

# Molecular detection of *Trypanosoma cruzi* from formalin fixed placentas and fetuses of Wistar rats

Cássia Mariana Bronzon da Costa, Luiz Miguel Pereira, José Clóvis do Prado Júnior, Ana Amélia Carraro Abrahão

Análises clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo; Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, ; Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo

✉ **Correspondence**  
marianabronzon@gmail.com

🔍 **Disciplines**  
Parasitology  
Molecular Biology

🔑 **Keywords**  
Chagas Disease  
Trypanosoma Cruzi  
PCR  
S35/S36  
Formalin-Fixed

📅 **Type of Observation**  
Follow-up

🔗 **Type of Link**  
Continuing the storyline

🕒 **Submitted** Mar 12, 2019  
📅 **Published** Jun 3, 2019



**Triple Blind Peer Review**  
The handling editor, the reviewers, and the authors are all blinded during the review process.



**Full Open Access**  
Supported by the Velux Foundation, the University of Zurich, and the EPFL School of Life Sciences.



**Creative Commons 4.0**  
This observation is distributed under the terms of the Creative Commons Attribution 4.0 International License.

## Abstract

Chagas disease is a tropical disease, endemic of the Americas and caused by the protozoan *Trypanosoma cruzi*. The classic transmission route of *T. cruzi* is based on the contact with feces from Triatominae insects containing trypomastigotes forms of the parasite. *T. cruzi* is also spread by oral and congenital forms, besides blood donation and organ transplant transmission. The parasite has a tropism to several organs and tissues, including heart, brain, digestive tract, muscles, placenta, and fetuses. The determination of parasite burden in organs is an important parameter for scientific assays related to parasite control and epidemiology. Among the procedures to detect and quantify *T. cruzi*, PCR demonstrates high sensitivity and specificity. In this study, we developed a simple and fast method to detect *T. cruzi* from fixed placentas and fetuses of Wistar rats. *T. cruzi* was detected only in tissues from infected animals, with different parasite burdens. Once the histology usually demonstrates some limitations such as low sensitivity and specificity to detect *T. cruzi* in fetuses, our methodology will be useful for fixed fetal tissues from organ banks. The detection of *T. cruzi* from gestational tissues will contribute to the elucidation of mechanisms related to congenital Chagas disease.

## Introduction

*Trypanosoma cruzi* is a flagellated protozoan (Trypanosomatidae family) with an important impact in populations of tropical and subtropical America. The parasite causes the Chagas disease in humans, usually leading to the degeneration of cardiac and/or digestive tissues in the chronic symptomatic phase of the infection [1]. Chagas disease is partially controlled by drugs (Benznidazole and Nifurtimox) and several attempts have been developed for the control of *T. cruzi* [2]. Most of the strategies for the evaluation of *T. cruzi* transmission, epidemiology and control are dependent on methods of detection in tissues. Samples from humans [3] [4], animal models [5] [6] [7] and Triatominae insects [8] [9] have been exhaustively analyzed for elucidation of mechanisms and control of Chagas disease.

The *T. cruzi* detection in tissues is usually performed using histology [10], immunohistochemistry [11], electron microscopy [12] and PCR [13]. Compared to the traditional detection techniques, PCR demonstrates higher sensitivity and specificity. Once standardized, PCR is easy to perform and allows the analysis of multiple samples with a superior reproducibility [14]. PCR was applied for the detection of *T. cruzi* in diverse tissue sources, such as from Triatominae insects [15], domestic and wild animals [16] [17], humans [18] and assays related to immunology [19], epidemiology [20] and chemotherapy [21]. However, the *T. cruzi* detection in fixed samples was usually restricted to histologic procedures. The use of PCR for *T. cruzi* detection in fixed tissues was mainly reported for wild animals (raccoon and monkeys) [22] [23] [24] and few attempts were developed for mice or rats, especially placentas and fetuses.

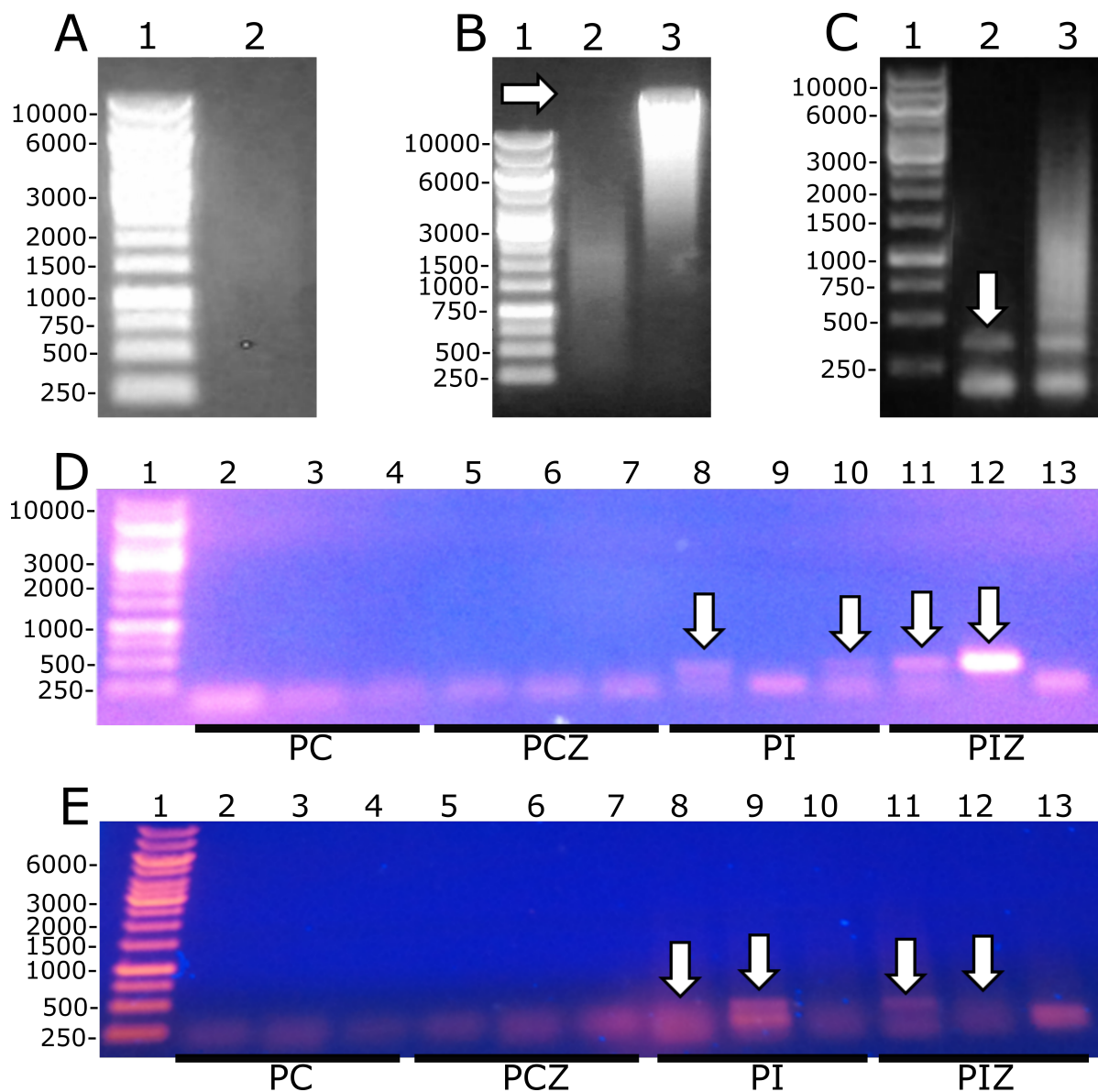
In this study, our aim was to detect *T. cruzi* in fixed placentas and fetuses from infected Wistar rats. The chronically infected animals were treated with zinc for 18 days and several immunologic parameters analyzed [25]. Placentas and fetuses were collected and fixed with formaldehyde 3.72% for evaluation of parasite burden. The samples were

treated with TE pH 9 buffer for breaking the cross-linkages between genomic DNA and proteins [26], which improved the integrity of purified material. The treated and purified genomic DNA was applied for detection of *T. cruzi* using the S35/S36 primers [27], amplifying fragments of 330 bp.

The mechanisms of congenital Chagas disease are not fully elucidated, demanding novel strategies for the control of the transmission of *T. cruzi* to fetuses. Moreover, there is interesting material from banks composed of fixed placenta and fetuses, primarily analyzed by histology. Therefore, the molecular analysis of fixed placentas and fetuses will expand the data applied for the comprehension of the congenital Chagas disease.

### Objective

The aim of this study is to detect *Trypanosoma cruzi* from fixed placentas and fetuses using conventional PCR.



**Figure Legend**

**Figure 1. *T. cruzi* is detected in fixed placentas and fetuses by PCR after genomic DNA extraction.**

The genomic DNA was extracted from fixed placentas and fetuses and applied for *T. cruzi* detection by PCR. The fixed tissues were treated with TE pH 9 buffer and the genomic DNA extracted using the Wizard SV Genomic DNA Purification System kit. The non-fixed samples were extracted with the same kit however, without the treatment with TE pH 9 buffer.

(A) (A, lane 2) Genomic DNA extracted from a fixed placenta without TE pH 9 treatment.

(B) (B, lane 2) Genomic DNA extracted from a fixed placenta after TE pH 9 treatment. (B, lane 3) Genomic DNA extracted from a non-fixed placenta.

(C) (C, lane 2) PCR amplification using genomic DNA from a fixed placenta infected with *T. cruzi*. (C, lane 3) PCR amplification using genomic DNA from *T. cruzi* epimastigote forms.

(D) PCR amplification of fixed placentas from animals infected with *T. cruzi* and/or treated with zinc. Lanes 2, 3, 4; 5, 6, 7; 8, 9, 10 and 11, 12, 13 represent PC, PCZ, PI and PIZ, respectively.

(E) PCR amplification of fixed fetuses from animals infected with *T. cruzi* and/or treated with zinc. Lanes 2, 3, 4; 5, 6, 7; 8, 9, 10 and 11, 12, 13 represent PC, PCZ, PI and PIZ, respectively. In all figures, lane 1 represents the DNA ladder (GeneRuler, 1kb DNA ladder, ThermoFisher Scientific). Arrows indicate the genomic DNA or the S35/S36 fragments.

## Results & Discussion

The first attempt to purify genomic DNA using a traditional method (Wizard SV Genomic DNA Purification System) from fixed placentas failed. When analyzed by agarose electrophoresis, a degraded DNA was observed (Fig. 1A, lane 2). The PCR reaction using this template was negative, generating only primer dimers (Figure not shown). Indeed, the Wizard SV Genomic DNA Purification System kit is not designed for fixed samples, which demanded a complementary procedure. The treatment with the TE9 buffer breaks the cross-linkages between genomic DNA and proteins [26], allowing the partial recovery of an integral material. After the treatment with TE9 buffer, a faint, but complete DNA band was observed (Fig. 1B, lane 2). The band was similar to the genomic DNA purified from the non-fixed placenta (Fig. 1B, lane 3). After the PCR reaction, a band of 330 bp was polymerized from samples using DNA from the fixed placenta (Fig. 1C, lane 2). The result was similar to the positive control composed by genomic DNA from epimastigotes (Fig. 1C, lane 3).

The PCR detection protocol was applied in fixed samples from animals infected with *T. cruzi* and treated with zinc [25]. *T. cruzi* was detected in placentas and fetuses from infected and/or treated animals, with different levels of parasite burden (Fig. 1D and E, lanes 8–13). Although no *T. cruzi* parasites were detected in all fetuses tissues analyzed by histology [28], the detection with PCR was inconclusive in only two samples (Fig. 1E, lanes 8 and 12). However, the use of complementary assays, such as nested, real-time PCR or higher percentages of agarose gels will be useful for inconclusive samples. The effect of zinc treatment on the *T. cruzi* parasite loads of placentas and fetuses was inconclusive compared to the non-treated and infected counterparts. This result confirms the role of zinc as an immunomodulatory rather than an anti-*T. cruzi* molecule. Indeed, zinc improved the immune response against *T. cruzi* in pregnant infected animals, decreasing the amastigotes nests in hearts. However, the parasite burden in placentas and fetuses was not fully determined due to the histology limitations [28]. For the complete elucidation of the effects of zinc on the parasite load of placentas and fetuses, quantitative assays (real-time PCR) will be required. Moreover, several methods have been described for DNA extraction from fixed samples, with different results. Besides the use of alkaline buffers for DNA extraction [29] [30], there are several kits with similar applications [31] [32] [33]. In further assays, we will perform an extensive comparison

between our method and the kits, concerning parameters such as cost, DNA yield, and purity. These parameters will guide a simple, effective and low-cost method for DNA extraction from fixed samples.

Our results indicate a procedure to detect *T. cruzi* from fixed placentas and fetuses of Wistar rats. There is an interesting bank of fixed organs from laboratories which samples were evaluated using histology. Our study may offer a specific and sensitive methodology to evaluate and confirm the observations reported using classic methods. Once the histology demonstrates limitations to determine the parasite burden of fetuses, the PCR detection on fixed organs is a sensitive, reproducible and specific option, complementing the studies related to the congenital Chagas disease.

## Conclusions

Our study allowed the detection of *T. cruzi* from fixed placentas and fetuses of Wistar rats. The methodology will be useful for fixed organs and tissues from banks.

## Limitations

The main limitation of the DNA extraction from fixed tissues is the low recovery of whole molecules. The quality of DNA is uncontrolled, which may generate false negative patterns. The use of complementary *T. cruzi* (e.g. TCZ) or rat primers (e.g. GAPDH) will be useful to confirm the DNA integrity.

## Alternative Explanations

## Conjectures

This study describes a qualitative form to detect *T. cruzi* in fixed placentas and fetuses. The next step will be the adaptation of our methodology for quantitative PCR (real-time PCR). The use of primers for rat tissue detection (GAPDH, for example) should be also validated.

## Additional Information

### Methods

#### Placental and fetal tissues

Female Wistar rats (5 animals/group) were used and housed in the Facility House of the São Paulo University Campus of Ribeirão Preto, with water and food at libitum. The animal manipulation protocol was approved by the local Ethics Committee (protocol number 11.1.1210.53.2). The animals were infected with *T. cruzi* and after 30 days allowed to mate. The pregnancy was confirmed by the presence of the vaginal plug. The animals were treated for 18 days (from the day 1<sup>st</sup> to 18<sup>th</sup> of pregnancy) with zinc sulfate (20 mg/Kg/day). At the 18<sup>th</sup> day of pregnancy, the animals were euthanized and the placentas and fetuses collected. The animals were divided into 4 groups: non-treated and non-infected (PC), treated and non-infected (PCZ), non-treated and infected (PI) and treated and infected (PIZ). The placentas and fetuses were fixed with 3.72% formaldehyde for 18 h at room temperature. The formaldehyde was replaced for 80% ethanol for conservation.

#### DNA extraction

The genomic DNA was extracted using the Wizard SV Genomic DNA Purification System kit (Promega), following the manufacturer's instructions. Firstly, the tissues (10



mg) were washed with PBS and used for DNA extraction without previous treatment. The placentas were cut into a wedge, while a part of the fetal thoracic region (heart) was collected. The same procedure was performed for the non-fixed controls (non-fixed placenta and  $10^6$  epimastigote forms). For the DNA-protein cross-linkages reversion, caused by the fixation process, a TE9 buffer (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0) was used [26]. The tissues (10 mg) were washed with PBS and incubated with 1 ml of TE pH 9 buffer for 24 h at 37°C. After the incubation, the samples were washed with PBS and the DNA extracted as previously described. The DNA samples were quantified in a NanoDrop 2000 (ThermoFisher Scientific), normalized for 25 ng/ml in TE buffer and 50 ng was applied in PCR assays.

## PCR

*T. cruzi* was detected by PCR using the primers S35/S36 (5'-AAATAATGTACGGGTGAGATGCATG - 3' and 5'-GGGTTCGATTGGGGTTGGTG - 3'), which amplify a fragment of 330 bp [27]. The PCR reaction (50 µl/reaction) was performed using the GoTaq Green Master Mix (Promega), following the manufacturer's instructions. The amplification was performed in a Eppendorf Mastercycler thermocycler, using a touchdown thermal profile consisting of 1 cycle of 94°C /2:00; 5 cycles of 94°C /0:30, 60°C /0:30, 72°C /1:00; 5 cycles of 94°C /0:30, 55°C /0:30, 72°C /1:00 and 25 cycles of 94°C /0:30, 50°C /0:30, 72°C /1:00 with a final extension at 72°C /10:00. The samples were visualized on 0.8% agarose gels stained with 0.5 µg/ml ethidium bromide.

## Funding Statement

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Grant number: 2013/04205-6).

## Acknowledgements

We would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

## Ethics Statement

The experimental protocols involving animals were approved by the local Ethics Committee (Protocol No 11.1.1210.53.2).

## Citations

- [1] World Health Organization. "Chagas disease (American trypanosomiasis)." In: <http://www.who.int/mediacentre/factsheets/fs340/en/> (accessed 10 March 2019) (2018).
- [2] Sanjay Varikuti et al. "Host-Directed Drug Therapies for Neglected Tropical Diseases Caused by Protozoan Parasites". In: *Frontiers in Microbiology* 9 (2018), p. 2655. DOI: 10.3389/fmicb.2018.02655. URL: <https://doi.org/10.3389/fmicb.2018.02655>.
- [3] Andréa Tieko Kinoshita-Yanaga et al. "Accidental infection by Trypanosoma cruzi follow-up by the polymerase chain reaction: case report". In: *Revista do Instituto de Medicina Tropical de São Paulo* 51.5 (2009), pp. 295–298. DOI: 10.1590/s0036-46652009000500011. URL: <https://doi.org/10.1590/s0036-46652009000500011>.
- [4] Mariane Barroso Pereira et al. "The detection of Trypanosoma cruzi by nested-PCR in elderly patients: relationship to the clinical and epidemiological profile". In: *Pathogens and Global Health* 110.6 (2016), pp. 228–232. DOI: 10.1080/20477724.2016.1232850. URL: <https://doi.org/10.1080/20477724.2016.1232850>.
- [5] Sabrina Cencig et al. "Parasitic Loads in Tissues of Mice Infected with Trypanosoma cruzi and Treated with AmBisome". In: *PLOS Neglected Tropical Diseases* 5.6 (2011), e1216. DOI: 10.1371/journal.pntd.0001216. URL: <https://doi.org/10.1371/journal.pntd.0001216>.
- [6] L V Kirchhoff et al. "Comparison of PCR and microscopic methods for detecting Trypanosoma cruzi." In: *Journal of Clinical Microbiology* 34 (1996), pp. 1171–1175.
- [7] Dawn M. Roellig and Michael J. Yabsley. "Infectivity, Pathogenicity, and Virulence of Trypanosoma cruzi Isolates from Sylvatic Animals and Vectors, and Domestic Dogs from the United States in ICR Strain Mice and SD Strain Rats". In: *The American Journal of Tropical Medicine and Hygiene* 83.3 (2010), pp. 519–522. DOI: 10.4269/ajtmh.2010.09-0663. URL: <https://doi.org/10.4269/ajtmh.2010.09-0663>.
- [8] P. L. MARCET et al. "PCR-based screening and lineage identification of Trypanosoma cruzi directly from faecal samples of triatomine bugs from northwestern Argentina". In: *Parasitology* 132.01 (2006), pp. 57–65. DOI: 10.1017/s0031182005008772. URL: <https://doi.org/10.1017/s0031182005008772>.
- [9] Miguel Saavedra et al. "Quantification by real-time PCR of Trypanosoma cruzi DNA in samples of Triatoma infestans used in xenodiagnosis of chronic Chagas disease patients". In: *Parasites & Vectors* 9.1 (2016), p. 382. DOI: 10.1186/s13071-016-1664-5. URL: <https://doi.org/10.1186/s13071-016-1664-5>.
- [10] Claudio R. F. Marinho et al. "Pathology Affects Different Organs in Two Mouse Strains Chronically Infected by a Trypanosoma cruzi Clone: a Model for Genetic Studies of Chagas' Disease". In: *Infection and Immunity* 72.4 (2004), pp. 2350–2357. DOI: 10.1128/iai.72.4.2350-2357.2004. URL: <https://doi.org/10.1128/iai.72.4.2350-2357.2004>.
- [11] Paulo Hernane Rabelo Azevedo et al. "Anti-serum validation for use in immunohistochemistry for Trypanosoma cruzi detection". In: *Revista da Sociedade Brasileira de Medicina Tropical* 51.4 (2018), pp. 467–474. DOI: 10.1590/0037-8682-0011-2018. URL: <https://doi.org/10.1590/0037-8682-0011-2018>.
- [12] P. T. V. Florentino et al. "An Historical Perspective on How Advances in Microscopic Imaging Contributed to Understanding the Leishmania Spp. and Trypanosoma cruzi Host-Parasite Relationship". In: *BioMed Research International* 2014 (2014), pp. 1–16. DOI: 10.1155/2014/565291. URL: <https://doi.org/10.1155/2014/565291>.
- [13] Luiz A. Benvenuti et al. "An autopsy-based study of Trypanosoma cruzi persistence in organs of chronic chagasic patients and its relevance for transplantation". In: *Transplant Infectious Disease* 19.6 (2017), e12783. DOI: 10.1111/tid.12783. URL: <https://doi.org/10.1111/tid.12783>.
- [14] Andrew S. Bell and Lisa C. Ranford-Cartwright. "Real-time quantitative PCR in parasitology". In: *Trends in Parasitology* 18.8 (2002), pp. 338–342. DOI: 10.1016/s1471-4922(02)02331-0. URL: [https://doi.org/10.1016/s1471-4922\(02\)02331-0](https://doi.org/10.1016/s1471-4922(02)02331-0).
- [15] Adriana Benatti Bilheiro et al. "First Report of Natural Infection with Trypanosoma cruzi in Rhodnius montenegrensis (Hemiptera, Reduviidae, Triatominae) in Western Amazon, Brazil". In: *Vector-Borne and Zoonotic Diseases* 18.11 (2018), pp. 605–610. DOI: 10.1089/vbz.2018.2266. URL: <https://doi.org/10.1089/vbz.2018.2266>.
- [16] Andréa Pereira da Costa et al. "Trypanosoma cruzi in Triatomines and wild mammals in the National Park of Serra das Confusões, Northeastern Brazil". In: *Revista da Sociedade Brasileira de Medicina Tropical* 51.4 (2018), pp. 445–451. DOI: 10.1590/0037-8682-0098-2018. URL: <https://doi.org/10.1590/0037-8682-0098-2018>.
- [17] Rachel Curtis-Robles et al. "Epidemiology and Molecular Typing of Trypanosoma cruzi in Naturally-Infected Hound Dogs and Associated Triatomine Vectors in Texas, USA". In: *PLOS Neglected Tropical Diseases* 11.1 (2017), e0005298. DOI: 10.1371/journal.pntd.0005298. URL: <https://doi.org/10.1371/journal.pntd.0005298>.
- [18] Alejandro Gabriel Schijman et al. "Early diagnosis of recurrence of trypanosoma cruzi infection by polymerase chain reaction after heart transplantation of a chronic chagas' heart disease patient". In: *The Journal of Heart and Lung Transplantation* 19.11 (2000), pp. 1114–1117. DOI: 10.1016/s1053-2498(00)00168-6. URL: [https://doi.org/10.1016/s1053-2498\(00\)00168-6](https://doi.org/10.1016/s1053-2498(00)00168-6).
- [19] Anita R. Schnapp et al. "Cruzipain Induces Both Mucosal and Systemic Protection against Trypanosoma cruzi in Mice". In: *Infection and Immunity* 70.9 (2002), pp. 5065–5074. DOI: 10.1128/iai.70.9.5065-5074.2002. URL: <https://doi.org/10.1128/iai.70.9.5065-5074.2002>.
- [20] Alejandro G. Schijman et al. "International Study to Evaluate PCR Methods for Detection of Trypanosoma cruzi DNA in Blood Samples from Chagas Disease Patients". In: *PLOS Neglected Tropical Diseases* 5.1 (2011), e931. DOI: 10.1371/journal.pntd.0000931. URL: <https://doi.org/10.1371/journal.pntd.0000931>.
- [21] Constança Carvalho Britto. "Usefulness of PCR-based assays to assess drug efficacy in Chagas disease chemotherapy: value and limitations". In: *Memórias do Instituto Oswaldo Cruz* 104.suppl 1 (2009), pp. 122–135. DOI: 10.1590/s0074-02762009000900018. URL: <https://doi.org/10.1590/s0074-02762009000900018>.
- [22] Michael J. James et al. "AMPLIFICATION OF TRYPANOSOMA CRUZI-SPECIFIC DNA SEQUENCES IN FORMALIN-FIXED RACCOON TISSUES USING POLYMERASE CHAIN REACTION". In: *Journal of Parasitology* 88.5 (2002), pp. 989–993. DOI: 10.1645/0022-3395(2002)088[0989:aotcsd]2.0.co;2. URL: [https://doi.org/10.1645/0022-3395\(2002\)088\[0989:aotcsd\]2.0.co;2](https://doi.org/10.1645/0022-3395(2002)088[0989:aotcsd]2.0.co;2).
- [23] James N. Mubiru et al. "Correlation between Presence of Trypanosoma cruzi DNA in Heart Tissue of Baboons and Cynomolgus Monkeys, and Lymphocytic Myocarditis". In: *The American Journal of Tropical Medicine and Hygiene* 90.4 (2014), pp. 627–633. DOI: 10.4269/ajtmh.13-0448. URL: <https://doi.org/10.4269/ajtmh.13-0448>.

- [24] Jeff T. Williams et al. "Polymerase Chain Reaction Detection of *Trypanosoma cruzi* in *Macaca fascicularis* Using Archived Tissues". In: *The American Journal of Tropical Medicine and Hygiene* 81.2 (2009), pp. 228–234. DOI: 10.4269/ajtmh.2009.81.228. URL: <https://doi.org/10.4269/ajtmh.2009.81.228>.
- [25] Cássia Mariana Bronzon da Costa et al. "Is the adaptive immune response in murine *Trypanosoma cruzi* infection influenced by zinc supplementation?" In: *European Journal of Pharmaceutical Sciences* 111 (2018), pp. 330–336. DOI: 10.1016/j.ejps.2017.10.014. URL: <https://doi.org/10.1016/j.ejps.2017.10.014>.
- [26] Susan E. Goelz, Stanley R. Hamilton, and Bert Vogelstein. "Purification of DNA from formaldehyde fixed and paraffin embedded human tissue". In: *Biochemical and Biophysical Research Communications* 130.1 (1985), pp. 118–126. DOI: 10.1016/0006-291x(85)90390-0. URL: [https://doi.org/10.1016/0006-291x\(85\)90390-0](https://doi.org/10.1016/0006-291x(85)90390-0).
- [27] Nancy R. Sturm et al. "Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease". In: *Molecular and Biochemical Parasitology* 33.3 (1989), pp. 205–214. DOI: 10.1016/0166-6851(89)90082-0. URL: [https://doi.org/10.1016/0166-6851\(89\)90082-0](https://doi.org/10.1016/0166-6851(89)90082-0).
- [28] Cássia Mariana Bronzon da Costa et al. "Zinc and pregnancy: Marked changes on the immune response following zinc therapy for pregnant females challenged with *Trypanosoma cruzi*". In: *Clinical Nutrition* 32.4 (2013), pp. 592–598. DOI: 10.1016/j.clnu.2012.10.012. URL: <https://doi.org/10.1016/j.clnu.2012.10.012>.
- [29] Burcu Sengüven et al. "Comparison of Methods for the Extraction of DNA from Formalin-Fixed, Paraffin-Embedded Archival Tissues". In: *International Journal of Medical Sciences* 11.5 (2014), pp. 494–499. DOI: 10.7150/ijms.8842. URL: <https://doi.org/10.7150/ijms.8842>.
- [30] Shan-Rong Shi et al. "DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution". In: *Histochemistry and Cell Biology* 122.3 (2004), pp. 211–218. DOI: 10.1007/s00418-004-0693-x. URL: <https://doi.org/10.1007/s00418-004-0693-x>.
- [31] A. Farrugia, C. Keyser, and B. Ludes. "Efficiency evaluation of a DNA extraction and purification protocol on archival formalin-fixed and paraffin-embedded tissue". In: *Forensic Science International* 194.1–3 (2010), e25–e28. DOI: 10.1016/j.forsciint.2009.09.004. URL: <https://doi.org/10.1016/j.forsciint.2009.09.004>.
- [32] Jianghai Lin et al. "High-quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples deparaffinized using mineral oil". In: *Analytical Biochemistry* 395.2 (2009), pp. 265–267. DOI: 10.1016/j.ab.2009.08.016. URL: <https://doi.org/10.1016/j.ab.2009.08.016>.
- [33] Sara Santos et al. "An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues". In: *Research in Veterinary Science* 86.3 (2009), pp. 421–426. DOI: 10.1016/j.rvsc.2008.08.007. URL: <https://doi.org/10.1016/j.rvsc.2008.08.007>.