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Pilar Hernández Pastor

M.Sc. Biology

Biochemisches Institut
der Medizinischen Fakultät
der Justus-Liebig-Universität Gießen

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Affordable diagnosis for Chagas disease

Supervisors:

Prof. Dr. Michael Martin
Immunologie
am Fachbereich Biologie und Chemie
Justus-Liebig-Universität Gießen

Prof. Dr. Ewald Beck
Institut für Biochemie
Fachbereich Humanmedizin
Justus-Liebig-Universität Gießen

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Finally, I would like to dedicate this work to all Chagas disease patients, which suffer in silence, and whose voices will be louder by this work.

“No estalla como las bombas,
ni suena como los tiros.
Como el hambre, mata callando.
Como el hambre, mata a los callados;
a los que viven condenados al silencio
y mueren condenados al olvido.
Tragedia que no suena,
enfermos que no pagan,
enfermedad que no vende.”

Eduardo Galeano

“It does not explode like a bomb,
or echo like gunfire.
Like hunger, it kills in silence.
Like hunger, it kills the silent:
those who live condemned to silence,
those who die condemned to oblivion.
Tragedy that makes no sound,
patients that can not pay,
illness that will not sell.”

“Sie explodiert nicht wie eine Bombe,
und klingt nicht wie Kanonenfeuer.
Wie Hunger, tötet sie still.
Wie Hunger tötet sie die, die schweigen:
Jene, die zum Schweigen verdammt sind,
jene, die verdammt sind, in Vergessenheit zu sterben.
Eine Tragödie, die keinen Lärm macht,
Patienten, die nicht bezahlen können,
eine Krankheit, die sich nicht verkauft.“

SUMMARY

Chagas disease, caused by the flagellate *Trypanosoma cruzi*, is endemic in Latin America where it imposes a high burden on disability and death combined with enormous economic losses for up to 18 million people. Useful tests for the diagnosis of the disease are available, but they are too expensive to be used in in large scale in most of the affected countries. Furthermore, many diagnostic kits cannot clearly discriminate between Chagas disease and visceral leishmaniasis, which can be tolerated for immunological screening of samples in blood banks, but not for patients, since both diseases can occur in the same areas, however, need to be treated with different drugs. In addition, it is essential to recognise infection with *T. cruzi* in time, i.e. before the onset of severe clinical symptoms, since the available drugs are effective in the early stages of the disease only. This demands developing more specific and less expensive diagnostic tools.

By means a bioinformatic approach, several recombinant antigens were produced, predominantly consisting of tandemly repeated amino acid sequences which occur in large numbers in different proteins of the parasite. Since the corresponding repeated DNA structures could not be maintained stably in *E. coli*, the whole range of possible base variations in codons was used to create varying coding sequences for up to nine tandem repeats of identical amino acid sequences. Some but not all of the expressed proteins were found to react strongly with sera from infected patients. To simplify purification as well as production, the most suitable antigens were fused. At the end a product composed of four different tandem repeat motives was obtained revealing an unexpected high diagnostic sensitivity, which was several orders of magnitude higher than with the antigens known so far. When used in ELISA, one milligram of the recombinant antigen is sufficient for one million single tests.

Immunoassays can frequently not discriminate between acute infection and overcome disease. Therefore, two different PCR assays were developed in addition to determine the number of parasites circulating in blood after therapy with drugs. Targets of the one PCR assay are the more than 200 fold amplified genes for 18S rRNA and, for the other assay, the kinetoplast minicircle DNA which occurs in approximately 10.000 copies per parasite. Both of these test can detect as few as 10 trypanosomes per millilitre of blood and can clearly discriminate *T. cruzi* infections from infections with other Trypanosomatides.

In conclusion, several highly sensitive and specific diagnostic procedures have been created, which are relatively simple to be performed and which can be produced for a low price. It is the goal to instruct scientist in Latin America to produce and to use these tests in the long term by their own means, independent of support from abroad.

ZUSAMMENFASSUNG

Die Chagas-Krankheit wird durch den Flagellaten *Trypanosoma cruzi* ausgelöst und ist in Lateinamerika endemisch. Sie betrifft dort bis zu 18 Millionen Menschen und führt in vielen Fällen zu schweren gesundheitlichen Schäden, nicht selten mit tödlichem Verlauf. Es gibt empfindliche diagnostische Nachweisverfahren für die Krankheit, aber sie sind entschieden zu teuer um in den betroffenen Ländern zum Einsatz zu kommen. Außerdem können viele dieser Verfahren nicht izwischen Chagas-Krankheit und viszeraler Leishmaniose unterscheiden. Das kann bei der Untersuchung von Proben in Blutbanken zwar toleriert werden, ist aber für die Diagnose von Patienten inakzeptabel, da beide Krankheiten in derselben Region auftreten können, jedoch mit unterschiedlichen Medikamente behandelt werden müssen. Darüber hinaus ist es wichtig, Infektionen mit *T. cruzi* rechtzeitig zu erkennen, lange bevor schwere Symptome auftreten, denn die verfügbaren Medikamente sind nur in der früheren Phase der Krankheit wirsam. Dies erfordert die Entwicklung neuer diagnostischer Methoden mit höherer Spezifität und niedrigeren Kosten.

Im Rahmen der Arbeit wurden mittels bioinformatischer Methoden mehrere rekombinante Antigene hergestellt, die hauptsächlich aus Wiederholungen von Aminosäure-sequenzen bestehen und in großen Zahl in verschiedenen Proteinen der Parasiten vorkommen. Weil die entsprechenden repetitiven DNA Segmente nicht stabil in *E. coli* etabliert werden konnten, wurden Gene mit möglichst vielen Austauschen in variablen Codons hergestellt. Auf diese Weise gelang es, codierende Bereiche für bis zu neun identische Aminosäurensequenz-Wiederholungen stabil in E. coli zu etablieren.. Manche der gereinigten Proteine reagierten stark mit Seren von infizierten Patienten. Um Produktion und Reinigung zu vereinfachen, wurden die am stärksten reaktiven Antigene fusioniert. Am Ende enstand ein Antigen mit vier unterschiedlichen repetitiven Motiven, das in verschiedenen immunologischen Tests zu einer extrem starken Reaktivität mit Patientenseren führte. Ein Milligram dieses rekombinanten Proteins ist ausreichend um eine Million Einzelversuche im ELISA durchzuführen.

Immundiagnose kann im Allgemeinen oft nicht zwischen akuter und überstandener Infektion unterscheiden. Dies ist aber wichtig um den Erfolg einer medikamentösen Behandlung verfolgen zu können. Daher wurden zusätzlich zwei verschiedene PCR-Nachweisverfahren für die Krankheit entwickelt. Mit der einen PCR werden die Gene der 18S rRNA amplifiziert, die in mehr als 200 Kopien im Genom von T. cruzi vorliegen, die andere PCR ist spezifisch für die mitochondriale Minicicle-DNA, die etwa 10.000fach im Parsiten vorliegt. Beide Tests haben eine Nachweisempfindlichkeit von ca. 10 Parasiten pro Milliliter Blut und können klar zwischen Infektionen mit den verschiedenen anderen auftretenden Trypanosomatiden unterscheiden.

Insgesamt wurden in Rahmen dieser Arbeit mehrere äußerst empfindliche und spezifische Diagnoseverfahren für die Chagas-Krankheit entwickelt. Sie können alle mit relativ geringem Aufwand durchgeführt und mit niedrigen Kosten hergestellt werden. Das weitere Ziel wird sein, Wissenschaftler in Lateinamerika anzuleiten, diese Diagnoseverfahren nicht nur zur Anzuwendung zu bringen, sondern sie auch in Eigenregie, d.h. ohne Hilfe von außen herzustellen.

CONTENTS

1. INTRODUCTION

1.1 What is Chagas disease? Background

Chagas disease (also known as American trypanosomiasis) is a parasitic disease endemic in Latin America, where it affects at present at least 10-14 million of people, probably more. Its pathogenic agent is a flagellate protozoan, *Trypanosoma cruzi*, which is transmitted to humans and other mammals mostly by blood-sucking bugs of the subfamily Triatominae (Family Reduviidae). Those insects are known by numerous common names varying by country, including benchuca, vinchuca, chipo, chupança and barbeiro.

DNA obtained from mummified human tissues from the northern coast of Chile indicates that Chagas disease was also prevalent 4.000 years ago in Latin America (Aufderheide et al., 2004). However, it was not until 1909 when a Brazilian physician and infectologist, Carlos Chagas, described the disease for the first time, which was later named after him (Chagas, 1909).

1.2 *Trypanosoma cruzi*

Trypanosoma cruzi is a parasitic protozoan that belongs to the order Kinetoplastida and the family Trypanosomatidae, characterised by the presence of one flagellum and a single mitochondrion in which the Kinetoplast is situated, a specialized DNA-containing organelle. *T. cruzi* is included in the section Stercoraria because it is the only human trypanosome to be transmitted by the feces of its invertebrate vector, as opposed to other trypanosomes transmitted by saliva (Salivaria section) such as *Trypanosoma rangeli,* a non-pathogenic species from South America transmitted by Triatominae and also able to infect humans; and *Trypanosoma brucei*, causing agent of the “sleeping sickness” in Africa (African trypanosomiasis). An important difference between this latter and *T. cruzi* is that *T. cruzi* is an intracellular parasite, whereas *T. brucei* lives and reproduces in the bloodstream. To the same family belongs another important human parasite, *Leishmania spp.*, which is responsible for the leishmaniasis (cutaneous, mucocutaneous and visceral), also called *kala-azar* in some regions, and very closely related to *Trypanosoma cruzi*.

The *Trypanosoma cruz*i population is not homogeneous and is composed of various strains. It has been demonstrated that this parasite undergoes some mechanisms of genetic exchange (Gaunt et al., 2003); however, its genetic diversity mainly results from the long-term

evolution of independent clones (Tibayrenc, 2003). After both isoenzyme and DNA analyses, a consensus has been reached to group most of the several existing clones into two principal subspecies, *T. cruzi* I and *T. cruzi* II (Fernandes et al., 1998) with marked heterogeneity, and five subdivisions within the *T. cruzi* II (II a-e). The *T. cruzi* strain selected for the genome sequencing project is a hybrid IIe strain. While both subspecies cause the human disease, *T. cruzi* II is more frequently associated with the domestic cycle, and *T. cruzi* I with the sylvatic cycle (see section 1.4). Furthermore, isolation and study of *T. cruzi* populations from different origins demonstrated the presence of a large range of strains with distinct characteristics. This intraspecific variation includes morphology of blood forms, curves of parasitemia, virulence, pathogenicity and sensitivity to drugs.

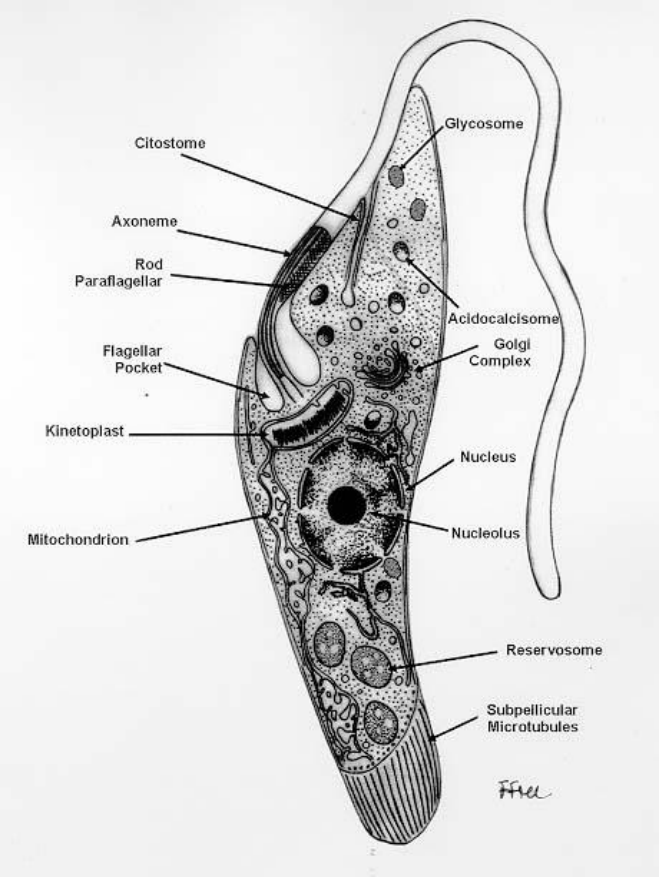

Figure 1.2-1 *Trypanosoma cruzi* morphology. *T. cruzi* has a single tubular mitochondrion where the kinetoplast is located. The kinetoplast is a fibrous network of DNA which constitutes 20-25% of the total parasite DNA. The flagellum is connected to the basal body and emerges from a specialized invagination, the flagellar pocket, which is apparently involved in the ingestion and uptake of nutrients from the external medium. Subpeculliar tubules are organized as cytoskeleton of the organism (Brener, 1992). (Image: http://www.scielo.br/img/fbpe/mioc/v94s1/html/8308.html).

1.3 Life cycle of *T. cruzi* and disease transmission

Infective metacyclic trypomastigotes contained in the feces of the Triatoma bug enter the human body through the skin at the site of bite or through mucosal surfaces such as the eyes, producing in some cases a local swelling. While some parasites stay at the bite site, other trypomastigotes reach the circulatory system, from where they penetrate different organs (especially heart, walls of the gastrointestinal tract, or skeletal muscle). Trypomastigotes can infect most vertebrate cells, especially leukocytes and macrophages. In the host cell, they convert to amastigotes, which reproduce by binary fission to form pseudocysts. Some amastigotes later transform into trypomastigotes that re-enter the blood circulation, where they can be taken up when another triatomine feeds, entering the bug's midgut. In the gut, the flagellates change to short epimastigote and rounded spheromastigote forms, in which they multiply profusely by binary fission. After one or two weeks, longer epimastigotes enter the rectum, where they form metacyclic trypomastigotes that are passed again to another human or animal host when the bug defecates.

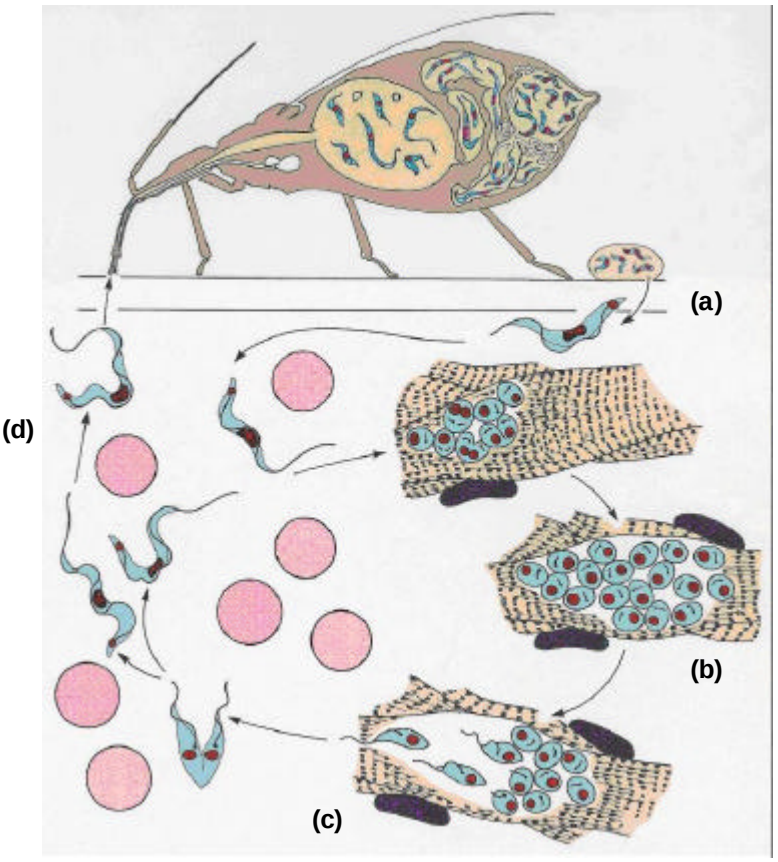

Figure 1.3-1 *Trypanosoma cruzi* life cycle. When introduced into the mammal host **(a)**, the metacyclic trypomastigotes must invade a cell to achieve its life cycle. After a latency time of 20-35 hours, amastigotes initiate a process of binary division that is repeated every 12-14 hours **(b)**. When the cell is saturated with parasites , amastigotes begin their differentiation into trypomastigotes. The intense movements of trypomastigotes disrupt the cell **(c)**, releasing free parasites that can invade other cells or can be sucked from blood by a vector, likewise completing the parasite life cycle **(d)**. (Image: adapted from Peters and Pasvol, 2006).

As described above, people can become infected with Chagas disease by contact with the insect vectors. This way of transmission accounts for about 80% of the cases, and it is only possible in endemic areas where the insects live. It occurs when the bugs directly deposit their infected feces on the skin of a person, and the parasite penetrates the skin through scratch wounds (bugs bites cause itching). It is also possible that the person gets infected, unknowingly touching their eyes, mouth, or open cuts after having come into contact with infective Triatomine bug feces. It takes place generally at night, when people sleep and the bugs are more active, getting out of the wall crevices where they live looking for food.

Blood transfusion and organ transplantation is the second most important way of infection, being responsible for 5 to 20% of the cases, depending on the region. It occurs while receiving an infected blood transfusion or organ transplantation.

In congenital infection, *Trypanosoma cruzi* crosses the placenta, and the foetus receives the infection from the mother during pregnancy. About 4% of the total Chagas disease cases have the origin on vertical transmission. This way of transmission does not have to be confused by the possibility of infection during birth through the contact of contaminated blood of the mother and open wounds or mucosas of the newborn.

Other minor transmission ways which account for less than 1% of the cases include laboratory accidents, while manipulating living parasites or infected blood; and ingestion of contaminated food, such as the outbreak in 2005 in Brazil, where many people got infected by eating contaminated sugar cane (Steindel et al., 2008). Other transmission modes have been reported including breast feeding and sexual transmission, but they where most probably due to open wounds, and therefore to contact with infected blood (Bittencourt et al., 1988; Nathan-Larrier, 1921).

1.4 Vectors

Vectors of Chagas disease are insects of the order Hemiptera, family Reduviidae, subfamily Triatominae. Members of this subfamily are blood-feeding insects and are called many different names including Triatomine bugs, reduvid bugs, kissing bugs, cone nosed bugs and assassin bugs. There are about 118 species of Triatominae, however, only a small number are significant *T. cruzi* vectors. The three most important vector species of the human Chagas disease are *Triatoma infestans, Rhodnius prolixus,* and *Triatoma dimidiata*. From Mexico to Venezuela and Colombia, *R. prolixus* and *T. dimidiata* are the principal vectors, while *T. infestans* is undoubtedly the main source of human Chagas disease below the Equator line.

Depending on the intermediate hosts and vectors, two different *T. cruzi* life cycles are to be distinguished, the “sylvatic cycle”, which involves the interaction between vectors and animal hosts; and the “domestic cycle”, that results from human-vector contact, which is responsible for Chagas disease. Generally, in the sylvatic cycle a balance between the vector, parasite and host (various mammals such as opossums and armadillos) is maintained. Human encroachment into the forest and destruction of the natural habitat forced the Triatomine and mammals to colonize human domiciles (poor quality rural housing) thereby initiating the domestic cycle. In this cycle, humans are the main domestic reservoirs, followed by dogs, cats and domestic rodents. It has to be noted that only mammals are susceptible to *T. cruzi* infection. Birds, amphibians and reptiles are naturally resistant, while pigs, goats, cattle and horses exhibit only transitory parasitemia and do not play an important role in transmission (Dias JCP,1992).

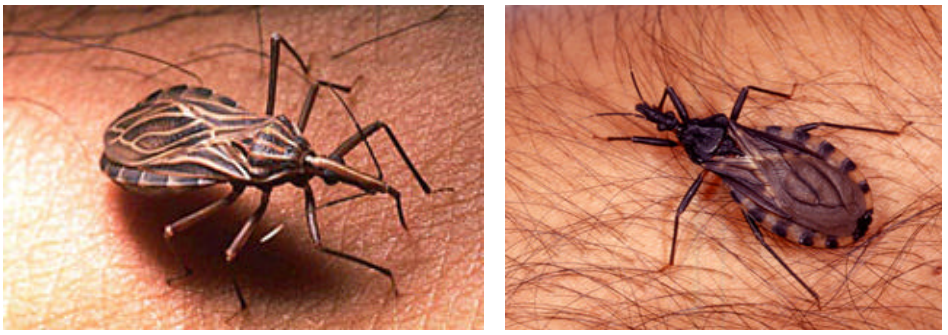

Figure 1.4-1 *Rhodnius prolixus* (left) and *Triatoma infestans* (right). Triatoma bugs are haematophagous insects of 3 to 5 cm size, varying between species. Their head is elongated and exhibits a tubular form, adapted to the blood-sucking function. Compound eyes are positioned laterally, and the projection of the head hairs allows to distinguish one species to the other. Vectorial transmission of *T.cruzi* to humans and other mammals is due to the contact of these vertebrates with the feces of the infected vector.

1.5 Pathogenesis of Chagas disease

1.5.1 Invasion and survival strategy of *T. cruzi.* Immune response

Trypanosoma cruzi is an intracellular parasite, and invades phagocytic cells, especially macrophages. The parasite recognizes lectin and integrin receptors at the cellular membrane. After binding of ß-integrin on the surface of the host cell, there is an increase of Ca^{2+} concentration in the cytoplasm of the cell (Tan and Andrews, 2000). The Ca^{2+} signal attracts the lysosomes to the places where the parasite is located. They fuse with the cellular membrane and form a surrounding that allows *T. cruzi* to enter the cell. Once inside the vacuole, the parasite secrets a trans-sialidase enzyme, that cleaves the sialic acid (that *T cruzi*

is not able to synthesize) from the lysosomal proteins and transfers them to its own surface molecules. On that way, *T. cruzi* is covered with sugars from the host cell, being then able to invade the cell. Finally, the parasite secrets a porus bilding-toxin, that lyses the vacuole, and the parasite is free in the cytoplasma where it reproduces. Once in the cytoplasma, the trypomastigotes, by means of the protease cruzipain, transform into amastigotes and divide. The amastigotes transform to trypomastigotes, start an intense movement and the cell bursts out, releasing hundreds of parasites to the bloodstream, where they can distribute through the entire organism and invade new cells again.

In the parasited cells, such as macrophages, *T. cruzi* uses different survival strategies. For example, it neutralises toxic oxygen derivatives, inhibits the nitric oxide synthesis (Clark et al., 1996), and modulates the programmed cell death (apoptosis) (Heussler et al., 2001), helping the trypomastigote forms to be released. The parasites are also able to alter the cytokine production of the host, in such way that they induce the secretion of cytokines that can inhibit the parasitic death, for example, IL-10 and TGFß. When the parasite is inside the cells, a T_H1 immune response predominates. But the host produces also an immune response against the extracellular forms of the parasite, playing the antigenic variability of the parasite, in this case, an important role in the evasion mechanisms.

Contradictory results have been reported on the participation of autoimmunity in experimental infection, and its role in the pathogenesis of Chagas disease remains controversial. However, the existence of molecular homologies between the parasite and host molecules, so called “molecular mimicry”, is indisputable, such as between the epitope B13 of *T. cruzi* and the heavy chain of cardiac myosin (Cunha-Neto et al. 1996), or between the ribosomal proteins of *T. cruzi* and some human P proteins and an extracellular functional loop of the human ß1-adrenergic receptor. The antibodies from patients with *T. cruzi* chronic infection, by recognising such human antigens, might contribute to worsening the cardiac dysfunction induced by the parasites (Tibbetts et al., 1994; Cunha-Neto et al., 2006).

The parasite plays a fundamental role in the genesis and development of organ lesions by sequentially inducing an inflammatory response, cellular lesions and fibrosis. Such pathological processes may occur in many organs but appear more frequently and more intensively in the heart, esophagus, and colon. The inflammatory response results from the rupture of infected cells releasing trypomastigotes and is intense in the acute phase. Cellular lesions affect the myocytes and the nervous cells (leading to an autonomic denervation), and fibrosis, which appear slowly and gradually, and is associated with chronic chagasic myocardiopathy (Carlier, 2003).

1.5.2 Clinical manifestations. Phases of Chagas disease

Three phases can be distinguished on the Chagas disease course: acute phase, indeterminate (or latent) phase and chronic phase. During the acute phase (1 to 4 months), the disease is characterised by active infection with circulating trypomastigotes in the blood. This phase frequently passes unnoticed, although there may be an inflamed swelling at the trypanosome entry site such as Romaña's sign (see Figure 1.5-1), which is characterised by periorbital edema and conjunctivitis (Romaña, 1935). An inflammatory lesion in the skin at the parasite entry site, known as chagoma, develops less frequently. Other symptoms, if they become apparent, will occur within seven to nine days post-bite and may include fever, tiredness and swelling of lymph nodes. These symptoms spontaneously resolve within three or four months. A small number of individuals develop severe complications associated with an acute myocarditis or meningoencephalitis, and die. During this stage, a large number of parasites are found in blood, although parasitemia levels vary between individuals.

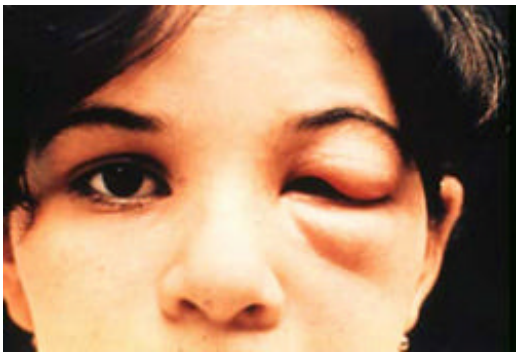

Figure 1.5-1 Romaña's sign. Conjunctival contamination with the vector's feces results in unilateral painless palpebral and periocular swelling. One of the few characteristic symptoms which occur in the acute phase of Chagas disease (Image: WHO/TDR).

During the indeterminate (or latent) phase, patients are clinically asymptomatic, and as good as no parasites are detectable in blood, however, they are seropositive. Some 50-70% of persons in this phase never develop recognizable chronic lesions and remain asymptomatic, but they are vulnerable to sudden death due to cardiac conduction abnormalities, sometimes without being diagnosed with Chagas disease.

The chronic phase develops 10-30 years after infection in approximately 30% of infected patients. During this phase, organs including heart, oesophagus, colon, and peripheral nervous

system can be affected. Chronic Chagas disease most often manifests itself as cardiomyopathy or autonomic neuronal dysfunction resulting in megasyndromes.

The heart is frequently affected in the chronic stage of disease, with significant destruction of the conduction system, myocytes, and parasympathetic cardiac nerves. This and the appearance of arrhythmogenic electric foci in the inflammatory areas are at the origin of arrhythmic syndrome (EKG abnormalities). The hypertrophy of remaining myocytes and the intense fibrosis replacing the destroyed myocytes predispose to cardiac dilatation and failure, and as a consequence, enlarged hearts are observed in patients. The left ventricular wall can become thinner, allowing the formation of an apical aneurysm, which is a feature of Chagas disease (Figure 1.5-2). Most of the “sudden death” cases by this disease are due to cardiac complications.

At digestive level, lesions (parasympathetic intramural denervation) are dispersed irregularly and mainly affect the esophagus and the colon. The affected segment may have a normal macroscopic appearance with only peristaltic alteration, leading frequently to constipation, which in many cases remains unnoticed by the patients. However, it may be dilated (megaesophagus or megacolon, Figure 1.5-2), or it may be dilated and elongated (dolichomegaesophagus). An increase of rigidity and tension of the cardia is present at the onset of esophageal dysfunction. The digestive pathology can be easily underdiagnosed, especially when the symptoms are mild, and therefore might be more frequent as previously described (Guevara et al., 1997).

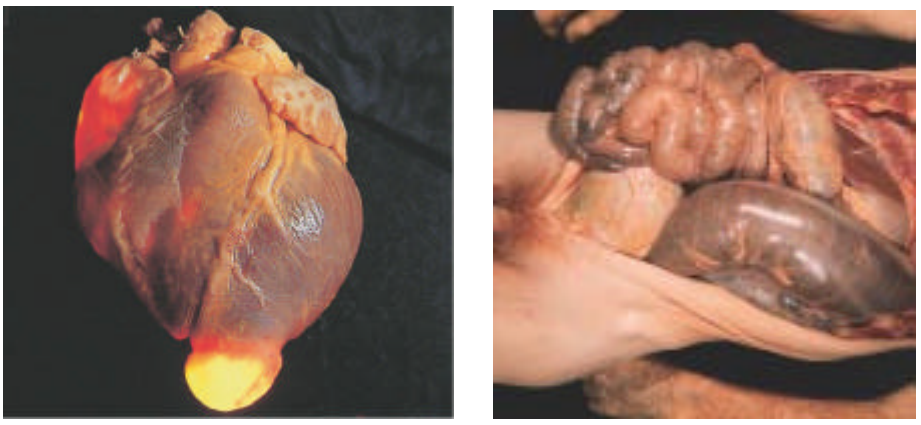

Figure 1.5-2 Apical aneurysm (left), and megacolon (right). The "apical aneurysm" is a very special heart lesion whose identification helps in the macroscopic diagnosis of the cardiopathy. Its location is the left heart apex, less frequently the right side. On acquired Chagas disease megacolon, degenerative changes occurs, including a quantitative reduction in the intramural ganglia of the entire intestinal tract. The denervated muscle layer becomes hypertrophic and lacking in propulsive efficiency owing to its in-coordinate contractions (aperistalsis). The result is fecal stasis, dilatation and finally hypoxic atony. (Images: Peters and Pasvol, 2006).

Chagas disease can be an opportunistic infection on HIV/AIDS individuals (Vaidian et al., 2004), occurring in endemic areas as well as in non-endemic areas, but frequently linked to social exclusion and poverty. The reactivation of the chronic Chagas disease, which can manifest in form of meningoencephalitis or myocarditis, has been observed in many of these cases. This happens because the immune response against *Trypanosoma cruzi* is mediated by T cells and, in HIV positive patients, this cell population is altered. In addition, coinfection of Chagas disease and HIV/AIDS shows difficulties on the immunodiagnosis, due to the diminished anti-*T. cruzi* antibody production. On the other hand, the elevated parasitemia level in these patients represents an advantage while using microscopy and xenodiagnosis to detect *T. cruzi* (Braz et al., 2001).

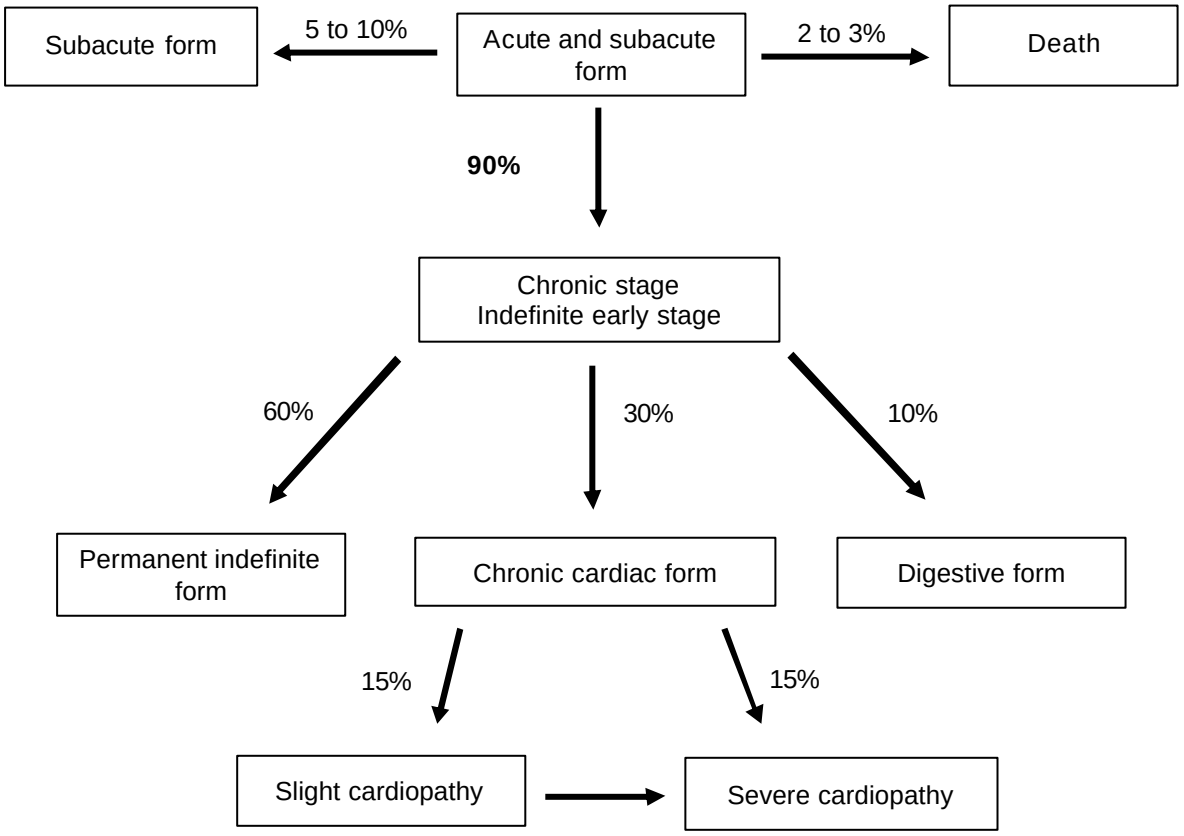

Fig 1.5-3 Diagram showing different Chagas disease stages. Almost all patients pass from the acute or subacute stage to a latent chronic stage, which can take 10 to 20 years. Most patients remain in an indefinite stage for the rest of their lives, but others develop cardiac or digestive complications. Cardiopathy severity varies between individuals. In some cases the complication level increases, leading to death. Taken together, patients which do not show any apparent symptoms, basically latent and mild foms, represent the majority of the cases (adapted from Albajar-Viñas et al., 2007).

1.6 Epidemiology of Chagas disease: geographical distribution, prevalence, incidence and mortality

Chagas disease is endemic in 18 countries in Central America (Belize, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua and Panama) and in South America (Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Paraguay, Peru, Uruguay and Venezuela). In addition, it is prevalent but less well documented in Guyana, French Guiana and Suriname (Figure 1.6-1). Originally, the disease was almost exclusively found in rural areas, characterized by poor sheltering, huts constructed with mud and covered by grass or palm leaves, where very poor population lives in contact with Triatomines (Figure 1.6-2). In such situation of poverty, people tend to emigrate to urban centres, spreading by this way the disease to cities.

Current estimates of the prevalence of Chagas disease range from 9.9 to 14 million infected individuals. This amounts to approximately 2-3% of the population of Latin America, with 100 million (25% of the Latin American population) at risk of acquiring the disease, which is killing around 50.000 people annually. Estimates of incidence range from 185.000 to 317.000 new cases per year, with a WHO-cited figure of 200.000 new cases per year. Due to the lack of an adequate reporting system, Chagas disease prevalence and mortality rates are chronically underestimated and dark numbers are higher as some published official statistics.

Figure 1.6-1 Geographical distribution of Chagas disease. The disease is distributed in the Americas, ranging from Mexico to Argentina, mostly in poor, rural areas of Central and South America. It predominates in warm areas were the insect vector lives. However, the new distribution tendency includes urban centres where other ways of transmission, different to vectorial, exist.
(Image:http://upload.wikimedia.org/wikipedia/commons/c/c3/Carte_maladie_Chagas.png)

Figure 1.6-2 Palm house in El Beni, Bolivia (left) and ”adobe“ house in Guatemala. These kinds of dwellings are characteristic of low-income settlements. Triatomine bugs live in the palm roofs and in crevices of adobe houses, getting out, mainly at night, to feed blood from sleeping people. (Image left: courtesy of Beck E; right: Médecines Sans Frontières, MSF).

1.7 Control of Chagas disease: vectors and blood banks

With no vaccine or specific treatment available for large scale public health interventions, the main control strategy still relies on prevention of transmission. Over the course of several decades, all available resources and planned activities targeted predominantly vector control. But with the arrival of the HIV/AIDS pandemic, this focus was extended to the monitoring of blood transfusions, and then large-scale screening of blood donors in Latin America began (Dias et al., 2002).

Through the Southern Cone initiative, which started in 1991, governments of the six Southern Cone countries (Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay) launched an ambitious initiative to control Chagas disease through both elimination of the main vector, *Triatoma infestans*, and large-scale screening of blood donors (Schofield et al., 1999). Stimulated by the Southern Cone success, two further regional strategies were launched in 1997 in Central America and the Andean Pact region. In both cases, the main targets are *R. prolixus* and *T. dimidiata*. More recently started in 2004 the Amazonic Initiative, that targets *Rhodnius* and *Panstrongylus*, vector species present in the Amazonic area. These initiatives have knocked down transmission rates dramatically primarily through insecticidal spraying of houses (Talerton et al., 2007). Unfortunately, the required repeated spraying to prevent re-infestation of houses by the insect vector, also promotes the development of insecticidal resistance, which has been already documented (Picollo et al., 2005). For this reason, improvement of housing would potentially reduce vectorial transmission permanently, but the economic costs of this strategy are too high for some regions, even though there are successful examples in Venezuela (Tonn, 1988).

1.8 Economic burden of Chagas disease

Chagas disease remains the most important vector-borne neglected disease in the Americas today (Inter-American Development Bank, 2005). As in all neglected diseases, Chagas disease creates financial and social burdens to the affected individuals and countries. The early mortality and substantial disability caused by this disease, which often occurs in the most productive population, young adults, results in devastating economic loss. Recent data demonstrate that globally, Chagas disease is associated with 0.7 million DALYs (Disability-Adjusted Life Years) constituting the sixth most important neglected tropical disease worldwide (Hotez et al. 2006).

In Latin America, Chagas disease holds the fourth place among the ailments with the largest burden of disease. The comparative burden of Chagas disease among infections and parasitic diseases in the region is less than diarrhoeal disease, HIV, and tuberculosis, but higher than meningitis, sexually transmitted diseases, hepatitis B and C, and malaria (Figure 1.8-1).

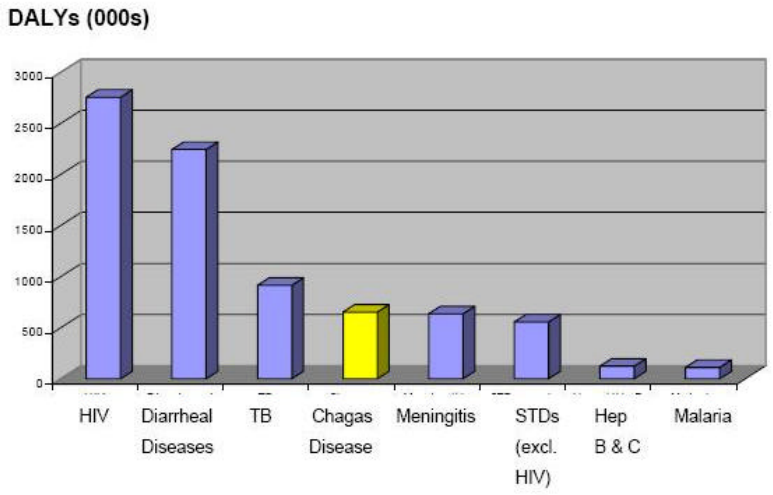

Figure 1.8-1 Infectious and parasitic disease burden in Latin America. The economic impact of Chagas disease during the chronic stage is very high, representing the first cause of cardiac lesions in young, economically productive adults in the endemic countries in Latin America. Data in DALYs (Disability-Adjusted Life Years). (Image: OneWorldHealth Institute, 2007)

Poor housing, lack of access to safe blood-bank supplies, and inadequate prenatal care are linked to social and health inequalities in many areas of Latin America. People living under these conditions are the ones at risk of acquiring Chagas disease. At the same time, this disease contributes to maintain the poverty cycle, keeping people in many areas unable to

work, stigmatised, and therefore poor and in underdevelopment. Additionally, *T. cruzi* infection impose an overwhelming load to the healthcare system due to hospitalizations and medical and surgical treatments especially as consequence of chronic manifestations such as chagasic cardiomyopathy, gastrointestinal dysfunction, and meningoencephalitis.

The annual economic loss in Latin America due to early morbidity and mortality from Chagas disease has been estimated to be as high as $18 billion. Countries with larger rural populations generally have higher prevalence rates, less vector control and less available resources for healthcare, and hence are more negatively impacted economically than countries with larger urban populations (Franco-Paredes, 2007).

1.9 International impact

In our days, travel of persons from one point of the planet to the another has become much easier than it was one century ago. People are constantly moving in their own countries, from the land to the cities, but are also traversing borders; and with them, their infections. Globalisation includes not only humans, but also pathogenic agents. Thus, diseases are not any longer only limited to their original endemic area, but are becoming cosmopolite. The so called tropical diseases exist not only in tropical regions, but can appear in almost every place on the planet linked to moving people. Therefore, according to this new tendency, the term “emergent infectious disease” has been defined. This means an infectious disease that has newly appeared in a population, where it originally did not exist, or a disease which has been already eradicated and it arises again, rapidly increasing in incidence or geographic range.

Chagas disease has been classified as emergent disease, not only due to the urbanization tendency, but also because migration is causing thousand of persons every year to abandon their homes, and leave for other places where they hope to have better opportunities. Many people from Chagas disease endemic countries in Latin America migrate to other rich countries, especially in North America (United States and Canada) and Europe. A minor group of migrants moves to Asia and Australia.

The fact that Chagas disease can be transmitted by blood transfusion and organ transplantation, together with the lack of specific test screening in blood banks and hospitals, has facilitated the spreading of the disease enormously. There are already several registered cases of infection by this ways in the United States and Europe (CDC, 2006; Young et al., 2007; Flores-Chavez et al., 2008).

Vertical transmission of the disease is an additional uncontrolled spreading factor, because children born with *Trypanosoma cruzi* infection remain undiagnosed in most of the cases, transmitting later the disease to other persons via blood and organ donation or, in the case of women, giving birth to infected children perpetuating congenital transmission (Muñoz et al., 2007).

Travel from tourists to Chagas disease endemic areas and infection there, is extremely rare, because people do not usually sleep in poor conditions or receive blood transfusions in these countries. However, it can occur, and people travelling to these areas are potentially exposed to infection and should be previously informed. Because physicians outside Latin American countries do often not recognize the disease, the probability of underdiagnosing the infection is extremely high.

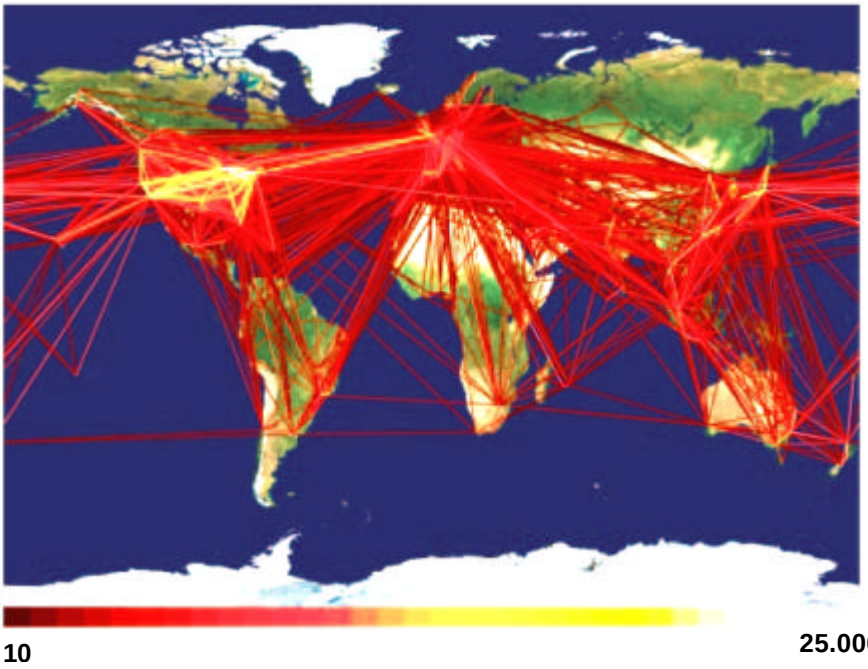

Figure 1.9-1 Global aviation network. A geographical representation of the civil aviation traffic among the 500 largest international airports in 100 different countries is shown. Each line represents a direct connection between airports. The colour encodes the number of passengers per day (see colour code at the bottom) travelling between two airports (Hufnagel et al., 2004). The expansion of international air travel has enhanced exponentially the hazard of spreading pathogens through human-to-human contact from a limited locality across the continents.

1.10 Current therapy and potential new therapies. Vaccines

At present, two drugs are available for the treatment of Chagas disease: Benznidazol (Rochagan® or Radanil®, Roche) and Nifurtimox (Lampit®, Bayer), both nitroheterocyclic compounds (Figure 1.10-1). They resulted from veterinary investigations in the 1970es, and have never been improved. These drugs show significant activity only in the acute and short-term chronic phase, with up to 80% parasitological cures in treated patients, and their effectiveness varies according to the geographical area. It has been observed that children tolerate these drugs better than adults. For this reason the early diagnosis and treatment leads to high chances to be cured.

Trypanosoma cruzi has been shown to be deficient in detoxification mechanisms for oxygen metabolites, particularly hydrogen peroxide, and is thus more sensitive to oxidative stress than are vertebrate cells (Docampo, 1990). Benznidazol acts via reductive stress, which implies the covalent modification of macromolecules by nitroreduction intermediates. Nifurtimox acts via the reduction of the nitro group to unstable nitroanion radicals, which react to produce highly toxic, reduced oxygen metabolites (superoxide anion, hydrogen peroxide). Side-effects of both compounds are probably resulting of their oxidative and reductive damage to the host's cells. Unfortunately, the antiparasitic activity of these compounds is obligatory bound to their toxicity to the vertebrate host.

Differences in drug susceptibility between different *T. cruzi* strains have been observed (Andrade et al., 1992). These differences are product of the natural susceptibility, but also drug-resistant *Trypanosoma cruzi* strains have been described (Buckner et al., 1998).

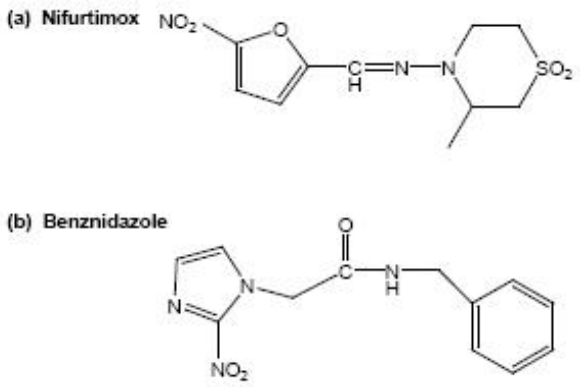

Figure 1.10-1 Nifurtimox (a) and Benznidazole (b) chemical structure. Both are nitroheterocyclic compounds, Nifurtimox is a nitrofuran, and Benznidazole a nitroimidazole derivative.

It is important to note that, due to the fact that the acute phase is frequently asymptomatic, many people do not realise to be infected until they are in a chronic phase, being then the effectiveness of the drugs very low. For this reason, there is an urgent need for a new drug (safer and more effective), with minimal side-effects and with activity also in the chronic phase.

It is easier to use drugs which are already in the market and are being used for other diseases, than to develop new ones. Efforts are being done in this direction. Some of the most promising new approaches for the development of drugs against Chagas disease, which have already completed pre-clinical studies, include sterol biosynthesis inhibitors. These are based on the fact that *T. cruzi* requires specific sterols for viability and proliferation in all stages of its life cycle, and the parasite has been shown to be very susceptible to inhibitors of ergosterol biosynthesis (Urbina et al., 2003). Cysteine protease inhibitors have been also tested. These are selective inhibitors for a protease (named cruzipain, cruzain or gp51/57), responsible for essential functions of the parasite. When it is blocked, proliferation of epimastigotes and amastigotes is arrested. Pyrophosphate metabolism inhibitors are other possible anti-trypanosomatid and anti-apicomplexan drugs, based on the specialized organelles that trypanosomatid and apicomplexan parasites contain, acidocalcisomes, which are involved in polyphosphate and cation storage, and in the adaptation of these microorganisms to environmental stress. Bisphosphonates are selectively accumulated in the parasite and inhibit enzymes involved in inorganic and organic pyrophosphate reactions.

Another promising possibility, still in pre-clinical studies, includes inhibitors of purine salvage. Trypanosomatids are absolutely deficient in the *de novo* synthesis of purines. Instead, they scavenge the essential compounds from their growth medium. Allopurinol, used since long time for the treatment of gout, acts as a purine analogue, and is incorporated into the parasite's DNA, disrupting the synthesis of RNA and proteins.

Other possible drugs against *Trypanosoma cruzi* include inhibitors of trypanothionine metabolism, based on the unique biochemical pathway to kinetoplastid protozoa, which replaces glutathione and glutathione reductase in the cell; inhibitors of phosphatidylcholine biosynthesis, such as miltefosine, already used for the treatment of leishmaniasis; transialidase inhibitors, Na^+H^+ Exchange inhibitors, and dihydrofolate reductase inhibitors.

At the moment, there is no existing vaccine for Chagas disease and its development has had a low priority, due to the high effective impact of other measures such as vector control and blood bank screening. However, question of cost-effectiveness increases the interest for the creation of a vaccine, but even though efforts are being made, progress is still slow. Current

strategies focus basically on recombinant subunit vaccines, live attenuated vaccines and DNA vaccines.

Recombinant subunit vaccines, such the immunization of mice with recombinant paraflagellar rod proteins (PAR) (Luhrs et al., 2003) and cruzipain (Schnapp et al., 2002), showed protective immunity in acute infection but did not block completely parasitemia. The different attempts to use a whole killed cell or attenuated vaccines produced only partial immunity. In this aspect, reduction of mortality and parasitemia in mice after immunization with *Trypanosoma rangeli* (Basso et al. 2004), and mucosal protective responses after oral feeding of mice with infected Triatomatide excreta (Hoft et al.,1996) have been observed.

DNA vaccines have shown to elicit both humoral and cellular immunity. Attempts using trypomastigote surface antigen I (TSA-I) (Wizel et al., 1998), Tc52 protein (virulence factor of *T. cruzi*), and trans-sialidases have been made. However, this kind of vaccines are still in a very early stage and it will take many years until they can be used in humans.

1.11 Diagnosis

Chagas disease is asymptomatic in most of its course in both, intermediate and chronic phases. This fact represents a major problem and is the reason why so many people are unconscious to be infected. In the acute phase some diffused and unspecific symptoms can appear, but not necessarily. Therefore, the diagnosis by symptoms is problematic and it is of great importance to apply other accurate diagnostic methods.

1.11.1 Direct microscopic methods

The classical direct microscopic observation of fresh anticoagulated blood or blood smears is highly specific, but its sensitivity varies, because the titre of circulating parasites can be very low and, in addition, a trained microscopist is required. Observation of fresh blood allows seeing *T. cruzi* living parasites in movement. Blood smears facilitate the differentiation of *T. cruzi* and *T. rangeli*, because it permits the easy visualization of the nucleus, kinetoplast and flagella, and can be used if the sample has to be transported.

In order to concentrate the parasites and facilitate their detection, other alternative methods can be used. The microhematocrit technique consists on the detection of *Trypanosoma cruzi* parasites in the “buffy coat” prepared from whole blood in heparinized

capillary tubes by centrifugation. Its use is specially extended in congenital Chagas disease, because only a little amount of blood is needed. However, this manipulation represents a risk of infection, and requires a special protection for the laboratory workers. The Strout method consists on the incubation of blood for 1 hour at 37ºC, collection of the serum by centrifugation to eliminate the erythrocytes, a second centrifugation of the obtained supernatant, and the examination of the precipitate from the last centrifugation under the microscope.

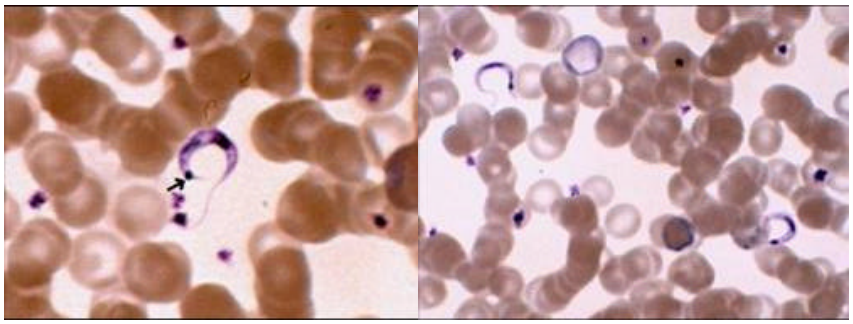

Figure 1.11-1 *Trypanosoma cruzi* microscopic observation in blood smears. Blood smears have been stained with Giemsa, which highlights DNA, allowing to distinguish kinetoplast in extracellular Trypomastigote forms, but also in Amastigotes that are inside the cells. Trypomastigotes can be observed especially in blood samples from patients in acute phase.

1.11.2 Indirect methods

Indirect methods consist on the multiplication of the parasites from the collected samples either in the Triatomine bug (xenodiagnosis) or in a culture medium (hemoculture). Xenodiagnosis is characteristic for Chagas disease. Excretes of sterile Triatomine insects breed in the laboratory are examined for *Trypanosoma cruzi*, after an incubation period (4 to 6 weeks) posterior to the bite of patients to be tested for Chagas disease. Alternatively, the insects can be put in contact with blood of the patients through a latex membrane. It is 100 % specific, but it takes long time to obtain the results and retards therefore the beginning of the treatment. It is, however, a widespread method in endemic countries, because it is quite inexpensive and does not require sophisticated laboratory equipment.

For the hemoculture, blood samples from patients are grown in liver infusion tryptose medium for a period of four to six months, and this is examined regularly for parasites. As xenodiagnosis, it is a highly specific method, but it takes long time to obtain the results. In addition, the sensitivity of these indirect methods is very low in the chronic stage of the disease, when the parasite level in blood decreases.

1.11.3 Immunological methods

Commercial serologic tests used routinely in the laboratory include indirect hemaglutination (IHA), indirect immunofluorescence (IIF) and enzyme linked immunosorbent assay (ELISA). Indirect hemaglutination is based on the agglutination of crude antigens (from *T. cruzi* epimastigotes) with antibodies contained in the blood of patients. It has a high sensitivity but low specificity. Indirect immunofluorescence uses antigens from epimastigotes and fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin as secondary antibody. It is less expensive than IHA, but it requires a UV microscope and a well trained person. In addition, readings are subjective, and cross-reactions especially with visceral leishmaniasis can occur. The ELISA technique can be performed using either crude extracts or recombinant antigens. The specificity increases while using recombinant antigens, because cross-reactions, obtained frequently with crude extracts, do not occur. It requires a microplate reader and washer, but it allows large amounts of samples to be tested, and results are objective.

A special test, the complement-mediated lysis (CoML), detects anti-*T. cruzi* lytic antibodies which are recognized by epitopes present on the surface of living trypomastigotes, in contrast to conventional serology, which detects antibodies that recognize epitopes present on the surface of fixed parasites, or parasite extracts. This is a time-consuming technique that requires manipulation of living parasites, which represents a high risk for the laboratory personnel, and therefore it is not used as routine method.

Western blot immunodiagnosis uses epimastigote antigens from *T. cruzi*. This technique has been used to compare the antigenic pattern recognized by different sera and distinguish the antibodies transmitted by the mother and the ones produced by the baby, allowing on that way the detection of congenital Chagas disease. Other western blot immunoassays have been developed with excreted/secreted antigens to distinguish the acute from the chronic phase of the disease. The specificity of this technique is not very high, because it uses crude antigens of the parasite.

Lateral flow devices are the most appropriate method for the detection of Chagas disease in the field. They have a user-friendly format, requiring only little amount of whole blood or serum, and results are obtained within few minutes. In addition, they can be stored for long time at room temperature in a relatively wide range of climates. However, specificity has proven to be not very high, and for this reason it is the method of choice for blood screening in the field, but the confirmation by a second method is recommended for diagnosis of patients (Figure 1.11-2).

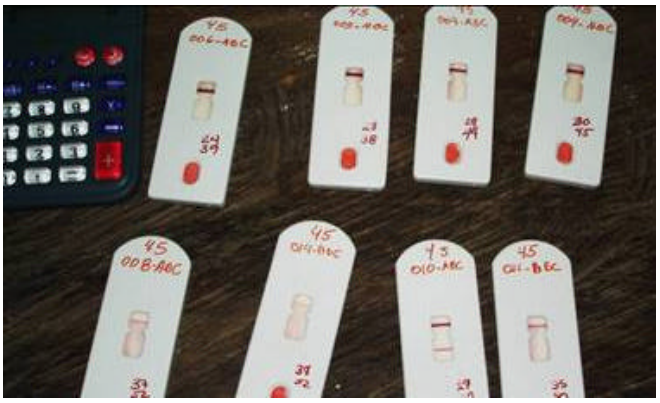

Figure 1.11-2 Lateral flow devices for diagnosis in the field. The only commercialised rapid tests for Chagas disease are used for large screening campaigns in the field (Image: MSF).

1.11.4 Molecular methods

Molecular methods for the diagnosis of Chagas disease are based on the detection of DNA of the parasite. The most important technique is the Polymerase Chain Reaction (PCR), which amplifies the parasite DNA in blood or tissue samples of patients. Many approaches including “nested” PCR or “real time” PCR (Piron et al., 2007) have been done, reaching highly specific and sensitive assays. However, this technique requires a laboratory equipped with a thermocycler, and the enzyme Taq DNA polymerase, which is usually quite expensive. PCR is also an appropriate method for the detection of congenital *T. cruzi* infection (Virreira et al., 2003), due to its sensitivity and the small amount of blood obtained in these cases.

To summarize, it can be concluded that direct microscopic examination, microhematrocrit, hemoculture and xenodiagnosis are generally specific techniques. Their major disadvantages rely on the fact that they are not very efficient in low parasitemia cases; there is a delay in obtaining results and, in the specific case of xenodiagnosis, that it causes stress to the patients. However, these are methods still widespread in the affected countries because they are inexpensive and do not require a high technology equipped laboratory.

Indirect hemagglutination and immunofluerescence as well as the complement mediated lysis and Western blot are quite sensitive but, in most of the cases, lack specificity, which can easily result in wrong diagnosis.

Serologic methods based on the detection of antibodies in blood of patients have the general limitation that antibodies persist for long time even after cure, and therefore it is not possible to distinguish an active infection from an already cured patient. With regard to the fact

that drugs used for Chagas disease are very toxic, the treatment time has to be reduced as much as possible. For these cases, PCR is highly recommended, even though this technique is not very popular in endemic countries, basically due to its equipment requirements and costs.

According to WHO recommendations, the general lack of specificity of many diagnostic assays for the detection of *T. cruzi* infection can at present be solved by the use of at least two different techniques for confirmatory diagnosis of Chagas disease. Low specific methods can be used for blood screening without major danger, because blood conserves are going to be discarded, but if the purpose is the diagnosis of the patient, then the technique must be accurate enough to distinguish infections with different pathogens.

Table 1.11-1 Chagas disease diagnostic methods summary table.

Method	Advantages	Disadvantages
Microscopic examination of blood	Highly specific	Trained microscopist needed Low sensitivity in chronic stage
Microhematocrit	A small amount of blood needed Specific	Extraction of “buffy coat” needed Limited sensitivity
Xenodiagnosis	Specific Inexpensive	Stressing for patient Takes long time to have a result
Hemoculture	Specific	Takes long time to have a result
Indirect Hemagglutination (IHA)	Highly sensitive	Low specificity
Indirect Immunofluorescence (IIF)	Sensitive	Not specific enough Requires training and expensive UV microscope
Complement-mediated lysis (CoML)	Sensitive	Handle of live parasites needed (high risk for laboratory staff)
Western blot	Sensitive	Low specificity
ELISA	Automatable Quantifiable	Requires a microplate reader Not able to distinguish chronic and acute phase
Lateral flow device	Field applicable Small amount of sample required Results within few minutes	Limited sensitivity and specificity
PCR	Highly specific and sensitive.	High risk of contamination

1.12 Bolivia, the most affected country

1.12.1 Health situation

In spite of its natural wealth, Bolivia is the poorest country in South America and 64% of the population lives below poverty line. The government heavily depends on foreign assistance to finance development projects.

Health conditions are notably poor, owing to inadequate hygiene conditions and insufficient number of medical doctors and hospitals, especially in rural areas, where 36% of the total population lives. The sustainable access to safe drinking water (only 85% of the population) and adequate sanitation (only 45% of the population) represents a main problem in these regions. In Bolivia, life expectancy at birth was reported to be of 65 years in 2005, and the country's population is young (38% of the population under 15), this latter due in part to high fertility rates (3.7 children per woman). However, maternal mortality accounts for 230 per 100.000 live births (WHO, 2004) and children deaths due to perinatal complications are very frequent.

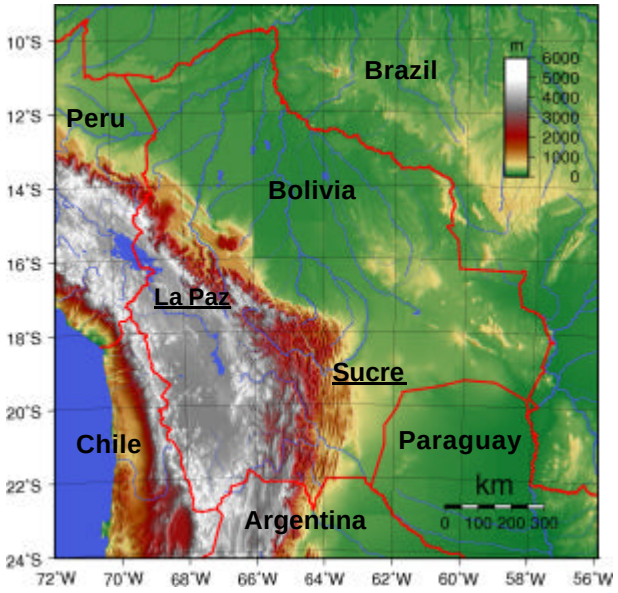

Figure 1.12-1 Bolivia's topography. Bolivia is a landlocked country in South America that borders with Peru, Brazil, Paraguay, Argentina and Chile. It has a territory of more than one million km^2 and a population of more than nine million inhabitants spread over three distinct topographies: highland plateaus and Andean mountain slopes, valley area, and plains. Almost the half of the population lives in the highlands.
(Image: http://commons.wikimedia.org/wiki/Image:Bolivia_Topography.png).

Leading death causes in Bolivia are cardiovascular problems (including those which are consequence of chagasic cardiopathy), communicable diseases and other causes such as diseases of the respiratory tract and affections originating in the perinatal period (PAHO, 2000). In 2003, 27% of children suffered from chronic malnutrition and of these, 8% from severe malnutrition. Children with chronic malnutrition are more likely to die from infectious diseases such as diarrhea and pneumonia, since their immune system is weak and they lack caloric reserves to fight off illnesses. At present, diarrhea causes 36% of deaths in children under five years in Bolivia.

Communicable diseases account for a high percentage of the deaths in the country, and the most common infectious disorders are acute respiratory diseases, tuberculosis, malaria, hepatitis, and Chagas disease. The HIV prevalence is 0.1% and it is currently increasing. The high rates of morbidity and mortality due to these diseases reduce the productive potential of Bolivia's human resources and reinforce the vicious cycle of poverty and hunger.

1.12.2 Chagas disease in Bolivia

Available information is not very accurate but, according to recent data, at present in Bolivia 1.6 to 2.1 million individuals are infected with *Trypanosoma cruzi* and 3.5 million are considered at risk to get infected. Chagas disease accounts for 13% of all the deaths in the country (MSF, 2004). The high disease prevalence, varying from 20 to 40% in some areas, is the largest infection rate in Latin America by far. The endemic area in Bolivia covers 60% of the country, and the rural population is considered as the main population at risk of infection. However, it has been recently shown that vectorial transmission is occurring in suburbs of the main cities, too. In addition, due to the large chagasic population, congenital and blood transmission are very abundant. Infection rates greater than 50% were reported in blood donors in Santa Cruz (Carrasco et al., 1990) and a prevalence of almost 10% congenital Chagas disease has been reported in some rural areas in Bolivia (Azogue, 1993).

Two major distinct monophyletic groups of clones (20 and 39) have been identified in the country in the domestic cycle, belonging to *T. cruzi* I and *T.cruzi* II respectively (Brenière et al., 1998), and mixed infections have been already described (Bosseno et al., 1996).

Some years ago, the Southern Cone Initiative, in which Bolivia was included, carried out spraying campaigns to eliminate the predominant insect vector, *Triatoma infestans*, and had a large impact in some areas, reducing enormously the infestation rates (Guillén et al. 1997). In addition, Bolivia's Ministry of Health, supported by the Pan-American Health Organization,

launched in the last years a campaign that aimed to control disease-bearing insects, treat Chagas disease in children under five years, safeguard national blood-bank supplies, and implement an epidemiological surveillance system. However, there is still a lot to do, and Chagas disease is still one of the most important public health problems in the country.

Figure 1.12-2. Chagas disease endemic area in Bolivia. The endemic area comprises around 55% of national territory (about 600,000 km^2) where about 3.5 million people are considered at risk to the infection. In highly endemic communities, especially in the Departments of Cochabamba, Chuquisaca, Tarija, Santa Cruz and Potosi, seroprevalence rates may reach close to 100% in older age groups (Image: IDBAmérica).

1.13 Purpose of this work

Infectious diseases continue to be the leading causes of disability and death in developing countries. In principle, these diseases would be preventable and treatable with our today's medical knowledge and are therefore listed as "preventable diseases" by the World Health Organisation. The causes for this unsatisfactory situation are manifold and complex, however, it is not always missing financial resources, but rather lack of rational strategies and appropriate tools. For the treatment of many of these diseases drugs are available, even at affordable prices. However, correct therapy presumes adequate diagnosis, and this is frequently more expensive than the drugs themselves, or the existing diagnostics are not applicable because they are not adapted to the needs of the specific conditions.

Chagas disease which belonged for many years to the category of “most neglected diseases” is exemplary in this respect. It has been called a "silent" disease because it kills people after years of hidden infection without significant forewarning. Médecins Sans Frontières called also a "silenced" disease because it affects mainly the poorest amongst the poor, and

neither the world nor even the governments of the concerned countries were interested to interfere. Only after blood and organ donations became a lucrative market, the disease turned to the attention of the scientific society, and more specific diagnostics were developed to prevent accidental infections with contaminated material. Consistently, these new diagnostics are appropriate for use in blood banks, but not for patients because they cannot clearly discriminate between Chagas disease and other diseases, which would be essential for appropriate therapy. The same holds for the only available drugs, once developed for use in animals and leading to severe side effects. There are no efforts from the site of the rich countries, who could afford it, to develop more appropriate drugs because there is no much money to earn.

However, when used on time, i.e. soon after infection, even these oldish drugs are useful and reveal a good cure rate combined with acceptable side effects. Under this aspect, a highly sensitive and specific diagnostic procedure would be highly beneficial when applied in regular periods of time for the whole population at risk. To be affordable for the impoverished countries even when used in large scale, it has to be inexpensive, too. Regarding the accumulated scientific data on Chagas disease, especially the almost complete genomic DNA sequence of *T. cruzi* and of several other Trypomastigotes, it looked very promising to develop new diagnostic procedures which would fulfil the conditions of being sensitive, specific and, at the same time, simple and inexpensive by combining molecular biological methods with contemporary bioinformatic tools.

Thus, it was purpose of this work, to design appropriate diagnostic procedures and to test their usefulness for the diagnosis of Chagas disease. To avoid cross-reactions, the new procedures should differentiate between related species such as *Leishmania species*, or *Trypanosoma rangeli*. In addition, the tests should be able to detect the disease in its acute as well as in its chronic phase, and they should also allow monitoring of the decrease of circulating parasites after treatment with drugs.

Two general techniques appeared to be appropriate: the production of *T. cruzi*-specific recombinant antigens for use in immunoassays and the design of primers for specific PCR diagnosis. Similar methods had been developed already by others, and it was therefore not needed to start form scratch. But the existing diagnostic tests appeared not good enough and were by far too expensive. Therefore, the new diagnostic procedures had to be as simple and inexpensive as possible and should not require very sophisticated laboratory equipment, otherwise their use in the affected countries would not be realistic.

2. MATERIALS AND METHODS

2.1 Instruments

Gel electrophoresis systems	
Horizontal minigel system (8 x 8 cm)	AGS, Heidelberg, Germany
Vertical minigel chamber (8 x 10 cm)	Keutz, Reiskirchen, Germany
Power supply EPS 500/400	Pharmacia, Freiburg, Germany
Shakers	
Horizontal shaker GFL 3020	Gesellschaft für Labortechnik, Burgweldel, Germany
Horizontal shaker GFL 3015	Gesellschaft für Labortechnik, Burgweldel Germany
Certomat R	Braun, Melsungen, Germany
Vortex Genie 2	Scientific Industries, Bohemia, NY, USA
Centrifuges	
Cooling centrifuge Beckman J2-21, (with Rotors: JA 14 and JA 20)	Beckman Instruments, Summerset, USA
Microfuge: Biofuge Pico	Heraeus Instruments, Hanau, Germany
Multifuge 3	Heraeus Instruments, Hanau, Germany
Waterbath	
GFL Wasserbad 1013	Gesellschaft für Labortechnik, Burgwedel, Germany
Thermocyclers	
T1 Thermocycler 96	Biometra GmbH, Göttingen, Germany
Primus 96	MWG Biotech AG, Ebersberg, Germany
Sonifier	
Sonoplus HD70	Bandelin, Berlin, Germany
Photographic equipment	
UV Transilluminator	Herolab GmbH, Wiesloch, Germany
UV transilluminator MW 312nm N36	Roth, Karlsruhe, Germany
Polaroid MP-4 Land Camera	Polaroid Corporation, Cambridge, MA, USA
Film: Polaroid 667 Professional	Polaroid Corporation, Cambridge, MA, USA
Olympus Camedia C-370 Digital camera	Olympus, Germany
Microplate reader	
Bio Rad Model 3550-UV	Bio Rad, Munich, Germany

2.2 Materials

2.2.1 Chemicals

Acrylamid	Serva, Heidelberg, Germany
Agarose for gel electrophoresis	Sigma-Aldrich, Munich, Germany
Ammoniumpersulfate (APS)	Serva, Heidelberg, Germany
Ammoniumsulfate	Carl Roth GmbH, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich, Munich, Germany
Adenil Triphosphate (ATP)	Carl Roth GmbH, Karlsruhe, Germany
Biorex 70 resin	BIO-RAD Laboratories, Hercules, USA
Bovine serum albumin fraction V (BSA)	Carl Roth GmbH, Karlsruhe, Germany
5-Bromo-4-chloro-3-indolylphosphat (BCIP)	Gerbu, Wiesloch, Germany
Coomassie Brilliant Blue R250	Serva, Heidelberg, Germany
Dithiothreitol (DTT)	Biomol, Heidelberg, Germany
Ethidium bromide	Serva, Heidelberg, Germany
Ethylenediaminetetraacetic acid (EDTA)	Serva, Heidelberg, Germany
Guanidiniumhydrochlorid (GHC)	ICN Biomedicals, Germany
Heparin sepharose	Pharmacia-LKB, Upsala, Sweden
Hydrogen peroxide 30% (w/v) (H_2O_2)	Sigma-Aldrich, Munich, Germany
Isopropyl-ß-D-thiogalactopyranose (IPTG)	Gerbu, Wiesloch, Germany
Kanamycin	Sigma-Aldrich, Munich, Germany
ß-Mercaptoethanol	Serva, Heidelberg, Germany
Nitro blue tetrazolium (NBT)	Gerbu, Wiesloch, Germany
N,N-dimethylacetamine (DMA)	Sigma-Aldrich, Munich, Germany
N,N,N‘,N‘- Tetramethylethylendiamine (TEMED)	Serva, Heidelberg, Germany
Phenylmethylsulphonylfluoride (PMSF)	Carl Roth GmbH, Karlsruhe, Germany
Polyethyleneimmine	Sigma-Aldrich, Munich, Germany
Polyethylenglycol (Mr 3500-4500)	Carl Roth GmbH, Karlsruhe, Germany
Sodiumdodecylsulfate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Tetracycline	Sigma-Aldrich, Munich, Germany
Tetrabutylammonium borohydride (TBABH)	Sigma-Aldrich, Munich, Germany 3,3’,5,5’-
Tetramethylbenzidine (TMB)	Gerbu, Wiesloch, Germany
Tween-20 (polyoxyethylen sorbitan monolaureat)	Serva, Heidelberg, Germany
Triton X-100	Merck, Darmstadt, Germany
Tris (hydroxymethyl) aminomethane	Carl Roth GmbH, Karlsruhe, Germany
Urea	ICN Biomedicals, Germany

Silica suspension for purification of nucleic acids

50 g of silica (SiO_2) powder (Sigma No. S 5631) were suspended in 250 ml of water, centrifuged for 3 min at 5000 x g, the milky supernatant was discarded, the sediment resuspended in another 250 ml of water and centrifuged again. The procedure was repeated 3-5 times more until supernatant became clear. The sediment was suspended in one volume of water and kept in 2 ml aliquots at -20°C.

Nitrocellulose membrane

Protran BA 85	Schleicher & Schuell Bioscience, Dassel, Germany

DNA size marker

Plasmid pSP 64 was cleaved a) with Hind III, b) with Dra I and c) with Hinf I. The individual cleavage assays were mixed in the relation 1:2:4 with respect to the amount of plasmid DNA, resulting in the following fragment sizes (bp):

2.2.2 Bacterial strains

Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB, lacIZ .M15* T*n10* (Tet)]
Escherichia coli XL1-Blue/pREP	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB, lacIZ .M15* T*n10* (Tet)]/pREP(Kan)

2.2.3 *Trypanosoma cruzi DNA*

A sample of *Trypanosoma cruzi* DNA (strain MIHOM/CH/00/Tulahuen C2) was obtained from the Bernhard-Nocht Institute in Hamburg, Germany. 80 µl of 3 M sodium acetate were added to the sample (700 µl in 70% ethanol) and the tube was incubated at -20ºC for 2 days. Afterwards, the sample was centrifuged 15 min at 13.000 rpm, and the small sediment washed once with 70% ethanol. DNA was redissolved in 400 µl TE buffer. The estimated final DNA concentration is 0.1 µg/µl.

2.2.4 Antisera

30 antisera from Chagas patients (Latin American immigrants living in Barcelona) were obtained from the parasitology department of the University of Barcelona. They were diluted with glycerol (50% final concentration) and kept at -20ºC.

400 additional Chagas disease patient sera were obtained from a diagnostic laboratory at the Servicio Departamental de Salud (SEDES), Cochabamba, Bolivia. They were diluted 1:1 with a saturated solution of ammonium sulphate, and kept as a suspension at 4ºC.

The leishmaniasis antisera from patients of cutaneous and visceral leishmaniasis used to test the cross-reactions of the recombinant antigens were obtained from a diagnostic laboratory in the Republic of Yemen, and were diluted 1:1 with a saturated solution of ammonium sulphate.

Control sera were derived from German blood donors in the Academic Hospital at the University of Giessen, Germany.

Phosphatase and peroxidase-conjugated goat anti-human IgG antibodies were bought from Dianova GmbH, Hamburg, Germany.

2.2.5 Enzymes

Restriction enzymes

Enzyme	U / µl	Buffer	Target sequence	Company
Bam HI	20	NEB 2 + BSA	G?GATCC	New England Biolabs®
EcoRI	20	NEB 2 + BSA	G?AATTC	New England Biolabs®
Hind III	20	NEB 2 + BSA	A?AGTCC	New England Biolabs®
PvuI	20	NEB 3 + BSA	CGAT?CG	New England Biolabs®

Other enzymes

Enzyme	Activity	Company
T4 DNA ligase	1 Weiss-U / µl	New England Biolabs®
Taq polymerase	5 U / µl	self-made (see below)
Proteinase K	0,3 U / µl	Sigma / Alldrich®
RNase A	0,5 Kunitz-U / µl	Sigma / Alldrich®
Lysozyme	100 U / µg	Sigma / Alldrich®
Polynucleotide kinase	10 U / µl	New England Biolabs®

2.3 Buffers and solutions

2.3.1 Buffers and solutions for protein gel electrophoresis

Tris-glycin electrophoresis buffer (TG):

25mM Tris; 192 mM glycin
0,1% SDS

Sample buffer (SB)
50 mM Tris-HCl (pH 6.8)
2% SDS
10% glycerol
1% ß-mercaptoethanol
12.5 mM EDTA
0.025% bromphenol blue

6 x SB buffer (10 ml)

2 ml 1M Tris-HCl pH 6,8
0.8 g SDS
0.4 ml glycerol
0.4 ml ß-mercaptoethanol
1 ml 0.5 M EDTA
1 ml 0.1% bromphenol blue
add H_2O to 10 ml

Coomassie Blue staining solution
50% ethanol
10% acetic acid
0.12% Coomassie Brilliant Blue

Coomassie Blue staining solution
2.4 g Coomassie Blue
1 litre ethanol 99.6%
200 ml acetic acid 96%
add H_2O to 2 litres

Distaining solution
7.2% acetic acid
5% ethanol

Distaining solution
375 ml acetic acid 96%
250 ml ethanol 99. 6%
add H_2O to 5 litre

2.3.2 Buffers and solutions for DNA gel electrophoresis

Agarose gel electrophoresis buffer (E-buffer)
40 mM Tris/acetate (pH 8,0)
40 mM Na acetate
2.0 mM EDTA

20 x E-buffer
193.8 g Tris-OH
131.2 g Na acetate
160 ml 0.5 M EDTA
adjust pH 8.3 with acetic acid
(approx. 55 ml of 96% HAc)
add H_2O to 2 litre

Acrylamide gel electrophoresis buffer (TBE-buffer)

90 mM Tris/borate pH 8.3
2.5 mM EDTA

10 x TBE-buffer
108 g Tris-OH
55 g boric acid
40 ml 0.5M EDTA
adjust pH 8.0 with acetic acid
(approx. 6 ml of 96% HAc)
add H_2O to 1 litre

Loading buffer
20 mM Tris/HCl pH 7.5
50% glycerol
0.02% bromphenol blue
0.02% xylene cyanol blue
20 mM EDTA

Loading buffer
0.2 ml 1 M Tris/HCl pH 7.5
5 ml glycerol
0.2 ml 10 mg/ml bromphenol blue
0.2 ml 10 mg/ml xylene cyanol blue

Ethidium bromide staining solution
1 µg ethidium bromide / 100 ml E-buffer

Staining solution
400 ml 1 x E-buffer
40 µl 10 mg/ml ethidium bromide solution

2.3.3 Buffers and solutions for methods of Molecular Biology

TE buffer
10 mM Tris/HCl pH 7.5
0.1 mM EDTA

TE buffer (100 ml)
1 ml 1 M Tris/HCl pH 7.5
20 µl 0.5 M EDTA
add H_2O to 100 ml

T4 Ligase buffer
30 mM Tris-HCl (pH 7,5)
10 mM $MgCl_2$
10 mM dithiothreitol (DTT)
10% polyethileneglycol (PEG)
1 mM ATP
100 µg /ml bovine serum albumin (BSA)

2 x T4 ligase buffer (1 ml)
30 µl 1 M Tris-HCl
10 µl 1 M $MgCl_2$
10 µl 1 M dithiothreitol
200 µl 50% polyethileneglycol
10 µl 0.1 M ATP
20 µl BSA (5 mg/ml)
720 µl H_2O

Restriction enzyme cleavage buffer NEB 2
10 mM Tris-HCl
10 mM $MgCl_2$
50 mM NaCl
1 mM dithiothreitol (DTT)

10 x NEB 2
0.1 ml 1 M Tris/HCl pH 8.0
0.1 ml 1 M $MgCl_2$
0.1 ml 5 M NaCl
0.1 ml 100 mM dithiothreitol

Restriction enzyme cleavage buffer NEB 3
50 mM Tris-HCl
10 mM $MgCl_2$
100 mM NaCl
1 mM dithiothreitol (DTT)

10 x NEB 3
0.5 ml 1 M Tris/HCl pH 8.0
0.1 ml 1 M $MgCl_2$
0.2 ml 5 M NaCl
0.1 ml 100 mM dithiothreitol

2.3.4 Buffers for total DNA extraction

GuHCl lysis buffer (DNA extraction buffer)
5 M Guanidinum hydrochloride (GuHCl)
40 mM Tris/HCl pH 7.0
20 mM EDTA
1% Tween 20

GuHCl lysis buffer
24 g GuHCl
2 ml 1 M Tris/HCl pH 7.0
2 ml of 0.5 M EDTA
0.5 ml Tween 20

Ethanol washing buffer
70% ethanol
10 mM TrisHCl pH 7.0

Ethanol washing buffer
70 ml ethanol
1 ml 1 M Tris/HCl pH 7.0
29 ml H_2O

RBC lysis buffer:
300 mM NH_4Cl
30 mM NH_4HCO_3
30 mM KCl
0.1 mM EDTA

RBC lysis buffer
4.1 g NH_4Cl
0.6 g NH_4HCO_3
0.5 g KCl
0.1 ml 0.5 M EDTA
add H_2O to 500 ml
adjust pH 7.2 with 1 M HCl

2.3.5 Buffers for alkaline lysis/silica method for plasmid preparation

Solution 1
100 mM Tris/HCl pH 7.5
10 mM EDTA

Solution 1
10 ml 1 M Tris/HCl pH 7.5
2 ml 0.5 M EDTA
88 ml H_2O

Solution 2
200 mM NaOH
1% SDS

Solution 2
4 ml 5 N NaOH
10 ml 10% SDS
86 ml H_2O

Solution 3
3 M Na acetate
2 M acetic acid

Solution 3
60 ml 5 M Na acetate
11.5 ml glacial acetic acid
28.5 ml H_2O

GuHCl lysis buffer, GuHCl washing buffer, and Ethanol washing buffer needed for the further purification steps are shown above.

2.3.6 Buffers for purification of His-tagged proteins with TALON®

Protein lysis buffer
6 M guanidinium hydrochloride (GuHCl)
40 mM Tris/HCl pH 8.0
250 mM NaCl

Lysis buffer
57.3 g GuHCl
4 ml 1M Tris/HCl pH 8.0
5 ml 5 M NaCl
add H_2O to 100 ml

Washing / equilibration buffer
8 M urea
40 mM Tris/HCl pH 8.0
250 mM NaCl

Washing/equilibration buffer
48 g urea
4 ml 1M Tris/HCl pH 8.0
5 ml 5 M NaCl
add H_2O to 100 ml

Elution buffer
8 M urea
40 mM Tris/HCl pH 7.0
250 mM NaCl
250 mM imidazol

Elution buffer
48 g urea
4 ml 1M Tris/HCl pH 7.0
5 ml 5 M NaCl
1.7 g imidazol
add H_2O to 100 ml

2.3.7 Buffers for immunoassays

TBS-buffer
10 mM Tris-HCl pH 8.0
150 mM NaCl

20 × TBS buffer (2 litres)
200 ml 1 M Tris/HCl pH 8.0
175 g NaCl
add H_2O to 2 litres

TBST-buffer
10 mM Tris-HCl pH 8.0
150 mM NaCl
0.05% Tween 20

20 × TBST buffer (2 litres)
200 ml 1 M Tris/HCl pH 8.0
175 g NaCl
10 ml Tween 20
add H_2O to 2 litres

Antigen dilution buffer
1 x TBS
0.2% Tween 20
10% glycerol
1 mM DTT

Antigen dilution buffer (100 ml)
5 ml 20 x TBS
2 ml 10% Tween 20
10 ml glycerol
1 ml 1M DTT
82 ml H_2O

Ab dilution buffer
1 × TBST
1% BSA
1 mM DTT

Ab dilution buffer (100 ml)
5 ml 20 x TBST
1 g BSA
1 ml 1M DTT
93 ml H_2O

AP buffer
100 mM Tris-HCl pH 9,5
100 mM NaCl
5 mM $MgCl_2$

AP buffer (200 ml)
20 ml 1M Tris-HCl pH 9,5
4 ml 5 M NaCl
1 ml 1 M $MgCl_2$
175 ml H_2O

NBT solution
50 mg/ml NBT (nitro blue tetrazolium)
in 70% dimethylformamide
keep at -20°C

BCIP solution
50 mg/ml BCIP (5-Bromo-4-Chloro-3-Indolylphosphate)
in 100% dimethylformamide
keep at -20°C

AP colour developing solution
60 µl BCIP solution and 60 µl NBT solution dissolved in 10 ml of AP buffer (stable for 1 hour)

ELISA Washing buffer (1 x PBS):

0.1 M phosphate buffer (pH 7.2)
0.15 M NaCl
2.5 mM KCl

10 x PBS
90 g of NaCl
14.4 g of Na_2HPO_4
2.4 g of KH_2PO_4
add H_2O to 1 litre

Blocking buffer
1% milk powder
1x PBS

Blocking buffer
1 g milk powder
100 ml 1 x PBS

Buffer A for ELISA assay
0.2 M potassium citrate pH 4.0
3 mM H_2O_2

Buffer A
2.2 g citric acid
2.5 g tri-Na-citrate (Mr = 294)
add H_2O to 100 ml
pH to 4.0 ± 0.1

before use, add 25 µl of 30% H_2O_2 to 8 ml of this pH 4.0 stock solution

Solution B for ELISA assay
41 mM tetramethylbenzidine (TMB)
8.2 mM TBABH in N,N-dimethylacetamide (DMA)
(absolute, H_2O content <0.01%)

Solution B:
100 mg TMB
20 mg TBABH
10 ml DMA

Substrate working solution for ELISA assay
Immediately before use,
add 200 µl Solution B to 8 ml Buffer A with freshly added H_2O_2

Stop Solution for ELISA assay
1 M H_2SO_4

Stop Solution:
5.4 ml 98% H_2SO_4
add H_2O to 100 ml

2.4 Methods

2.4.1 DNA purification

Isolation of nucleic acids using guanidinium-hydrochloride/silica

The following procedure is appropriate to isolate total DNA (1-5 µg) from a variety of bacteria, eukaryotic cells, or homogenised tissue (10 to 20 mg). 500 µl of guanidinum hydrochloride (GuHCl) lysis buffer (5 M GuHCl, 40 mM Tris/HCl pH 7.0, 20 mM EDTA, 1% Tween 20) were added to 10-20 µl sedimented cells, vortexed and incubated for 10 min at 65°C. After 2 min centrifugation at 13.000 rpm in a minifuge, the supernatant was transferred to a new tube and 5 µl of silica suspension were added, mixed with vortex and incubated for 5 min at room temperature. The sample was centrifuged for 30 s at 13.000 rpm, the pellet resuspended in 300 µl 70% ethanol washing buffer, centrifuged for 30 s at 13.000 rpm and the supernatant removed. The pellet was washed in the same way three more times with ethanol washing buffer (70% ethanol, 10 mM TrisHCl pH 7.0). The last pellet was centrifuged again and the rest of ethanol removed. 5 µl of TE buffer were added, incubated for 5 min at 55°C and centrifuged for 1 min at 13.000 rpm. The supernatant was collected and the pellet eluted with another 5 µl TE buffer. The supernatants were combined.

For the particular case of blood samples conserved in guanidinium hydrochloride, 400 µl of the Gu-blood were mixed with 600 µl of guanidinium lysis buffer. Then, the general procedure for DNA extraction with silica (described above) was followed.

Alkaline lysis/silica method for plasmid preparation

The plasmid DNA purification procedure is a combination of the alkaline lysis protocol originally published by Ish-Horowicz and Burk (1981) and the above described silica adsorption method. It can be used for mini plasmid preparations (1.5 ml cultures) as well as for bigger preparations (50 ml cultures or more). In the following, the volumes of the different solutions needed in each step are given for mini plasmid preparations and for 50 ml cultures in brackets. 1.5 ml [50 ml] of over night culture were centrifuged for 3 min. The supernatant was removed by aspiration and the pellet resuspended in 100 µl [2 ml] of Solution 1. 10 µl [100 µl] of Rnase A (5 mg/ml; kept at -20°C in 50 % glycerol) were added, and the sample was incubated at room temperature for 2 min. 200 µl [4 ml] of Solution 2 were added, quickly mixed (it is important not to vortex) and incubated for 2 min at room temperature (RT). Then, 150 µl [3 ml] of Solution 3 were added and again well mixed (without vortexing) and incubated for 1 min. The sample was

centrifuged at 13.000 x g for 10 min at RT and the supernatant transferred to a new tube. Then, 400 µl [7 ml] of GuHCl lysis buffer and 7 µl [100 µl] of silica suspension were added. The sample was mixed by vortexing, incubated at RT for 5 min, centrifuged for 1 min and the supernatant removed. To the pellet, 400 µl [7 ml] of ethanol washing solution were added. The sample was mixed by vortexing, centrifuged for 1 min and the supernatant discarded. The washing steps were repeated twice. Finally, the wet silica was centrifuged again for 1 min and the residual washing solution removed completely. To elute the DNA, silica was resuspended in 20 µl [200 µl] TE buffer, incubated 10 min at 55°C, centrifuged for 2 min and the supernatant collected in a new tube. Silica was eluted once more with 20 µl [200 µl] TE buffer (only vortexed, not incubated at 55°C), centrifuged and the supernatants combined.

DNA purification from agarose gels

To purify DNA fragments from agarose gels the following protocol (Vogelstein et al., 1979) was used. The corresponding part of the gel was cut out under UV, and incubated with 2-3 volumes of 4 M NaI at 60°C until the agarose was molten. 5-10 µl of silica suspension were added and incubated at room temperature for 5 min. After centrifugation for 30 s, the silica pellet was washed three times with ethanol washing solution and the DNA eluted in the minimal volume of TE buffer as described above.

Buffy coat DNA extraction

2-3 ml of citrate or EDTA-blood were centrifuged for 15 minutes at 1000 x g. Then, supernatant plasma was collected and kept in the refrigerator. The yellow thin “buffy coat” layer on top of the sedimented erythrocytes was collected with a pipette (200 to 300 µl) and transferred to an Eppendorf tube. 1.2 ml of RBC lysis buffer were added, mixed shortly by vortexing, incubated 10 minutes at room temperatures, inverting the tube several times. Afterwards, the tube was centrifuged 1 minute at 2000 x g, and the supernatant removed. A pellet of 10-20 µl was obtained and mixed with guanidinium hydrochloride lysis buffer. DNA was extracted as described above.

2.4.2 Purification of recombinant Taq DNA polymerase

Taq DNA polymerase was purified from a recombinant strain of *E. coli* (*E. coli* XL-1 Blue/pQE-Taq) expressing an exonuclease-free mutant of the enzyme. The strain was constructed previously in the laboratory of E. Beck (unpublished results). The thermostable enzyme can also be purified without the cooling steps described in the following protocol.

1 litre of LB medium containing 25 µg/ml kanamycin and 50 µg/ml ampicillin was inoculated with 100 ml of an over night culture of XL-1 Blue pQE-Taq cells. The cells were grown at 37°C with optimal aeration for 1-2 hours. At an OD_{600} of 1.8, 1 ml of 1 M IPTG was added and then the culture vigorously shaked for another 4 hours. Cells were harvested by centrifugation (yield ~5 g) and frozen at -20°C. Freezing of the cells can be omitted, but is recommended if the enzyme preparation is not performed immediately. The cells were resuspended in 15 ml TMN buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 20 mM $(NH_4)_2SO_4$) together with 8 mg of lysozyme (from 10 mg/ml stock solution) and incubated for 15 min at 20-25°C. After adding 200 µl of 10% Triton X-100 and 200 µl of 10% Tween 20, the culture was mixed and incubated at 80°C for 20 min in a 50 ml screw-cap Falcon tube. After the heating step, 250 µl of 100 mM PMSF were added to prevent proteolytic degradation.

DNA was sheared by 1 min sonification (it is also possible to do it by repeated pressing through a syringe first with a 1 mm diameter needle and then with smaller needles) and centrifuged 15 min at 20.000 x g at 4°C. The supernatant (~15 ml) was collected and 0.6 ml of 5 M NaCl (0.2 M final concentration) were added.

The DNA was precipitated with polyethyleneimine (PEI) by adding dropwise 500 µl of 5% PEI solution, mixing and incubating in ice for 10 min. The sample was centrifuged at 10.000 x g for 5 min. 4 aliquots of 500 µl of supernatant were transferred to Eppendorf tubes each and mixed with increasing amounts (2-8 µl) of 5% PEI solution. They were incubated 5 min on ice, centrifuged and the amount of pellet compared. The minimal amount of PEI to precipitate DNA quantitatively was determinated and added to the bulk extract (usually 100-200 µl; use 1/100 volume of 5% PEI solution in excess). The suspension was left 20 min on ice and centrifuged at 20.000 x g for 20 min. The supernatant was collected and diluted 6 fold with KTA buffer (20 mM Tris/HCl pH 8.5, 10 mM beta-mercaptoethanol, 10 % (w/v) glycerol, 0.1 mM EDTA, 0.05 % Triton X 100, 0.05 % Tween 20).

The excess of PEI was removed by passing the extract through a 2 ml BioRex 70 column equilibrated in KTA buffer + 30 mM ammonium sulphate. A disposable 4 ml plastic column (International Sorbent Technology, Hengoed, Mid Glamorgan, UK) was used. The column was rinsed with 2 ml KTA buffer and the flow-through loaded on another plastic column

containing 2 ml heparin sepharose equilibrated with KTA buffer, 30 mM ammonium sulphate. The column was washed first with 50 ml KTA buffer, 40 mM ammonium sulphate. Thereafter, the column was washed with 20 ml KTA buffer, 40 mM ammonium sulphate, 50% glycerol. Taq DNA polymerase was eluted with KTA buffer, 150 mM ammonium sulphate, 50% glycerol. 0.5 ml fractions were collected and 3 µl aliquots analysed on a 12.5% SDS polyacrylamide gel. The enzyme was found in fractions 4 to 7. The enzyme was stored at -20°C. For long term storage, Tween 20 to 1% final concentration was added. The yield was approximately 30.000 units of enzyme at a concentration of 80 units/µl in the peak fraction.

2.4.3 Standard cleavage assay

Cleavage of DNA with BamHI and HindIII in the same assay was performed with NEB2 buffer, and 500 mg / ml BSA (end concentration) for 1 to 2 hours at 37°C. For PvuI, NEB3 buffer was used under the same conditions. In general, 20 U of each restriction enzyme were used in a 50 µl assay.

2.4.4 Standard ligation assay

Ligation of fragments to be cloned in expression vectors was performed by T4 ligase (1 Weiss-U / µl) with ligation buffer containing 5% PEG. To the assay were added the previously with BamHI and HindIII cleaved vector and fragment. The ligation assay was incubated over night at 12ºC, and used the next day, after purification with silica, for the transformation of the electroporation competent cells.

2.4.5 Transformation of *E. coli* cells by electroporation

Transformation by electroporation was performed essentially as described by Sambrook et al. (1989). 5 ml of a fresh pre-culture (over night culture is not recommended) were taken to inoculate 200 ml pre-warmed LB medium. The cells were grown at 37°C until OD_{600} of 0.6 was reached. The culture was chilled on ice as quickly as possible and the cells centrifuged for 5 min at 10.000 x g. The pellet was resuspended in 25 ml ice-cold water. Centrifugation and resuspension were repeated two times and finally the pellet was resuspended in 400 µl ice-cold water and 10% glycerol (final concentration) added. The cells were aliquoted in 40 µl portions and kept frozen at -70°C.

For transformation, 40 µl competent frozen cells were thawn in ice. 10 µl of silica purified ligase assay were added and the tube left in ice for 1 min. The cells were transferred in a pre-cooled 2 mm electroporation cuvette and electroporated at 2500 V. Immediately after, 1 ml of LB medium was added, the cells transferred in an Eppendorf tube and incubated for 1 h at 37°C. 100 µl of the content of the Eppendorf tube were spread in one half of an agar plate containing selective antibiotics. The rest of the cells was centrifuged, resuspended in approx 100 µl of medium and spread on the other half of the plate. The agar plate was incubated over night at 37°C.

2.4.6 Expression and purification of His-tagged proteins

Expression and purification of His-tagged proteins was performed essentially as described by Hochuli et al. (1988). The recombinant antigens were cloned in pQE-30 (Qiagen, Hilden, Germany). This vector provides a histidine tag to the N-terminus of the recombinant proteins (see Figure 2.4-1). Expression from the strong phage T5 promotor of this vector is regulated by a lac repressor provided in excess by the plasmid pREP (maintained in the cells by kanamycin selection).

E. coli XL-1Blue/pREP cells transformed with a gene cloned in pQE-30 were grown in LB medium containing 50 µg/ml ampicillin and 25 µg/ml kanamycin up to 5 x 10^8 cells per ml (OD_{600} = 0.8-1.0). At that point, IPTG was added to a final concentration of 1 mM, and the cells (250 ml culture in a 1 litre Erlenmeyer flask) were further incubated under vigorous agitation on a shaker for 3-4 hours at 37°C. The induced cells were harvested by centrifugation and frozen at -20°C. Next day, the cells were thawn, resuspended in 10 ml of Lysis buffer (6 M guanidinium hydrochloride, 40 mM Tris/HCl pH 8.0, 250 mM NaCl) and incubated for 30 min at RT, shaking from time to time. The lysed cells were centrifuged 20 min at 20.000 x g in a cooling centrifuge, the pellet was discarded and the supernatant transferred to a 50 ml Falcon tube containing 1 ml suspension (0,5 ml packed volume) of TALON® (equilibrated in Washing buffer). The solution was mixed gently in a rotating wheel for 1 hour and centrifuged for 1 min at 3.000 x g in a swinging bucket centrifuge. The supernatant was removed carefully and 50 ml of Washing buffer (8 M urea, 40 mM Tris/HCl pH 8.0, 250 mM NaCl) were added. The solution was shaked 5 min, centrifuged again for 1 min at 3.000 x g and the supernatant removed carefully. The washing steps were repeated two times. The resin was then suspended in 4 ml of Washing buffer and loaded on a disposable plastic column.

The column was rinsed with 5 ml of Washing solution and the proteins eluted by adding stepwise 0.5 ml of Elution buffer (8 M urea, 40 mM Tris/HCl pH 7.0, 250 mM NaCl, 250 mM imidazol). There was a break of 2 min between the single steps leading to elution of more concentrated protein. Aliquots of 3 µl and 15 µl of each of the 0.5 ml fractions were analysed with SDS PAGE. Protein concentration was determined by the Bradford assay (Bradford, 1976).

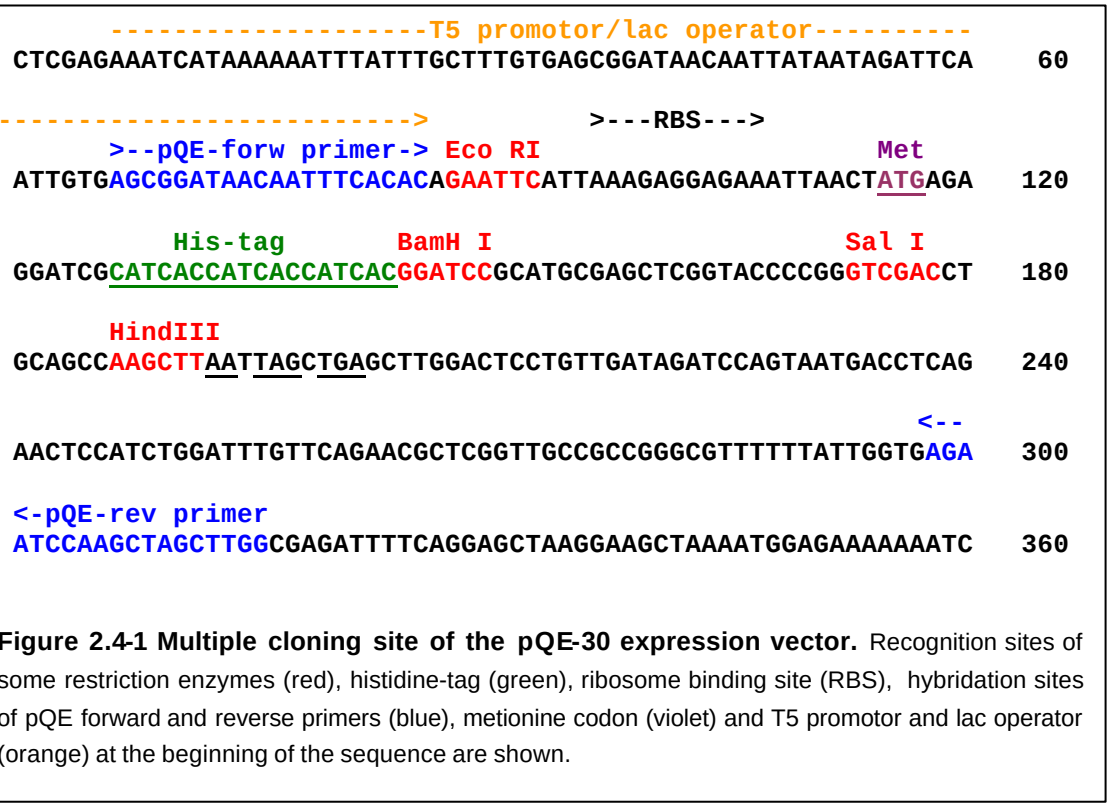

Figure 2.4-1 Multiple cloning site of the pQE-30 expression vector. Recognition sites of some restriction enzymes (red), histidine-tag (green), ribosome binding site (RBS), hybridation sites of pQE forward and reverse primers (blue), metionine codon (violet) and T5 promotor and lac operator (orange) at the beginning of the sequence are shown.

2.4.7 Line blot

The line blot is a simple immunoassay similar to the well known Western blot and was performed essentially as described by Klinkert *et al*., 1991. In contrast to the Western blot, antigens are not electrotransferred but applied manually to the nitrocellulose membrane.

Preparation of antigen test stripes

Antigen was applied to nitrocellulose sheets as a line with a 1.0 mm tip of an ink pen (Rotring Radiograph, Rotring GmbH, Hamburg, Germany), using a ruler as a guide. Alternatively, a 200 µl plastic pipette tip was used whose opening was squeezed with pliers to reduce the speed of flow. The antigen solution was prepared by mixing recombinant antigen (in

7 M urea as eluted from the TALON column) with different amounts of antigen dilution buffer (1xTBS, 0.2 % Tween 20, 10 % glycerol, 1 mM DTT). The concentration of protein is in the range of 0.1 - 0.01 mg/ml, however, the appropriate dilution has to be determined empirically for each batch of antigen by serial two-fold dilutions. The concentration just not yet reactive with negative control sera is correct. Lines of individual antigens were placed 0.5 cm apart. Non-specific binding sites were blocked by placing the air-dried nitrocellulose sheet in TBS buffer, 1% Tween 20 for 1 hour. Sheets were then washed in TBS, 0.1% Tween 20 (TBST), cut perpendicularly to the antigen lines in 4 mm stripes, dried and stored at ambient temperature.

Immunodetection

The nitrocellulose stripes were re-hydrated in TBST and incubated in disposable incubation trays (Schleicher & Schuell, item No. 10448017) with 0.5 ml of a primary antibody solution (patient or control serum) diluted 1:100 - 1:200 in Ab dilution buffer (1 × TBST, 1 % BSA, 1 mM DTT) for 0.5-1 hours at room temperature or over night at 4°C on a shaker. The antibody was removed from the solution and saved at 4°C. The nitrocellulose stripes were washed three times for 10 minutes each with TBST and 0.5 M NaCl. Then, they were incubated with goat anti human IgG conjugated to alkaline phosphatase (Dianova, Hamburg, Germany; 1:7000 diluted in Ab dilution buffer) for 1-2 hours at room temperature on a shaker. Finally, the stripes were washed three times for 10 minutes in TBST and once with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The "disposable" incubation trays can be re-used many times when washed carefully with detergent using a brush.

AP staining protocol

To visualise the bound AP-conjugated antibodies, the following method was used. An AP colour developing solution was prepared just before use by adding 60 µl BCIP solution (50 mg/ml BCIP in 100% dimethylformamide) and 60 µl NBT solution (50 mg/ml NBT in 70% dimethylformamide) to 10 ml of AP buffer. The membrane stripes were added to the AP colour developing solution with the protein side up and it was waited for colour to develop on the membrane. When bands became clearly visible, the membranes were transferred to 10 mM Tris-HCl pH 8.0, 1 mM EDTA to stop the reaction. After drying at room temperature, the test stripes were photographed, or scanned into a computer.

2.4.8 ELISA

Flat-bottom 96 well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were incubated over night at 4°C with 0.1 ml of antigen TcBCDE diluted in PBS to a concentration of 1 ng/ml. The wells were washed three times with 0.35 ml PBS each and then incubated with 0.2 ml blocking solution (1% milk powder in PBS) for 1 hour at ambient temperature. The blocked plates were washed three times with PBS, dried and stored at room temperature. For long term storage (more than one month), the antigen-coated plates were sealed in plastic sheets. 100 µl of serum samples diluted 1:200 in blocking solution were added to each well and incubated for 1 hour at room temperature. The plates were washed three times with PBS, the wells filled with a 1:5.000 dilution of goat anti human IgG-HRP (horse radish peroxidase) conjugate (Dianova, Hamburg, Germany) and incubated again for 1 hour at room temperature. The plates were washed three times with PBS as before, then 75 µl of HRP substrate working solution were added to each well, incubated at room temperature, and the staining reaction stopped after 30 minutes with 100 µl of 1 M sulphuric acid and the results read at 450 nm. The HRP substrate working solution was prepared immediately before use as follows: 25 µl of a 30 % solution of H_2O_2 and 200 µl of 41 mM 3,3',5,5'-tetramethylbenzidine, 8.2 mM tetrabutylammonium borohydride in N, N-dimethylacetamide were added to 8 ml of 0.2 M potassium citrate buffer pH 4.0 (Frey et al., 2000).

2.4.9 PCR procedures

Standard PCR conditions

For all PCR assays, the same Taq DNA polymerase buffer was used. It is prepared as a 10 x buffer and has the following composition:

1 x Taq buffer
40 mM Tricine-KOH (pH 8.7 at 25°C)
15 mM K acetate
3.5 mM acetate
0.1 % gelatine
0.05 % Tween 20

10 x Taq buffer (10 ml)
4 ml 1 M Tricine-KOH
1.5 ml 1 M K acetate
350 µl 1 M acetate
2.5 ml 4% heated gelatine solution
500 µl Tween 20
1.15 ml H_2O

Standard PCR reaction assay

Using efficient standard thermocyclers such as T1 thermocycler 96 from Biometra, or Primus 96 from MWG Biotech which are appropriate for 200 µl reaction tubes, have a heated lid and heating/cooling rates of at least 2-3°C/s, highly reproducible results were obtained with assays as small as 10 µl. A standard PCR reaction had the following composition:

1 µl 10 x Taq buffer
1 µl mixture of 4 dNTPs (2.5 mM each)
0.5 µl 10 µM forward primer
0.5 µl 10 µM reverse primer
1 µl template DNA
5.8 µl H_2O
0.2 µl Taq DNA polymerase (5 units/µl)

The standard program of the thermocycler was set as follows:

step 1: 2 min at 94°C (denaturing of DNA template)
step 2: 35 cycles of [94°C 20 s, 60°C 1 min, 72°C 1 min]
step 3: 5 min 72°C (optional, to fill-inn the DNA ends completely)

The same conditions were applied for nested PCR. 1 µl of a 1:10 dilution (with H_2O) of the result of first PCR assay was used as DNA template.

2.4.10 Gel electrophoresis

Agarose gels

Agarose gel electrophoresis was performed according to standard procedures (Sambrook et al. 1989). Plasmid DNA and PCR fragments were analysed on horizontal 1% or 2% agarose gels using a Tris/acetate electrophoresis buffer (E-buffer). To check migration of the DNA fragments, the samples were mixed with 20% loading buffer containing 50% glycerol and marker dyes. In 1% agarose gels, bromphenol blue migrates at the position of 200 bp, whereas xylenecyanol blue migrates at the position of 300 bp. The gels were stained for 20 min with ethidium bromide staining solution. Then they were rinsed with tape water, the DNA bands visualised on an UV transilluminator and photographed by a Polaroid camera. Alternatively, a simple digital camera in combination with a yellow filter was used.

Polyacrylamide gels

For the analysis of proteins, discontinuous polyacrylamide gels (10 x 8 x 0.1 cm) containing SDS (Lämmli, 1970) were prepared in different concentrations according to the following recipe:

	Stacking gel	Separating gel	
	6%	12,5%	15%
40% Acrylamide / 1.3% bisacrylamide solution	1,5 ml	5,0 ml	6,0 ml
4 x buffer	2,5 ml	4,0 ml	4,0 ml
H_2O	6,0 ml	7,0 ml	6,0 ml
Ammonium persulphate	10 µg	10 µg	10 µg
TEMED	15 µl	20 µl	20 µl
	10 ml	**16 ml**	**16 ml**

Stacking gel buffer:

125 mM Tris-HCl (pH 6.8)
0.1% SDS

4 x Stacking gel buffer
12.14g Tris-OH
8 ml 10% SDS
adjust pH 6.8 with HCl (~8 ml HCl 37%)
add H_2O to 200 ml

Separating gel buffer:

375 mM Tris-HCl (pH 8.8)
0.1% SDS

4 x Separating gel buffer
45.43 g Tris-OH
10 ml 10% SDS
adjust pH 8.8 with HCl (~6 ml HCl 37%)
add H_2O to 250 ml

3. RESULTS

The lack of appropriate diagnostic tools and the regular screening of the population at risk are still one of the main obstacles for the control and eradication of the Chagas disease (Talerton et al., 2007). In the last years, several diagnostic methods have been developed, mainly for the use in blood banks. In this case, the specificity of the diagnostic tools is not much taken into account, because these methods are assigned to the screening of donated blood which can be discarded in case of infection. On the contrary, for the direct diagnosis of persons and particularly in areas where Chagas disease coexists with other diseases, and especially visceral leishmaniasis (Bastrenta et al., 2003), the specificity of diagnosis plays an important role. Discrimination between *T. cruzi* and *Leishmania* infections represents a problem, since the cross-reactions between them are very usual, due to their close relation. For this reason, false positives are often obtained, and therefore wrong diagnoses are done. In addition, it has also been thought that there is an underestimated number of visceral leishmaniasis patients in Latin America, being some of these cases confused with Chagas disease.

As mentioned above, regular screening of all the population at risk (about 90 million of people) would be necessary for efficient control of Chagas disease. This is not possible at present, primarily due to the high price of the available commercial diagnostic kits. For this reason, the goal of this work was to develop highly sensitive and specific and at the same time, inexpensive diagnostic methods, with the perspective, to establish these techniques in near future in a highly affected country, Bolivia.

The principal methods developed in the present work are immunodiagnostics using recombinant proteins as antigens. Most part of the work consists in the selection, cloning and expression of these antigens, and, finally, the test of them with a large number of chagasic patient samples provided from a Bolivian laboratory.

Immunodiagnosis is, on the other hand, not sufficient for monitoring the treatment of the disease. While taking drugs against Chagas disease, patients must be followed up. As immunodiagnosis is based on the detection of antibodies in blood which have been shown to persist many years after cure, this is only possible by means of PCR, which detects the presence of the living parasite (or rests of its DNA) in blood. Due to the toxicity of the drugs, the treatment period has to be as short as possible. For this reason, it is very important to have an effective method for the confirmation of cure, able to detect an “active” infection, and not only the persisting antibodies in blood. Two different PCR assays were established during this work.

However, they may have still to be improved and tested with a large amount of samples, which is only feasible in a laboratory of an endemic country.

3.1 Establishing immunodiagnostics

Most of the commercial kits for diagnosis of Chagas disease employ the ”crude” antigens, obtained from the lysis of trypomastigote or epimastigote forms of *Trypanosoma cruzi.* They show a high sensitivity, but, unfortunately, are not specific enough, leading in many cases to false positive reactions. Therefore tests using recombinant antigens were chosen for this work. Such tests have shown to be much more specific, and they can be as as sensitive as the ones using crude extracts.

3.1.1 Selection of appropriate diagnostic antigens

Establishing of new immunoassays began with the searching for the most appropriate antigens in the existing publications, mainly from groups in Latin America. The corresponding genomic sequences were obtained from different DNA data banks, preferentially the Sanger Gene Data Base (GeneDB) (Hertz-Fowler et al., 2004). This data bank contains most of the available data of *Trypanosoma cruzi* CL Brener strain genomic sequence that was published in 2005 (El-Sayed et al., Science). The genomic sequence is incomplete. The *T. cruzi* release v4.0 consists of 4,008 annotated contigs (continuous sequences of up to more than 50.000 base pairs) totalling 60.4 Mb, plus a further 28737 unannotated contigs totalling 29.2 Mb. The contigs have never been put together to chromosomes, and there are redundant sequences.

The similarity of the sequences with other organisms was checked by means of the BLAST program (http://www.genedb.org/genedb/tcruzi/blast.jsp). This program finds similarity regions between biological sequences. It compares nucleotide or protein sequences to sequence data bases and calculates the statistical significance of matches.

The genetic information extracted from GeneBank (http://www.ncbi.nlm.nih.gov/Genbank) was used to clone the antigen 1F8 (Gonzalez et al.,1985), by amplifying the corresponding DNA sequence with PCR by using the genomic DNA of the parasite as template. Previous preparation of cDNA is not needed in the case of *T.cruzi*, because no introns have been found in the genes of this organism so far (El-Sayed et al., 2005). By the same strategy, several unsuccessful repeated attempts were done to clone another antigen, A13 (Paranhos et

al., 1990). The failure was most probably due to the fact that A13 does not exist in the genome of the *T. cruzi* strain (Tulahuen C2) used in our laboratory, and it was also not contained in the CL Brener strain sequenced by the Sanger consortium.

The next antigens to be cloned were CRA (Cytoplasmic Repetitive Antigen) and FRA (Flagellar Repetitive Antigen), reported to be highly immunogenic (Krieger et al., 1992; Pereira et al., 2003), even though the exact sequence of the recombinant antigens has never been disclosed. The natural gene products are huge, having calculated molecular masses of 120 kDa (CRA) and 500 kDa (FRA), and contain multiple tandemly repeated amino acid sequences of 14 residues (CRA) and 68 residues (FRA). It is generally presumed that the tandem repeats represent major immunogenic epitopes for the induction of strong humoral response.

Attempts to clone representative parts of the antigens in *E. coli* failed, in spite the fact that PCR fragments containing several copies of tandem repeats were obtained. It was only feasible to maintain single copies of the repetitive DNA sequences stably in plasmid vectors, even trying with *recA* minus strains of *E. coli* for transformation (Troester et al., 2000). It appears that *E. coli* prevents efficiently the amplification of tandemly repeated sequences, which may represent a defence strategy against the amplification of structures such as transposable elements.

Cloning of sequences for tandem repeated amino acids was only possible by using synthetic DNA, whereby different nucleotides in variable codons were included in such way that repeats of more than eight identical nucleotides were prevented. The open reading frames were constructed by overlapping complementary oligonucleotides which were coupled by DNA ligase. On this way, it was possible to stably maintain three copies of the 14mer CRA repeat and two copies of the 68mer of the FRA repeat in an *E.coli*-specific expression vector. At the same time, the coding sequence of the B13 antigen (Gruber et al., 1993), consisting of a trimer of 13 repetitive amino acids, was constructed and successfully expressed.

All three synthetic antigens showed an excellent sensitivity of immunoreaction with chagasic patient sera. However, some drawbacks prevent their direct use to establish a good diagnostic system. One disadvantage of the synthetic B13 and CRA antigens was that production and purification of the resulting polypeptides was demanding. In addition, they did not stick efficiently on the nitrocellulose membrane or plastic surface of mictrotiter plates due, most probably, to their small size. Part of this problem was solved by fusing them into a bigger single antigen, which led, in addition, to an unexpected increase of sensitivity in immunoassays.

The FRA antigen, at the other hand, turned out to cross-react with sera from patients with *Leishmania* infections. The reason for this became transparent in comparing the

corresponding genes between *T. cruzi* and *L. infantum* as well as *L. major*: the 68mer tandem repeats were conserved to almost 80% identity between the different species. Thus the FRA antigen was of no use to discriminate these two diseases, even though it was highly immunoreactive.

Therefore additional potential diagnostic antigens were searched in the literature as well as in the genomic sequence of *T. cruzi* as contained in the Sanger GeneDB. The search was concentrated on antigens containing repeat motives in their genes. This limitation on repetitive antigenic motives was not only due to the high antigenicity of the sequences successfully cloned so far, but was also supported by reports in the recent literature. Several authors had shown that most of the highly immunoreactive antigens contain such tandemly repeated amino acid sequences (Ibañez et al., 1988; Hoft et al., 1989; Frasch et al., 1991). By means of a bioinformatic approach, a large number of additional potential diagnostic antigens consisting of tandemly repeated amino acid sequences were identified in the *T. cruzi* Sanger GeneDB using a computer program predicting tandem repeats in DNA (Tandem Repeats Finder: http://tandem.bu.edu/trf/trf.html). Analysis of genomic sequences of *T. cruzi* is demanding, because there are not yet arrayed chromosomes. Screening several hundred contigs statistically with TR Finder software, a number of new tandem repeats was found. However, many of them had low repeat numbers only, or were also present in related form in the genomes of *L. infantum* and *L. major*. Several of the repeats found by this way corresponded to antigens already described in the literature. At the end, six different tandem repeats in addition to B13, CRA and FRA appeared to be appropriate as candidates for diagnostic antigens. These sequences are listed in Table 3.1-1.

DNA sequences with open reading frames were constructed for all of these repeats by means of synthetic oligonucleotides. A great help in this approach was the software program DNA Works (http://mcl1.ncifcrf.gov/dnaworks/dnaworks2.html, Hoover and Lubkowski, 2002). The program designs automatically oligonucleotides for PCR-based gene synthesis just by inserting the desired protein sequence and the preferred host for gene expression. By means of this program it was easy to calculate how many repeats of a given amino acid sequence could be designed without to include repetitive DNA sequences longer than 8-10 nucleotides. The genes were synthesised using the oligoncleotides predicted by DNAWorks program according to Stemmer et al., 1995. This method relies on DNA polymerase to build increasingly longer DNA fragments from overlapping oligonucleotides during the assembly process. By this way, the coding sequences for up to nine amino acid repeats (in case of TcD) could be constructed and stably maintained in the expression plasmid.

antigen	sequence of synthetic antigen	genomic repeats	homology with *Leishmania*
MAP	PRHVDPDHFRSTTQDAYRPVDPSAYKRALPLEEEEDVG PRHVDPDHFRSTTQDAYRPVDPSAYKRALPLEEEEDVG PRHVDPDHFRSTTQDAYRPVDPSAYKRALPQEEEEDVG	135	40 % homology with *L. infantum*
JL8	AAEATKVAEAEKQR AAEATKAVEAEKQR AAEATKVAEAEKQK	140	very weak homology
CRA	KVAEAEKQKAAEAT KVAEAEKQKAAEAT KVAEAEKQKAAEAT	130	no homology
B13	PFGQAAAGDKPS PFGQAAAGDKPS PFGQAAAGDKPK	103	no homology
FRA	AFLDQKPEGVPLRELPLDDDSDFVAMEQERRQLLEKDPRRNAREIAAL EESMNARAQELAREKKLADR AFLDQKPEGVPLRELPLDDDSDFVAME QERRQLLEKDPRRNAKEIAALEESMNARAQELAREKKLADR AFLDQK	14	80% homology with *L. infantum*
TcD	PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE	430	no homology
TcE	PAKAAA PPAKAAA PPAKAAA PPAKAAA PPAKAAA PPAKAAAP	70	no homology
SAPA	PVDSSAHGTPST PVDSSAHGTPST PVDSSAHSTPST PVDSSAHSTPST PADSSAHSTPST	>130	no homology
TcMyo	LAQREADNEKLAED LAQREADNEKLAEE LAQREADNEKLTED LAQREADNEKLAED	>170	no homology

Table 3.1-1. Synthetic tandem repeat antigens of *T. cruzi*.

3.1.2 Example of cloning by PCR amplification: 1F8 antigen.

Design of PCR primers

1F8 is the only not-repetitive antigen that was cloned. The gene sequence, which codes for a 24 kDa Ca^{+}-binding protein, was obtained from the Sanger GB and is shown in Figure 3.1-2. Only part of the gene coding for 1F8 was amplified by PCR using primers derived from the sequence in GenBank.

For the ease of the cloning of different recombinant antigens, it was decided to use always the same restriction sites at the beginning (BamHI) and at the end (HindIII) of the open reading frames. Furthermore, the sequences should not contain stop codons at the end to allow the fusion of different antigens as polyproteins in a later stage of the project. As BamHI and HindIII restriction sites should not occure inside of the open reading frames, a search for these sequences within the genomic sequence was performed. This was possible by using "The Sequence Manipulation Suite" software (http://www.bioinformatics.org/sms2/, Stothard, 2000), that allows many different analysis of the selected sequence. By using the "Restriction Summary" option and introducing the 1F8 DNA sequence, the program returned the number and positions of commonly used restriction endonuclease cut sites. As a result, one BamHI and one HindIII restriction sites were found within the sequence. Because the cloning strategy was based on the use of these two enzymes, the amplified fragment had to exclude the C-terminal coding part of the gene.

Secondly, codons “rarely used” by *E. coli* had to be searched. These codons and their frequencies are shown in the *Escherichia coli* codon usage (see Figure 3.1-5). There is large experimental evidence on the fact that the presence of non-optimal codons (i.e., those translated by rare tRNAs) can reduce the translation rate of proteins (Robinson et al., 1984; Bonekamp et al., 1985), which is used by the bacteria as strategy for the regulation of protein expression (Grantham et al., 1981). Therefore, in order to avoid these unfavourable codons as much as possible, a search in the 1F8 DNA sequence was done. The results are shown in Figure 3.1-2.

Gene sequence:

```
  1 ATG GGT GCT TGT GGG TCG AAG GGC TCG ACG AGC GAC AAG GGG TTG GCG AGC GAT AAG GAC
 61 GGC AAG AAG GCC AAG GAC CGC AAG GAA GCG TGG GAG CGC ATT CGC CAG GCG ATT CCT CGT
121 GAG AAG ACC GCC GAG GCA AAA CAG CGC CGC ATC GAG CTC TTC AAG AAG TTC GAC AAG AAC
181 GAG ACT GGG AAG CTG TGC TAC GAT GAG GTG CAC AGC GGC TGC CTC GAG GTG CTG AAG TTG
241 GAC GAG TTC ACG CCG CGA GTG CGC GAC ATC ACG AAG CGT GCG TTT GAC AAG GCG AGG GCC
301 CTG GGC AGC AAG CTG GAG AAC AAG GGC TCC GAG GAC TTT GTT GAA TTT CTG GAG TTC CGT
361 CTG ATG CTG TGC TAC ATC TAC GAC TTC TTC GAG CTG ACG GTG ATG TTC GAC GAG ATT GAC
421 GCC TCC GGC AAC ATG CTG GTC GAC GAG GAG GAG CTC AAG CGC GCC GTG CCC AAG CTT GAG
481 GCG TGG GGC GCC AAG GTC GAG GAT CCC GCG GCG CTG TTC AAG GAG CTC GAT AAG AAC GGC
541 ACT GGG TCC GTG ACG TTC GAC GAG TTT GCT GCG TGG GCT TCT GCA GTC AAA CTG GAC GCC
601 GAC GGC GAC CCG GAC AAC GTG CCG GAG AGC GCG TGA
```

Protein sequence:

```
MGACGSKGSTSDKGLASDKDGKKAKDRKEAWERIRQAIPREKTAEAKQRRIELFKKFDKNETGKLCYDEVHSG
CLEVLKLDEFTPRVRDITKRAFDKARALGSKLENKGSEDFVEFLEFRLMLCYIYDFFELTVMFDEIDASGNML
VDEEELKRAVPKLEAWGAKVEDPAALFKELDKNGTGSVTFDEFAAWASAVKLDADGDPDNVPESA*
```

Figure 3.1-2. Gene and protein sequence of 1F8 antigen. Codons very rarely used in *E. coli* are highlighted in red, less rarely used in orange. BamHI and HIndIII restriction sites are shown in green, and start and stop codons in violet.

Only two very rare codons were contained in the sequence. Therfore, the N-terminal 70% of the sequence could be used for cloning. As overexpressed recombinant proteins are insoluble in most of cases and have to be purified with caotropic agents under denaturating conditions anyway, omitting of parts of a protein must not be deleterious for its use as antigen. Only regions with a stable secondary structure were expected to function as good epitopes, and such regions could occur in any part of the protein.

To design appropriate PCR primers for amplification, the selected largest possible fragment without BamHI and HindIII restriction sites, but still containing some rare codons, was entered in the Primer3 program (Rozen et al., 2000, http://frodo.wi.mit.edu/). This software allows the designing of optimal primers for PCR and sequencing, but also for hybridization probes within an selected sequence. In order to adapt the results to the own needs, different

parameters such annealing temperature, primer size, fragment size, CG percentage can be selected. The program also predicts the creation of hairpins, by detecting self complementary regions. In the case of the 1F8 antigen, only few of the many possible parameters were adjusted. Product size ranges were selected from 450 to 468 basepairs (length of the introduced sequence), primer size in a range of 18 to 20 basepairs, and optimal annealing temperature of 60ºC. Forward and reverse primers with similar size and annealing temperature were preferred. By this way, standardisation of PCR conditions was facilitated. According to the selected parameters, the programme returned the position of optimal forward and reverse primers within the included region and the size of the resulting length of the product of 456 basepairs. The results are shown on Figure 3.1-3.

ATGGGTGCTTGTGGGTCGAAGGGCTCGACGAGCGACAAGGGGTTGGCGAGCGATAAGGACGGCAAGAAGGCCAAGGAC
CGCAAGGAAGCGTGGGAGCGCATTCGCCAGGCGATTCCTCGTGAGAAGACCGCCGAGGCAAAACAGCGCCGCATCGAG
CTCTTCAAGAAGTTCGACAAGAACGAGACTGGGAAGCTGTGCTACGATGAGGTGCACAGCGGCTGCCTCGAGGTGCTG
AAGTTGGACGAGTTCACGCCGCGAGTGCGCGACATCACGAAGCGTGCGTTTGACAAGGCGAGGGCCCTGGGCAGCAAG
CTGGAGAACAAGGGCTCCGAGGACTTTGTTGAATTTCTGGAGTTCCGTCTGATGCTGTGCTACATCTACGACTTCTTC
GAGCTGACGGTGATGTTCGACGAGATTGACGCCTCCGGCAACATGCTGGTCGACGAGGAGGAGCTCAAGCGCGCCGTG

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER          5   18   60.25   61.11  4.00  0.00 GTGCTTGTGGGTCGAAGG
RIGHT PRIMER       460   19   60.24   63.16  6.00  1.00 GCTTGAGCTCCTCCTCGTC
SEQUENCE SIZE: 468
INCLUDED REGION SIZE: 468

PRODUCT SIZE: 456, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 3.00
```

Figure 3.1-3 Primer3 results. The sequence included in the search was 468 bp, without BamHI and HindIII restriction sites. The forward and reverse primers returned by the software are highlighted in blue. The length of the selected primers, annealing temperature and size of the final product are given by the program as well.

In order to allow cloning, restriction sites for BamHI (g|gatcc) and HindIII (a|agctt) were added manually to the primers designed by the Primer3 software. By PCR amplification using the corresponding synthetic primers, these sites were included as a part of the fragment, and later used to create “sticky ends” for cloning of the sequence in the expression vector. To facilitate cleavage of the PCR product by the restriction enzymes, three additional nucleotides were included at the ends of the primers each. The restriction sites were introduced in such a way that the open reading frame (ORF) of 1F8 could be fused to the ORF of the His-tag in the vector pQE-30 at the N-terminal part, and to the ORF of another antigen at the C-terminal part.

The final design of the 1F8 primers, which resulted after these procedures were two primer of 27 base pares each, and their sequences are shown below.

Tcru-1F8-F: ATA **GGA TCC** GGT GCT TGT GGG TCG AAG

Tcru-1F8-R: CGG **AAG CTT** CTT GAG CTC CTC CTC GTC

The amplification of the fragment product of the 1F8-primers was made by PCR at standard conditions (see Materials and Methods) using *T. cruzi* DNA as template. However, due to the length of the fragments, the annealing temperature was increased to 70ºC and the extension time to 1 minute and 30 seconds. After checking that the expected band (456 bp) was correctly amplified, it was cut out of the gel and diluted by using the NaI/silica DNA extraction method (see Materials and Methods).

Cloning in pQE vector and protein expression

For the cloning of 1F8 fragment in pQE-30 expression vector, “sticky ends” with BamHI and HindIII restriction enzymes had to be created. Then, the cleaved 1F8 fragment was ligated in pQE vector (also containing sticky ends). After purification with silica, the DNA of the ligation assay was electroporated in competent cells which were therafter grown on selection plates containing ampiciline and kanamicine antibiotics. Resulting colonies were analysed by PCR, using the 1F8 forward primer and the pQE reverse primer. Finally, one of the positive clones was selected for protein purification, as shown in Figure 3.1-4.

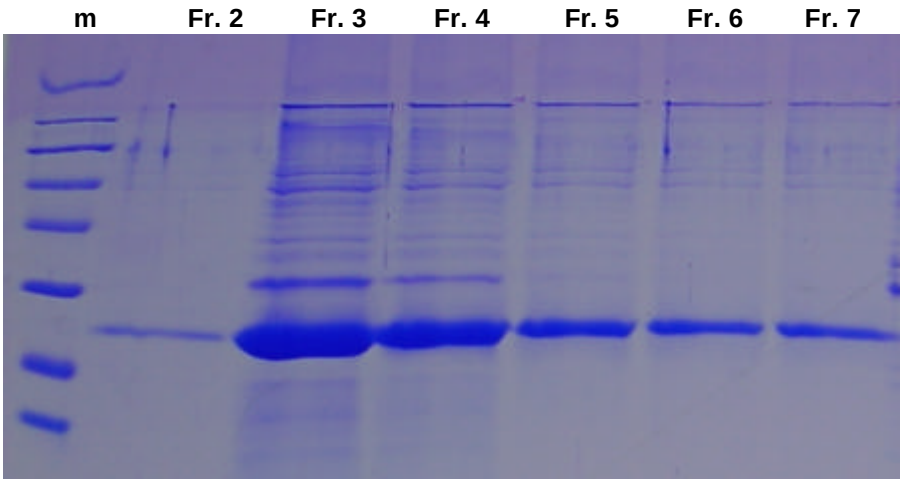

Figure 3.1-4 Protein expression of pET-1F8 clone. Fractions 3 and 4 contain the most part of the protein 1F8.

3.1.3 Synthetic genes

Tandem repeated sequences

Production of tandemly repeated recombinant antigens showed some problems while trying to clone and express them stably in *E. coli*. This was solved by constructing genes with different codons in the nucleotide sequences for the repetitive sequences by means of synthetic oligonucleotides avoiding codons rarely used by *E. coli* (see Figure 3.1-5). The two different procedures used during the work in this thesis will be next described.

TTT F 0.58	TCT S 0.17	TAT Y 0.59	TGT C 0.46
TTC F 0.42	TCC S 0.15	TAC Y 0.41	TGC C 0.54
TTA L 0.14	TCA S 0.14	TAA * 0.61	TGA * 0.30
TTG L 0.13	TCG S 0.14	TAG * 0.09	TGG W 1.00
CTT L 0.12	CCT P 0.18	CAT H 0.57	CGT R 0.36
CTC L 0.10	CCC P 0.13	CAC H 0.43	CGC R 0.36
CTA L 0.04	CCA P 0.20	CAA Q 0.34	CGA R 0.07
CTG L 0.47	CCG P 0.49	CAG Q 0.66	CGG R 0.11
ATT I 0.49	ACT T 0.19	AAT N 0.49	AGT S 0.16
ATC I 0.39	ACC T 0.40	AAC N 0.51	AGC S 0.25
ATA I 0.11	ACA T 0.17	AAA K 0.74	AGA R 0.07
ATG M 1.00	ACG T 0.25	AAG K 0.26	AGG R 0.04
GTT V 0.28	GCT A 0.18	GAT D 0.63	GGT G 0.35
GTC V 0.20	GCC A 0.26	GAC D 0.37	GGC G 0.37
GTA V 0.17	GCA A 0.23	GAA E 0.68	GGA G 0.13
GTG V 0.35	GCG A 0.33	GAG E 0.32	GGG G 0.15

Figure 3.1-5 Genetic standard code of *Escherichia coli*. Codons are followed by the amino acid they code for, and the corresponding usage frequencies. Codons marked in red present the lowest frequencies, and another group of condons with low frequencies in orange. Stop codons are indicated with an asterisc.

Example 1: Construction of synthetic B13 antigen

B13 (CA-2) is localised on the surface of *T. cruzi* and is one of the immunodominant antigens containing many repeats of the 12 amino acid motive PSPFGQAAAGDK (Gruber et al., 1993). In the Sanger GeneDB, two different alleles of the gene with together 103 highly conserved repeats were observed. Its performance in immunoassays of different groups has been found to be very good. The following strategy was used to express a representative fragment of the repetitive sequence as a recombinant antigen.

It was presumed that three repeats of the conserved sequence PSPFGQAAAGDK may represent a good antigen to be recognised by sera from chagasic patients. As three identical repeats of the coding region were not stably to be maintained in *E. coli*, an open reading frame for three repeats was designed manually including as many sequence variations between the three repeats as possible. This was easily feasible by using the whole spectrum of possible base exchanges in codons for specific amino acids with the only restriction not to select codons rarely used by *E. coli*. The sequence was designed as follows:

```
 P   S   P   F   G   Q   A   A   A   G   D   K
CCA TCA CCA TTT GGA CAG GCC GCA GCA GGT GAC AAA
CCG AGC CCG TTC GGT CAA GCA GCC GCA GGA GAT AAG
CCT TCT CCC TTT GGC CAG GCA GCT GCA GGC GAT AAA
```

Nucleotides differing from the sequence in the first repeat are underlined in the two other repeats. The complete sequence was constructed by means of the following nine complementary overlapping synthetic oligonucleotides:

```
TcruB13-F1:  GATCACCATCACCATTTGGACA
TcruB13-F2:  GGCCGCAGCAGGTGACAAACCGAGCCCGTT
TcruB13-F3:  CGGTCAAGCAGCCGCAGGAGATAAGCCTTCT
TcruB13-F4:  CCCTTTGGCCAGGCAGCTGCAGGCGAT
TcruB13-F5:  AAACCTGGATCCTTACCGA

TcruB13-R1:  AGCTTCGGTAAGGATCCAGGTTTATCGCCTGCA
TcruB13-R2:  GCTGCCTGGCCAAAGGGAGAAGGCTTATCTC
TcruB13-R3:  CTGCGGCTGCTTGACCGAACGGGCTCGGT
TcruB13-R4:  TTGTCACCTGCTGCGGCCTGTCCAAATGGTGATGGT
```

To allow ligation of the synthetic DNA fragment into the vector pQE-30 via a BamHI site at the 5' end and a HindIII site at the 3' end, sequences matching the corresponding "sticky" sites in the vector were added to the terminal oligonucleotides (underlined). The sequence at the 5' end was designed in such a way (gatc**a** instead of gatc**c**) that the BamHI site would not exist anymore after ligation. Instead, another BamHI site was added at the end of the repeat in front of the HindIII site (in fragments TcruB13-F5 and TcruB13-R1) which would allow to fuse B13 trimer later with other genes synthesised according to the same principle.

Except of TcruB13-F1 and TcruB13-R1, all oligonucleotides were phosphorylated at the 5' ends by means of polynucleotide kinase and ATP and ligated in stoichiometric amounts to a single DNA fragment with T4 ligase. Omitting the phosphorylation of the terminal oligonucleotides prevented the polymerisation of the fragment, but did not interfere with later ligation into the vector.

```
3′ GATCACCATCACCATTTGGACAGGCCGCAGCAGGTGACAAACCGAGCCCGTTCGGTCAAGCAGCCGCAGGAGAT
5′     TGGTAGTGGTAAACCTGTCCGGCGTCGTCCACTGTTTGGCTCGGGCAAGCCAGTTCGTCGGCGTCCTCTA

   AAGCCTTCTCCCTTTGGCCAGGCAGCTGCAGGCGATAAACCTGGATCCTTACCGA 5′
   TTCGGAAGAGGGAAACCGGTCCGTCGACGTCCGCTATTTGGACCTAGGAATGGCTTCGA 3′
```

The ligation product was separated on a 2% agarose gel, a band of correct size (120 bp) excised and ligated into pQE-30. By this way, an N-terminal His-tag was added to the B13 trimer which allowed easy purification of the protein by affinity chromatography on TALON® resin.

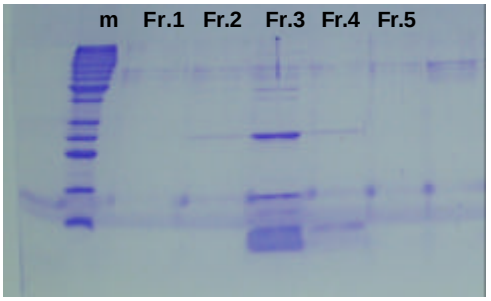

Figure 3.1-6 Preparative expression of B13 trimer. Most of the protein is contained in fraction 3. The size of the obtained protein corresponded with the expected size of approximately 5 kDa

Fusion of B13 and CRA antigens

CRA (Cytoplasmic Repetitive Antigen) has been described as another highly immunoreactive repetitive antigen (Lafaille et. al., 1989) and three of its tetradecameric repeats occurring 130 times in highly conserved form in the genome of *T. cruzi* had been synthesised with oligonucleotides using the same strategy as for the B13 antigen (for the sequence, see Table 3.1-1). CRA was also obtained in minute amounts only upon expression in *E. coli* and affinity purification. The CRA antigen also contains a BamHI restriction site at the 5' end and a

HindIII site at the 3' end of the coding sequence. It was combined with the B13 antigen by ligation to the BamHI site near the end of B13. The fusion product has the following sequence:

```
M   R   G   S   H   H   H   H   H   H   G   S   P   S   P   F   G   Q   A
ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC GGA Tca cca tca cca ttt gga cag gcc

A   A   G   D   K   P   S   P   F   G   Q   A   A   A   G   D   K   P   S
gca gca ggt gac aaa ccg agc ccg ttc ggt caa gca gcc gca gga gat aag cct tct

                  <---------B13------------ BamHI  --------- CRA -------->
P   F   G   Q   A   A   A   G   D   K   P   G   S   K   V   A   E   A   E
ccc ttt ggc cag gca gct gcg ggc gat aaa cct gga tcc aaa gtg gcg gag gcc gaa

K   Q   K   A   A   E   A   T   K   V   A   E   A   E   K   Q   K   A   A
aag cag aag gca gct gaa gcc acg aag gtt gcc gaa gcg gag aag cag aaa gcg gca

E   A   T   K   V   A   E   A   E   K   Q   K   A   A   E   A   T   K   L
gaa gct gca gag gta gct gag gca gaa aaa caa aag gct gcg gag gcc act aag ctt
                                                                HindIII
```

Figure 3.1-7 Fusion of B13 and CRA antigens.The nucleotides exchanged in the different repeats are underlined. In addition, the N-terminal hexahistidin-tag added by the pQE-30 vector is shown. The BamHI site used for the fusion and the HindIII site at the 3' end are indicated.

The B13-CRA fusion antigen was expressed to a much higher extent in the induced *E. coli* cells than the individual antigens alone. That supports the idea that very small recombinant proteins are not stable in *E. coli*.

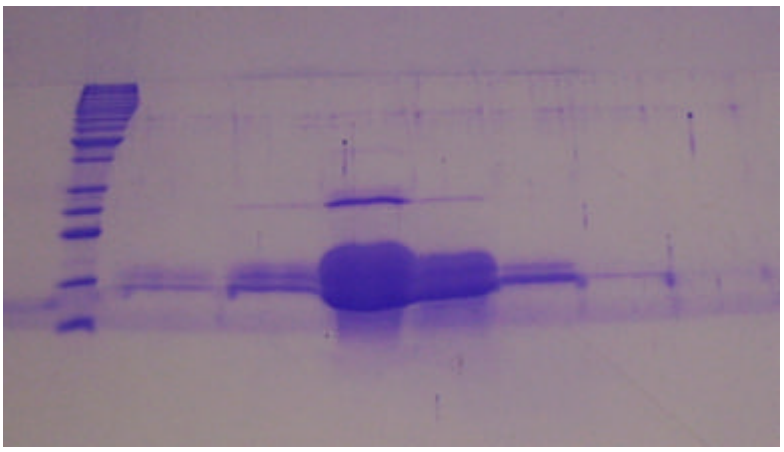

Figure 3.1-8 Purification of Tcru-B13-CRA fusion antigen. 5 µl of each 500 µl fraction of the B13-CRA fusion antigen eluted from the TALON® affinity column was analysed on a 12 % SDS polyacrylamide gel and stained with Coomassie Blue.

Example 2: SAPA antigen cloning

SAPA (shed-acute-phase-antigen) has an enzymatic trans-sialidase activity which is described only for trypanosomes and is involved in the invasion of host cell. The enzyme is made of two domains, the C-terminal region containing immunodominant amino acid repeats that define the SAPA antigen. The SAPA antigen induces a strong humoral response detected shortly after infection, both in humans and in mice. This response is directed to the immunodominant domain but is irrelevant in terms of neutralization of trans-sialidase activity (Affranchino et al., 1989). Five different genes containing varying numbers of a repeat of 12 amino acid residues (PVDSSAHGTPST) were detected in the Sanger GeneDB. The sequence of the gene with the highest number of these repeats is shown in Figure 3.1-9.

MGKTVVGASRMFWLMFFVPLLLALCPSEPAHALAPESSRVELFKRQSSKVPFEKDGKVTDRVVHSFRL
PALVNVDGVMVAIADARYETSNDNSLIDTVAKYSVDDGETWETQIAIKNSRVSSVSRVVVPTVIVKGN
KLYVLVGSYNSSKSYWTWQPDGSDWDILLAVGEVTKSTAGGKTTASIKWGSPVSLKKFFPAEMEGMHT
NQFLGGAGVAIVASNGNLVYPVQVTNKRKQVFSKIFYSEDEGKTWKFGKGRSDFGCSEPVALEWEGKL
IINTRVDRARRLVYESSDMGNTWVEAVGTLSRVWGPSPKSDQPGSQSSFTAVTIEGMRVMLFTHPLNF
KGRWLRDRLNLWLTDNQRIYNVGQVSIGDENAAYSSVLYKDDKLYCLHEINTNEVYSLVFARLVGELR
IIKSVLQSWKNWDSHLSSICTPADPAASSSERGCGPAVTTVGLVGFLSGNASQNVWEDAYRCVNASTA
NAERVPNGLKFAGVGGGALWPVSQQGQNQRYRFANHAFTLVASVTIHEVPSVASPLLGASLDSSGGKK
LLGLSYDKKHQWQPIYGSTPVTPTGSWETGKRYHVVLTMANKIGSVYIDGEPLEGSGQTVVPDGRTPD
ISHFYVGGYKRSDMPTISHVTVNNVLLYNRQLNAEEIRTLFLSQDLIGTEAHMDSSS DSSAHGTPST
PVDSSAHGTPST PVDSSAHSTPST PADSSAHGTPST PVDSSAHGTPST PVDSSAHSTPST
PVDSSAHGTPST PVDSSAHGTPST PVDSSAHSTPST PVDSSAHGTPST PVDSSAHGTPST
PVDSSAHSTPST PVDSSAHSTPST PADSSAHGTPST PVDSSAHGTPST PVDSSAHSTPST
PVDSSAHSTPST PADSSAHGTPST PVDSSAHSTHST PVDSSAHSTPST PVDSSAHGTPST
PVDSSAHGTPST PVDSSAHSTPST PVDSSAHGTPST PVDSSAHGTPST PVDSSAHGTPST
PVDSSAHSTPST PVDSSAHGTPST PVDSSAHGTPST PVDSSAHSTPST PVDSSAHGTPST
PVDSSAHSTPST PVDSSAHSTPST PVDSSAHSTPST PVDSSAHSTPST PVDSSAHGTPST
PVDSSAHGTPSA PVDSSAHSTPST PVDSSAHSTPST PVDSSAHGTPST PVDSSAHSTPST
PAGNSATRMFLILPDGAAISAFSGGGLLLCACALLLHVFFTAVFF

Figure 3.1-9 Amino acid sequence of *T. cruzi* SAPA antigen (trans-sialidase). Tandem repeats are highlighted in red. Changes in single amino acid residues in the repeated sequences are shown in black.

As can be seen from Figure 3.1-9, the repeat is not completely conserved but contains variations in the second position (V-A) and in the eight position (G-S). The longest possible DNA sequence (without repetitions longer than eight nucleotides) for a synthetic multimer of the amino acid repeat, consisted of five complete repeats. Variations to the conserved sequence are underlined:

PVDSSAHGTPST PVDSSAHGTPST PVDSSAHSTPST PVDSSAHSTPST PADSSAHSTPST

The DNA sequence for the corresponding open reading frame using the genetic code preferred by *E. coli* was designed by means of the DNAWorks computer program described above. One of the solutions calculated by this program suggested the following sequence of 180 nucleotides.

```
  1 CCTGTGGACTCCTCTGCCCATGGTACCCCTAGCACTCCGGTCGACTCTAGCGCGCACGGC
 61 ACCCCATCTACCCCAGTTGATTCTTCTGCGCACTCTACTCCATCCACGCCGGTTGACTCT
121 TCCGCGCACAGCACCCCTTCCACTCCTGCAGACAGCTCTGCTCATAGCACGCCGTCTACG
```

The program designs the oligonucleotides to be synthesised. The maximal lengths of these oligonucleotides has to be selected at the beginning. As longer the nucleotides, as easier is the experimental assembly, but there are technical limitations with most providers of synthetic oligonucleotides. 6 oligonucleotides were synthesized:

```
SAPA2-1 ATAAGATCTCCTGTGGACTCCTCTGCCCATGGTACCCCTAGCACTCCG  (48 bp)
SAPA2-2 AGATGGGGTGCCGTGCGCGCTAGAGTCGACCGGAGTGCTAGGGGTACC  (48 bp)
SAPA2-3 GCACGGCACCCCATCTACCCCAGTTGATTCTTCTGCGCACTCTACTCC  (48 bp)
SAPA2-4 TGTGCGCGGAAGAGTCAACCGGCGTGGATGGAGTAGAGTGCGCAGAAG  (48 bp)
SAPA2-5 TGACTCTTCCGCGCACAGCACCCCTTCCACTCCTGCAGACAGCTCTGC  (48 bp)
SAPA2-6 TATAAGCTTCGTAGACGGCGTGCTATGAGCAGAGCTGTCTGCAGGA    (46 bp)
```

The sequences marked in blue were added manually and provide the restriction sites needed for cloning in the vector pQE-30, as previously described for other antigens. In addition, the program provides a scheme of the assembly of these oligonucleotides:

```
   1 --->                                                3 --->
 1 CCTGTGGACTCCTCTGCCCATGGTACCCCTAGCACTCCG               gcacggc
                       CCATGGGGATCGTGAGGCCAGCTGAGATCGCGCGTGCCG

    P  V  D  S  S  A  H  G  T  P  S  T  P  V  D  S  S  A  H  G

                                                         5 --->
61 accccatctaccccagttgattcttctgcgcactctactcc            TGACTCT
   TGGGGTAGA             gaagacgcgtgagatgaggtaggtgcggccaactgaga
     <--- 2
    T  P  S  T  P  V  D  S  S  A  H  S  T  P  S  T  P  V  D  S


121 TCCGCGCACAGCACCCCTTCCACTCCTGCAGACAGCTCTGC
   aggcgcgtgt             AGGACGTCTGTCGAGACGAGTATCGTGCGGCAGATGC
     <--- 4                                          <--- 6
    S  A  H  S  T  P  S  T  P  A  D  S  S  A  H  S  T  P  S  T
```

Furthermore, the program calculates the annealing temperature for the overlaps of the oligonucleotides, which in this case was 62°C:

Tm Range	# of Overlaps
61	1
62	4

The DNAWorks program uses the same principles for selection of codons as described in example 2, to avoid repetitions of more than eight nucleotides in the DNA. This is shown in Figure 3.1-10, where the sequences of the open reading frames for the five repeats were written one upon the other.

```
 BamHI* P   V   D   S   S   A   H   G   T   P   S   T
AGA TCT CCT GTG GAC TCC TCT GCC CAT GGT ACC CCT AGC ACT

       P   V   D   S   S   A   H   G   T   P   S   T
       CCG GTC GAC TCT AGC GCG CAC GGC ACC CCA TCT ACC

       P   V   D   S   S   A   H   S   T   P   S   T
       CCA GTT GAT TCT TCT GCG CAC TCT ACT CCA TCC ACG

       P   V   D   S   S   A   H   S   T   P   S   T
       CCG GTT GAC TCT TCC GCG CAC AGC ACC CCT TCC ACT

       P   A   D   S   S   A   H   S   T   P   S   T   HindIII
       CCT GCA GAC AGC TCT GCT CAT AGC ACG CCG TCT ACG AAG CTT
```

Figure 3.1-10 Synthetic SAPA antigen pentamer. The sequence codons are shown in black under the corresponding amino acid residues (in red). In blue, the restriction sites of BamHI and HindIII added later. Note that different codons are coding for the same amino acid, and the amino acid sequence remains unaltered, even though nucleotide sequence changes in each repeat.

To synthesise the gene, the 6 oligonucleotides were mixed in standard PCR buffer in the presence of desosoxyribonucleotide triphosphates in different concentrations, 0.15 µM for the inner oliconucleotides SAPA-2, SAPA-3, SAPA-4 and SAPA-5, and 1 µM for the flanking oligonucleotides SAPA-1 and SAPA-6. After 2 minutes incubation at 62°C, Taq DNA polymerase was added and the temperature kept at 62°C for another 2 minutes. Then followed 3 cycles of denaturation at 94°C for 20 seconds and DNA synthesis at 60°C for 1 minute, thereafter 30 cycles of heating to 94°C for 20 seconds and DNA synthesis at 72°C for 30 seconds, and finally 5 minutes at 72°C to fill in the ends of the fragment completely.

The product of this amplification reaction was separated on a 2% agarose gel and stained with ethidium bromide. As can be seen from Figure 3.1-11, the expected fragment of 190 base pairs was the major product.

Figure 3.1-11 Synthetic SAPA antigen. It was analysed by electrophoresis on a 2 % agarose gel. The expected size (190 bp) was obtained.

The fragment was cut out of the gel, the DNA extracted with NaI and silica, digested with Bam HI and HindIII, and finally ligated in the vector pQE-30. Upon transformation of *E. coli* cells by electroporation and selection of several resulting colonies by PCR, one appropriate clone was selected and induced to express the recombinant protein. The protein was extracted, purified by metal affinity chromatography and analysed by SDS polyacrylamide gel electrophoresis. After staining with Coomassie Blue, a protein band with the expected size of approximately 7 kDa was observed (Figure 3.1-12).

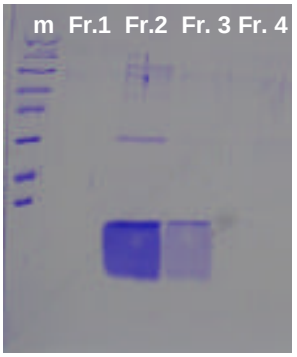

Figure 3.1-2 Purified recombinant SAPA antigen. On the left side, the size marker (m), and from left to right, metal affinity column eluted fractions 1 to 4 are shown. Protein is contained basically in fraction 2.

The repetitive motives of the antigens MAP, TcD, TcE and TcMyo were synthesised according to the same principle. MAP (micrutubule-associated antigen) is a trimer of the sequence PRHVDPDHFRSTTQDAYRPVDPSAYKRALPQEEEEDVG, TcD is a nonameric

repeat of the five amino acid residues PKPAE, TcE is a hexameric repetition of PPAKAAA and TcMyo corresponds to 4 repeats of the tetradecameric sequence LAQREADNEKLAED whereby the last the residues are slightly variable. The complete sequences of these antigens are shown in Table 3.1-1 above.

3.1.4 Sensitivity and specificity of antigens

Efficiency of the purified recombinant diagnostic antigens was tested using an immunoblot technique called line blot (described in the Materials and Methods section). This technique allowed reaching basically two objectives: to determine the optimal concentration of the antigens in immunoassays, and to detect possible cross-reactions. The line blot allows the comparison of the reactivity of different antigens and of different concentrations of these antigens in a single assay. To determine the optimal concentration, the antigen is applied in serial dilutions on the membrane, and then this later is either incubated with chagasic patient serum or with control serum. At the optimal concentration, the antigen leads to a clear reaction with specific sera, but not with the control sera which were obtained from healthy German persons. A too high concentration of the antigen leads to false positive reactions with control sera, and a too low concentration reduces sensitivity of the test.

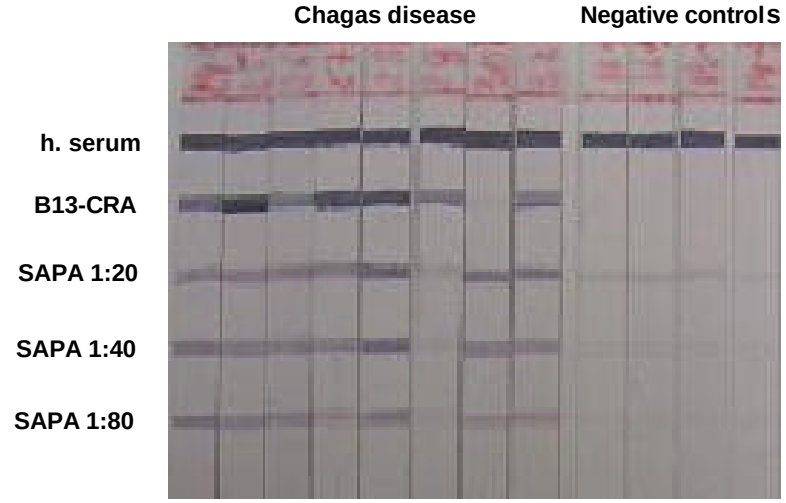

Figure 3.1-13 Line blot test of different SAPA concentrations and quantification. Different SAPA proteins were tested on a line blot in different concentrations and with chagasic patient sera and negative control sera, and a line of positive antigen control (B13-CRA fusion). The upper line is human serum diluted 1:70 as a control for the staining reaction, the next line is the B13-CRA antigen as a positive marker, and the three lower lines correspond to three different dilutions of the SAPA antigen. The middle dilution (1:40) is appropriate for further tests.

Intensity of reaction with patient sera is a property of the antigen and depends on the concentration of specific antibodies. Some antigens have to be used at concentrations as high as 200 µg per ml, others lead to strong positive reactions at concentrations less than 1 µg per ml. The most reactive antigens were a dimer of the FRA repeat which reacted strongly with more than 95 % of all infection sera, and the fusion B13-CRA. The antigens 1F8, MAP, SAPA, TcD, TcE and JL8 revealed a lower or moderate immunoreactivity.

Some antigens show a strong background reaction with control sera from healthy persons. This holds mainly for antigens with low specific reactivity which have to be used at high concentrations. As the affinity purification procedure does not remove bacterial components such as lipopolysaccharid completely, the high background reaction with the control sera may be due to antibodies directed against these components. *E. coli*-specific antibodies are present in all human sera and prevail even more in sera from people in developing countries. Bacterial contaminations in the antigen preparations cannot be removed completely without great effort. However, as more diluted an antigen can be used in immunoassays, as lower the cross-reaction with bacterial contaminations will be.

Line blot was also used to determine the specificity of the immunoreaction of the recombinant antigens. The test stripes were incubated with sera from patients suffering from cutaneous and visceral leishmaniasis, syphilis, or brucellosis. The latter sera were obtained from patients from Mongolia, and the leishmaniasis sera were from the Republic of Yemen. These countries are all free of Chagas disease. Thus, accidental infection with *T. cruzi* could be excluded. None of the antigens led to reactions with the syphilis and brucellosis sera, but with the antigens JL8, 1F8 and FRA cross-reactivity with sera from visceral leishmaniasis patients was clearly to be recognised (Figure 3.1-14).

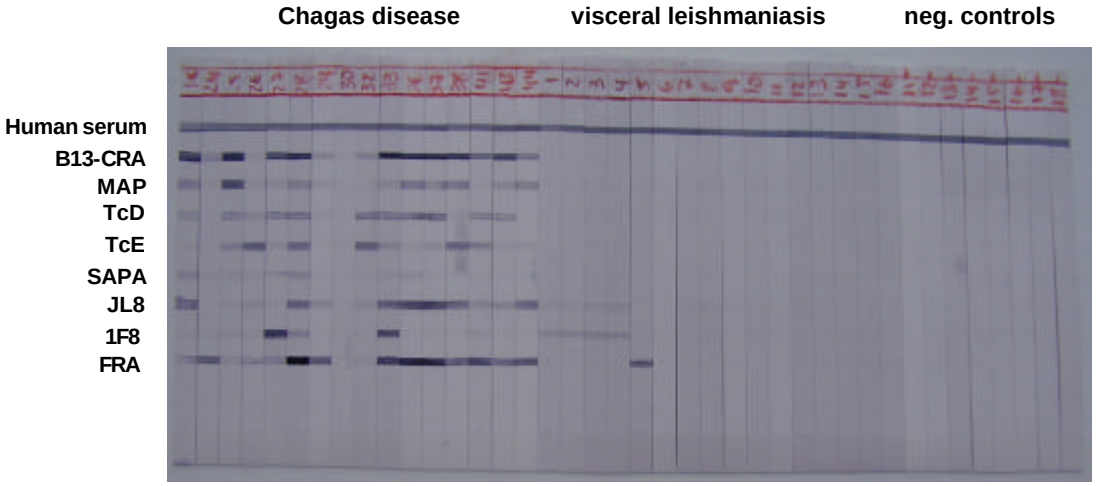

Figure 3.1-14 Cross-reaction tests for the different obtained antigens. The first line is a colorimetric reaction positive control (human serum), and the second lane a positive control for Chagas disease antigens (fusion B13-CRA). All obtained antigens were tested on lanes with Chagas disease and visceral leishmaniasis patients, and healthy German donors. A strong FRA reaction with visceral leishmaniasis patients is to be seen, as well as further cross-reactions of JL8 and 1F8.

To search for the reason for the observed cross-reactivity with leishmaniasis patient sera, the genomes of *L. infantum* and *L. major* in the Sanger GeneDB were analysed with the BLAST software (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for potential homologies. For all cross-reactive antigens significant homologies were detected with *Leishmania* genes containing similarly arranged tandem repeats (accidental homologies with single sequences were disregarded).

The original version of the recombinant FRA antigen, consisting of two copies of the 68 amino acid residue repeat, led to a strong cross-reactivity with sera from leishmaniasis patients. It turned out that a related tandem repeat occurs also in *Leishmania*, thus the cross-reactivity became understandable. A much weaker cross-reactivity was observed with sera from patients with cutaneous leishmaniasis, even though the repeat is also present in *L. major* in almost identical form as in *L. infantum*. However, there are fewer copies of the repeat in *L. major* which may lead to a weaker immune response as compared to *L. infantum* where the repeat is three times more frequent than in *T. cruzi* (Fig. 3.1-15).

Recombinant FRA antigen is used in several commercial diagnostic kits for Chagas disease, most probably because of its high reactivity. As it was not clear whether the FRA antigen in such kits corresponds to the complete length of the repeat or to a subregion only, a trimer of the least conserved part of the repeat was produced (Fig. 3.1-15). In order to allow the molecule to fold in a structure similar to the complete repeat, short spacer peptides containing two proline residues each were included. However, the resulting antigen FRA2 was still cross-reactive with leishmaniasis patient sera, even though at a lower degree. Therefore, this antigen was not included in further tests.

```
L. infantum: LDPKPEGVPLRCVPLDEDAEFVALEDEWRGLLQ-DPQRNSMPLKDLERRMNDRAHDVACGKKWADRDRV
identity:    LD KPEGVPLR +PLD+D++FVA+E E R LL+ DP+RN+  +  LE  MN RA ++A +KK ADR
T. cruzi:    LDQKPEGVPLRELPLDDDSDFVAMEQERRQLLEKDPRRNAREIAALEESMNARAQELAREKKLADRAF
  (FRA2)                           MEQERRQLLEKDPRRNAREIAALEESMNARAQELAREKKLADRAF
```

Fig 3.-15 Similarity of FRA antigen between *T. cruzi*, *L. infantum* and *L. major*. 80 % homology between the *T. cruzi* FRA antigen and a repeat in a calpain-like cystein protease of *L. infantum* (LinJ27.0400) and, in identical form, of *L. major* (LmjF27.0490) was found. The repeat is present 43 times in *L. infantum* and 23 times in *L. major*. The synthetic FRA2 antigen (lower line) corresponds to the least conserved part of the repeat.

Similarly, the BLAST program detected up to 50 % homology of 1F8 antigen to a sequence corresponding to a putative flagellar calcium-binding protein in the genome of *Leishmania infantum* (LinJ16.0960). For the JL8 antigen only a weak sequence homology to *L. infantum* and *L. major* was found. Nevertheless, a clear cross-reactivity was observed in the line blot. Due to their obvious unspecificity, the three antigens FRA, 1F8 and JL8 were excluded from further use in immunodiagnosis, even though they have been described as good recombinant antigens in the literature by others (Umezawa et al., 1999; Gomes et al., 2001).

Also for the MAP repeat of *T. cruzi*, homology with a microtuble-associated protein of *L. infantum* (LinJ05.0380) was detected. The protein of *L. infantum* contains 27 repeats of a sequence with 40 % homologous amino acid residues. However, no cross-reactions were observed in the immunoblots, and the MAP antigen was further used in the following assays.

No related sequences were detected for the tandem repeats of the antigens B13, CRA, TcD, TcE and SAPA, and there was also no doubt on their specificity for *T. cruzi* from the experimental results.

3.1.5 Fusion of the selected antigens

After the individual recombinant antigens had been analysed thoroughly in line blot assays, they were tested in ELISA assays. Even though ELISA assays depend on special equipment such as optical readers and washers, they are more appropriate for tests in large scale because they can be automated. It turned out that some of the recombinant antigens showed low immunoreactivity in ELISAs, in spite of the fact they performed well in the immunoblots. A possible explanation for this phenomenon was that the small peptides did not bind properly to the plastic surface of the microplate and were lost during the washing steps. Another aspect was that the production of these small proteins in *E. coli* and/or their purification was not very efficient. To overcome these problems, it was attempted to combine the small peptides to larger fusion proteins.

In contrast to the simple mixture of individual recombinant antigens in immunoassays (Umezawa et al., 2003), their fusion in a unique polypeptide has some advantages. The production is simplified, because only one protein, instead of several, has to be purified. Furthermore, by the fusion of the selected recombinant antigens, it turned out that their efficiency was not only summed up, but enhanced. This could be explained by an altered conformation of the final polypeptide, leading to better recognition by specific antibodies.

In order to allow the synthetic proteins to adopt the natural conformation needed to function as antigenic determinants, the selected synthetic genes were fused with the help of “adapter” oligonucleotides. These adapters had several functions. First, they had to join the 3’ terminal HindIII site of the anterior antigen in the correct reading frame with the BamHI site of the following antigen. The nucleotide sequence was designed in such way that both restriction sites were destroyed by the fusion. Second, the adapters served as spacers between the different antigens. In order to allow maximal flexibility between the fused antigens, one or two proline residues were introduced in the spacer elements. Proline residues are frequently present in flexible areas of proteins (e.g. in the "hinge" regions of immunoglobulins). It was assumed that such flexible elements would prevent – or reduce at least – the interference of secondary structures of the individual antigenic regions between each other, and thus to facilitate the individual antigenic motives to fold in the correct structure. To avoid repetitions of homologous sequences in the fused genes, three different adaptor fragments were constructed (Figure 3.1-16).

Hind-Bam-AD1

```
          HindIII                              BamHI

5' NNN A       AG CTG CCG AGC CTG AGC A       GA TCC NNN 3'
3' NNN TTC GA       C GGC TCG GAC TCG TCT AG       G NNN 5'

    X   K          L   P   S   L   S   R          S   X
```

Hind-Bam-AD3

```
5' NNN A       AG CTC TCT CCG CTG CCG A       GA TCC NNN 3'
3' NNN TTC GA       G AGA GGC GAC GGC TCT AG       G NNN 5'

    X   K          L   S   P   L   P   R          S   X
```

Hind-Bam AD4

```
5' NNN A       AG CTT CCA GAT CCG TTA A       GA TCC NNN 3'
3' NNN TTC GA       A GGT CTA GGC AAT TCT AG       G NNN 5'

    X   K          L   P   D   P   L   R          S   X
```

Figure 3.1-16 Adapter sequences used to fuse antigen coding regions. Nucleotide sequences of the adapter oligonucleotides are depicted in complementary form. Residues providing "sticky ends" for the restriction site HindIII and BamHI are in blue, and amino acid sequences encoded by the different adaptors are shown below the nucleotide sequences in red. All three adapters bind between BamHI and HindIII restriction sites, but have different sequences.

At first, the coding regions of TcD and TcE antigens were fused with the adapter Hind-Bam-AD1. With this objective, the plasmid pQE-TcD was cleaved with HindIII and then ligated with the two non-phosphorylated oligonucleotides of adapter Hind-Bam-AD1. By this way, the negative strand of the adapter was covalently linked to the 3' ends of the HindIII site, whereas the positive strand was bound by hydrogen bonds only. Then, the vector was cleaved with PvuI in a region some 700 basepairs outside of the cloning site. In parallel, the plasmid pQE-TcE was cleaved in a double-digest with BamHI and PvuI. The digestion products of both plasmids were separated by agarose gel electrophoresis and the 1.1 kb PvuI-HindIII fragment from pQE-TcD and the 3 kb BamHI-PvuI fragment from pQE-TcE were isolated.

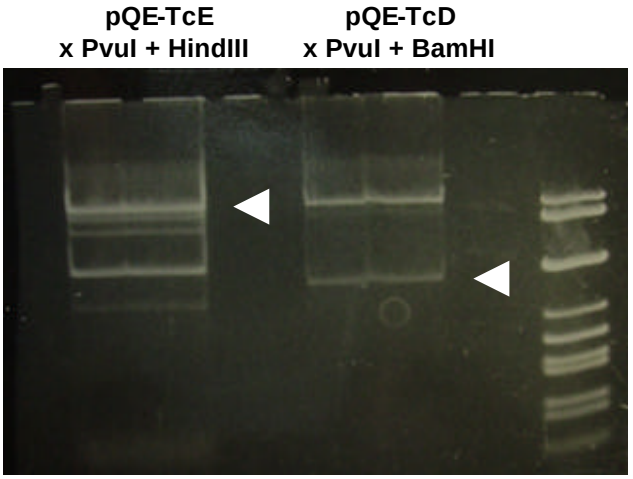

Figure 3.1-17 Gel electrophoresis of the digested pQE-TcD and pQE-TcE plasmids. The positions of the eluted fragments are indicated.

The two fragments were ligated whereby the positive strand of the adapter was added again since it was not covalently linked and probably lost during the isolation of the DNA fragments. The resulting construct was a pQE vector containing the desired fusion protein. According to the same principle also the antigens MAP and SAPA were combined to a MAP-SAPA fusion protein. To prevent sequence repetitions, the different adapter Hind-Bam-AD-3 was used. The antigens B13 and CRA had been fused before by means of an artificial BamHI site at the end of the open reading frame of B13 (see above). The immunoreactivity of the fused dimeric antigens was tested in ELISA and was found to be markedly higher than the reactivity of the monomeric antigens alone (data not shown).

For the ease of purification and in the hope to increase the immunoreactivity even more, the TcD-TcE dimer was fused to the B13-CRA dimer in the same way, this time using the Hind-Bam-AD1 adapter. The final construct included 3 repeats of B13, 3 repeats of CRA, 9 repeats of

TcD and 6 repeats of TcE antigens, designated TcBCDE with a molecular weight of 20 kDa. The fusion was expressed with good yield in *E. coli* cells (approximately 2 mg antigen per litre of culture) and was easily purified by metal affinity chromatography.

An unexpected extremely high increase of the immunoreactivity was observed with the tetrameric fusion product. It was needed to dilute the antigen 1:100.000 for coating the microtiter plates, otherwise the staining reaction exceeded the range of the photometer within few minutes. The calculated optimal protein concentration was as little as one nanogram per assay only, a result never seen in any immunoassay before. This result could not be explained solely by a better adhesion of the antigen to the microtiter plate. Rather the protein conformation must have been changed so that the epitopes were exposed to the specific antibodies in an optimal way. It is not clear whether this effect was due to the absence of the histidine-tag in front of the second fusion partner, or the introduction of a flexible spacer region, or a combination of both.

Finally, a very large polypeptide was constructed by fusing the the B13-CRA-TcD-TcE tetramer antigen and the MAP-SAPA dimer. However, the reactivity of the long hexameric fusion was not higher than that of the tetrameric fusion. For this reason, and to avoid instability of the construct, it was decided to use the tetrameric antigen for further assays. The different antigen fusions are depicted in Table 3.1-18.

T. cruzi antigens	amino acid sequence	sensitivity
Fusion B13-CRA	MRGSHHHHHHGSPSPFGQAAAGDKPSPFGQAAAGDKPSPFGQAAAGDKPGSKVAEAE KQKAAEATKVAEAEKQKAAEATKVAEAEKQKAAEATKL	**high**
Fusion TcD-TcE	MRGSHHHHHHGSPKPAEPKPAEPKPAEPKPAEPKPAEPKPAEPKPAEPKPAEPKPAEK LPSLSRSPAKAAAPPAKAAAPPAKAAAPPAKAAAPPAKAAAPPAKAAAPPAKAAAPKL	**high**
Fusion B13-CRA-TcD-TcE (TcBCDE)	MRGSHHHHHHGSPSPFGQAAAGDKPSPFGQAAAGDKPSPFGQAAAGDKPGSKVAEAE KQKAAEATKVAEAEKQKAAEATKVAEAEKQKAAEATKLPSLSRSPKPAEPKPAEPKPA EPKPAEPKPAEPKPAEPKPAEPKPAEPKPAEKLPSLSRSPAKAAAPPAKAAAPPAKAA APPAKAAAPPAKAAAPPAKAAAPPAKAAAPKL	**extremely high**
Fusion MAP-SAPA	MRGSHHHHHHLPRHVDPDHFRSTTQDAYRPVDPSAYKRALPLEEEEDVGPRHVDPDHF RSTTQDAYRPVDPSAYKRALPLEEEEDVGPRHVDPDHFRSTTQDAYRPVDPSAYKRALP QEEEEDVGKLSPLPRSPVDSSAHGTPSTPVDSSAHGTPSTPVDSSAHSTPSTPVDSSAH STPSTPADSSAHSTPSTKL	**very high**
Fusion B13-CRA-TcD-TcE-MAP-SAPA	MRGSHHHHHHGSPSPFGQAAAGDKPSPFGQAAAGDKPSPFGQAAAGDKPGSKVAEAE KQKAAEATKVAEAEKQKAAEATKVAEAEKQKAAEATKLPSLSRSPKPAEPKPAEPKPA EPKPAEPKPAEPKPAEPKPAEPKPAEPKPAEKLPSLSRSPAKAAAPPAKAAAPPAKAA APPAKAATcEPAPPAKAAAPPAKAAAPPAKAAAPKLPDPLRSPRHVDPDHFRSTTQDAY RPVDPSAYKRALPLEEEEDVGPRHVDPDHFRSTTQDAYRPVDPSAYKRALPLEEEEDVG PRHVDPDHFRSTTQDAYRPVDPSAYKRALPQEEEEDVGKLSPLPRSPVDSSAHGTPSTP VDSSAHGTPSTPVDSSAHSTPSTPVDSSAHSTPSTPADSSAHSTPSTKL	**extremely high**

Table 3.1-18 Antigen fusions table. On the left column, fusions of the individual antigens, a tetrameric and an hexameric fusions are indicated. In the middle, the corresponding sequences. Metionine codons, histidine-tag and amino acids coded by adapters and intermediate sequences (in black and underlined). Antigen sequences that form the fusions are depicted in different colours.

3.2 Test of the antigens with patient sera

3.2.1 ELISA

A large number of sera had been tested in line blots during this work to characterise the quality of the recombinant antigens. However, line blot is not a very practical method for the routine in a diagnostic laboratory. In most laboratories, ELISA is the method of choice due to its capacity to be automated, and the possibility to test large amounts of samples. Furthermore, the results are more objective, since they can be exactly quantified by photometry.

At the beginning, alkaline phosphatase-conjugated second antibodies were used for the immunostaining. Alkaline phosphatase assays are highly sensitive and the conjugates have the advantage to be very stable during storage. However, the tests can be too sensitive and sometimes false positive values were obtained, most probably due to accidental contaminations. Phosphatases are ubiquitous and very resistant enzymes which are also contained in body fluids such as sweat, saliva etc. They can be easily spread by handling reaction tubes and microtiterplates even wearing gloves. For this reason, the ELISA tests were adapted later to be used with horse radish peroxidase conjugated second antibodies, and the corresponding colour assays. Peroxidase assays are widely used and have the same sensitivity as alkaline phosphatase assays. They have the disadvantage that the enzymatic activity is not as stable as the activity of phosphatase, i.e. the conjugates have a limited halftime. However, this is compensated by the lower risk of contamination, since peroxidases occur less frequent in nature than phosphatases.

To obtain more exact values in the ELISA tests, double determinations were done for each sample, and then the average value was calculated. By this way test-to-test variations were reduced. To discriminate positive from negative results, usually a cut off value is established by using the following formula:

Cut off = average of negative controls + 2-5 x standard deviation (SD) of negative controls.

To determine how many SD had to be used in the formula, a test was performed with a large number of *T. cruzi* positive sera and negative sera from healthy persons calculating the cut off value differently with 2, 3, 4, or 5 x SD. Taking 2 x SD, several false positives were obtained and taking 5 x SD, several false negative were observed. In contrast, neither false positive nor false negative results were obtained using 3 and 4 x SD. For this reason, it was decided to multiply factor 4, increasing thereby slightly the stringency of the results. Using negative patient sera from Bolivia as negative controls, a slightly increase in the cut off value was observed, as compared with the value obtained with German negative sera.

When the ELISA tests were carried out with 1:10.000 diluted TcBCDE as antigen, a slight background was obtained with visceral leishmaniasis sera from the Republic of Yemen Therefore, the concentration of the TcBCDE was reduced to 1:100.000 corresponding to one nanogram of antigen per well in the microtiter plates. Under these conditions, clear negative results with these sera, whereas chagasic sera reacted still to strongly positive.

3.2.3 Comparison of TcBCDE ELISA with commercial kits. Cross-reactions

The ELISA assay developed so far was compared with to types of commercial diagnostic kits for Chagas disease. The one kit (BiosChileIII, Ingeniería Genética S.A., Chile) uses *T. cruzi* crude extracts as antigen, and the other kit (bioelisa CHAGAS, Biokit S.A., Spain) uses a mixture of several *T. cruzi* recombinant proteins (B13 (PEP-2), TcD, TcE and TcLo1.2 (trans-sialidase protein) in a different form as the antigens developed during this work. For the test, approximately 400 sera provided from a laboratory in Cochabamba, Bolivia (SEDES, Servicio Departamental de Salud) were used. These sera had been tested in Bolivia before with the BiosChileIII diagnostic kit containing whole extracts of *Trypanosoma cruzi* of the two strains Tulahuen and Mn as antigen. Approximately 45% of the sera were positive, another 45% were negative, and 10% were determined as "inconclusive" due to ambiguous results with this diagnostic test kit. The ambiguous samples had been tested with an agglutination immunoassay and many of them were positive in this second test. However, since the agglutination assay was also based on crude extract antigens from *T. cruzi*, similar results were to be expected.

65 positive and 65 negative Bolivian sera, tested previously with the BiosChileIII commercial kit, were analysed by the TcBCDE ELISA with 5 µl of serum. The cut off of TcBCDE assay was calculated as explained above, and the cut off of the BiosChileIII kit was determined according to the instruction of the supplier, which is:

(mean absorbance reading of positive controls + mean absorbance reading of negative controls) x 0.35

None of the samples defined as ambiguous in the previous analysis in Bolivia were used in this experiment, however, weakly positive and borderline negative samples were included. The ambiguous samples were analysed later in another set of tests (see below). The same positive and negative sera were also tested Bioelisa CHAGAS kit from Biokit S.A., Spain, which uses recombinant proteins as antigens. The results of all these comparative ELISAs are shown in Figure 3.2-1.

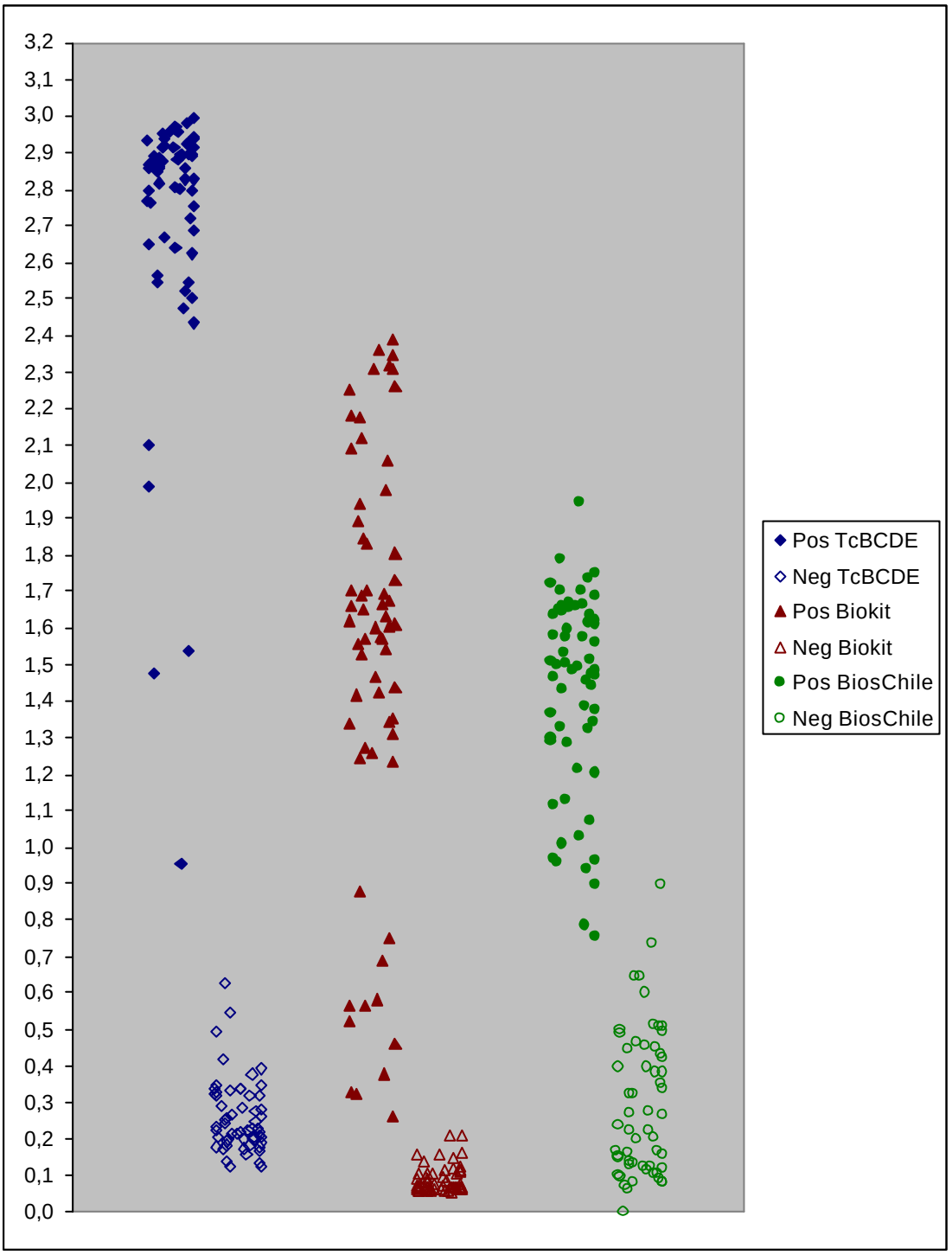

Figure 3.2-1 Comparison of TcBCDE ELISA with commercial kits. On the left (blue) results obtained with TcBCDE ELISA. Observe the high values of most positive samples. Only 5 samples showed weaker values, but still clearly positive. The group of negative samples show clear negative results, but with higher values than the negative group of bioelisa Chagas Biokit (commercial kit using recombinant antigens). Therefore, the cut off value is higher too. In the middle, Biokit results (red) with the positive samples group, that resulted in lower values. However, most values are clearly positive, and only a small group show weaker values. Finally, on the right side, results of BiosChileIII performed in Bolivia (green), have lower values than the previous assays, and the discrimination between positive and negative is not well defined. Observe, in the group of negative serum samples, several values similar to the positives, which could be diagnosed as false positives. The possible explanations will be further discussed.

The results obtained with the two ELISAs using recombinant antigens appeared to be very similar, even though the OD values obtained with TcBCDE ELISA were clearly higher than the ones obtained with Biokit Chagas ELISA. In contrast, with the BiosChile crude extract ELISA several samples led to ambiguous results. In this test, there is also a more or less continuous transition from negative to positive results and there is no clear decision feasible in many cases.

It is generally accepted that diagnostic methods using recombinant antigens show a higher specificity than tests using crude extracts of the parasite. This has been corroborated by the results obtained in this work. In the graphic, a clear separation between OD values corresponding positive and negative samples obtained with the TcBCDE ELISA contrasts to the results of BiosChileIII ELISA. This lack of specificity with crude extract immunoassays represents a problem for the definitive diagnostic of a patient. Therefore, and according to WHO recommendations, at least two tests must be performed to conclude whether the patient is positive for *T. cruzi* or not. In the laboratory in Cochabamba an indirect hemagglutination (HAI) assay is used as a confirmatory technique. However, also this test is based on crude extract antigens and can lead to uncertain results. Some of the samples test negative by hemagglutination and positive by BiosChileIII ELISA, being then urged to be analysed by a third technique.

Some of the samples clearly negative with both ELISAs using recombinant antigens were positive with the BiosChile crude extract ELISA. A possible explanation for the high OD values observed by the latter immunoassay could theoretically be a technical error, or mistaken serum number labels. However, the more probable explanation is a cross-reaction with visceral leishmaniasis. This is easily possible due to the fact that the region where the Bolivian laboratory is located (Cochabamba) is endemic for leishmaniasis too. In the same laboratory, cutaneous leishmaniasis is diagnosed by optical observation (typical skin ulcer). However, no tests for visceral leishmaniasis are available, being most probably undiagnosed therefore. Due to the well known cross-reactivity of antisera between both organisms, it is possible that the patients were wrongly diagnosed as Chagas disease patients instead of leishmaniasis patients. Since cutaneous leishmaniasis is self-healing after few months, but the specific antibodies may persist for many years, cross-reactions with both, cutaneous as well as visceral leishmaniasis may occur. Furthermore, there is some anecdotic evidence that *T. cruzi* antigens may cross-react with sera from syphilis patients.

Therefore, 30 serum samples classified as "inconclusive" by previous assays were analysed in another approach with four different diagnostic tests: the TcBCDE ELISA, the BiosChile ELISA, a self-made ELISA with crude extract from *Leishmania major* (kindly provided

by Michael Heimann) and a line blot assay with recombinant antigens from *Treponema pallidum* (E. Beck, unpublished).

Whereas none of the sera led to a positive result in the *Treponema pallidum*-specific line blot (data not shown), the majority of samples ambiguous or even positive in the BiosChile ELISA, but negative in the TcBCDE ELISA, were clearly positive with the *Leishmania*-specific crude extract ELISA as shown in Figure 3.2-2.

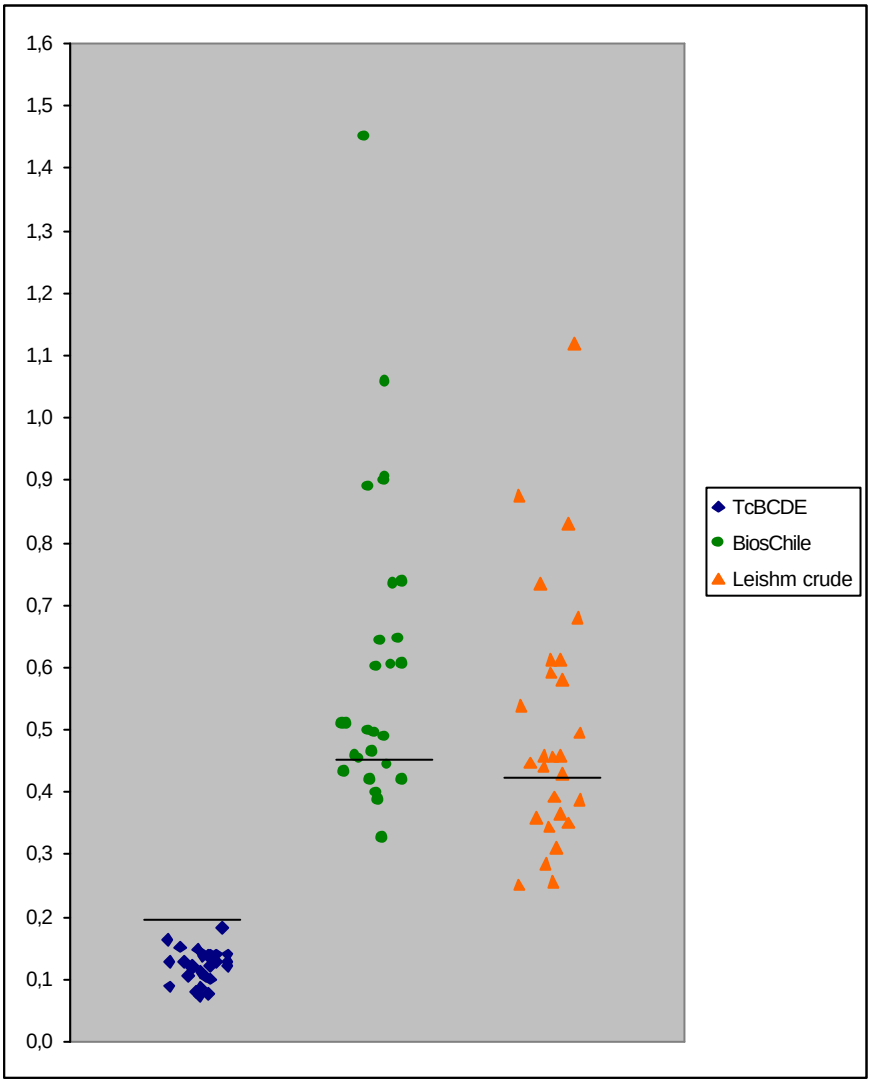

Fig 3.2-2 TcBCDE ELISA assay compared with commercial *T. cruzi* crude extracts and self-made *Leishmania* crude extracts test. On the left (blue), TcBCDE ELISA results, which are all under the calculated cut off. On the middle (green), results obtained with the commercial BiosChile which uses *T. cruzi* crude extracts. Observe the high values obtained with some samples. On the right (orange) the same samples were tested with a self-made *Leishmania* crude extracts test. Note the similar distribution of both *T. cruzi* and *Leishmania* crude extracts tests, in contrast to the clearly negative results by TcBCDE recombinant antigens. The corresponding cut off values are shown as a lane by each test.

Thus, it can be concluded that the reason why some samples clearly negative in immunoassays with recombinant antigens test positive in assays using crude extracts, is the cross-reaction between *Trypanosoma cruzi* and different *Leishmania* species. In the laboratory in Cochabamba, several people are diagnosed regularly for cutaneous leishmaniasis, thus at least the cutaneous form of the disease exists in the area. Once infected, the *Leishmania* antibodies may persist for long time, even if the disease appears to be clinically cured. It is these antibodies which show a high reactivity against the antigens from *T. cruzi* crude extracts. For this reason, not only samples from people suffering from undetected visceral leishmaniasis are prone to show cross-reactivity, but also samples from people who suffered from cutaneous leishmaniasis in the past.

	TcBCDE ELISA (our test)			**BiosChileIII ELISA (crude extract)**			**Chagas Bioelisa Biokit (recombinant antigens)**		
Sample set	**positive**	**negative**	**unclear**	**positive**	**negative**	**unclear**	**positive**	**negative**	**unclear**
Chagasic patients (n=65)	65	0	0	58	0	7	65	0	0
Leishmaniasis patients (n=30)	0	30	0	----	----	----	----	----	----
Syphilis patients (n=40)	0	40	0	----	----	----	----	----	----
Healthy individuals (n=65)	0	65	0	0	2	63	0	65	0

Table 3.2-3 Summary of the results with three different *T. cruzi*-specific ELISA tests. This panel of results shows the clear positive results obtained by recombinant antigens test (TcBCDE and Biokit) in front of the some unclear values obtained with the crude extract antigens (BiosChile). Tests with leishmaniasis patients were only performed with TcBCDE ELISA, which lead to clearly negative results, indicating no cross-reactions. Additional syphilis samples were tested with the same TcBCDE assay, again with negative reactivity. Finally, the negative samples group clearly negative values were obtained with recombinant antigens assays, contrasting with the large amount of unclear results by using crude extracts.

In summary it can be stated that the TcBCDE ELISA has an excellent performance. It reveals a high specificity and has probably a much higher sensitivity than other immunoassays available at present. However, the test has to be validated with larger amounts of samples (in order of thousands) under local conditions. This was not feasible in the frame of this work, since the access to such an amount of sera is not (yet) possible in Europe. However, this will be done in the next future during a long stay in Bolivia, a country most heavily affected by Chagas disease.

3.3 Molecular methods: PCR

PCR has been shown to be useful in different areas of Chagas disease control. In this work, the focus is on the diagnosis of *T. cruzi* in blood of patients, but this assay has also been used to detect *the parasite* in Triatomine bug feces, being more sensitive than traditional microscopy (Pizarro et al., 2007). Congenital *T. cruzi* infection is also diagnosed by means of this technique (Virreira et al., 2003), because it represents an advantage as compared with serologic tests, which have problems to distinguish antibodies from the mother and from the child. PCR represents an important contribution also for paleobiology. As previously mentioned, the detection of *T. cruzi* DNA in tissues from 9000 years old Chilean mummies has been reported (Aufderheide et al., 2004).

In cases of low parasitemia such as in the chronic form of the disease, a highly sensitive technique is needed. Common used methods in this phase include xenodiagnosis, blood culture and serology. The two first methods show a high specificity but a low sensitivity, and in serological tests cross-reactions are commonly observed with samples of patients infected with other trypanosomatides. PCR has a higher sensitivity and specificity, and therefore it is a good alternative to these techniques (Salomone et al., 2003).

Monitoring of the success of treatment with drugs has been a problem since long time and still represents a challenge due to the lack of appropriate methods (de Castro, 1993). Tools commonly used for the assessment of the therapy are mainly serological, following the patient until the negative “seroconversion”. This can take years, since the antibodies against *T. cruzi* persist in blood for long time. The PCR technique has the advantage that it detects the DNA of the parasite in the patient sample which directly demonstrates the presence of the agent, not indirectly as specific antibodies. Thus, it is able to distinguish an “active” infection from a “past” infection, allowing discriminating if a person is already cured or not (Galvão et al., 2003). Therefore, PCR diagnosis complements the immunodiagnostic tools developed during this work and covers the gap of serologic methods.

3.3.1 18S ribosomal RNA-specific nested PCR

To increase the sensitivity of the PCR it is recommended to use sequences occuring several times in the genome of a pathogenic agents as target. For this reason, the amplification of the 18S ribosomal RNA sequence was thought to be a good option, since it is a repeatedly existent fragment. The genome of *T. cruzi* contains an estimated number of 218

genes for ribosomal RNA (El-Sayed et al, 2005). The 18S rRNA sequence of *T. cruzi* differs significantly from the corresponding genes in humans or other organisms. However, there is a high sequence homology within the Trypanosomatide family, and different pathogenic and apathogenic forms of this family co-exist in Latin America (*T. cruzi*, *T. rangeli* and several *Leishmania* species) making the design of specific primers demanding. Comparison of the 18S rRNA sequences of these forms reveals that several parts in the central part of the genes differ sufficiently to derive species-specific primers. These sequences are shown for several representative Trypanosomatides in the following Figure 3.3-1.

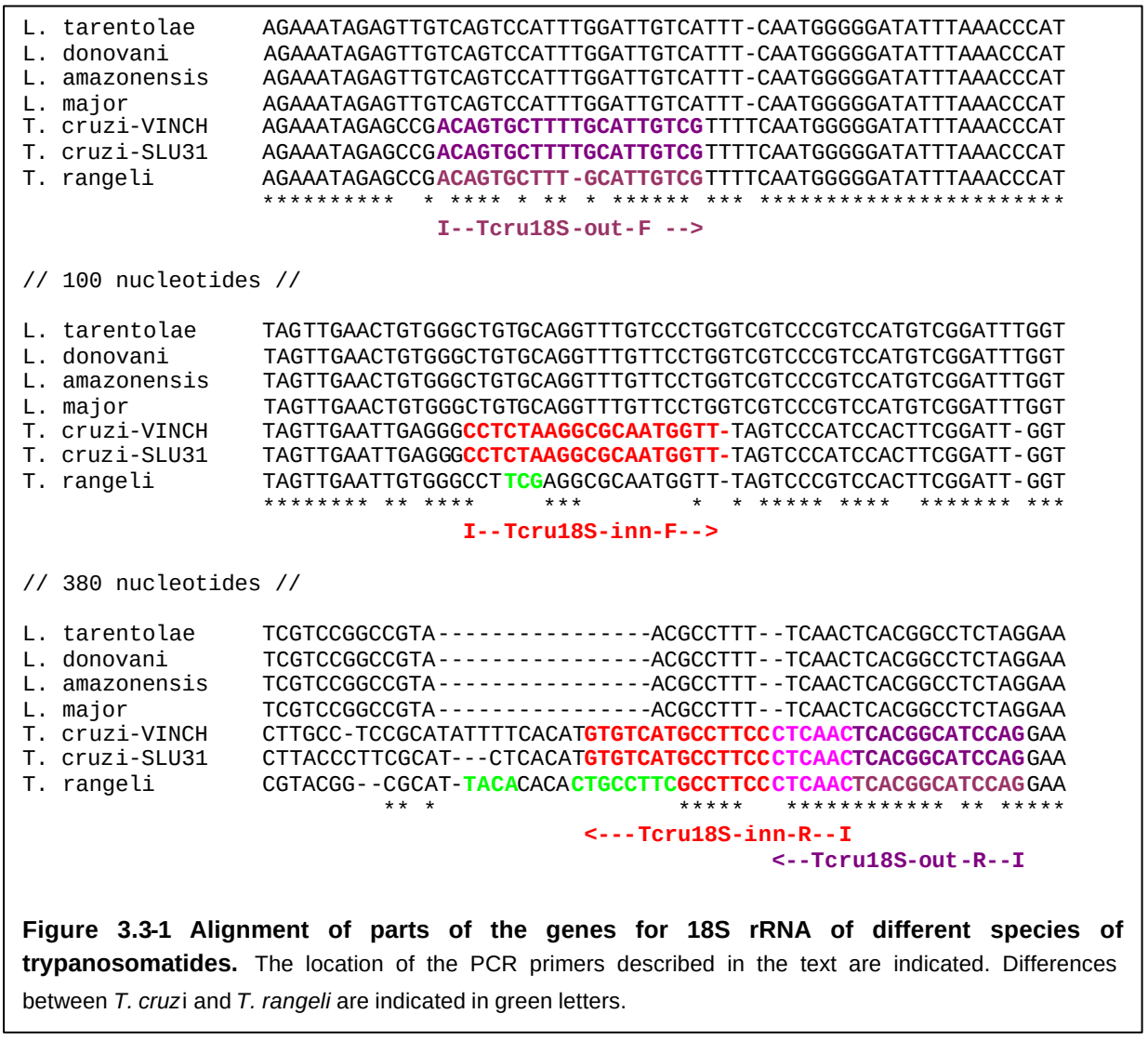

Figure 3.3-1 Alignment of parts of the genes for 18S rRNA of different species of trypanosomatides. The location of the PCR primers described in the text are indicated. Differences between *T. cruzi* and *T. rangeli* are indicated in green letters.

PCR primers sequences were selected from regions differing most between the Trypanosomatides. Since it was not possible to select completely different sequences for the oligonucleotides, the 3' ends of the oligonucleotides were chosen in a way that no hybridisation

to the DNA of the other species was possible. By this way the oligonucleotides could not be extended by the Taq DNA polymerase, even if the would bind to the template. Such a selection was feasible easily for one set of primers (Tcru18S-inn-F and Tcru18S-inn-R). In this case, Primer3 software could not be used to select optimal sequences as in case of cDNA synthesis described above, but the program was used to predict the physical properties of the chosen oligonucleotides such as annealing temperature and self-complementarity.

In order to increase sensitivity of the PCR assay, a set of outer primers was selected additionally. No highly specific sequences were found for these outer primers (Tcru18S-out-F and Tcru18S-out-R), so that PCR with these primers would not lead with certainty to a specific discrimination of the species. However, the function of the outer primers was solely to amplify the target region in a first step, and the specificity would then be provided by a nested PCR with the inner primers.

Primers for *T. cruzi*-specific 18S rRNA PCR		
Name	**Sequence**	**Fragment length**
Tcru18S-out-F	ACAGTGCTTTTGCATTGTCG	642 bp
Tcru18S-out-R	CTGGATGCCGTGAGTTGAG	
Tcru18S-inn-F	CCTCTAAGGCGCAATGGTT	467 bp
Tcru18S-inn-R	GTTGAGGGAAGGCATGACAC	

The specificity of the primers was shown in an experiment including purified DNAs from *Trypanosoma cruzi*, *Leishmania major* and *Leishmania infantum*.

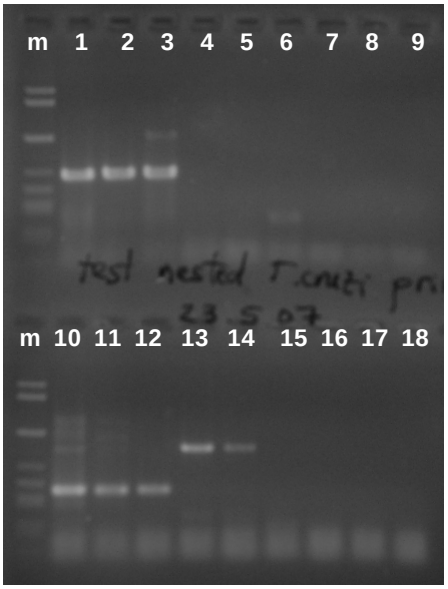

Figure 3.3-2 Test of Tcru18S outer PCR primers (upper part) and inner primers (lower part) with DNA from *T. cruzi*, *L. infantum* and *L. major* with different concentrations of DNA at 60°C annealing temperature. Lane 1 to 3 and 10 to 12 *T. cruzi* DNA; lanes 4 and 5 *L. infantum* DNA; lanes 6 to 8 and 15-17 *L. major* DNA; lanes 1,6, 10 and 15 10 ng DNA each, lanes 2, 4, 7, 11, 13, and 16 1 ng each; lanes 3, 5, 8, 12, 14, and 17 0.1 ng DNA each; lanes 9 and 18 no DNA.

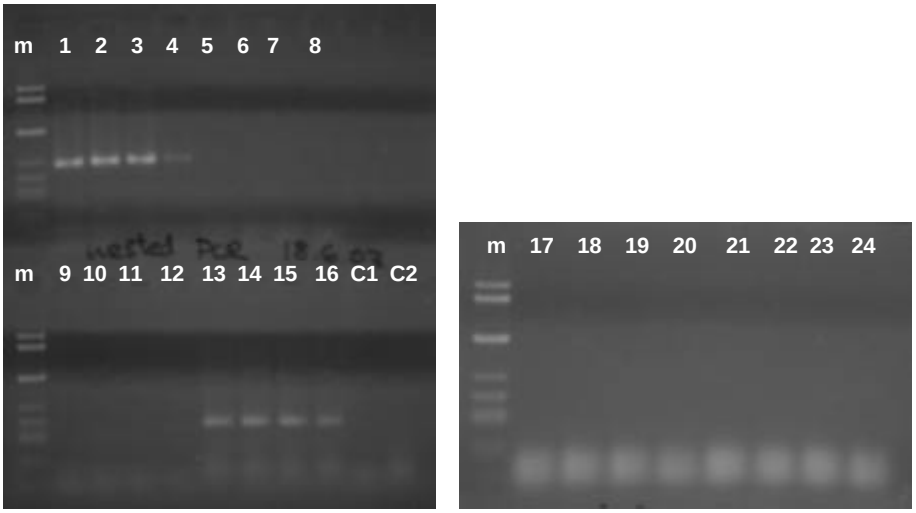

Figure 3.3-3 Specificity of 18S rRNA-specific nested PCR at higher temperatures. To determine the optimal hybridisation conditions, PCRs were performed with different annealing temperatures for 18S rRNA outer and inner primers using 0.1 ng of the individual parasitic DNAs per assay. Four annealing temperatures were selected: 64°C (lanes 1, 5, 9, 13, 19 and 23); 66°C (lanes 2, 6, 10, 14, 20, 24); 68°C (lanes 3, 7, 11, 15, 21, 25); 70°C (lanes 4, 8, 12, 16, 22, 26); *T. cruzi* DNA: lanes 1 to 4 and 13 to 16; *L. infantum* DNA lanes 5 to 8 and 19 to 22; *L. major* DNA lanes 9 to 12 and 23 to 26; lanes 17 and 18: no DNA.

The PCR with the outer primers performed at 60°C annealing temperature was specific for *T. cruzi* leading to a single 640 bp band, but the inner primers led in addition to the specific band with 470 base pairs to an artificial product of approximately 900 bp with the DNA of *L. infantum*. Therefore, the stringency of the reaction conditions was increased and the PCR was repeated at different higher annealing temperatures between 64°C and 70°C.

The result shows that at an elevated annealing temperature of 68°C, the PCR assay is equally sensitive as before, but there are no more cross-reactions with *L. infantum*.

The efficiency of this 18S rRNA-specific PCR was tested with 20 blood samples of chagasic patients. After isolation, the blood of these patients had been mixed with one volume of 5 M guanidinium hydrochloride and was stored for several weeks at 4°C. DNA was extracted from 400 µl of these samples with the guanidinium-silica method and dissolved in 20 µl each. Two µl of these DNA samples were used in each PCR assay

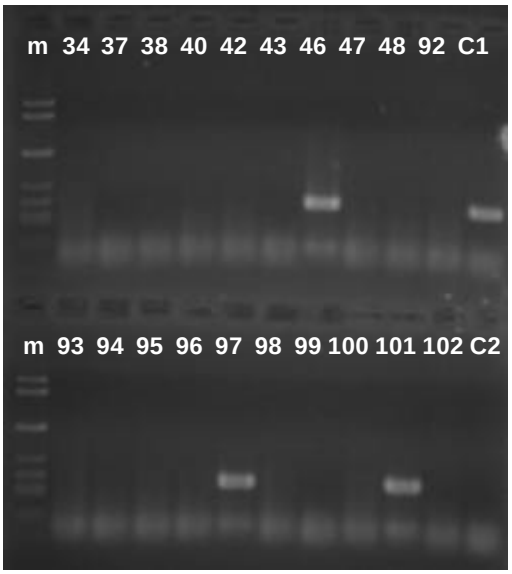

Figure 3.3-4 Tcru-18S rRNA-specific nested PCR with blood samples. PCR was performed with DNA extracted from 20 blood samples of chagasic patients at 68°C annealing temperature in both, primary and secondary cycles. The numbers refer to patient samples, C1 is a positive control with purified *T. cruzi* DNA, and C2 is a negative control with water.

Only some of the patient samples led to a positive result in this assay. This may have been due to the fact that the samples had been stored before DNA extraction for several months in the refrigerator. It is well known that DNA is degraded in guanidinium-blood upon extended storage. As described in the Materials section, an alternative method of DNA extraction starting from buffy coat was developed. In future experiments, this new procedure will be used. During work on this thesis, no alternative blood samples were available.

However, the same blood samples tested positive several months before with another PCR assay in a laboratory in Barcelona. Therefore, it was not to be excluded that the sensitivity of the 18S rRNA-specific PCR was not sufficiently sensitive.

3.3.2 Kinetoplast minicircle DNA-specific PCR

In order to test whether the sensitivity of the molecular biological approach could be increased, another PCR assay was designed based on minicircle DNA of the parasite. Minicircle DNA occurs in more than 10.000 copies in each tryptomastigote and is very heterogeneous in size, but it contains a conserved sequence of approximately 380 base pairs in each circle. Alignment of three representative examples of many different minicircle DNA sequences contained in gene data bases are shown in Figure 3.3-5.

Several primers amplifying different regions of the kinetoplast minicircle DNA have been largely used. First described, the primers 121, 122 (Wincker et al. 1994) amplify a 330 bp fragment of the minicircle kDNA. This original protocol has been improved by using additional primers to create a nested PCR assay, leading to an increase of sensitivity and specificity (Ribeiro-Dos-Santos et al., 1999). Analysis of the primers used in these former assays by Primer3 software revealed that the annealing temperatures differed between forward and reverse primers by more than 20°C. This can negatively interfere with the PCR assay, since the low annealing temperature needed for one primer easily leads to unspecific hybridisation of the other primer with the template which may result in artefact bands. In order to optimise the described nested PCR protocol, additional new primers with similar predicted annealing temperatures were designed by means of Primer3 software. These primers are shorter than the formerly used oligonucleotides and are termed “new” in the following Figure 3.3-6.

Figure 3.3-5 Alignment of representative examples of *T. cruzi* minicircle DNAs

name	sequence	Tm	comments
TcMC-121	AAATAATGTACGGGKGAGATGCATGA	66°C	(old outer-reverse)[1]
TcMC-122	GGTTCGATTGGGGTTGGTGTAATATA	65°C	(old inner–forward)[1]
TcMC-122-short	TCGATTGGGGTTGGTGTAAT	60°C	(new inner-forward)
TcMC-89	GGGAGGTGGGGTTCGATTGGGGTTGG	78°C	(old outer–forward)[2]
TcMC-89-short	GGGAGGTGGGGTTCGATTG	65°C	(new outer-forward)
TcMC-90	GGGAGGTGGGGGTTCGATTGGGGTTGG	89°C	(old outer–forward)[2]
TcMC-90-short	GGGAGGTGGGGGTTCGATTG	66°C	(new outer-forward)
TcMC-91	GSCCCMAAAKTTGAACGCCCCTCCC	72°C	(old inner-reverse)[2]
TcMC-91-short	AAAKTTGAACGCCCCTCCCA	67°C	(new inner reverse)

Figure 3.3-6 Nested PCR primers for *T. cruzi* minicircle DNA
[1]Wincker et al., 1994; [2] Ribeiro-Dos-Santos et al., 1999; **M** = A+C; **K** = G+T

These primers were tested in PCR assays with different annealing temperatures and different concentrations of purified *T. cruzi* DNA. It has to be noted that, while performing PCR amplifying kDNA, the kinetoplast network (present in the cell in highly concatemeric form) has to be cleaved in order to allow the homogeneous distribution of minicircle molecules in blood samples. This can be reached by chemical methods, but also by physical means, as heating, whose results are the same, and are less expensive. This latter method was used in this work, that is, the purified DNA was boiled for 10 minutes.

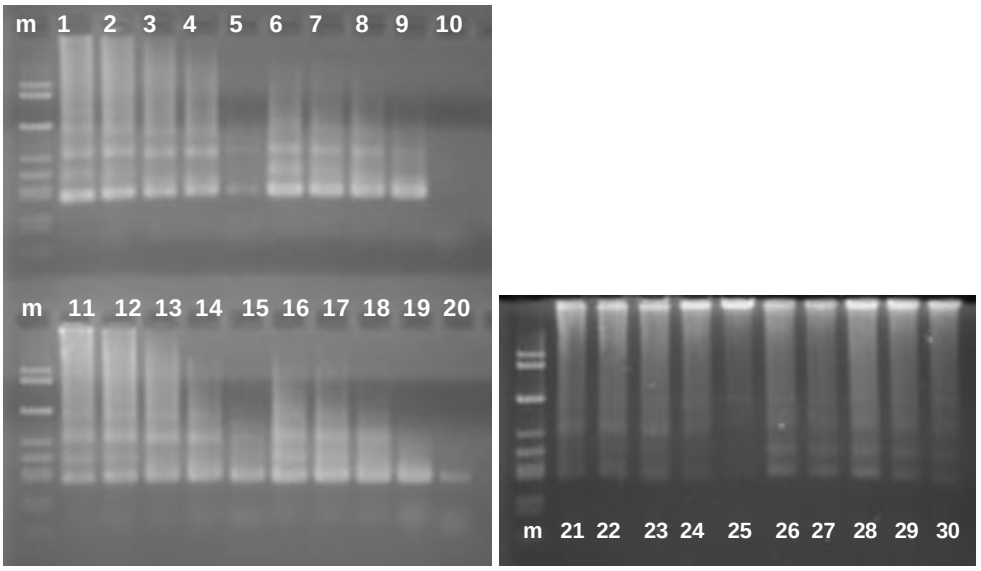

Figure 3.3-7 Annealing conditions for the outer and inner primers for *T. cruzi* minicircle DNA. Four different combinations of forward and reverse primers have been tested under standard PCR conditions except of varying annealing temperatures with *T. cruzi* DNA as template.: Combination I: primers 121 + 122 (lanes 1 to 10); combination II: primers 89/90 + 121 (lanes 11 to 20); combination III: primers 122 short + 121 (lanes 21 to 25); combination IV: primers 89 short/90 short + 122 (lanes 26 to 30). DNA concentration was 1 ng/assay in lanes 1 to 5, 11 to 15 and 21 to 30; and 0.1 ng/assay in lanes 6 to 10 and 16 to 20. Annealing temperatures: 58°C (lanes 1, 6, 11, 16, 21, 26); 60°C (lanes 2, 7, 12, 17, 22, 27); 62°C (lanes 3,8, 13, 18, 23, 28); 64°C (lanes 4, 9, 14, 19, 24, 29); 66°C (lanes 5, 10, 15, 20, 25, 30).

The results from this experiment clearly revealed that the published longer versions of primers for minicircle-specific PCR performed better than the computer-predicted new shorter versions. As only exception, the new primer TcMC-91-short led to equivalent results as the longer (old) version (data not shown). However, in contrast to the published protocol, it is needed to use an annealing temperature of 66°C for both, outer as well as inner primers.

A nested PCR under the determined conditions was performed with 10 DNA samples extracted from the above mentioned chagasic patients guanidinium-blood. For the primary PCR, the outer primers TcMC-89/90 and TcMC 121 were used. In the second PCR, one microlitre of the primary product was amplified with two combinations of inner primers each,

TcMC-122 + TcMC-91 in one set of samples, and TcMC-122 + TcMC-91-short in another set of samples. As can be seen from Figure 3.3-8, only the longer inner primers led to a positive result. In this case, 5 out of 10 blood samples tested clearly positive in the assay. Faint bands are also to be observed in lanes 1 and 2. Thus, the minicircle-based nested PCR appears to be more sensitive as the 18S rRNA-based nested PCR described before.

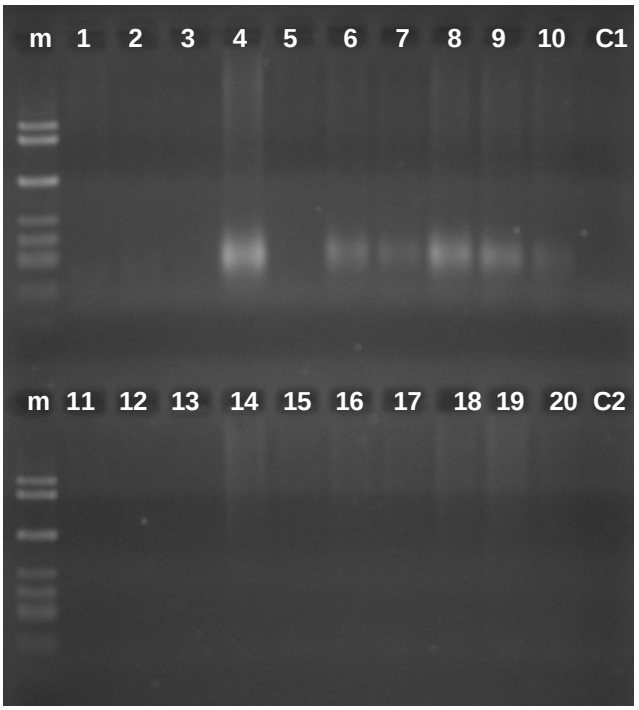

Figure 3.3-8 Minicircle-specific nested PCR with DNA from 10 patient sera. In the primary PCR the primers TcMC-89/90 + TcMC-121 were used. In the secondary PCR, the inner primers TcMC-122 + TcMC-91 (lanes 1 to 10) or TcMC-121 + TcMC-91-short (lanes 11 to 20) were used. C1 and C2 are positive controls with 0.1 ng purified *T. cruzi* DNA.

4. DISCUSSION

Chagas disease is the most important parasitic disease of the Americas accounting for 670,000 disability-adjusted life years per annum. In principle, the disease can be cured efficiently, but most infected people do not know that they have become infected, as the symptoms are usually unspecific or mild in the early stages. Infections remain largely asymptomatic often for years or even decades, until up to 30% of patients develop chronic disease, i.e., cardiac or gastrointestinal complications, which are severely debilitating and in many cases fatal. In contrast to the possibility of successfully treatment early after infection, cure becomes ineffective in the late chronic stage with the drugs available at present. With a timely diagnosis – which has to be affordable for the affected and mostly impoverished population – the magnitude of this disease could be largely reduced. Diagnostic procedures for the diagnosis of Chagas disease are available, however, they either lack specificity and sensitivity, or they are too expensive and not appropriate for laboratories in developing countries, due to the sophisticated equipment they require.

The purpose of this work was to develop new diagnostic procedures which are not only highly sensitive and specific, but could also be produced in an inexpensive and independent way in countries endemic for Chagas disease, so that, in future, patients can be detected in time to start treatment as soon as possible.

The necessary basic bioinformatic tools for developing new diagnostics such as the genomic sequence of the parasite, or computer programs to manipulate these sequences are all freely available in the internet and were used in this project to design recombinant diagnostic antigens. In contrast to diagnostic tests that use crude extracts as antigens, assays using recombinant proteins show high specificity. It turned out that the predominant structures of the parasite that elicit a strong immune response were tandemly repeated amino acid sequences. Therefore such repetitive amino acid sequences were produced during this work as recombinant antigens.

Fusion of the recombinant antigens into a single polypeptide led to an extraordinary and unexpected high reactivity with sera from patients infected with *T. cruzi*. Furthermore, and in contrast to the results obtained with commercial diagnostic kits, it was observed that the specificity was very high, being helpful to discriminate the so called “weak positives”, which are the main problem in the diagnosis of patients with the available methods at present. Application of the assay developed during this work for a large number of patient samples revealed that some of the samples diagnosed as Chagas disease “positive” by other tests, were actually

samples from patients infected with *Leishmania* species, organisms closely related to *Trypanosoma cruzi*, and therefore prone to show cross-reactions.

In conclusion, this work led to three important results. At first it was demonstrated that favourable protein folding can strongly increases the sensitivity of immunoassays. The second main result is the finding that some commercial diagnostic kits lack specificity, being not able to distinguish between *T. cruzi* and *Leishmania* infections. This is especially problematic in areas where leishmaniasis and Chagas disease coexists since wrong diagnosis may have fatal consequences. The third important result is that the new diagnostic assay combining the properties of high sensitivity and high specificity can be produced by simple means and at low costs, which makes it affordable also for the poor population in the affected countries.

4.1 Production of recombinant antigens

Establishing the almost complete DNA sequence of the genome of *Trypanosoma cruzi* (El-Sayed et al., 2005) was crucial for this work. The complete genetic information is online available in the Wellcome Trust Sanger Data Bank. Also all other bioinformatic tools necessary for this work such as the designing of synthetic oligonucleotides, designing of PCR primers, aligning of sequences, or searching for restriction sites are freely accessible in the internet.

Recombinant antigens produced during this work consist almost exclusively on tandemly repeated amino acid sequences. This kind of sequence repetitions occurs also in other protozoa such as *Leishmania* species and *Plasmodium* species, and in all these organisms they represent efficient antigens for inducing specific antibodies. As these parasites replicate inside of cells, the strong humoral immune response may not affect them very much, but rather deviates the immune system from a protective cellular response.

Cloning and expression of the genes for repetitive sequences in *Escherichia coli* presented some difficulties, probably due to the fact that they were not tolerated by the repair system of the cells. First it was tried to clone the antigens in *E. coli* strains defective in homologous recombination (recA$^-$ strains), but without success. The problem could only be solved by the construction of synthetic genes avoiding direct repeats of more than 8 nucleotides. By combining four different tandemly repeated amino acid sequence motives by means of flexible spacer regions, an extremely immunoreactive antigen was obtained. A standard protein preparation containing approximately one milligram protein per millilitre has to be diluted 100.000 fold for use in immunoassays. By this way, there is as good as no contaminating bacterial contamination present and correspondingly no immunological cross-

reaction with *E. coli* antibodies which usually causes background problems in other immunoassays involving recombinant proteins. Production of the polyvalent antigen is easily reproducible. Several batches of the protein have been prepared in the laboratory and all performed exactly equally.

4.2 Specificity of the diagnosis

Cross-reactions between *T. cruzi* antibodies and antigens from other organisms are widely known. Strongest cross-reactivity is observed with related pathogenic and apathogenic members of the Trypanosomatide family. While this can be tolerated in screenings of samples in blood banks since suspicious samples have to be eliminated anyway, wrong diagnosis of patients may lead to fatal consequences. Traditional diagnostic protocols using crude parasite extracts as antigens cross-react especially with sera from persons infected with different species of *Leishmania*, or with the apathogenic parasite *Trypanosoma rangeli* (Saldaña et al., 1996). *T. cruzi* and *T. rangeli* are closely related and occur within the same geographic regions, and both species use the same *Triatomine* bugs as vectors. Humans are infected by *T. rangeli* directly by the bite of the vector, whereas *T. cruzi* occurs in the feces of the insect and can enter the body via mucosa or little wounds only. Co-infections with both species are also possible. However, no homologies of the *T. cruzi*-specific recombinant proteins used in the TcBCDE antigen have been detected so far in *T. rangeli* sequences by BLAST homology searches, thus a reactivity of sera from persons infected with *T. rangeli* with the recombinant antigen appears to be highly improbable. Even though no *T. rangeli* infected samples were available in the laboratory, the antigens synthesised had already been tested against this organism by other groups without finding any cross-reaction (Ferreira et al., 2001). In addition, this view is supported by the fact that commercial diagnostic kits including recombinant antigens of *T. cruzi* related to the amino acid structures of TcBCDE have been shown to be highly specific and did not react with sera from *T. rangeli*-infected persons.

The high specificity for *T. cruzi* does not hold for all produced recombinant antigens. By line blot assays, it turned out that one of the highly reactive recombinant antigens, FRA, cross-reacted with leishmaniasis sera samples. FRA is included in several commercial diagnostic kits, most probably due to its high sensitivity. However, FRA was discarded in this project because of its lack of specificity. The antigen might be included in diagnostic kits in areas free of leishmaniasis but, according to our results, should not be used elsewhere in order to avoid false positive results. Other diagnostic antigens described previously, JL8 and 1F8 were found to cross-react with leishmaniasis sera as well.

Clear discrimination between Chagas disease and leishmaniasis is a major problem in many parts of Latin America. As shown above, immunoassays with crude extracts of *Leishmania major* as antigens led to a strong cross-reaction with sera of *T. cruzi* patients, and it became clear that the difference between patient sera positive with *T. cruzi* crude extract antigen, but negative with recombinant antigens was due to this fact. In order to prove that the TcBCDE antigen does not cross-react with leishmaniasis sera, it was of great importance that the origin of leishmaniasis samples was from the Republic of Yemen which is an area free of *T. cruzi.* On that way, co-infections were excluded, and positive results obtained with the Bolivian sera could be evaluated as cross-reaction.

Using recombinant antigens, clear discrimination between Chagas disease and leishmaniasis is feasible only for *T. cruzi* at present. The clear identification and differentiation of infections with different species of *Leishmania* remains problematic. While the relatively harmless cutaneous form of leishmaniasis can easily be diagnosed by observation of skin ulcers, there is no good diagnosis for the mucocutaneous and visceral forms of the disease. Leishmaniasis is most probably highly underdiagnosed in Latin America, and even co-infections with *T. cruzi* and *Leishmania* species are possible. Chagas disease has an overlapping geographic distribution with leishmaniasis, and mixed infections have been demonstrated (Bastrenta et al., 2003). The visceral form of leishmaniasis represents a major problem, because, as Chagas disease, it does not present clear clinical symptoms. Some of the drugs indicated for leishmaniasis (antimonial derivatives) are strongly disrecommended for patients with severe chagasic cardiopathy, and there are some registered cases of sudden death and electrocardiograph changes derived from this situation (Sampaio et al., 1988; Antezana et al., 1992). With respect to the observed high cross-reactivity of chagasic sera with crude extract antigen from *L. major*, the use of such crude extracts for diagnosis of leishmaniasis is problematic in areas with high prevalence of Chagas disease. Some recombinant antigens for the specific diagnosis of visceral leishmaniasis have been produced and tested. However, they still have limited sensitivity and specificity with respect to the discrimination of the different forms of the disease. Due to these limitations and the high costs of corresponding diagnostic test kits, most cases of visceral leishmaniasis remain undiagnosed in Latin America.

It has been reported that *T. cruzi* antigens may also cross-react with sera from patients with other diseases such as leprosy, malaria and multiple myeloma (Guevara and Ouaissi, http://www.cdfound.to.it/html/trip1.htm#tc15). However, this may hold for crude extract antigens, but not for carefully selected recombinant antigens. To analyse possible cross-reactions with other widespread infectious diseases, the TcBCDE antigen was tested with sera from syphilis patients and brucellosis patients. These sera were from Mongolian patients who cannot be

accidentally infected with *T. cruzi* or any *Leishmania* species. No cross-reactions were observed. Complementary tests, incubating chagasic sera with recombinant antigens from *Treponema pallidum* were also negative (data not shown).

4.3 ELISA

For most infectious diseases, diagnosis is usually performed only when patients suffer from any evident clinical symptoms, because only then patients search for medical care. At present, this holds also for the diagnosis of Chagas disease. However, when clinical symptoms such as cardiac or digestive dysfunctions become apparent, cure with the available drugs is not possible anymore in most cases. At the other hand, cure with these drugs is highly effective, if the infection is recognised early, this means long before the onset of clinical symptoms. Therefore, the only way for timely recognition of the disease would be screening of large populations in endangered regions in regular periods of time. This demands diagnosis in large scale, including millions of persons rather than hundreds or thousands per year. For this reason, diagnostic procedures appropriate for use in large scale have to be selected.

The ELISA technique is one of the most widespread methods for the diagnosis of large amounts of samples, essentially due to its capacity to be automated. In addition, by this technique, results are quantifiable. Thus, the recombinant antigens purified in this work were adapted to be used in this kind of assay. The first synthesised antigens did not bind sufficiently to the plastic surface of the microtiter plates, probably due to their small size. Therefore, it was decided to fuse the single antigens to larger fusion proteins. The longer fusions bound better to the plastic, indeed, but unexpectedly the immune reaction was more increased than the added reaction of the individual antigens. Probably, the antigens were better accessible to the antibodies when presented as a larger protein. This effect was even more pronounced when a fusion of four different diagnostic antigens was constructed. By this way, the strength of the immunoreaction increased by a factor of one hundred, or more.

Fusing different antigens to a single protein compared with the simple mixing of the same antigens has additional advantages. It facilitates the production of the recombinant proteins, because purification of just one polypeptide, instead of several ones, is necessary. By this way, there is less content of bacterial contaminations leading to low background reactions. In addition, large-scale purification is simplified and the production costs are reduced.

The TcBCDE ELISA assay developed during this work was compared with two different commercial diagnostic kits for Chagas disease, one (BiosChileIII, Chile) using a crude extract of

T. cruzi epimastigotes as antigen, and a second one (Bioelisa Chagas from Biokit, Spain) using the recombinant fusion protein TcF (Houghton et al., 1999; Ferreira et al., 2001) which contains in part the same antigens as used in TcBCDE, but in a different structure. The TcF antigen contains two repeats of the antigens B13, TcD, TcE and TcLo1.2 (related, but not identical to SAPA) as direct fusions, i.e. not separated by proline containing linker sequences like in TcBCDE. Using equal amounts of patient sera in the tests, the TcBCDE ELISA led to much higher OD values than the two commercial diagnostic kits, and led to better discrimination between negative and positive sera. From 65 chagasic sera tested with the TcBCDE ELISA, most reacted strongly positive and only very few showed a weak positive reaction (which was still well to be discriminated from negative sera), whereas 20% of the same serum samples led to weak positive signals with the Biokit ELISA. With the BiosChile crude extract ELISA, the same sera led also all to positive results, even though many of them were weakly positive only. The problem with the crude extract ELISA was rather the negative control sera which led in part to results which were in the range of weakly positive sera. This phenomenon is well known and some authors describe the OD range where negative and positive sera result in similar values as "gray zone" (Ferreira et al., 2001). As shown in the Results section, most of the weakly positive signals obtained with the control sera were caused by immune reactions against leishmaniasis infections which are frequent in many areas of Latin America. At present, such ambiguous immunological results have to be tested with another diagnostic technique, which in many cases leads unfortunately to similarly inconclusive results. TcBCDE ELISA is expected to avoid these problems in the future. However, the results have to be validated with larger amounts of sera in endemic areas in Latin America.

4.4 Polymerase Chain Reaction

The polymerase chain reaction is not only the most sensitive and specific procedure for diagnosis of many infectious diseases, it is, in principle, also one of the most inexpensive diagnostic tests. Furthermore, a positive PCR result is a direct indication of the presence of the pathogen, i.e. an active infection, whereas a positive result in an immunoassay does not discriminate between acute and overcome infections. Unfortunately, this technique is not very popular in developing countries, due to the need of a thermocycler, the need of purchasing thermostable DNA polymerases and the risk of false positive results due to smallest contaminations occurring easily under suboptimal conditions in poorly equipped laboratories.

However, PCR is the only objective way to measure the success of treatment of chagasic patients with drugs. Upon elimination of the parasites with drugs, the titre of specific

antibodies drops very slowly during a period of many months or even years and can therefore not be used as a measure to determine when the drug therapy can be stopped. As many patients suffer much from the side effects produced by the only available drugs benznidazole and nifurtimox, treatment should last only as long as absolutely needed. Thus, in addition to a sensitive immunological method for the general detection of *T. cruzi* infections, another sensitive procedure to measure the presence of parasites in the circulation is needed. As the titre of circulating parasites can be very low, depending on the time of infection, microscopy is ineffective in most cases. Xenodiagnosis takes at least a month to obtain results and is therefore also inappropriate. The detection of the DNA of the parasites with PCR remains for these reason as the only suitable alternative diagnostic procedure.

The sensitivity of a PCR assay depends on the number of DNA molecules available at the beginning of the test. Theoretically, a single DNA molecule would be sufficient, however, some hundred molecules as targets for a selected pair of primers are needed under the suboptimal conditions prevailing normally in reality. With titres as low as ten parasites per millilitre of blood, the lower limit for a reliable PCR result is quickly reached. It is therefore reasonable to select amplified target sites in the genome such as the genes for ribosomal RNAs (in *T. cruzi* approximately 220 per haploid genome) or conserved telomeric sequences (approximately 60 per haploid genome) as targets for PCR amplification. The kinetoplast of trypanosomatides contains up to 10.000 copies of the so called minicircle DNA and has therefore been the preferred target for developing specific PCR assays.

In this work, a new PCR assay for the detection of the 18S rRNA genes has been established, and a minicircle-specific test described in the literature (Wincker et al., 1994; Ribeiro-Dos-Santos et al., 1999) has been modified. Respecting the expected low titres of circulating parasites in blood, both assays have been designed as nested PCRs to increase sensitivity. Both assays turned out to function well with the blood samples available for the tests, however they have to be tested more accurately with more samples in the future. During work on this thesis it was not possible to obtain larger amounts of such blood samples.

A major concern in PCR assays is the quality of the DNA used in the tests. At the one hand, the DNA is frequently fragmented in short pieces in clinical samples due to inappropriate isolation and storage. PCR assays comprising long amplification products are more affected than those amplifying short sequences only. DNA fragments shorter than 200 base pairs are difficult to separate clearly by simple electrophoresis on agarose gels, which is the preferred procedure in developing countries. Fragments shorter than 100 base pairs are perfect targets for light cyclers, but this technology is not affordable. At the other hand, contaminations in the DNA can severely interfere with the activity of the Taq DNA polymerase. It is well known that

hemin is a strong inhibitor of the polymerase, and other contaminations such as polysaccharides, glycolipids and other cellular components are inhibitory as well. Therefore, it is essential to use an appropriate procedure to purify DNA carefully for use in PCR assays.

Many kits for the purification of DNA are available from different commercial sources, but the purification costs using these kits are in the range of one to two dollars per sample which is unacceptable for poor countries. Therefore, new and inexpensive procedures for the purification of DNA from blood samples have been developed in this work. They are based on the principle that DNA binds selectively to crystalline silica (SiO_2) under anhydrous conditions. Dehydration of DNA can be reached in the presence of high concentrations of salt, or chaotropic substances such as guanidinium hydrochloride, or in the presence of alcohols such as ethanol or isopropanol. The procedures used in this work include lysis of the tissue in guanidinium hydrochloride, addition of a small amount of silica to bind the solubilised DNA and washing cellular contaminations away with 60% ethanol. At the end, the DNA is dissolved in water and separated from silica by centrifugation. This procedure is simple, fast and inexpensive and yields a highly pure DNA. The only costs are the plastic material used (two reaction tubes and several pipette tips). This method has not only been used in the DNA preparations for the PCR assays, but also for all plasmid constructions described above. The procedure is highly reproducible and robust and therefore perfectly applicable in developing countries.

For simplicity reasons, blood samples for PCR analysis are usually mixed with guanidinium hydrochloride which conserves DNA for some time also at ambient temperature and inactivates potential pathogens at the same time. However, the disadvantage of this method is that the DNA is fragmented to small pieces after some weeks, and the extraction of DNA is messy due to the large amount of protein derived from the lysed erythrocytes. A more comfortable procedure was developed during this work which includes collecting of blood in the presence of citrate or EDTA and separating plasma and blood cells by short centrifugation, or, in absense of a centrifuge, keeping the syringe in vertical position over night. The blood components are separated by this way in three parts, the upper part is plasma and can be used for immunoassays, the lower part is the sedimented red blood cells, and on top of these cells is a yellow layer of leucocytes and platelets, called buffy coat, which can be harvested by a pipette upon removal of the plasma. Even if a fraction of the erythrocytes is co-collected with the buffy coat, DNA can be extracted much easier and cleaner and with better yields than from whole blood. Since during the chronic phase of the disease most parasites are inside of cells, they are present in highly enriched form in the buffy coat. DNA extraction is by far faster and efficient by this way, and the method has to be applied when large numbers of blood samples are to be analysed by PCR.

The DNA sequence of the genes for the 18S ribosomal RNA vary sufficiently from the corresponding sequences in other eukaryotes and bacteria, but are quite conserved between the different members of trypanosomatides. It was not feasible to design sequences for the outer primers for a nested PCR which would warrant absolute specificity for *T. cruzi*. However, the experimental data revealed that the discrimination between *T. cruzi* and *Leishmania* species was satisfactory at higher annealing temperatures. Amplification with the outer primers worked perfectly with 1 nanogram of purified *T. cruzi* DNA, but was borderline with 0.1 nanogram. One nanogram of DNA corresponds to 50 molecules of the haploid genome, or 25 parasites. A detection limit of 10-50 parasites per millilitre of blood is not sufficient since this is the maximal number of parasites circulating during chronic infection. Therefore, PCR with the outer primers alone is not sufficient, but it serves to amplify enough target DNA for the nested PCR with an inner set of primers. The calculation indicates also that use of less than one millilitre of blood would be limiting for the efficiency of the assay. This amount of blood has to be extracted to make sure that enough parasites are contained, even though the DNA is fragmented in chromosomes, and there are more than 200 target sites for the PCR primers in each parasite. As the major content of DNA extracted from blood is derived from leukocytes (several micrograms DNA per millilitre of blood), only part of the purified DNA can be added to single PCR assay anyway (maximal one to two microgram total DNA per assay).

The use of minicircle DNA for PCR can increase the sensitivity of the test because of the high number of target sites for the primers. However, even if there are 10.000 minicircle sequences in a single parasite, the minimal amount of blood to warrant a sufficient number of parasites is subjected to the same limitations as discussed above. Other constraints of the minicircle DNA concern the sequence heterogeneity of the individual DNA sequences found in the cell which lead to a slightly diffuse appearance of the PCR product and the fact that the circles exist as a highly concatenated network which is hardly to be dissolved homogeneously in buffer. The isolated DNA has to be fragmented by 10 minutes of boiling before it is sufficiently accessible for PCR amplification. Because only a short part of the minicircle DNA is conserved, there is limited choice for the selection of primers. Different sequences can be found for the reverse primers, but overlapping sequences have to be used for outer and inner forward primers. Most of the possible primer combinations have been tested by others already (Wincker et al., 1994; Ribeiro-Dos-Santos et al., 1999), however, the calculated annealing temperatures differed as much as 20°C between forward and reverse primers. This may lead to mishybridisations of the primers with the higher temperature resulting in amplification of DNA fragments with heterogeneous size. Therefore it was tried to use shorter primers with more similar annealing temperatures. However, testing of different combinations of forward and reverse primers revealed that except of one of the new short primers, the oligonucleotides

designed before by others were superior to the new primers, even though annealing temperatures markedly higher than described had to be used for optimal results.

Minicircle PCR turned out to be more sensitive than 18S rRNA-specific PCR. However, this may be due rather to the bad quality of the DNA extracted from the blood samples than to the selected target sites. The minicircle PCR yielded fragments of approximately 380 base pairs, whereas the 18S rRNA-specific PCR led to 640 and 460 base pair fragments with outer and inner primers, respectively. The latter procedure led to much clearer results and may reveal 100% sensitivity when used with freshly prepared DNA. The blood samples available for the tests had been stored for almost half a year as a suspension in guanidinium hydrochloride before DNA extraction.

The inner primers of the nested 18S rRNA PCR were selected in a way that no cross-reaction with *T. rangeli* should occur. This problem had not been solved by earlier approaches using the 18S rRNA target. Instead, another group has developed a PCR specific for the telomeric region of the chromosomes (Chiurillo et al., 2003) which differs between these species.

4.5 Conclusion

The present work is an example how available knowledge of informatic tools and molecular biology can be used to adapt medical problems such as the diagnosis of infectious diseases to the real needs of developing countries. The World Health Organisation assured in 1996, that Chagas disease would be eliminated by the year 2010, however, this is by large not going to happen. In contrast, a clear picture of the real extent of this highly neglected disease is just emerging. Strong effort has to be made to restrict the huge magnitude of this disease using the available drugs and diagnostics efficiently. Diagnosis as well as therapy have to be improved in the future. At present, only two drugs for the treatment of Chagas disease are available, and they are effective only in the primary stages. For this reason, early diagnosis of Chagas disease becomes a priority.

Some objectives were reached during this work. First, a highly sensitive and specific diagnostic assay has been developed. And second, this diagnostic tool has been created in a way which is adapted to the limited economic and suboptimal technical conditions that occur unfortunately very often in developing countries. New and inexpensive PCR assays complementary to the immunoassays were also designed during this work. Due to the lack of blood samples of infected patients it was not possible to test and improve them sufficiently. It

has to be kept in mind that PCR is the only technique which allows monitoring the success of the treatment with the available drugs. In the case of Chagas disease, treatment has to be reduced as much as possible in order to avoid severe side effects. Some adjustments will be necessary to adapt the PCR assays to the use under poor laboratory conditions. To avoid contamination, separated areas for DNA extraction and performing the PCR assays have to be established, the laboratory personnel has to be trained to use different pipetters in the different places and, so far affordable filter tips. To reduce further sources of contaminations, all components of the PCR assays except of the DNA sample can be pre-mixed, divided in test tubes and lyophylised. Finally, the Taq DNA polymerase can be self-made with a recombinant clone reducing markedly the final cost of the assays (E. Beck, unpublished).

The necessary diagnostic screening of large populations can only be managed using immunodiagnostic assays since they can be automated. Use of the existing commercial ELISA assays for this purpose is not feasible due to the enormous costs. However, the TcBCDE antigen described in this work can be produced inexpensive and in a very simple way. A single batch of the antigen is sufficient for one million assays, and the efficiency of different batches of the antigen was highly reproducible. This will help to reduce the costs of the diagnosis greatly.

Because most of the population suffering from Chagas disease lives in rural areas, where medical facilities are insufficient, there will be an urgent need of additional diagnostic tools such as rapid lateral flow assays in the future (Villa et al., 2007). These diagnostic assays can be performed without any special equipment. Such a lateral flow test is already on the market (StatPak®), however, this test is not yet specific and sensitive enough and cannot be used in areas where co-infections with *Leishmania* and *T. rangeli* occur. This test stick is based on recombinant antigens used also in other immunoassays. It should not be too difficult to develop such a lateral flow device on the basis of TcBCDE. Efforts will be done in the future in this direction.

As a next future goal, the methods developed during this work have to be validated with larger amounts of patient samples in a country endemic for Chagas disease. This will show whether the developed assays function under real conditions and is planed to be done within the next months in Bolivia. In addition, the technology has to be transferred to the local scientists to enable them to solve the urgent health problems of their population by own means. Finally, it has to be emphasized that the rich countries with their enormous developing capacity should try to contribute more to the real needs of poor countries, and not only to their own ones.

5. LITERATURE

Affranchino JL, Ibañez CF, Luquetti AO, Rassi A, Reyes MB, Macina RA, Aslund L, Pettersson U, Frasch AC. 1989. "Identification of a Trypanosoma cruzi antigen that is shed during the acute phase of Chagas' disease." Mol Biochem Parasitol. 34:221-228.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P. 2004. Molekularbiologie der Zelle. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

Andrade SG, Rassi A, Magalhaes JB, Ferriolli Filho F, Luquetti AO. 1992. "Specific chemotherapy of Chagas disease: a comparison between the response in patients and experimental animals inoculated with the same strains." Trans R Soc Trop Med Hyg. 86:624-626.

Antezana G, Zeballos R, Mendoza C, Lyevre P, Valda L, Cardenas F, Noriega I, Ugarte H, Dedet JP. 1992. "Electrocardiographic alterations during treatment of mucocutaneous leishmaniasis with meglumine antimoniate and allopurinol." Trans R Soc Trop Med Hyg. 86:31-33.

Aufderheide AC, Salo W, Madden M, Streitz J, Buikstra J, Guhl F, Arriaza B, Renier C, Wittmers LE Jr, Fornaciari G, Allison M. 2004. "A 9,000-year record of Chagas' disease." Proc Natl Acad Sci USA (PNAS) 101:2034-2039.

Avila HA, Pereira JB, Thiemann O, De Paiva E, DeGrave W, Morel CM, Simpson L. 1993. "Detection of Trypanosoma cruzi in blood specimens of chronic chagasic patients by polymerase chain reaction amplification of kinetoplast minicircle DNA: comparison with serology and xenodiagnosis." J Clin Microbiol. 31:2421-2426.

Azogue E. 1993. "Women and congenital Chagas' disease in Santa Cruz, Bolivia: epidemiological and sociocultural aspects." Soc Sci Med. 37:503-11.

Basso B, Cervetta L, Moretti E, Carlier Y, Truyens C. 2004. "Acute Trypanosoma cruzi infection: IL-12, IL-18, TNF, sTNFR and NO in T. rangeli-vaccinated mice." Vaccine. 22:1868-1872.

Bastrenta B, Mita N, Buitrago R, Vargas F, Flores M, Machane M, Yacsik N, Torrez M, Le Pont F, Brenière F. 2003. "Human Mixed Infections of Leishmania spp. and Leishmania-Trypanosoma cruzi in a Sub Andean Bolivian Area: Identification by Polymerase Chain Reaction/hybridization and Isoenzyme." Mem Inst Oswaldo Cruz 98:255-264.

Benson G. 1999. "Tandem repeats finder: a program to analyze DNA sequences." Nucleic Acids Research 27:573-580.

Betônico GN, Miranda EO, Silva DA, Houghton R, Reed SG, Campos-Neto A, Mineo JR. 1999. "Evaluation of a synthetic tripeptide as antigen for detection of IgM and IgG antibodies to Trypanosoma cruzi in serum samples from patients with Chagas disease or viral diseases." Trans R Soc Trop Med Hyg. 93:603-606.

Bittencourt AL, Sadigursky M, Da Silva AA, Menezes CA, Marianetti MM, Guerra SC, Sherlock I. 1988. "Evaluation of Chagas' disease transmission through breast-feeding." Mem Inst Oswaldo Cruz. 83:37-39.

Bonekamp F, Andersen HD, Christensen T, Jensen KF. 1985. "Codon-defined ribosomal pausing in Escherichia coli detected by using the pyrE attenuator to probe the coupling between transcription and translation." Nucleic Acids Res. 13:4113-4123.

Bosseno MF, Telleria J, Vargas F, Yaksic N, Noireau F, Morin A, Brenière SF. 1996. "Trypanosoma cruzi: study of the distribution of two widespread clonal genotypes in Bolivian Triatoma infestans vectors shows a high frequency of mixed infections." Exp Parasitol. 83:275-282.

Bradford MM. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." Anal. Biochem. 72:248-254.

Braz LM, Amato Neto V, Carignani FL, De Marchi CR. 2001. "Trypanosoma cruzi parasitemia observed in immunocompromised patients: the importance of the artificial xenodiagnosis." Rev Inst Med Trop Sao Paulo. 43:113-115.

Brener Z. 1973. "Biology of Trypanosoma cruzi." Annu. Rev. Microbiol. 27:347-382.

Brenière SF, Bosseno MF, Telleria J, Bastrenta B, Yacsik N, Noireau F, Alcazar JL, Barnabé C, Wincker P, Tibayrenc M. 1998. "Different behavior of two Trypanosoma cruzi major clones: transmission and circulation in young Bolivian patients." Exp Parasitol. 89:285-295.

Britto C, Cardoso MA, Marques P, Fernandes O, Morel CM. 1999. "Polymerase chain reaction detection: new insights into the diagnosis of chronic Chagas disease." Mem Inst Oswaldo Cruz. 94:305-306.

Buckner FS, Wilson AJ, White TC, Van Voorhis WC. 1998. "Induction of resistance to azole drugs in Trypanosoma cruzi." Antimicrob Agents Chemother. 42:3245-3250.

Burns JM Jr, Shreffler WG, Rosman DE, Sleath PR, March CJ, Reed SG. 1992. "Identification and synthesis of a major conserved antigenic epitope of Trypanosoma cruzi." Proc Natl Acad Sci USA. 89:1239-1243.

Caballero ZC, Sousa OE, Marques WP, Saez-Alquezar A, Umezawa ES. 2007. "Evaluation of Serological Tests To Identify Trypanosoma cruzi Infection in Humans and Determine Cross-

Reactivity with Trypanosoma rangeli and Leishmania spp." Clinical and Vaccine Immunology 14:1045–1049.

Camargo ME. 1988. "American trypanosomiasis (Chagas' disease)." Pp. 744-753 in Laboratory diagnosis of infectious disease-principles and practice., edited by W. J. H. A. Balows, and E. H. Lennette (ed.). New York: Springer-Verlag.

Carlier Y. 2003. "Chagas Disease (American Trypanosomiasis)." eMedicine.com:1-24.

Carrasco R, Miguez H, Camacho C, Echalar L, Revollo S, Ampuero T, Dedet JP. 1990. "Prevalence of Trypanosoma cruzi infection in blood banks of seven departments of Bolivia." Mem Inst Oswaldo Cruz. 85:69-73.

Centers for Disease Control and Prevention (CDC). 2006. "Chagas disease after organ transplantation--Los Angeles, California, 2006." MMWR Morb Mortal Wkly Rep. 55:798-800.

Chagas C. 1909. "Nova espécie mórbida do homem produzida por um trypanozoma. Nota prévia." Brasil Méd 23:161.

Chiaramonte MG, Zwirner NW, Caropresi SL, Taranto NJ, Malchiodi EL. 1996. "Trypanosoma cruzi and Leishmania spp. human mixed infection." Am J Trop Med Hyg. 54:271-273.

Chiurillo MA, Crisante G, Rojas A, Peralta A, Dias M, Guevara P, Añez N, Ramírez JL. 2003. "Detection of Trypanosoma cruzi and Trypanosoma rangeli infection by duplex PCR assay based on telomeric sequences." Clin Diagn Lab Immunol. 10:775-779.

Cimo PL, Luper WE, Scouros MA. 1993. "Transfusion-associated Chagas' disease in Texas: report of a case." Tex Med. 89:48-50.

Coura JR. 2007. "Chagas disease: what is known and what is needed – A background article." Mem Inst Oswaldo Cruz 102:113-122.

Cunha-Neto E, Bilate AM, Hyland KV, Fonseca SG, Kalil J, Engman DM. 2006. "Induction of cardiac autoimmunity in Chagas heart disease: a case for molecular mimicry." Autoimmunity. 39:41-54.

Cunha-Neto E, Coelho V, Guilherme L, Fiorelli A, Stolf N, Kalil J. 1996. "Autoimmunity in Chagas' disease. Identification of cardiac myosin-B13 Trypanosoma cruzi protein crossreactive T cell clones in heart lesions of a chronic Chagas' cardiomyopathy patient." J Clin Invest. 98:1709-1712.

da Silveira JF, Umezawa ES, Luquetti AO. 2001. "Chagas disease: recombinant Trypanosoma cruzi antigens for serological diagnosis." Trends Parasitol. 17:286-291.

de Castro SL. 1993. "The challenge of Chagas' disease chemotherapy: an update of drugs assayed against Trypanosoma cruzi." Acta Trop. 53:83-98.

Dias JCP. 1992. "Chagas' disease and blood transfusion in endemic areas." Pp. 135-142 in Chagas' Disease (American Trypanosomiasis): its Impact on Transfusion and Clinical Medicine, edited by Z. B. S Wendel, ME Camargo & A Rassi (eds). São Paulo: ISBT.

Dias JCP, Silveira AC, Schofield CJ. 2002. "The impact of Chagas disease control in Latin America: a review." Mem Inst Oswaldo Cruz. 97:603-612.

Docampo R. 1990. "Sensitivity of parasites to free radical damage by antiparasitic drugs." Chem Biol Interact. 73:1-27.

Dorn P, Buekens P, Hanford E. 2007. "Whac-a-mole: future trends in Chagas transmission and the importance of a global perspective on disease control." Future Microbiology 2:365–367.

El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, Ghedin E, Worthey EA, Delcher AL, Blandin G, Westenberger SJ, Caler E, Cerqueira GC, Branche C, Haas B, Anupama A, Arner E, Aslund L, Attipoe P, Bontempi E, Bringaud F, Burton P, Cadag E, Campbell DA, Carrington M, Crabtree J, Darban H, da Silveira JF, de Jong P, Edwards K, Englund PT, Fazelina G, Feldblyum T, Ferella M, Frasch AC, Gull K, Horn D, Hou L, Huang Y, Kindlund E, Klingbeil M, Kluge S, Koo H, Lacerda D, Levin MJ, Lorenzi H, Louie T, Machado CR, McCulloch R, McKenna A, Mizuno Y, Mottram JC, Nelson S, Ochaya S, Osoegawa K, Pai G, Parsons M, Pentony M, Pettersson U, Pop M, Ramirez JL, Rinta J, Robertson L, Salzberg SL, Sanchez DO, Seyler A, Sharma R, Shetty J, Simpson AJ, Sisk E, Tammi MT, Tarleton R, Teixeira S, Van Aken S, Vogt C, Ward PN, Wickstead B, Wortman J, White O, Fraser CM, Stuart KD, Andersson B. 2005. "The genome sequence of Trypanosoma cruzi, etiologic agent of Chagas disease." Science 309:409-415.

Fernandes O, Souto RP, Castro JA, Pereira JB, Fernandes NC, Junqueira AC, Naiff RD, Barrett TV, Degrave W, Zingales B, Campbell DA, Coura JR. 1998. "Brazilian isolates of Trypanosoma cruzi from humans and Triatomines classified into two lineages using mini-exon and ribosomal RNA sequences." Am. J. Trop. Med. Hyg. 58:807–811.

Ferreira AW. 1992. "Tests for Chagas disease serodiagnosis: a review." Pp. 179-193 in Chagas disease (American trypanosomiasis): its impact on transfusion and clinical medicine., edited by Z. B. S. Wendel, M. E. Camargo, and A. Rassi e (ed.). São Paulo: International Society of Blood Transfusion.

Ferreira AW, Belem ZR, Lemos EA, Reed SG, Campos-Neto A. 2001. "Enzyme-Linked Immunosorbent Assay for Serological Diagnosis of Chagas' Disease Employing a Trypanosoma cruzi Recombinant Antigen That Consists of Four Different Peptides." Journal of Clinical Microbiology 39:4390-4395.

Flores-Chávez M, Fernández B, Puente S, Torres P, Rodríguez M, Monedero C, Cruz I, Gárate T, Cañavate C. 2008. "Transfusional chagas disease: parasitological and serological monitoring of an infected recipient and blood donor." Clin Infect Dis. 46:e44-47.

Franco-Paredes C, Von A, Hidron A, Rodríguez-Morales AJ, Tellez I, Barragán M, Jones D, Náquira CG, Mendez J. 2007. "Chagas disease: an impediment in achieving the Millennium Development Goals in Latin America." BMC Int Health Hum Rights. 28;7:7 28:7.

Frasch AC, Cazzulo JJ, Aslund L, Pettersson U. 1991. "Comparison of genes encoding Trypanosoma cruzi antigens." Parasitol Today. 7:148-151.

Frey A, Meckelein B, Externest D, Schmidt MA. 2000. "A stable and highly sensitive 3,3',5,5'-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays." J Immunol Methods. 233:47-56.

Galvão LM, Chiari E, Macedo AM, Luquetti AO, Silva SA, Andrade AL. 2003. "PCR Assay for Monitoring Trypanosoma cruzi Parasitemia in Childhood after Specific Chemotherapy." J Clin Microbiol. 41:5066-5070.

Gaunt MW, Yeo M, Frame IA, Stothard JR, Carrasco HJ, Taylor MC, Mena SS, Veazey P, Miles GA, Acosta N, de Arias AR, Miles MA. 2003. "Mechanism of genetic exchange in American trypanosomes." Nature 421:936-939.

Gomes YM, Pereira VR, Nakazawa M, Rosa DS, Barros MD, Ferreira AG, Silva ED, Ogatta SF, Krieger MA, Goldenberg S. 2001. "Serodiagnosis of chronic Chagas infection by using EIE-Recombinant-Chagas-Biomanguinhos kit." Mem Inst Oswaldo Cruz. 96:497-501.

Gonzalez A, Lerner TJ, Huecas M, Sosa-Pineda B, Nogueira N, Lizardi PM. 1985. "Apparent generation of a segmented mRNA from two separate tandem gene families in Trypanosoma cruzi." Nucleic Acids Research 13.

Grantham R, Gautier C, Gouy M, Jacobzone M, Mercier R. 1981. "Codon catalog usage is a genome strategy modulated for gene expressivity." Nucleic Acids Res. 9: r43-74.

Gruber A, Zingales B. 1993. "Trypanosoma cruzi: characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease." Exp Parasitol. 76:1-12.

Guevara AG, Eras JW, Recalde M, Vinueza L, Cooper PJ, Ouaissi A, Guderian RH. 1997. "Severe digestive pathology associated with chronic Chagas' disease in Ecuador: report of two cases." Rev Soc Bras Med Trop. 30:389-392.

Guillen G, Diaz R, Jemio A, Cassab JA, Pinto CT, Schofield CJ. 1997. "Chagas disease vector control in Tupiza, southern Bolivia." Mem Inst Oswaldo Cruz. 92:1-8.

Gürtler RE. 2007. "Combining Residual Insecticide Spraying Campaigns with Targeted Detection and Specific Chemotherapy for Trypanosoma cruzi Infection in Children." PLoS Negl Trop Dis. 1:e168.

Hertz-Fowler C, Peacock CS, Wood V, Aslett M, Kerhornou A, Mooney P, Tivey A, Berriman M, Hall N, Rutherford K, Parkhill J, Ivens AC, Rajandream MA, Barrell B. 2004. "GeneDB: a resource for prokaryotic and eukaryotic organisms." Nucleic Acids Res. 32.

Heussler VT, Küenzi P, Rottenberg S. 2001. "Inhibition of apoptosis by intracellular protozoan parasites." Int J Parasitol. 31:1166-1176.

Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. 1988. "Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent." *Bio/Technology* 6:1321-1325.

Hoft DF, Farrar PL, Kratz-Owens K, Shaffer D. 1996. "Gastric invasion by Trypanosoma cruzi and induction of protective mucosal immune responses." Infect Immun. 64:3800-3810.

Hoft DF, Kim KS, Otsu K, Moser DR, Yost WJ, Blumin JH, Donelson JE, Kirchhoff LV. 1989. "Trypanosoma cruzi expresses diverse repetitive protein antigens." Infect Immun. 57:1959-1967.

Hoover DM, Lubkowski J. 2002. "DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis." Nucleic Acids Res. 30:e43.

Hotez PJ, Molyneux DH, Fenwick A, Ottesen E, Ehrlich Sachs S, Sachs JD. 2006. "Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria." PLoS Med. 3:e102.

Houghton RL, Benson DR, Reynolds LD, McNeill PD, Sleath PR, Lodes MJ, Skeiky YA, Leiby DA, Badaro R, Reed SG. 1999. "A multi-epitope synthetic peptide and recombinant protein for the detection of antibodies to Trypanosoma cruzi in radioimmunoprecipitation-confirmed and consensus-positive sera." J Infect Dis. 179:1226-1234.

Hufnagel L, Brockmann D, Geisel T. 2004. "Forecast and control of epidemics in a globalized world." Proc Natl Acad Sci U S A. 101:15124-15129.

Ibañez CF, Affranchino JL, Macina RA, Reyes MB, Leguizamon S, Camargo ME, Aslund L, Pettersson U, Frasch AC. 1988. "Multiple Trypanosoma cruzi antigens containing tandemly repeated amino acid sequence motifs." Mol Biochem Parasitol. 30:27-33.

Ish-Horowicz D, Burke JF. 1981. "Rapid and efficient cosmid cloning." Nucleic Acids Res. 9:2989-2998.

Kerner N, Liégeard P, Levin MJ, Hontebeyrie-Joskowicz M. 1991. "Trypanosoma cruzi: antibodies to a MAP-like protein in chronic Chagas' disease cross-react with mammalian cytoskeleton." Exp Parasitol. 73:451-459.

Kirchhoff LV. 1990. "Trypanosoma species (American trypanosomiasis, Chagas disease): biology of trypanosomes." Pp. 2077-2084 in Principles and practice of infectious diseases., edited by R. G. D. G. L. Mandell, and J. E. Bennett (ed.). New York: Churchill Livingstone.

Kirchhoff LV, Votava JR, Ochs DE, Moser DR. 1996. "Comparison of PCR and microscopic methods for detecting Trypanosoma cruzi." J Clin Microbiol. 34:1171-1175.

Klinkert MQ, Bommert K, Moser D, Felleisen R, Link G, Doumbo O, Beck E. 1991. "Immunological analysis of cloned Schistosoma mansoni antigens Sm31 and Sm32 with sera of schistosomiasis patients." Trop Med Parasitol. 42:319-324.

Krieger MA, Almeida E, Oelemann W, Lafaille JJ, Pereira JB, Krieger H, Carvalho MR, Goldenberg S. 1992. "Use of recombinant antigens for the accurate immunodiagnosis of Chagas' disease." Am J Trop Med Hyg. 46:427-434.

La Fuente Zeraín CA. 2006. "Inmunología de la infección por T. cruzi y de la enfermedad de Chagas." Enf Emerg 8:17-19.

Laemmli UK. 1970. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature. 227:680-685.

Lafaille JJ, Linss J, Krieger MA, Souto-Padrón T, de Souza W, Goldenberg S. 1989. "Structure and expression of two Trypanosoma cruzi genes encoding antigenic proteins bearing repetitive epitopes." Mol Biochem Parasitol. 35:127-136.

Leary JJ, Brigati DJ, Ward DC. 1983. "Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots." Proc Natl Acad Sci USA. 80:4045-4049.

Levin MJ, Mesri E, Benarous R, Levitus G, Schijman A, Levy-Yeyati P, Chiale PA, Ruiz AM, Kahn A, Rosenbaum MB, et al. 1989. "Identification of major Trypanosoma cruzi antigenic determinants in chronic Chagas' heart disease." Am J Trop Med Hyg. 41:530-538.

Levy MZ, Kawai V, Bowman NM, Waller LA, Cabrera L, Pinedo-Cancino VV, Seitz AE, Steurer FJ, Cornejo Del Carpio JG, Cordova-Benzaquen E, Maguire JH, Gilman RH, Bern C. 2007. "Targeted Screening Strategies to Detect Trypanosoma cruzi Infection in Children." PLoS Negl Trop Dis. 1:e103.

Luhrs KA, Fouts DL, Manning JE. 2003. "Immunization with recombinant paraflagellar rod protein induces protective immunity against Trypanosoma cruzi infection." Vaccine. 21:3058-3069.

Mair G, Shi H, Li H, Djikeng A, Aviles HO, Bishop JR, Falcone FH, Gavrilescu C, Montgomery JL, Santori MI, Stern LS, Wang Z, Ullu E, Tschudi C. 2000. "A new twist in trypanosome RNA metabolism: cis-splicing of pre-mRNA." RNA 6: 163–169.

Médecins Sans Frontièrs (MSF) 2004. "Bolivia: A killer that preys on the poor: Chagas disease." Activity Report 2003/2004 Medicins Sans Frontieres.

Moncayo A, Luquetti AO. 1990. "Multicentre double blind study for evaluation of Trypanosoma cruzi defined antigens as diagnostic reagents." Mem. Inst. Oswaldo Cruz Rio de J. 85:489-495.

Moser DR, Kirchhoff LV, Donelson JE. 1989. "Detection of Trypanosoma cruzi by DNA amplification using the polymerase chain reaction." J Clin Microbiol. 27:1477-1482.

Mundo Sano, Organización Panamericana de la Salud. 2007. La enfermedad de Chagas, a la puerta de los 100 años del conocimiento de una endemia americana ancestral.

Muñoz J, Portús M, Corachan M, Fumadó V, Gascon J. 2007. "Congenital Trypanosoma cruzi infection in a non-endemic area." Trans R Soc Trop Med Hyg. 101:1161-1162.

Nathan-Larrier L. 1921. "La Schizotripaniose americaine, peut elle être transmise par contagiou genitale." Compt. Rend. Soc. Biol. 84:773–775.

PAHO. 2000. "Situation Analysis of Mortality in Bolivia." Epidemiological Bulletin 23.

Paranhos GS, Cotrim PC, Mortara RA, Rassi A, Corral R, Freilij HL, Grinstein S, Wanderley J, Camargo ME, da Silveira JF. 1990. "Trypanosoma cruzi: cloning and expression of an antigen recognized by acute and chronic human chagasic sera." Exp Parasitol. 71:284-293.

Peralta JM, Teixeira MG, Shreffler WG, Pereira JB, Burns JM Jr, Sleath PR, Reed SG. 1994. "Serodiagnosis of Chagas' disease by enzyme-linked immunosorbent assay using two synthetic peptides as antigens." J Clin Microbiol. 32:971-974.

Pereira VR, de Lorena VM, Nakazawa M, da Silva AP, Montarroyos U, Correa-Oliveira R, Gomes Yde M. 2003. "Evaluation of the immune response to CRA and FRA recombinant antigens of Trypanosoma cruzi in C57BL/6 mice." Rev Soc Bras Med Trop. 36:435-440.

Peters W, Pasvol G. 2007. Atlas of Tropical Medicine and Parasitology. Sixth Edition.: Elsevier Mosby.

Picollo MI, Vassena C, Santo Orihuela P, Barrios S, Zaidemberg M, Zerba E. 2005. "High resistance to pyrethroid insecticides associated with ineffective field treatments in Triatoma infestans (Hemiptera: Reduviidae) from Northern Argentina." J Med Entomol. 42:637-642.

Pirard M, Iihoshi N, Boelaert M, Basanta P, López F, Van der Stuyft P. 2005. "The validity of serologic tests for Trypanosoma cruzi and the effectiveness of transfusional screening strategies in a hyperendemic region." Transfusion. 45:554-561.

Piron M, Fisa R, Casamitjana N, López-Chejade P, Puig L, Vergés M, Gascón J, Gómez i Prat J, Portús M, Sauleda S. 2007. "Development of a real-time PCR assay for Trypanosoma cruzi detection in blood samples." Acta Trop. 103:195-200.

Pizarro JC, Lucero DE, Stevens L. 2007. "PCR reveals significantly higher rates of Trypanosoma cruzi infection than microscopy in the Chagas vector, Triatoma infestans: high rates found in Chuquisaca, Bolivia." BMC Infect Dis. 7:66.

Pollevick GD, Sanchez DO, Campetella O, Trombetta S, Sousa M, Henriksson J, Hellman U, Pettersson U, Cazzulo JJ, Frasch AC. 1993. "Members of the SAPA/trans-sialidase protein family have identical N-terminal sequences and a putative signal peptide." Mol Biochem Parasitol. 59:171-174.

Ponce C, Ponce E, Vinelli E, Montoya A, de Aguilar V, Gonzalez A, Zingales B, Rangel-Aldao R, Levin MJ, Esfandiari J, Umezawa ES, Luquetti AO, da Silveira JF. 2005. "Validation of a rapid and reliable test for diagnosis of chagas' disease by detection of Trypanosoma cruzi-specific antibodies in blood of donors and patients in Central America." J Clin Microbiol. 43:5065-5068.

Reyes MB, Lorca M, Muñoz P, Frasch AC. 1990. "Fetal IgG specificities against Trypanosoma cruzi antigens in infected newborns." Proc Natl Acad Sci USA. 87:2846-2850.

Ribeiro-dos-Santos G, Nishiya AS, Sabino EC, Chamone DF, Saez-Alquézar A. 1999. "An improved, PCR-based strategy for the detection of Trypanosoma cruzi in human blood samples." Ann Trop Med Parasitol. 93:689-694.

Riera C, Guarro A, Kassab HE, Jorba JM, Castro M, Angrill R, Gállego M, Fisa R, Martin C, Lobato A, Portús M. 2006. "Congenital transmission of Trypanosoma cruzi in Europe (Spain): a case report." Am. J. Trop. Med. Hyg 75:1078–1081.

Robinson M, Lilley R, Little S, Emtage JS, Yarranton G, Stephens P, Millican A, Eaton M, Humphreys G. 1984. "Codon usage can affect efficiency of translation of genes in Escherichia coli." Nucleic Acids Res. 12:6663-6671.

Romaña C. 1935. "A area de un sintoma inicial de valor para el diagnóstico de forma aguda de la enfermedad de Chagas." MEPRA 22:16-30.

Rozen S, Skaletsky H. 2000. "Primer3 on the WWW for general users and for biologist programmers." Methods Mol Biol. 132:365-386.

Saldaña A, Sousa OE. 1996. "Trypanosoma rangeli: epimastigote immunogenicity and cross-reaction with Trypanosoma cruzi." J Parasitol. 82:363-366.

Salomone OA, Basquiera AL, Sembaj A, Aguerri AM, Reyes ME, Omelianuk M, Fernández RA, Enders J, Palma A, Barral JM, Madoery RJ. 2003. "Trypanosoma cruzi in persons without serologic evidence of disease, Argentina." Emerg Infect Dis. 9:1558-1562.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning a laboratory manual., vol. 1, Edited by C. S. Harbor. NY: Cold Spring Harbor Laboratory Press.

Sampaio RNR, Netto EM, Faria EA, Sampaio JHD, Freitas LCF, Marsden PD. 1988. "Morte súbita causada por glucantime." An Bras Derm 63:35-37.

Schnapp AR, Eickhoff CS, Sizemore D, Curtiss R 3rd, Hoft DF. 2002. "Cruzipain induces both mucosal and systemic protection against Trypanosoma cruzi in mice." Infect Immun. 70:5065-5074.

Schofield CJ, Dias JC. 1999. "The Southern Cone Initiative against Chagas disease." Adv Parasitol. 42:1-27.

Steindel M, Kramer Pacheco L, Scholl D, Soares M, de Moraes MH, Eger I, Kosmann C, Sincero TC, Stoco PH, Murta SM, de Carvalho-Pinto CJ, Grisard EC. 2008. "Characterization of Trypanosoma cruzi isolated from humans, vectors, and animal reservoirs following an outbreak of acute human Chagas disease in Santa Catarina State, Brazil." Diagn Microbiol Infect Dis. 60:25-32.

Stemmer WP, Crameri A, Ha KD, Brennan TM, Heyneker HL. 1995. "Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides." Gene. 164:49-53.

Stothard P. 2000. "The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences." Biotechniques. 28:1102-1104.

Stramer S, Dodd RY, Leiby DA et al. 2007. "Blood Donor Screening for Chagas Disease --- United States, 2006--2007." MMWR Morb. Mortal. Wkly Rep., Centers for Disease Control and Prevention 56:141–143.

Strosberg AM, Barrio K, Stinger VH, Tashker J, Wilbur JC, Wilson L, Woo K. 2007. "CHAGAS DISEASE: A Latin American Nemesis." Institute for OneWorld Health.

Tan H, Andrews NW. 2002. "Cell entry of T. cruzi: Don't bother to knock - the cell invasion strategy of Trypanosoma cruzi." Trends in Parasitology 18:427-428.

Tanowitz HB, Kirchhoff LV, Simon D, Morris SA, Weiss LM, Wittner M. 1992. "Chagas' disease." Clin Microbiol Rev. 5:400-419.

Tarleton RL, Reithinger R, Urbina JA, Kitron U, Gürtler RE. 2007. "The Challenges Of Chagas Disease - Grim Outlook Or Glimmer Of Hope?" PLoS Medicine 4:e332.

Tibayrenc M. 2003. "Genetic subdivisions within Trypanosoma cruzi (Discrete Typing Units) and their relevance for molecular epidemiology and experimental evolution." Kinetoplastid Biol Dis. 2:12.

Tibbetts RS, McCormick TS, Rowland EC, Miller SD, Engman DM. 1994. "Cardiac antigen-specific autoantibody production is associated with cardiomyopathy in Trypanosoma cruzi-infected mice." J Immunol. 152:1493-1499.

Tonn RJ. 1988. "Review of recent publications on the ecology, biology and control of vectors of Chagas' disease." Rev Argent Microbiol. 20:4-24.

Troester H, Bub S, Hunziker A, Trendelenburg MF. 2000. "Stability of DNA repeats in Escherichia coli dam mutant strains indicates a Dam methylation-dependent DNA deletion process." Gene 258:95-108.

Umezawa ES, Bastos SF, Camargo ME, Yamauchi LM, Santos MR, Gonzalez A, Zingales B, Levin MJ, Sousa O, Rangel-Aldao R, da Silveira JF. 1999. "Evaluation of Recombinant Antigens for Serodiagnosis of Chagas' Disease in South and Central America." J Clin Microbiol 37:1554–1560.

Umezawa ES, Bastos SF, Coura JR, Levin MJ, Gonzalez A, Rangel-Aldao R, Zingales B, Luquetti AO, da Silveira JF. 2003. "An improved serodiagnostic test for Chagas' disease employing a mixture of Trypanosoma cruzi recombinant antigens." Transfusion. 43:91-97.

Umezawa ES, Luquetti AO, Levitus G, Ponce C, Ponce E, Henriquez D, Revollo S, Espinoza B, Sousa O, Khan B, da Silveira JF. 2004. "Serodiagnosis of Chronic and Acute Chagas' Disease with Trypanosoma cruzi Recombinant Proteins: Results of a Collaborative Study in Six Latin American Countries." J Clin Microbiol. 42:449-452.

Umezawa ES, Silveira JF. 1999. "Serological Diagnosis of Chagas Disease with Purified and Defined Trypanosoma cruzi Antigens." Mem Inst Oswaldo Cruz 94:285-288.

Urbina JA, Docampo R. 2003. "Specific chemotherapy of Chagas disease: controversies and advances." Trends Parasitol. 19:495-501.

Vaidian AK, Weiss LM, Tanowitz HB. 2004. "Chagas' disease and AIDS." Kinetoplastid Biol Dis. 3:2.

Vergara U, Veloso C, Gonzalez A, Lorca M. 1992. "Evaluation of an enzyme-linked immunosorbent assay for the diagnosis of Chagas' disease using synthetic peptides." Am J Trop Med Hyg. 46:39-43.

Villa L, Morote S, Bernal O, Bulla D, Albajar-Vinas P. 2007. "Access to diagnosis and treatment of Chagas disease/infection in endemic and non-endemic countries in the XXI century." Mem Inst Oswaldo Cruz. 102:87-94.

Virreira M, Torrico F, Truyens C, Alonso-Vega C, Solano M, Carlier Y, Svoboda M. 2003. "Comparison of polymerase chain reaction methods for reliable and easy detection of congenital Trypanosoma cruzi infection." Am J Trop Med Hyg. 68:574-582.

Vogelstein B, Gillespie D. 1979. "Preparative and analytical purification of DNA from agarose." Proc Natl Acad Sci USA. 76:615-619.

Wincker P, Britto C, Pereira JB, Cardoso MA, Oelemann W, Morel CM. 1994. "Use of a simplified polymerase chain reaction procedure to detect Trypanosoma cruzi in blood samples from chronic chagasic patients in a rural endemic area." Am J Trop Med Hyg. 51:771-777.

Wizel B, Garg N, Tarleton RL. 1998. "Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal Trypanosoma cruzi infection." Infect Immun. 66:5073-5081.

Young C, Losikoff P, Chawla A, Glasser L, Forman E. 2007. "Transfusion-acquired Trypanosoma cruzi infection." Transfusion. 47:540-544.

CURRICULUM VITAE

Name:	Pilar Hernández Pastor
Birth date and place:	22 July 1981, Barcelona
Nationality:	Spanish

2005-2008	Doctoral thesis work in the group of Prof. Ewald Beck at the Institute of Biochemistry, Faculty of Medicine, Justus-Liebig-Universität Gießen, Germany.
2005-2008	Teaching of practical classes in biochemistry at the Justus-Liebig-Universität Gießen, Germany. Practical laboratory work on different infectious diseases in developing countries (for several weeks each) at the Universidade Federal do Rio Grande do Norte, Natal, Brazil, at the Health Science University of Mongolia, Ulaanbaatar, Mongolia, at the Universidad de Chiriquí, David, Panamá, at the Universidad Mayor de San Andrés, La Paz, Bolivia, and at the Universidad Mayor de San Simón, Cochabamba, Bolivia.
2004-2005	University of Barcelona Master Degree in Experimental Biology prepared in the group of Prof. Ewald Beck at the Institute of Biochemistry, Faculty of Medicine, Justus-Liebig-Universität Gießen, Germany, with the title “Affordable diagnostics for low-income countries: detection of sexually transmitted diseases using new molecular biological procedures in Mongolia”.
2003-2004	Laboratory work on tuberculosis diagnosis at the Microbiology and Parasitology Department, Medicine Faculty, Universitat de Barcelona, Spain.
1999-2004	Biology Master degree at the Universitat de Barcelona, Spain.

MEETINGS AND WORKSHOPS

Oral presentation and poster at the Spring Meeting of the English Society of Parasitology in Belfast, with the title: “Visceral leishmaniasis or Chagas disease? Flaws in diagnosis of Trypanosomatides”.

Participation in the annual Workshops of Chagas disease, years 2006, 2007 and 2008, organised by the CRESIB Foundation, Barcelona.