Title: Molecular detection and parasite load of *Trypanosoma cruzi* in digestive tract tissue of Chagas disease patients affected by megacolon.

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Abstract

Chagas disease, caused by the Trypanosoma cruzi parasite, in Americas ~7 million people are infected. 30% with cardiac tissue damage, 10-20% very severe alterations . In this study we write a protocol to detect the presence of the parasite and the parasitic load in segments of resected dysfunctional tissue, in chronically infected patients with digestive megacolon, of 43 individuals, 38 with positive serology for Chagas and five with negative serology and digestive syndromes not related to T. cruzi.

Digestive tissue samples of 1.5 to 2.0 cm3 were taken from three different points from dysfunctional tissue, in specialized centers of Cochabamba, Bolivia.

Inoculation with NNN-LIT culture medium was performed. DNA was extracted proceding semi automatic and by multiplex real-time PCR with TaqMan probes directed at the specific sequence of the satellite nuclear DNA, for quantification of T. cruzi loads in clinical samples a synthetic DNA molecule containing a satellite DNA (satDNA) repeat unitwas the standard for quantification.

Mean age was 61 years, predominant clinical sign was dolicomegacolon. No parasites could be isolated NNN-LIT culture medium DNA extracction showed the endogenous control human RNAse P without significant differences.

With three sample points and analysed in triplicate with the qPCR obtained a sensitivity of 42.1% (16/38) , this sensitivity was higher than that obtained with one or two sampling points. Parasitic load ranged from 2.2 x 10^{2} to 1.0 x 10^{6} satDNA copies/µl. The positive samples from the distal end showed a higher parasitic load. Our results support those previously obtained by others, and bring further evidences on the relevance of the digestive tract in the persistence of the parasite. These characteristics support the establishment of a user-friendly protocol in future investigations of digestive Chagas disease.

Keywords: Chagas disease, *Trypanosoma cruzi,* megacolon, molecular diagnosis, real-time PCR.

1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi,* is the neglected tropical disease (NTD) with the highest burden in the Western hemisphere. It is endemic in 21 countries in the Americas, where reside the majority of the ~7 million people infected (WHO 2021). Nonetheless, the disease epidemiology changed in the last few decades due to population flows and it is now considered a global threat (Gascon J et al., 2010; Requena-Méndez et al., 2015). The main routes of infection in endemic countries are through triatomine vectors transmission and congenitally from mother to child. Other described transmission routes are the intake of parasite contaminated food or drinks, through blood transfusion or organ transplant (Marin-Neto et al., 2008; Rassi et al., 2012).

The clinical course of the disease includes an initial acute stage that is usually asymptomatic (WHO 2021; Prata, 2001). Thus, it goes unnoticed, undiagnosed and untreated, despite the fact that the efficacy of the two available drugs (benznidazole and nifurtimox) is greater the earlier the infection is treated (WHO 2021).

Although achieving spontaneous cure is infrequent (Francolino et al., 2003), infected subjects experience a reduction of the circulating parasite levels within four to eight weeks, entering in the so-called asymptomatic (indeterminate) chronic stage of the infection (Prata et al., 2001; Rassi et al., 2010).

Symptomatology is mostly observed during this chronic stage, and it can take several years until it overtly manifests, thus hindering clinical diagnosis. Cardiac tissue damage is the main clinical sign affecting around 30% of those chronically infected (A. Rassi et al., 2012). Disruption of digestive tract tissues is described in between 10% to 20% of the patients, and it can be observed on its own or accompanying cardiac damage (Pinto et al. 2019). Central neurological alterations have also been described to affect a lower proportion of infected individuals (Carod-Artal and Gascon, 2010)

T. cruzi infection can affect any part of the digestive tract, but the most commonly involved are the esophagus and the colon (Pinto et al., 2019; Matsuda et al., 2009). Such infection can lead to gastrointestinal complications, which can be highly morbid and seriously influence the quality of life of those suffering them. Overall, pathogenesis of *T. cruzi* infection is yet a controversial matter (Pinazo et al., 2010), but the fundamental damage that occurs and that ultimately results in megavisceral presentations is the destruction of the neurons of the enteric nervous system (Iantorno et al., 2007) and an important reduction in the number of interstitial cells of Cajal (Hagger et al., 2000). Histopathological changes evident in whole-mount specimens of chagasic/megacolonic tissues appear as prototypical signs of degeneration most clearly in the dilated as prototypical signs of degeneration most clearly in the dilated megacolonic region and in the anal region (non-dilated).(Jabari et al., 2014)

Current knowledge points to mixed mechanisms that entail the participation of the parasite (Lages-Silva et al., 2001), autoimmune-mediated phenomena (López et al., 2006), microvascular alterations and autonomic denervation (Oliveira et al., 2009; Pinazo et al., 2010). Regarding digestive involvement, there is limited data on the parasite persistence in chronically infected intestinal tissues. Former histological studies identified parasite persistence in gastrointestinal samples in 20% to 50% of megaesophagus cases (De Castro Côbo et al., 2012).Other authors reported the presence of *T. cruzi* kinetoplastid DNA (kDNA) in 100% of the samples analyzed by conventional PCR (da Silveira AB et al., 2005).

On the other hand, in animal models of *T. cruzi* infection, evidence of the continuous presence of parasites in the digestive tract has been described (Lewis et al., 2014). In fact, *ex vivo* imaging of chronically infected mice showed that the digestive tract is constantly acting as a parasite reservoir, particularly the colon and the stomach, at least in that model of animal infection (Lewis et al., 2014).

However, little is known about the characteristics of the damage to the digestive tract in patients suffering from chronic Chagas disease. With the aim to aid in the analysis of digestive Chagas, we have developed and evaluated techniques for the detection and quantification of *T. cruzi* DNA in digestive samples. These were dysfunctional histological segments resected from chronically infected patients undergoing surgery due to the severity of their digestive affection.

2. Materials and Methods

2.1 Ethics

All procedures complied with the statements of the Declaration of Helsinki. The study protocol and informed consent form were revised and approved by the ethical committees of Fundación CEADES (No. 0990-0279/FWA:00024189; Cochabamba, Bolivia) and that of the Hospital Clínic of Barcelona (2013/8736; Barcelona, Spain).

2.2. The Study

It was designed as a transversal case control study that prospectively included patients attending for megacolon surgical treatment requiring resection of the dysfunctional segments. Study participants were recruited in three specialized centers located in the department of Cochabamba (Bolivia): Hospital Gastroenterológico Boliviano Japonés (Cochabamba), Fundación Pietro Gamba (Anzaldo) and Hospital Manuel Asensio Villarroel (Punata). Participants were confirmed to be chronically infected with *T. cruzi* by means of two commercial conventional serological assays: Chagatest ELISA® Recombinante v3.0 (Wiener lab, Rosario, Argentina) and Chagatek ELISA based on whole parasite lysate antigens (Laboratorio Lemos, Buenos Aires, Argentina).

Sample collection took place between July 2014 and July 2016. Participation was totally voluntary and a written informed consent was obtained from all participants, compulsory for their inclusion in the study. In total, 38 chronic Chagas disease patients (positive anti-*T. cruzi* serology) with different digestive clinical signs were included. Patients suffering from other digestive syndromes distinct from Chagas disease were included as controls (n = 5).

Samples were collected in triplicate from three different sites within the resected dysfunctional segment: distal, central, and proximal segments; with an approximate size of 1.0 to 2.5 cm^2. Of each piece, half was preserved for culture in a 15 mL conical propylene

tube with a solution of 0.9% sodium chloride (NaCl) and 100 µg/mL of gentamicin, and another half was preserved in a conical tube with 70% ethanol,(Shokralla et al., 2010; (Patricia García et al., 2006)for multiplex Real Time-PCR.

2.3 Tissue culture

Samples in NaCl and gentamicin buffer were kept at room temperature and, upon arrival to the laboratory, immediately washed three times with the same buffer under a laminar flow hood to eliminate potential contaminants. Next, four to five tissue fragments with a diameter of 3 mm were taken from different sites of the samples, crushed in a glass mortar and the solution obtained inoculated into three tubes with NNN-LIT culture medium. Tubes were incubated at 26 °C and examined microscopically 30, 60, and 90 days after inoculation. This procedure was performed in the Parasitology laboratory of the Biomedical Research Institute (IIBISMED; Faculty of Medicine, University Mayor de San Simón, Cochabamba, Bolivia).

2.4 Sample processing and DNA extraction from colorectal resected tissue

Samples preserved in ethanol 70% were washed three times with abundant 70% ethanol to remove contaminants on the same day of re-collection. Each piece of tissue was placed in a 15 ml conical tube with 70% ethanol and stored at -80 °C until needed.

In stored samples, differences in size, pathological alterations and disease progression were observed in the tissue samples of included patients, therefore they were subjected to fragmenting into smaller pieces with a sterile scalpel, which was sprayed with liquid nitrogen to further contribute to the fragmentation and homogenization ,

approximately 40 mg of each sprayed tissue was stored in 1.5 mL Eppendorf tubes, at -20 ºC.

To minimize the risk of contamination between samples, all equipment used was thoroughly washed with a 10% sodium hypochloride solution and UV irradiated. Each pulverized sample was digested overnight at 55 ºC in 400 µL of a solution containing 4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA and 40 µL of proteinase K (20 mg/mL, Roche Applied Science) at pH = 7.4.

DNA extraction and purification was performed following a semiautomatic procedure based on the commercial DNA MultiSample ultra 2.0 magnetic bead kit (Applied Biosystems, Lithuania) and the KingFisher robot (Thermo Fisher, Lithuania) following the manufacturer's instructions, with a final elution volume of 100 µL. Purified DNA was stored at -20 °C for molecular analysis.

2.5 *T. cruzi* DNA amplification

We followed the protocol described by Piron and co-workers that targets the parasite's satellite DNA sequence (Piron et al., 2007), an alternative to nested polymerase chain reaction (Nested-PCR), with similar sensitivity and higher throughput, and could help determine ongoing parasitemia in *T. cruzi*-infected patients. Parasite specific DNA amplification was made using the following primers (5'-3'): Cruzi 1 STCGGCTGATCGTTTTCGA; Cruzi 2 AATTCCTCCAAGCAGCGGATA; and the TaqMan probe Cruzi 3 FAM-CACACACTGGACACCAA-NFQ-MGB. Real-time PCR was designed to amplify a fragment of 166 bp of the satellite repeats of the *T. cruzi* nuclear genome, (Piron et al., 2007).

An endogenous internal amplification control (IAC) targeting the human RNAse P gene (Applied Biosystems) was included to monitor the process (Duffy et al., 2013). Controls of positive amplification of 10 fg/µl and 1 fg/µl purified parasite DNA from *T. cruzi* strain CL-Brener (discrete typing unit VI) were included in all experiments, and as a control, a tissue sample taken from a patient with negative serology. Real-time PCR was performed in a thermocycler CFX-96 (BioRad, Hercules, USA) according to the following incubation steps: 2 min at 50 ºC; 1 min at 95 ºC; 40 cycles of 15 sec at 95 ºC and 1 min at 58 ºC.

2.6 Estimation of parasitic load in qPCR positive samples.

We used a standard curve made of serial dilutions of a synthetic satDNA oligonucleotide repeat to estimate the parasite load in Real Time-PCR positive samples, positive tissue samples, regardless of parasite strain, allowing the direct comparison of loads in samples (Muñoz-Calderón et al., 2021). To build this standard curve, total DNA obtained from a pool of digestive tissues from seronegative individuals as a matrix equivalent to the clinical specimens was mixed with synthetic *T. cruzi* satDNA oligonucleotide and serial 1/10 dilutions were made for the points on the standard curve.

2.7 Statistical Analysis

The analysis of results was performed in Microsoft Office Excel (version 2010) where frequencies and/or percentages of events were calculated. Generally, values are shown as mean with standard deviation (SD). Due to the potential presence of Real-Time PCR inhibitors or poor DNA extraction yield, we applied Tukey criteria to detect those samples with atypical outlier values (Vago et al., 2003).

3. Results

Mean age of participants was 61 years old (range 40 to 80 years), 42% of them were women (n = 18) and 58% (n = 25) were men. According to the clinical classification, the predominant sign observed within the group of patients with Chagas disease was dolicomegacolon (26/38; 68.4%) which is characterized by colonic dilatation. On the other hand, 6/38 (15.7%) were clinically diagnosed with megasigmoid or sigmoid colon; 4/38 (10.5%) were diagnosed with volvulus and could reestablish their intestinal transit by endoscopic decompression. Only 5.3% (2/38) presented mixed cardiac and digestive symptoms. In the control group, two people managed to reestablish their intestinal transit through laparoscopic Hartmann, while three presented dolicomegacolon (Table 1).

Table 1. Classification of the study cohort in terms of clinical and serological readouts.

	Dolicomegacolon		**Megasigmoid**		**Volvulus**		**Megacolon/ cardiomegaly**		**Total**
Sex/Chagas serology	M	F	M	F	M	F	M	F	
Positive	15	11	4	2	1	3	1	1	38
Negative	3	0	0	0	0	2	0	0	5
Total	18	11	4	2	1	5	1	1	43

M, male. F, female.

Despite culturing efforts, no parasites could be isolated, likely due to the intestinal nature of the samples. A heavy contamination with bacteria was usually observed, and it was not possible to grow parasites in any of the three samples from each of the three distinct sites of the resected segments subjected to culture.

Tissue samples preserved in 70% ethanol at -80 °C were used to extract DNA for subsequent multiplex real-time PCR evaluation. Overall, the isolation of DNA from the

samples with the semi-automated magnetic bead-based system performed well, the A260/A280 ratio between 1.8 and 2.0 corresponds to a highly pure DNA (90-100%); the mean obtained with the samples is 1.95 ± 0.06, the amount in ng/µL with a mean of 399.48 ± 374.93, a variability attributable to the diversity of tissue samples from included patients.

Furthermore, the extracted DNA showed no signs of degradation according to the average amplification cycle threshold (Cq) readings of the endogenous human RNAse P gene (Table 2). This would support the viability of parasite DNA amplification when processed and stored in 70% ethanol. Notably, most samples were stored for more than two years and parasite DNA amplification from them was still possible (Table 2).

All samples from seronegative patients were negative by qPCR. In comparison, it was possible to amplify the parasite satDNA in 16 out of 38 cases of digestive Chagas disease (Table 2). Remarkably, such rate of genetic detection was feasible due to the performance of one DNA extraction per tissue region followed by qPCR in triplicate of each DNA sample from the three different regions of the resected tissue. In fact, the sensitivity of the qPCR, i.e., the number of positive *T. cruzi* DNA detections among the total number of serologically positive cases, was influenced by the number of technical replicates.

Table 2. Multiplex real-time PCR results obtained with samples from all *T. cruzi*-infected participants.

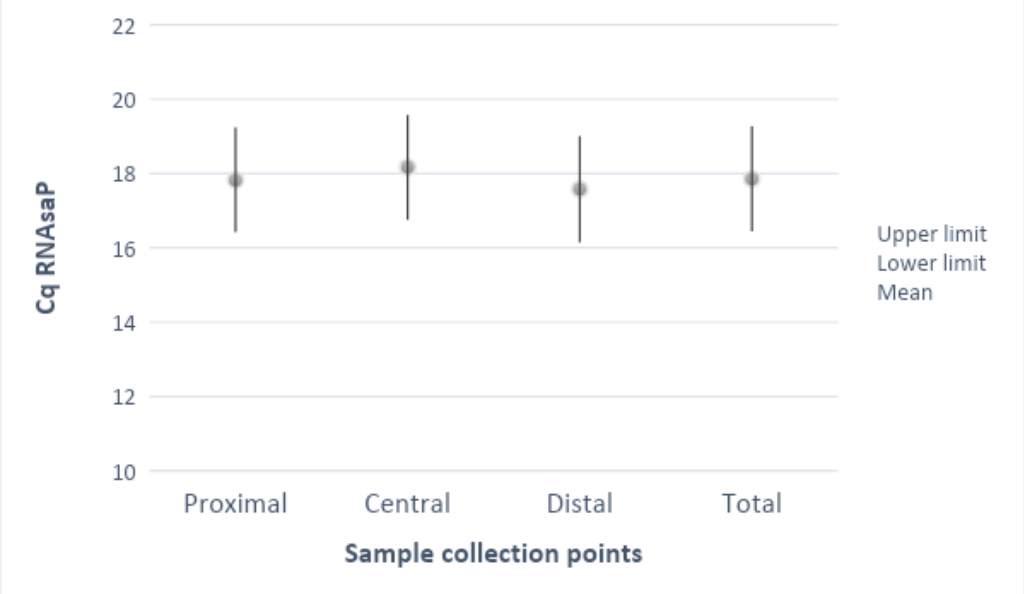
Study No.	Chagas serology	Proximal end ascending colon			Central end transverse colon			Descending end distal colon			Result
		Mean Cq *T. cruzi* (SD)	Mean copies/µL	Mean Cq RNAseP	Mean Cq *T. cruzi* (SD)	Mean copies/µL	Mean Cq RNAseP	Mean Cq *T. cruzi* (SD)	Mean copies/µL	Mean Cq RNAseP	

01	+	ND	ND	18.47	ND	ND	17.16	34.25 (0.88)	440.83	20.53	+
02	-	ND	ND	19.23	ND	ND	18.2	ND	ND	16.2	-
03	+	31.14 (0.10)	$3.8x10^3$	17.85	ND	ND	18.2	ND	ND	18.24	+
04	+	ND	ND	16.34	ND	ND	18.34	ND	ND	19.09	-
05	-	ND	ND	20.0	ND	ND	18.32	ND	ND	20.54	-
06	+	ND	ND	18.39	36.69 (0.51)	63.0	16.24	ND	ND	20.2	+
07	+	ND	ND	17.81	ND	ND	19.8	ND	ND	16.17	-
08	+	37.78 (0.00)	27.0	17.32	ND	ND	17.72	21.70 (0.14)	$4.6x10^6$	16.15	+
09	+	ND	ND	19.45	35.99 (0.91)	110.0	16.28	23.28 (0.89)	$1.4x10^6$	16.1	+
10	+	31.01 (0.11)	$4.3x10^3$	17.14	23.20 (0.10)	$1.4x10^6$	15.99	36.33 (1.34)	80.0	16.71	+
11	+	28.85 (0.52)	$2.2x10^4$	16.29	38.64 (1.79)	21.0	16.23	ND	ND	16.02	+
12	+	ND	ND	18.65	ND	ND	18.84	ND	ND	17.4	-
13	+	38.20 (1.69)	28.0	17.22	ND	ND	18.21	35.87 (2.02)	670.0	16.56	+
14	+	35.86 (0.67)	124.0	19.32	ND	ND	18.02	25.10 (0.04)	$4.4x10^5$	17.59	+
15	+	ND	ND	18.3	ND	ND	18.24	ND	ND	19.14	-
16	+	ND	ND	21.05	ND	ND	17.16	ND	ND	17.8	-
17	+	ND	ND	20.75	ND	ND	17.84	ND	ND	19.77	-
18	+	ND	ND	17.67	31.98 (2.22)	$3.7x10^3$	16.79	ND	ND	17.29	+
19	+	ND	ND	16.08	ND	ND	20.03	ND	ND	19.65	-
20	-	ND	ND	17.58	ND	ND	21.45	ND	ND	16.62	-
21	+	ND	ND	16.39	ND	ND	21.09	ND	ND	16.57	-
22	+	ND	ND	19.2	ND	ND	21.12	ND	ND	14.5	-
23	+	ND	ND	18.71	ND	ND	17.99	ND	ND	16	-
24	+	ND	ND	18.8	ND	ND	17.53	ND	ND	15.49	-
25	+	ND	ND	19.77	ND	ND	17.66	ND	ND	18.3	-
26	+	ND	ND	20.03	36.24 (3.10)	340.0	17.22	ND	ND	15.55	+
27	+	ND	ND	15.34	ND	ND	16.78	ND	ND	17.17	-
28	+	ND	ND	17.29	ND	ND	17.88	ND	ND	19.95	-
29	+	ND	ND	16.33	ND	ND	19.25	ND	ND	18.47	-
30	+	ND	ND	14.94	ND	ND	18.19	ND	ND	17.32	-
31	+	ND	ND	18.31	ND	ND	20.61	ND	ND	16.38	-
32	-	ND	ND	16.91	ND	ND	22.66	ND	ND	15.26	-
33	+	ND	ND	18.01	ND	ND	21.6	ND	ND	16.56	-
34	+	31.98 (2.06)	$4.6x10^3$	16.64	ND	ND	17.86	ND	ND	17.71	+
35	+	33.51 (2.22)	$1.1x10^3$	18.25	ND	ND	17.99	ND	ND	18.29	+

36	-	ND	ND	16.7	ND	ND	18.48	ND	ND	15.83	-
37	+	ND	ND	17.34	ND	ND	18.87	ND	ND	16.9	-
38	+	ND	ND	17.51	ND	ND	18.67	ND	ND	17.95	-
39	+	ND	ND	16.19	ND	ND	19.38	ND	ND	18.68	-
40	+	ND	ND	18.59	ND	ND	18.16	29.06 (0.71)	$2.0x10^4$	17.39	+
41	+	ND	ND	16.68	35.10 (0.49)	210.0	16.26	ND	ND	18.36	+
42	+	38.36 (5.02)	24.66	16.86	ND	ND	18	ND	ND	18.63	+
43	+	ND	ND	18.17	ND	ND	17.22	ND	ND	17.37	-

ND, not determined; +, positive; -, negative; SD, standard deviation; Cq, average amplification threshold cycle.

Figure 1. Endogenous control analysis of qPCR tissue samples by point of collection. CI95% statistical significance upper and lower limits are shown for each mean Cq RNAseP value.



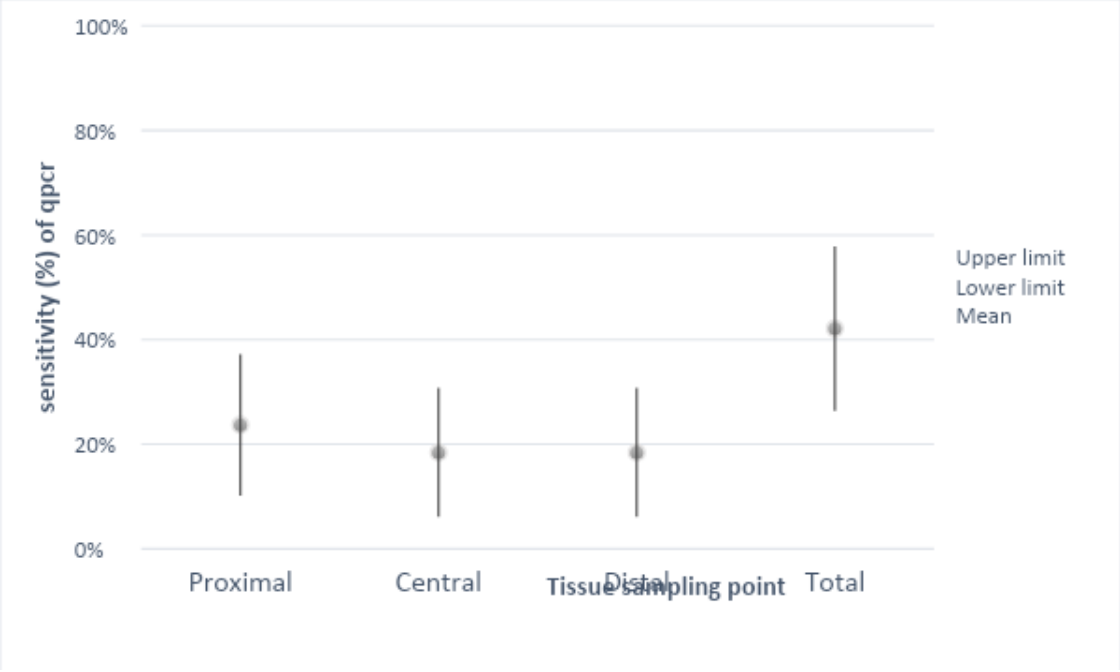
Regardless of whether the tissue sample was proximal, central or distal, qPCR performed in triplicate (three replicates per DNA sample), resulted in an average of RNAseP-DNA Cq value of 17.86 (±1.40) for the proximal end samples, 18.33 (±1.58) for

the central end, and 17.45 (±1.54) for the distal; altogether giving a mean 17.86 (±0.29). Figure 1 shows the differences obtained at the three collection points were not significant.

The qPCR sensitivity for *T. cruzi* DNA obtained with all three sampling points was 42.1% (16/38), in comparison, when a single replicate was performed, the sensitivity lowered to ≤ 25% all three sampling points.

Figure 2. qPCR sensitivity in relation to tissue sampling point.

Statistical significance around the value of the proportions is shown by drawing CI95% upper and lower limits.



It is observed that sensitivity increases with increasing sampling points at all three sites; for the detection of *T. cruzi* in tissue samples, this difference in overall sensitivity versus estimated sensitivity per sampling point, with a 95% CI around the proportions, is not statistically significant with the study population.

Table 3. *T. cruzi* parasitic loads at tissue collection sites in different clinical forms of digestive Chagas disease.

Clinical diagnosis	Colon tissue segment (mean Cq values and parasitic loads)								
	Ascending proximal end Men (±SD)			Transverse central end Men (±SD)			Descending distal end Men (±SD)		
	Cq *T. cruzi*	Copies satDNA/µL	Cq RNAseP	Cq *T. cruzi*	Copies satDNA/µL	Cq RNAseP	Cq *T. cruzi*	Copies satDNA/µL	Cq RNAseP
Volvulus	33.51 (9.85)	1.1E+04 (2400)	17.54 (1.40)	33.54 (12.37)	9.9E+02 (85.23)	18.61 (1.53)	ND	0	17.35 (1.37)
Dolicomegacolon	36.58 (14,77)	1.1E+04 (21.04)	17.83 (1.41)	36.11 (13.51)	2.2E+02 (66.09)	18.17 (1.41)	28.77 (11.80)	1.0E+06 (128.64)	17.58 (1.43)
Megasigmoid	30.33 (14.52)	1.0E+04 (21.98)	17.86 (1.46)	32.84 (13.26)	4.9E+04 (61.23)	18.28 (1.42)	32.69 (11.32)	1.0E+04 (115.31)	17.44 (1.39)
Megacolon/ cardiomegaly	35.86 (0.67)	1.2E+03 (65.22)	19.32 (0.43)	ND	0	0	25.1 (0.043)	4.4E+05 (11762.33)	17.70 (0.12)

*ND, **not determined.**

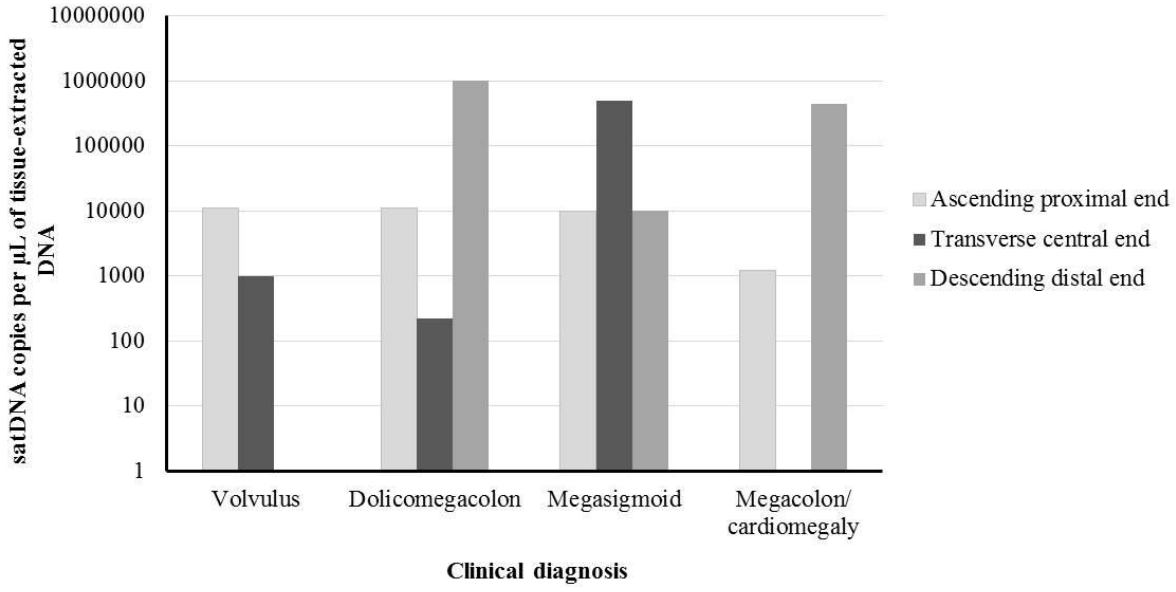
If the qPCR results are compared to the clinical status of each study case, then the samples that showed a higher rate of positivity were those from megasigmoid (5/6) and intestinal transit restitution by volvulus (3/4). In contrast, the most numerous clinical group (dolicomegacolon) registered seven positive detections of the parasite DNA out of 26 cases.

The ratio between clinical diagnosis and mean parasite load, expressed as satellite DNA copies/µL, ranged from $2.2x10^2$ at the central transverse end to $1.0x10^6$ at the distal

descending end. In addition, quantification of parasite load in tissue samples shows high heterogeneity of *T. cruzi* DNA burden, with higher values in the distal regions in all clinical scenarios (Table 3, Figure 3).

Figure 3. Clinical diagnosis and parasite loads by region of tissue analyzed.

Parasitic Load values in y-axis are expressed as copies of satDNA/µL of tissue-extracted DNA.



4. Discussion

Between 10 and 20% of subjects chronically infected with *T. cruzi* in Cochabamba, Bolivia, is affected by digestive symptoms (Pinto et al., 2019). However, the available data on the presence of parasites in the intestines is very limited as the demonstration of the presence of *T. cruzi* in the gastrointestinal tract is difficult due to the lack of robust methodologies to detect the rare presence of parasites. (Prata, 2001).

Several authors agree on the fact that the distinct clinical outcomes observed in Chagas disease, including the differential involvement of target organs is influenced by host and pathogen factors as the parasite genetic (Dutra and Gollob, 2008; Martins et al., 2008).

In a recent series of studies derived from the establishment, of an innovative experimental model of acute and chronic *T. cruzi* infections in mice, determined that the persistence of the parasite was prominently observed in digestive tissues (Lewis et al., 2014). If such observation is extrapolated to humans, the phenomenon could play a role in the development of Chagas disease digestive forms. Nonetheless, to be able to better understand the host-pathogen interactions and the clinical outcome of digestive Chagas disease, it is very important to count with established methodologies for the collection, storage and molecular analysis of the parasite presence in biological samples.

In this regard, in the present study we adapted the recommendations by Shokralla and co-workers (Shokralla et al., 2010), taking into consideration the environment and work routines of the collaborating centers involved. Such procedures helped to optimally collect the tissues and preserve their cellular integrity towards DNA extraction despite of their highly contaminated nature. It was due to this contamination that no parasites could be isolated from any of the samples set to grow in NNN LIT media.

This characteristic did not allow us to isolate any parasites, and we were unable to observe their growth at 30, 60 and 90 days with the methodology followed. The culture of

335 samples of this nature, in the presence a higher percentage and more diverse presence of
336 antibiotics that could improve the results.

337 A first and fundamental step towards the molecular and genomic identification of
338 samples is the extraction and purification of DNA from them (Eguiarte et al., 2007);
339 Dittrich-Schroder et al., 2012). In this study, tissue samples rich in mucosal content were
340 subjected to a pulverizing pre-treatment with liquid N_2 before digestion with proteinase K.

341

342 Such pulverization pre-treatment step was very relevant, especially taking into
343 account the origin of the samples, that favored their solubility and homogenization the
344 mucus layers they carried within, to take the 40 mg and easily extracted the DNA from the
345 tissues following a semi-automated DNA extraction procedure based on the use of
346 magnetic beads that contributed to reduce the time and led to clean and reproducible
347 extractions (Milligan et al., 1998;Chen et al., 2010). The obtained DNA was shown to be
348 very suitable for qPCR amplification, with the ratio of A260/A280 showing a mean value
349 of 1.95 ± 0.06, indicator of a good yield of DNA obtained in the extraction.

350 There is only a limited amount of data on the detection of *T. cruzi* presence in the
351 intestinal tissue of humans. Virreira and co-workers typified the *T. cruzi* discrete typing
352 units (DTUs) in megacolon samples from 18 bolivian patients using kDNA probes specific
353 for TcI, TcII, TcV and TcVI . Most samples (16/18) were classified as DTU TcV. (Virreira
354 et al., 2006).

355 He work carried out by Marcon and co-workers (2011) aimed at detecting and
356 quantifying *T. cruzi* in paraffin-embedded cardiac and gastrointestinal tissue samples from
357 chronic patients using Nested-PCR and Sybr Green-qPCR methods, demonstrating that the

presence of the parasite in the tissues is essential for the pathogenesis of Chagas disease (Marcon et al. 2011).

In another study by da Silveira and co-workers, esophagus tissue samples were obtained from six chagasic patients with megaesophagus, eight chagasic patients without megaesophagus and six control individuals submitted to necropsy or surgery procedures. *T. cruzi* presence was detected in 100% of the Chagas cases with megaesophagus and in 60% of patients without megaesophagus, which tissue samples were analyzed following a qPCR targeted to kDNA (da Silveira AB et al., 2005).

Also relying on a kDNA targeted PCR, Vago and collaborators detected *T. cruzi* DNA in tissue samples from 55% (5/9) of the patients affected by megaesophagous (Vago et al., 2003). Nevertheless, previous standardization studies carried out in human blood specimens, showed that kDNA may lead to a higher proportion of false positive findings with respect to satellite DNA (Schijman AG et al., 2011).

Accordingly, for the present work, we relied on a satDNA based real-time PCR in a duplex format that included an endogenous internal RNase P gene sequence from humans as control of integrity of the obtained DNA (Piron et al., 2007; Duffy et al., 2009). Following that protocol, maximum sensitivity was achieved from *T. cruzi* satDNA amplification in triplicate, in DNA samples extracted from three different points from each tissue sample obtained (42.1%; 16/38).

Testing of a single sample from these tissues decreased the possibility of diagnosing *T. cruzi* in an infected patient, and testing of samples collected from different tissue sites is recommended to improve sensitivity, and generate more accurate data on the presence of *T. cruzi* in tissues of patients with digestive disease.

To the best of our knowledge, this is the first time that the parasitic burden in digestive tissues has been addressed by qPCR, employing a standard curve made of a satDNA synthetic molecule embedded in total DNA from non-infected digestive tissues as a matrix.

This standard according to work by Muñoz et al., allowed reflecting the parasitic burden as copies of satDNA/µL of DNA extract, which rendered direct comparison of loads in samples infected with different discrete typing units (Muñoz-Calderón et al., 2021).

Positive tissue DNA samples for *T. cruzi* showed parasite loads that were highly variable between samples, ranging from 2.2 x x10^2 to 1.0 x 10^6 satDNA copies/µL, between the three sampling points. Differences in variability could be attributable to the diversity of the tissue samples for degree of digestive involvement, peculiarity of each of the patients included, as the extracted DNA showed no signs of degradation as evidenced the similar Cq readings of the human RNAseP gene at all three points, illustrated in figure 1, the differences obtained at the three collection points were not significant.

The use of a highly conserved satDNA synthetic sequence as a standard for quantification allows more accurate quantification of parasites belonging to any discrete typing unit (Muñoz-Calderón et al., 2021), and therefore direct comparison of qPCR loads in samples infected with different DTUs can be done. If the DTU of a clinical sample could be identified, an equivalence between the dosage in copy numbers of the satDNA repeats and parasite genome equivalents per unit of sample volume could be estimated if the satDNA copy dosage per parasite cell is known for the DTU under study (Vargas et al., 2004).

In Cochabamba, a high prevalence of TcV genotype has been described (Parrado et al., 2019). In this regards, assuming most samples would be infected with Tc V strains, one

parasite equivalent would harbor between 49,000 and 87,000 satDNA copies, on the basis of a relative estimation of satDNA content reported by Vargas and coworkers (Vargas et al., 2004).In contrast to formerly cited studies, we analyzed a larger study cohort of chronic Chagas disease cases with digestive mega-syndrome manifestations.

Our results support those previously obtained by others, and bring further evidences on the relevance of the digestive tract in the persistence of the parasite. Findings by Lewis and co-workers on a mouse experimental model of chronic *T. cruzi* infection have pointed at the digestive tract as a permissive niche of continuous parasite presence (Lewis et al., 2014). Such finding strongly suggests the relevance of focusing on better understanding the host-pathogen interaction in digestive tissues, particularly considering their high immunological relevance and role in the disease physiopathology and treatment response.

In that model, depending on the parasite strains (belonging to distinct genotypes) used to infect the animals, variable tissue tropism, type and extent of clinical manifestations were observed (Lewis et al., 2014).The next step of our research will be to attempt genotyping *T. cruzi* positive samples in order to further comprehend the role of the different parasite genotypes (Macedo et al., 2004) in the development of one clinical outcome or another.

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