. M.M., Guimaro, M.C., Sousa, A.O., Nitz, N. Pathogenesis of Chagas Disease: Parasite Persistence and Autoimmunity. *Clinical Microbiology Review*. **24** (3), 592-630 (2011).
15. Murcia, L et al. Risk factors and primary prevention of congenital Chagas disease in a nonendemic country. *Clinical Infectious Diseases*. **56** (4), 496-502 (2013).
16. Chagas, C. New human trypanosomiasis. Morphology and lifecycle of *Schizotrypanum cruzi,* the cause of a new human disease. *Memórias do Instituto Oswaldo Cruz.* **1**, 159 (1909).

17. Vianna, G. Contribution to the study of the Pathology of Chagas disease. *Memórias do Instituto Oswaldo Cruz*. **3**, 276 (1911).
18. Teixeira, A.R.L., Roters, F., Mott, K.E. Acute Chagas disease. *Gazeta Médica da Bahia***. 3**, 176-186 (1970).
19. Teixeira, A.R.L. et al. Prevention and Control of Chagas Disease – An Overview. *International STD Research, Reviews*. **7** (2), 1-15(2018).
20. Rios, A. et al. Can sexual transmission support the enzootic cycle of Trypanosoma cruzi? *Memórias do Instituto Oswaldo Cruz.* **113** (1), 3-8 (2018).
21. Lenzi, H. L et al. Trypanosoma cruzi: compromise of reproductive system in acute murine infection. *Acta Tropica*. **71** (2), 117-29 (1998).
22. Carvalho, L.O.P. et al. *Trypanosoma cruzi* and myoid cells from seminiferous tubules: Interaction and relation with fibrous components of extra cellular matrix in experimental Chagas' disease. *International Journal of Experimental Pathology*. **90** (1), 52-7 (2009).
23. Hecht, M.M. et al. Inheritance of DNA transferred from American trypanosomes to human hosts. *PLoS One*. **12**, e9181 (2010).
24. Alarcon, M. et al. Presencia de epimastigotes de *Trypanosoma cruzi* en el plasma seminal de ratones con infección aguda*. Boletín Malariología y Salud Ambiental.* **51**, 237 (2011).
25. Teixeira, A.R.L. et al. *Trypanosoma cruzi* in the Chicken Model: Chagas-Like Heart Disease in the Absence of Parasitism. *PLoS Neglected Tropical Diseases*. **5** (3), e1000 (2011).
26. Guimaro, M.C. et al. Inhibition of Autoimmune Chagas-Like Heart Disease by Bone Marrow Transplantation. *PLoS Neglected Tropical Diseases*. **8** (12), e3384 (2014).
27. Oliveira, C.I. et al. *Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice. *Microbes and Infection*. **6** (11) 977–84 (2004).
28. Mendes, D.G. et al. Exposure to mixed asymptomatic infections with *Trypanosoma cruzi*, *Leishmania braziliensis* and *Leishmania chagasi* in the human population of the greater Amazon. *Tropical Medicine, International Health*. **12**, 629 (2007).
29. Moser, D.R., Kirchhoff, L.V., Donelson, J.E. Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction. *Journal of Clinical Microbiology.* **27** (7), 1477-82 (1989).
30. Da Silva, A. R. Sexual transmission of *Trypanosoma cruzi* in *mus musculus* [MsD thesis] Brasília: University of Brasília – UnB; 2013. Available from http://repositório.unb.br/handle/10482/14829.
31. Ribeiro, M. et al. Can sexual transmission support the enzootic cycle of Trypanosoma cruzi? *Memórias do Instituto Oswaldo Cruz*. **113** (1), 3-8 (2018).
32. Billingham, R.E., Brent, L., Medawar, P.B. Actively acquired tolerance of foreign cells. *Nature.* **172** (4379), 603-6 (1953).
33. Burnet, F., Fenner, F. *The production of Antibodies.* MacMillan, Melbourne, Australia (1949).
34. Hasek, M. Parabiosis of birds during their embryonic development. *Chekhoslovatskaia Biology*. **2** (1), 29-31 (1953).
35. Coura, J.R., Junqueira, A.C.V., Fernades, O., Valente, S.A., Miles, M.A. Emerging Chagas Disease in Amazonian Brazil. *Trends Parasitology* **18** (4), 171-6 (2002).

36. Teixeira, A.R.L. et al. Emerging Chagas disease: Trophic network and cycle of transmission of *Trypanosoma cruzi* from palm trees in the Amazon. *Emerging Infectious Diseases*. **7** (1), 100112 (2001).

37. Hazebroek, M., Dennert, R., Heymans. S. Idiopathic dilated cardiomyopathy: possible triggers and treatment strategies. *Netherland Heart Journal.* **20** (7-8), 332–35 (2012).

38. Arimura, T., Hayashi, T., Kimura, A. Molecular etiology of idiopathic cardiomyopathy. *Acta Myologica*. **26** (3), 153-8 (2007).

39. Dec, W. G., Fuster, V. Idiopathic Dilated Cardiomyopathy. *New England Journal of Medicine*. **33**, 1564-1575 (1994).

40. Niederkorn, J.Y. See no evil, hear no evil, do no evil: the lessons of immune privilege. *Nature Immunology*. **7** (4), 354-9 (2006).

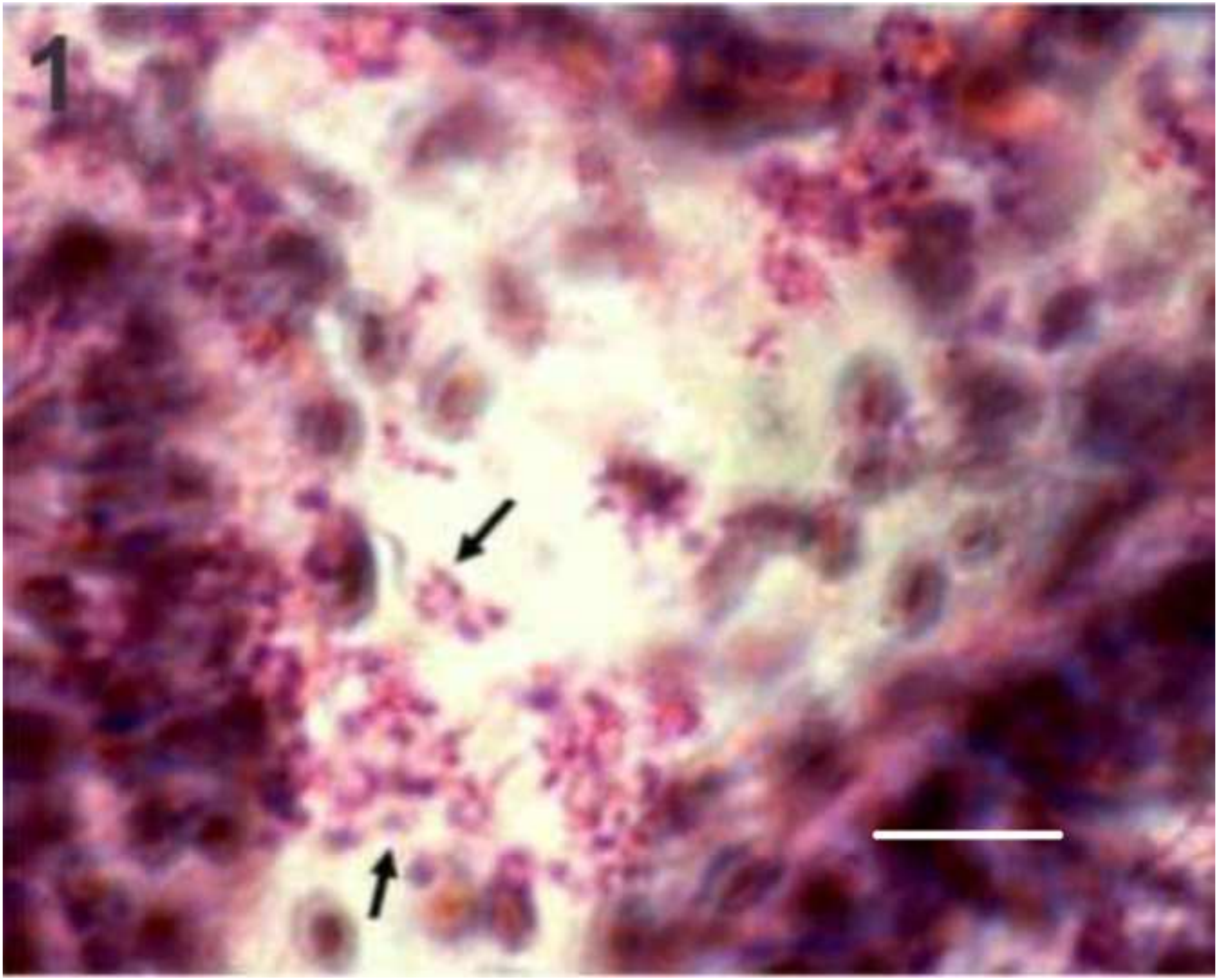
41. Smith, B.E., Braun, R.E. Germ cell migration across Sertoli cell tight junctions. *Science*. **338** (6118), 798-802 (2012).

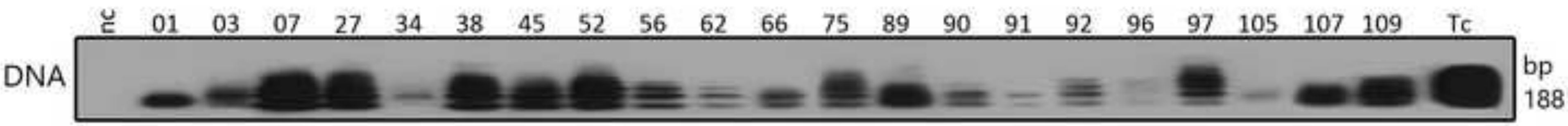
42. Garth, A., Wilbanks, J., Streilein,W. Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation *via* a mechanism involving transforming growth factor-β. *European Journal of Immunology*. **22** (4), 1031-1036 (1992).

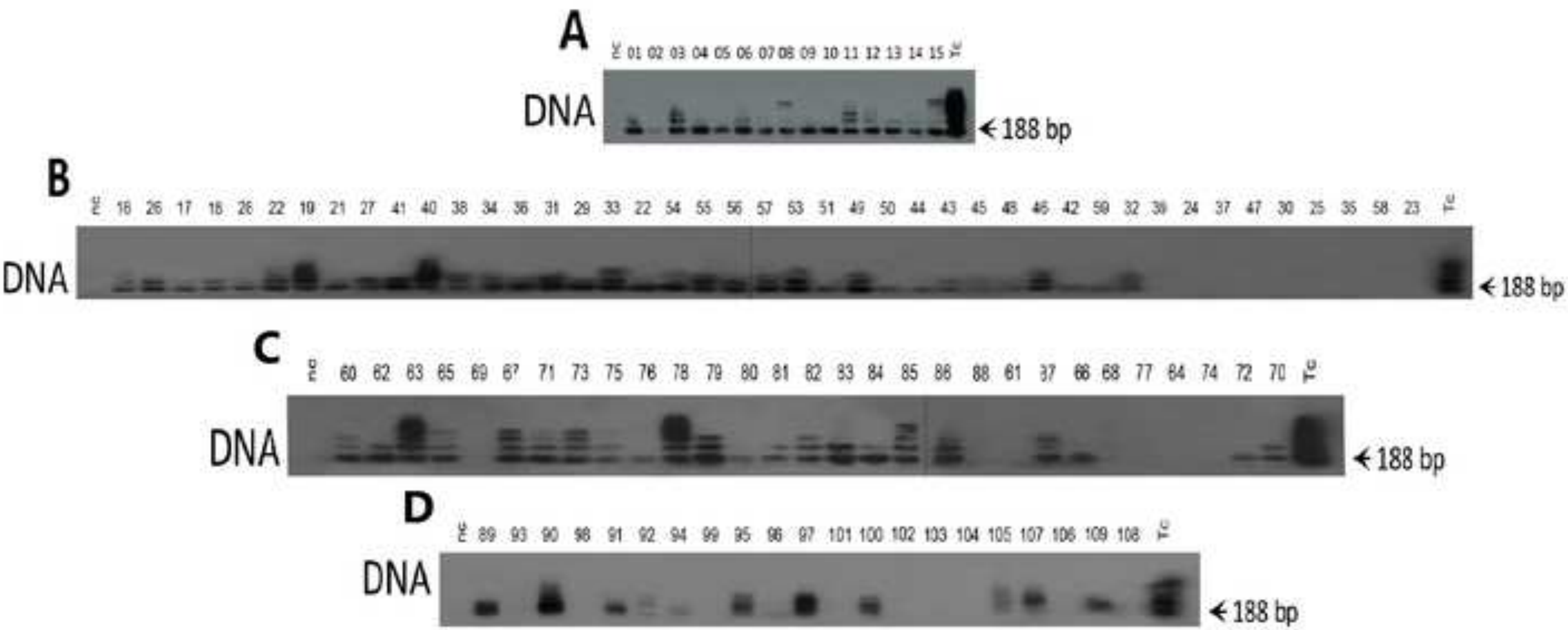
43. Meng, J., Anne, R., Greenlee, C., Taub, J., Braun, R.E. Sertoli Cell-Specific Deletion of the Androgen Receptor Compromises Testicular Immune Privilege in Mice. *Biology of Reproduction*. **85** (2), 254-60 (2011).

44. Fujisaki, J. et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature.* **474** (7350), 216-9 (2011).

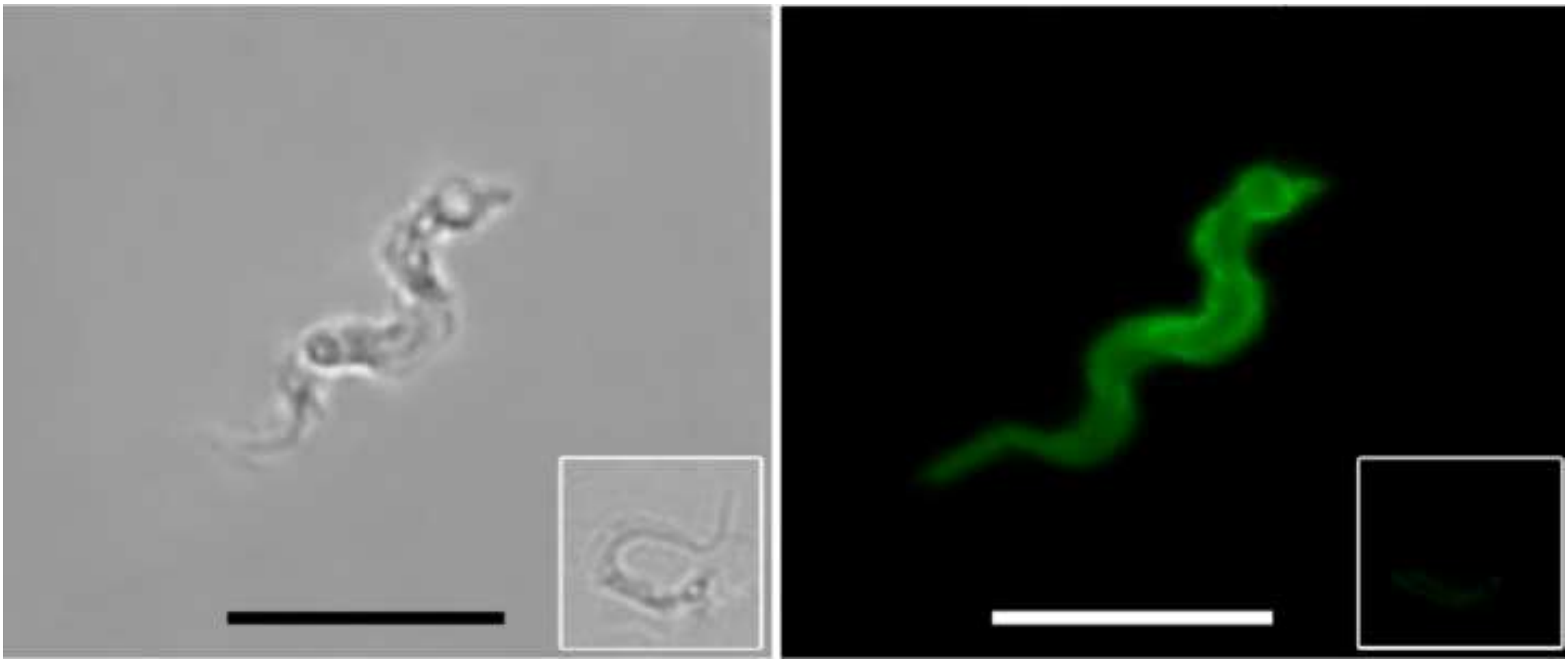
45. Wood, K.J., Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nature Review Immunology*. **3** (3), 199-210 (2003).


1


nc
01
03
07
27
34
38
45
52
56
62
66
75
89
90
91
92
96
97
105
107
109
Tc
DNA
bp
188

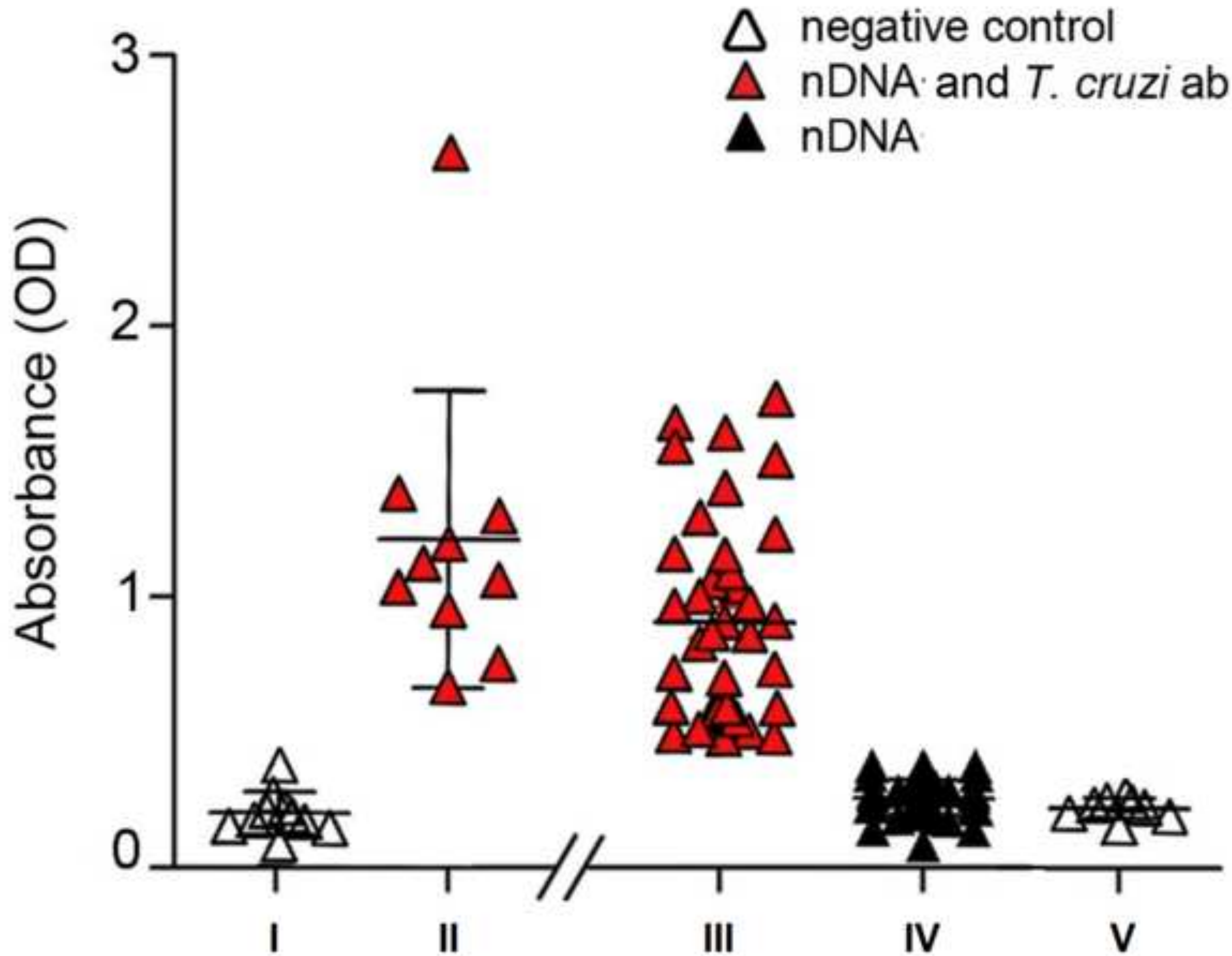

A
DNA
188 bp
B
DNA
188 bp
C
DNA
188 bp
D
DNA
188 bp


nc
5
9
11
12
13
21
22
26
28
36
42
53
59
63
67
72
73
92
95
Tc
nDNA
188 bp



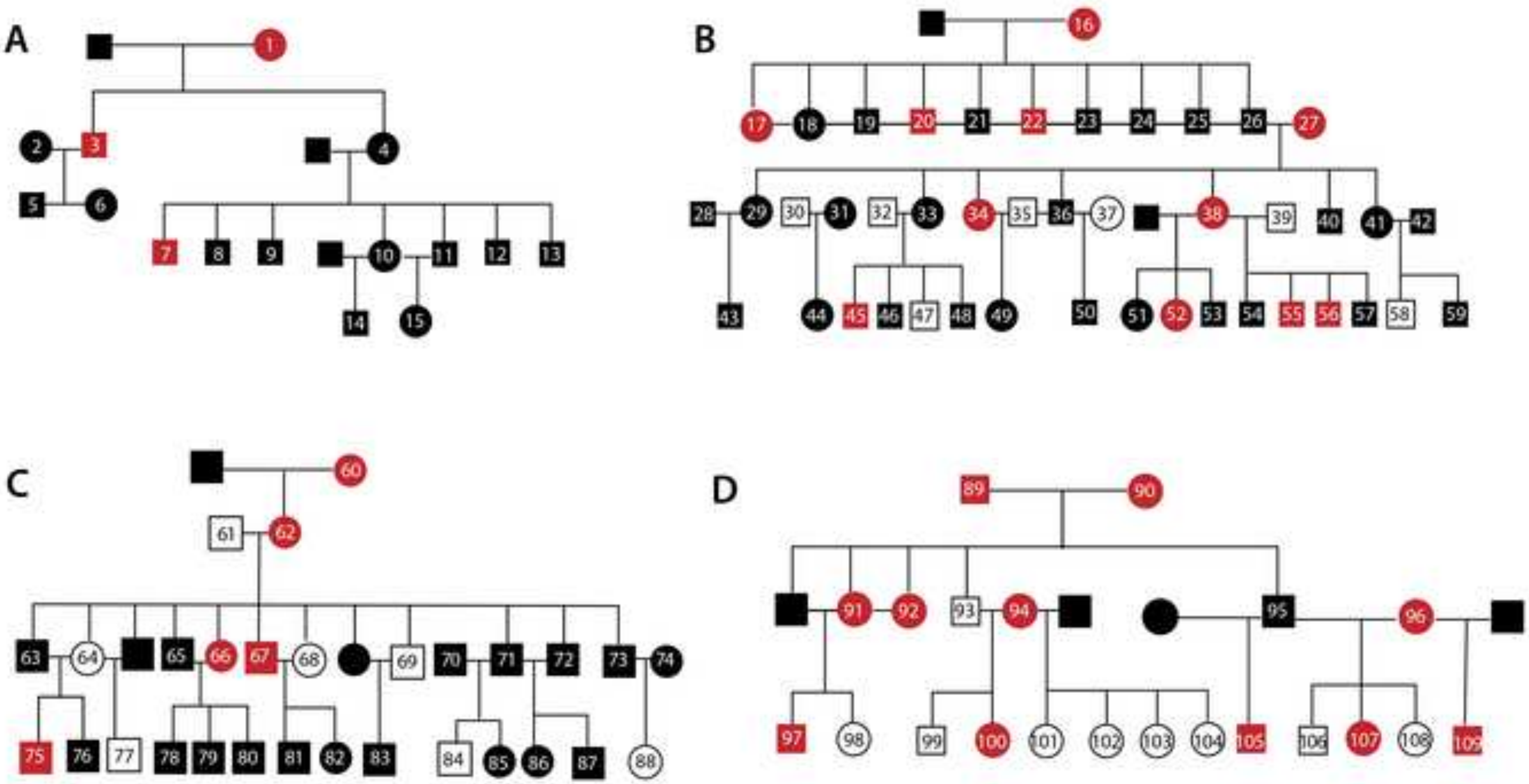
Figure

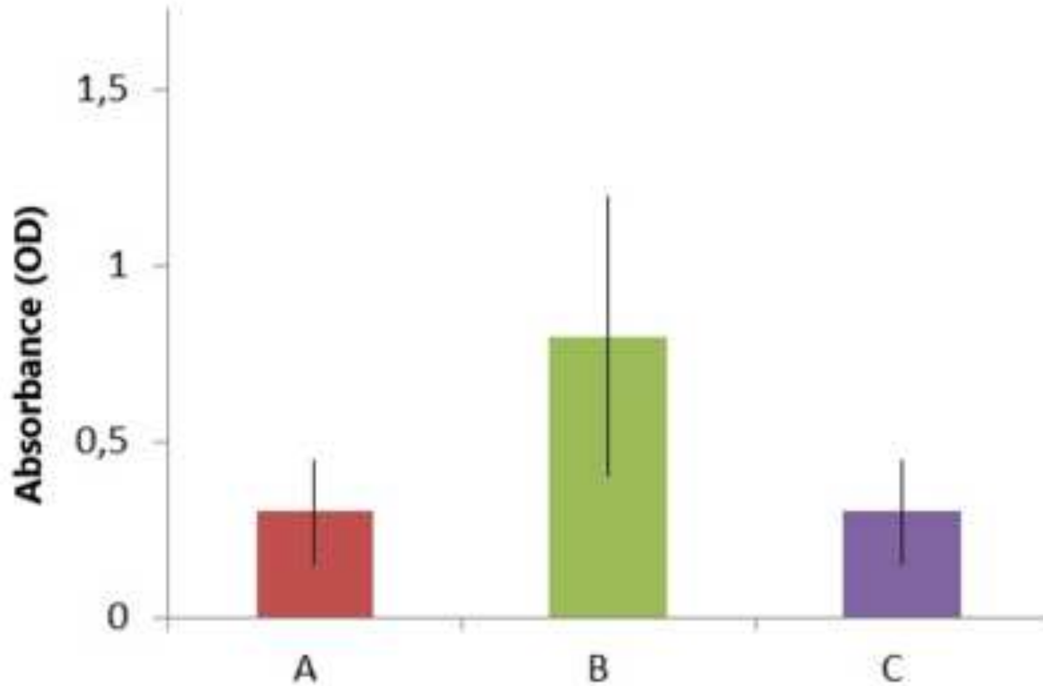
Click here to access/download;Figure;Figure 6.tif

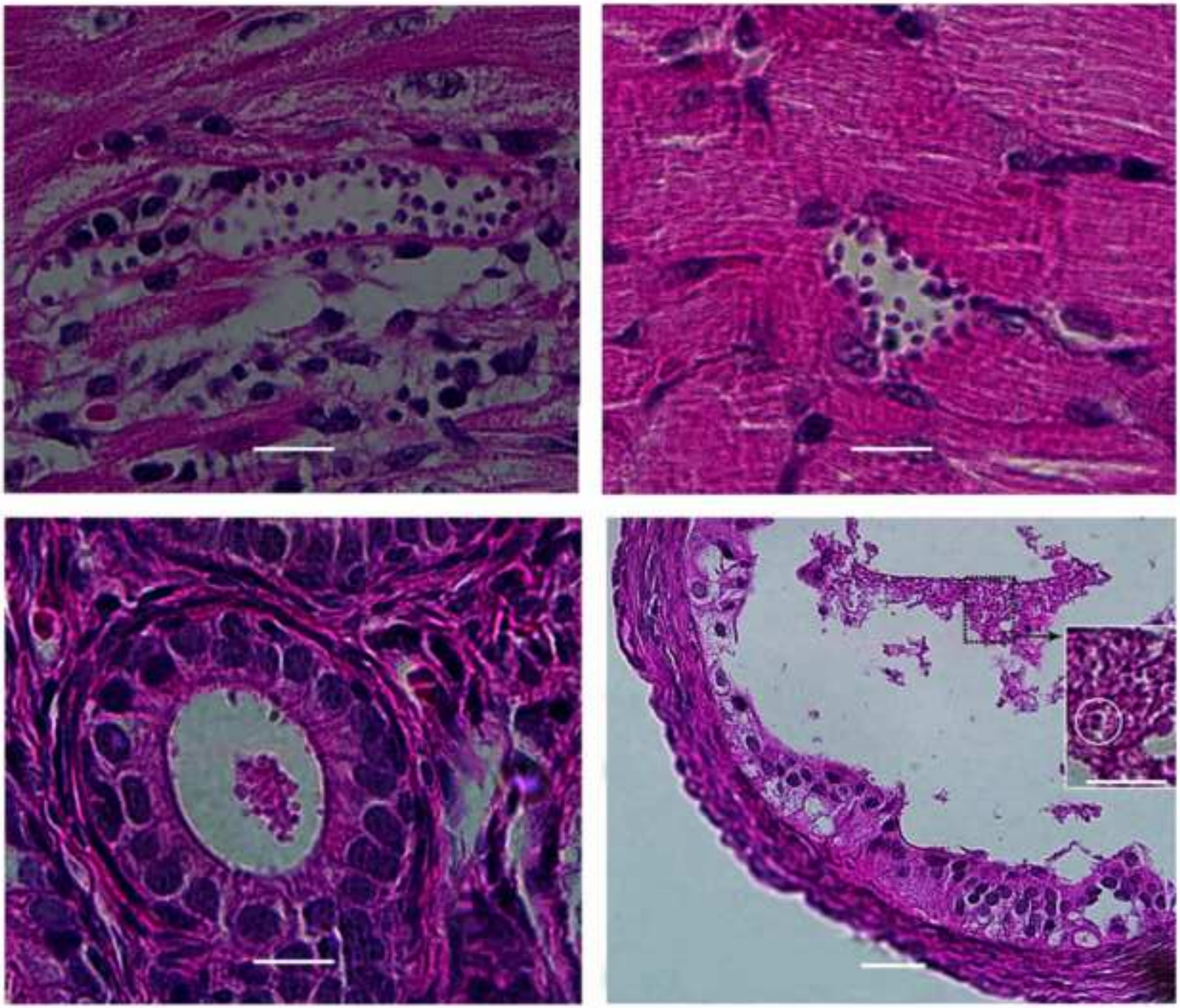


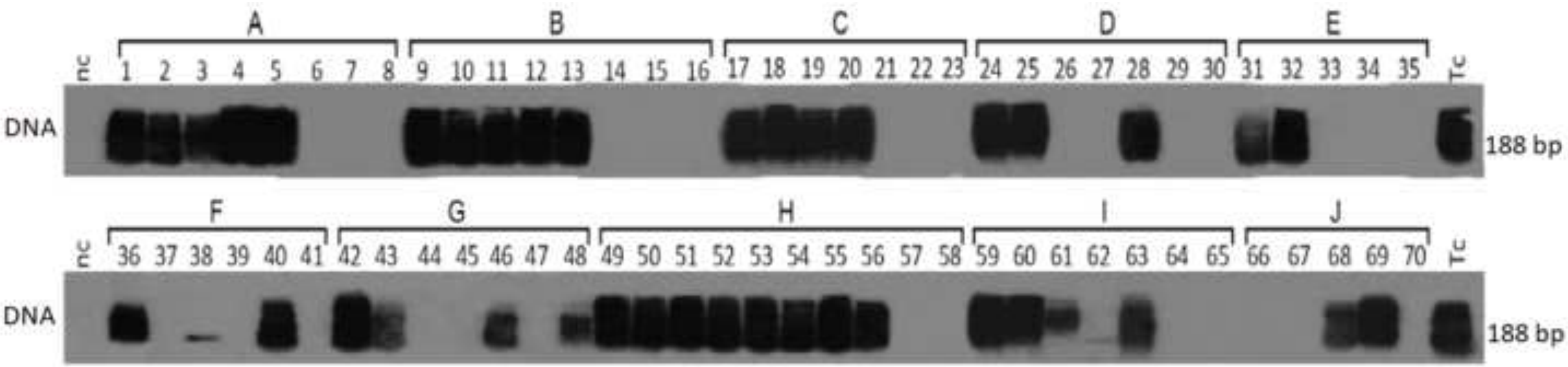
Figure

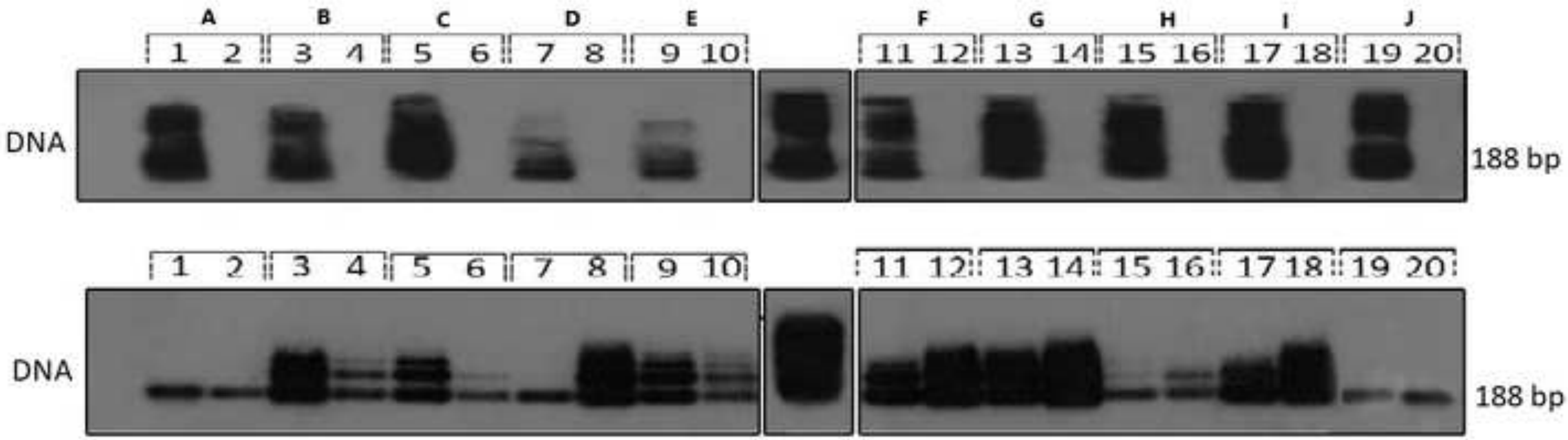
Click here to access/download;Figure;FIGURE 7. heredogram 1.png




Absorbance (OD)
1,5
1
0,5
0
A
B
C




A
B
C
D
E
nc 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 Tc
DNA
188 bp
F
G
H
I
J
nc 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 Tc
DNA
188 bp


A
B
C
D
E
F
G
H
I
J
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
DNA
188 bp
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
DNA
188 bp

Figure

Click here to access/download;Figure;Figure12 .tif

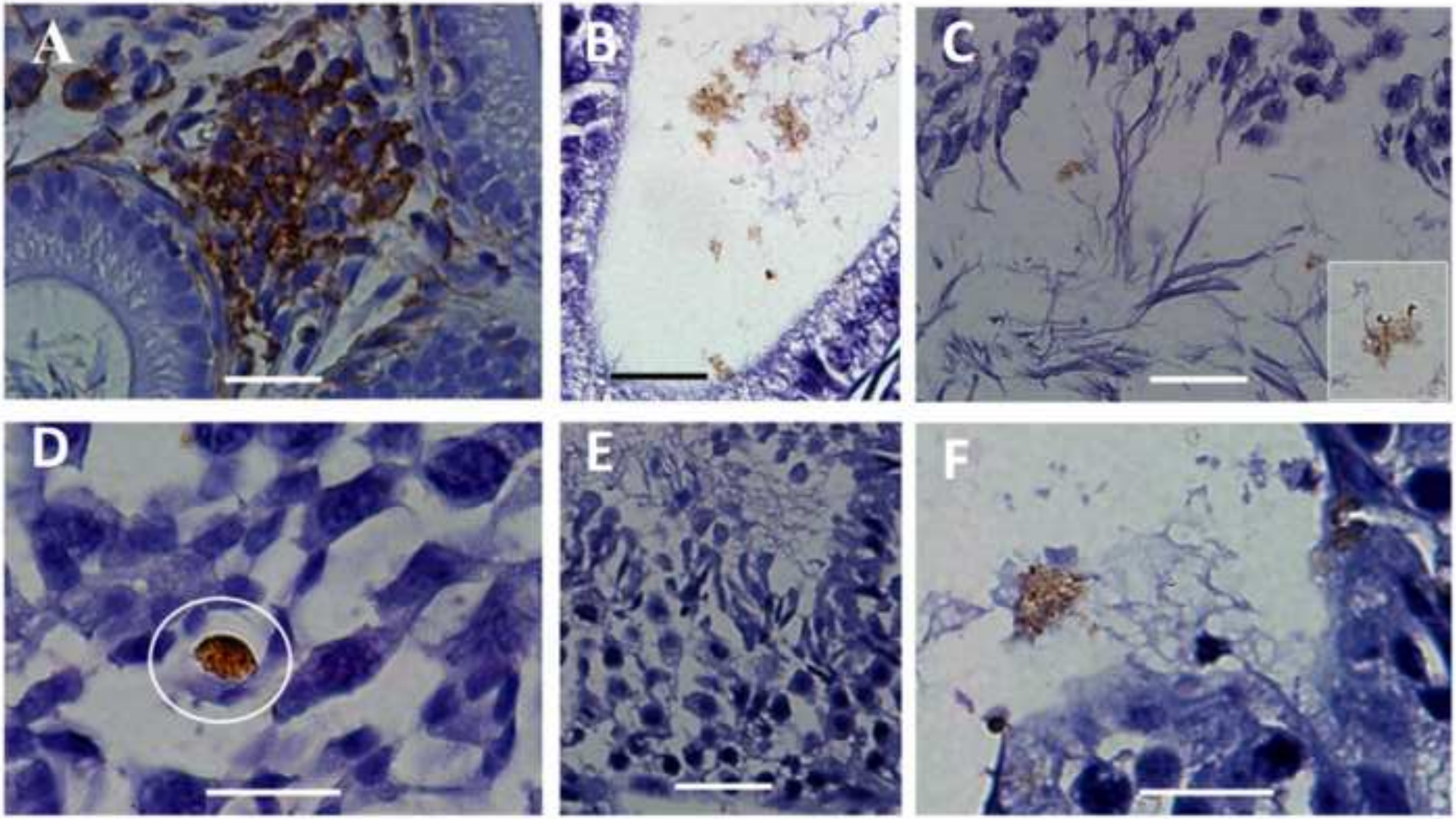


Table 1. The discrepancies between the ratios of positive IIF and ELISA exams and those of nDNA-PCR assays in the samples collected from the human study families A-to-D*

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+ $^{\delta}$ (n = 31)	31/109	28.4	31/109	28.4
IV- Chagas Ab- PCR+ $^{\delta}$ (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 ($p < 0.05$).

$^{\delta}$ 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.

Name of the reagent	Company	Catalogue number
BCIP and NBT redox system	Sigma-Aldrich	681 451 001
Blood DNA Purification columns	Amersham Biosciences	27-9603-01
d-ATP, [**α-**^{32}P], 250 μCi.	Perkin Elmer	BLU012H
DNA, Solution Salt Fish Sperm	AMRESCO	064-10G
dNTP Set, 100 mM Solutions	GE Healthcare	28-4065-51
Eco RI	Invitrogen	15202-021
Goat anti-human IgG- alkaline phosphatase conjugated	Southern Biotech	2040-04
Goat anti-human IgG- FITC conjugated	Biocompare	MB5198020
Hybond – N+ nylon membrane	GE Healthcare	RPN303B
Hybridization oven	Thomas Scientific	95-0031-02
Micro imaging software cell Sens software	Olympus, Japan	
Molecular probes labeling System	Invitrogen	700-0030
Nsi I	Sigma-Aldrich	R5584 1KU
Plasmid Prep Mini Spin Kit	GE Healthcare	28-9042-70
Plate reader	Bio-Tek GmBH	2015
Rabbit anti-chicken IgG-alkaline phosphatase conjugated	Sigma-Aldrich	A9171
Rabbit anti-chicken IgG-FITC conjugated	Sigma-Aldrich	F8888
Rabbit anti-mouse IgG- alkaline phosphatase conjugated	Sigma Aldrich	A2418
Rabbit anti-mouse IgG-FITC conjugated	Biorad	MCA5787
Spin Columns for radio labeled DNA purification, Sephadex G-	Sigma-Aldrich	G25DNA-RO
Taq DNA Polymerase Recombinant	Invitrogen	11615-010
Thermal cycler system	Biorad, USA	1709703
Vector Systems	Promega	A1380

jove JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Sexual transmission of the American trypanosomes from males and females to naive mates

Author(s): Adriana B. Almeida, Perla F. Araujo, F.M. Bernal, Ana C. Rosa, Sebastião A. Valente, Antonio R.L. Teixeira

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Antonio R. L. Teixeira
Department:	Pathology
Institution:	University of Brasilia
Title:	Professor Emeritus, MD., Ph.D.
Signature:	Antonio Teixeira — Date: 08/10/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Brasília, July 27, 2018.

From: Antonio Teixeira
To: Vineeta Bajaj, Editor **JoVE**
Ref.: manuscript JoVE57985R2

Dear Editor:

Thank you for your extensive and excellent editing job. Please, find my answers and comments on the specific questions and comments of the reviewers:

Editorial and production comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: the manuscript was reviewed by an English first Language.

2. Please complete the first Page of the Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.
Answer: The filled-in and signed ALA uploaded.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.
Answer: Copyright permissions obtained and sent to the Editorial Manager account*.

4. Figure 1: Please add a scale bar to provide context to the magnification used. Scale bar is defined in the figure legend but missing from the figure. Please also explain the black arrows in the figure legend.
Answer: The scale bar introduced in the figure legend and the black arrows explained. Note that the high-resolution figures 1 to 13 are 300 pixels.

5. Figure 2: There are no panels G, H and I. Please revise the figure legend.
Answer: Certainly, panels G, H and I replaced by D, E and F in the figure legend.

6. Figure 9: Please define error bars in the figure legend.
Answer: Error bars defined in the figure legend.

7. Please define all abbreviations before use.
Answer: Abbreviations defined in the text.

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
Answer: Abbreviations now appear accordingly with SI

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
Answer: Yes. The space included between numbers and their units.

10. Please change centigrade to °C.
Answer: The change made in the text.

11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Answer: The formatting is now in agreement with the JoVE Instructions for Authors.

12. For culture media and buffer such as DMEM, etc., please spell out at first use and provide composition. If they purchased, please cite the materials table.
Answer: The purchased culture ingredients (such as DMEM) defined in the text and cited in the table Materials and Equipment.
13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:
Line 98: What are the symptoms of acute Chagas disease?
Answer: symptoms of acute Chagas disease defined at lines 95 and 96 of the Protocol

Line 100: Please describe how to obtain serum and blood mononuclear cells.
Answer: please find description at lines 98 to 100 of the Protocol.

Line 101: Please describe how to isolate the wild type T. cruzi from the blood.
Answer: please find description at lines 103 to 126 of the Protocol.

Lines 103-105: What container is used in this step? What volume of DMEM is used? What do haploid cells refer to? Please specify which supernatant.
Answer: those definitions made at lines 114 to 120 of the Protocol.

Lines 117-118: Please specify growth conditions.
Answer: the growth conditions defined at lines 114 to 120 of the Protocol.

Lines 119-121, 139-142: Please describe how this is actually done.
Answer: Actually, this made as described at lines 103 to 126 of the Protocol.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Answers: the steps a) to e) explicitly covered in a full paragraph at lines 550 to 575 of the Discussion.

15. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.
Answer: journal titles are no longer abbreviate, and the volume, issue numbers are included for all references.

16. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.
Answer: sorry, but lot numbers and RRIDs of antibodies are no long available.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.
Answer: the homogeneity between the written protocol and the narration in the video achieved to considerable extent, according with the Editor’s instruction. For example, see description at lines 92 to 100 of the Protocol, which now appear in the video section on the ‘Study population and isolating the wild type *Trypanosoma cruzi’*. These changes appear at red lines in the current article text.

2. The details in the video are not the same as the details in the written manuscript. For example:
01:28: The video mentions the physicians delivered health care for a period of five years while the written manuscript does not mention this.

Answer: gap is filled in at line 97 of the protocol, which appears in the video: ‘the research team delivered health care for the study population’.

01:41-02:23: The video does not contain any actions but only shows figures. Steps in lines 99-121 of the written manuscript not covered in the video.
Answer: actually, the video shows actions described at lines 92 to 122 of the Protocol.

02:26-03:52: Steps 3 and 4 of the video are difficult to follow because they do not match the written manuscript.
Answer: those steps of the video now match the manuscript.

02:48: The section title card shows now we are at step 3 (DNA extraction), while the details stated in the video are provided in step 5 of the written manuscript.
02:59: The video says 1.5 while the written manuscript states 15 μM $MgCl_2$. Please revise to be consistent.
Answer: The video now says 15 μM $MgCl_2$ in agreement with the sentence at lines 152 to 154 of the Protocol.

03:07: The video mentions and shows centrifugation while the written manuscript does not state centrifugation.
Answer: the narration in the video is in agreement with lines 129 and 130 of the Protocol.

07:45: The video says that “instill … into the peritoneal cavity or into the vagina”, while the written manuscript states that “into the peritoneal and an equal amount into the vagina of group-B mice”.
Answer: The description in the protocol (lines 139 and 140) now coincides with the audio – ‘instill semen aliquots into the peritoneal cavity and into the vagina”.

3. Please show and describe representative results after the protocol, not during the protocol.
Answer: The modification made in the text and in the video, such as for the growth of the wild type parasites (lines 103 to 126 of the Protocol) that proceeds in the next step of the investigation.

4. Please upload a revised high-resolution video. Answer: The high-resolution video uploaded here: http://www.jove.com/files_upload.php?src=17685473

Video Production Notes 5/1/18:

Formatting issues
• 0:00-0:10 - The opening 10 seconds of the title card can be cut out. We recommend starting the music at the beginning of the video. Answer: The recommendation is ok.

Audio issues
• The audio quality clearly jumps around throughout the video. It sounds like different pieces were recorded in different sessions. All of the audio is sufficient for our minimum quality standards, but it does sound like an audio patchwork. If it is possible to re-record in one session, this would be a large improvement.
Answer: Sure, the audio patchwork recorded at different laboratories in different sessions because the laboratory no long exists after my retirement.

• 1:03 - There is some stray audio here that should be cut out.
Answer: Some stray audio was cut out.

• 1:20 - The beginning of this sentence fades in while "The" is being spoken. The fade in should be removed in the audio.
Answer: The fade is resolved.

• 7:19-7:20 - The punch-in audio recording here sounds too different for it to work as a mid-sentence patch. This entire sentence should be re-recorded.
Answer: The punch-in is resolved in the new sentence recorded.

Animal use issues
• 1:01 - Please give the name of the institution in the disclaimer card.
The name of the institution (Faculty of Medicine of the University of Brasilia) now appear in the Study population disclaimer card.

Editing issues
• 4:03, 6:45, 6:48, 6:50, 6:53, 6:55, 7:03 - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.
Answer: jump cuts and jarring effect smoothed out.

• 5:06-5:11 - The combination of the shaky camera and the quick edits makes this part seem chaotic and difficult to follow. Either the shots should be redone, or they should at least be allowed to play out for a bit longer so that the viewer doesn't feel rushed.
Answer: the video plays a bit longer in order the viewer doesn´t feel rushed.

• 3:20-3:26, 7:42-7:50 - The edits during this time are too rapid. The viewer's eye never gets to settle on any shot, which makes it very difficult to follow what is happening on screen. The pacing of the edits should be slowed down.
Answer: The pacing of the edits slowed down.

• 8:27 - There needs to be more space between the audio statements here.
Answer: more space added.

Video quality issues
• 7:56-8:06 - Rather than 3 video clips that shake a bit with one that is 90° different from the other two, we recommend taking still photos of the placards and using those in the video. It will be easier for the viewer to read and will look more uniform.
Answer: this query solved with the following demand.

• 7:56-8:31 - This section talks about performing specific steps but none are shown in the video. The only visual is slowed down B-roll of mice in cages. We recommend cutting this section out and adding a reference to the text in the previous section of the video.
Answer: this full section cut and replaced by a card reference to the text Protocol section.

In agreement with the JoVE's Video Technical team, the Introduction and the Discussion video and audio recorded again in order to obtain homogeneity. In this new look, the Discussion is now six paragraphs in length, because it includes the suggestion of the Reviewer to cover explicitly the details: a) Critical steps within the protocol; b) Modifications ant troubleshooting; c) Limitations of the technique and significance; d) future applications of the technique. In this regard, the video is over 16 min now.

Dear Editor, I look forward to hearing from you.
Sincerely,
Antonio Teixeira,
Corresponding Author.

*Annexes subjected to the JoVE Manager account:
- Letters of permissions from the Author and the publishers:
- A) Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, Brazil (ref. 1).
- B) Acta Venezolana de Malariología y Salud Publica (ref. 24).
- C) International Journal of STD Research & Reviews (ref. 19)
- D) PLoS Neglected Tropical Diseases (ref. 26).

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes

Title: Sexual transmission of the American trypanosomes from males and females to naive mates during intercourse

1	Laboratory mice	No corrections in the video	NO	Yes	The research Ethical Committee approved the protocol 054/09: the mice received humane care; the animals were sacrificed under anesthesia
2		Mice aren’t housed in hoods	N	Y. line 237	Remove “kept in hoods”
		The diet should be whatever is offered at the facility.	N	y. line 238	Remove “Purina Chow”
		Mice should be under anesthesia before performing a cardiac puncture.	N	Y. Lines 260-262	Draw 0.5 mL of blood by heart puncture from the parental (FO) and progeny (F1) mice under anesthesia; then submit the body tissue sections for pathology analysis.

URL: https://www.jove.com/video/57985/title?status=a59991k

Reference 19

Brasília, September 7, 2018.

To
Vineeta Bajaj, Ph.D.
Editor, JoVE
Ref. JoVE57985

Dear Editor:

Please, find the revised JoVE57985 uploaded manuscript and the new high definition video downloaded at the Journal website and, thus addressing to each of the Editorial comments.

Answers to the Editorial Comments:

1) The Journal style is retained.
2) The Corresponding author addressed to all the specific comments with words and sentences marked red in the text article.
3) The redundancies were removed from the protocol section and from the discussion. Now, new sentences marked red in the manuscript describe all specific details about the carried out experiments.
4) Please, find new sentences (marked red) in the current download version of the protocol, mainly at steps 1 to 5. Accordingly, the sentences were re-written aiming at someone interested in the reproduction of the experiments in the protocol, and now all the details about collection of biological samples, volumes and concentrations, time of exposure, centrifugations, etc., are included.
5) The formatted Protocol is now seven pages long.
6) The extensive rewriting of the protocol critical sections 1 to 10 secured agreement between the video and the manuscript.

Video Production Comments:

- The length of the video increased from 16:12 to 18:27 min but it does not exceed the JoVE's video length limit, according with the instructions to authors.
- After the inclusion of all the details in the protocol sections we proceeded with the extensive cuts at the introduction (last paragraph) and at the discussion (>25% cuts).
- 0:57 an Ethics disclosure card naming the official body which provided oversight is included at the position suggested by the Editor.
- Frame size/proportion issues: The suggested blocks of images, which had black borders on the top and bottom of the frame were enlarged, and now fill the frames.

  Audio issues
- Although in the lack of a professional audio equalizer, the audio was almost totally recorded once more from the beginning to the end of the video and the narration is readily audible.

  Editing issues
- 9:35-11:06 the representative results section now appear with narration.
- 8:18, 8:21 the jarring effects are smoothed out.

Other video comments

1) Please, find out the title is changed as per the text.
2) 00:08 definitely, the word Doctor removed from the text box.

3) An ethics statement for human and animal study is included after the Protocol announcement.
4) The video was worked up accordingly with the direction in the manuscript. For example, the quantities of biological samples are specified in the sections' steps, as requested.
5) 9:27 the VOs were included.
6) Representative results now show narrations along each figure length.
7) The discussion is now rather short (>25%) in comparison with the previous version.
8) As a result of much narration of details and entire sentences, as suggested by the video Editor, I cannot make the video shorter than 18:27 min in length but within the limit set forth in the instructions for authors.

I hope the high definition new version of the JoVE57985 video is suitable for publication, despite of the adverse local conditions for the video production, in lack of a permanent laboratory set up. However, I will not hesitate to conduct any further task in case that the JoVE's Editor suggests further improvement of the video and article publication.

Sincerely,

Antonio Teixeira
Corresponding author.

Brasília, October 8, 2018.

To
Vineeta Bajaj, Ph.D.
Editor, JoVE
Ref. JoVE57985R4

Dear Editor:

Please, find the new version of the JoVE57985R4 uploaded manuscript and the new high definition video downloaded at the Journal website and, thus addressing to each of the Editorial comments.

Answers to the Editorial Comments:

1) Presently, the video is 14':13".
2) 5':23''-5':29''. The video cut backs are corrected in the best of our skills.
3) 5':30''-5:33", 11:09-11:17. The pacing of the actions is slowed down.
4) 10':20". The jump cuts are smoothed out with crossfades.

Frame size proportion issues

5) 5:23-5:33, 8:12, 10:00-10-30, 5:11-5:53, 6:04-6:37, 6:48-7-54, 8:00;8-31. The video clips are enlarged and now fill in the frame.

Audio issues

11':13". The entire narration with new images and audio replaces the previous version bad sounds all through the video.

Other video issues

DNA extraction and PCR analysis. The imperative tense is used throughout the narration and the research protocol step used (E.g. 5:29) wherever necessary.

Legends.

At 0:35, 4:39, 8:20 please, find corrections of the misspellings (d**e**sease by disease; Tce by Tcz1/2; and, Sheme by S**c**heme.

Southern hybridization section

The imperative tense used throughout the protocol as done in the text protocol.

6:13. The writings style now follows the examples as per the Editor's letter.

The imperative tense is used in the Immunofluorescence section in order to shortening the video.

9:52. The Biotek mark is eliminated, as suggested.

The discussion section is short enough to keep the video at the time limit.

A ALA is signed for open access.

Table 1 xlsx is uploaded in the 57985R4 submission file.

6) The extensive rewriting of the protocol critical sections 1 to 10 secured style in the video and in the manuscript.

A high definition new version of the JoVE57985R$ video downloads at the Jove's website.
Thanks so much to the Jove's Editors team.

Sincerely,

Antonio Teixeira
Corresponding author.

To
Nam Nguyen, Ph.D.
Manager of Review
JoVE,

Dear Nham:

Our manuscript, JoVE57985R5 "Sexual transmission of the American trypanosomes from males and females to naive mates," was subject to our careful attention, as per the Editor comments and specific points addressed.

The language problems were resolved by the AJE. The editing of the video was thoroughly worked up. Please, find below a list with the answers to the specific points and comments. Hopefully the publication criteria are met by the article, as follows:

The AJE professional copy-editing services assure publication grade. Additional comments are in the attached manuscript. The references list of articles is OK.

The revisions are accomplished and the video is below 15 minute limit:

• 0:00-0:05 - The black silence is removed here.
• 3:59-4:18 - The thin black borders on the top and bottom removed and the shots fill in the frame.
• 4:17-4:26 - The length of some of the shots are either extended or removed.
• 4:19-4:26 - The black borders on the top and bottom removed and the shots fill in the frame.
• 4:29-4:58 -. The slow motion effect is removed and some of the shots extended.
• 5:55, 6:18, 6:20 - The jump cuts resolved with crossfades.
• 6:22-6:24 - The black borders removed and the shots fill in the frame.
• 6:30-7:42 – The problems with this section are removed according with the instructions.
• 6:42, 7:24 - The shots are smoothed out with crossfades.
• 7:45-8:13 -. Now the shots fill the frame.
• 7:54, 7:58, 8:01, 8:03, 8:12 - The jump cuts are smoothed out with cross fades.
• 8:41 - Instead of "sheme", you read, "scheme".
• 8:49-9:14 - The thin black borders on the top and bottom of frame fill the frame, which now are slowed down.
• 12:11 - A title card "Conclusion" is inserted.

Please, find a high definition update version of the JoVE57985R 12112018 video uploaded at the Jove’s website.

Sincerely,

Antonio Teixeira
Corresponding author.

Title: Sexual transmission of the American trypanosomes from males and females to naive mates during intercourse

URL: https://www.jove.com/video/57985/title?status=a59991k

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes
1	Laboratory mice	No corrections in the video	NO	Yes	The research Ethical Committee approved the protocol 054/09: the mice received humane care; the animals were sacrificed under anesthesia
2		Mice aren't housed in hoods	N	Y. line 237	Remove "kept in hoods"
		The diet should be whatever offered at the facility.	N	y. line 238	Remove "Purina Chow"
		Mice under anesthesia before performing a cardiac puncture.	N	Y. Lines 260-262	Draw 0.5 mL of blood by heart puncture from the parental (FO) and progeny (F1) mice under anesthesia; then submit the body tissue sections for pathology analysis.

Copyright permission, reference 1.

Rio de Janeiro, February 26, 2018.

Dr Antonio Teixeira
antonioteixeirarl@gmail.com

Ref.: "Sexual transmission **of** American trypanosomiasis **in** humans**:** **a** new potential pandemic route **for** Chagas parasites"

DOI: **10.1590/0074-02760160538**

Regarding your request of permission to use the figures that were published in *Memórias do Instituto Oswaldo Cruz* Vol. 112(6) 437-446, 2017 by Teixeira ARL et al., we would like to inform that the permission is granted but all images must be accompanied by a visible credit line.

Best regards,

Claude Pirmez
Editor

C) Reference 19

International STD Research & Reviews 7(2): 1-15, 2018; Article no.ISRR.42594 ISSN: 2347-5196, NLM ID: 101666147 Prevention and Control of Chagas Disease – An Overview. A. R. L. Teixeira1*, C. Gomes2 , A. C. Rosa1 , P. F. Araujo1 , C. E. Anunciação2 , E. Silveira-Lacerda2 , A. B. Almeida1 and S. Petrofeza .

http://www.sciencedomain.org/issue/3637,

DOI : 10.9734/ISRR/2018/42594

amaritza@ula.ve

Traduzir mensagem
Buenos días Dr. Antonio Teixeira

Yo Maritza Alarcón autora principal del artículo publicado en Bol Mal Salud Amb 51: 237 (2011) autorizo al Dr. Antonio Teixeira para re-publicar las microfotografías del plasma seminal NMRI infectado con Trypanosoma cruzi que muestram: (A) amastigotes dentro de celulas fagociticas. (B) Amastigotes extracelulares. (C y D) tripomastigotes; (E, > F > y G), epimastigotes; (H e I) epimastigotes en division. Giemsa, 1000X)

Att. Maritza Alarcón
amaritzaa@gmail.com

PD: por favor escribir a amaritzaa@gmail.com porque este es un correo ULA y no siempre funcion

PLOS Neglected Tropical Diseases por 4pmth8tvx1wyxkke.4jwuaga.u-ifismac.na48.bnc.salesforce.com
para mim

Dear Dr. Teixeira,

Thank you for your email. PLOS applies the Creative Commons Attribution (CC-BY) license to all of our articles: http://journals.plos.org/plosntds/s/content-license

Under the CC BY license, authors retain ownership of the copyright for their article, but allow anyone to download, reuse, reprint, modify, distribute, and/or copy articles in PLOS journals, as long as the original authors and the source are cited. No permission is required from the authors or the publishers.

Please don't hesitate to contact us if you have any further queries.

All my best,
Charlotte

PLOS | OPEN FOR DISCOVERY
Charlotte Bhaskar | Publications Manager, PLOS Neglected Tropical Diseases
1160 Battery Street, Suite 225, San Francisco, CA 94111
plosntds@plos.org

Case Number: 05881117

--------------- Original Message ---------------
From: Antonio Teixeira [antonioteixeirarl@gmail.com]
Sent: 7/26/2018
To: plosntds@plos.org
Subject: Request of reprint permission

Dear Editor:

I will appreciate receiving the signed permission for the reprint of Figure 8 in our article:

PLoS Negl Trop Dis. <https://www.ncbi.nlm.nih.gov/pubmed/25521296#> 2014 Dec 18;8(12):e3384. doi: 10.1371/journal.pntd.0003384. eCollection 2014 Dec.
Inhibition of autoimmune Chagas-like heart disease by bone marrow transplantation. Guimaro MC
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Guimaro%20MC%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Alves RM
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Alves%20RM%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Rose E
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Rose%20E%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Sousa AO
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Sousa%20AO%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, de Cássia Rosa A
<https://www.ncbi.nlm.nih.gov/pubmed/?term=de%20C%C3%A1ssia%20Rosa%20A%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Hecht MM
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Hecht%20MM%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>

1, Sousa MV
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Sousa%20MV%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
2, Andrade RR
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Andrade%20RR%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Vital T
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Vital%20T%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Plachy J
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Plachy%20J%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
3, Nitz N
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Nitz%20N%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, Hejnar J
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Hejnar%20J%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
3, Gomes CC
<https://www.ncbi.nlm.nih.gov/pubmed/?term=Gomes%20CC%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1, L Teixeira AR
<https://www.ncbi.nlm.nih.gov/pubmed/?term=L%20Teixeira%20AR%5BAuthor%5D&cauthor=true&cauthor_uid=25521296>
1
Sincerely,
Antonio Teixeira
Professor Emeritus, University of Brasilia, Brazil.
:

PLOS | OPEN FOR DISCOVERY
Charlotte Bhaskar | Publications Manager, PLOS Neglected Tropical Diseases
1160 Battery Street, Suite 225, San Francisco, CA 94111
plosntds@plos.org
ref:_00DU0Ifis._5000BlxP7U:ref