**Trypanosoma cruzi Persistence in Crevicular Fluid from Inflamed Gum of Chronic Chagasic Patients**

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**Abstract**

The presence of *Trypanosoma cruzi* in crevicular fluid from gingival inflammation foci of chronic chagasic patients (CCPs) was detected for the first time using polymerase chain reaction (PCR) assays. Samples taken from 11 unquestionable CCPs, who had suffered from acute infection between seven months and 27 years before the endpoint evaluation, revealed the presence of specific *T. cruzi*-DNA in all of them. This fact demonstrates that *T. cruzi* persistence in human hosts is a long-standing phenomenon and the oral tissue is frequently involved. In addition, *T. cruzi* persistence in crevicular fluid of the studied CCPs occurred independently of the degree of gum inflammation, the time of the infection and/or the patient clinical profiles. The potential of this new non-invasive, fast and easy sampling technique for further epidemiological studies in Chagas disease endemic areas is discussed. The present results may alert dentists to avoid the risk of accidental infection while attending patients from areas where Chagas disease is endemic or when they have migrated to non-endemic countries.

**Keywords:** *Trypanosoma cruzi*; Persistence; Crevicular Fluid; Chagas Disease

**Introduction**

*Trypanosoma cruzi*, the etiological agent of Chagas disease, is a well-adapted and ecologically successful euryxenic (sustained by over 70 genera of mammals) and paninfective (able to invade any kind of cells) protozoan of the family Trypanosomatidae, originated in the American continent, nowadays infecting a great population (> 10^9 people) exposed in Central and South America [1]. Although the parasite primarily affects poor rural populations, recent globalisation and migratory tendencies have provoked the dissemination of Chagas disease from endemic to non-endemic areas suggesting a worldwide spread [2-6].

Chagas disease is characterized by both an acute and a chronic phase. The former is an active infection showing blood-circulating tissue forms of *T. cruzi* and amastigote- forms invading any tissue of the human host. This phase is followed by the chronic phase which may appear as early as one to two months. Both the acute and chronic phases are associated to specific humoral response in which IgM and IgG predominate, respectively [7,8]. One of the most distinguishing features of Chagas disease is that it covers a wide spectrum of pathological outcomes, which range from asymptomatic to severe which include cardiac syndromes that may provoke death [7-9].

Although the use of specific drugs (Nitro derivates compounds) associated with the host’s immune anti-*T. cruzi* response may keep the disease in check, avoiding parasitic proliferation, none of them are able to eliminate the entire population, allowing the tissue parasite to persist for long periods of time during the chronic phase. The persistence of *T. cruzi* tissue forms in chronic chagasic patients (CCPs) was detected for the first time in the myocardium of seropositive individuals following endomyocardial biopsies (EMBs) processed by immuno-histochemical techniques and PCR assay [8]. Despite the scientific advantages this study provided, allowing: i. Detecting *T. cruzi* persistence in the myocardium of living CCPs, which made it possible to correlate the presence of persistent parasites with clinical findings characterizing myocarditis; ii. Increasing the possibility of detecting heart abnormalities not detected by electrocardiogram (EKG) or echocardiogram (ECHO), and iii. Demonstrating that the finding of normal EKG/ECHO is not indicative of a healthy myocardium, it was not fully accepted. However, taking into account the findings of *T. cruzi* tissue forms in organs other than the heart and its association to inflammation processes, Anez, et al [10,11], lead investigations to demonstrate *T. cruzi* persistence at oral inflammatory foci of CCPs, finding positive results in gum biopsies using PCR assays and immuno-histopathological techniques. Having evidence of the presence of *T. cruzi* itself and/or part of its genome in gingival inflammatory foci of CCPs, the present study deals with the demonstration of *T. cruzi*-DNA, using PCR assay, in samples of crevicular fluid circulating into inflamed gums hoping to shed some light on how the parasite can reach this part of the anatomy and also alert dentists professionally working in this area to prevent the risk of accidental infections.

**Patients and Methods**

**Patients**

Eleven chronic chagasic patients (CCPs) were selected during the present study. The patients, five women (45%) and six men (55%) with an average age 48 ± 14 years (range 28-79 years) came from localities of Merida (27%) and Barinas (73%) western Venezuela where Chagas disease is endemic. They had been previously diagnosed clinically, parasitologically, serologically and molecularly (PCR) when suffering the acute phase of Chagas disease from seven months to 27 years before the present study. Patients were regularly re-evaluated every 6-12 months at the cardiologic units of the “Luis Razetti” and University of Los Andes General Hospitals in Barinas and Merida, Venezuela respectively, to monitor their progress and clinical condition. In addition, six healthy individuals were selected as control, from which five crevicular fluid samples were taken from non-endemic villages’ patients of the State of Merida, and the remaining one from a locality of Barin as where Chagas disease is endemic. The former were taken at the Periodontal Unit of the Faculty of Dentistry, University of Los Andes-Merida, and the latter at “Luis Razetti” General Hospital in Barinas, Venezuela from a patient showing heart disease caused by etiology other than *T. cruzi*. 
Inclusion criteria

Several conditions were established to select the patients included in the present study. Firstly, patient history had to show evidence of having suffered a previous acute episode of Chagas disease and secondly, recent evaluations had to demonstrate a chronic condition either clinically or serologically. To confirm their condition as CCP, clinical examination including electrocardiogram (EGK) and echocardiogram (ECO) were performed considering criterion established previously [11]. In addition, blood samples were taken to be processed for serology using a direct agglutination test (DAT) and indirect immunofluorescence antibody test (IPAT) for both polyvalent and specific IgM/IgG subtypes, as described previously [10,11]. On the other hand, selected patients with chronic Chagas disease were previously confirmed to have gingival T. cruzi persistence [11]. They were regularly evaluated and then referred to dentistry units to be examined to determine whether they fulfilled the established inclusion criterion of having gingivitis. The condition of the patient’s oral cavity according to the degree of gingival inflammation was established by a dentist at the dentistry unit of “Luis Razetti” General Hospital for patients from the State of Barinas and at the Periodontal Unit of the Faculty of Dentistry, University of Los Andes for patients from Merida, following the index previously reported [12].

All the control patients, including healthy ones and that suffering from other etiology, must show a different degree of gingivitis to be selected and included in the study.

Collection and processing of crevicular fluid

After confirming that the selected CCPs fulfilled with the established inclusion criteria, the crevicular fluid was collected to be processed. Once the inflamed area of the chosen tooth was selected and isolated with cotton rolls, the saliva on the surface was carefully dried and the exceeding bacterial plaque material removed, the gingival sulcus was stimulated using a periodontal probe 1 mm depth during five to ten seconds to increase the flow of crevicular fluid, avoiding bleeding. From 30 seconds to one minute after sulcus stimulation, an N© 15 absorbent sterile Peripaper strip was introduced into the gingival sulcus for two to three min to let the fluid fill the cone by capillarity. After collecting the sample, cones containing crevicular fluid were placed into Eppendorf tubes that were maintained at -20°C until they were processed for PCR at the Center for Parasitological Research “J.F.Torrealba”, Faculty of Science, University of Los Andes, Merida, Venezuela. Collecting process for crevicular fluid in CCPs is shown in figure 1.

To detect the presence of T. cruzi-DNA in the collected crevicular fluid from inflamed gums of CCPs a specific PCR assay was carried out using primers S35 (5’- AAA TAA TGT ACG GGT GAG ATG CAT GA-3’) and S36 (5’- GGG TTC GAT TGG GGT TGG TGT-3’) following protocol previously established [13]. In addition, to confirm observations with the mentioned PCR assay for kDNA, a Sat-DNA was performed using the primers Cruzi 1 (5’- AST CGG CTG ATC GTT TTC GA 3’) and Cruzi 2 (5’- ATT TCC TCC AAG CAG CGG ATA 3’) as previously indicated [10]. In all cases, the periopaper strip cones impregnated with crevicular fluid of each CCP were treated with a volume of 150 µL lyses buffer containing 10 mM Tris-HCl; 150 mM NaCl and 3 µL proteinase K (20 µg/mL). The extraction of T. cruzi-DNA was carried out by the classical phenol-chloroform method and the PCR amplified product was separated by electrophoresis in 2% agarose gels and stained with ethidium bromide as previously reported [14].

Ethical considerations

This study was approved by the ethical committee of the “Luis Razetti” General Hospital, Ministry of Health, Barinas, Venezuela. A written consent, including agreement previous information (API-form) was obtained from each patient to comply with the criteria established by the Biomedical Committee of the National Research Council of Venezuela.

Results

The present study was carried out on eleven unquestionable chronic chagasic patients whose infections have been monitored since they were suffering from acute phase of Chagas disease. Having observed different clinical conditions in symptomatic chagasic patients ranging from mild to severe profiles, as well as different time post primary infection including periods as early as seven months to 27 years, gave us enough confidence for obtaining representative samples to accomplish the task to initiate the study. The patient baseline characteristics during the acute chagasic infection detected using clinical, parasitological, serological and molecular (PCR) methods are shown in table 1. This background together with detection of the gingival inflammation degree confirmed by a dentist, allowed us to select the included patients to be examined during the endpoint evaluation. Examination of patient’s gums detected different degrees of inflammation including severe (9%), moderate (73%) and mild (18%) profiles (Table 1). In addition, results of the confirmatory serology revealed seropositivity in 55% of CCPs both with immunofluorescence technique (IFAT) and direct agglutination test (DAT) methods, showing IgG titers ranging from 16 to 512. These patients also showed EKG and ECO abnormalities. The remaining five patients (45%) showed seronegative results. However, they presented EKG and ECO abnormalities when were examined cardiologically, reason why they were maintained as candidates for the following molecular test. In all cases control patients showed seronegative results as expected.

Detection of Trypanosoma cruzi-DNA persistence in crevicular fluid of chronic chagasic patients

The presence of T. cruzi persistence in gingival inflammation foci was demonstrated and confirmed with specific PCR assays carried out in DNA samples taken from crevicular fluid circulating into the CCP’s affected gum. During this process, specific bands of 330 bp and 166 bp were amplified respectively when used S35/S36 and Cruzi1/Cruzi2 primers, which matched with that of T. cruzi-DNA controls used in the PCR assays (Figure 2) evidencing the presence of a portion of T. cruzi genome in the crevicular fluid circulating into the inflamed gum of CCPs. It also shows T. cruzi persistence in patients bearing different inflammation profiles identified as mild (18%), moderate (73%) and severe (9%). The fact that all of the studied CCPs received specific treatment with nitroderivate drugs is also relevant. This included the eight patients from Barinas who were treated with Benznidazole more than 20 years ago, and the three from Merida, receiving Nifurtimox only seven months previous to the endpoint.

Figure 1: Collection of crevicular fluid from gingival inflammation foci of chronic chagasic patients. Note the insertion of an absorbent periopaper strip cone into the gingival sulcus to collect the sample.
in gingival inflammation is followed by an increase in crevicular fluid flow, making it possible to establish differences between stable and progressing gum disease [19]. Basically, crevicular fluid is an oral cavity-specific liquid containing pro-inflammatory cytokines and a large number of other proteins present in high levels during infectious processes, being also characteristic during early infections the findings of lymphoid cell infiltrate predominating T and B lymphocytes, plasma cells as well as macrophages [17]. The constituents of crevicular fluid have been studied to determine whether they could be used as biomarkers for periodontal disease diagnosis and even to recognize molecular patterns predictive for disease development [17]. On the other hand, some authors have recognized that oral infection may affect the course and pathogenesis of several systemic diseases including cardiovascular affection, among others [20].

Regarding Trypanosoma cruzi and gum disease association, in a previous report the presence of this parasite at oral inflammatory foci of CCPs was demonstrated using PCR assays. In the mentioned report 22.5% of the gingival biopsies revealed the presence of T. cruzi-DNA [18]. In addition, in a follow up study of 60 CCPs evaluated from one to 23 years after suffering the acute phase of Chagas disease, the persistence of T. cruzi in oral tissue taken from infected gums, was detected in 40% of them when different techniques were used to visualize the parasite itself, T. cruzi antigenic deposits and/or part of the parasite genome [11]. These consistent findings were evidence enough to consider the persistence of T. cruzi in oral tissues as a relatively frequent factor in CCPs and its significance must be a matter of concern for those dentists professionally working in areas where Chagas disease is endemic [14].

In the present study, analyses by PCR assays carried out in samples of crevicular fluid obtained from gum inflamed foci of CCPs who suffered acute Chagas disease for 0.6 to 27 years at different endemic regions of western Venezuela, revealed the presence of specific T. cruzi-DNA in all of them, demonstrating that T. cruzi persistence in the human host is a long-standing phenomenon and the oral tissues a frequent involvement, supporting previous reports [8,10,11,14]. Several interesting features derived from this particular biological behavior showed by T. cruzi in its hosts: i. the fact that even when 45% CCPs showed negative results for specific anti-T. cruzi antibodies, they revealed parasite persistence evidenced by detection of specific T. cruzi-DNA in crevicular fluid. ii. T. cruzi persistence seems to be a phenomenon occurring irrespective of the patient clinical profile and gum disease association, in a previous report the presence of this parasite at oral inflammatory foci of CCPs was demonstrated using PCR assays. In the mentioned report 22.5% of the gingival biopsies revealed the presence of T. cruzi-DNA [18]. In addition, in a follow up study of 60 CCPs evaluated from one to 23 years after suffering the acute phase of Chagas disease, the persistence of T. cruzi in oral tissue taken from infected gums, was detected in 40% of them when different techniques were used to visualize the parasite itself, T. cruzi antigenic deposits and/or part of the parasite genome [11]. These consistent findings were evidence enough to consider the persistence of T. cruzi in oral tissues as a relatively frequent factor in CCPs and its significance must be a matter of concern for those dentists professionally working in areas where Chagas disease is endemic [14].

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during the acute phase and/or the time of evolution of chagasic infection, a fact demonstrated by the presence of part of *T. cruzi* genome in crevicular fluid of patients who suffered mild (27%) or severe (73%) acute symptoms seven months to 27 years before the endpoint evaluation, and iii. the detection of *T. cruzi* persistence in crevicular fluid of CCPs may occur independently of the degree of gum inflammation, being revealed in patients with severe, mild and moderate processes in 9%, 18% and 73%, respectively.

Gathering results on *T. cruzi* persistence in crevicular fluid of CCPs obtained in the present study together with previous reports regarding persistent infection in inflamed gum foci in patients with Chagas disease as referred above, allows to alert dentists and assistant personnel to avoid risk of accidental infection while working with patients from areas where Chagas disease is endemic as previously suggested [21]. This fact makes it particularly necessary to use bio-security barriers such as personal protection and instrument sterilization during invasive procedures for surgical intervention in people suspected to come from Chagas disease endemic areas.

Detection of *T. cruzi* persistence in crevicular fluid of CCPs reported here, for the first time, adds a new non invasive, fast and easy sampling technique which may result useful in further epidemiological studies in populations in risk areas where Chagas disease is endemic, or in those suspected clinical cases observed in patients living in non-endemic areas.

**Conclusion**

- In the present study the detection of *T. cruzi* persistence in crevicular fluid from inflamed gum of chronic chagasic patients is demonstrated, for the first time, using molecular (PCR assay) methodology.
- The detection of *T. cruzi* persistence in crevicular fluid of the study CCPs occurred independently of the degree of gum inflammation, the time of evolution of chagasic infection and/or patient clinical profiles.
- The present findings may serve to alert dentists and assistant personnel to avoid risk of accidental infection while working with patients from areas where Chagas disease is endemic.

**Acknowledgements**

The technical assistance of dentistry units at Faculty of Dentistry, University of Los Andes, Merida, and “Luis Razetti” General Hospital, Barinas, Venezuela is gratefully acknowledged. This work was supported by CDCHTA-ULA [Grant C-1821-13-07- AA (NA); Grant C-1820-13-07-B (GC)] and FONACIT (FUNDACITE-Barinas-Grant 2013001529-LOCTI, Sabaneta Hospital). We thank the Administrative-Vice-Chancellor of University of Los Andes for supporting us. We are indebted to Ernesto Anez and Tessa Swanson on their criticism of the manuscript.

**Conflict of Interest**

We confirmed that this article content has no conflict of interest.

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