The detection of *Trypanosoma cruzi* by nested-PCR in elderly patients: relationship to the clinical and epidemiological profile

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Chagas disease, which is caused by *Trypanosoma cruzi*, is transmitted primarily by triatomine bugs, although the incidence of new cases has decreased as a result of vector control. In Brazil, most of those affected have the chronic form of the disease and are generally elderly individuals who require appropriate clinical follow-up. In this work, we undertook a descriptive study in which 85 patients were interviewed and blood samples were collected for molecular analyses based on the amplification of parasite satellite DNA. The cardiac form of the disease was the most prevalent among the patients and hypertension was the most frequent comorbidity; polypharmacy was detected in 34% of the cases. Serological tests were positive in 95% of cases while 36% were positive in nested-polymerase chain reaction. These findings indicate an increased use of medications and a larger number of age-related diseases in elderly patients with Chagas disease, even in patients with low parasitemia. We conclude that elderly patients with Chagas disease require special attention and that further studies should be done with elderly individuals who carry this disease.

Keywords: Chagas disease, Elderly, Polymerase chain reaction

Introduction

Human American trypanosomiasis or Chagas disease, named after the Brazilian physician and epidemiologist Carlos Chagas, who first described the disease in 1909, is caused by the protozoan *Trypanosoma cruzi* that is transmitted to humans primarily by triatomine bugs.¹ Chagas disease can also be transmitted to humans by non-vectorial mechanisms, such as blood transfusion, and vertically from mother to infant.² Chagas disease classically presents in an acute or initial phase in which the symptoms are usually mild and nonspecific, and can include fever, malaise, hepatosplenomegaly and atypical lymphocytosis.³ The acute clinical manifestations disappear in 2–4 months, after which the parasites are seldom detected in peripheral blood. This second phase is known as the chronic phase and starts as an indeterminate form that can last for 10–30 years or throughout life. Some patients with the indeterminate form can present complications such as an abnormal heart rhythm and digestive disorders.^{4,5} When transmission of the infection is reduced or interrupted, a

cohort effect is generally observed, with a higher prevalence of *T. cruzi* infection among older individuals.⁶

Brazil has experienced important changes in its demographic and population age structure, with increased life expectancy, and aging of the population. Various studies have shown that hypertension, diabetes, and arthritis/osteoarthritis are the most prevalent diseases in this population.^{7,8} Elderly patients with Chagas disease show a greater number of diseases associated with old age and have overuse of medicines beyond those that are normally used for digestive and heart disorders.⁹ The fact that Chagas disease is one of the leading causes of death among the elderly in Brazil indicates that early acquired infection is still a major problem.¹⁰

Most of the human infections with *T. cruzi* are only detected in the chronic phase, which is characterized by subpatent parasitemia and limited tissue parasitism.¹¹ In this context, a better knowledge of the role of *T. cruzi* and its persistence in small numbers in peripheral blood could provide insights into the evolution of the disease.¹² In this context, the polymerase chain reaction (PCR) may be a useful tool in the diagnosis of Chagas disease and nested-polymerase chain reaction (N-PCR) shows good

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Figure 1 Clinical form of Chagas disease in the patients studied.

sensitivity and specificity for detecting *T. cruzi* in patients with the chronic form of the disease.^{13,14}

The aim of this study was to evaluate the relationship between the detection of Chagas disease and the clinical and epidemiological profiles of elderly patients with this disease who attended a reference center at the teaching hospital of the State University of Campinas (HC/UNICAMP) in the state of São Paulo, southeastern Brazil. Chagas disease was detected by screening blood samples from these patients for the presence of *T. cruzi* DNA.

Materials and methods

This descriptive study was approved by the institutional Ethics Committee at UNICAMP (protocol no. CEP 254/2010). The data were collected from July 2010 to December 2011. During treatment at the hospital's outpatient clinic, all carriers of Chagas disease >60 years old of both sexes were asked to participate in the study. The 85 patients who agreed to participate provided written informed consent prior to the collection of any information or blood samples.

Blood samples were collected for serology and N-PCR. Enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) were done in the Clinical Pathology Laboratory and N-PCR was done in the Laboratory for the Diagnosis of Infectious Diseases of the university teaching hospital at UNICAMP.

DNA extraction from blood samples

DNA was extracted from blood samples using the phenol-chloroform extraction protocol. Blood with seronegative results for Chagas disease was used as a negative control. The samples were centrifuged (800 g, 10 min, 4 °C) and the red blood cells obtained were then treated with lysis buffer (0.0114 M NH_4Cl and 0.01 M NH_4HCO_3) and washed with TKM1 buffer (10 mM Tris-HCl, pH 7.6, containing 10 mM KCl, 10 mM MgCl_2 and 20 mM EDTA), followed by TKM2 buffer (10 mM Tris-HCl, pH 7.6, containing 10 mM KCl, 0.4 M NaCl, 10 mM MgCl_2 and 2 mM EDTA) and 10% sodium dodecyl sulfate. The samples were incubated at 55 °C for 30 min and 5 M NaCl was added to the supernatant. DNA was extracted with

phenol/chloroform and precipitated with ethanol/3 M sodium acetate. After storage overnight at 20 °C, DNA was washed with 70% ethanol and solubilized in 30 μl of sterile deionized water.^{15,16}

Nested-PCR assay

N-PCR was used to amplify parasite satellite DNA. The first reaction used the primers TCZ1 (5'-CGAGCTCTTGCCACACGGGTGCT-3') and TCZ2 (5'-CCTCCAAGCAGCGGATAGTTCAGG-3') to amplify a region of 188 bp, while the second reaction used 0.4 μl of the amplicons from the first reaction and the primers TCZ3 (TGCTGCA 5'-(G/C)TCGGCTGATCG TTTTCGA-3') and TCZ4 (5'-CA(a/G)G(C/G)TTGTTT GGTGTCCAGTGTGTGA-3') which amplified a region of 149 bp.^{14,16}

After the second thermal cycle, 5 μl of each sample was electrophoresed in a 2% agarose gel stained with ethidium bromide and the amplicon was visualized with a UV light transilluminator. The human β -globin gene was amplified in each sample in order to check the DNA quality and confirm the absence of inhibitors since the phenol/chloroform extraction procedure can leave traces of contaminants.¹⁶ The sensitivity of the technique was assessed by extracting DNA from *T. cruzi* cultivated in LIT medium (10^{-5} – 10^7 parasites/ml) followed by amplification by N-PCR; this procedure was capable of detecting 1 parasite/ml.

Interview and medical records

Socio-demographic and epidemiological data such as gender, origin, and contact with the vector were obtained by interview. Additional information, such as associated diseases and medication use, was confirmed by consulting the patients' hospital records.

Data analysis

All the data collected were analyzed descriptively using the software package BioEstat®5.0 (Instituto Mamirauá, Tefé, Amazonas, Brazil).

Results

There was no significant difference in the number of males ($n = 41$) and females ($n = 44$) recruited for the study. The mean age of the patients was 65.5 ± 5.4 years (median: 64 years). Figure 1 shows that the cardiac form was the most common clinical form of Chagas disease among the patients studied. Megaeosophagus was present in 52% of patients with the digestive form of the disease. In the group with the mixed form, seven patients (54%) had the cardiac form and megaeosophagus.

Most patients (89.5%) also had other diseases and were on medications (87%), with polypharmacy (≥ 5 medications) occurring in 34%. Figure 2 shows the comorbidities and Table 1 the most commonly used medications. The use of diuretics, antiulcer drugs, antiarrhythmics and thyroid

Figure 2 Most common comorbidities in elderly patients with Chagas disease.

Table 1 Medications most commonly used by elderly patients with Chagas disease

Medications	Frequency (%)
Antihypertensive	65
Diuretics	37
Antiulcer	34
Antiplatelet	23.5
Antilipemic	16.5
Antiarrhythmic	15
Mineral supplement	14
Thyroid hormone	12

hormone was more frequent in females, while the use of antiplatelet drugs was more frequent in males.

Most of the patients originally had lived in rural areas and migrated to urban areas. The mean time lived in rural areas was 26.6 years, compared to 41 years in urban areas. Eighty-three respondents reported knowing the transmitter of the disease. Nearly 70% (69.5%) said they did not recall having been bitten by a triatomine bug. Of those who remembered, the age at which they were bitten ranged from 6 to 26 years old. The antecedents for Chagas disease were also investigated: 28% of the patients had already undergone a blood transfusion and 66% had a relative with the disease.

Based on the serological results, 95% of the patients showed consistent findings (positivity for Chagas disease) in the two serological tests (ELISA and IFA). The remaining 5% were considered doubtful since they had only one test that was positive for Chagas disease.

Of the 85 samples, 10 (11%) showed no amplification of the human β -globin gene, possibly because of the presence of inhibitors, and were therefore not analyzed by N-PCR. The N-PCR was positive for *T. cruzi* satellite DNA in 27 of the 75 samples, indicating a positivity of 36%. All PCR tests were done in triplicate to confirm the results, with the sample being considered positive for Chagas disease if there was at least one positive reaction; a sample was considered negative after three negative results.

Discussion

Over the past 30 years, systematic surveillance and control in endemic areas have altered the profile of patients with

Chagas disease.¹⁷ Most of the elderly subjects in this study were in the 60–69-year-old age group and were considered ‘young elderly individuals.’¹⁸ This expected ‘young elderly’ age range agreed with that described by Almeida *et al.*¹⁹ and Alves *et al.*¹⁸. The longevity of the disease remains a major public health problem among the elderly.⁶

Cardiomyopathy is the most important clinical consequence of *T. cruzi* infection and the most serious and frequent manifestation of chronic Chagas disease.¹ Most elderly Chagas disease patients develop electrocardiographic abnormalities during the indeterminate form of the disease.²⁰ The findings reported here corroborate the studies of Almeida *et al.*,¹⁹ Alves *et al.*¹⁸ and Guariento *et al.*,²¹ who also found the cardiac form to be the most prevalent in the same outpatient clinic where the present study was done. In addition, the cardiac form was the main clinical form in another study done in the northeastern Brazilian state of Ceará.⁹ Aging is known to cause histopathological changes in the heart and to contribute to cardiovascular pathologies such as arterial hypertension and coronary and valvular pathologies that are common in elderly patients.²² However, in elderly Chagas disease subjects, electrocardiographic abnormalities and cardiac lesions may be caused by a combination of Chagas cardiomyopathy and other conditions often seen in elderly persons without Chagas disease, e.g. ischemic and hypertensive heart disease.²³

In an endemic area, 15–20% of patients with Chagas disease develop alterations in gastrointestinal motility, secretion and absorption, especially in the esophagus and colon.⁴ In hospital-based observations, megaesophagus is more frequently diagnosed than megacolon,¹ as also observed here. In most countries, the development of megaesophagus usually precedes heart and colon disease, but the exact prevalence of the cardiodigestive form of Chagas disease is unknown because of a lack of appropriate studies.²

Comorbidity, defined as the co-occurrence of multiple chronic or acute diseases, can provide some idea of the possible impact of the diseases on an individual’s physiological reserve.²⁴ According to Guariento *et al.*²¹ and Lima e Costa *et al.*,⁶ the number of comorbidities may constitute an indicator of poor health in elderly patients with Chagas disease and lead to a greater use of health services.

Hypertension, the most common comorbidity among the patients in this study, would likely have manifested itself with advancing age, regardless of the evolution of Chagas disease. According to Gurgel *et al.*,²⁵ the frequency of hypertension among Brazilian patients with chronic Chagas disease is similar to that of the general population. This was also the case in the study of Lima-Costa *et al.*,⁸ in which hypertension was found in almost 44% of the 29,976 elderly patients studied. However, Vicco *et al.*²⁶ reported an important association between Chagas disease and high blood pressure. According to Bertanha *et al.*,²⁷ hypertension is a risk factor for the development of cardiovascular disease; these findings emphasize the

relevance of studying Chagas disease. The increased survival of individuals with Chagas disease poses additional challenges to health professionals because of the association and interaction with other diseases, such as hypertension and diabetes mellitus.¹⁷

Polypharmacy is considered when there is a use of multiple medications and the administration of more medications is clinically indicated. The use of many medications can cause problems such as the risk of drug interactions, non-adherence, and adverse effects.²⁸ Polypharmacy is an important issue among elderly individuals as a whole.⁹ Normally, older adult patients use more drugs compared to other age groups.²⁹ A study in the Brazilian state of Minas Gerais found that a high intake of drugs (five or more) was observed in 14.3% of 1598 elderly subjects (10.5% among men and 16.9% among women).³⁰ In our study, almost 90% of the elderly patients evaluated used some medication and polypharmacy was found in 34% of these subjects. This finding indicated that polypharmacy was more prevalent in older people with Chagas disease. An increased use of medicines is expected as the population ages because of the demand for medications that can delay and treat diseases, relieve pain and improve the quality of life.³¹

Studies done in Brazil and the United States have shown that the major medications used by older people include cardiovascular agents such as antihypertensive, diuretic, antiarrhythmic and antiplatelet drugs.^{30,32} In the case of patients with Chagas disease, our findings were similar to those of Alves *et al.*¹⁸ and Pereira *et al.*,⁹ who reported that cardiovascular drugs were the most commonly used medicines. Based on our findings and previous reports, we conclude that elderly individuals with and without Chagas disease show a similar use of cardiovascular drugs.

The rural-urban migration in recent decades has displaced millions of infected people to urban areas, where vector transmission does not occur, and this has changed the epidemiological pattern of Chagas disease.¹⁷ The effect of migration was also noted in the patients studied here. In a study done in the Brazilian state of Mato Grosso do Sul, Pompilio *et al.*³³ reported that 50% of those surveyed had been in contact with the insect vector, whereas Silva *et al.*³⁴ reported a lower percentage of contact (14%) in the Brazilian state of Minas Gerais. To ascertain whether there had been contact with the vector, we sought to determine the patients' knowledge of the vector and whether they had been bitten. Knowledge was the easiest to determine, with most patients having some understanding of the vector. Few were able to tell when they were bitten, probably because the majority of them were too young to remember when it happened.

Serological tests for Chagas disease usually involve two methods, one of them being necessarily an ELISA. Hemagglutination inhibition tests, IFA, and ELISA can determine the diagnosis in virtually 100% of cases.³⁵ In the present study, patients with two positive tests were

considered to be infected with *T. cruzi*, whereas those with only one positive result were considered to be inconclusive or questionable.³⁶ The serological test results obtained in this study were expected since serological tests have good sensitivity for detecting the parasite.

PCR-based diagnostic tests have been widely used to diagnosis and monitor the progression of Chagas disease. These methods have revealed highly variable levels of sensitivity and specificity, depending on a number of technical factors, such as the sample volume collected, the sample storage conditions, the methods used to isolate DNA, the parasite sequences and primers selected, the reagents used and the thermocycling conditions.³⁷ Piron *et al.*³⁸ reported that qPCR (quantitative real-time PCR) was sensitive to 0.8 parasites/ml. Campos *et al.*³⁹ reported that a PCR for kDNA detection in mice was able to detect 2–4 parasites/ml. The latter study also showed that for smaller numbers of parasites the assay (extraction and PCR) should be done several times to confirm the result. As shown here, although the N-PCR was repeated several times, only low positivity (36%) was observed among elderly individuals. Positivity was higher in the studies reported by Aguiar *et al.*,¹⁵ Batista *et al.*¹⁶ and Marcon *et al.*¹⁴. However, in these studies, the patients were not elderly. There are no other studies in the literature that have used molecular biology as a tool to verify the parasitemia in elderly individuals. Variability in PCR sensitivity can be partially explained by the intermittent presence and quantity of circulating parasites at the time of blood collection.³⁷ Since the technique used in this study can be considered sensitive, we conclude that the individuals examined here had few parasites circulating in the bloodstream. Possible solutions to increase the low frequency of positivity, especially when using conventional PCR, include the collection of serial blood samples or increasing the blood sample volume.³⁵ Another possibility would be to use real-time PCR to quantify these parasites in elderly individuals.

Conclusions

Analysis of the socio-demographic characteristics of the patients in this study showed that most of them were born in rural areas, but subsequently migrated to urban areas. The profile of medications used and the occurrence of comorbidities in elderly people with Chagas disease were similar to those of elderly patients in general, but polypharmacy was more prevalent in chagasic patients. The majority of elderly patients had the expected cardiac form, in agreement with outpatient clinical studies showing that this is the most prevalent form. The low rate of N-PCR positivity may reflect the fact that the patients had been in the chronic phase for least 40 years.

Conflict of interest

No potential conflict of interest was reported by the authors.

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Supplementary material

Supplemental data for this article can be accessed [<http://dx.doi.org/10.1080/20477724.2016.1232850>].

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