Université de Montréal

Development of an ELISA test using different antigens of *Trypanosoma cruzi* for the diagnosis of Chagas' disease

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Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de Philosophiae Doctor (Ph.D)

en sciences vétérinaires

option microbiologie

Août, 2005

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée :

Development of an ELISA test using different antigens of *Trypanosoma cruzi* for the diagnosis of Chagas' disease

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RÉSUMÉ

Les infections par *Trypanosoma cruzi* sont généralement transmises par contact de la peau avec du matériel fécal de triatomines infectées pendant leur piqûre. Cette transmission a lieu du sud des États-Unis jusqu'à la Pampa argentine. L'infection des humains est connue sous le nom de la maladie de Chagas. La phase amastigote du parasite détruit les muscles cardiaques et de l'intestin et est responsable de cardiomyopathies et de mégasyndromes intestinaux. La maladie se divise en trois phases : aigüe, indéterminée et chronique. Le traitement de la maladie n'est efficace que pour la phase aiguë. Le diagnostic de l'infection se base sur l'analyse microscopique du sang (phase aiguë) ou par des méthodes sérologiques (phases indéterminées et chroniques) mais ces techniques ne sont pas efficaces à 100% et manquent de sensibilité et de spécificité. Le but de notre travail a été de développer une méthode d'EIA plus sensible et spécifique pour le diagnostic de la maladie de Chagas.

Nos premiers tests d'EIA ont utilisé des organismes complets et ont inclus différentes formes du parasite (épimastigotes, trypomastigotes et amastigotes). Trois cent soixante échantillons de sérum ont été testés (70 individus infectés, 114 individus avec des autres infections parasitaires et 176 contrôles non-infectés). Après une période d'optimisation, nous avons obtenu une sensitivité de 100% et une haute spécificité (97.6%

pour les amastigotes, 98.3% pour les épimastigotes et 99.3% pour les trypomastigotes). Le test restait efficace après 4 mois de stockage à 4° C ou à température ambiante (20°C).

Une deuxième classe d'EIA a été développée en utilisant des extraits des antigènes excrétés-secrétés des trypomastigotes de *T. cruzi* appartenant à la souche Brazil et Tulahuen (TESA total). Un panel de 709 échantillons de sérum des individus avec des infections par *T. cruzi* confirmées (n=195), non-infectés (n=400) et des patients avec des autres infections parasitaires (n =114), a été utilisé. L'essai TESA total a montré une excellente sensitivité et spécificité malgré la presence d'une réactivité croisée avec du sérum de patients infectés par *Leishmania.* Les protéines TESA ont été purifiées par chromatographie d'affinité ($TESA_{IA}$) afin d'augmenter la spécificité de l'essai. Les analyses de Western blots ont révélé une série de polypeptides de poids moléculaire de 60-220 kDa, reconnus par un pool de sérum de patients avec la maladie de Chagas. L'EIA avec les antigènes $TESA_{IA}$ présentait une sensitivité un peu diminuée (98.6%) mais une spécificité de 100%, par rapport à l'EIA TESA total. Un polypeptide de 60 kDa a été identifié comme la plus importante bande réagissant avec des sérums de patients infectés par *Leishmania.*

Afin de valider la performance de nos EIAs sur le terrain, un panel de 2038 sérums ont été testés, provenant d'une banque de sang au Venezuela, pays où la maladie de Chagas reste endémique. Les EIAs avec des épimastigotes et trypomastigotes fixés des souches Brazil ou Tulahuen se sont montrés supérieurs à une trousse commerciale utilisée au Venezuela pour le diagnostic de la maladie de Chagas. Une sensibilité de 100% et une

spécificité supérieure à 99% ont été observées. En comparant nos résultats avec ceux du centre national de référence de diagnostic de Chagas, nos essais ont identifié 7 échantillons faux négatifs et discriminé 2 échantillons avec de réactions positives fausses . Malgré l'amélioration pour le diagnostic sérologique de nos essais, nous avons eu de réactions fausses positives. Nous proposons l'utilisation d'une stratégie à deux essais comme alternative jusqu'à l'obtention d'un essai de qualité supérieure.

Mots-clés : Maladie de Chagas, ***Trypanosoma cruzi*,** epimastigote, trypomastigote, amastigote, antigènes sécrétées/ excretées, EIA, validation, banque du sang, immunoaffinité, transfusion.

ABSTRACT

Trypanosoma cruzi infections are usually transmitted by faecal soiling of the mammalian skin during the act of feeding of blood-sucking triatomine bugs. Such transmission occurs from the southern United States to the Argentine Pampas. Chagas' disease is the term used for human infection. The tissue amastigote phase of the parasite destroys cardiac and gut smooth muscles causing cardiomyopathies and gut megasyndromes. The disease is divided into acute, indeterminate and chronic phases. Treatment of the disease is only effective in the acute phase. The diagnosis is based on parasitological methods such as microscopy (acute phase) and serological methods (indeterminate and chronic phase) but none of these techniques is 100% sensitive and specific. The aim of this work was to develop an EIA test with improved sensitivity and specificity for the diagnosis of Chagas' disease.

Our first EIA tests used fixed, whole organisms and included different forms of *Trypanosoma cruzi* (eg: epimastigote, trypomastigote and amastigote). A panel of 360 sera was used (70 infected subjects, 114 subjects with other parasitic diseases, 176 healthy controls). Following optimization, the EIAs using whole fixed parasites showed good sensitivities (100%) and specificities (97.6% amastigote, 98.3% epimastigote, and 99.3% trypomastigote). The trypomastigote-based assay was still effective following 4 months

storage at both 4°C and room temperature (20°C). A second type of EIA assay was developed using concentrated extracts of excretory-secretory antigens from trypomastigotes from the Brazil and the Tulahuen strains (total TESA). A panel of 709 sera from subjects with confirmed Chagas' disease (n=195), healthy controls (n=400), and patients with other parasitic diseases (n=114) was used. The total TESA assays had excellent overall sensitivity (100%) and specificity (> 95%), although some cross-reactivity with sera from patients infected with *Leishmania* were observed. Immunoaffinity chromatography was used to purify TESA proteins ($TESA_{IA}$) and thus increase the specificity. Western blot analysis revealed a series of polypeptide bands with molecular weights ranging from 60-220 kDa recognized by pooled sera from Chagas' disease patients. An EIA based on $TESA_{IA}$ proteins had slightly lower sensitivity (98.6%) but improved specificity (100%) compared to the total TESA EIA. A 60 kDa polypeptide was identified as a major contributor to the cross-reactivity with sera from subjects with leishmaniasis. In order to validate the performance of these tests, we assessed our most-promising candidates in blood banks in an endemic country (Venezuela). Using a panel of 2038 sera, EIAs with fixed-epimastigote, fixed-trypomastigote, and TESA with the Brazil and Tulahuen strains were all superior to the commercial kit currently used in Venezuela, achieving 100% sensitivity and >99% specificity at optimal cut-off values. Based on results from the National Reference Centre for Chagas Inmunodiagnosis, the novel assays identified between 7 false-negative samples and 2 false-positive samples compared with the commercial kit performed under routine conditions. Although the results with our EIAs

were excellent in terms of sensitivity and specificity, problems with false positive reactions persisted. The implementation of a two-test strategy for the screening of Chagas' disease appears to be necessary until a true 'gold-standard' test can be developed.

Keywords : Chagas' disease, *Trypanosoma cruzi*, epimastigote, trypomastigote, amastigote, trypomastigoste excreted/secreted antigens, EIA, validation, blood bank, immunoaffinity, transfusion.

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LIST OF ABREVIATIONS

ADCC	cell-mediated cytotoxicity
APCs	antigen presenting cells
ASEA	alkaline soluble epimastigote antigen
A&T	purified trypomastigote antigen
BHC	benzene hexachloride
BSA	bovine serum albumin.
CEIA	competitive antibody enzyme-linked immunoabsorbent assay.
C-EIA	competitive enzyme-linked immunoabsorbent assay.
CFT	complement fixation test
CM	carboxymethyl
CPI	cysteine protease inhibitors
CRA	cytoplasmatic repetitive antigen
CV	variation coefficient
DCs	dendritic cells
DEAE	diethylaminoethyl
Dig-EIA	difusion-in-gel enzyme linked immunoabsorbent assay
DNA	deoxyribose nucleid acid
DDT	dichloro diphenyl trichloroethanol
EIA	enzyme immunoassays
ELISA	enzyme-linked immunoabsorbent assay
EMEM	Eagle's minimum essential media
EpEx	complex of epimatigote antigen
Fab	antigen binding fraction of antibody
FBS	fetal bovine serum
FCaBP	flagellar calcium-binding protein

FC	crystalysed fraction of antibody
FRA	flagellar repetitive antigen
IHA	indirect hemagglutination
HCH	hexachlorocyclohexane
IFA	indirect immunofluorescence assay
IFN-γ	interferon gamma
IIF	indirect immunofluorescence
IgG	immunoglobulin G
IgM	immunoglobulin M
Mab	monoclonal antibody
MEM	minimum essential medium
NK	natural killer
NLCI	National Laboratory for Chagas Immunodiagnosis
OD	optical density
PBS	phosphate buffer saline
PBST	phosphate buffer saline tween 20
PCR	polymerase chain reaction
PEF	purify *Trypanosoma cruzi* epimastigote
PPV	positive predictive value
QC	quality control
RIPA	radioimmunoprecipitation assay
RT	room temperature
rTc24	24 kDa recombinant protein
SAPA	shed acute-phase antigen
SDS-PAGE	sodium dodecyl sulphate polyacrilamide gel electrophoresis
SQS	quinuclidine based squalene synthase
TESA	trypomastigote excreted/secreted antigens
$TESA_{IA}$	immunoaffinity purified TESA proteins

TS	transialidase
UnSRNPs	small nuclear ribonucleoproteins

A mi esposo: Ricardo, mis hijos : Adriana Elisa, Ricardo Emilio, María Estefanía y a mis padres : Emilio y Olga.

ACKNOWLEDGMENTS

I thank all those people who contributed to the execution of this research project; my gratitude is directed especially to:

Dr. Marcelo Gottschalk, full Professor of the Department of Pathology and Microbiology belong to the Medicine Veterinary Faculty (Université de Montréal), and Dr. Brian Ward, associate Professor of the Immunology and Microbiology Department (McGill University) who supported and guided all these years of study.

Dr. Momar Ndao, director of the National Reference Center of Parasitology who shared his knowledge with me contributing to my development as a researcher, and also for bringing me his friendship.

Dr. José Bubis, for receiving me in his laboratory and providing his support and experience that were very important for the culmination of the last phase of my doctoral studies. My gratitude also to Dr. Bubis' investigation team who gave me their help and friendship.

Dr. Alberto Aché, agreagate professor of the Sciences Veterinary Faculty (Universidad Central de Venezuela) Public Health Department, whose unconditional support and good disposition facilitated the development of all the phases of this research.

Angela Brewer, Natalie Martel and Fabio Vásquez, assistant researchers from Dr. Brian Ward's laboratory, and Sonia Lacouture, assistant researcher, from Dr. Marcelo Gotschalk's laboratory (Université de Montréal) for their technical contribution and friendship.

To the professors of the University Simón Bolívar (Venezuela): Dr. Lilian Spencer, Dr. Karen Noris, Dr Mary Isabel Gonzatti and Dr.Thelma Slezynger for their support and friendship during the last phase of my studies.

To Dr. Edgar Izarra (Public Health Institute, Bolivar State, Minister of Health), Dr. Omaira Gotopo (Blood bank, University Hospital Ruiz and Paez, Universidad de Oriente, Ministry of Health). Dr. Geomar de Valenzuela (Enviroment Health Direction, Minister of Health), Lic. Mehudy Medina (National Laboratory for Chagas Immunodiagnosis, Minister of Health, Maracay, Venezuela, whose interested aid permitted me to carry out this project by facilitating acces to all of the sera used for this project.

To all the personnel of the blood banks in Portuguesa state (University Hospital Dr. Miguel Oraa and University Hospital Dr. Jesús María Casal Ramos) and Bolivar state (Ruiz and Paez Hospital) whose contribution permitted the culmination of the last phase of my studies.

To all my friends of Canada who became my family, their support and friendship helped make the separation from my relatives and country less painful.

To the members of my family who have always supported and encouraged me to begin and accomplish my doctoral studies.

I received a scholarship from the Universidad de Oriente (Venezuela) for the execution of these studies.

I. INTRODUCTION

Chagas' disease is caused by the protozoan flagellate *Trypanosoma cruzi*. It was first described by Carlos Chagas in 1909. The parasite is transmitted to the host by hematophagous insects (genus *Triatoma*). The disease is broadly distributed in the Western hemisphere from the southern United States to the north of Argentina. It is estimated that between 16 to 18 million people are infected with the parasite, and some 120 million (about 25% of the population of Latin America) are at risk of acquiring Chagas' disease.

Chagas' disease is characterized by three different clinical stages: acute, indeterminate and chronic. The acute phase appears mainly in children and the symptoms are typically non-specific resembling a viral infection (fever, myalgias, vomitting, and diarrhea). Rarely, two more specific signs can appear. The Chagoma of inoculation, a swelling around the insect bite, and Romaña's sign, unilateral ocular swelling. Complications of this stage of infection include meningitis, myocarditis and meningoencephalitis. During the indeterminate phase, there are no clinical signs of infection, and the patients have only parasitological and/or serological evidence of disease. Ten to twenty years after initial infection, Chagas' disease patients can develop the chronic phase of the disease, characterized by cardiac (eg: dilated cardiomyopathy, hearth failure) or digestive track complications (eg:mega-esophagus, mega-colon).

There are few therapeutic options for *T. cruzi* infection at the present time. Although two drugs are available (Nifurtimox and Benznidazole), they are only effective during the acute phase of the disease, and both have severe side effects. As a result, public health authorities in endemic regions have focused on vector control programs such as house improvement and insecticide spraying. The transmission of *T. cruzi* by transfusion has been controlled largely by using a range of serological tests (eg: ELISA, immunofluorescence, and indirect hemagglutination).

In the last decades, economic and social problems in Latin America have prompted increased migration of people to developed countries such as Canada, the United States, and Europe, where Chagas' disease is not endemic. As a consequence, Chagas' disease is now regularly reported in these countries, following blood transfusions or organ transplant.

There is no gold standard test to diagnose Chagas' disease. The diagnosis of the infection is currently based on parasitological methods (eg: microscopy, xenodiagnosis, hemoculture, PCR) and serological assays (eg: ELISA, indirect hemagglutination, indirect immunofluorescence, complement fixation). However, no test for blood bank screening is currently licensed in either Canada or the USA. The Pan American Health Organization recommends the use of two different serological tests in parallel to confirm the diagnosis of Chagas' disease and to screen donated blood.

The goal of this doctoral project was to develop, evaluate, and validate an EIA test for the diagnosis of Chagas' disease using different *T. cruzi* antigens. Our hypothesis was that a sufficiently sensitive, specific, practical, and economical ELISA (EIA) test could be developed for use in the blood banks of both endemic and nonendemic countries.

General Aim: To develop an ELISA (EIA test) sensitive and specific enough to detect antibodies against *T. cruzi* in patients during the indeterminate and chronic phases of Chagas' disease.

Specific Objectives:

1. To develop a family of first generation EIA assays using fixed, whole organisms (trypomastigotes, amastigotes and epimastigotes).

2. To develop a family of second generation EIA assays using trypomastigote excreted-secreted antigens (TESA antigens) obtained from of *T. cruzi* trypomastigote cultures.

3. To validate the best of the first and second generation EIA assays in an endemic region.

II. LITERATURE REVIEW

1. Chagas' disease

American trypanosomiasis, or Chagas' disease, is caused by the flagellate protozoan parasite, *Trypanosoma cruzi*, and is transmitted to humans by triatomine insects (185). It is estimated that between 16 and 18 million Latin Americans are infected with this organism (188). Of the chronically infected patients, 27% develop late cardiac lesions, 6% digestive disorders and 3% peripheral neurological lesions. These chronic complications typically develop 10-20 years after initial infection if no treatment is provided (www.who.int/tdr/publications/publications/pdf/pr13/chagas.pdf).

1.1 Historical background

In 1908, Carlos Chagas was commissioned to Lassance (Rio das Velhas Valley, Brazil) to combat malaria. After a year of intense work, he learned of the existence of hematophagus insects (barbeiro) that fed on humans and infested their homes (www.dbbm.fiocruz.br/tropical/chagas/chapter.html). Chagas was intrigued by the possibility that this insect was transmitting a parasite to humans or other vertebrates. He observed a group of patients with an illness, the symptoms of which did not correlate with the pathophysiology of any known disease. Studying the insect, Chagas discovered flagellates in the hindgut. He then exposed a few primates, and found the same flagellates

in their blood (15). Chagas went on to describe the agent, reservoirs, vectors, and clinical signs in human beings and animals. The new human trypanosomiasis transmitted by the barbeiros that he had discovered was appropriately termed Chagas' disease (183).

In 1916, Chagas faced open opposition from the prominent microbiologist, Rudolf Kraus, who during the 1st Pan-American Congress in Buenos Aires denied Carlos Chagas' findings with the argument that he had been incapable of finding cases of Chagas' disease in Chaco, Argentina (183). Almost 40 years later, Mazza in Argentina revived Chagas' disease, describing over a thousand cases in regions included in Kraus' investigations (107). Mazza (1936) was also the first to mention the possibility of transfusion-transmitted Chagas' disease (108), a fact that would be confirmed in 1952 by Freitas *et al.* in blood and blood products (67). In 1953, Nussenzwieg *et al.* proposed gentian violet as a chemoprophylactic agent against transfusion-transmitted Chagas' disease (120). Romaña described the "unilateral schysotrypanosomic conjunctivitis", later designated "Romaña sign" by Chagas and Dias (146). The inoculation chagoma was also first described by Mazza *et al.* in 1940 (112). In the 1960's, Köberle *et al.* showed that infection by *T. cruzi* resulted in the reduction of neurons in the autonomic nervous system (95), and Teixeira *et al.* proposed the role of autoimmunity in the pathogenesis of Chagas (173).

These early controversies in Chagas' disease not limited to the clinic. Laboratory diagnostic efforts began in 1913 with a complement fixation (CFT) test introduced by Guerreiro and Machado (121), and continued in 1914 with the introduction of the xenodiagnosis test (by Joseph Emilie Brumpt) (121). Fife and Muschel described an indirect immunofluorescence test in 1959 (67), and in 1975, Voller applied the first EIA test for the diagnosis of Chagas' disease (182). Between 1980 and 1985, the standardization of serological tests and the criteria for diagnosis of human *T. cruzi* infection were established (121) and in 1986 the first genes for *T. cruzi* were cloned (136).

1.2 *Trypanosoma cruzi*

T. cruzi is a flagellate that belongs to the subkingdom Protozoa, phylum Sarcomastigophora, subphylum Mastigophora, class Zoomoastigophorea, order Kinetoplastida, family Trypanosomatidae, genus *Trypanosoma* and species *cruzi* (116). It is characterized by the presence of flagella, an undulant membrane, and unique mitochondria located inside the kinetoplast (Figure 1), a specialized organelle containing DNA. The parasite has three different stages or forms: amastigote, epimastigote and trypomastigote (23). *T. cruzi* is a heteroxenous and pleomorphic trypanosome, having one phase of its life cycle in a mammalian host and another phase in the insect vector (186).

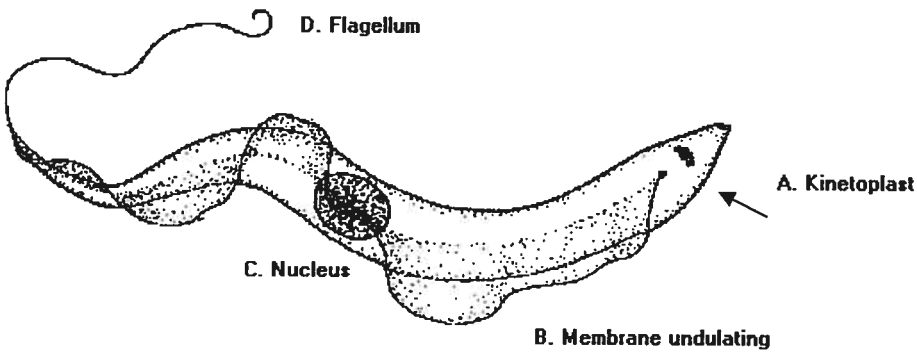

Figure 1. Trypomastigote form of *T. cruzi*.
(http://sciences.aum.edu/bi/bi2033/thomson/trypanosoma.htm)

1.2.1 Developmental stages

The two stages of the parasite in the vertebrate host are trypomastigotes and amastigotes. Trypomastigotes are extracellular (12-20 μm long), nondividing, and transitory. This stage circulates in the blood and invades muscle cells by attaching to specific receptors (60). There are two forms of the trypomastigote, broad and slender. The broad form is shorter and less motile and has a posterior or terminal kinetoplast (Figure 2B), whereas the slender form has an elongated nucleus and a subterminal kinetoplast. The former (broad) has a tendency to stay within a circumscribed area. The latter (slender) usually moves quickly through the microscopic field (186).

Once inside a host cell, the trypomastigotes transform into spheroid amastigotes, 1.5 μm to 4.0 μm wide, with no flagellum (Figure 2A). Amastigotes grow in myocytes and various other tissues including the testis, ovaries, thyroid, adrenal glands, and the central nervous system. The amastigotes tend to cluster themselves forming a nest that is directly or indirectly responsible for the Chagas' disease pathology. When multiplying amastigotes fill the host cell, they differentiate into trypomastigotes and the cell ruptures releasing the parasite back into circulation to invade local tissues or spread hematogenously to distant sites (107, 184).

In the insect vector, trypomastigotes transform into epimastigotes. This stage is also extracellular (6-15 μm in length) and located in the mid-intestine of the insect. Epimastigotes are the stage observed in axenic culture. The epimastigote is a multiplication stage that permits an increase in the population of parasites in the vector's gut or during hemoculture. This form has little mobility, the kinetoplast is located anteriorly and it has a short flagellum (Figure 2C) (26).

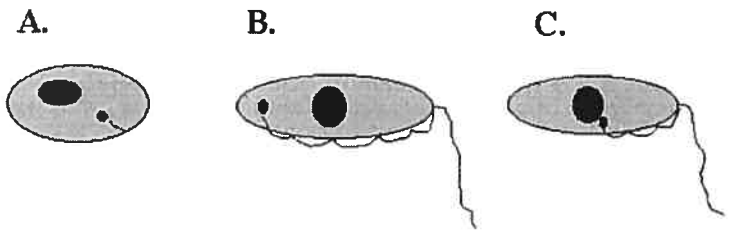

Figure 2. Developmental stages of *T. cruzi*. Amastigote form (A). Trypomastigote form (B). Epimastigote form (C).

(http://flowers.cvm.ncsu.edu/vmm932/lectures/protozoa/leishmania.ppt)

1.2.2 Life cycle

Triatomine insects ingest blood containing trypomastigotes from a vertebrate host (Figure 3A). The ingested parasites transform themselves into epimastigotes in the stomach of the insect where they adhere to the epithelium before they differentiate (Figure 3B). Epimastigotes arrive rapidly at the insect rectum where they divide by longitudinal binary fission becoming metacyclic trypomastigotes (infecting form) (22) (Figure 3C).

During the night, the insects feed on sleeping people. The vectors usually defecate during or after biting, so the bite wound is frequently contaminated (Figure 3C). The metacyclic trypomastigotes reach the blood by penetrating through the bite wound in the skin or directly through conjunctival membranes (15). Once in the mammalian bloodstream, they can either be taken with the blood meal of another insect vector (hence maintaining the cycle) or be phagocytosed by macrophages. Inside the macrophage, the trypomastigotes become amastigotes, dividing actively by binary division (Figure 3D). As a result, the amastigotes rupture the phagocytic cells, again becoming trypomastigotes that travel through the blood to the muscle cells of the heart, esophagus, and intestine where they transform themselves back into amastigotes (123) (Figure 3E and F). The most frequently invaded cells are reticuloendothelial cells of the spleen and liver, the skeletal,

cardiac and smooth muscle cells, as well as those of the nervous system, intestinal mucosa and bone marrow. Placental trophoblasts can be also infected (116, 123, 154).

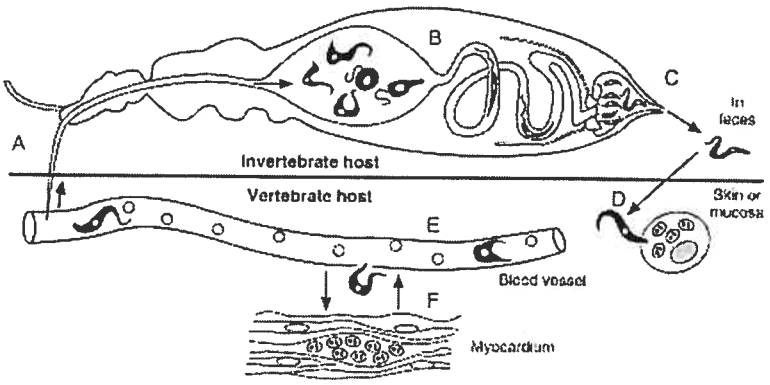

Figure 3. Life cycle of *T cruzi* in the intestine of an invertebrate (triatomine bug) and in the vertebrate host. When the bug takes contaminated blood (A) the trypanosomes convert to epimastigotes in the stomach and midgut. B) Epimastigotes fix to the walls of the rectal sac and generate infective metacyclic trypomastigotes, which are purged with feces (C) and go into the vertebrate host through a rupture in the skin. The parasites change to amastigotes within local cells (D), and reproduce to liberate blood trypanosomes, which infect other tissues (E & F).

Adapted from: (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=mmed.figgrp.4356)

1.3 Clinical presentation

During the acute phase, a local inflammatory lesion may appear which represents the entrance site of the parasite (chagoma). There may also be a focal lymphadenopathy when the inflammatory process extends regionally. The majority of patients are asymptomatic or only mildly symptomatic during this phase. A small proportion of children and even fewer adults develop severe symptoms after 7 to 14 days of incubation (60). The acute symptoms include Romaña's sign (ophthalmo-ganglial complex) and conjunctivitis. Other symptoms may suggest a viral illness including: fever, lymphadenopathy, hepatosplenomegaly, nausea, vomiting, diarrhea, rash, anorexia, lassitude, and meningeal irritation. A small number of patients with acute disease die of complications related to acute myocarditis or meningoencephalitis (168).

Evolution from the acute to chronic phase of Chagas' disease corresponds with a decrease in parasite numbers from the tissues and bloodstream (subpatent parasitemia) (164). This period, called the indeterminate phase, can extend for 20 plus years during which people present with only parasitological and/or serological evidence of infection. During this phase, infected individuals are asymptomatic and do not show electrocardiographic signs of heart involvement or X-ray abnormalities of the digestive tract (6).

Approximately twenty percent of infected individuals eventually develop one of the chronic forms of Chagas' disease. The heart is the organ most commonly affected (107). The early signs of cardiomyopathy are electrocardiographic, extra systoles or first-degree atrioventricular block. In some cases, patients can develop a total right bundle branch block with left anterior hemiblock and eventually complete atrioventricular block resulting in Stokes-Adams attacks (130). The pathologic presentation of the chronic cardiac form of Chagas' disease consists of a progressive myocarditis and congestive heart failure that are related to myocardial hypertrophy, chronic myositis with degeneration of myocytes, severe interstitial fibrosis and thickening of the cardiac basement membranes (6).

The digestive tract can also be affected by *T. cruzi*. The destruction of the autonomic enteric innervation caused by the infection leads to dysfunction of the digestive system. Both anatomical and functional alterations can be observed at different levels. Abnormalities are most frequently found in the oesophagus and in the colon, apparently because both handle harder material, such as the alimentary bolus and stools. Mega-colon and mega-esophagus are most common in Brazil and Bolivia (123, 186). The intrinsic denervation of the oesophagus caused by *T. cruzi* infection results in a loss of oesophageal peristalsis and achalasia of the lower esophageal sphincter. The initial symptom is typically dysphagia. With progression of the disease, this is followed by thoracic pain, active and passive regurgitation, heartburn, hiccups, cough, ptyalism, enlargement of the salivary glands, and emaciation. The other major disturbance of the digestive tract presents during

the chronic phase of Chagas' disease is megacolon. Dilation occurs mainly in the sigmoid colon and extends to the rectum in about 80% of cases. The main signs and symptoms that suggest a diagnosis of megacolon are associated with retention of faeces and gas. Constipation is the most frequent symptom followed by meteorism, uncomfortable abdominal distension, and sometimes abdominal cramps. In addition, patients complain of difficulty in expelling stools, even when they are of normal consistency (186). Due to lack of peristalsis, food can remain in the gut for long periods of time causing the intestine to progressively enlarge. This atonic constipation of the bowel can lead to tissue necrosis and death (15).

1.4 Epidemiology

Chagas' disease is distributed widely in the American continent from the Great Lakes of the USA to southern Patagonia in Argentina (roughly 42 °N to 46 °S) (158). In some regions on the world the cycle of *T. cruzi* is exclusively sylvatic. The introduction of human beings into the cycle is a result of civilization. Endemic regions are always rural and contain the kind of houses where the vectors have the best chance to survive (26). In the Americas, it is estimated that between 16 to 18 million people are infected by the parasite. Approximately 25% of the Latin American population is at risk of acquiring the disease (~ 35 million people) (188). The annual incidence of the disease is approximately 565,000 and >50,000 deaths from Chagas' disease each year (96, 127, 154).

The highest prevalence of this disease occurs in rural areas and among the poorest segment of the population residing in thatched adobe huts. Because the insects are photosensitive, poor quality of housing favors their survival. Cracked walls, thatched roofs and trash-filled rooms are perfect for the infestation and proliferation of the insects (187). The age of the exposed individuals is also very important in the epidemiology of the Chagas' disease. The majority of acute infections occur in children younger than two years old, and the acute phase is frequently fatal at this age (154). Other epidemiological factors that increase the risk of human infection in a region are type of climate, family size and earnings, intradomiciliary animals, poor lighting conditions, poor domestic hygiene and the presence of insect predators (130, 191).

There are two major classifications of *T. cruzi* (groups I and II). *T. cruzi* I is common within the sylvatic cycle (associated with opossums). It is rarely found in the southern cone countries (Argentina, Brazil, Chile, Paraguay and Uruguay) but is the only form found in human infections above Ecuador. *T. cruzi* II is associated with rodents and with *Triatoma infestans*. Both classifications (*T. cruzi* I and II) can cause cardiac involvement but only *T cruzi* II results in digestive tract alterations (141). As a result, the clinical presentation of Chagas' disease varies in different geographic areas. For example, there is no evidence of mega-colon or mega-esophagus in Venezuela and Colombia. However, these syndromes are frequently observed in Brazil and Bolivia (123).

It is possible that *T. cruzi* transmitted man-to-man via domesticated triatomine insects is more pathogenic than strains that are transmitted from other mammalian species. The most important domestic reservoirs for *T. cruzi* are dogs, cats and guinea pigs (191).

The prevalence of Chagas' disease varies in the different countries of Latin America. In a study done in western Venezuela in 1999, the prevalence of the disease was a staggering 59.9% (7). In the Mexican state of Guerrero, the prevalence of the disease was reported to be 1.7% in 1990 (5). The current prevalence in north-eastern Brazil (state of Piaui) varies slightly depending on the serological test employed: 14.3% by the IFA test, 14.7% by EIA and 13.2% by competitive enzyme-linked immunosorbent assay (C-EIA) (68). In countries where this disease is not endemic, historical migration is the key factor that determines prevalence (184).

1.5 Treatment

Two nitroheterocyclics have been used for the treatment of Chagas' disease; nifurtimox, known commercially as Lampit® (Bayer), and benznidazole, known as Rochagan® (Roche) (36). Nifurtimox first appeared in 1972 and has antiprotozoal activity. It is also utilized in the treatment of leishmaniasis and African trypanosomiasis. Benznidazole was discovered in 1974 and released for use in 1978 (15).

Nifurtimox has been used for more than 2 decades. In congenital and acute Chagas' disease, this drug can reduce the severity of the illness and decrease mortality. However its use results in parasitologic cure in only ~ 70% of patients (107). The efficacy of benznidazole is similar to that of Nifurtimox. Both of these drugs are useful only in acute stage (48, 55). Both drugs have high toxicity and low efficacy during the indeterminate and chronic stages, making chemotherapy for late Chagas' disease unsatisfactory and controversial (62).

The mode of action of nifurtimox is metabolic reduction of nitro groups by nitroreductases, leading to the production of nitroanion radicals that *T. cruzi* cannot eliminate. However, nifurtimox has been discontinued and benznidazole is the only treatment currently available. Benznidazole acts by interfering with the synthesis of macromolecules via covalent binding or through nitroreduction intermediates, such as lipids, proteins and DNA (52). The trypanocidal effect of benznidazole can be broadened when this drug is prescribed in combination with IFN-γ (139).

Both of these drugs produce severe side effects. Nifurtimox generates anorexia, weight loss, mental alterations (excitability or sleepiness), and digestive manifestations (nausea, vomit, colic, and diarrhea). The adverse reactions to benznidazole include symptoms of hypersensitivity (dermatitis, cutaneous eruptions), fever, edema, articular and muscular pain, depression of the bone marrow (thrombocytopenic purpura,

agranulocytosis), and polyneuropathy (paresthesia and polyneuritis of peripheric nerves) (43). The side effects of both medications are probably due to the oxidative or reductive damage in host tissues (179).

Several drugs have been experimentally evaluated for the treatment of Chagas' disease. Cysteine protease inhibitors (CPI) are capable of curing experimental *T. cruzi* infection in mice infected with a lethal inoculum (62). Treatment with CPI also reduced parasitemia, and/or cured mice in the chronic stage of disease. Urbina *et al.*, (180) have demonstrated the antiparasitic activity of quinuclidine-based squalene synthase (SQS) inhibitors: E5700 and ER-119884. These candidate drugs provide total protection against *T. cruzi* infection in a murine model. Another drug recently tested is etanidazole that has shown anti-*T. cruzi* activities against both circulating trypomastigotes and intracellular amastigotes without affecting host cell viability *in vitro* (137). Castro (52) has reviewed the drugs evaluated for the treatment of Chagas' disease between 1980 to 1992 (Table 1).

In 2005, no drug is considered to be safe, effective and inexpensive. As a result, the chemotherapy of Chagas' disease remains an unanswered problem, and the search for alternate therapies continues. At the moment, ketoconazole represents the best hope among the licensed products because successful cure rates in infected mice approach 80% (154).

TABLE 1. Drugs tested for the treatment of Chagas' disease between 1980 to 1992 (36).

Antibiotics	Actinomycin D, amphotericin B
Cationic amphiphilic drugs	Phenothiazines, acridines, phenazine metasulphate, crystal violet
Azole derivatives	Ketoconazole, itraconazole, RS-49, 676, ICI-195,739
Nitroimidazoles	Metronidazole, CL-64,855, MK-436, HOE-239
Purine derivatives	allopurinol, allopurinol riboside, For A
Naphthoquinones	β-Lapachone),
Metallic complexes	DDP
Natural products	Taxol, alkaloids, magainin derivatives, violacein derivatives, propolis
Other drugs	Carboxamide derivatives, fluoro arginine derivatives, ellipticin and derivatives, benzoisoquinolin-1-1-dione derivatives, lonidamine, lovastatin, P-536, gossypol, antioxidants, 3-mercaptopicolinic acid, mytochondrial fluorescent dyes

1.6 Control

The control of Chagas' disease is based on primarily on: vector control, improved housing or some combination of these two activities (51). Improved house construction consists of replacing palm roofs with metal, elimination of mud houses, plastering adobe walls and cementing dirt floors (1). Improvement of homes is economically impractical however. In many rural regions, the principal method for combating the disease is the elimination of domestic reduviid insects through the use of insecticides. In 1950, the control of Chagas vectors began using DDT. This agent had a high rate of success in controlling malaria but it failed in the control of Chagas' disease (192). DDT was replaced by hexachlorocyclohexane (HCH) but this agent was also discontinued due to a low residual effect (64). Later benzene hexachloride (BHC) and dieldrin (mainly used in the Venezuelan campaigns) were applied in controlled trials and campaigns for vector elimination (57). In the early 1980's, the first generation of pyrethroids was introduced. Although these agents had triatomicidal activity in laboratory bioassays, they had no success in the field (57). The current implementation of third generation pyrethroids (deltamethrin, lambdacyhalothrin and beta-cyfluthrin) for Chagas' disease vector control has dramatically reduced house triatomine infestation (51, 192). The ideal insecticide needs to have the ability to penetrate into the walls with long-term residual capability to destroy eggs and to have a low probability of the development of resistance (116).

The Southern Cone Initiative for the elimination of transmission of Chagas' disease was initiated in 1991 by the Ministers of Health of Argentina, Bolivia, Chile, Paraguay and Uruguay (117). The Andean countries (Bolivia, Ecuador, Colombia, Peru and Venezuela) also proposed an initiative to interrupt the transmission of Chagas' disease in 1999 (www.icp.ucl.ac.be/~opperd/parasites/press4.htm). These initiatives have resulted in the reduction or interruption of vectorial transmission in several countries of Latin America such as Venezuela, Chile, Colombia and Uruguay, and in different regions of Brazil (1, 81, 106, 117, 162).

In areas where vectorial transmission has been reduced or eliminated, the principal risk for disease acquisition is transfusion. The transmission of *T. cruzi* by transfusion is currently addressed by screening all donations and/or the addition of gentian violet dye to all donated blood at a concentration of 0.025% to kill the parasite (123).

1.7 Vectors

Potential *T. cruzi* vectors include 129 species from five families and seventeen different genera. The vast majority of these species are limited to the American continents; the exception being *Triatoma rubrofasciata* found in tropical regions of the African continent. There is no evidence of infection of *Triatoma rubrofasciata* by *T. cruzi* however. The most important vectors from the epidemiological point of view are from three genera:

Triatoma, *Rhodnius* and *Panstrongylus* (60). These insects all belong to the order Hemiptera, family Reduviidae and subfamily Triatominae (186). There are 72 species inside the genus *Triatoma*, but the principal vector is the *Triatoma infestans*, which is primarily domestic and shows the highest parasitic infection rates. It has a wide geographic distribution from Mexico to Peru and the entire Pacific coast. This insect is responsible for half of the infections in Latin America (mainly in Argentina, Chile, Bolivia, Brazil and Uruguay) (187). The most important vector after *Triatoma infestans* is *Rhodnius prolixus* which is involved in the transmission of the disease in Central America and in the Northern half of South America (Venezuela, Colombia). *Triatoma diminata* is the most important vector in Central America and parts of Mexico (186). *Pastrongylus megistus* was the first vector species identified by Carlos Chagas in 1909. It is now just of historical interest because it has been displaced by the arrival of *Triatoma infestans* into its territory (60).

T. cruzi infection is enzootic in the Amazon basin, wild mammals and at least 10 of the 16 sylvatic triatomine insect species can carry the parasite. However, human cases of Chagas' disease are rising, indicating that the disease may be emerging as a public health concern in the Amazon region (40, 44, 56, 58, 181).

1.8 Transmission

The primary means of *T. cruzi* transmission is through the bite of a vector in an endemic region. In addition to vectorial and transfusion transmission, Chagas' disease can also be spread through contaminated food, human milk, beverages, organ transplantation or congenitally (4, 20, 28, 66, 83, 125, 147).

Mazza *et al.* first cited the possibility that Chagas' disease can be transmitted through blood transfusion in 1936 (113). Soon after, Freitas *et al.* proved this fact in 1952 (70). Post-transfusional American trypanosomiasis is the second most common route of infection in endemic zones, and the only route of infection in non-endemic countries such as Canada and the United States (37, 77, 124). A subject with chagasic infection in the chronic phase does not display symptoms but can have transitory parasitemia and is therefore a dangerous donor. The period of incubation of post-transfusional Chagas' disease is longer than when transmitted by the vector. Lasting between 40 days to 3 months and acute disease is less evident (143) (See details in section 2.2).

1.9 Immunity

Interaction between the immune human response system and *T. cruzi* is highly complex. While infected individuals usually acquire resistance to severe pathology upon

subsequent exposures (15), factors that determine susceptibility or resistance to the various manifestations of Chagas' disease remain poorly understood. Genetic resistance and susceptibility has been clearly documented in animal models however. Both innate and acquired immune responses are involved in protection against *T. cruzi*. Immune responses initiated by *T. cruzi* antigens have been implicated in autoimmune or immunopathologic actions. Finally, *T. cruzi* has found a wide range of more or less sophisticated mechanisms for evading the human immune response.

Macrophages are the first host cells to be invaded by *T. cruzi*. The parasite is phagocytosed into the cell, escapes the phagolysosome, and replicates. However, only trypomastigotes can escape from the phagolysosome and multiply in the cytosol, epimastigotes are destroyed (115, 126). Furthermore, macrophages activated by cytokines (principally IFN-γ), can rapidly kill intracellular trypomastigotes. The main role of the IFN-γ *in vivo* appears to be the activation of the respiratory burst and generation of toxic oxygen intermediates (nitric oxide). Interleukin 12 (IL-12), the cytokine that stimulates NK cells for IFN-γ production, seems to trigger a Th1 type response in the acute phase of infection. Other cell types such as lymphocytes (eg: Thy-1+CD4-CD8-, CD4+ and CD8+) are also involved in IFN-γ production (32). Treatment of mouse resident peritoneal macrophages with the IFN-γ and IL2 increased the capacity of these cells to kill internalized trypanosomes (190).

The role of IL-10 in the inhibition of macrophage killing of *T. cruzi* has been reported by several investigators. Reed *et al.* have shown that genetically susceptible mice produced more IL-10 during *T. cruzi* infection than resistant mice suggesting an association between IL-10 production and disease susceptibility (144). Highly susceptible C57BL/6 mice can be protected from acute disease and death from *T. cruzi* by the administration of neutralizing anti-IL-10 monoclonal antibody (mAb) (144). Furthermore Silva *et al* demonstrated that exogenous IFN-γ protects mice from fatal infection with *T. cruzi*. Resistant B6D2 mice develop fatal *T. cruzi* infections when treated with a neutralizing anti-IFNγ (mAb) (160). IL-10-specific mRNA is produced in the spleens of mice with acute *T. cruzi* infections showing that IL-10 blocks the ability of IFN-γ to inhibit the intracellular replication of *T. cruzi* in mouse peritoneal macrophages. These results demonstrate that IL-10 is produced early during infection with a protozoan parasite and suggest a regulatory role for this cytokine in the mediation of susceptibility to acute disease (160).

A protective role for NK cells in *T. cruzi* infection has also been described (31). In experimental *T. cruzi* infection, the early response of NK cells reduces parasitemia and augments survival (178). These cells are the major source of IFN-γ that limits the replication of *T. cruzi* in host macrophages during the early acute phase of the infection (31). NK cells also control the propagation of the parasite by a contact-dependent, perforin-independent action against circulating trypomastigotes (102). While NK cells may have a protective effect in early *T. cruzi* infection, some investigators have proposed that NK

production of IFN-γ against *T. cruzi* antigens may favors the development of a strong Th1 response in cardiac Chagas' disease patients and progression of heart disease (13, 74).

Toll-like receptors (TLR) are evolutionarily conserved, germline encoded receptors that recognize specific molecular patterns associated with microbes. Recent evidence suggests that TLRs play an important role in controlling adaptive immune responses (14). TLRs are expressed primarily on macrophages and dendritic cells (DCs) and control the activation of these APCs (114). Oliveira *et al.* have recently demonstrated that TLR4-mutant mice were hypersusceptible to *T.cruzi* infection, as evidenced by a higher parasitemia and earlier mortality. These results suggest that natural resistance to *T. cruzi* is TLR4 dependent, most likely due to TLR4 recognition of *T. cruzi* glycoinositolphospholipids (GIPLs) (129).

T. cruzi antigen stimulated B cell proliferation and differentiation into plasma cells producing parasite-specific antibodies (15). However, the humoral response to *T. cruzi* is polyclonal, and the majority of the activated B lymphocytes do not recognize parasite antigens (reviewed in 91). The production of IgM is the first adaptive immune reaction to *T. cruzi* but has little or no effect. IgG, which appears later, is more efficient against the trypomastigote form (15). The strategies used by *T. cruzi* to evade the immune response are varied, complex and not totally understood. Several theories have been proposed, including the expression of many antigenic immunodeterminants at the same time, antigenic mimicry, the down-regulation of IFN-γ production by Th1 lymphocytes, the complexity and variety

of receptors needed for internalization, escape from complement-mediated lyses, the endocytosis of antibodies by the parasite, genetic polymorphism and the polyclonal activation of B and T cells (167). One well-documented mechanism is the binding of host antibodies to Fc receptors on the surface of the parasite (15). By reorienting host antibodies in this way complement cannot be activated, and lyses of the parasite is prevented.

While the human immune response to *T. cruzi* can protect the host to some extent, there is evidence that this small response contributes to the pathogenesis of Chagas' disease. There are currently two controversial theories for immune pathology: 1) That chronic inflammation is due to the persistence of *T. cruzi* at specific sites in the infected host and 2) that *T. cruzi* infection provokes immune responses that cross-react with self tissues and eventually become autonomous of the parasite (170). On the one hand, the absence of parasites in the heart lesions of patients with chagasic chronic cardiac pathology raises doubts about the direct participation of *T. cruzi* in tissue lesions (47). On the other hand, patients who are treated and cured do not progress to severe disease. This last observation means that Chagas fails to meet the most important criteria for being considered an autoimmune disease (reviewed in 169).

Nonetheless, there are direct and indirect data to support several of the autoimmune theories, including molecular mimicry, cross-reacting antigens, anti-idiotipic reactions, polyclonal activation and imbalance of immune regulation (164). For example, some strains

have antigens that elicit antibodies that cross-react with antigens from the cardiac muscle. Furthermore, cardiac myocytes have receptors for *T. cruzi* that make it possible for the parasite to insert itself into these cells (148). Others have suggested that the autoimmune response in murine Chagas' disease is due to deposition of proinflamatory laminin-binding cytokines (161). Bach *et al.* have demonstrated the presence of autoantibodies against small nuclear ribonucleoprotein (UnsRNPs) in sera from Chagasic patients (12), and Giordanengo *et al.* found that cruzipain antigen-immunized mice produce antibodies to the immunogen and to myosin (210 kDa antigen from skeletal muscle) (71).

In fact, it is quite likely that there is some truth to both theories (72, 118). Tarleton *et al.* transplanted neonatal hearts into mice chronically infected with *T. cruzi* and failed to observe signs of autoimmune rejection or any significant inflammatory response (171). *In situ* PCR analysis in murine models demonstrates an absolute correlation between the persistence of the parasites and the occurrence of disease in muscle tissue (179). The direct destruction of tissues by the parasite or by parasite-derived molecules, in the acute phase may rupture the heart fibers and expose new antigens to the immune system (148).

2. Diagnosis of Chagas' disease

There is no gold-standard test for the diagnosis of Chagas' disease. Indeed, many dozens of tests have been developed/reported including: classic parasitologic, serologic, and nucleid acid-based assays.

The diagnosis of Chagas' disease in clinical practice is typically based on conventional parasitological methods (thin and thick blood smear, fresh blood examination, hemoculture and xenodiagnosis) and serological methods. The parasitological methods are efficient in diagnosing the acute phase of the disease and they allow for direct examination of the parasites in the blood (23). In acutely infected patients, examination of blood preparations is the cornerstone of detecting *T. cruzi*. However, other specimens such as lymph node and bone marrow aspirates, pericardial fluid, and cerebrospinal fluid may also be examinated microscopically (107). When these methods fail to detect *T. cruzi* in a patient whose clinical history suggests that the parasite is present, methods of concentration or culture of the parasite can be undertaken (108). Hemoculture is performed by culturing whole blood in NNN medium. Xenodiagnosis consists of feeding reduviid insects with blood from the suspected patient. Then, 30 to 40 days after the blood meal, the insect's intestinal tract is microscopically checked for parasites (168). There are several problems with these two methods for diagnosing acute Chagas' disease. They take at least a month to complete, they are no useful for blood bank screening, both have low sensitivity (less than

50%), and they are time consuming (23, 39, 107). Maintenance of a colony of naïve reduviid bugs is also far beyond the capabilities of most routine clinical laboratories.

Although polymerase chain reactions (PCR) have been useful for the diagnosis of Chagas' disease (27, 92, 189), the use of PCR in diagnostic laboratories and blood banks is not optimal because this assay is laborious, expensive and may not be suitable for the screening of large numbers of samples. PCR may also have limited sensitivity in the setting of chronic infection (97). The methodology used for screening Chagas' disease in blood banks has to have characteristics that include: simplicity, specificity, sensitivity, low cost and performance by automated laboratory equipment. For blood security, sensitivity is far more important than specificity (84). At the present time no PCR-based test for the detection of *T. cruzi* is available commercially (107).

In conclusion, the parasitological methods are insufficient for the indeterminate and chronic phases of Chagas' disease because of the intermittency of *T. cruzi* in the blood. At these disease phases, serology becomes critical in combination with clinical and epidemiological data (25, 152)

The first serological diagnostic test for the diagnosis of Chagas' disease was introduced by Machado and Guerreiro in 1913 (complement fixation test or CFT) (80). Later, Fife & Muschel (1959) described the indirect immunofluorescence test for Chagas

diagnosis (67), and Romaña (1961) used indirect hemagglutination (145). Most recently the enzyme-linked immunoabsorbent assays (ELISA) have become the most important (182).

The diagnosis of Chagas' disease in Central and South America is complicated due to antigenic similarities of the *Trypanosomatidae* (41), principally when an individual has multiple infections with *Leishmania* species and *Trypanosoma rangeli*. Prevalence data from different seroepidemiological studies in Latin America may be confused due to the presence of these parasites in the same region. Cross-reactions have been observed with all of the more common serological tests used in the field such as CFT, immunofluorescence, hemagglutination and immunoenzymatic assays (82). In order to solve this problem, the Pan American Health Organization proposes the use of at least two methods for the diagnosis of Chagas' disease (34).

The CFT (known as Machado-Guerreiro) has a low sensitivity in acute Chagas' disease (35%). Although easy and inexpensive, it has been largely replaced by other methods (184). Immunofluorescence has been widely used for diagnosis of Chagas' disease. Although this method is very sensitive, it is time consuming and prone to subjective interpretation resulting in a large number of false-positive results, primarily in individuals with other parasitic infections (34, 84).

The enzyme-linked immunoabsorbent assay (ELISA), first described by Engvall and Perlamann in 1971 (63) have been shown to be useful in detecting antigens or antibodies in many infectious diseases. The advantages of EIA tests include: simplicity, sensitivity, relatively inexpensive, results can be read objectively, and adaptable to field conditions (166). EIA assays are, in fact, the tests of choice and are the most frequently employed by blood banks for the diagnosis of Chagas' disease. The following sections will resume in all the antigen preparations and techniques that have been reported using the EIA format for the diagnosis of Chagas' disease.

2.1 Serologic diagnosis with different *T. cruzi* antigens preparations and techniques

Serologic tests have been developed and some of them have become commercial kits (127). However, a persistent problem with these assays has been the occurrence of false-positive reactions. Although the reported sensitivities and specificities have been high, many of the studies have not used appropriate controls, samples sizes have typically been small, different parasite biodemes have been not used, and very few assays have undergone validation studies to demonstrate their true utility. Furthermore, the majority of commercially available tests are based on the epimastigote form (whole or semi-purified antigenic fraction). Significant discrepancies in the reproducibility and reliability of these tests have been described by different laboratories, mainly due to cross-reactions with other parasites and standardization of the reagents (29).

2.1.1 Fixed forms of *T.cruzi*

Different forms of *T. cruzi* have been fixed with formalin or formaldehyde, and used in several formats for the diagnosis of Chagas' disease (8, 30). Whole organisms are attractive for developing diagnostic tests since the 'antigens' are relatively easy to prepare, can be stable for long periods of time, and are generally safe. The use of fixed parasites avoids the use of sonication with the activation of proteases that can lead to antigen degradation (10).

In the indirect immunofluorescence format (IIF), fixed-amastigotes have stronger signal compared to epimastigotes (9, 111). Pappas *et al.* in 1983 described a dot-immunoassay using fixed promastigotes from *Leishmania donovani* (131). This method was reported to be very sensitive but the specificity was limited in the diagnosis of Chagas' disease. This approach is quite attractive as a qualitative method in field studies because it is inexpensive and simple to perform, and it can be implemented in laboratories with few resources (53, 54). Fixed epimastigotes have also been employed in diffusion-in-gel enzyme-linked immunosorbent assay (Dig-EIA), a modification of the EIA test. First described by Elving and Nygren in 1979 (61), this method adsorbs whole *T. cruzi* onto a plastic surface (semi-solid medium). Castilla *et al.* used the Dig-EIA method for in a study in Mexico (35), and found that all of the patients positive for *T. cruzi* by xenodiagnosis or repeated positive serological tests were also positive by Dig-EIA. There were no cross-

reactions with the healthy control sera or with samples from patients with other parasite infections. However, the number of sera included in this study was too low to validate the approach.

Given the advantages of fixed-forms of *T. cruzi* for the diagnosis of Chagas' disease and the absence of validation studies for these antigens, we decided to develop, compare and validate EIAs based on the three forms of *T. cruzi* (17-19).

2.1.2 Whole protein extracts of *T. cruzi*

Reported sensitivities and specificities for various tests using whole extract antigens range from 99-100% and 96-98% respectively. Whole extract of *T. cruzi* is obtained mainly by simple sonication of specific parasite forms. Sonicated trypomastigote antigen in an EIA format was found to have a sensitivity of 98.8%. Specificity for 405 serum samples from healthy blood donors was 98.3%, and 96.8% for a limited number of sera from patients with Kala-azar and muco-cutaneous leishmaniasis (11). Similarly, a Dot-EIA based on an alkaline soluble *T. cruzi* epimastigote antigen (ASEA) was reported to be 100% sensitive and 95.6% specific (103). A slightly more complex whole extract antigen was prepared by Cuna *et al.* (46) using chloroform followed by ethanol precipitation of disintegrated epimastigotes. In a competitive antibody enzyme immunoassay (CEIA) format using a monoclonal antibody specific for component 5, this test detected 94% of chronic chagasic

patients and no reactions were observed with 96.6% of the control sera (46). Using epimastigote antigen (EpEx) or purified trypomastigote antigen (A&T) Almeida *et al.* have reported excellent sensitivity (100%) and specificity (100% and 99.7% respectively) in a chemiluminescent enzyme-linked immunosorbent format (3). Whole lysated antigen prepared from *Crithidia luciliae* has also been used in an EIA format. This non-pathogenic trypanosomatid reduces the risk of accidental laboratory infections (119). Whole organism extract antigen has also been used in an EIA format for testing saliva but with only limited success (140).

2.1.3 Trypomastigote excreted-secreted antigens (TESA)

T. cruzi trypomastigotes excrete and secrete a wide range of polypeptides that can be harvested from culture medium. The use of TESA (trypomastigote excreted-secreted antigens) has been described in a number of different formats (90, 110, 122, 176, 177). Many of these antigens belong to the *T. cruzi* transialidase (TS) family that are responsible for the transfer of exogenous sialic acid to acceptor molecules on the trypomastigote surface, an event critical to parasite penetration (45). The N-terminal region of the TS includes the catalytic domain and the C-terminal end contains 12 amino acid repeats. Both regions stimulate B-cell responses in patients with acute and chronic Chagas' disease (45). In contrast, the shed acute-phase antigens (SAPA) present in TESA elicit the production of antibodies detectable mainly during the acute phase of the disease (89).

There are numerous reports that describe the use of TESA antigens in different EIA formats with excellent sensitivities and specificities with these tests. A good example of the power of TESA antigens is the work by Umezawa *et al.* (176). They evaluated 145 chagasic patients (congenital, acute and chronic), 111 healthy individuals, and 256 chronic chagasic subjects from three different areas of Brazil. Using TESA in immunoblot format both IgM and IgG from acute-phase and congenital Chagas' disease patients reacted with bands in the 130 to 200 kDa antigen range, whereas the sera (IgG) from chronic patients recognized primarily the 150 to 160 kDa bands. Sera from healthy subjects, and sera from patients with leishmaniasis, did not react with these antigens in this report. TESA blots were 100% sensitive and specific demonstrating the usefulness in diagnosing acute and congenital cases of Chagas' disease (176). Others investigators have evaluated the utility of TESA using four *T. cruzi* strains from different biodemes. Chronic chagasic sera reacted to 150 to 170 kDa bands on TESA-blot from Y, WSL, 12SF, and Colombiana strains. Polypeptides below 150 kDa were responsible for cross-reactions with visceral and cutaneous leishmaniasis (122). Kesper *et al.*(90) used TESA immunoblots to demonstrate unique banding patterns that seems for each strain or isolate suggesting that immunoblotting may be helpful tool for the characterization of *T. cruzi* strains and isolate. Immunoblotting is not practical for blood bank screening but could certainly be used as confirmatory test. This is the case in the work conducted by Silveira *et al.* (163) in which TESA-blot was used as confirmatory test for inconclusive sera from a Brazilian blood bank. From 348 donors whose blood had been diagnosed as doubtful for *T. cruzi* (initially

inconclusive), the TESA-blot was positive in 2.87% (n=10) and negative in 97.12% (n=338) (163).

Using the EIA format, Umezawa *et al.* (177) evaluated TESA antigens for the diagnosis of acute and chronic Chagas patients. The sera from 120 cases (67 chronic and 53 acute cases), and 164 nonchagasic subjects (30 blood donors, 53 leishmaniasis patients and 81 with unrelated diseases) were tested using TESA antigens from the Y strain. The sensitivity and specificity of this assay were 100% and 96.34% respectively. All of the IgM antibodies present in the acute sera recognized TESA antigens (TESA-EIA IgM sensitivity: 100%). Unfortunately, these results were obtained using only a single biodemes of *T. cruzi* (177). Other investigators have evaluated the utility of TESA antigens using different biodemes in the EIA format. The sensitivity and specificity with the Y strain was 100% and 95.78% respectively using a total of 360 samples (124 chronic chagasic patients, 205 healthy, 14 with cutaneous and 17 with visceral leishmaniasis), and similar results were obtained for the other strains (122).

Although excellent sensitivities and specificities have been obtained with TESA antigens in different serological assays, TESA antigens have not been validated in large field trials. At the present time, no TESA-based test for the detection of *T. cruzi* is available commercially, and the use of this type of antigen is limited to Brazil. Given the unexplored

potential of TESA antigens, we decided to develop, validate and field test TESA-based EIAs using antigen from two distinct biodemes (eg: Tulahuen and Brazil) (17, 18).

2.1.4 Purified proteins

A wide variety of techniques is available to purify proteins and other components from complex mixtures such as whole organisms. Chromatographic techniques are among the most powerful for the separation and purification of proteins (138). Affinity chromatography has been particularly useful to purify proteins for the development of tests for Chagas' disease. For example, Silber *et al.* used an erythrocyte ghost affinity column to isolate a 67 kDa molecule. This protein is present in both epimastigote and trypomastigote lysates, and is involved in the recognition of host-cell receptors (156). While biologically interesting, the method used by these authors has no practical value for obtaining the amounts of protein required to perform serological assays. The 67-kDa lectin-like protein was subsequently purified using chromatography on agarose and was evaluated in an EIA format. The sensitivity and specificity of this test are shown in TABLE 2 but no sera from patients with leishmaniasis were evaluated (109). Similar results have been obtained with *Trypanosoma cruzi* epimastigote antigen purified by ion exchange chromatography (PEF) and a 90-kDa surface glycoprotein obtained from a lectin-affinity column. The reported sensitivity and specificity, for these assays are found in Table 2 but neither assay was undergone rigorous validation (132, 152).

Radiolabeled surface glycoproteins of *T. cruzi* purified by immunoprecipitation (72 and 90 kDa) or Con-A Sepharose columns (Gp25) were able to discriminate among Chagas positive and negative controls as well as patients with visceral leishmaniasis in a RIPA format. However, the radioactivity involved makes these assays processes suitable only for a research facility rather than for a routine diagnostic laboratory (94, 151).

Several other proteins that have been purified by different protocols for the diagnosis of Chagas' disease are described in Table 2. Examples include: ubiquitin and epimastigotes ribosomal fraction of *T. cruzi* purified by biochemical protocols, the polypeptides Tc 46 and Tc58 obtained from three different strains of *T. cruzi* (Y, WSL and Colombiana) by electroelution from SDS-PAGE, and two synthetic peptides (TcD and PEP 2) synthesized on the basis of repeat units from *T. cruzi* trypomastigotes. However, EIA assays based on these purified proteins have limited sensitivity and adequate numbers of sera generally have been not used (Table 2) (134, 135, 165, 172).

TABLE 2. Different purified proteins used in the diagnosis of Chagas' disease

Technique use for purification	Antigen obtained	Test	Sensitivity (%)	Specificity (%)	Reference
Chromatography Techniques					
Chromatography on Agarose	67 kDa	EIA	98	98.11	(109)
Ion exchange chromatography	PEF	EIA	98.6	98.7	(132)
Lectin affinity column	90 kDa	EIA	96.6	91.9	(152)
Con A Sepharose column	25 kDa	RIPA	97.8	99.2	(151)
Other Purification Techniques					
Immunoprecipitation	72 and 90 kDa	RIPA		100	(94)
Biochemical protocols	Ubiquitin	EIA	89.4	93.8	(172)
Electroelution	Tc46, TC58	EIA	> 97	90-100	(135)
Synthetic peptides	TcD	EIA	93.8	97	(134)
	PEP2		91.6	98	
	TcD + PEP2		99	99	

2.1.5 Recombinant proteins

Molecular biology has allowed the development of highly purified and well-standardized recombinant antigens in large quantities that can be tested in the EIA format (49, 79, 87).

Overall, recombinant antigens have several significant advantages including: 1) greater specificity than crude parasite extracts avoiding cross-reactions with other parasitic diseases, most importantly leishmaniasis and, 2) several recombinant antigens can be combined in the same test to improve sensitivity (128). However, recombinant antigens also have limitations. For instance, not all individuals will have a strong immune response to a specific antigen and the recombinant antigens may lack post-translational modifications (eg: glycosylation) necessary for immunogenicity. Preabsorbtion may also be needed for recombinant antigen preparations in order to remove antibodies against residual bacterial antigens (38).

Nonetheless, reported specificities in the 95-100% range and sensitivities > 97-98% are typical for recombinant antigen based assays. Investigations of these serological tests are summarized in Table 3.

Good examples of very high sensitivity and specificity for recombinant based assays have been reported by Krieger *et al.* (97) and Godsel *et al.* (73). The sensitivities and specificities of these assays (100%) may to be artificially high since a limited numbers of sera were tested (73, 97).

Limitations of recombinant antigen-based assays are exemplified by Oelemann *et al.* and (128) Pastini *et al.* (133). Although the sensitivity and specificity of these assays are also reported to be over 99% (Table 3), both require interpretation by a subjective reader as a result these tests are not recommended for blood bank screening since variability can be introduced based on the skill of the readers (128, 133). However, these tests could be used as confirmatory assays or in epidemiological surveys (149).

Sensitivity is one of the most important limitations when a single recombinant protein is used for the diagnosis of Chagas' disease. Examples include the works of Cetron *et al.* (sensitivity 36-66%), Matsumoto *et al* (sensitivity: 82.2%), and Umezawa *et al* (sensitivity: 87.1-99%) (38, 110, 174). However, the mixture of several recombinant proteins can improve both the sensitivity and specificity of recombinant-based tests. Examples of the use of mixed recombinant proteins include: Umezawa *et al.* (175) (mixed recombinant proteins: B13, 1F8 and H49), Krieger *et al* (mixed recombinant proteins: FRA+CRA) (97), Ferreira *et al* (65) (recombinant proteins: PEP-2, TcD, TcE and TcoL1.2), Gomes *et al.* (76) (EIE-Recombinant-Chagas-Biomanguinhos kit: CRA + FRA),

and Houghton *et al* (85) (mixed recombinant proteins: (PEP2, TcD, TcE, TcLo1.2). Although all of these assays have excellent sensitivity and specificity (see Table 3), several limitations should be acknowledged. The positive sera used in these studies were known to be reactive in a baterry of other serological tests. As a consequence, the estimates for test sensitivity are artificially high. Also the number of sera used in these studies was limited and none of these tests has been evaluated in large field trials. Finally the production of recombinant requires adequate equipment and reagents that are not available in many Latin America laboratories. As a result, the production of recombinant proteins for the diagnosis of Chagas' disease is not feasible in the majority of laboratories in endemic countries.

TABLE 3 Diagnostic performance of serodiagnostic assay using different *T. cruzi* recombinant proteins

Antigens	Assay	Type of antigen	Sensitivity (%)	Specificity (%)	Refs
PEP-2+TcD+ TcE+TcLo2.1	EIA	Linear assembly	100	98.94	(65)
FcaBP	EIA	Fusion proteins	100	100	(73)
CRA+FRA	EIA	Recombinant Chagas Biomanguinhos kit (Fussion proteins)	100	100	(76)
Ag1+Ag2+Ag13, Ag30+SAPA	Enzyme immunoassay (immunodot)	Fusion protein	99.6	99.1	(133)
(CRA+FRA+ Tc-24+SAPA+MAP+TcD+Ag39)	Line immunoassay	Recombinant proteins and synthetic peptides	100	99.3	(128)
TcD+TcE+PEP-2 multi-epitope	EIA	Multiepitope Synthetic Peptide	99.6	99.3	(86)
CRA+FRA mixture	EIA	Fusion protein	100	100	(2, 34, 97)

Continuation TABLE 3……..

Antigens	Assay	Type of antigen	Sensitivity (%)	Specificity (%)	Refs
TESA-1	Immunoblotting	Recombinant protein	82.2	93.5	(110)
H49, JL7, A13, B13, JL8, and 1F8	EIA	Recombinant protein	87.1-99	96.2-99.6	(174)
B13+1F8+H49	EIA	Multiantigenic reombinant protein	99.7	98.6	(175)
F1-160, SA 85-1.1, SA 85-1.2	EIA	Recombinant protein	36-66	82-96	(38)

2.1.6 Comparison of tests for the diagnosis of Chagas' disease

Comparison among various commercial tests or different assay formats (serological, molecular, parasitologic) for Chagas' disease demonstrates that no "gold standard" test exists. Most tests report high sensitivities and specificities but discrepancies between assays are always present (25, 75, 104, 127).

The discrepancies observed in the published data for the different serological tests for Chagas' disease are likely due to clonal and strain variations, the type of antigen preparations used in each test, and the different procedures used to obtain the antigens and to culture the parasites. Any or all of these factors may determine the quantity and quality of epitopes that are present in antigenic preparations. Moreover, binding of these molecules to solid surfaces can hide or expose epitopes that have different affinities for specific and non-specific antibodies (153). Such disagreements among the commercial tests have been extensively reported (101, 104, 127).

For example Oelemann *et al.* (127) reported different sensitivities and specificities in the widely used commercial kits with sera from four regions of Brazil (listed in Table 4), but the differences were not significant. The biozima Chagas kit had the highest sensitivity whereas the bioeiacruzi kit was the most specific. Lorca *et al.* compared the sensitivity and specificity of 10 different Chagas' disease assays in the blood banks of Chile (Table 4) (105). They found the most reliable were the commercial EIA kits, with the best agreement, the best sensitivity (between 94-97%) and the greatest specificity (93-100%). They could not recommend the hemagglutination or latex tests. Later tests had the lowest specificities, and their sensitivities were < 80%. Assays with such low sensitivity present an unacceptable risk of transfusion-associated transmission (Table 4).

In contrast, other authors have found no significant differences among various EIA tests, CFT and indirect immunofluorescence (IIF) (25). However, Larrouy *et al.* (98) has suggested that immunofluorescence is neither sensitive nor specific, and that the use of EIA tests give cross-reactions with the sera of patients with *Leishmania* infections. Thus, these authors recommended the hemagglutination test due to its high specificity in discriminating sera of patients with other parasitic diseases (98).

TABLE 4. Different commercial and serological tests used for the diagnosis of Chagas' disease (105, 127)

Performance of different commercial reagent for the diagnosis of Chagas' disease reported by Lorca *et al* (105)		
Reagent	**Sensitivity (%)**	**Specificity (%)**
Chagas test EIA	96	98
Ortho Chagas EIA	95	99
Bio Chagas Hi Quick	64	100
Estabilgen Hemo Chagas	91	100
Hemave Chagas	88	98
Chagatest Latex	79	100
Chagas test HAI screening	70	94
Abbot Chagas EIA	94	100
EIA Chagas-Bios Chile	97	99
EIA Gull-Bios Chile	94	93
Performance of commercial EIAs reported by Oelemann *et a.l* (127)		
Abbot Chagas EIA	98.5 - 100	95.3 – 100
(BIOEIACRUZI kit)	98.5 - 100	98.8 – 100
BIOZIMA Chagas kit	100	93.6 – 99.6

The radioimmunoprecipitation assay (RIPA) has been used as a confirmatory test in several studies among blood donors in the United States but this test has some major disadvantages. This includes its laborious nature, relatively high cost, the manipulation of live parasites, the use of radioactive iodine, and the lack of reagents outside of developed world (101). A study comparing the RIPA assay with several commercial tests (IHA and four different enzyme-linked immunoabsorbent assay kits: EIA) revealed that the RIPA was more sensitive and more specific. However, it is unclear if the differences observed were truly significant (101). With all the disadvantages of the RIPA and the fact that similar results can be achieved with other serological tests, the use of RIPA for routine blood bank screening cannot be recommended.

2.2 Chagas' disease and blood donors

The probability of receiving an infected transfusion varies widely in different countries and in different regions of the same country. The highest risk has been reported from Nicaragua and Bolivia (1048-1096/10,000) while Honduras and Venezuela have much lower risk (13/10,0000). At the current time, only Venezuela and Honduras screen 100% of donated blood for Chagas' disease. Information from Costa Rica, Peru and Mexico demonstrated that these countries have not yet introduced routine screening for *T. cruzi* in blood banks (155). In Argentina, screening is not complete and, in Brazil, screening is performed in public sector blood banks but no information is available for private banks.

In Latin America, overall seropositivity for *T. cruzi* varies from 0.20% (Ecuador) to 14.8% (Bolivia) (155).

Several studies have directly assessed the prevalence of Chagas' disease among blood donors in Latin America. For example, in a non-endemic area of Mexico (Puebla state), 2140 random blood samples were evaluated for anti-*T. cruzi* antibodies using EIA and IHA. The antigen used for both serological tests was a *T. cruzi* autochthonous preparation (Puebla strain RyC-V1). Samples that were positive for both tests were considered (7.7%) to be true positives (150). It is not clear why the prevalence of Chagas' disease among these donors in this non-endemic area was so high when compared with the prevalence of the disease in the rest of Latin America (156). Studies carried out in Mexico city among blood donors reveal that the prevalence of the disease is ~ 0.3% screened by EIA, IFA, Dot-blot and Western blot (120, 143).

The highest rate of *T. cruzi* infection among blood donors in Latin America is found in Bolivia (157). The donor prevalence has been evaluated in seven regions of this country using IIF and EIA (n=1298). The study revealed very high prevalence rates in all the different regions: Sta. Cruz (51%), Tarija (45%), Cochabanba (28%), Sucre (39%), La Paz (4.9%), Oruro (6%) and Potosi (24%). There is no obligatory serological screening of donors and the blood is not treated with gentian violet in Bolivia blood banks (33).

Lower but impressive prevalence rates among blood donors were observed in a study conducted in Sao Paulo (1.7%) and in central Brazil (13.1%) using three different serologic tests (EIA, IFA and IHA) (50). Similar but generally lower rates have been found using EIA tests in several other countries of Latin America: Ecuador (Quito: 0.01%, 0.04% and 0.02% in 1994, 1995 and 1996 respectively), Argentina (Buenos Aires: 1.46%), Chile (northern Chile 22 hospitals: 2.7%), Belize (Belize city: 0.5%), Colombia (33 departments around the country: 1.2%) (16, 21, 42, 78, 88).

Until quite recently, the risk of Chagas' disease was entirely limited to Latin America. However, with increasing emigration to North America and Europe, the disease has been introduced to many non-endemic countries by blood transfusion (184). Screening for Chagas' disease is not-mandatory in the northern hemisphere. Despite the fact that, several studies have shown the presence of *T. cruzi* in blood donors in these settings. For example, a study conducted in Berlin evaluated 100 immigrants by IIF, EIA and interview for risk factors (eg: contact with the vector, rural origin). The seroprevalence was 2% (69). Two cases of transfusion-associated Chagas have been reported in non-travellers Canadians, both in Manitoba (124). A seroepidemiological study carried out in two Red Cross regions revealed that the prevalence of *T. cruzi* was 0.15% and 0.09% among blood donors from Los Angeles and Miami, respectively (100). Later, this same group of investigators performed the largest study to date on the seroprevalence of *T. cruzi* in U.S blood donors in these same areas over a period of four years. Donors were initially

classified based on their response to a risk question concerning possible *T. cruzi* exposure (7.3% Miami and 14.3% Los Angeles). The final seropositivity rates were 1 in 9000 and 1 in 7500 donations, respectively (99). Another study conducted in Los Angeles during a 29–month period reported that 1311 of 3320 donors were judged to be at risk for *T. cruzi* infection, and 7 donor sera, were reactive by EIA (confirmed by RIPA) (159). The prevalence *T. cruzi* among blood donors in the south-western and western United States was 0.105% in a study performed with 13,309 samples from 7 cities in California and Texas (24). A higher prevalence was found among Central American immigrants from El Salvador and Nicaragua living in the Washington, D.C. (4.9%). Parasites could be isolated in 50% of the seropositives by xenodiagnosis (93). These findings clearly reveal that the risk of transmitting Chagas' disease by blood transfusion is present throughout North America and Europe. Thus, routine screening for antibodies to *T. cruzi* should be performed in blood donated by immigrants from Latin America countries.

An alternate solution to prevent transmission of Chagas' disease through blood transfusion is the use of gentian violet. The dye kills trypomastigotes *in vitro* at 4°C at a concentration of 125 mg/500 mL for 24 h (reviewed in 155). An improved method that uses less gentian violet (62.5 ug/mL) in combination with ascorbic acid and visible light (75 W) has been proposed (142) since concentrations of the dye higher than 25 g/Kg/day is carcinogenic in rodents (59).

However, it is imperative to further advance the diagnosis of Chagas' disease for epidemiological, social, and clinical reasons as well as to reduce the wastage of blood (34). Although there have been many studies on the different diagnosis methods using different kinds of T. *cruzi* proteins, a "gold standard" test for Chagas does not exist. Many of the tests reported achieve 100% sensitivity and > 98% specificity yet problems with the diagnosis of Chagas' disease persist. There are several reasons: 1) no test has truly reached 100% sensitivity and specificity, 2) discordant results are found when different serological tests are applied, 3) many studies have used only a limited number of sera, inadequate controls or antigen from single strain of *T. cruzi*. Furthermore, in some cases the protocols to produce the test antigens are not simple, reproducible, and inexpensive. Many of these protocols result in antigen yields that are not sufficient for large-scale diagnostic application. Finally, and most importantly very few of these tests have been validated in large studies in the field to assess their true performance and potential for commercial kits development. For all of these reasons, we decided to develop and validate an EIA assay for the diagnosis of Chagas' disease: that would be simple, cheap, reproducible, sensitive, specific, and stable.

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II. MATERIAL AND METHODS AND RESULTS

ARTICLE I

Development and Comparison of Enzyme Immunoassays for Diagnosis of Chagas' disease Using Fixed Forms of *Trypanosoma cruzi* (Epimastigotes, Amastigotes, and Trypomastigotes) and Assesment of Antigen Stability for the Three Assays

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Journal of Clinical Microbiology. 2004. 42(4):1766-1769

Role of the candidate in conception of this article:

I was the main author of this article. I have actively participated in the initiative and design of the experiments, and the standardization techniques. I carried out all the laboratory work and also the analysis of results. Finally, I wrote the manuscript

ABSTRACT

Three enzyme immunoassays (EIAs) for diagnosis of Chagas' disease were developed with fixed forms of *Trypanosoma cruzi* using a panel of 435 sera from the following groups: Venezuelan subjects positive by immunofluorescence (n=70), Venezuelan healthy controls (n= 85), healthy Canadians (n= 166), and subjects with other parasitic diseases (n= 114). All assays achieved 100% sensitivity and reasonable specificity for amastigotes (97.6%), epimastigotes (98.3%), and trypomastigotes (99.3%). The fixed-trypomastigote assay was stable over 4 months at 4°C and room temperature. These data suggest that a fixed-trypomastigote EIA may be a suittable candidate for blood bank screening.

INTRODUCTION

American trypanosomiasis or Chagas' disease is caused by the protozoan flagellate, *Trypanosoma cruzi (8)*. It is estimated that more than 16 - 18 million people are infected by *T. cruzi* in Latin America (22, 33).

A major effort to interrupt transmission in rural South America during the last 2-3 decades has dramatically altered the epidemiology of Chagas' disease (1, 20, 23, 35). At the current time, the major route for transmission of Chagas' disease in many previously endemic regions is blood transfusion. The migration of large numbers of asymptomatically infected individuals in recent decades has extended the risk of transfusion-associated Chagas' disease to all parts of the developed world (15, 16, 18, 19, 25, 34). To our knowledge, no jurisdiction in the developed world routinely screens blood and blood products for Chagas' disease.

Although many screening methods have been evaluated, the EIA format has significant attractions in its simplicity, low cost and potentially excellent sensitivity (37). During the last 30 years, many different EIA-based assays using a wide range of antigens have been developed (2, 5, 7, 9, 10, 12-14, 24, 27, 28, 31, 36). Several studies have reported particularly promising results using fixed whole organisms (ie: integral antigens) (5, 6, 11, 16, 17). However, theses studies have been relatively small and have focused on epimastigote-form antigens.

Since the human immune system is exposed almost exclusively to trypomastigote- and amastigote-form antigens, we sought to determine which integral antigen preparation of *T. cruzi* offered the best combination of sensitivity and specificity for the diagnosis of the American trypanosomiasis. We also assessed the stability of the fixed-form EIA under conditions of possible use (ie: long-term storage at various temperatures).

We used a panel of 435 sera to evaluate the candidate assays. The cases were Venezuelan blood donors (n=70) defined as positive by a battery of tests including immunofluorescence, indirect hemagglutination and EIA. Negative controls included 85 Venezuelan blood donors and 166 non-traveling and healthy Canadians. Specificity was assessed using sera from 114 subjects with other parasitic diseases: leishmaniasis (n=20), ascariasis (n=6) fasciolosis (n= 8), malaria (n=23), toxoplasmosis (n= 17), trichinosis (n= 11), filariasis (n= 8), cysticercosis (n= 8) and schistosomiasis (n=13).

T. cruzi antigens

Epimastigotes (Tulahuen and Brazil strains) were grown in LIT broth as previously described (11). Parasites were washed three times in 1M phosphate-buffered saline (PBS; pH 7.4) and fixed in 2% formaldehyde (Fisher, Lawn, NJ)-PBS (pH 7.4) for 1 hour at 37°C. To obtain trypomastigote and amastigote forms, Vero cell monolayers (ATCC CCL-81) were infected with Tulahuen or Brazil strain epimastigotes and supernatants were harvested at either 7 days or 15 days respectively as previously described (7, 21). Harvested

amastigotes were placed in LIT medium at 37°C for a further 48 hours. Final purification was achieved by column (17) or gradient as previously described (4) before formaldehyde fixation (1.5% trypomastigotes and 2% amastigotes) as above. The organisms were counted in a Neubauer chamber and final antigen preparations were produced by mixing the Tulahuen and Brazil strains (50/50). The antigens were stored in aliquots at –20°C until used. Morphology (Giemsa-stained preparations) was used as the principal criterion for assessing antigen purity: epimastigote 100% (yield: $1x10^8$-$x10^9$ per mL), trypomastigote 97% (yield: 0.6–7 x 10^7 per mL) and amastigotes 100% (yield: $1x10^8$-$x10^9$ per mL) (data not shown).

RESULTS AND DISCUSSION

Polystyrene 96-well plates (Immulon 2, Thermo Labsystems, Franklyn, MA) were coated overnight with 100 µL/well of fixed *T. cruzi* antigen ($1x10^6$ epimastigotes, $1x10^5$ amastigotes or $1x10^5$ trypomastigotes per mL) at 4 °C in 1M sodium carbonate buffer (pH 9.6). Plates were incubated with blocking buffer (PBS-5% bovine serum albumin (Sigma)-0.1% Tween for 1 hour at 37°C. Sera were diluted 1:400 in blocking buffer, added in duplicate to wells (100 µL/well) and incubated 1 hour at 37°C. The assays were completed with HRP-conjugated goat, anti-human IgG for 30 minutes at 37 °C (Perkin Elmer Life Science, Boston, MA: 100 µL/well) and then 3,3′, 5,5′-tetramethylbenzidine (100 µL/well) for ten minutes at room temperature. The reaction was stopped with IN sulphuric acid

(H_2SO_4: 50 μL/mL) and read at 450 nm (Titertek Multiskan MCC/340, Labsystem and Row Laboratories, Finland). Results from the 176 healthy control sera were used to establish cut-off values that yielded optimal sensitivity and specificity for each assay.

After assay optimization, negative control values were low and tightly clustered in all three fixed-organism EIAs. Mean OD values for the Canadian negative control sera were 0.09 ± 0.06, 0.07 ± 0.03 and 0.09 ± 0.05 for the epimastigote, trypomastigote and amastigote assays respectively. Sera from Venezuelan negative control had significantly higher background 'noise' in the amastigote ($P < 0.03$), trypomastigote ($P < 0.0001$) and for epimastigote ($P < 0.001$) assays. Among the healthy controls sera, a linear model indicated a significant difference among the three antigens ($P < 0.0001$). Tukey's post hoc tests revealed the following order among the assays (from least reactive to most reactive): trypomastigote < epimastigote = amastigote (Table 1). As expected, the mean OD values for the sera from subjects with other parasitic diseases were more variable: epimastigote 0.09 ± 0.19, trypomastigote 0.07 ± 0.17 and amastigote 0.12 ± 0.21. For this group of patients the linear model indicated that the OD values did not differ among antigens ($P = 0.14$). The mean ODs for the known positive sera were 1.98 ± 0.32, 1.65 ± 0.42 and 1.79 ± 0.49 for the epimastigote, trypomastigote and amastigote assays respectively. ANOVA showed significant differences between the mean ODs among the three assays ($P < 0.0001$). Tukey's post hoc tests revealed the following order among antigens: (most reactive to least reactive) epimastigote> trypomastigote = amastigote (Table 1).

Cut-off values between OD = 0.20 - 0.40 yielded excellent sensitivity (100%) with a range of specificities for the fixed-antigen EIAs: epimastigote (86.2 – 98.3%), amastigote (84.8 – 97.6%) and trypomastigote (93.8 - 99.3%). When specificity was assessed for each serum group at a cut-off value of 0.40 (Table 1) the trypomastigote-based assay performed best with both healthy patient sera (100%) and sera from patients with other parasitic diseases (98.2%). The presumed 'false-positive' reactions (epimastigote = 5, trypomastigote = 2 and amastigote = 7) occurred with sera from subjects with known protozoan (eg: leishmaniasis, malaria) or helminth infections (eg: ascariasis, fascioliasis, schistosomiasis) and one healthy control (Table 1).

Our first hypothesis that a simple EIA based upon fixed, whole organisms could be sensitive, specific and practical for field application, proved to be correct. Using readily accessible materials and straightforward techniques, we were able to generate large numbers of essentially pure epimastigote, trypomastigote and amastigote forms. These assays performed well with our panel of sera, confirming the earlier observations of several groups (5, 10, 13, 14). As noted above, these early studies focused almost exclusively on epimastigote antigens. Only Araujo & Guptill included amastigote forms (6) but they tested only a small number of positive and negative sera and no sera from subjects with other parasitic diseases were included.

The second of our initial hypotheses that fixed amastigote or trypomastigote antigens would be superior to epimastigote antigens in the EIA format, proved to be largely incorrect. The trypomastigote-based assay was slightly better than the other two assays in terms of specificity and this advantage was accentuated at lower arbitrary cut-off values (Table 1). These data suggest that there is extensive sharing of antigens among the three forms of the parasite (3). It is also possible that the *in vitro* culture systems used by us and by others do not generate the antigenic diversity that may exist in the different forms in natural infection (26, 32). We found it relatively simple to generate large numbers of morphologically homogeneous trypomastigote-forms and the potential for trypomastigote antigens has not been well studied. Indeed, we could only find a single trial that used a crude extract antigen in an EIA format (7). To our knowledge, no studies using fixed, whole trypomastigote-forms have been published to date.

Since antigen stability and assay consistency are major concerns for many of the reported Chagas' disease assays, we sought to determine the stability of the fixed trypomastigote assay over time under realistic storage conditions. Half of the wells of EIA plates were pre-coated and stored with desiccant sachets at 4°C or room temperature (RT) for up to four months. At monthly intervals, the unused wells were coated with frozen stock trypomastigote antigen and the EIA procedure outlined above was completed for pre-coated and freshly coated wells in parallel. Using multiple replicates of pooled negative and positive sera, we observed excellent maintenance of reactivity without upwards drift in the

negative controls over the 4-month period. Indeed, the positive samples had generally higher absorbance values in the pre-coated than the freshly coated wells on plates stored either at 4°C (2.39 ± 0.235 vs 1.52 ± 0.326 respectively; $P < 0.006$) or at room temperature (2.13 ± 0.169 vs 1.60 ± 0.234; $P < 0.0001$) respectively. Compared with the freshly-coated wells, there was also less variation in the precoated wells stored either at 4°C (CV positives: 21.5 vs 9.83, CV negatives: 87.0 vs 23.58) or RT (CV positives:14.6 vs 7.95, CV negatives: 88.7 vs 19.38). Similar assay stability has recently been reported for up to 12 months and at temperatures up to 50°C by Rebeski *et al* in a whole, fixed *T. congolense* EIA (29, 30).

Several limitations of the current study should be acknowledged. Most importantly, the Chagas' disease-positive sera used were known to be reactive in a battery of other serodiagnostic tests. As a result, our estimates for assay sensitivity are almost certainly artificially high. This fact may also contribute to the very high signal: noise ratios we observed. Second, no samples from subjects with visceral leishmaniasis were included in our panel of 'other parasitic diseases', so our specificity estimates are also likely to be slightly elevated for regions where *T. cruzi* and *L. chagasi* are both endemic. Although two different *T. cruzi* strains were used to generate the antigens we used, the fact that all of the Chagas' disease-positive sera were obtained from a single geographic region should also be considered in interpreting these data.

In conclusion, we believe that the use of formalin-fixed, whole-organism antigens has several advantages compared with most other antigen preparations reported to date for use in the EIA format. These include ease of preparation and quantification (eg: hemocytometer) and stability for prolonged periods of time at a range of temperatures. We have shown that fixed, whole-organism EIAs based on epimastigote, trypomastigote or amastigote forms of *T. cruzi* can have excellent sensitivities and specificities. These assays were reproducible and robust under various storage conditions. The trypomastigote-based test has advantages over the assays in terms of specificity in our initial evaluation. As with all novel assays for *T. cruzi*, the performance of these assays in the field will determine their eventual clinical utility.

ACKKNOWLEDGMENTS

This work was supported by Health Canada grant # HT070-010033.

We thank Dr. Herbert Tanowitz (Einstein University of Medicine, New York) for providing the *T. cruzi* strains. Evelyn Kokoskin (McGill Centre for Tropical Diseases, McGill University, Montreal, Canada) for her technical support and Guy Beauchamp (Université de Montréal) for the statistic support.

Doctoral scholarship support for M. Berrizbeitia was provided by the Universidad de Oriente (UDO), Venezuela.

TABLE 1. Mean, standard deviation (SD), range optical density (OD) and specificity estimates for serum groups in EIAs based on formalin-fixed forms amastigote, epimastigote or trypomastigote forms of *T. cruzi*.

Antigen	Mean (OD) ± SD[a]	Range OD Min – max	Specificity% (OD 0.200)	Specificity% (OD 0.400)
Canadians Healthy patients (n = 166)				
Amastigote	0.088± 0.048	0.025-0.445	97.59	99.40
Epimastigote	0.088 ± 0.065	0.034 – 0.551	92.77	99.40
Trypomastigote	0.068 ± 0.033	0.027-0.310	99.40	100
Venezuelan Healthy patients (n= 85)				
Amastigote[b]	0.121±0.055*	0.055-0.254*	90*	100*
Epimastigote	0.121±0.037	0.061-0.263	95.29	100
Trypomastigote	0.108±0.034	0.070-0.293	97.65	100
Patients with other parasite diseases (n = 114)				
Amastigote	0.116 ± 0.206	-0.042-2.040	65.79	94.74
Epimastigote	0.085 ± 0.190	-0.045-1.864	75.44	96.49
Trypomastigote	0.070 ± 0.168	-0.026-1.638	85.09	98.24
Chagas positive patients (n = 70)				
Amastigote	1.790± 0.496	0.832-2.771	-	-
Epimastigote	1.981± 0.315	1.143-2.619	-	-
Trypomastigote	1.648± 0.423	0.648-2.280	-	-

[a]Values are expressed as means ± standard deviation (SD). The following numbers of presumed 'false positive' reactions using a cut-off OD 0.40: amastigote-based assay, four subjects with cutaneous leishmaniasis, one with ascariasis, one with fascioliasis, and one who was healthy; trypomastigote-based assay, one subject with cutaneous leishmaniasis, and one with schistosomiasis; epimastigote-based assay, two subjects with cutaneous leishmaniasis, two with malaria, and one who was healthy subject.

[b]Only 10 sera from healthy Venezuelan blood donors were tested in the amastigote-based assay.

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ARTICLE II

Purified excreted-secreted antigens from *Trypanosoma cruzi* trypomastigotes as diagnostic tools for Chagas' disease

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Role of the candidate in conception of this article:

I was the main author of this article. I have actively participated in the initiative and design of the experiments, and the standardization techniques. I carried out all the laboratory work and also the analysis of results. Finally, I wrote the manuscript

ABSTRACT

There is currently no 'gold standard' test for the diagnosis of late-stage Chagas disease. As a result, protection of the blood supply in Chagas endemic areas remains problematic. A panel of 709 sera from subjects with confirmed Chagas' disease (n=195), healthy controls (n=400), and patients with other parasitic diseases (n=114), was used to assess enzyme-linked immunosorbent assays (EIA) based on a concentrated extract of excretory-secretory antigens from the Brazil or Tulahuen strains *Trypanosoma cruzi* trypomastigotes (total TESA). The total TESA assays had excellent overall sensitivity (100%) and specificity (>94%), except for cross-reactivity with *Leishmania* sera. In an attempt to increase specificity, immunoaffinity chromatography was used to purify TESA proteins ($TESA_{IA}$). By Western blot, a series of polypeptide bands with molecular weights ranging from 60-220 kDa were recognized by positive Chagas' disease pooled sera. An EIA based on $TESA_{IA}$ proteins had slightly lower sensitivity (98.6%) but improved specificity (100%) compared to the total TESA EIAs. A 60 kDa polypeptide was identified as a major contributor to the cross-reactivity with *Leishmania.* These data suggest the need for field validation studies of TESA- and $TESA_{IA}$-based assays in endemic regions.

INTRODUCTION

Trypanosoma cruzi is the causative agent of Chagas' disease, a major health problem in Central and South America (31). A large number of antigen preparations have been used for the serological diagnosis of this infection (1-5, 7, 8, 11, 12, 15, 18-20, 22, 27, 28). Of these preparations, trypomastigote excretory-secretory antigens (TESA) appear to provide good sensitivity and specificity as diagnostic reagents for Chagas' disease. Major components of TESA proteins are transialidases secreted into the culture supernatant (27, 29). Parasite transialidases are implicated in penetration of host cells by transferring exogenous sialic acid to acceptor molecules located on the trypomastigote surface. Transialidases are highly immunogenic and both the C- and N-terminal regions stimulate strong B-cell responses (9, 24). Transialidase-like proteins are predominant antigens on the surface of bloodstream trypomastigotes, metacyclic trypomastigotes and intracellular amastigotes (13).

Both enzyme-linked immunoabsorbent (EIA) and immunoblot assays have been reported for the diagnosis of Chagas' disease using TESA proteins. Immunoblots have typically been used in parasite diagnostics to rule out false positive EIA results (27). However, immunoblots are expensive, difficult to standardize and time consuming. In contrast, the EIA format is inexpensive, simple to automate and rapid (20). To date, the TESA proteins evaluated for diagnostic use in Chagas' disease have been complex mixtures that cross-react with sera from subjects with other parasitic diseases, particularly leishmaniasis (28). We evaluated the performance of an EIA based on concentrated or immunoaffinity-purified TESA proteins for the diagnosis of latent Chagas disease. Both assays performed

well compared with current serologic tests employed in Central and South American blood banks. Of particular note, we identified a 60 kDa TESA protein that contributes significantly to the known cross-reactivity with *Leishmania* sera.

MATERIALS AND METHODS

Serum samples: A panel of 709 sera was used in this study. One hundred ninety five samples were obtained from Venezuelan subjects with Chagas disease confirmed by a battery of three different serological tests including immunofluorescence, indirect hemagglutination and EIA in the National Chagas Immunodiagnosis Laboratory (NCIL: Maracay, Venezuela). Samples were considered 'positive' if 2/3 assays were positive. Sera from 114 subjects suffering with the following parasitic diseases were obtained from the Canadian Reference Centre for Parasitology (NRCP) serum bank: leishmaniasis (n=20), ascariasis (n=6) fasciolosis (n= 8), malaria (n=23), toxoplasmosis (n= 17), trichinosis (n= 11), filariasis (n= 8), cysticercosis (n= 8) and schistosomiasis (n=13). Sera from 234 consecutive healthy Venezuelan blood donors negative by in all three serological tests performed by the NCLI and 166 healthy, non-travelling Canadians were also tested as controls.

TESA Proteins: TESA proteins from two *T. cruzi* strains (Tulahuen and Brazil) were obtained from infected Vero cells supernatants, as described by Umezawa *et al.* (27) with slight modifications. Briefly, Vero cell monolayers at 65% confluence were infected with *T. cruzi* trypomastigotes (1×10^9 parasites/mL/175cm^2) and incubated at 37°C with 5% CO_2 for 4 days in Eagle's minimum essential media (EMEM; Wisent, St. Bruno, Quebec, Canada), supplemented with 1M Hepes (1%) and gentamycin reagent solution (0.1%) without fetal

bovine serum (FBS) and phenol red. After four days of incubation, the infected monolayers were washed twice with the media and re-incubated for 18-20 hours at 37°C in 5% CO_2 in complete media. Supernatants were then harvested and centrifuged at 2,800 x *g* for 15 minutes at 4°C, and filtered through a Millipore membrane (0.22 μm) (Bradford, MA). Supernatant proteins were concentrated ~32-fold (Amicon Ultra device: 30,000 MWCO, Bradford, MA) and either used immediately or stored at –80°C. Total concentrated TESA retained the high molecular weight polypeptide bands (150-170 kDa), which correspond to the most immunogenic antigens (20). Protein content of the FBS-free TESA was quantified using the Micro-BCA protein assay reagent kit (Pierce Co, Rockford, IL).

Purification of chagasic antibodies: Antibodies with specificity for *T. cruzi* were purified following the procedure described by Curtis and Chase (10), with some modifications. A pool of sera from subjects with serologically confirmed Chagas disease (10 mL) and confirmed clinical and serological negatives for leishmaniasis in NCLI was precipitated with 50% ammonium sulphate at 4 °C, for 24 hours, then centrifuged at 3000 x *g*, for 5 minutes. The resulting pellet was re-suspended in 10 mL distilled water, then, 37% ammonium sulphate (vol/vol) was added and incubated for 24 hours. After centrifugation at 3000 x g for 5 minutes, the final pellet was re-suspended in 10 mL of 0.02 M phosphate buffer (pH 8.0), and the residual ammonium sulphate was removed by dialysis (0.02 M phosphate buffer for 24 hours, at 4 °C). The final antibody concentration was determined by spectrophotometry using the extinction coefficient (1.4) as previously described (14). Antibodies were purified by chromatography on a DEAE-cellulose column, equilibrated with 0.02 M phosphate buffer (pH 8.0). Column elution fractions were spectrophotometrically

monitored at 280 nm; and protein purity was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing antibodies were pooled, and final protein concentration was determined as reported by Bradford (6). Purified antibodies were dialysed 24 hours against 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.5) at 4 °C, and coupled to CNBr-activated Sepharose 4 Fast Flow following the manufacturer's instructions (Amersham Biosciences)

Immunoaffinity purification of TESA proteins: Aliquots of the total concentrated TESA (850 μg/mL) were mixed with the chagasic antibodies-Sepharose resin, and incubated overnight in a rotator, at 4°C. After washing unbound proteins with 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.5), adsorbed antigens were eluted with 0.1 M glycine (pH 2.5). Fractions of 1 mL were collected in tubes containing 0.1 mL of 1M Tris-HCL (pH 8.0) to neutralize the eluting buffer. Fractions were read at 280 nm and the purity of eluted proteins was assayed by SDS-PAGE followed by silver staining. Fractions containing the immunoaffinity-purified proteins ($TESA_{IA}$) were dialysed against PBS (pH 7.4 for 24 hours).

TESA-based EIAs: Coating of 96-well polystyrene plates (Immulon 2; Thermo Labsystems, Franklin, Mass.) was accomplished by incubating either total TESA protein (1 μg/mL) or $TESA_{IA}$ (3 μg/mL), at 4°C, overnight (100 μL/well), in 1M sodium carbonate buffer (pH 9.6). Plates were washed four times with phosphate-buffered saline pH 7.4 (PBS, 0.01 M phosphate buffer, 0.14 M NaCl) containing 0.05% Tween 20 (PBST) (A&C, American Chemicals LTD, St-Laurent, Quebec), blocked for one hour at 37°C with either PBS containing 5% BSA (Sigma, St Louis, MI) and 0.1% Tween 20, in the case of the total

TESA proteins, or with PBS containing 5% skim milk (Parmalat, Quebec, Canada) and 0.1% Tween 20, in the case of $TESA_{IA}$. Sera were diluted at 1:800 (total TESA) or 1:200 ($TESA_{IA}$) in the corresponding blocking solution (100 µl/well), and incubated for 1 hour at 37°C. These dilutions were selected to permit optimal differentiation between positive and negative control sera, as tested by checkerboard titration (data not shown). Assays were completed with an optimal dilution of HRP-conjugated goat, anti-human IgG (30 minutes at 37°C) (Perkin Elmer Life Science, Boston, MA, 100 µL/well), four washes with PBST and a final incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) (Serologicals Corporation, MA, USA) (100 µL/well) for 10 minutes at room temperature. The reaction was stopped with 1N H_2SO_4 (50 µl/well). Optical density was measured at 450 nm using an automated EIA reader (Titertek Multiskan MCC/340, Labsystem and Row Laboratories, Finland or TECAN EIA reader). All experiments were performed in duplicate on different days and pooled, NCLI-confirmed positive and negative controls were included on each plate. Results were accepted only when the coefficient of variation within and among plates was ≤15%; otherwise, the samples were tested again. Results from healthy control sera were used to establish cut-off values that yielded optimal sensitivity and specificity results for each assay.

SDS-PAGE: Protein purity was evaluated by SDS-PAGE carried out on 1.5 mm thick slabs containing 7% (for $TESA_{IA}$) or 11% (for purified antibodies) polyacrylamide according to Laemmli (16). Electrophoresis was performed for 2 hours, in Tris-glycine electrode buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3), at a constant voltage of 100 V. Coomassie blue R-250 (for purified antibodies) or silver staining (for $TESA_{IA}$) was employed

for protein visualization. Broad range molecular weight markers (Promega, Madison, WI) and pre-stained protein standards (Invitrogen, Carlsbad, CA) were included in each run.

Western blot analyses: Proteins separated by SDS-PAGE were transferred to nitrocellulose sheets (MFS, Pleasanton, CA) according to Towbin *et al.* (26) using a minitank electroblotter (Owl Scientific, Woburn, MA). Transfer was performed for 1 hour, at 4°C, in 25 mM Tris, 192 mM glycine, 20% v/v methanol (pH 8.3) at a constant current of 0.25 A. Membranes were blocked overnight, at 4°C, with PBS containing 5% skim milk (Parmalat) and 0.1% Tween 20, and were incubated for 2 hours with sera diluted 1:400 in the blocking solution. Following four 5 minute washes with PBS containing 0.05% Tween 20 (PBST), the antigenically reacting proteins were incubated for 2 hours with the appropriate dilution of horseradish peroxidase (HRP)-conjugated goat, anti-human IgG diluted in PBS containing 5% skim milk and 0.1% Tween 20. Nitrocellulose filters were then washed four times with PBST, and the immune complexes were revealed by chemiluminescence (Super signal West Pico, Pierce, Rockford, IL).

Statistical analyses: statistical analyses were performed using SAS [SAS: V.8.2 (Cary, N.C)]. Sensitivity and specificity were determined by examining all possible cut-off values. Analyses of variance (linear model, ANOVA) were utilized to examine the effect of parasite type on optical density in each study group. Tukey's post hoc test was employed to evaluate differences between parasite antigens. Comparison among tests was determined using the kappa index. Values of $P < 0.05$ were considered significant. The Chi squared test

was used to determine significant differences in specificity. The sensitivities and specificities of the tests were evaluated over a range of arbitrary optical density (OD) cut-off values (OD 0.2 to 0.4).

RESULTS

Concentrated TESA fraction: The total TESA was concentrated by an Amicon Ultra-device that maintains the high molecular weight polypeptide bands (150-170 kDa), which correspond to the most immunogenic and specific antigens from this fraction (20). The protein concentration determined using Micro BCA for both TESA before (550 μg/ml) and after concentration (850 μg/ml) was very similar for both strains. However, the optical density (OD) values obtained for total TESA from the Brazil- and Tulahuen-based EIAs before and after concentrating were significantly different for a pool of positive sera by ANOVA (*P* values < 0.0001; TABLE 1). In all cases, the assays based on concentrated TESA had higher OD values than the corresponding assays based on unconcentrated antigen. The increment in OD values was not the result of changes in background signal (Table 1).

EIA based upon total TESA proteins: The sensitivity of the EIAs based on total TESA was excellent for antigens from both strains (100%), at several cut-off values (OD = 0.200 to 0.400) (Table 2). The assay based upon antigens derived from the Tulahuen strain showed slightly higher specificity at the various cut-off values. However, when results from all the negative controls were combined for evaluation, these differences did not reach statistical significance (Table 2). There were minor differences in the specificity estimates observed between Tulahuen- and Brazil-based assays in the individual control populations at

various cut-off values (Table 3). However, the agreement between tests (Kappa index) was excellent and increased progressively at the higher cut-off values (Table 2). No differences were observed in terms of OD between the assays based on the different strains for sera from Chagasic patients, healthy patients from an endemic area or for patients with other parasitic diseases. The mean OD values for sera from patients with leishmaniasis were generally higher than those observed in the other groups of healthy and parasitic disease controls (Table 3).

Purification of total TESA proteins by immunoaffinity chromatography: $TESA_{IA}$ proteins from the total Tulahuen and Brazil TESA had essentially the same pattern of purified bands in the peak fraction (Tulahuen: peak fraction 9, Brazil: peak fraction 23) (Figure 1). When total TESA proteins were compared with the corresponding $TESA_{IA}$, far fewer polypeptide bands were observed by silver staining (Figure 1A and B). Western blots of total TESA and $TESA_{IA}$ antigen preparations using a pool of positive chagasic sera also revealed far fewer bands with prominent polypeptide bands at 220, 170, 120, 85 and 60 kDa for both strains (Figure 2, data for Brazil strain not shown).

The $TESA_{IA}$ proteins were further evaluated by Western blot using a panel of 17 sera from NCLI-confirmed Chagas patients, 9 sera from healthy NCLI-seronegative individuals from an endemic area, and 10 sera from patients with clinically-confirmed mucocutaneous leishmaniasis. These last subjects had no clinical evidence of Chagas disease and had strongly positive serologic tests (indirect immunofluorescence and indirect hemagglutination) for leishmaniasis. Polypeptides with molecular weights of 170,000 (17/17), 120,000 (16/17) 85,000 (17/17) and 60,000 (15/17) were recognized by the Chagasic sera (Table 4). Sera from

healthy individuals and subjects with leishmaniasis did not react with the 170-, 120- or 85-kDa polypeptide bands but 3/10 of the sera from the leishmaniasis patients reacted with the 60-kDa polypeptide band. This observation suggests that this polypeptide is partially responsible for the known serologic cross-reactivity between these two parasitic diseases. Further evaluation with a larger number of sera from patients with leishmaniasis is required to confirm this result (Figure 3).

EIA based upon immunopurified TESA proteins ($TESA_{IA}$): We used a panel of 166 sera to perform a preliminary evaluation comparing the total TESA- and $TESA_{IA}$-based EIAs (Tulahuen strain). Seventy-four out of 75 sera from subjects with confirmed Chagas' disease were reactive in the $TESA_{IA}$-based assay using a cut-off OD value of 0.300 (Sensitivity 98,67%, 95%CI 96-100%). None of the sera from healthy individuals (endemic region) (n=75) or from patients with leishmaniasis (n=16) were reactive in this assay (Specificity 100%). The mean OD values for Chagasic sera, sera from healthy patients and leishmaniasis positive sera were 1.043±0.334 (range 0.298-1.735), 0.166±0.046 (range 0.086-0.284) and 0.145±0.048 (range 0.083- 0.242), respectively. The mean OD for the *Leishmania*-positive sera was considerably lower in the optimized $TESA_{IA}$–based EIA (0.145±0.048) compared to the mean values obtained in the total TESA-based assays using either Tulahuen (0.253± 0.481) or Brazil strain (0.300± 0.481). These differences did not reach statistical significance however due to the wide variability seen in the total TESA-based assays. Compared to results from the optimized EIAs based on total TESA, the optimized assay based on $TESA_{IA}$ had slightly lower sensitivity (98.7% vs 100%) but a slightly higher specificity (100% vs total TESA Brazil and Tulahuen: 97.8 and 96.7 respectively) (Table 5).

DISCUSSION

The use of TESA proteins for the diagnosis of Chagas' disease has been reported with generally good results in terms of specificity and sensitivity (15, 18, 20, 27, 28). However, most studies have tested relatively small numbers of samples (18, 20, 28), and some have used TESA proteins from a single strain (27, 28). In the current study, we used a large panel of well-defined sera to evaluate EIA assays based on TESA proteins from two *T. cruzi* strains from different geographic regions (Brazil and Tulahuen). We demonstrated for the first time the utility of concentrating the most antigenic components of TESA, presumably by eliminating interfering low molecular weight peptides/proteins. In our hands, this simple manipulation increased the signal-to-noise ratio dramatically for the total TESA Brazil antigen in particular (~15-fold). We also show, for the first time, that immunoaffinity-purification of TESA proteins can significantly enhance specificity with only a slight loss of sensitivity. Finally, our observations implicate a 60 kDa protein in the known serologic cross-reactivity that confounds the evaluation of patients and the screening of blood in regions where both Chagas disease and leishmaniasis are endemic.

We chose to test our concentrated total TESA EIAs using samples obtained from a blood bank in a Chagas disease endemic region (ie: a real life situation in which any Chagas disease assay would be likely to be used). Both the Brazil- and Tulahuen-based assays had excellent sensitivity for the confirmed positive blood bank specimens (100%) and excellent specificity for samples from healthy control subjects from both endemic and non-endemic

regions (99-100%). Furthermore, the tests maintained very reasonable specificity even when large numbers of samples from subjects with other parasitic diseases were tested (94-99%). As has been previously reported (19, 27), sera from patients infected with *Leishmania spp* were the most likely to cross-react in our TESA assays (2/20 presumed false-positive results for patients with cutaneous leishmaniasis in both the Brazil and Tulahuen antigen-based assays). We only analysed positives samples from one endemic region (Venezuela) a further study could be conducted to know the performance of these strains with samples from other endemic regions of Latin America. Moreover, our specificity estimates of ~90% for samples from leishmaniasis patients is likely to be an over-estimate since no visceral leishmaniasis samples were included in our panel. Our data suggest that the concentrated total TESA antigen from both Brazil and Tulahuen strains includes conserved epitopes that are highly immunogenic, meeting one of the principal criteria for Chagas disease tests suggested by the WHO (32).

During the course of these studies, in addition to confirming the potential utility of the TESA antigens in Chagas disease diagnosis, we were also able to address several practical issues relevant to the use of these assays. Large amounts of TESA proteins could be harvested with simple and inexpensive manipulations of relatively small volumes of trypomastigote cultures (two 175cm^2 Vero cell monolayer yielded ~5 mL of concentrated TESA proteins at ~850 μg/mL). Pooled total and concentrated TESA proteins were stable when frozen as aliquots at –80°C for at least 18 months, suffering no loss of reactivity or increase in background 'noise' (data not shown). The anticipated yield from only two infected 175cm^2 flask would permit the coating of approximately 420 EIA plates and the performance of

16,800 tests. The 32-fold concentration of total TESA proteins that we achieved with a 30,000 molecular weight cut-off filter resulted in marked increases in the signal-to-noise ratio, simplifying assay interpretation and the establishment of optimal cut-off values for specificity. It is likely that this procedure eliminates small molecular weight proteins/peptides that interfere with the assay.

To our knowledge, the potential impact of immunoaffinity purification of TESA has not been previously reported. In our hands, this step eliminated almost all of the complexity present in the total TESA protein mixtures, reducing the preparation from many bands to essentially 5 bands of molecular weights 220, 170, 120, 85 and 60 kDa. The purification procedure consisted of a single step using a Sepharose column coupled to pooled antibodies from Chagasic patients. This approach resulted in a high yield of purified proteins (190 μg/mL from an initial 850 μg/mL concentrated TESA) and had the advantage that the immunoaffinity column could be re-used several times. Data for the $TESA_{IA}$-based EIA should be considered preliminary however because we performed only a small-scale purification that yielded enough material to test 166 of the 709 sera in our panel. A larger-scale purification of $TESA_{IA}$ and broader field studies of the $TESA_{IA}$ EIA with different strains of *T. cruzi* are planned.

Many TESA antigens have recently been recognized as transialidases. These enzymes may be virulence factors and they participate in binding to non-infected epithelial, fibroblast and muscle mammalian cell lines (9, 24). They are strong immunogens, eliciting polyclonal antibody responses and are implicated in the induction of both inflammation and

autoimmunity (21, 30). A monoclonal antibody (mAb39), that recognizes parasite transialidases detects polypeptide bands ranging from 85- to 170-kDa in TESA proteins (23). Several of these transialidase bands are of similar molecular weight to the polypeptides present in our $TESA_{IA}$ preparation. Silber and colleagues employed chromatography to purify *T. cruzi* proteins from total parasite extracts for diagnostic purposes in Chagas disease. They identified a 67-kDa lectin-like protein that binds to human erythrocyte membranes in a galactose-dependent fashion (25) and evaluated its potential as a diagnostic reagent (17). Although they reported good sensitivity (98%) and specificity (98%) in an EIA format, relatively small numbers of positive sera were tested and only sera from healthy subjects were included as negative controls. Studies to identify the proteins present in our $TESA_{IA}$ preparation are currently underway.

To our knowledge, we are the first to implicate a 60 kDa protein in the serologic cross-reactivity between *Leishmania* and Chagas disease samples. Cross-reactivity in an EIA based on TESA antigens has previously been reported for subjects with visceral and cutaneous leishmaniasis (20). In this study, Nakazawa and colleagues (20) implicated polypeptides $\leq$ 150 kDa as cross-reactive but did not carry the characterization any further. Our immunoaffinity purified $TESA_{IA}$ antigen also contains a 60 kDa protein yet no cross reactivity was observed in the 16 *Leishmania* samples tested including the samples presumed to be false-positive in the total TESA EIA. The mean ODs for the Leishmania positive sera were higher in the total TESA antigen EIAs than in the $TESA_{IA}$ EIA suggesting that immunoaffinity purification may eliminate background 'noise' that interferes with assay specificity. Alternately, the improved specificity of the $TESA_{IA}$ EIA despite the presence of a

60 kDa band in the $TESA_{IA}$ preparation by Western blot raises the possibility that two proteins of similar molecular weight are present in the total, concentrated TESA. Studies are underway to determine whether or not removal of the reactive 60 kDa protein(s) from total TESA by molecular exclusion chromatography can achieve improved specificity while maintaining high sensitivity.

In summary, we have demonstrated that concentrated total TESA proteins are simple and inexpensive to produce, are stable at –80°C for prolonged periods of time and have excellent sensitivity and specificity in an EIA format. Using a simple protocol based on pooled Chagasic antibodies, we have shown for the first time that immunoaffinity purified TESA proteins can be used to significantly enhance EIA specificity. These observations raise the possibility of sequential total TESA EIA followed by $TESA_{IA}$ EIA testing as a simple and cost-effective strategy for Chagas disease screening in blood bank samples.

ACKNOWLEDGMENTS

This work was supported by Health Canada grant # HT070-010033 and partially supported by a grant from FONACIT N° LAB-2000001639, Caracas, Venezuela, to Dr. José Bubis. We thank Dr. Herbert Tanowitz (Einstein University of Medicine, New York) for providing the *T. cruzi* strains. Deisy Perdomo, Rafael Medina and Dra. Lilian Spencer (Departamento de Biología Celular, Universidad Simón Bolívar, Caracas, Venezuela) for their technical support, Dra. Mary Isabel Gonzatti (Departamento de Biología Celular, Universidad Simón Bolívar, Caracas, Venezuela), for let us used the EIA reader in her laboratory (TECAN), and Guy Beauchamp (Université de Montréal) for the statistic support. Doctoral scholarship support for M. Berrizbeitia was provided by the Universidad de Oriente (UDO), Venezuela.

TABLE 1. Differences in optical densities (OD) in the EIA assays based on total *T. cruzi* TESA proteins prior to and following concentration.

	Total TESA (OD)		Total TESA concentrate (OD)	
	Mean + (OD)± SD	Mean – (OD) ± SD	Mean + (OD) ± SD	Mean - (OD) ± SD
TESA Tulahuen	0.596 ± 0.033	0.078 ± 0.021	2.162 ± 0.143	0.150 ± 0.018
TESA Brazil	0.152 ± 0.013	0.059 ± 0.015	2.237 ± 0.102	0.130 ± 0.003

ANOVA single factor: TESA Tulahuen $P < 0.0001$

TESA Brazil $P < 0.0001$

TABLE 2. Sensitivity, specificity and agreement in the enzyme-linked immunoabsorbent assay using total secreted-excreted antigens (TESA) of *T. cruzi* at different arbitrary optical density (OD) cut-off values (Chagasic patients = 195, non-chagasic = 514).

	TESA Tulahuen	TESA Brazil
EIA cut-off 0.2		
Specificity (%)	96.30	95.33
Sensitivity (%)	100	100
Agreement (kappa)	0.93	
EIA cut-off 0.300		
Specificity	99.03	98.83
Sensitivity	100	100
Agreement (kappa)	0.99	
EIA cut-off 0.400		
Specificity	99.61	99.42
Sensitivity	100	100
Agreement (kappa)	0.99	

Presumed false positive tests that occurred at the 0.4 OD cutoff were: TESA Tulahuen assay - 1 subject with cutaneous leishmaniasis, and 1 healthy control; TESA Brazil assay - 1 subject with cutaneous leishmaniasis, 2 with toxoplasmosis and 1 healthy control.

TABLE 3. Mean, standard deviation (SD) and range of optical density (OD) values as well as specificity estimates for serum groups in EIA based on total secreted and excreted antigens (TESA) from trypomastigote forms of *T. cruzi*.

Antigen	Mean (OD) ± SD	Range OD Min – max	Specificity% (OD 0.200)	Specificity (OD 0.300)	Specificity% (OD 0.400)
Canadian healthy patients (n = 166)					
TESA B	0.069± 0.040	0.019-0.275	98.29	100	100
TESA T	0.060 ± 0.032	0.018 – 0.181	100	100	100
Venezuelan healthy patients (n= 234)					
TESA B	0.088±0.060	0.031-0.820	97.44	99.57	99.57
TESA T	0.087±0.052	0.028-0.476	95.30	99.14	99.57
Patients with other parasite diseases (n = 114)					
TESA B	0.134 ± 0.200	0.027-2.077	94.02	97.86	98.72
TESA T	0.107 ± 0.194	0.018-2.038	96.58	98.72	99.57
Chagas positive patients (n = 195)					
TESA B	1.479± 0.506	0.450-2.628	-	-	-
TESA T	1.558± 0.465	0.437-2.60	-	-	-

Footnote for TABLE 3. Mean OD for patients with other parasitic diseases: Ascariasis (n=6) TESA B (0.145); TESA T (0.127), Cysticercosis (n= 8) TESA B (0.088); TESA T (0.064), Fasciolosis (n= 8) TESA B (0.085); TESA T (0.070), Filariasis (n= 8) TESA B (0.092); TESA T (0.070), Leishmaniasis (n=20) TESA B (0.255); TESA T (0.216), Malaria (n=23) TESA B (0.128); TESA T (0.099), Toxoplasmosis (n= 17) TESA B (0.133); TESA T (0.091), Trichinosis (n= 11) TESA B (0.084); TESA T (0.065), Schistosomiasis (n=13) TESA B (0.082); TESA T (0.073)

TABLE 4. Western-blot of immunopurified TESA proteins ($TESA_{IA}$) using sera from Chagasic, healthy and leishmaniasis patients.

Group of study	Western-blot with $TESA_{IA}$ (% reactive)			
	170 kDa	120 kDa	85 kDa	60 kDa
Chagasic (n = 17)	17 (100.0)	16 (94.1)	17 (100.0)	15 (88.2)
Healthy (n = 9)	0 (0)	0 (0)	0 (0)	0 (0)
Leishmaniasis (n = 10)	0 (0)	0 (0)	0 (0)	3 (30.0)

TABLE 5 Comparison of EIA reactivity in assays based on either total TESA proteins or immunopurified TESA proteins

Antigen	Chagasic sera (n = 75)		Non-chagasic sera (n = 91)		Sensitivity (%)	Specificity (%)
Reactivity	+	-	+	-		
Total TESA T	75		3	88	100	96.70
Total TESA B	75		2	89	100	97.80
Immunopurified TESA	74	1		91	98.67	100

($TESA_{IA}$) using Chagasic and negative control sera (cut-off 0.300).

The following numbers of presumed false positive reactions occurred using an OD cutoff of 0.300: TESA Tulahuen assay - 2 subjects with cutaneous leishmaniasis, and 1 healthy control; TESA Brazil assay - 2 subjects with cutaneous leishmaniasis. Mean OD for the group of positive *Leishmania* patients were 0.253 ± 0.481, 0.300 ± 0.481, 0.145 ± 0.048 for total TESA Tulahuen, total TESA Brazil and $TESA_{IA}$ respectively.

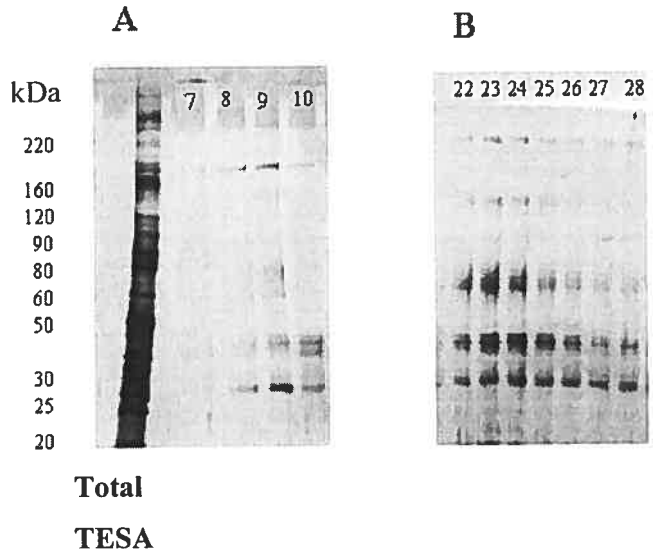

Figure 1. SDS-PAGE separation of the immunopurified proteins from total TESA ($TESA_{IA}$) visualized by silver staining. **A**, Tulahuen strain (fraction 7 to 10, peak in fraction 9); **B**, Brazil strain (fractions 22 to 28, peak in fraction 23).

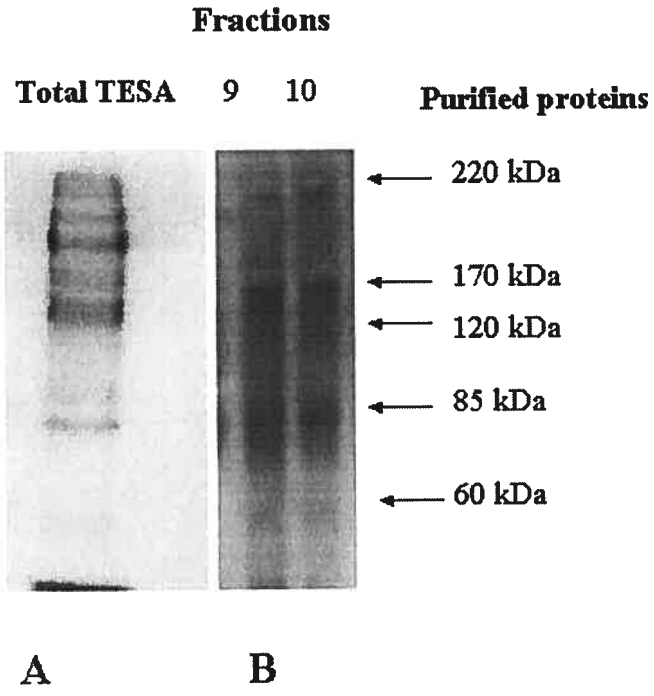

Figure 2. Western blot analysis of sodium dodecyl-polyacrylamide gel electrophoresis of Total TESA (A) and immunopurified fractions from total TESA of *T. cruzi* (B). Tulahuen strain. The dot was probed with a pool of chagasic sera

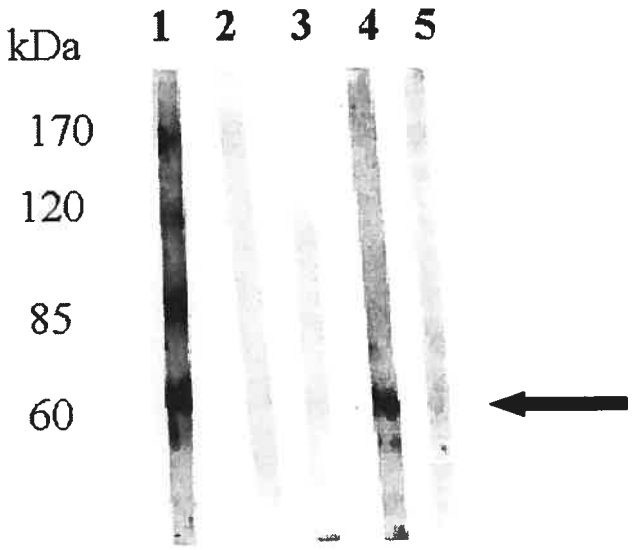

Figure 3. Western blot analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins purifiedby affinity chromatography from total TESA of *T. cruzi* (Tulahuen strain). Chagas positive sera (1), Healthy patient sera (2, 3). *Leishmania* positive sera (4, 5). Arrow (60 kDa band that cross reacts)

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ARTICLE III

Field evaluation of four novel enzyme immunoassays for Chagas' disease in Venezuela blood banks: Comparison of assays using fixed-epimastigotes, fixed-trypomastigotes or trypomastigote secreted-excreted antigens (TESA) from two *T. cruzi* strains

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Role of the candidate in conception of this article: I was the main author of this article. I have actively participated in the initiative and design of the experiments, and the standardization techniques. I carried out all the laboratory work and also the analysis of results. Finally, I wrote the manuscript

BACkGROUND: Many serological tests have been developed for the diagnosis of Chagas' disease but few have been subjected to a rigorous field evaluation. We have recently described several novel enzyme immunoassays (EIA) based on fixed whole organisms or trypanosome excretory-secretory antigens (TESA) from different *T. cruzi* strains (Tulahuen or Brazil). This study evaluated the most promising of these novel assays (eg: fixed-epimastigotes, fixed-trypomastigotes, TESA Brazil and TESA Tulahuen) in a field study of Venezuelan blood bank specimens.

STUDY DESIGN AND METHODS: The assays were tested in an operator-blinded fashion using 2038 blood bank samples obtained from low and high *T. cruzi* prevalence regions of Venezuela (n= 1050 from Bolivar and n = 988 from Portuguesa states respectively).

RESULTS: Based on National Laboratory for Chagas Immunodiagnosis (NLCI) 'gold standard' results, all of novel EIAs were superior to the commercial kit currently used in Venezuela, achieving 100% sensitivity and >99% specificity at optimal cut-off values. The novel assays identified 7 false-negative samples compared with the routine screening performed by the Venezuelan blood bank although 2-3 samples were also misclassified as positive. Minor differences in the performance of the four novel assays were observed at lower arbitrary cut-off values.

CONCLUSION: This study confirms the potential utility of both the fixed-organism and TESA-based assays in the diagnosis of *T. cruzi* infection.

INTRODUCTION

Almost one hundred years after the description of American trypanosomiasis by Carlos Chagas in 1909, this infection remains a major public health problem affecting between 16 to 18 million people in the Americas (61). The disease, caused by the protozoan flagellate *Trypanosoma cruzi*, has an annual incidence of 561,000 cases and results in between 45,000-50,000 deaths each year (33, 46).

Historically, *T. cruzi* infection most commonly occurred following percutaneous exposure to the feces of infected reduviid bug (genus *Triatoma*). However, in the last 2-3 decades, control programs have successfully interrupted vectorial transmission in many regions of Latin America (2, 30, 34, 54). As a result, blood transfusion is now the major risk for transmission in many areas of Latin America. However, transfusion-related Chagas' disease is not limited to endemic areas since tens of thousands of chronically infected subjects have migrated throughout the world in the last half-century and the parasite can be passed vertically from mother to child for several generations (22, 23, 28, 29, 37, 50). In some endemic areas of Latin America, the proportion of *T. cruzi*-infected blood in blood banks can be startlingly high (3% - 53%); much higher than the seroprevalence rates for hepatitis B, hepatitis C or HIV (60). More typically, the *T. cruzi* seroprevalence rates range from 0.20-37.7 in major Latin American blood banks (1, 13, 14, 42, 48, 49, 51, 52), and up to 0.06-4.9 in developed world cites with large migrant populations (9, 22, 25, 27-29). As a result, the screening of blood for Chagas' disease should be a high priority in both endemic regions as well as regions that have welcomed migrants from endemic areas (38).

Since first described by Engvall and Perlmann in 1971 (20), the enzyme immunoassay format (EIA or EIA) has been applied to the diagnosis of virtually all infections including Chagas' disease (56). In fact, an extraordinarily wide range of EIAs based on different *T. cruzi* antigens have been described. These include crude sonicated epimastigotes (3, 10, 35, 40), proteins extracted from epimastigotes (17, 39, 45, 55), fixed whole epimastigotes (5, 12, 18, 19), fixed and sonicated amastigotes (6), sonicated and purified trypomastigote glycoconjugate antigens (3, 7) and trypomastigote excretory-secretory antigens (TESA) (26, 31, 36, 57, 58). Epimastigote antigens have been used in most of these studies (84%) due to ease of culture and good antigen yield (35). Although the reported sensitivities and specificities have been excellent for many of these assays, the large majority of the studies have been relatively small and some of them have not included appropriate controls for specificity assessment. Very few of these candidate assays have been validated in the field. We have recently described several novel EIAs for Chagas' disease based on fixed whole organisms (eg: trypomastigote, epimastigote and amastigote forms) (8) as well as TESA antigens from two *T. cruzi* strains (eg: Brazil and Tulahuen) (submitted for publication). Several of these assays have excellent sensitivities (99-100%) and very acceptable specificities (94-99%) using a large panel of well-defined control sera. The objective of the current study was to assess the most promising of these novel assays (eg: fixed-epimastigotes, fixed-trypomastigotes, TESA Brazil and TESA Tulahuen) in a field study of blood bank specimens in low and high *T. cruzi* prevalence areas of Venezuela.

MATERIALS AND METHODS

Serum samples

We used a panel of 2038 test sera to evaluate the candidate assays. Consecutive serum samples were collected from three different blood banks in Venezuela between September 2003 to May 2004. The Guanare and Acarigua blood banks are located in Portuguesa State West-Central part of the country (n = 988), a region known to have a relatively high prevalence of Chagas' disease (1). The remaining samples were obtained from the Ciudad Bolivar blood bank in Bolivar State, a region in the South of Venezuela close to the Amazon region with low prevalence (n = 1050) (1). We also used a large number of well-defined sera for quality control (QC) in this study including 75 positive- and 75 negative-control samples from Venezuelan patients. These samples were confirmed by the National Laboratory for Chagas Immunodiagnosis (NLCI) in Maracay, Venezuela, using immunofluorescence, indirect hemagglutination and EIA (see definition of positive and negative sera below). An additional 166 samples from non-travelling Canadians were also included as negative controls. All test samples were collected into separator tubes (Vacutainers, Sarstedt, QC, Canada), stored at -20° C and shipped to the laboratory at Universidad Simon Bolivar (Caracas, Venezuela) on dry ice. Samples were subsequently thawed once for distribution in aliquots without personal identifiers and stored at –20°C until used. The age and sex of each donor were obtained from blood bank records.

Fixed whole *T. cruzi antigens*

The epimastigote form of *T. cruzi* (Tulahuen and Brazil strains) was grown in LIT broth: LIT broth 20 g (Difco Labs, Detroit, MI); tryptose (Difco), 5 g; NaCl, 4 g, KCl, 0.4 g; Na_2HPO_4, 8 g; Dextrose, 2 g in one litre of distilled water, pH 7.4 and sterilized by autoclave or filtration. After sterilization, heat-inactivated fetal bovine serum (FBS, Wisent, Inc. St-Bruno, Qc, Canada: final 10%) was added, penicillin/streptomycin (Wisent, Inc: final 1%) and 25 mg/L of hemin (Sigma, St-Louis, MO) dissolved in 50/50 (v/v) triethanolamine (Sigma) and distilled water and sterile filtered. Epimastigotes were collected during the logarithmic growth phase as previously described (15) and washed three times in 1M phosphate-buffered saline (PBS; pH 7.4) by centrifugation at 850 *g* for 15 minutes at 4°C. The parasites were then fixed in 2% formaldehyde (Fisher, Lawn, NJ,)-PBS (pH 7.4) for 1 hour at 4 °C and washed three more times with 1M PBS (pH 7.4).

To obtain trypomastigote forms, tissue culture flasks (175 cm^2) of Vero cells (ATCC CCL-81) were infected with fresh Tulahuen or Brazil strain epimastigotes at a multiplicity of infection of $1x10^9$ parasites/mL. Trypomastigotes were concentrated and purified from the culture supernatant after 7 to 10 days as previously described (24). Briefly, culture supernatants were centrifuged in 50 ml polypropylene tubes (500 x *g* for 10 minutes at 20°C) after which the tubes were incubated at 37 °C for 30 minutes to permit the highly motile, viable trypomastigotes in the pellet to move into the supernatant. The supernatant was then decanted and centrifuged at 1000 x *g* for 20 min at 4 °C. This pellet was resuspended in Cellgro minimal essential medium (Wisent Inc) supplemented with 10% heat-inactivated FBS

and passed through a CM 23-cellulose column (Serva, Heidelberg, Germany). After column purification, trypomastigote forms were washed three times in 1M PBS (pH 7.4) by centrifugation (850 *g* for 15 min at 4ºC) and fixed in 1.5% formaldehyde (Fisher)-PBS (pH 7.4) for 1 hour at 4 ºC. After fixation, parasites were washed three more times with 1M PBS (pH 7.4). The final integral antigen preparations were made by mixing equal volumes of the fixed Tulahuen and Brazil strains and counting in a Neubauer chamber. Fixed, mixed antigens were stored in aliquots at –20ºC until used.

TESA Proteins

TESA proteins from two *T. cruzi* strains (Tulahuen and Brazil) were obtained from infected Vero cells supernatants, as described by Umezawa *et al.*, (57), with slight modifications. Briefly, Vero cell monolayers at 65% confluence were infected with *T. cruzi* trypomastigotes (1 x10^9 parasites/ml/175cm^2) and incubated at 37 ºC with 5% CO_2 for 4 days in Eagle's minimum essential media (EMEM; Wisent Inc), supplemented with 1M Hepes (1%) and gentamicin reagent solution (0.1%) without FBS and phenol red. After four days of incubation, the infected monolayers were washed twice and re-incubated for 18-20 hours, at 37 ºC, in 5% CO_2, in complete media. Supernatants were then harvested and centrifuged at 2,800 x *g*, for 15 minutes at 4 ºC, and filtered through a Millipore membrane (0.22 µm) (Bradford, MA). Supernatant proteins were concentrated ~32-fold (Amicon Ultra device: 30,000 MWCO, Bradford, MA) and either used immediately or stored at –80 ºC. Total concentrated TESA retained the high molecular weight polypeptide bands (150-170 kDa), which correspond to the most immunogenic antigens (36). Protein content of the FBS-free

TESA was quantified using the Micro-BCA protein assay reagent kit (Pierce Co, Rockford, IL).

Routine and Reference Serologic Assays

Routine screening of test sera for Chagas' disease was performed in each of the three blood banks by designated personnel not associated with the study (Pharmatest-EIA Chagas IgG, Guarenas, Venezuela). The NLCI in Venezuela uses a panel of three different serological tests to classify sera: indirect immunofluorescence (IIF) according to the method described by Camargo (11), indirect hemagglutination (IHA) followed the procedure described by Cerisola *et al.*, (16), and a commercial EIA kit obtained from Tecnosuma Centro de Inmunoensayo (UMEIA Chagas (E2), La Habana, Cuba). Reference testing was performed for all samples found to be positive in the standard blood bank screening assay (n = 8) or any of the test EIAs (an additional 9-10 samples). All sera that yielded borderline results in any of our novel EIAs (optical densities (OD) between 0.300-0.400, n= 28) and 140 randomly selected samples negative by the routine screening assay and our four EIAs were also tested by the NLCI. A sample was considered to be a true positive if it was positive in at least two reference tests as recommended by the Pan American Health Organization (14).

Tests Assays

All test assays (eg: fixed-epimastigote, fixed-trypomastigote, TESA Tulahuen and TESA Brazil) were performed as previously described (8, 58). Briefly, 96-well polystyrene

plates (Immulon 2, Thermo Labsystems, Franklyn, MA) were coated overnight with 100 µL/well of *T. cruzi* antigen ($1x10^6$/mL epimastigotes, $1x10^5$/mL trypomastigotes or 1µg/mL of TESA Tulahuen or Brazil antigen) at 4 °C in 1M sodium carbonate buffer (pH 9.6). Plates were washed four times with PBS-0.05% Tween 20 (PBST) (A&C, American Chemicals LTD, St-Laurent, Qc, Canada), blocked with PBS-5% BSA (Sigma)-0.1% Tween for one hour at 37 °C. Sera were diluted 1:400 (fixed epimastigotes and trypomastigotes) and 1:800 for TESA antigens in PBS-5% BSA-Tween 0,1%, added in duplicate to wells (100 µL/well) and incubated 1 hour at 37 °C. After washing four times with PBST, all assays were completed by a 30-minute incubation with HRP-conjugated goat, anti-human IgG at 37°C (Perkin Elmer Life Science, Boston, MA). Plates were washed four times with PBST and H_2O_2 and 3,3′, 5,5′-tetramethylbenzidine (TMB) was added (100 µL/well), permitted to develop for 10 minutes at room temperature and stopped with IN sulphuric acid (H_2SO_4: 50 µL/mL). Optical density was measured at 450 nm by an automated EIA reader (TECAN). All the experiments were carried out in duplicate on two different days; positive and negative control sera were included on each plate. The average optical density obtained was only accepted when the coefficient of variation within and between plates was below 15%; otherwise, samples were re-tested. The performance characteristics of the test EIAs were confirmed using the panel of 316 positive and negative control samples. Based on our previous work (8) and evaluation of the control samples, we selected an OD cut-off of 0.400 to define positive samples. OD values between 0.300 and 0.399 were considered indeterminate and were routinely re-tested before final classification. Samples with OD <0.300 were classified as negative. For the evaluation of specificity, cut-off values between

0.200 and 0.400 were also considered. All test assays were carried out by operators blinded to sample origin and routine blood bank screening EIA results.

STATISTICAL ANALYSES:

All statistical analyses were performed using a statistical software package [(SAS: V.8.2 (Cary, N.C)]. Optimal sensitivity and specificity were determined by examining all possible arbitrary cut-off values between 0.200 – 0.400. We used analyses of variance (linear model, ANOVA) to examine the effect of the type of parasite over the mean optical densities for each test EIA. Tukey's post hoc test was used to determine significant differences among different parasite antigens. The McNemar test was applied to determine significant differences in terms of specificity and kappa test to determine agreement among tests. Unless otherwise indicated, all numbers are expressed as means ± standard deviation (SD). *P* values $\leq .05$ were considered to be significant. Evaluations at different arbitrary cut-off were performed to establish the best sensitivity and specificity of the EIAs.

RESULTS

The majority of blood donors were male (88.77%) between the ages of 18 to 58 years (mean: 29 years, SD: 9.07 years). Each of the four test EIAs classified all 316 QC samples correctly.

As we observed in our previous studies (9), there were small but significant differences between the overall optical density (OD) values obtained with the four test EIAs for the negative sera (ANOVA: $P < 0.0001$), except between the fixed-trypomastigote and TESA Brazil antigens ($P= 0.369$) (Table 1). For the samples classified as true positive by the NLCI, the mean OD values from our four EIAs were not significantly different (Table 1).

Sensitivity and Specificity

The commercial kit used by the Venezuelan blood banks classified a total of 8/2038 (0.39%) samples as positive and 2030/2038 (99.61%) as negative. In contrast, the TESA Tulahuen assay identified 16/2038 (0.78%) positive samples while 15/2038 (0.74%) were classified as positive in the remaining three test assays. There was complete agreement between the blood bank screening assay and all four test assays used in this study for only 6 positive samples. All of these samples were confirmed as positive by the NCLI. Among the discordant samples for which at least one of the screening or test EIAs was negative, the majority were resolved in favour of the test assay results. Most importantly, 7 sera classified as negative by the routine screening assay were correctly identified as positive by all four novel assays. The novel assays also identified between 0-2 samples falsely classified as positive by the screening EIA. However, each test assays classified 2-3 samples as positive that were negative in the blood bank screening EIA and were subsequently confirmed as negative by the NCLI (Table 2). The battery of NCLI assays did not identify any new positives among the 28 samples classified as 'borderline' ($0.300 < OD < 0.399$) in the test

assays or among the 140 randomly-selected samples that had been classified as negative by all of the test EIAs and the blood bank screening assay.

For TESA Tulahuen EIA, 13 of 16 positive samples were confirmed as positive for IHA and IIF and 14 confirmed as positive by E2. For the TESA Brazil, fixed-trypomastigote and fixed-epimastigote EIAs, 13 of the 15 positives samples were confirmed as positive for IHA and IIF and 14 for E2 (Table 2). It is worth noting that even the NCLI reference assays did not achieve 100% concordance. The E2 EIA test classified one serum from Bolivar State as positive that was negative by both IFA and IHA. This serum specimen was also classified as positive by all of the novel EIAs but not by the EIA in routine use by the Venezuelan blood banks (Table 2).

All of the test EIAs were highly specific at the 'optimal' cut-off value of OD = 0.400 (99.85% - 99.9%) with excellent positive predictive values (81.3% – 86.7%). In an attempt to look for subtle advantages and disadvantages of individual tests assays, we calculated the specificity and positive predictive value (PPV) of each assay over a range of arbitrary cut-off values (Table 3). Using the McNemar test to compare test assay pairs, the TESA Tulahuen EIA was found to be the least specific at cut-off values lower than OD = 0.400. The fixed-whole organisms EIAs and TESA Brazil assay retained much greater specificity and PPV at the lower cut-off values that were highly significant compared to the TESA Tulahuen assay ($p < .0001$) (Table 4). Other, subtler differences between the latter 3 test assays also reached statistical significance (Table 4). The agreement between test EIA pairs was quite low at cut-

offs between OD 0.2-0.25 (Kappa test 0.26-0.53), improved at OD 0.30 – 0.35 (0.53 - 0.97) level and was excellent at OD 0.400 (.97 – 1.00) (Table 5).

Using the results of our test EIAs with confirmation by the NLCI, we applied Bayes's theorem to estimate the prevalence of Chagas' disease in the low (Bolivar State) and high (Portuguesa State) prevalence regions where this study was conducted. Our results revealed that the prevalence rates of Chagas' disease among blood donors in these two states of Venezuela (Bolivar state P = 0.62, and for Portuguesa state P = 5.47) have not changed significantly during the last 12 years (1). If the serum sample from Bolivar classified as positive by the E2 reference assay and our 4 novel assays is, in fact, a true positive result, the calculated prevalence rate in Bolivar state would be slightly higher (P = 0.64).

DISCUSSION

Despite several decades of effort, there is still no ideal test for the diagnosis of late-stage Chagas' disease. Reference laboratories still employ a battery of tests in an attempt to make a definitive diagnosis. As occurred in the present study, there is often incomplete agreement between these 'reference' tests, even in expert hands (Table 2). Although a large number of tests with promising estimates of sensitivity and specificity have been described in the literature, very few have undergone validation in field studies. To our knowledge, the current work assessing our four different *T. cruzi* antigen preparations in the EIA format represents the largest field study of any Chagas' disease diagnostic test in Venezuela to date.

This study demonstrated that each of our tests was superior to the commercial kit currently used in Venezuelan blood banks in terms of sensitivity. Each of our test EIAs identified seven false-negative samples from a pool of only 2038 blood donors (false-negative rate of 0.34%). Although the absolute number of false-negative samples is relatively small, each unit of such misclassified blood may reasonably be presumed to have been transfused, placing the recipients at high risk of transfusion-related disease. The potential magnitude of the problem revealed by this study is evident by extrapolation to the total number of blood transfusions that take place in regions of Venezuela where Chagas' disease has been historically endemic. The most recent data available from the National transfusion service suggests that ~369,440 units were transfused in these regions in 2001/2002 (47), of which 1256 may have been false negative in the screening assay. Our findings contradict those of Schmunis *et al.* (47, 51) who estimated the total risk of transfusion-transmitted Chagas' disease in Venezuela from 1993 to 2002 to be zero. This finding can be explained by the fact that few *T. cruzi*-positive cases may have been missed when only one test for screening was used (47). This is the case for Venezuelan blood banks where just the EIA test is performed for the diagnosis of Chagas' disease in blood donors. Furthermore, the number of cases reported in Venezuela could be underestimated due to a poor quality information system from public institutions. In addition, the association between Chagas' disease and a previous blood transfusion is not always easy to establish, and clinical manifestations may not be recognized since a large proportion of infected recipients are asymptomatic (59). Therefore, the data we present suggest that the national estimates for Chagas' disease prevalence, which are based on Schmunis' findings, are far too low. The discrepancy is almost explained by an overestimate of the sensitivity of the EIA screening test used by the Venezuelan blood bank from 1994 to

the present. Although 6/8 of the missed true-positive samples occurred in the Guanare facility (Portuguesa State), it is not likely that simple poor technical performance of the routine assay accounts for our findings. True-positive samples were missed in all three of the regional laboratories employing the routine EIA.

Our results have shown the limitations and risks associated with performing just the EIA for diagnosis of Chagas in the blood banks, and the use of any of the novel EIAs or using a secondary tests in parallel with the routine assay (either our EIAs or any of the reference tests: eg: IHA E2 EIA or IIF) would increase both the sensitivity and specificity of blood screening for Chagas' disease in Latin America blood banks. Pirard *et al.* (41) have demonstrated that routine blood donor testing with a single test results in undesirable numbers of false-negative samples in Mexico. Although the implementation of a screening protocol with an EIA and either IHA or IFA would be technically challenging and more expensive, routine screening with two different EIAs would not be much more expensive than the use of a single EIA. Although some Latin America countries have adopted two-test screening strategies (44, 53), single-test protocols persist in many countries and regions, including all regions of Venezuela to our knowledge. Even the so-called 'reference' assays used by the NCLI did not achieve 100% concordance. These observations are a further demonstration of the need to develop alternate diagnostic tests for latent Chagas' disease.

All novel EIAs achieved excellent specificity at the optimal cut-off value of OD 0.400 (98.7 – 99%). However, modest differences in specificity between the tests assays were revealed at lower cut-off values (TABLE 4). While such differences may not be clinically

relevant, it is possible that the generally better specificity of the fixed-trypomastigote and TESA Brazil EIAs at low cut-off values reflect a greater robustness of these assays in terms of discriminative power between true-positives and true-negatives. Such tests might have significant advantages in environments with many endemic parasitic diseases. These data confirm our previous results with both the fixed-organism (8) and TESA-based EIAs (submitted for publication). It is interesting that Kesper *et al.*, (26) found that the 150-160 kDa TESA band on Western blot was present for all strains but varied widely in intensity. Such variability in the individual components of TESA preparations may account for some of our findings with the TESA-based EIAs. One the other hand, our whole organism EIAs directly meet the criteria suggested by Zingales *et al.*, that antigens from *T. cruzi* isolates from different endemic areas should be present (62). Many of the studies of different antigens previously reported for Chagas' disease have focused on antigens from a single strain (5-7, 12, 32, 57, 58).

All the novel assays had some degree of difficulty in terms of false-positive results compared to both the commercial assay used by the blood banks and the NCLI reference tests (2-3/2038). In the absence of a true 'gold-standard' assay, it is impossible to approach 100% confidence unless the results of all tests are concordant. As mentioned above, one of the three sera that we are considering false positive was classified as positive by the E2 EIA reference assay as well. While false-positive results are less problematic than false-negatives, each misclassified sample can lead to significant direct and indirect costs for a blood banking system. Each unit of blood lost to transfusion systems of Latin America can cost as much as $60 (US) (14). This estimate does not include the life-long loss of the otherwise willing donor

to the donor pool. Misclassification of 0.10-0.15% units of blood as positive would result in the rejection of 262-393 units of blood in the historically endemic regions of Venezuelan at a cost of $15,720- $23,580/ per year (plus the costs associated with donor loss). However, almost 100% of these misclassified units could be recuperated in using the two-step testing with almost any combination of the novel EIAs, the reference assays or even the currently used EIA.

Although none of the antigens used in our novel EIAs is particularly challenging to produce, the fixed-organism antigens are certainly easier to prepare and are subject to fewer *in vitro* manipulations (ie: no concentration or purification steps needed). Furthermore, our previous work (8) as well as that of Rebeski *et al.* (43) suggests that the fixed-organism antigens can also be stable over prolonged periods at room temperature if stored with an appropriate desiccant. Although there were subtle differences between the performance of the fixed-epimastigote and fixed-trypomastigote EIAs (Table 4), it is not clear whether or not the slight specificity advantage of the trypomastigote-based test is clinically relevant or trumps the simpler protocol for growing epimastigotes in culture. Epimastigotes grow easily and rapidly in broth media and high yield is obtained after the logarithmic growth phase. In order to produce enough antigen to screen 300,000 units (Venezuela's national requirement), the production of 750 mL of epimastigote antigen is required with a parasite density per flask of approximately 2.5 x 10^8 parasites/mL that can be produced using only 30 flasks of axenic culture (175 cm^2). Trypomastigotes (and TESA) antigens need to be produced *in vitro* cell culture and their yield is less than that obtained for epimastigote. The quantity of cell culture flasks that have to be used to cover screening of blood donations in all blood banks in

Venezuela is much higher and expensive (~ 1000 flasks with a parasite density of $1x10^7$ parasite/mL). In the case of TESA, 82.5 mL of these antigens will be needed for the same purpose (fourteen $175cm^2$ Vero cell monolayer; each harvested 4 times, at ~850 μg/ml) but TESA antigens will be more laborious to produce due to purification, concentration and protein determination procedures. Therefore, the cost to produce TESA antigens is higher than that for fixed-parasites. Cost is very important in Latin America laboratories where the resources for research are limited and are very expensive.

Although the vectorial transmission of Chagas' disease in Venezuela has decreased considerably in the last half-century (2), our work suggests that disease prevalence has not changed significantly in either Bolivar (0.62%) or Portuguesa States (5.47%) in the last 12 years (1). There are many possible explanations for this observation including: a) reduction in government funding for Chagas programmes, b) decreased surveillance & case finding due to the rapid political decentralization c) elimination of the vertical programme for Chagas' disease and finally, d) the re-emergence of other infectious diseases such as dengue and malaria has diluted the efforts of field workers dedicated to Chagas control activities (2). Although Chagas' disease can persist in populations at low levels in the absence of vectorial transmission through transplacental and transfusion-related transmission, the mean age of three confirmed positive donors in our study (27 years: range 25-28) suggests that vectorial transmission has actively continued to occur in Venezuela during the last three decades. Considering together all the aspects discussed above, we support a previous opinion indicating an active transmission in some regions of Venezuela in recent years for the reduction of the vector control due to the suspension of dwellings sprayings with insecticide,

and the findings of the infected vectors, lead to conclude that the susceptible population living in endemic areas, transmission could now be increasing and the re-emergence of Chagas' disease in Venezuela is imminent in the near future (4, 21).

In summary, the performance of each of our four novel EIAs was superior to that of the commercial EIA kit currently employed in Venezuelan blood banks in terms of sensitivity. Although there were subtle differences between the test assays in terms of specificity, their performance at optimal cut-off values was very similar. These assays are trivial to produce at bench-scale and would be relatively simple to scale-up. Inclusion of any one of these assays in the Venezuelan screening program would be expected to significantly reduce transfusion-associated Chagas' disease in this country. Finally, the data presented in this study are an eloquent argument for implementation of two-test strategies for Chagas' disease screening until a true 'gold-standard' test can be developed.

ACKNOWLEDGMENTS

This work was supported by Health Canada grant # HT070-010033 and partially supported by a grant from FONACIT N° LAB-2000001639, Caracas, Venezuela, to Dr. José Bubis. We thank Dr. Herbert Tanowitz (Einstein University of Medicine, New York) for providing the *T. cruzi* strains. Dr. Omaira Gotopo, Dr. Edgar Izarra, Dr. Geomar de Valenzuela, Lic. Irene Couvel provided us the samples from the blood banks to carry out this work. To all the personnel of the blood banks in Portuguesa state (University Hospital Dr. Miguel Oraa and University Hospital Dr. Jesús María Casal Ramos) and Bolivar state (Ruiz and Paez Hospital) whose provided us all the blood bank samples to carry out this work.

Dr. Mary Isabel Gonzatti (Departamento de Biología Celular, Universidad Simón Bolívar, Caracas, Venezuela) kindly permitted us to used the EIA reader in her laboratory (TECAN). Dr. Guy Beauchamp (Université de Montréal) and Dr. Michel Bigras provided key statistic support. The Universidad de Oriente (UDO), Venezuela, provided a doctoral scholarship for M. Berrizbeitia.

TABLE 1. Optical density means (OD), standard deviations and OD ranges in the four novel EIA assays applied to Venezuelan blood bank sera confirmed as positive or negative by the Venezuelan National Laboratory for Chagas Immunology.

	True negatives	
ANTIGEN	Mean (OD) ± SD	Range (OD) Min-Max
TESA Tulahuen	0.114 ± 0.053	0.031 – 0.674
TESA Brazil	0.097 ± 0.041	0.028 – 0.622
Fixed-Trypomastigote	0.099 ± 0.042	0.035 – 0.509
Fixed-Epimastigote	0.109 ± 0.042	0.035 – 0.711
	True positives	
TESA Tulahuen	1.337 ± 0.340	0.734 – 1.813
TESA Brazil	1.132 ± 0.353	0.564 – 1.912
Fixed-Trypomastigote	1.181 ± 0.157	0.916 – 1.452
Fixed-Epimastigote	1.352 ± 0.184	1.037 – 1.352

* Tukey's post tests revealed significant differences between mean OD for fixed-epimastigote and TESA Brazil ($P < 0001$), fixed-epimastigote and TESA Tulahuen (P 0.0023), fixed-epimastigote and fixed-trypomastigote ($P < 0001$), TESA Brazil and TESA Tulahuen ($P<.0001$), TESA Tulahuen and fixed-trypomastigote ($P <.0001$).

† The linear model shows no significant effect of antigen type on optical densities ($P = 0.17$).

TABLE 2. Positive results in Venezuelan blood banks using novel EIAs (fixed-epimastigote, fixed-trypomastigote and TESA Tulahuen and TESA Brazil), the commercial kit in current use in Venezuelan blood banks and the battery of NCLI tests.

Blood bank states	Positives by Fixed-epimastigote	Positives by Fixed-Trypomastigote	Positives by TESA Tulahuen	Positives by TESA Brazil	Positives by the Commercial kit (blood bank)	NLCI Results		
						IIF Positives	IHA Positives	E2 Positives
Guanare (Portuguesa state)	7	7	7	7	1	6	6	6
* Acarigua (Portuguesa state)	6	6	7	6	*7	6	6	6
Ciudad Bolivar (Bolivar state)	2	2	2	2	0	1	1	2
†Total of positives samples	15	15	16	15	8	13	13	14

*In Acarigua blood bank 2 samples that were positives in the blood bank screening, were negatives for our four EIAs and confirmed as negative by the NLCI. † Our four EIAs reported 2-3 false-positives and no false-negatives; the commercial EIA reported 7 false-negatives and 2 false-positives.

TABLE 3. Specificities and positive predictive value (PPV) for Chagas' disease using the novel EIAs to test Venezuelan blood bank samples (n = 2038).

	TESA T		Tesa B		Fixed-Trypomastigote		Fixed-epimastigote	
Cut-off	Specificity (%)	PPV (%)	Specificity (%)	PPV (%)	Specificity (%)	PPV (%)	Specificity (%)	PPV (%)
0.2	93.48	7.12	97.58	20.97	97.53	20.64	96.49	15.48
0.30	99.01	39.39	99.56	59.09	99.51	56.52	99.60	61.90
*0.40	99.85	81.25	99.90	86.67	99.90	86.67	99.90	86.67

*Sensitivity for the four novel EIAs at OD 0.200 to 0.400 cut-offs: 100%

TABLE 4. Discordant data applying the McNemar test to evaluate the specificity of the novel *T. cruzi* EIAs (fixed-trypomastigotes, fixed-epimastigotes, TESA Tulahuen and TESA Brazil) at different OD cut-offs in Venezuelan blood bank sera (n=2038).

Paired-data	Discordant data	*P* value
	Cut-off 0.2	
Tesa T vs fixed epi	61	< 0.0001
TesaT vs Tryp	82	< 0.0001
Tesa T vs TESA B	83	< 0.0001
Epi vs. Tesa B	22	< 0.03
Epi vs. Tryp	21	< 0.026
Tesa B vs. Tryp	1	Not significant
	Cut-off 0.25	
Tesa T vs fixed epi	35	< 0.0001
TesaT vs Tryp	31	< 0.0001
Tesa T vs TESA B	31	< 0.0001
Tesa B vs. Epi		Not significant
Tesa B vs. Tryp		Not significant
Tryp vs. Epi		Not significant
	Cut-off 0.30	
Tesa T vs. fixed epi	12	< 0.01
TesaT vs. Tryp	10	< 0.04
Tesa T vs. TESA B	11	< 0.01
	Cutoff 0.35	
Tesa T vs. TESA B	6	0.01

TABLE 5. Agreement among the novel *T. cruzi* EIAs for the diagnosis of Chagas disease in two different blood bank regions of Venezuela.

	Agreement among test at cutoff 0.2 (Kappa test)		
	Fixed-Trypomastigote	Fixed-Epimastigote	TESA Brazil
TESA Tulahuen	0.2765	0.2398	0.3491
TESA Brazil	0.3150	0.2618	-
Fixed-trypomastigote	-	0.3724	0.3150
	Agreement among test at cutoff 0.30 (Kappa test)		
	Fixed-Trypomastigote	Fixed-Epimastigote	TESA Brazil
TESA Tulahuen	0.5295	0.5874	0.6132
TESA Brazil	0.7079	0.6465	-
Fixed-trypomastigote	-	0.6784	0.7979
	Agreement among test at cutoff 0.40 (Kappa test)		
	Fixed-Trypomastigote	Fixed-Epimastigote	TESA Brazil
TESA Tulahuen	0.9675	0.9675	0.9675
TESA Brazil	1.00	1.00	-
Fixed-trypomastigote	-	1.00	1.00

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IV. DISCUSSION

1. Use of fixed-forms of *Trypanosoma cruzi* in the diagnosis of Chagas' disease. First generation EIA assay.

As outlined at the end of the literature review, there is a continued interest to develop assays for Chagas' disease to satisfy the needs of both the developed and developing world. There are many tests for the diagnosis of Chagas' disease but no gold standard. At the current time, EIA tests are the best choice for use in blood bank screening. Although there are several commercial EIA kits licensed in Latin America, the majority are based on epimastigote-form antigens. Inconclusive and discordant results are frequently reported with the use of different commercial EIA tests in Latin America blood banks.

Excellent sensitivities and specificities have been reported in different EIA formats using a wide range of different antigens (whole, fixed, purified and recombinant proteins). However, the tests developed to date have several important limitations including: reproducibility, low sample size, the use of adequate controls, limited use of different strains of *T. cruzi*, discordant results, among others. One of the most important limitations is that the majority of these tests have been produced only in laboratory scale, and no validation studies have been conducted in the field.

The ideal serological technique for Chagas' disease should be easy to perform, quick in reading results, reliable and inexpensive. In addition, sensitivity and specificity should be

high. The main objective of this study was to develop an EIA test that met all the criteria mentioned above. One of our initial hypotheses was that a fixed-form EIA based on the trypomastigote or amastigote forms would yield superior tests. Our reasoning was simple: good results have been reported using fixed-form assays but epimastigote antigens have been used almost exclusively. Trypomastigotes and amastigotes are the form to which the human immune response is exposed. Finally, a *T. congolense* assay developed by Rebeski *et al.* (40, 41) suggested that a fixed form assay could be very stable under field conditions. Our first generation EIAs tests based on fixed-forms of *T. cruzi* (Article I) (10) confirmed that such sensitive, simple, and relatively and inexpensive EIA assay could be developed. In addition, the plates could be precoated and stored for at least four months without loss of the antigen reactivity (Article I). Such pre-sensitized plates could be easily stored at a central location and sent to different field laboratories.

The majority of commercial tests that are currently on the market used sonicated epimastigote antigens (3, 36, 49). The reason for this is simple; epimastigotes are very easy to produce since they can be cultured in broth, and no purification steps are needed resulting in excellent yield. However, EIAs using sonicated epimastigote antigens have had significant problems with specificity, and variable results have been obtained when these kits are applied in different blood banks in endemic countries (35). To our knowledge, no kit based on fixed-form antigens have ever been commercialized. Table 1 lists the studies that have reported the use of fixed forms *T. cruzi* for assay development. These studies all have several limitations: the Dot EIA, the Dig EIA and the immunofluorescence assays are all highly subjective and

operator-dependent. Therefore, these techniques may be more appropriate for field studies than for blood bank screening. Several of the studies listed in TABLE 1 are based on only limited numbers of samples (n= 40-78) or the sensitivities and specificities were not clearly reported (7, 8, 11). Our first study (Article I) has several advantages over those listed in Table 1. We used an EIA format that is more suitable to be employed in blood banks, 2) we employed a mix of two different strains of *T. cruzi* antigen (Brazil and Tulahuen), 3) we used a large panel of well-defined sera (Chagas positive sera, and healthy controls) from endemic and non-endemic regions that were classified based on the results of three different serological tests EIA, IIF and IHA, and well as sera from patients with other parasitic diseases. Finally, we are the first to describe the used of fixed-trypomastigote form and a direct comparison among the three forms of *T. cruzi* in an EIA format for the diagnosis of Chagas' disease. We could find only one publication that used a crude extract in an EIA format trypomastigote (9). The sensitivity of this test was 98.8% and the specificity using control serum samples from nonendemic area (n= 405) was 98.3%. Our EIAs achieved 100% sensitivity and reasonable specificity 97.6%, 98.3% and 99.3% for amastigotes, epimastigotes and trypomastigotes respectively (Article I) (10). To our surprise however, our initial hypotheses that fixed amastigote or trypomastigote antigens would be superior to epimastigote antigen in the EIA format, proved to be incorrect. As we explained in Article I, it is possible that the procedures to extract and purify these forms could modify the antigens on the trypomastigote and amastigote cell surfaces, resulting in the slightly lower optical densities observed (45).

Another innovation in our fixed-parasite first generation EIA assay was the use of pure evolutive forms (Annex 1). In the previous work with fixed form antigens purification procedures were not well-defined and purity was rarely reported (8, 30).

TABLE 1. Different studies that have used fixed-forms of *T. cruzi* for the diagnosis of Chagas' disease.

Antigen	**Test**	**Sensitivity (%)**	**Specificity (%)**	**Reference**
Fixed-epimastigote.	Immunofluorescence	100	89.9	(30)
Fixed-amastigote		100	95.0	
Fixed-epimastigote	EIA capture IgM	98	?	(7)
Fixed-epimastigote	Dot-EIA	100	92.8	(11)
Fixed epimastigote	EIA	?	?	(8)
Fixed amastigote	Immunofluorescence			
Fixed epimastigote	DIG-EIA	100	100	(12)
Fixed epimastigote	Dot-EIA	100	100	(14)

Our results demonstrate that the use of formaldehyde or formalin to fix parasites is relatively simple, reproducible, sensitive and inexpensive. This technique can be used in laboratories with limited budgets such as those in the rural areas of Latin America where techniques to purify proteins are not always available or feasible. Moreover, the quantity of

fixed-form antigen needed to coat the plates is determined by the hemocytometer. As a result, protein concentration procedures that are time consuming, and lysis of organism, which can be dangerous, are avoided (8, 12).

The data we generated with our first generation, fixed-form EIAs gave us the confidence to validate these assays (Venezuela); such field tests are the only way to know the real performance of any novel assay (see details below).

2. Use of trypomastigote excreted-secreted antigens from *Trypanosoma cruzi* (TESA) in the diagnosis of Chagas' disease. Second generation EIA assays.

Our parallel work with TESA-based EIAs had a similar grounding to the fixed antigen products. Although a number of other groups had worked with TESA and reported very promising results, there were significant problems with these studies. We hypothesized that an improved TESA-based assay could be generated by testing a number of reasonable simple manipulations: using different strains of *T. cruzi*, concentrating the TESA, and using immunoaffinity for the first time to purify TESA proteins targeted by the immune response.

The sensitivities and specificities of the second generation EIAs were generally excellent at the different cut-offs evaluated. Antigens from both strains showed excellent

agreement and no significant differences in terms of specificity were observed. Our work (Article II) had several simple advantages over the studies in the literature (51): 1) large number of samples were tested, 2) the tests were more specific using appropriate control sera, 3) antigens from two different strains were used. Finally, concentrating the higher molecular weight protein using the Amicon Ultra device, we markedly improved the antigenicity of the TESA proteins (Article II).

Perhaps most importantly, we were able to purify proteins involved in immune recognition in Chagas positive patients. Using immunoaffinity chromatography, we were able to isolate 5 bands (220, 170, 120, 85 and 60 kDa) that were present in TESA from both strains of *T. cruzi* (Brazil and Tulahuen) (Article II). Such purification of TESA has not been previously described.

The purified proteins were evaluated using 166 samples from chagasic and healthy patients as well as a limited number of patients with leishmaniasis. In this preliminary study, $TESA_{IA}$-based was 98% sensitive and 100% specific. The Western-blot using the same purified proteins showed cross-reaction with some of the sera from patients with leishmaniasis, specifically a 60-kDa protein. We are the first to implicate this TESA antigen protein in cross reactivity with *Leishmania sp* (Article II). Other authors have suggested that polypeptide bands in TESA proteins below 150 kDa are responsible for the cross-reactivity with visceral and cutaneous leishmaniasis but the exact size of these implicated proteins was never specified (34).

Our laboratory evaluation of the second generation EIAs based on TESA antigens proved that these assays can be very sensitive, specific and stable for long periods of time. The results of this study are discussed in Article II. Although production of these antigens is a little more laborious than producing the fixed organisms, it is still relatively easy to perform. The performance of the TESA proteins in the EIA format encouraged us to take these assays to field trials as well (next section).

3. Validation and comparison of four different *Trypanosoma cruzi* antigens in the EIA assay at two different blood banks in Venezuela.

After standardization, purification and laboratory evaluation of the two generations of EIAs (fixed-form epimastigotes or trypomastigotes, and TESA antigens from Tulahuen and Brazil strains), we decided to validate these antigens in the field in blood banks located in an endemic country (Venezuela). The vast majority of assays reported for the diagnosis of Chagas' disease have never progressed beyond the laboratory where they have been developed to be validated in field studies (3, 7-9, 11-13, 16, 17, 19, 21-25, 28-34, 37, 38, 42-44, 46, 47, 50, 51, 54). This failure explains, in part, why only a few tests have become commercial kits (18, 24, 26, 32).

To validate the results of our first and second generation EIAs, we used a representative sample (n=2038) from three different blood banks in Venezuela, two located in

the West-central part of the country (high prevalence: Acarigua and Guanare cities, in the Portuguesa state) and the other in the Southern part of Venezuela (low prevalence: Ciudad Bolivar city, in the Bolivar state). The performance of our four antigens was superior to the commercial EIA kit (Pharmatest-EIA Chagas IgG, Guarenas, Venezuela) used routinely in these blood banks. Our tests (at cutoff 0.400) were able to detect seven false negative and two false positive reactions compared with the routine blood bank assay. The positive samples and the discordant sera were confirmed in the Chagas Immunodiagnosis Laboratory (National Reference Center) (Article III). In our tests all of the antigens had essentially identical sensitivity, specificity, positive and negative predictive values and very good agreement (kappa test) at an OD cut-off of 0.4 (Article III). However, fixed-trypomastigote and TESA Brazil were the most specific at lower cut-off values. TESA Tulahuen was the least specific at cut-off values lower than 0.35. Choosing which antigen should eventually be used for the development of a new commercial kit will depend on several criteria such as cost, antigen yield, simplicity of the assay as well as sensitivity and specificity.

The use of fixed-epimastigote antigens may be most appropriate where cost of goods and material is paramount since this antigen is very easy to produce (axenic culture), and fixed-epimastigotes do not need purification procedures. The quantity of antigen to be produced (discussed in article III) would be only 30 flasks of axenic culture (175 cm^2) (2.5 x 10^8 parasites/mL/per flask) to permit the screening of all donations in Venezuela (~ 300,000 units/per year). However, it is important to take into account that epimastigote forms have been reported to have problems with specificity (3, 36). Moreover, fixed-epimastigote EIA

(Article I) was less specific than the fixed-trypomastigote assay when patients with other parasitic diseases and healthy patients were evaluated at different cut-offs values (10). The use of the fixed-epimastigote EIA for blood bank screening lead to the inappropriate loss of large numbers of blood units representing an economic lost. Fixed-trypomastigotes are more expensive to produce than fixed-epimastigote since cell culture, purification procedures and more manipulations are required for this form. The yield obtained after purification in the CM column is very much lower than the yield obtained from epimastigote axenic cultures. To cover the screening needs for all blood donations in Venezuela, ~ 1000 flasks (with a parasite density of $1x10^7$ parasite/mL) would need to be processed. In order to make a final decision, it will be important to know if the increased cost of producing of the trypomastigote antigens would be offset by the expense of discarding units inappropriately due to the lower specificity of the epimastigote antigen EIA ($60 per each unit discarded).

TESA antigens are much more expensive to produce than fixed-parasites. In addition to cell culture production, these antigens need purification, concentration and determination of protein concentration that require specialized equipments and costly reagents. However, the most important advantage of the TESA proteins is the yield obtained. The quantity of protein needed to coat an EIA plate is only 1 μg/mL. As a result, only fourteen $175cm^2$ flasks, harvested 4 times each, and later concentrated (32-fold) yield 62.5 mL of TESA at a concentration of ~850 μg/mL. This quantity of TESA antigens would be sufficient to screen all blood donations in Venezuela for a year.

From the literature reviewed, very few studies have validated antigens for *T. cruzi* diagnostics with the goal of developing a new commercial test. Umezawa *et al* evaluated six *T. cruzi* recombinant antigens by EIA from nine countries of South and Central America (48). Three of the best antigens were selected and combined in a single EIA resulting in a multi-antigen test with very good sensitivity and specificity (49). Based on these results, this group developed and commercialized an immunochromatography assay (Chagas Stak-Pak). This test was 98.5% sensitive and 94.8% specific with samples from Brazil and 100% sensitive and 98.6% specific with samples from different countries of Latin America (27). This test is very easy to manipulate and to read but the results are still subjective, and, as a consequence, reading errors can occur. This assay is ideally suited for epidemiological studies but could not to be employed in blood bank screening. Krieger *et al* have also developed an EIA test using the recombinant antigens CRA and FRA (24), commercialized as the EIE-Recombinant-Chagas-Biomanguinhos kit (18). Both of these commercial tests above were evaluated using smaller numbers of samples than those employed in our validation study (Article III, n = 2038). Moreover, the samples used in the evaluations of these tests were previously classified and chosen based on their responses in three different reference assays. In our validation study, we used consecutive samples which also included borderline samples. This approach reflects more accurately the real situation in a blood bank. Therefore, the results obtained in our validation study are likely to be more accurate than those obtained in the evaluation studies for the commercial kits. The pre-selection of samples based on known reaction introduces an unacceptable bias in the validation of any assay. For example, our TESA EIAs were initially evaluated with confirmed Chagasic and non-Chagasic samples (Article II) and no significant differences were found in terms of specificity between the two strains

(Tulahuen and Brazil). However, in the validation study, significant differences between the two groups in terms of OD and specificity were found between strains. These results confirmed our initial assumption that the real performance and utility of diagnostic tests, developed and optimized in research laboratories, can only be demonstrated in the field using large numbers of unselected samples.

Our data certainly support the suggestion of Zingales *et al.* that any assay for Chagas' disease intended for use in different locations should incorporate antigens from at least 2 parasites strains (53). The potential impact of strain antigenic variation on assay performance has been addressed by few groups. Verbisck *et al* showed that surface epitopes on fixed parasites among different isolates of *T. cruzi* was diverse using monoclonal antibodies (mAbs) in a fixed-organism EIA format (52). Depending on the kind of mAb used, each strain had different responses in terms of optical densities. For example, the expression of the target for mAb 4B5 was higher on Tulahuen amastigotes than the rest of the strains (52). Kesper *et al.* found 13 different immunoblotting patterns from TESA antigens in 5 strains and 10 isolates of *T. cruzi* (20). Such differences among the antigenic profiles of different strains may account for the differences in specificities found in our validation study using Brazil and Tulahuen antigens in the EIA-based assays.

In addition to the validation of the assays, this project was able to determine the actual prevalence of Chagas' disease among donors in states of Venezuela (Bolivar and Portuguesa). Our work showed that prevalence has not changed in the last 12 years, despite official control

program over the last decades (1). In fact, ours is the largest study ever conducted for the validation of a Chagas diagnostic test in the blood banks of Venezuela. In 1999, Añez *et al* reported the results of a clinical, seroparasitologic and epidemiological study in six different states of Western Venezuela to detect acute Chagas' disease in these regions (4). Fifty-nine new cases were found between 1988 and 1996. These authors also found discrepancies in the data on the prevalence of Chagas' disease in Venezuela demonstrating that active vector transmission was still a problem in endemic areas thought to be controlled by the 1960s campaign against indoor triatomine insects (2). The same investigators subsequently, performed another study in five states (Barinas, Cojedes, Falcon, Merida and Portuguesa) considered to be highly endemic in Western Venezuela. Almost 16% of the evaluated patients (n = 1251) were positive using three serologic methods (direct agglutination, IFA and EIA) (6). A very recent study (2004) showed that 56.8% of patients with a presumptive clinical diagnosis of Chagas' disease were seropositive, and that a serologic examination of 3,835 individuals from rural areas revealed 11.7% seroprevalence (5). Although the Chagas control program in Venezuela successfully reduced the annual incidence of infection from ten per 1000 in the 1950s to one per 1000 in the 1980s, the goal of suspending transmission has not been accomplished, and no real progress has occurred to further reduce the risk in the last decade. The recent lack of progress may be explained, in part, by the initial successes in reducing infection and infestation rates to low levels. This early accomplishment resulted in the downgrading of the high priority control program. The re-emergence of malaria (1982) and dengue (1989) in Venezuela also contributed to the diversion of important resources from the Chagas' disease control program (15). The constant prevalence rates for the last 12 years

and the mean age of three confirmed positive donors in our study (27 years: range 25-28) suggest a reactivation of active vectorial transmission in these areas.

In summary we were able to validate four different EIAs in the largest study of any Chagas' disease ever conducted in Venezuela. Each of our assays was superior to the current assay used in Venezuela blood banks. All the EIAs performed well although the fixed-trypomastigote and TESA Brazil assays were the most specific at lower cut-off values. Whether or not the expense of producing the fixed-trypomastigote or TESA antigens is counter-balanced by the costs of inappropriately refused units of blood will be the subject of future evaluations. Fixed-form EIAs certainly could be immediately introduced in Latin America blood banks because these antigens are very inexpensive and easy to produce and standardize. In contrast, the TESA antigens based-assays may be good candidates in blood banks of developed countries were more facilities for production of these antigens are available and cost is less important. Our validation study also confirmed the fact that no test is perfect (Article III). Although each showed excellent sensitivity and specificity, false-positive reactions were still observed. Therefore, we continue to support the World Health Organization recommendation that two serological tests should be performed in parallel to confirm the real status of any sample. Based on previous reports, the use of an EIA test plus IHA is less cost-effective than EIA plus IIF (39). In addition, a third generation of EIA assay could be developed by cloning and expressing the bands purified by immunoaffinity chromatography (170, 120 and 85 kDa bands) (Article II). Unique bands could be obtained by molecular exclusion chromatography, and tested individually or in combination to evaluate the sensitivity and specificity of a third generation of EIA. The search for the perfect antigen

for the diagnosis of Chagas' disease will continue. Until this goal is reached, the application of two different serological tests in parallel represents the best option available. One or more of the assays that we have developed could be used for this purpose with confidence in Venezuela.

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V. CONCLUSION

We have demonstrated that

- The EIA generation assays using fixed forms of *T. cruzi* had excellent sensitivities and specificities. However, the fixed-trypomastigote assay had the greatest specificity at different cut-off values suggesting that this format is more robust. Additionally pre-coated plates with this antigen were found to be stable for long periods of times under different conditions of storage.

- The second generation EIA assays using excreted-secreted antigens with two different stains of *T. cruzi* (TESA) were also very sensitive and specific.

- Immunopurified proteins from TESA antigens (220, 170, 120, 85 and 60 kDa) were described for the first time revealing potential new tools for diagnosis that could be cloned and assayed in a third generation EIA test.

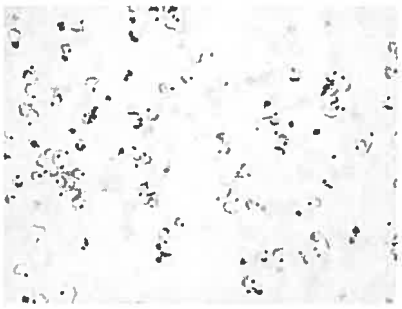
- The 60-kDa immunopurified TESA proteins is responsible of some of the known cross reactivity with leishmaniasis samples.

- Each of the four antigens validated in the field using the EIA format are superior to the commercial test currently use in Venezuelan blood banks.

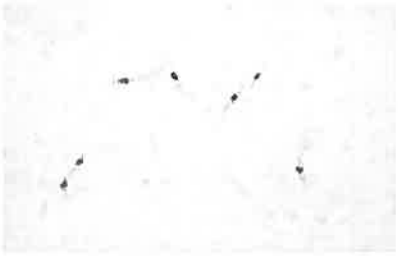
- The validation study revealed that a new test for diagnosis of Chagas' disease should be implemented in Venezuelan blood banks. From these antigens fixed-organisms should be introduced in Latin America blood banks. TESA antigens based-assays may be good candidates in blood banks of develop countries.

We were able to standardize, evaluate and validate several different antigens of *T. cruzi* for the diagnosis of Chagas' disease. However, problems with the specificity of these antigens still remain. Further studies to develop a third generation of EIA tests using recombinant proteins; are feasible possibly the immunopurified TESA proteins identified in this study could contribute to the search the perfect test for the diagnosis of Chagas' disease.

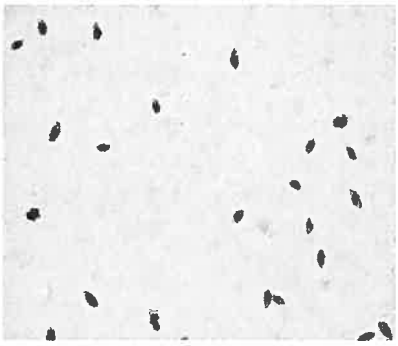
VI. ANNEX



A.



B.



C.

Figure 1. Aspect of the suspension of the different forms of the parasite. A) Trypomastigotes obtained after purification in a CM-cellulose column. B) Aspect of the suspension of amastigotes obtained after purification in a percoll gradient. C) Epimastigotes obtained during the logarithmic grow of phase in LIT medium. Giemsa-stained preparations.

PERMISSION DE L'ÉDITEUR D'UNE REVUE OU D'UN LIVRE[1]

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DESCRIPTION DE L'ARTICLE

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Titre

Development and Comparison of Enzyme Immunoassays for Diagnosis of Chagas' Disease Using Fixed Forms of *Trypanosoma cruzi* (Epimastigotes, Amastigotes, and Trypomastigotes) and Assessment of Antigen Stability for the Three Assays.

Numéro de la revue	Pages	Date
42(4)	1766-1769	April 2004

IDENTIFICATION DE LA REVUE OU DU LIVRE

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Journal of Clinical Microbiology	
Adresse	**Date**

DECLARATION DE L'EDITEUR

Nom complet de l'éditeur

[redacted]

Déclaration

À titre d'éditeur, j'autorise le microfilmage ~~du mémoire~~ de la Thèse et je suis d'accord que (nom de l'étudiant) *inclut cet article dans sa thèse de doctorat*

Éd[redacted] Date 3/15/2005

[1] *Annexe II du Guide de présentation et d'évaluation des mémoires de maîtrise et des thèses de doctorat, mars 2001*

ACCORD ET PERMISSION DES COAUTEURS D'UN ARTICLE[1]

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DESCRIPTION DE L'ARTICLE

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Titre	
Development and Comparison of Enzyme Immunoassays for Diagnosis of Chagas' Disease Using Fixed Forms of *Trypanosoma cruzi* (Epimastigotes, Amastigotes, and Trypomastigotes) and Assessment of Antigen Stability for the Three Assays.	
Revue	**Date de publication**
Journal of Clinical Microbiology 42((4): 1766-1769	April, 2004

DECLARATION DES COAUTEURS

Déclaration		
À titre de coauteur de l'article identifié ci-dessus, j'autorise le microfilmage de la thèse et je suis d'accord que *Mariolga Berrizbeitia Uztátiz inclut cet article dans sa thèse de doctorat qui a pour titre* :Development of an ELISA test using different antigens of Trypanosoma cruzi for the diagnosis of Chagas' disease.		
Coauteur Brian Ward	[redacted]	**Date** 05/08/29
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Coauteur Marcelo Gottschalk		**Date**
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DESCRIPTION DE L'ARTICLE

Auteurs
Mariolga Berrizbeitia, Momar Ndao, José Bubis, Marcelo Gottschalk, Alberto Aché, Sonia Lacouture, Mehudy Medina and Brian J. Ward.
Titre
Purified excreted-secreted antigens from Trypanosoma cruzi trypomastigotes as diagnostic tools for Chagas' disease.

Revue	**Date de publication**
Journal of Clinical Microbiology	Submitted for publication (01/06/05)

DECLARATION DES COAUTEURS

Déclaration

À titre de coauteur de l'article identifié ci-dessus, j'autorise le microfilmage de la thèse et je suis d'accord que (Mariolga Berrizbeitia Uztáriz) inclut cet article dans sa thèse de doctorat qui a pour titre : Development of an ELISA test using different antigens of *Trypanosoma cruzi* for the diagnosis of Chagas' disease.

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ACCORD ET PERMISSION DES COAUTEURS D'UN ARTICLE[1]

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Sigle du programme	**Titre du programme**	**Option**
Ph.D.	Sciences vétérinaires	Microbiologie

DESCRIPTION DE L'ARTICLE

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Mariolga Berrizbeitia, Momar Ndao, José Bubis, Marcelo Gottschalk, Alberto Aché, Sonia Lacouture, Mehudy Medina and Brian J. Ward.
Titre
Field evaluation of four novel enzyme immunoassays for Chagas Disease in Venezuela blood banks: Comparison of assays using fixed-epimastigotes, fixed-trypomastigotes or trypomastigote secreted-excreted antigens (TESA) from two *T. cruzi* strains.

Revue	**Date de publication**
Journal of Clinical Microbiology	In preparation

DECLARATION DES COAUTEURS

Déclaration
À titre de coauteur de l'article identifié ci-dessus, j'autorise le microfilmage de la thèse et je suis d'accord que Mariolga Berrizbeitia Uztáriz inclut cet article dans sa thèse de doctorat qui a pour titre : Development of an ELISA test using different antigens of *Trypanosoma cruzi* for the diagnosis of Chagas' disease.

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