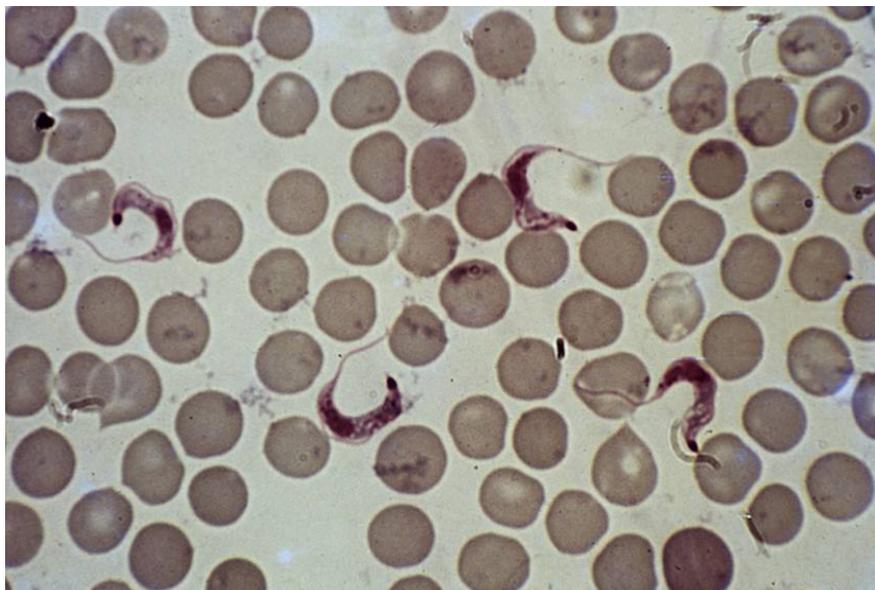




WHO Consultation on International Biological Reference Preparations for Chagas Diagnostic Tests

WHO, Geneva, 2–3 July 2007



© Four *T. cruzi* in blood sample - Coura J.R. LDP-IOC-Fiocruz

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Abbreviations

ELISA	enzyme-linked immunosorbent assay
HIV	human immunodeficiency virus
IgG	immunoglobulin G
IgM	immunoglobulin M
IHA	indirect haemagglutination assay
IFA	indirect immunofluorescence assay
PCR	polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RR	repeatedly reactive
<i>T. cruzi</i>	<i>Trypanosoma cruzi</i>
TESA	trypomastigote excretory–secretory antigen

1 Introduction

The World Health Organization (WHO) Consultation on International Biological Reference Preparations for Chagas Diagnostic Tests was held from 2 to 3 July 2007 at WHO headquarters, Geneva, Switzerland. Dr Lembit Rägo, Acting Coordinator, Quality Assurance and Safety: Blood Products and Related Biologicals, opened the meeting on behalf of WHO and welcomed the participants, who included representatives of reference and clinical laboratories, blood centres, regulatory agencies and manufacturers of diagnostic tests (see Annex 1). He outlined the WHO procedure for the development of international reference preparations, which is undertaken on behalf of the WHO Expert Committee on Biological Standardization, with contributions from the relevant WHO collaborating centres and from consultations and working groups convened by the Organization. The Expert Committee and the WHO collaborating centres have expressed the view that the development of international reference preparations for diagnostic tests for Chagas disease is of high priority. WHO therefore convened the present Consultation to discuss the scientific issues involved and to define the characteristics that will be required. Dr Hans Hogerzeil, Director, Department of Medicines Policy and Standards joined the Consultation for its final session. He thanked the participants for their valuable contribution to WHO's important work on biological standardization, and highlighted the importance of the development of reference materials for the quality control of Chagas diagnostic tests globally.

Dr Gabriel Schmuñis and Professor Felipe Guhl were elected Chairman and Rapporteur, respectively.

The conclusions and recommendations from the Consultation were presented at the meeting to launch the WHO Global initiative "Revisiting Chagas Disease: from a Latin American Health Perspective to a Global Health perspective", which took place at WHO headquarters, Geneva, Switzerland from 4 to 6 July 2007.

2 Transmission and epidemiology of *Trypanosoma cruzi* infection

Chagas disease, or American trypanosomiasis, is a protozoan zoonotic disease caused by the haemoflagellate *Trypanosoma cruzi* (*T. cruzi*). It has been estimated that there are 7.6 million people infected with *T. cruzi* in the Americas. The trypanosome is transmitted to the vertebrate hosts by infected dejections of haematophagous insects, the triatomine bugs.

More than 120 species of triatomines have been reported. However, only a dozen are epidemiologically important, three in particular: *Rhodnius prolixus* and *Triatoma infestans*, which are domiciliary species, and *Triatoma dimidiata*, which, although belonging to the sylvatic habitat, may invade houses and the peridomicile area. Vectorial transmission is still the most common way of acquiring the infection, from Mexico, in the north, to Argentina, in the south.

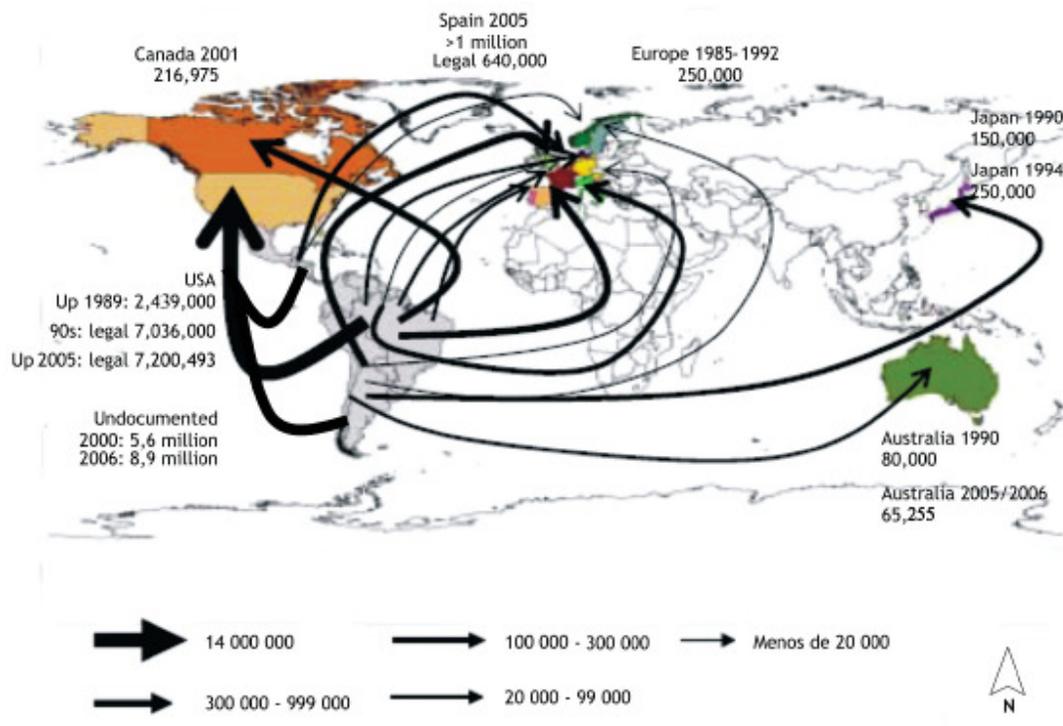
T. cruzi is also present in the Amazon Basin, a region that spreads into nine South American countries. Three epidemiological patterns have been found in the region: oral transmission through contaminated food, causing frequent outbreaks; imported disease through infected migrants from endemic *T. cruzi* areas; and vectorial autochthonous infection, especially among individuals entering the rain forest. *T. rangeli* is also present in the Amazon region, and co-infections with the two trypanosomes have been reported.

Years after the infection, *T. cruzi* may be detected in the blood in as many as 50% of those infected and untreated. Therefore, it may be expected that *T. cruzi* in the bloodstream could be transferred to a noninfected person by blood transfusion. This mode of transmission is considered to be the

second most common way of acquiring the infection. Fortunately, only around 20% of those receiving infected blood may become infected. In Bolivia, however, the rate may be higher, while in the United States of America it seems to be much lower. *T. cruzi* can also be transmitted by plasma, platelets and red blood cell concentrates.

Economic hardship in Latin America has stimulated migration to urban areas in the last six decades. What used to be a rural disease is therefore now often seen in Latin American cities, where more than 70% of the population lives. Migration from rural to urban areas, while decreasing the rural population exposed to infected vectors, increases the possibility of *T. cruzi* infection in blood transfusions given in cities. Economic hardship and/or political turmoil have also increased migration between countries within Latin America, and from Latin America to Australia, Canada, Japan and the United States and to countries in Europe (Figure 1).

Figure 1: Number of immigrants from Chagas disease endemic countries to non endemic countries in the last decades (Schmuñis, 2007)



More than 7 million legal immigrants from Latin America have arrived in the United States in recent decades, and it is estimated that the number of undocumented immigrants is even higher. In Europe, Spain is the country with the highest number of Latin American immigrants: 1.8 million in 2006 (Figure 1). It is therefore not surprising that *T. cruzi* infection acquired by transfusion or organ transplantation is now a potential problem in these historically non-endemic countries.

The risk of acquiring Chagas disease by transfusion depends on a number of epidemiological factors: the level of parasitaemia of the donor, the number and volume of transfusions received, the time between blood collection and transfusion, the immunological state of the recipient, etc. Cases have been reported in recipients of blood products in Canada, Spain and the United States.

Patients who have received organs infected with *T. cruzi*, taken from dead or living donors infected with *T. cruzi*, have themselves become acutely infected. This has occurred most frequently following kidney transplantation. Heart, bone marrow and pancreas transplantation are also possible causes of transmission, as has been shown in Argentina, Brazil, Chile, Spain, the United States and Venezuela.

Another way of acquiring the infection naturally is through congenital transmission. Because of migration from the countryside, large numbers of infected women of fertile age are residing in cities where there is no vector transmission, but they may nevertheless give birth to a child infected with *T. cruzi*. The prevalence of *T. cruzi* infection in women varies widely in the different endemic countries, but congenital Chagas disease is more widespread than previously believed, and has been reported from Argentina, Bolivia, Brazil, Chile, Colombia, Guatemala, Honduras, Mexico, Paraguay, Uruguay and Venezuela. Because of migration, it has also been reported in non-endemic countries like Spain and Sweden. Transplacental infection may occur in different pregnancies in the same woman, but siblings of congenitally infected children are not usually infected. In newborn twins, both may or may not be infected. Most published information suggests that, depending on the geographical area, 1–10% of mothers infected with *T. cruzi* give birth to an infected child. Most of the cases are oligosymptomatic or asymptomatic at delivery.

Another route of transmission that is becoming important is the oral route, and outbreaks resulting from the ingestion of contaminated food have been found in Brazil, Colombia, French Guiana and Mexico. Most cases, however, are believed to have originated through a locally made beverage in the Brazilian Amazon region.

Accidental transmission of *T. cruzi* has been reported in several situations, e.g. in laboratories and hospitals in endemic and non-endemic countries. More than 70 well-documented cases have been recorded among technicians, doctors and research workers known to have handled different types of contaminated materials, such as triatomine dejections, parasite cultures, and infected human and animal blood.

Because of chemical control of domiciliary triatomine bugs, vectorial transmission by *Triatoma infestans* was interrupted in Uruguay in 1997, in Chile in 1999 and in Brazil in 2006. Considerable progress has also been made in the control of this domestic vector in Argentina, Paraguay and Bolivia. Substantial progress has also been made towards the elimination of domiciliary transmission by *Rhodnius prolixus* in Guatemala and El Salvador.

At present, screening of blood donors by blood banks has been made compulsory by law in most Latin American countries in order to prevent the transmission of *T. cruzi* by blood transfusion. As a general rule, the implementation of effective national blood-bank policies, including mandatory screening for *T. cruzi* using an enzyme-linked immunosorbent assay (ELISA) test of high sensitivity, results in a drastic reduction in the risk of transfusion-associated transmission of *T. cruzi*.

Congenital transmission depends directly on the prevalence of the infection in fertile women, who have usually been infected by vectorial transmission. In endemic areas, subject to vector control, a progressive decrease in congenital disease can be expected over the medium or long term.

3 *Trypanosoma cruzi* heterogeneity: *T. cruzi* I and II

As a consequence of infection with *T. cruzi*, up to 30% of those infected, may develop the cardiac symptoms and/or dilation of hollow viscera that characterize chronic Chagas disease. The disease

shows a wide spectrum of pathological manifestations in different geographical areas in Latin America.

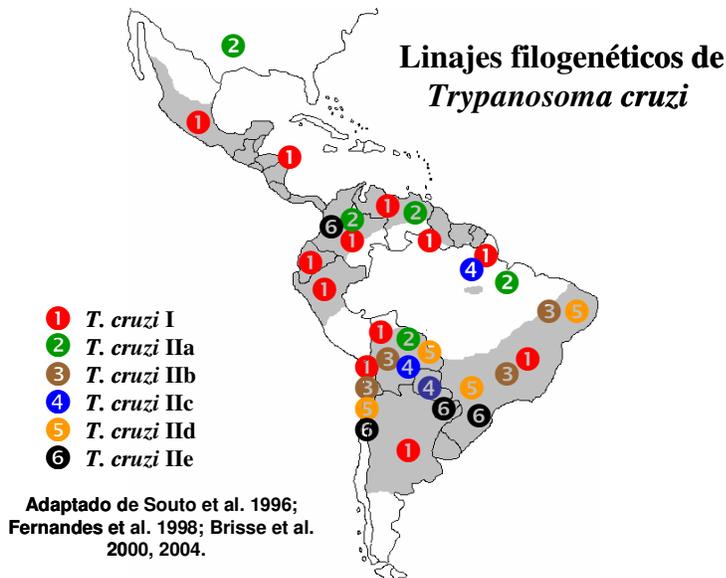
There are several possible explanations for the clinical differences. It is widely accepted that the *T. cruzi* strains found in human infection are heterogeneous. Evidence to support this view includes the lack of mega syndromes (enlargement of intestines and oesophagus) in the chronic forms of Chagas disease in Central American countries as compared with Southern Cone countries, and differences in the parasite susceptibility to drug treatment.

The heterogeneity present within isolates labelled as *T. cruzi* has been shown to take various forms, and phenotypic and genotypic differences have been reported. Recent studies indicate that there are at least two main groups of *T. cruzi* populations, *T. cruzi* group I (TCI) and *T. cruzi* group II (TCII). The first is closely linked to the sylvatic cycle and human disease in the Amazon basin and Central American countries. The second is closely related to the domestic cycle, distributed south of the Amazonas, and produces major morbidity in humans (Figure 2).

There is some evidence that the distribution of such parasite populations is related to the distribution and other characteristics of the vector species, and this may have epidemiological consequences for human Chagas disease. Subgroups have been reported in both groups: four haplotypes (1, 2, 3 and 4) in TCI and five subgroups (a–e) in TCII. Each subgroup corresponds to a particular epidemiological pattern.

Clearly the goal of defining the number of relevant subdivisions in *T. cruzi* should relate back to biological, medical and pathological parameters. The recognition and identification of biologically distinct subgroups of *T. cruzi* may help the prediction and alleviation of the clinical manifestations of Chagas disease in infected individuals. However, the geographical distribution of *T. rangeli* overlaps with that of *T. cruzi*, and the two species infect the same vertebrate and invertebrate hosts. *T. rangeli* infection is thought to be entirely non-pathogenic and mixed infections are relatively common in humans. Differential diagnosis of these two trypanosomes is therefore important.

Figure 2: Distribution of TCI and TCII (including subgroups a, b, c, d and e) in Latin America (Guhl, 2007)



4 Diagnostic tests for the detection of Chagas disease

Diagnosis should include compatible epidemiological, clinical and laboratory data. Laboratory diagnosis of the acute phase comprises demonstration of the presence of the parasite *T. cruzi*. This is performed using a wet-smear or concentration technique (the Strout test or microhaematocrit). Stained preparations can be used to detect parasites when parasitaemia is high, as in the acute phase, or in cases of transfusion-associated transmission in immunosuppressed hosts. The acute phase can be confirmed indirectly through the detection of antibodies against *T. cruzi* of the immunoglobulin M (IgM) class, or detection of the conversion from negative to positive serology (immunoglobulin G (IgG) class). IgM detection should be interpreted with caution – it may not be specific owing to cross-reaction with rheumatoid factor. There is also a lack of tests and positive controls. Detection of the parasite is easier during the first 30 days after the start of symptoms.

In the chronic phase, parasites are usually scarce, and laboratory diagnosis relies mainly on serological tests. Antibodies are present in more than 98% of infected individuals. In most Latin American countries, specific antibodies are currently measured by the so-called “conventional tests”, which have been in use since 1975 (see below Table 1). A variety of proprietary assays are available commercially. Parasites can also be detected through xenodiagnosis, haemoculture and using the polymerase chain reaction (PCR). These tests are not available commercially, and give positive results in $\leq 50\%$ of infected individuals; they are therefore indicated only in limited situations, such as in cases of doubtful serology or during follow-up after specific treatment.

Conventional tests include the indirect immunofluorescence assay (IFA), the indirect haemagglutination assay (IHA) and the enzyme-linked immunosorbent assay (ELISA). The use of two conventional tests in parallel is recommended, and provides an accurate diagnosis in more than

95% of samples, i.e. gives two negative or two positive tests. In cases of discordance, it is useful to repeat both tests to try to resolve the discrepancy.

The cut-off level should be determined carefully for each test and each lot of reagent using panels of sera of well-known reactivity. A grey zone should be recognized, and results falling within this zone should be reported as inconclusive. Sera from patients in the acute phase, from those receiving treatment or from newborn infants infected through passive transmission of antibodies from an infected mother usually fall into this category.

The ideal single serological test, with 100% specificity and 100% sensitivity, is unlikely to be attained, but with a combination of one test of high specificity (such as IHA, or recombinant antigens) and one of high sensitivity (such as IFA or ELISA), it is possible to diagnose most cases serologically. By using three serological tests in parallel, specialized laboratories can reduce the level of indeterminate results to <2%. Analysis of inconclusive results shows that almost half of them may be identified as cross-reactions with leishmaniasis (mainly the visceral form), or as chagasic patients who have received treatment, or by passive transmission of IgG with anti-Chagas antibodies. A special problem may exist with samples from the Amazon region, where parasitaemia and antibody titres are normally low; the latter close to the cut-off level. For samples from this region, the IFA does not always present a uniform pattern, which necessitates a quantitative and qualitative study with a detailed description. Where the recombinant ELISA is not helpful in clarifying doubtful or inconclusive cases, the western blot with trypomastigote excretory–secretory antigen (TESA) needs to be used to achieve results. Moreover, clinical manifestations have closely followed serological profiles: low serological titres with low morbidity/mortality rates are characteristic of this region.

For several reasons, including the need to achieve rapid results and to increase specificity, a number of other serological tests, such as complement-mediated lysis, latex agglutination, immunochromatography, TESA and radioimmuno-precipitation assay (RIPA), have also been used. Newer tests, based mainly on purified, recombinant or synthetic peptide antigens have been used recently, after successive multicentre evaluations. New devices and the inclusion of several recombinant antigens have led to the production of commercial rapid tests with acceptable sensitivity and specificity, which are useful in certain circumstances.

It is necessary to distinguish several different contexts for serological diagnosis (Table 1). The first is diagnosis of Chagas disease in a patient, for which tests of high specificity should be used to avoid misdiagnosis of infection in those who are not infected. In contrast, diagnosis for the exclusion of chagasic blood donors in order to ensure security of donated blood requires tests of high sensitivity, such as ELISAs. In Brazil, commercial ELISA kits have demonstrated excellent sensitivity and the recommendation for blood banks is therefore to use a single ELISA test. A third situation concerns the diagnosis of congenital infection. When a pregnant woman has tested positive, parasites should be looked for in the newborn infant. However, it has proved more practical to look for antibodies (of the IgG class) in serum from the baby after the age of six months. If the serum tests positive at that age, the infection is proven and the child deserves specific treatment. Other special situations are sero-epidemiological surveys, for which a test of high sensitivity (such as IFA or ELISA) is recommended; and the follow-up of treated patients, for which it is recommended that as many tests as necessary are done to reach diagnosis, with comparison with serum samples taken before treatment to look for the decline in antibody titres that is indicative of cure. For inconclusive results, it is recommended that serum samples are sent to specialized laboratories for further investigation.

Table 1: Diagnostic procedures for the detection of *T. cruzi* infection ¹⁾

Objectives/Applications	Conventional			Non-conventional	
	ELISA	IFA	IHA	Rec. antigens	PCR
Serological evidence (two tests recommended)	✓	✓	✓	✓	—
Blood bank screening (one test recommended)	✓	—	—	—	—
Transplacental and perinatal; transmission (two tests recommended)	✓	✓	—	✓	✓
Treatment follow-up (two tests recommended)	✓	✓	✓	—	✓

¹⁾ Second report of the WHO Expert Committee on the Control of Chagas Disease. Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 905), page 28.

Rec. = recombinant

Sensitivity and specificity performances of the various types of tests available in Brazil that have been evaluated are displayed in tables 2A-D. Tests are recommended by the Ministry of Health in Brazil if they are shown to have a sensitivity of $\geq 99\%$ and a specificity of $\geq 97\%$.

Table 2A: Chagas diagnostic tests evaluated by the Ministry of Health, Brazil in 2005, ELISAs ¹⁾

Kit ²⁾	Sensitivity (%)	Specificity (%)
Adaltis	100	60
Bio-manguinhos convencional ^a	100	93
Bio-manguinhos recombinante ^b	97	98
Biomérieux	100	95
Bioschile	99	98
Biozima Chagas	100	97
Ebram	99	97
Hemagen Diagnostics	100	97
Pathozyme-Chagas	99	97
REM Gold	99	97
Wama diagnóstica	99	98
Wiener	100	95

¹⁾ Ministry of Health, Rio de Janeiro, 2006 *Nota Técnica N.º 03/06*; <http://portal.saude.gov.br/portal/arquivos/pdf/>.

²⁾ Kits bought in 2005. Some of the kits were changed after then.

^a Conventional; ^b Recombinant

Table 2B: Chagas diagnostic tests evaluated in Brazil in 2005, IHA ¹⁾

Kit	Sensitivity (%)	Specificity (%)
Imunoserum	94.64	95.42
Ebram	88.69	59.92
Wama Diagnostics	100.00	95.80
Hemagen Diagnostics	93.45	87.79
Biolab Diagnostics	99.40	97.33

¹⁾ From: Schmuñis GA. *Memórias do Instituto Oswaldo Cruz*, 2007, 102(Suppl. 1):1–11.

Table 2C: Multicenter evaluation of IHA assays for anti-*T. cruzi* available in Brazil ¹⁾

Kit ²⁾	Sensitivity (%)	Specificity (%)
BioChagas (or Biokit or Imunolab)	84.8	98.1
Biomerieux Rio Janeiro (formerly Biolab)	91.2	100.0
Cecon Sao Paolo	82.2	93.1
Ebram, Sao Paulo	90.7	90.3
Hemagen, Sao Paulo	44.2	96.6
Biomanguinhos Fiocruz	94.3	92.7
Lemos (formerly in Brazil Imunoserum)	96.0	98.3
Salk, Sao Paulo	93.5	97.1
Trilab, Sao Paulo	71.5	97.1
Wama, Sao Paulo	94.6	96.5
Wiener, Sao Paulo	96.1	91.4

¹⁾ Saez-Alquezar et al., *Revista de Patologia Tropical*, Vol. 26(2): 343-365, 1997.

²⁾ Data are from kits bought in 1996. Tests are no more available. Due to the results of this study, tests were discontinued or meanwhile after 11 years, companies have withdrawn products or developed improved test versions.

Table 2D: Chagas diagnostic tests evaluated in Brazil, Particle agglutination assays ¹⁾

Kit	Sensitivity (%)	Specificity (%)
Serodia	100.00	97.71
ID-PaGIA – Diamed	98.81	98.85

¹⁾ Rabello A et al., *Mem Inst Oswaldo Cruz*, Rio de Janeiro, Vol. 94(1): 77-82, Jan./Feb. 1999.

5 Experiences in screening blood for antibodies to *Trypanosoma cruzi* in endemic and non-endemic areas

5.1 Countries where Chagas disease is endemic

Although there has been a tendency to improve the screening for *T. cruzi*, as for the other transfusion-transmissible infections, screening has still not reached 100% coverage in endemic countries in the Region. In 2005, 86.6% of blood donations in continental Latin American countries were screened, up from 79 % in 2000. In 2005, there were 11 countries, including Belize and Trinidad and Tobago, which reported universal screening for *T. cruzi*, up from six in 2000. There were 919,163 blood donations not screened for *T. cruzi* in 2005, 93.6% of these were collected in Mexico and 6.1% in Chile.

The prevalence rates of anti-*T. cruzi* antibodies vary widely across the Region. In 2005, the figures ranged from 0.01% in Ecuador and 0.09% in Costa Rica, two countries that report results obtained by confirmatory testing, to 8.61% in Bolivia. Variations with time are also observed. For instance, the prevalence rates decreased from 0.61% in 2001 to 0.27% in Chile, while the opposite trend was reported by Mexico, where the rate increased from 0.05% in 2001 to 0.50% in 2005.

Screening donated blood for anti-*T. cruzi* antibodies has prevented an estimated 188,750 infections since 2000. However, lack of screening has resulted in an estimated 16,533 infections.

In order to establish how the serological tests were being conducted in Latin America, a programme of performance evaluation was introduced in 1995, with the support of the Pan American Health Organization (PAHO). Panels of 24 unknown samples were sent from Sao Paulo, Brazil, to 21 reference centres in different countries. Each country also prepared a performance evaluation panel for its own national institutions. However, not all blood banks participated in national performance evaluation. Training courses of 10 days have been held in several countries, starting in Honduras in 1995 and followed by Colombia in 1996, Brazil and Paraguay in 1997, and Venezuela in 2000. An operation manual for the data analysis software was prepared in Colombia in 2006 [in Spanish].

5.2 Informing individuals of their serological status

In most blood banks in South America, blood donors can be excluded on the basis of a single positive test, which should be an ELISA with a high sensitivity for Chagas. Donors found to be positive should be informed of the result of their serological test and referred to a health facility capable of diagnosing Chagas disease with further tests and, if necessary, providing clinical management. For instance in Central Brazil, Chagas disease is confirmed in 80–85% of such cases; around 10% are found to be negative and are provided with certification that they are fit to donate blood; in the case of an inconclusive result, the patient is called in and asked to give a further blood sample, and tests are repeated. If appropriate, the patient is referred for clinical examination and an electrocardiogram; in 95% of cases there is no cardiopathy.

5.3 Countries where Chagas disease is not endemic

5.3.1 United States of America

The United States are considered to be non-endemic for *T. cruzi* despite rare reports of autochthonous transmission. However, over recent decades, transmission of *T. cruzi* by blood transfusion has become an increasing concern owing to the change in donor demographics resulting from continuing immigration from Latin America. It has been estimated that up to 100,000 Latin

Americans infected with *T. cruzi* now reside in the country, many of whom routinely donate blood. The potential for transmission by transfusion has been demonstrated by the five cases that have been reported; it is likely that many other cases have gone unrecognized.

Beginning in August 2006, the American Red Cross (ARC) conducted a clinical trial of the Ortho *T. cruzi* ELISA Test System in blood donors from California and Arizona, areas where *T. cruzi* positive donors were likely to be found. Blood donations from participating donors that were repeatedly reactive (RR) were confirmed by supplemental RIPA testing. Based in part on the results of this clinical trial, the United States Food and Drug Administration (FDA) approved the assay for blood screening use in December 2006. The ARC and several other blood organizations began blood screening in late January 2007. All blood donations are screened by ELISA, and RR samples are confirmed by RIPA. Retrospective investigations have been conducted to identify recipients of blood products from confirmed positive donors for follow-up testing. Follow-up samples have also been collected from positive donors for testing by PCR and haemoculture. Additionally, seropositive donors complete a risk factor questionnaire to determine the likely source of infection. During the initial clinical trial, 148,969 blood donors were tested, 63 (0.042%) were RR and 32 were confirmed positive. The overall rate was 1:4,655 and the specificity of the test was 99.98%.

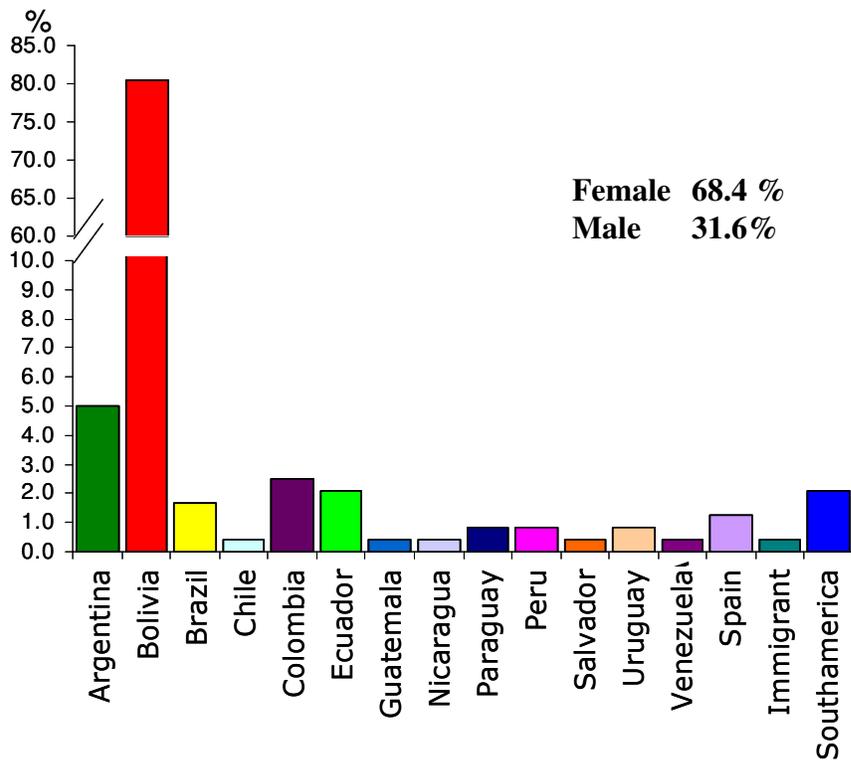
Since the initiation of blood screening, approximately 3.5 million donations have been tested. Of these, 328 were RR, with 112 confirmed by RIPA as positive; positive predictive value, 28%. Confirmed positives have been identified in 29 states and the current nationwide seroprevalence rate is 1:25,000. Follow-up testing of seropositive donors from both, the screening and the confirmed positives, has shown that seven of 46 (15%) tested positive using PCR and three of 16 (19%) using haemoculture. A total of 39 recipients from 18 RIPA-positive donors have been tested and one recipient of a platelet product was found to be positive for *T. cruzi*.

To date, blood screening for *T. cruzi* has proved to be an effective blood safety measure. Approximately one in 25,000 blood donors nationwide has been found to be positive. However, only 70% of the blood supply is being screened at present. The current algorithm of testing by ELISA and confirmation by RIPA has proved its specificity and sensitivity. Of confirmed positive donors >85% were born in countries endemic for *T. cruzi*; nine cases of possible autochthonous transmission have also been identified. While only a limited number of samples have been tested, the observation of donors that are positive in PCR and haemoculture suggests a significant transmission risk. Indeed, one presumptive case of transfusion-associated transmission has been identified.

5.3.2 Spain

Imported Chagas disease is a new reality for Spanish public health. Demographic data indicate a sustained growth in the number of foreigners resident in Spain, which has risen from 923,879 in 2000 to 4,144,166 at the end of 2006. Of these, 1,916,672 (46.2%) are from countries endemic for Chagas disease, with Ecuador (28.6%), Colombia (17%), Argentina (12.2%), Bolivia (8.8%) and Peru (6.1%) being the most frequent countries of origin. Figure 3 shows the origins of the seropositive population.

Figure 3: Origin of seropositive population in Spain (Cañavate, 2007)



More than five years ago, and as a result of the new situation, some blood centres began studying the detection of *T. cruzi* antibodies in candidate blood donors. After the entry into force of new legislation (Royal Decree 1088/2005), donors from endemic countries could be accepted provided that an analytical *T. cruzi* antibody test proved negative.

A survey was sent to all Spanish blood centres with the aim of compiling information about the current criteria for the acceptance of blood donors from areas endemic for Chagas disease and, in particular, the application of anti-*T. cruzi* antibody detection assays. The data refer to the total number of donations screened since the practice of testing began in each centre, up to 30 May 2007. The regions with lower numbers of immigrants from endemic areas, Castilla-La Mancha (2.1%) and Extremadura (0.6%), have opted to exclude candidates from endemic zones and do not perform antibody tests. Madrid (8.2%), Catalonia (5.8%) and Valencia (4.4%) account for 58% of the population at risk of being infected with *T. cruzi* nationally. These three autonomous communities were among the first to commence screening donations to detect anti-*T. cruzi* antibodies and have analysed the most samples. Blood donors from endemic areas represent approximately 1.3% of the total blood donor population. The prevalence of anti-*T. cruzi* antibodies among 29,846 blood donors was 0.9%.

The majority of Spanish blood centres use anti-*T. cruzi* antibody tests on a routine basis. In six blood centres, donors are not permitted to donate until the results are known and in 10 the donation is accepted and the test is performed as a requirement to validate the donation. Almost all centres have arranged for the follow-up of seropositive donor by a tropical disease unit.

The techniques most frequently employed for antibody detection are the DiaMed ID-PaGIA assay, followed by four commercial ELISAs (manufactured by Biokit, Certest-Abbott, Ortho Diagnostic Systems and Dade Behring). Some blood centres submit reactive samples to the Carlos III Health

Institute laboratory, Madrid, where an in-house ELISA and IFA are conducted to validate the original results.

Table 3: Evaluation of different assays for antibodies to *T. cruzi* in Spain: Performance of ELISAs and rapid tests ¹⁾

	ELISA in house	Certest²⁾	Ortho²⁾	Biokit²⁾	ID-Pagia³⁾	Operon⁴⁾
Sensitivity	100	100	100	100	92.4	92.4
Specificity	100	100	100	98.7	98.6	97.3
Prevalence	47.5	47.5	47.5	47.1	47.1	47.1
PPV	100	100	100	98.5	98.4	96.8
NPV	100	100	100	100	93.6	93.5

¹⁾ Cañavate C et al., Instituto de Salud Carlos III, Madrid, Spain 2007

²⁾ ELISA

³⁾ Particle Gel Immunoassay

⁴⁾ Rapid

PPV = positive predictive value

NPV = negative predictive value

Screening for the detection of anti-*T. cruzi* antibodies in blood donors from areas endemic for Chagas disease helps to improve the safety of blood transfusions without reducing the panel of eligible donors. This is therefore an acceptable public health measure in a society undergoing constant demographic evolution and diversification.

6 Test development

Participants representing several manufacturers associations informed the Consultation that in the last 20 years, there have been substantial developments in tests for the detection of antibodies. The most frequently used methods, IFA, IHA and ELISA, use antigens derived from parasite lysates (which may or may not have been purified), synthetic peptides and/or recombinant antigens. This results in diverse levels of performance, which several manufacturers are trying to improve.

Several serological tests to detect antibodies to *T. cruzi* use crude extracts or soluble antigens from *T. cruzi* epimastigotes from a specific strain, such as Y, Tulahuen or C. However, because of their nature, these antigen preparations may interfere with the sensitivity or specificity of serological tests. On occasion this may result in batch-to-batch variations. This is a limiting factor for test standardization.

Several manufacturers have developed ELISAs that use recombinant antigens. The mixtures have a sensitivity of >99–100% and a specificity of >98–99%, and have few cross-reactions with other diseases. Tests can be performed manually or with automated equipment. An ELISA for use with dried blood spots, which is useful for the screening of newborn infants and for epidemiological studies, has also been developed. The test again employs recombinant antigens representative of the different stages of the parasite, which have been selected to ensure reproducibility, sensitivity and low cross-reactions. A rapid immunochromatography test based on recombinant antigens is

currently being improved, while a test for detection of *T. cruzi* nucleic acids by PCR is in the initial stage of testing.

The Consultation emphasized the importance of developing reliable confirmatory assays with well-defined antigen preparations, such as TESA (trypomastigote excreted secreted antigen) or recombinant antigens associated with immunoblotting, or rapid tests with molecular amplification. The Consultation suggested that standardization of Chagas disease immunoassay tests for antibody detection should be based on the following:

1. A panel of positive sera with samples obtained from blood donors that have undergone serological screening.
2. The positive material should represent the two major *Trypanosoma cruzi* types, i.e. Type I from Mexico and Type II from Brazil.
3. A negative sample should be included as well to define the background signal level and to discriminate potential cross-reactivity.

The representatives of the manufacturers associations indicated their support for the concept of an international approach to the standardization of Chagas testing. They participated in discussions to define what that standard might be. They also indicated that the members of their associations would be willing to work with WHO to develop a panel or standard for testing.

7 Standardization of diagnostic tests for Chagas disease

The goals of developing standards for screening assays are to ensure in-process control during the development of an assay; and to ensure lot-to-lot consistency after the test is approved. The purpose of in-process testing is to validate the analytical characteristics (sensitivity, specificity and reproducibility) and clinical characteristics of the assays. Standard reference panels of sera with varying degrees of reactivity can be used to ensure lot-to-lot consistency of the antigen composition in a crude parasite lysate that is used as an analyte in the test kit, and to validate acceptable limits for the final product release. In addition, panels of sera with different dilutions can also be useful in determining the limits of detection. Standard reference panels can comprise dilutions of highly positive serum samples or sera from seroconversion panels obtained from patients. For testing the reproducibility and proficiency of test kits, it is preferable to have a panel of sera that contains samples with high, medium and low concentrations of the analytes, and negative samples. The panel is used to test three lots of each kit, at three different testing sites and with three different operators.

Manufacturers generally develop their own panels for in-process testing and for lot release. However, the FDA develops its own reference panels, which are used to test manufacturers' kit lots before they are released to the market. FDA reference panels consist of positive samples with high, medium and low concentrations of the analytes, and negative samples. The FDA also provides such panels to the manufacturers to validate their tests.

The following would need to be considered in developing a WHO Chagas reference panel that can be used to validate tests manufactured in different parts of the world:

- the size of the panel
- the geographical source of samples
- the extent of cross-reactivity among different endemic regions
- whether sera can be pooled (by region or type)
- the titres of the final panel

- minimum stability requirements
- the need to ensure that the panel can be used with all testing platforms
- the logistics for development
- the coordination of the panel.

The European Directive on in vitro diagnostic medical devices (98/79/EC) has been the sole regulation in the European Union countries since December, 2003. The regulation makes manufacturers responsible for the design, manufacture and performance of IVD devices, which must conform to the essential requirements, listed in Annex I of the Directive. This compliance allows CE conformity marking and free circulation of the device concerned within the European Union. The European regulations promote harmonized standards among manufacturers. If a manufacturer attests that a product conforms to a harmonized standard whose reference is published in the *Official Journal of the European Communities*, the member states shall presume compliance with the essential requirements of the directive concerned. For the time being, there are no product specific standards for anti-Chagas assays within the framework of the IVD Directive. In the European Union, only Chagas devices that are CE marked are permitted. In 2007, notifications reported to the French competent authority (Agence française de sécurité sanitaire des produits de santé) include five ELISAs with native antigens, four ELISAs with recombinant antigens, one immunofluorescence assay, one particle gel immunoassay, and five rapid tests.

The Consultation agreed that any reference panel suitable for helping Member States to confirm the capacity of commercial tests would need to be appropriate for all tests available – a “minimum reference panel” allowing common information. Questions as to whether geographical location was relevant for sample collection, whether the use of sera or plasma is more appropriate, and whether individual or pooled samples should be used were not answered definitively. It seems, however, that some samples would need to be collected also from Mexico, as some reports indicate that assays with antigen of strains isolated from Mexico are better for detecting antibodies from infected Mexicans.

8 The process for the preparation, characterization and establishment of WHO International Biological Reference Preparations

The recommendations for the production of international standard preparations are given in the fifty-fifth report of the WHO Expert Committee on Biological Standardization (WHO Technical Report Series, No. 932, Geneva, World Health Organization, 2006, Annex 2). In the first instance, a rationale for an international standard has to be established. The following should then be considered: the type and volume of the designated material; the appropriate antibody class/subclass for use in serodiagnosis; the suitable potency of the material; and suitable serodiagnosis test(s). The minimum safety criteria for the material should also be defined.

A collaborative study is then designed. This involves the selection of participants, often specialized or national reference laboratories that are representative of the geographical range of the disease, the selection of one or more tests, and the selection of samples for distribution. The material is aliquoted into ampoules and freeze dried. Freeze dried samples of the candidate standard and additional positive and negative samples are distributed to the participants. These samples are coded and tested blindly in the designated tests. The results are collated and analysed.

Statistical analysis will indicate the inter-laboratory variability and the inter- and intra-test variability for the samples. The geometric mean potency and the geometric coefficient of variation for the standard can then be calculated. This enables the assignment of an arbitrary unitage to the

candidate standard material. A stability study, usually over a 12-month period, also forms part of the collaborative study.

A report with all findings, including the complete data set, is sent to all participants. After comments have been received, the revised report is submitted to the WHO Expert Committee on Biological Standardization for final approval.

Several of the processes described here can be carried out by the United Kingdom National Institute for Biological Standards and Control (NIBSC). These include filling and freeze drying of ampoules, storage and distribution, stability studies and statistical analysis.

9 Proposals for the development of WHO International Reference Preparations for Chagas diagnostic tests

Proposals for the development of a reference panel for use in the quality control of the analytical sensitivity of Chagas diagnostic tests were considered on the basis of the following questions:

- How many samples should be included?
- Samples from which geographical areas should be included?
- What is the extent of cross-reactivity to other diseases in samples from different endemic regions?
- Can sera be pooled (by region or type) if there is shortage of availability of sera from particular individuals?
- What should be the range of antibody titres for the panel samples?
- What are the minimum stability requirements?
- How can it be ensured that the panel meets the needs of all testing platforms (manual, semiautomatic and fully automatic)?
- How can it be assured that samples from the panel are tested in the same way that a “routine” sample is tested?
- Which test(s) should be considered as the gold standard?
- Which test(s) are mandatory and which tests are voluntary?
- What are the logistic needs for the development of a reference panel?
- How should testing of the panel be coordinated?

T. cruzi exhibits considerable genetic variability throughout the endemic countries in Latin America. It is important to consider the significance of that variability for diagnostic tests and therefore for the geographical representation of the source samples for the reference panel. It was decided that the reference panel should comprise:

- Plasma units that are positive for antibodies to *T. cruzi* in ELISA, IHA, IFA and RIPA/western blot tests, obtained from one country in the north of Latin America, e.g. Colombia or Mexico (where the parasite is predominantly *T. cruzi* I).
- Plasma units that are positive for antibodies to *T. cruzi* in ELISA, HAI, IFA and RIPA/western blot tests, obtained from one country in the south of Latin America, e.g. Bolivia or Brazil (where the parasite is predominantly *T. cruzi* II).
- Plasma units that are negative in all tests, obtained from an endemic area.

In considering the relative merits of the pooling of samples, it was decided that the aim should be to obtain, for each member of the panel, a sufficient volume of plasma from one individual. Samples should be obtained with the informed consent of donors and with local ethical approval in the source country, and should be certified free of HIV-1, HIV-2 and hepatitis viruses A, B and C. It

was agreed that a suitable batch size would be approximately 3000 freeze-dried ampoules per panel member, with a volume of 1 ml per ampoule.

A coordinating group is needed to develop the reference panel, and an appropriate WHO collaborating centre will need to be involved in developing the production processes and the production of the panel.

10 Conclusions and recommendations

10.1 Conclusions

1. The epidemiology of Chagas disease is changing because of the greater movement of people from endemic to non-endemic areas.
2. Some donated blood in non-endemic countries has been shown to be positive for antibodies to *T. cruzi*.
3. Countries are currently using a wide range of serological tests for blood screening for *T. cruzi* and for the clinical diagnosis of Chagas disease. The tests have different levels of sensitivity and specificity and some show cross-reactions with other diseases, in particular visceral leishmaniasis.
4. A variety of reference panels are used in the evaluation of the sensitivity and specificity of the tests. The panels are composed of samples collected from different geographical locations and are taken from people with different stages of the disease. The lack of comparability of these panels makes it difficult to compare test sensitivity and susceptibility.
5. There is a global need for the regulation and control of in vitro Chagas diagnostic tests.

10.2 Recommendations

1. The Consultation supported the WHO proposal that International Biological Reference Preparations for antibodies against *T. cruzi* for use in the quality control of diagnostic tests should be developed.
2. The Consultation agreed that a reference panel should be established comprising three samples obtained from endemic countries in Central and South America: two samples positive for Chagas disease (one from the *T. cruzi* I region, i.e. Mexico; one from the *T. cruzi* II region, i.e. Brazil; and one sample that is clearly negative for the disease (negative in all tests).
3. The Consultation established a coordinating group to support the development of the reference panel. The following agreed to serve as members of the group: Dr A.O. Luquetti (Chairman), Dr M. Guzman and Dr M. Otani.
4. It is expected that the coordinating group will be able to select candidate materials for the reference panel within three months. The coordinating group will draw up the timetable for the project once the appropriate materials have been selected, and design a collaborative study to test the panel.
5. The WHO Collaborating Centre for Biological Standardization in UK (NIBSC) will develop production processes for the reference panel. A meeting of experts may need to be convened to confirm the suitability of the proposed materials before production of the reference panel. In collaboration with the WHO Secretariat, the coordinating group should prepare the list of expert laboratories that will participate in the collaborative study to test the reference panel. The list should include manufacturers, regulatory authorities and reference laboratories involved in blood screening and management of Chagas disease from a wide range of countries.
6. The composition of the reference panel and, if possible, a proposal for the design of the collaborative study, will be reported to the WHO Expert Committee on Biological Standardization at its fifty-ninth meeting in October 2007.

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