Invest Clin 53(4): 378 - 394, 2012

Electrocardiography repolarization abnormalities are characteristic signs of acute chagasic cardiomyopathy.

Edilmar Alvarado-Tapias[1], Rumania Miranda-Pacheco[1], Claudina Rodríguez-Bonfante[2], Glenda Velásquez[4], Jorge Loyo[3], Marianyeliz Gil-Oviedo[3], Nora Mogollón[5], Mary Carmen Pérez-Aguilar[5], Giannina Recchimuzzi[6], Raul Espinosa[7], Hernán José Carrasco[6], Juan Luis Concepción[5], Rafael Armando Bonfante-Cabarcas[3].

[1]Hospital Rafael Medina Jiménez, estado Vargas;
[2]Unidad de Investigaciones en Parasitología Médica y [3]Unidad de Bioquímica, Decanato de Ciencias de Salud, Universidad Centroccidental Lisandro Alvarado, Barquisimeto, Lara;
[4]Centro de Investigaciones Biomédicas, Universidad de Carabobo, Valencia, Carabobo;
[5]Laboratorio de Enzimología de Parásitos, Facultad de Ciencias, Universidad de los Andes, Mérida,
[6]Laboratorio de Biología Molecular de Protozoarios, Instituto de Medicina Tropical, Universidad Central de Venezuela,
[7]Hospital Pérez Carreño, Caracas, Distrito Capital. Venezuela.

Keywords: *Trypanosoma cruzi*, acute Chagas´ Disease, electrocardiographic repolarization abnormalities, microvascular involvement, myocardial ischemia.

Abstract. Chagas disease is a tropical parasitic disease caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*), whose reemergence as oral outbreaks is currently a public health problem in Venezuela. *T. cruzi* infection induces myocardial damage; which according to the microvascular theory, is derived from parasite-mediated disruption of the endothelium, inducing platelet aggregation and ischemia. In order to determine whether ventricular repolarization disorders observed in human patients are characteristic signs of the disease that can be reproduced in NMRI mice; we studied 12 patients with a well documented diagnosis of acute Chagas disease, based on epidemiological, clinical, parasitological and molecular data. Also, *T. cruzi* isolates from the blood of human patients from other Venezuelan geographical regions were characterized and inoculated in albino NMRI mice. A standard 12-lead and bipolar electrocardiogram configuration were done in human patients during the acute phase of the disease and in mice, after three weeks of

Corresponding author. Rafael Armando Bonfante-Cabarcas. Av. Libertador con Andrés Bello, Unidad de Bioquímica, Decanato de Ciencias de la Salud, Universidad Centro-Occidental "Lisandro Alvarado". Barquisimeto, estado Lara, Venezuela. Código Postal: 3001 Teléfono: 58-251-2591854, Fax: 58-251-2591950 E-mail: rcabarca@ucla.edu.ve

infection. Results in human showed repolarization disorders, characterized by: negative, bimodal or biphasic T waves, ST segment depression or elevation and early repolarization. In mice a significant increase in T wave amplitude, increased QT interval duration and elevation or depression of ST segment were observed. These findings were evidenced in all infected mice, suggesting that electrocardiographic repolarization abnormalities in a well documented clinical and epidemiological context are signs that increase the sensitivity for the diagnosis of acute Chagas´ disease.

Los trastornos de la repolarización ventricular son signos característicos de la cardiomiopatía chagásica aguda.

Invest Clin 2012; 53(4): 378 - 394

Palabras clave: *Trypanosoma cruzi*, Chagas agudo, trastornos de la repolarización ventricular, isquemia miocárdica, trastornos de la microvasculatura.

Resumen. La enfermedad de Chagas es una hemoparasitosis causada por *Trypanosoma cruzi* (*T. cruzi*), cuya re-emergencia como epidemias por contaminación oral es actualmente un problema de salud pública en Venezuela. La infección por *T. cruzi* causa miocarditis; que de acuerdo con la teoría microvascular deriva del daño del endotelio vascular, al inducir agregación plaquetaria e isquemia. Con el objetivo de demostrar que los trastornos de repolarización son signos propios de la miocarditis chagásica aguda (MChA) reproducibles en modelos animales, estudiamos 12 pacientes humanos con diagnostico bien documentado de MChA, basado en datos epidemiológicos, clínicos, parasitológicos y moleculares. A partir de la sangre de los pacientes obtuvimos los aislados de *T cruzi*, los caracterizamos molecularmente y los inoculamos en ratones albinos NMRI; paralelamente, aislados de *T cruzi* provenientes de otras regiones de Venezuela fueron también ensayados. Tanto en los pacientes humanos como en los ratones con Chagas agudo, se realizaron estudios electrocardiográficos en 12 derivaciones estándares y en configuración bipolar, respectivamente. En humanos observamos trastornos de la repolarización ventricular caracterizados por: onda T negativa, bimodal o bifásica; elevación o depresión del segmento ST y despolarizaciones tempranas. En ratones observamos incrementos en la amplitud de la onda T, aumento en la duración del intervalo QT y elevación o depresión del segmento ST. Estos hallazgos fueron evidenciados en todos los ratones infectados con los diferentes aislados, sugiriendo que los trastornos de repolarización, en un adecuado y bien documentado contexto epidemiológico y clínico, son signos que aumentan la sensibilidad para el diagnóstico de MChA.

Recibido: 17-09-2012. Aceptado: 22-11-2012

INTRODUCTION

Currently, it is considered that Chagas disease is locally transmitted in 19 countries in the Americas, and there are between 8 and 15 million of infected individuals, with an overall prevalence rate of 1.45% (1-4). In a report from PAHO (2006)(4), it was estimated for Venezuela, based on a total population of 26,749,000 inhabitants, that 4,944,000 individuals are at risk for infection and 310,000 are all ready infected individuals; also there are 1,400 new cases by vector transmission with an incidence of 0.005% and prevalence of 1.16%. The incidence of congenital transmission was projected at 0.102% with 68,000 infected women in the ages between 15 and 47 years and the seroprevalence in blood banks was estimated at 0.78%. Our group in several seroepidemiological studies done in the central-western region of Venezuela have reported prevalences between 1.57 (5) and 7.24% (6).

Vectorial transmission of Chagas disease has decreased all over Latin-America; on the contrary *T. cruzi* oral-accidental transmission is becoming increasingly common. Since 1965, several outbreaks caused by oral accidental routes have occurred in many Brazilian states and in other Latin American countries (references in 7 y 8). Recently in Venezuela, in the north-central region there have been several outbreaks of acute Chagas disease, specifically in Chacao (Miranda State), Antímano (Capital District) and Chichiriviche de la Costa (Vargas State) between 2006 and 2010 (8-10).

In endemic areas, the primary infection usually occurs in children. It is estimated that *T. cruzi* acute infection is symptomatic in about 5-10% of the affected individuals. Acute Chagas´ disease is a predominant nonspecific usually prolonged febrile syndrome; with the exception of face and lower limbs edema, other signs and symptoms are nonspecific and they constitute elements for diagnostic mistakes in endemic areas, where other tropical and infectious disease are prevalent or appear as outbreaks, for example influenza, dengue, infectious mononucleosis and malaria, among others (11).

Mortality during an acute phase of Chagas´disease is about 5-10%; death is mostly caused by myocarditis and meningoencephalitis. Oral infection with *T. cruzi* is associated with higher mortality rates, usually in the first two weeks after infection. Myocarditis is present in 80% of patients presenting severe symptoms of acute Chagas disease, it causes myocardial dyskinesis, heart enlargement, pericardial effusion and heart failure (references in 7). Electrocardiographically it is frequently noticed disturbances of ventricular repolarization in acute Chagas´ disease (7, 10-13), which indicate that acute Chagas´ myocardiopathy could be considered an ischemic disease; indeed pattern of wall motion abnormalities and delayed enhancement determined by Cardiac magnetic resonance in Chagas' disease patients may mimic ischemic cardiomyopathies, with especial predilection for the apical and inferolateral segments of the left ventricle (14).

According to the microvascular theory, *T. cruzi* infection causes different structural and functional alterations of the coronary microvasculature, due to platelet aggregation stimulation, vascular tone increase and microvascular hypoperfusion, which lead to cardiac ischemia and multifocal necrosis, triggering inflammatory mechanisms with subsequent repair and fibrosis (15).

In the present paper with the aim to analyze ventricular repolarization disturbances as hallmark sign in acute Chagas disease, we recollected clinical and electrocardiographic data from human patients

with documented acute Chagas disease, from which we isolated and characterized *T. cruzi* strains and inoculate them in NMRI mice to reproduce repolarization disturbances. Likewise to observe whether repolarization disturbances are related to specific geographical strains, we tested isolates obtained from different Venezuelan geographic areas. Our results confirmed that repolarization disturbance is an even present electrocardiographic sign during the acute phase of the Chagas´ disease in humans and mice independent of the parasite genetic profile or geographical origin.

PATIENTS AND METHODS

Sample

Human patients consisted of 12 individuals, aged between 8 and 14 years, 50% were males and 50% females; with a clinical and parasitological diagnosis of acute Chagas´ disease. Patient used to live in "Chichiriviche de la Costa" town located at 10°33'00'' north latitude and 67°14'01'' west longitude, in the coast of Vargas State (Venezuela), where an oral outbreak of Chagas disease was confirmed in April 2009. Animal model consisted of 232 albino mice NMRI strain, with 2 months of age and 34.19 ± 0.527 g average weight, which were divided into: control group (n = 17) and 9 experimental groups named according to the geographical origin of *T. cruzi* isolates: Guarico (n = 22), Chabasquén (n = 22), Barinas (n = 22), p6 (n = 22), p11 (n = 24), p13 (n = 25), p14 (n = 20), p16 (n = 23) and CHHP (n = 22).

Chabasquén and Guarico isolates were obtained from *Panstrongylus geniculatus* specimens captured in those populations. Chabasquen is located in Portuguesa state at 9°37'07'' north latitude, 69°47'52'' west longitude and 1115 meters above sea level altitude. Guarico located in Lara state at 9°25'41'' north latitude, 69°57'14'' west longitude and 698 meters above sea level altitude, respectively. Isolates called "p" were obtained from blood of patients hospitalized with the diagnosis of acute phase Chagas´ disease. CHHP isolate was obtained from a specimen of *Panstrongylus geniculatus* captured in Chichiriviche de la Costa town. Barinas strain is a reference strain isolated from an acute case of Chagas´disease in Barinas state and recorded in the WHO strains bank as M/HOM/VE/92/YBM. All isolates were propagated by inoculating weanling NMRI mice with blood obtained from patients or with vectors´ dejection; they were maintained in cycles of mouse-vector-mouse passages. *Rhodnius prolixus* three stage nymphs were used as a vector.

Experimental mice were inoculated with 1000 bloodstream trypomastigotes/g via intraperitoneal (ip) and parasitemia tested after three weeks of inoculums application. Mice were maintained in stainless steel cage (30×20×13.5 cm; 10 animals/cage), with free access to water and food (Ratarina ®, Protinal, Venezuela), light-dark cycles of 12 hours each and temperature between 24 and 28°C.

Electrocardiographic protocol

12-lead resting electrocardiograms were done in human patients during the time of hospitalizations before treatment. Mice were anesthetized with 40 mg/kg weight of sodium pentobarbital administered via ip. The electrocardiographic recordings were performed under a bipolar configuration, where all electrodes were placed in the subcutaneous tissue: the positive on the xiphoid process, the negative on the right shoulder joint and the reference on the left shoulder joint. Each electrode was connected to a BioAmp Amplifier (ADInstruments) and analog signals were converted to digital signals by Powerlab/8sp interface (ADInstruments) connected

to a personal computer using Chart v4.2.1 software (ADInstruments); signal uptake was performed at 1000 events/s frequency and filtered at 60 Hz.

Extraction of DNA and polymerase chain reaction (PCR) conditions

Five milliliters of blood were mixed with an equal volume of 6 M guanidine hydrochloride and 200 mM EDTA, pH 8. DNA purification was performed using the kit for blood samples (Axyprep ™ Blood Genomic DNA Miniprep kit, Axygen Bioscience, California, USA). DNA integrity was evaluated through agarose gel electrophoresis and quantified spectrophotometrically. 500 ng of DNA resuspended in Green GoTaq® Flexi Buffer (Promega) was PCR amplified using *T. cruzi* specific minicircle primers (121: 5'-AAATAATGTACGGGKGAGATGCATGA - 3' and 122: 5'- GGTTCGATTGGGGTTGGTGT AATATA - 3') (16). The reaction mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.0 mM MgCl2, 250 μM dNTPs Mix (Promega), 4 μM of each oligonucleotide primer, and 0.6 units of GoTaq® Flexi DNA Polymerase (Promega) in a final volume of 25 μL. PCR was conducted in a thermal cycler Mastercycler gradient Eppendorf, using five cycles at 94°C for one minute, 68°C for one minutes and 72°C for one minute, 35 cycles at 94°C for forty-five seconds, 64°C for forty-five seconds and 72°C forty-five seconds, followed by one extension step at 72°C for 10 minutes. The positive control was done with DNA extracted from *T. cruzi* and negative control DNA extracted from confirmed (without epidemiological and clinical history of Chagas´ disease, non evidence of another disease and seronegative to *T. cruzi* antigens) nonchagasic healthy individuals.

Serology

Specific IgM antibodies to *T. cruzi* were measured by an enzyme linked immunosorbent assay (ELISA), using commercial kits CruziELISA produced by Diagen (Merida, Venezuela). These were performed in accordance with manufacturer's instructions.

***T. cruzi* excreted secreted antigens (TESA):** TESA proteins were obtained from supernatant of *T. cruzi* infected Vero cells. When Vero monolayer cells cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum achieved 60% confluence were infected with 1×10^7 parasites /mL and placed in 5% CO_2 atmosphere at 37°C for 4 days. After that, cells were washed 3 times with phosphate saline buffer pH 7.2; DMEM without FBS were then added and incubated again in the same conditions until trypomastigotes release. In this moment the culture medium was collected and centrifuged at 1500 × g for 15 min, the supernatant was passed through a 0.22 mm membrane filter and a protease inhibitor cocktail (Sigma Chemical Company, USA) was added. The supernatant containing the antigenic proteins was stored at –80°C until use. TESA proteins were concentrated by precipitation with 8% trichloroacetic acid/ 1.25% sodium deoxycholate. Proteins were quantitatively assayed by Lowry's method as modified by Schachterle and Pollack (17) with bovine serum albumin as standard.

SDS-PAGE and Western blotting

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (18). For Western blotting experiments, TESA proteins were transferred to Polyvinylidene difluoride (PVDF) membrane (Thermo Scientific, U) as described elsewhere (19). The membrane was blocked with PBS containing 5% casein and incubated with serums from acute or chronic chagasic patients diluted 1:200 for 1h at room temperature. After three washings with PBS, the membrane was incubated

with goat anti-human IgM or IgG conjugated with peroxidase diluted 1:4000 and revealed by adding diaminobenzidine and H_2O_2.

T. cruzi genotyping

After growing the *T. cruzi* isolates in supplemented RPMI 1640 medium as describe by Miles (20) and Carrasco *et al.* (21), the parasites were harvested by centrifuging 20×10^6 cells at 4°C, 2500 g, during 5 min. DNA was extracted using the Nucleon BACC2 DNA extraction Kit (Amersham Life Science) following the instructions of the manufacturer. DTU of the parasites was obtained by the Random Amplified Polymorphic DNA (RAPD) technique as in Carrasco *et al.* (21). PCR reactions for RAPD typing were achieved using primers A1 and A2. Each reaction was conducted in a 20 μL final volume containing 10 mM Tris HCl (pH 8.8) buffer, 0.2 mM each dNTP, 20 pg of primer, 1.0 unit of Taq DNA polymerase (Invitrogen, Brazil) and included 5 ng of whole genomic DNA. Reaction conditions were as follows: two cycles at 95°C for 5 min, 30°C for 2 min and 72°C for 1 min, 32 cycles at 95°C for 1 min, 40°C for 2 min, and 72°C for 1 min, and a final extension cycle at 72°C for 5 min.

Data analysis

All values are expressed as mean ± standard error (SEM). Since the variances between the analyzed animals groups was significantly different (Bartlett's test, $P < 0.05$), the statistical significance of the observed difference between the values of the control group compared to the values of the experimental group was determined using the Kruskal-Wallis test followed by Dunns post test; p value less than 0.05 was considered as significant. Calculations were performed using GraphPad Prism 4 (Graph Pad Software Inc, La Jolla, California).

Ethics

The study protocol was approved by the Ethics Committee at the School of Health Sciences, "Lisandro Alvarado" University, Barquisimeto, State of Lara, Venezuela, in accordance with the Helsinki Declaration of 1964, as revised in 1975, 1983, 1989, 1996, and 2000. Data were collected after the participants signed the informed consent. The animals used in this study were manipulated in compliance with APS guiding principles concerning the care and use of laboratory animals, published by the US National Institute of Health and following the experimental animal handling protocol of the Ministry of the Popular Power for Science and Technology (Venezuela).

RESULTS

Clinical findings

Hospitalization time of the patient ranged between 2 and 17 days, with an average about 13 days. The clinical symptoms more frequently observed amongst patients were fever and abdominal pain followed by headache and facial edema. The main findings were evidenced by physical examination, chest radiography and echocardiography: lymphadenopathy, hepatomegaly, splenomegaly, cardiomegaly, pericardial effusion in almost all patients and pleural effusion in a lesser degree. Increased heart silhouette was mainly due to pericardial effusion, because the volumes of cardiac chambers were normal in most patients. All patients received oral treatment with Benznidazole 5-10 mg/Kg in two divided doses with meals, during 60 days. No mortality was observed among patients included in this study. Electrocardiographic records were obtained from 8 patients in the acute phase of infection. Clear evidences of EKG disturbances were ventricular repolarization disorders, represented by

specific alterations of T wave morphology, elevation or depression of ST segment and J point elevation. Also we observed first degree AV block, incomplete right bundle branch block, sinus and supraventricular tachycardia, premature ventricular complex, sinus bradycardia, ventricular and right atrial enlargement (see Table I and Fig. 2).

All patients had genomic *T. cruzi* DNA in their bloods as demonstrated by PCR technique (Fig. 1).

For all studied patients, specific IgM antibodies to *T. cruzi* were detected by ELISA, also positive PCR amplification products with primers that annealed to *T. cruzi* kDNA were demonstrated. Fig. 1 shows PCR amplification in four patient samples.

Animal model

Because in all mice, T wave decay has two components: fast and slow, being the fast component more reliable, we decided to measured repolarization disturbances in *T. cruzi* infected mice using the following parameters: T wave maximum amplitude (TMA) measure from Q wave to the maximum peak amplitude of T wave, QT1 amplitude and QT1 length both measured from Q wave to the end of the first component of T wave decay.

TMA values in *T. cruzi*-infected groups revealed a significant augment (Fig. 3 panel A) in all isolates, recording the highest variable average in p16, Guarico and Chabasquén isolates, whose differences with the control group reached the highest statistical significance (Table II). Also, the values of QT1 amplitude in mice inoculated with the parasite proved to be much higher than in healthy mice, however a statistical significance were reached by mice inoculated with p13, p14, p11, p16, p6 and CHHP. Likewise, QT1 length proved to be an indicator of repolarization abnormalities in infected mice, displaying a marked increase, a statistical significance were reached by mice inoculated with: p11, p16, YBM and CHHP (Table II). In Fig. 3 panel B we can observe qualitative repolarization disturbances as peaked and prolonged T waves, where can be noticed that T wave remain elevated for a long period. In Fig. 4 a histopathological section of heart from acute chagasic mouse is shown, where a

TABLE I
ELECTROCARDIOGRAPHIC CHARACTERISTICS OF HUMAN PATIENTS WITH ACUTE CHAGAS´ DISEASE

Patient	Sinus Rhythm	Heart Rate	PR segment	QRS segment	QT segment	Axis (°)	Conduction Disturbances	Rhythm Disturbances	Repolarization Disorders	Hypertrophy
1	Present	<60-100	0.16	0.08	0.36	30	IRBB	B/VE	Present	Absent
2	Absent	125	-	0.08	0.34	90	Absent	SVT	Present	Absent
3	Present	88	0.22	0.08	0.36	60	IDAVB	Absent	Present	Absent
4	Present	107	0.2	0.08	0.32	50	IDAVB	ST	Present	Absent
5	Present	68	0.16	0.08	0.32	30	Absent	Absent	Present	RAH
6	Present	65-93	0.16	0.08	0.4	60	Absent	Absent	Present	LVH
7	Present	107	0.12	0.08	0.32	60	Absent	Absent	Present	LVH
8	Present	107	0.14	0.08	0.36	0	Absent	Absent	Present	Absent

IRBB: Incomplete Right Bundle Block; IDAVB: First Degree Atrio-Ventricular Block; B: Bradycardia; VE: Ventricular Extrasystoles; SVT: Supraventicular Tachycardia; ST: Sinus Tachycardia; RAH: Right Atrium Hypertrophy; LVH: Left Ventricular Hypertrophy.

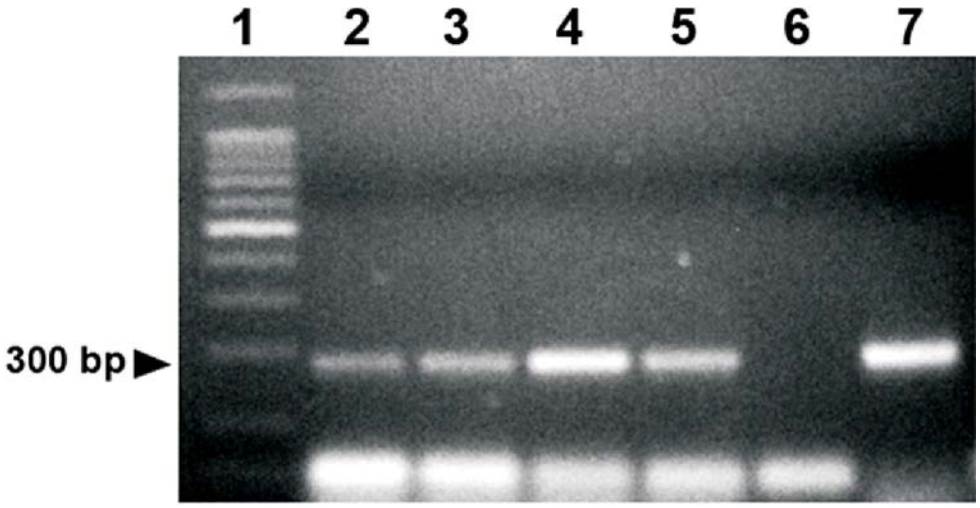

Fig. 1. Representative results of polymerase chain reaction (PCR) amplification of variable regions of the *T. cruzi* minicircle molecule from blood samples. The 330-basepair (bp) band is the expected *T. cruzi* specific product. Molecular weight markers (100-bp ladder) are shown in lanes 1; lanes 2, 3, 4 contain positive samples from patients with acute Chagas´disease; lane 5 contains a positive control from a confirmed chronic chagasic human patient; lane 6 contain sample from seronegative control and Line 7 contain DNA sample isolated from *T. cruzi*.

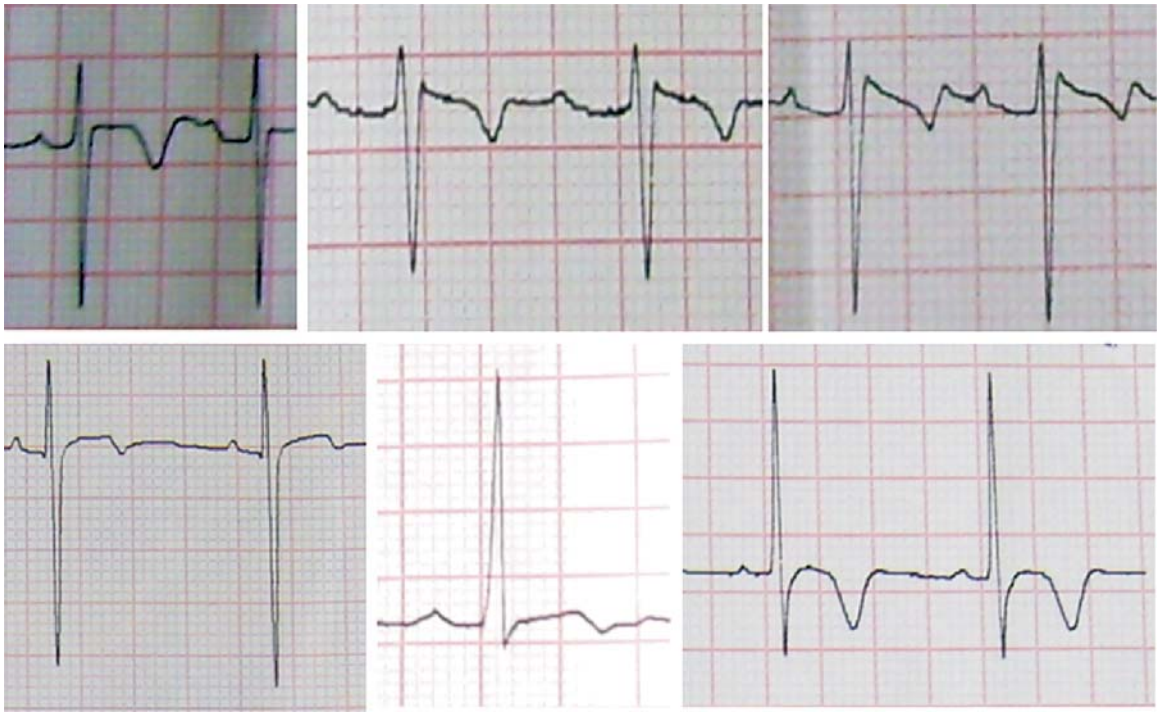

Fig. 2. Electrocardiographic traces obtained from patients with acute Chagas´ disease. Electrocardiographic were obtained using standard equipment, parameters and paper. The six traces came from six different patients and represents precordial derivations upper than V2. Observe that all patients displayed repolarization disturbances represented as negative or bimodal T wave, elevation of the QT segment and J point elevation.

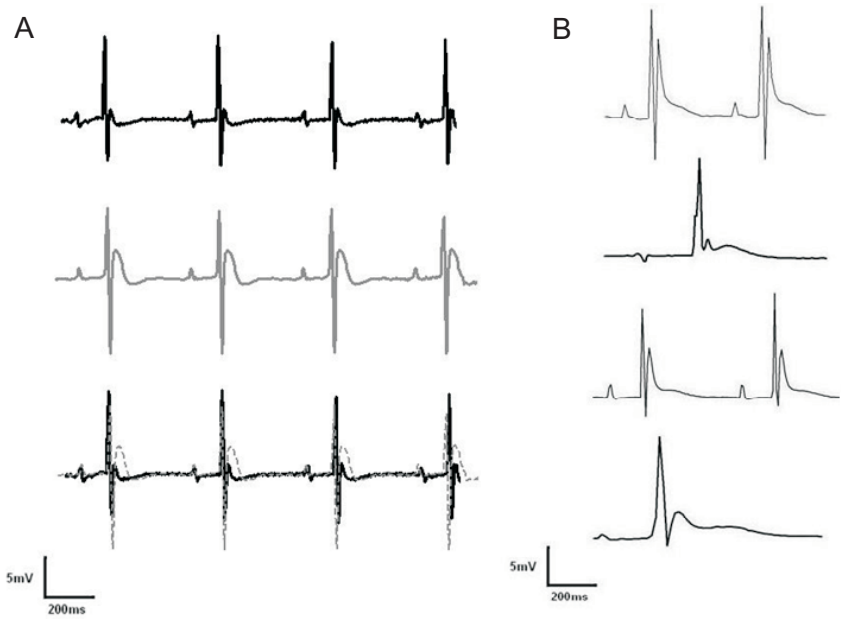

Fig. 3. Electrocardiographic traces obtained from mice infected with strains isolated from patients with acute Chagas´ disease. In A is shown electrocardiographic traces obtained from a control mouse (upper trace) and infected mouse (middle trace), where a repolarization disorder represented as an increase in the T wave amplitude and length are displayed (see in A the lower trace, where both traces are superimposed). In B electrocardiographic abnormalities of repolarization manifested as alterations in the T wave morphology are demonstrated; note that T wave in all cases are bimodal with a fast higher first component and slow second component when depolarization is maintained for a long period (compare these traces with the upper figure at panel A).

TABLE II
T WAVE AND QT SEGMENT ELECTROCARDIOGRAPHIC CHARACTERISTICS IN MICE INFECTED WITH DIFFERENT *Trypanosoma cruzi* STRAINS

Strain´s Groups	T wave Maximun Amplitude (μV)	QT1 segment amplitude (μV)	QT1 segment length (ms)
Control	200,7 ± 22,65	40,35 ± 5,21	18,35 ± 0,47
Guarico	390,5 ± 56,34*	72,77 ± 12,81	24,64 ± 1,18
Chabasquén	372,3 ± 29,39*	61,45 ± 7,6	26,68 ± 1,26
P13	344,8 ± 29,35*	71,64 ± 8,04	21,96 ± 0,64
P14	310,9 ± 31,84	84,70 ± 6,43*	21,95 ± 1,24
P11	336 ± 21,21*	103,6 ± 6,49*	22,5 ± 0,54*
P16	526,5 ± 45,41*	129,3 ± 13,21*	28,83 ± 1,95*
P6	372 ± 32,38*	82,50 ± 11,93	21,82 ± 0,84
Barinas	292,6 ± 31,69	52,69 ± 8,25	29,19 ± 1,97*
CHHP	350 ± 28,63*	67,64 ± 9,78	24,68 ± 0,92*

Control group was not infected with *T. cruzi;* Data presented are $\bar{x}$ ± SEM; *means $p < 0.05$ analized by Kruskal-Wallis followed by Dunns post hoc against control group.

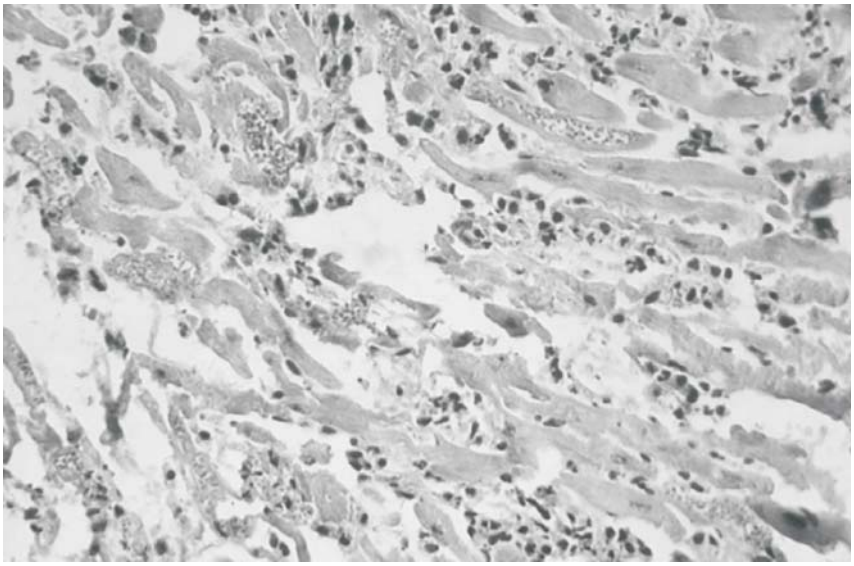

Fig. 4. Histopathology of mouse cardiac tissue. Cardiac tissue samples from mice infected with *T. cruzi* strains isolated from patients with acute Chagas´ disease were fixed in formalin, embedded in paraffin wax, cut in 200 μm pieces and stained by hematoxylin-eosin. Observe an intense mononuclear infiltrate, cardiomyocyte degeneration and many *T. cruzi* amastigotes into complete or broken nests.

classical picture of *T. cruzi* nests, mononuclear inflammatory infiltrate, interstitial edema and myofibrillar lesions compatible with myocarditis can be seen.

In Fig. 5 five TESA profile is presented. In panels A and B antigenic proteins were disclosed using serum from acute Chagas´disease patients and revealed by anti-IgM (panel A) or anti-IgG (panel B) secondary antibodies. Observe, that antigenic protein profile are different for P11, P14, CHHP and P6 when compared each other, either when anti-IgM or anti-IgG secondary antibodies were used; this difference is even more evident for each strain when the profile revealed by anti-IgM is compared with the profile revealed by anti-IgG. Also, notice that although P13 antigens were not unveiled by acute serum samples, but by serum samples from patients with chronic Chagas´disease. On the other hand, serums from chronic chagasic patients tend to give a similar pattern for all strains (panel C, D and E), independent whether patients are in I, II o III clinical phase of the disease.

Fig. 6 shows the RAPD profile generates with five different *T. cruzi* isolates obtained from acute Chagas´ disease human cases from the oral outbreak in Chichiriviche de la Costa (Fig. 6, lines 1 to 5). Bands´ patterns reveal that all isolates have the same profile as the TcI DTU reference strain (Fig. 6, line 8). Likewise, two isolates from *P. geniculatus* recollected in Lara and Portuguesa states (Fig. 6, lines 6 and 7), also shows RAPD profiles that correspond to TcI genotype when compared with the TcI reference strain (Fig. 6, line 8). It is important to notice the size and number of bands variation in the range of 0.8 to 1.5 kb between the isolates.

DISCUSSION

Ventricular repolarization disorders have not received enough attention as basic sign for the diagnosis of acute Chagas disease, although there are sufficient data in the literature to support its value. Laranja *et al.* (12) were the first to describe

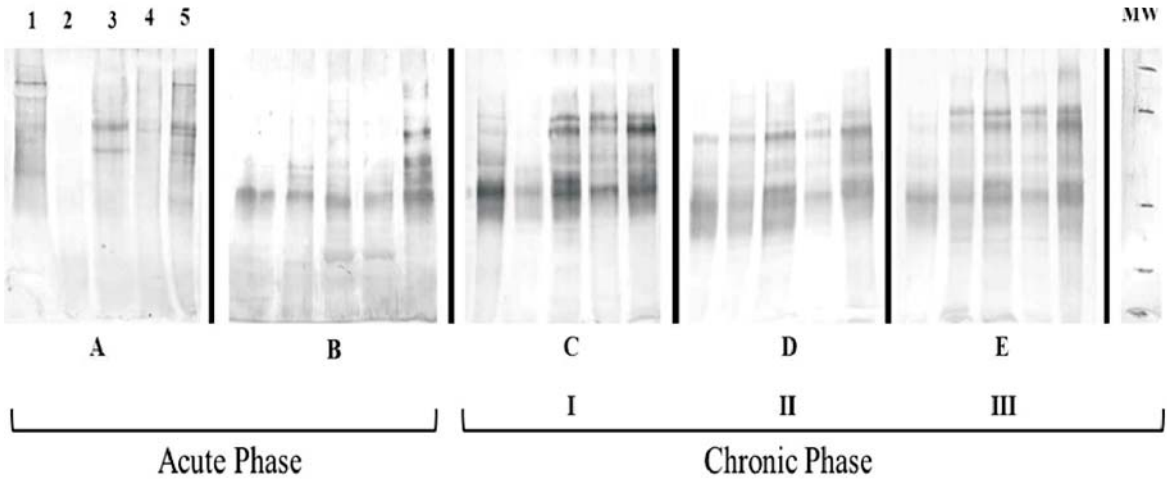

Fig. 5. Immunoblot of TESA antigens against serum samples from acute and chronic chagasic patients. TESA antigens were obtained from P11 (line 1), P13 (line 2), P14 (line 3), CHHP (line 4) and P6 (line 5) *T. cruzi* isolates cultured in Vero cells. Antigenic proteins were detected by serum from acute chagasic patients (panels A and B) or from chronic chagasic patients in different phase of the disease (panel C, D and E for I, II and III phases, respectively). Anti-IgM (panel A) or anti-IgG (panels B, C, D and E) were used as secondary antibody. At the right are shown highlighted pre-stained molecular weight markers from pierce.

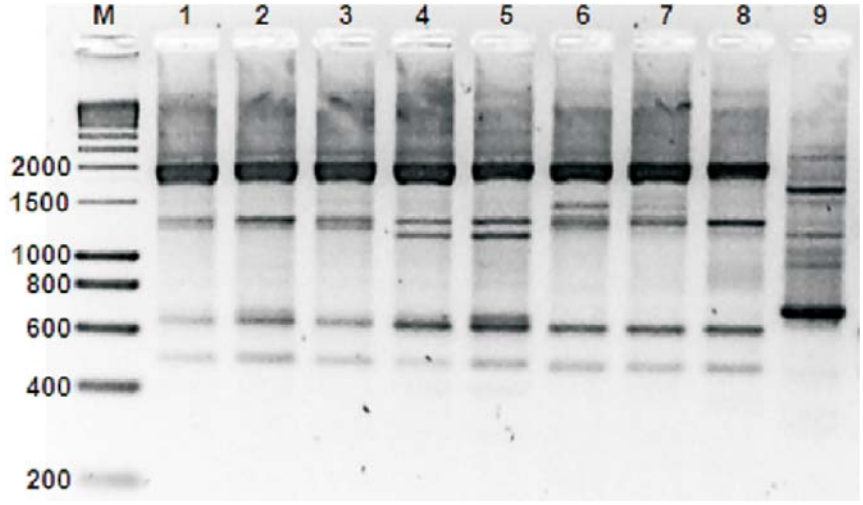

Fig. 6. RAPD profile of *T. cruzi* isolates from human and Triatomine bugs. Primer A2. Lines: M = Molecular *Marker Hyper Ladder* I; 1= P6; 2= P11; 3= P13; 4= P14, 5= P16; 6= Guárico; 7= Chabasquén; 8= TcI (WA250 cl10B, Reference Strain); 9= TcIV (CanII, Reference Strain).

repolarization disorders in patients in this phase, finding that 19.4, 7.2 and 4% had T wave changes, prolonged QT and QT interval changes, respectively. Pinto-Dias (13) reviewed 369 cases of acute Chagas disease in Minas Gerais (Brazil), between 1940 and 1969, finding that 43.3% of patients had electrocardiographic abnormalities, 19.4% had abnormal T-wave, 7.2% had a prolonged QT interval and 4.4% had ST segment changes. Das Neves *et al.* (22) studied 188 patients diagnosed with acute Chagas dis-

ease, between 1988 and 2005, of which 96 (51.1%) had electrocardiographic abnormalities, 40 (41.66%) of the later cases showed ventricular repolarization abnormalities and 2 (2.08%) left ventricular overload. Bastos *et al.* (7) found ventricular repolarization disorders in all patients (n = 12), while Barbosa-Ferreira *et al.* (23) observed no ventricular repolarization disorder in 5 patients.

In Venezuela, in acute Chagas´ disease outbreaks related to oral transmission, repolarization abnormalities are frequent among patients with EKG disturbances (24). Alarcon *et al.* (10) reported that 59% of the patients had at least one EKG abnormalities, 33% had ST segment changes, 39% had T wave changes significantly associated with age under 19 years old and 1.94% had a prolonged QTc. In outbreaks related with vectorial transmission, Ochoa *et al.* (25) reported a 60% of changes on ST segment and T wave, which return to normality after benznidazol treatment, with the exception of 1 patient. Paradas *et al.* (26) found only 7% of repolarization abnormalities in patients with vectorial acute Chagas´ disease.

In the present paper the entire human patients had impaired ventricular repolarization and the strains isolated from these patients were able to induce similar disorders in NMRI infected mice, confirming that impaired ventricular repolarization is a hallmark sign of acute Chagas´ disease.

Because outbreaks are unexpected phenomena, the etiological early diagnosis determines the rates of mortality and disability. In the case of Chagas´ disease, the diagnosis is difficult, since transmission of the infection has been successfully reduced in endemic countries; therefore medical training is not sufficient, due to lack of cases in a daily medicine practice to discuss clinical diagnosis. Moreover, acute Chagas´ disease clinically manifests as a febrile non-specific infectious syndrome, similar to diseases with high incidence in Chagas´ disease endemic areas, for example dengue, influenza and mononucleosis, among others.

Recently in our country, in the north-central region there have been several outbreaks of acute Chagas disease, specifically in Chacao (Miranda State), Antímano (Capital District) and Chichiriviche de la Costa (Vargas State) between 2006 and 2010 (8-10). All of these outbreaks were surprising, because the areas affected were residential areas of the capital city Caracas or coastal towns, where triatomine infestation and Chagas´ disease prevalence were considered negligible; as a consequence, etiologic diagnosis was understandably delayed.

For that reason, in an epidemiological context, a clinical and/or paraclinical tests suggestive of Chagas´ disease is required, which could allow an accurate diagnosis *in situ*. Undoubtedly, microscopic observation of the parasite in blood samples and the presence of anti-*T. cruzi* IgM antibodies are essential elements; but the observation of the parasite has low sensitivity related to the observer expertise, while serological diagnosis requires specific and sensitive antigen to recognize IgM anti-*T. cruzi* antibodies. We evaluated the presence of IgM anti-*T. cruzi* in the sera using the kit CruziELISA carrying the recombinant antigen SAPA, however, in situ serological diagnosis of Chagas disease in the acute phase require dipstick technology, which is not yet available in Venezuelan public health systems. Thus disorders of ventricular repolarization, in an integral framework of clinical and epidemiological data, could be considered an electrocardiographic sign that might strongly guide to chagasic etiology.

The electrocardiographic signs of repolarization disorders are related to ischemia and ventricular overload. The

term "ischemia" in an electrophysiopathological sense, refers specifically to a disorder of cell repolarization. Ischemia is represented by the alteration of T wave, QT interval and ST segment (Figs. 2 and 3). Ischemic T waves are mainly due to a delay or a change in the direction of repolarization charges in the myocardium due to anoxia, it could be abnormally high or peaked, or on the contrary deeply inverted, as well QT segments associated with ischemic T waves are usually prolonged (27).

As mentioned above, another acute ischemia characteristics feature is a deviation of the ST segment, following the occurrence of so-called injury current, which is caused by current flow between normal and ischemic zone areas. These currents are manifested in the electrocardiogram as a ST segment elevation or depression, as consequence of subepicardial and subendocardial ischemia, respectively (27, 28) In subendocardial ischemia, there is a delay in subendocardial cardiac cells repolarization; in consequence repolarization proceeds as a normal condition, from the epicardium to the endocardium, but is delayed in the ischemic subendocardial area causing a prolonged QT interval and a positive symmetrical, high and pointed T wave (28-32). According to the results presented here, mice predominantly display subendocardial ischemia, because T wave had peak amplitude, area and length higher than control healthy animals.

On the other hand, in subepicardial ischemia there is a delay of cardiac subepicardial cells repolarization, as result, repolarization begins at the endocardium and moves into the opposite direction from the endocardium to the epicardium, decelerating upon reaching subepicardical ischemic area, this causes a prolonged QT interval and negative, symmetrical and deep T-wave (28-32). According to our results, human patients predominantly display subepicardial ischemia, because T wave were deep, negative and symmetrical.

In patients with Chagas´ disease different theories that explain cardiac lesions caused by *T. cruzi* infection have been described, one of these called microvascular theory, explains the myocardial damage dependent on coronary microcirculation. This is determined by an increase platelet activity, which may contribute to thrombosis in the coronary microvasculature, compromising vascular perfusion. This phenomenon does not occur in specific areas of the coronary vascular tree, but equally affects all the endothelial cells lining the heart microcirculation, triggering diffuse ischemic disorders that compromise the entire myocardial tissue (15).

The histopathological findings shown in Fig. 4 are similar to those observed in a post-mortem study of an adult female patient from the same outbreak, where it was observed an important microvasculature commitment, arterioles showed wall edema, endothelial hypertrophy and wall permeation of inflammatory lymphomononuclear cells (33). Microvascular compromise with their corresponding ischemic sequela, suggest an early immunologic inflammatory mechanism that together with a parasite direct effect could explain pathogenic events of the disease.

In addition to the T-wave changes as a consequence of ischemia product, the characteristics of heart muscle tissue per se also determine the different patterns of myocardial response to depolarizing currents, and represent an important element in the generation of electrocardiographic repolarization abnormalities. Indeed, it is shown that ventricular myocardium is homogeneous from the histological point of view; but not from the electrophysiological perspective. This difference is mainly explained by changes in the morphology and

duration of action potential at the three cell types that build up myocardial tissue: endocardial, epicardial and M cells. The latter is characterized by longer action potentials as compared to the formers, which are shorter and intermediate, respectively. These results in voltage gradients that give a T wave specific characteristics in different conditions. Ischemia causes time-dependent effects on the electrical properties of these three cell types, slowing action potential with the subsequent increase in T wave duration. The occurrence of these disorders on M cells is the reason for the prolongation of the QT interval, since these cells develop more prolonged action potentials and determine the duration of this interval, specially related to T wave decay phase (31, 34). In the present paper the second component of T wave consistently observed in chagasic mice could be related to pathological disturbances in M cells that remain depolarized for long period (Fig. 3).

As mentioned before, another possible cause of disorders related to repolarization is represented by ventricular overloading phenomena, referred as an increase in pressure and /or volume in the heart chamber, which causes growth and dilation. These phenomena of ventricular overload experienced by acute *T. cruzi* infected patients are mainly consequence of cardiac chambers hemodynamic changes as result of parasite-mediates myocardial cells inflammation, this causes a reduction of myocardial contractility and decrease in cardiac ejection fraction. This ventricular dysfunction leads to increased ventricular residual volume after systole, leading to an increase in the end diastolic volume, which finally causes ventricular volume overload (35).

Ventricular overload is electrocardiographically characterized by ST segment alterations, which usually elevate or undulate in the middle part; this overload is often accompanied by ventricular hypertrophy, since a ventricle fighting against resistance hypertrophies in an attempt to redress. ST segment depression and the T-wave inversion together, constitute a ventricular overload characteristic pattern (28) (Fig. 2).

Results shown in Fig. 6, clearly show that the *T. cruzi* isolates obtained from infected patients, belong to the TcI genotype as well as the two isolates obtained from insect vectors. When compared all *T. cruzi* isolates from humans and triatomine bugs (Fig. 6), amplified bands variation in the range of 0.8 to 1.5 kb, reveal genomic polymorphism between the parasites as demonstrated by Carrasco *et al.* (21). Both TESA protein and genetic profile were heterogeneous; while profile related to electrocardiographic repolarization disturbances tended to be homogeneous, indicating that repolarization disorders is a sign that is independent of the strain subtype able to infect individuals.

In chronic chagasic patients, it has been reported that electrocardiographic repolarization parameters are markers of left ventricular systolic dysfunction and predictors for mortality in patients with Chagas' disease (36, 37). Likewise, repolarization variability, evaluated by beat-to-beat T-wave amplitude variability is independently related to the risk of death (38). Since all of our patients were hospitalized due to homeostatic disturbances that threatened their life, and all had impaired ventricular repolarization, we could then suggest that repolarization disorders could be associated with disease severity, assumption that could sustained by the severe microvascular changes observed in histopathological studies (33).

Ventricular repolarization rhythm disorders are more frequently in patients whose sera have muscarinic acetylcholine antibodies with agonist activity; in these patients, ventricular repolarization heterogeneity is increased significantly and maxi-

mum corrected QT intervals is an independent predictors of cardiac death (39).

Finally, repolarization EKG disturbances are characteristics sign of acute chagasic myocarditis that would allow early diagnosis and treatment, reducing mortality and disability. Since acute Chagas disease affects mainly children, who often do not develop cardiac ischemic disorders during non-chagasic febrile infectious diseases, the finding of impaired myocardial repolarization could be a sign that addresses the diagnosis of acute Chagas disease. Consequently, general physicians serving in primary levels of health care should be trained to detect electrocardiographic signs of myocarditis and ischemia, and interpret them in an appropriate clinical and epidemiological context of Chagas disease.

ACKNOWLEDGMENT

Study funded by National Fund for Science and Technology (FONACIT) under the Ministry of Popular Power for Science and Technology (Venezuela), Project No 2007001425. The molecular analysis was done under support of the Project FONACIT N° G-2005000827.

REFERENCES

1. **Armaganijan L, Morillo CA.** Chagas disease: 101 years of solitude! Time for action. Stroke 2010; 41: 2453-2454.
2. **WHO.** Chagas disease (American trypanosomiasis) fact sheet (revised in June 2010). Wkly Epidemiol Rec 2010; 85:334-36.
3. **WHO.** Chagas disease: control and elimination. Report of the Secretariat. EXECUTIVE BOARD 124th Session 27 November 2008 Document EB124/17. http://apps.who.int/gb/ebwha/pdf_files/EB124/B124_17-en.pdf (accessed on 21/May/2011).
4. **OPS.** Estimación cuantitativa de la enfermedad de Chagas en las Américas. Montevideo, Uruguay: Organización Panamericana de la Salud; 2006. OP5/HDM/CD/425-0G. http://www.bvsops.org.uy/pdf/chagas19.pdf (accessed on 21/May/2011).
5. **Rojas ME, Várquez P, Villarreal MF, Velandia C, Vergara L, Morán-Borges YH, Ontiveros J, Yelitza Calderón M, Chiurillo-Siervo MA, Rodríguez-Bonfante C del C, Aldana E, Concepción JL, Bonfante-Cabarcas RA.** An entomological and seroepidemiological study of Chagas' disease in an area in central-western Venezuela infested with Triatoma maculata (Erichson 1848). Cad Saude Publica 2008, 24:2323-2333.
6. **Bonfante-Cabarcas R, Rodríguez-Bonfante C, Vielma BO, García D, Saldivia AM, Aldana E, Curvelo JL**. Seroprevalence for Trypanosoma cruzi infection and associated factors in an endemic area of Venezuela. Cad Saude Publica 2011; 27:1917-1929.
7. **Bastos CJ, Aras R, Mota G, Reis F, Dias JP, de Jesus RS, Freire MS, de Araújo EG, Prazeres J, Grassi MF.** Clinical outcomes of thirteen patients with acute chagas disease acquired through oral transmission from two urban outbreaks in northeastern Brazil. PLoS Negl Trop Dis 2010; 4(6):e711.
8. **Toso M A, Vial UF, Galanti N.** Oral transmission of Chagas' disease. Rev Med Chil 2011; 139:258-266
9. **Alarcón de Noya B, Martínez J.** Transmisión oral de la enfermedad de Chagas en Venezuela: un segundo brote escolar. Salus on line 2009; 13: 9-10.
10. **Alarcón de Noya B, Díaz-Bello Z, Colmenares C, Ruiz-Guevara R, Mauriello L, Zavala-Jaspe R, Suarez JA, Abate T, Naranjo L, Paiva M, Rivas L, Castro J, Márques J, Mendoza I, Acquatella H, Torres J, Noya O.** Large urban outbreak of orally acquired acute Chagas disease at a school in Caracas, Venezuela. J Infect Dis 2010; 201:1308-1315.
11. **Pinto AY, Valente SA, Valente Vda C, Ferreira Junior AG, Coura JR.** Acute phase of Chagas disease in the Brazilian Amazon region: study of 233 cases from

Pará, Amapá and Maranhão observed between 1988 and 2005. Rev Soc Bras Med Trop 2008; 41:602-614.

12. **Laranja FS, Dias E, Nobrega G, Miranda A.** Chagas' disease; a clinical, epidemiologic, and pathologic study. Circulation 1956; 14:1035-1060.
13. **Pinto-Dias JC.** Revisão geral e evolução imediata de casos agudos de doença de Chagas estudados no Posto Avançado Emmanuel Dias (Bambuí, MG, Brasil) entre 1940 e 1969. Rev Med Minas Gerais 2009; 19: 325-335.
14. **Regueiro A, García-Álvarez A, Sitges M, Ortiz-Pérez JT, De Caralt MT, Pinazo MJ, Posada E, Heras M, Gascón J, Sanz G.** Myocardial involvement in Chagas disease: Insights from cardiac magnetic resonance. Int J Cardiol. 2011, Sep 8 [Epub ahead of print].
15. **Rossi MA, Tanowitz HB, Malvestio LM, Celes MR, Campos EC, Blefari V, Prado CM.** Coronary microvascular disease in chronic Chagas cardiomyopathy including an overview on history, pathology, and other proposed pathogenic mechanisms. PLoS Negl Trop Dis 2010, 4(8): e674.
16. **Carvalho CM, Andrade MC, Xavier SS, Mangia RH, Britto CC, Jansen AM, Fernandes O, Lannes-Vieira J, Bonecini-Almeida MG.** Chronic Chagas' disease in rhesus monkeys (Macaca mulatta): evaluation of parasitemia, serology, electrocardiography, echocardiography, and radiology. Am J Trop Med Hyg 2003; 6:683-691.
17. **Schachterle GR, Pollack RL.** A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal. Biochem 1973; 351:654-655.
18. **Laemmli UK.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-685.
19. **Sambrook J, Fritsch EF, Maniatis T.** Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1989.
20. **Miles MA.** Culturing and biological cloning of Trypanosoma cruzi. Methods Mol Biol 1993; 21:15-28.
21. **Carrasco HJ, Frame IA, Valente SA, Miles MA.** Genetic exchange as a possible source of genomic diversity in sylvatic populations of Trypanosoma cruzi. Am J Trop Med Hyg 1996; 54: 418-424.
22. **Das Neves Pinto AY, Gomes Ferreira Jr A, Da Costa Valente V, Saburo Harada G, Da Silva Valente SA.** Urban outbreak of acute Chagas disease in Amazon region of Brazil: four-year follow-up after treatment with benznidazole. Rev Panam Salud Publica 2009; 25: 77-83.
23. **Barbosa-Ferreira JM, Guerra JA, Santana Filho FS, Magalhães BM, Coelho LI, Barbosa MG.** Cardiac involvement in Acute Chagas' Disease cases in the Amazon region. Arq Bras Cardiol 2010; 94: 147-149.
24. **Mendoza I, Marques J.** Una nueva epidemia de arritmias. La enfermedad de Chagas aguda por transmisión oral. Avances Cardiol 2008; 28: 70-72.
25. **Ochoa O, Anselmi G, Machado I, Febres C, Villalobos L, Gontran E, Gomez JR.** Acute Chagas myocarditis in children. Diagnosis and current treatment. Acta Pediatr Mex 1995; 16: 187-196.
26. **Parada H, Carrasco HA, Añez N, Fuenmayor C, Inglessis I.** Cardiac involvement is a constant finding in acute Chagas´ disease: a clinical, parasitological and histopathological study. Int J Cardiol 1997, 60:49-54.
27. **De Micheli A, Medrano GA:** En torno al concepto electrofisiopatológico y las manifestaciones electrocardiográficas de isquemia, lesión y necrosis. Arch Inst Cardiol Mex 2009; 79:2-4.
28. **Huszar RJ.** Arritmias. 3ª edición, Ediciones Harcourt, S.A, Madrid, España, Capitulo 3 (p: 34-69), Capítulo 15 (p: 315-340), 2002.
29. **Handjani AM.** Significance of positive, tall and peaked electrocardiographic T waves in early diagnosis of ischemic heart disease. Chest 1972; 62:24-28.
30. **Cowan JC, Hilton CJ, Griffiths CJ, Tansuphaswadikul S, Bourke JP, Murray A, Campbell RW.** Sequence of epicardial repolarization and configuration of the T wave. Br Heart J 1988; 60:424-433.

31. **Yan GX, Antzelevitch C.** Cellular basis for the normal T wave and the electrocardiographic manifestations of the long-QT syndrome. Circulation 1998; 98:1928-1936.
32. **Higuchi T, Nakaya Y.** T wave polarity related to the repolarization process of epicardial and endocardial ventricular surfaces. Am Heart J 1984; 108:290-295.
33. **Suarez J, de Suarez C, Alarcón de Noya B, Espinosa R, Chiurillo MA, Villaroel A, De Martin F, Paiva M, Díaz-Bello Z, Valderrama E, Estrada D, Vivas E.** Enfermedad de Chagas sistémico en fase aguda por transmision oral: diagnostic integral de un caso autopsiado. Gac Med Caracas 2010; 118: 212-222.
34. **Sicouri S, Civetta M, Chiale P, Elizari M.** El papel de la heterogeneidad electrica celular del miocardio ventricular en la génesis de las arritmias cardiácas. Rev Argent Cardiol 2003; 71: 372-379.
35. **Simon MA**. Right ventricular adaptation to pressure overload. Curr Opin Crit Care 2010; 16:237-43.
36. **Salles GF, Cardoso CR, Xavier SS, Sousa AS, Hasslocher-Moreno A.** Electrocardiographic ventricular repolarization parameters in chronic Chagas' disease as predictors of asymptomatic left ventricular systolic dysfunction. Pacing Clin Electrophysiol 2003; 26:1326-1335.
37. **Salles G, Xavier S, Sousa A, Hasslocher-Moreno A, Cardoso C.** Prognostic value of QT interval parameters for mortality risk stratification in Chagas' disease: results of a long-term follow-up study. Circulation 2003; 108:305-312.
38. **Ribeiro AL, Rocha MO, Terranova P, Cesarano M, Nunes MD, Lombardi F.** T-wave amplitude variability and the risk of death in chagas disease. J Cardiovasc Electrophysiol 2011; 22:799-805.
39. **Medei E, Pedrosa RC, Benchimol Barbosa PR, Costa PC, Hernández CC, Chaves EA, Linhares V, Masuda MO, Nascimento JH, Campos de Carvalho AC.** Human antibodies with muscarinic activity modulate ventricular repolarization: basis for electrical disturbance. Int J Cardiol 2007; 115:373-380.