High Prevalence Anti-*Trypanosoma cruzi* Antibodies, among Blood Donors in the State of Puebla, a Non-endemic Area of Mexico

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Blood transfusion is the second most common transmission route of Chagas disease in many Latin American countries. In Mexico, the prevalence of Chagas disease and impact of transfusion of Trypanosoma cruzi-*contaminated blood is not clear. We determined the seropositivity to* T. cruzi *in a representative random sample, of 2,140 blood donors (1,423 men and 647 women, aged 19-65 years), from a non-endemic state of almost 5 millions of inhabitants by the indirect hemagglution (IHA) and enzime linked immunosorbent assay (ELISA) tests using one autochthonous antigen from* T. cruzi *parasites, which were genetically characterized like TBAR/ME/1997/RyC-V1* (T. cruzi *I) isolated from a* Triatoma barberi *specimen collected in the same locality. The seropositivity was up to 8.5% and 9% with IHA and ELISA tests, respectively, and up to 7.7% using both tests in common. We found high seroprevalence in a non-endemic area of Mexico, comparable to endemic countries where the disease occurs, e.g. Brazil (0.7%), Bolivia (13.7%) and Argentina (3.5%). The highest values observed in samples from urban areas, associated to continuous rural emigration and the absence of control in blood donors, suggest unsuspected high risk of transmission of* T. cruzi, *higher than those reported for infections by blood e.g. hepatitis (0.1%) and Aids (0.1%) in the same region.*

Key words: Chagas disease - seroprevalence - blood donors - antibodies to *Trypanosoma cruzi* - autochthonous antigens - Puebla - Mexico

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is now ranked as the most serious parasitic disease of the Americas with an economic impact far outranking the combined effects of the other parasitic diseases such as malaria, schistosomiasis and leishmaniasis (Dias & Schofield 1999).

Current World Health Organization estimation indicates about 16 to 18 millions people infected with *T. cruzi* (WHO 1991). Sixty percent of them live in urban areas, and about 50% are in a latent period. Therefore, since many infected people are potential blood donors, one can expect that a further 100 millions are at risk of contracting the disease (Schmunis 1991, Moncayo 1992, Moraes-Souza & Bordin 1996, Dias & Schofield 1998).

Following the recent emphasis of public health policies on vector control (Hayes & Schofield 1990), blood transfusion has now become one of the most significant routes for Chagas disease transmission (Dias 1992, Dodd 1998).

During the past decades, because of the migration from rural to urban areas, while decreasing the rural population exposed to infected vectors (Quintero et al. 1990), Chagas disease became frequent in cities and a health problem in a non endemic countries, where it can be transmitted vertically and by blood transmission (Schumunis 1999a) or organ transplantation (Carvalho et al. 1997). This, thus increases the possibility of alternative pathways to disease contamination.

To date, the status of Chagas disease in Mexico is not clear, although an increasing number of cases of Chagas disease has been reported (Cuartero et al. 1967, Velasco & García 1970, Gloss et al. 1990) since this disease was first recognized in 1940 by Mazzotti. Thus, while the National Seroepidemiological Survey (NSS) (Velasco et al. 1992) realized by the official Secretaría de Salud (SSa) showed a prevalence of 1.6%, independent studies carried out, mainly in rural areas, have shown that between 20-30% of the population was infected by the pathogen (Monteon et al. 1989, Tay et al. 1992). Moreover, positive blood donors have been detected at percentages of 0.2-17% of infected people (Goldsmith et al. 1978, Trujillo et al. 1993, Ramos-Echevarria et al. 1993, Rangel et al. 1998). This reports highlights the need for a comprehensible evaluation of the prevalence and risk of transmission of *T. cruzi* in endemic and non-endemic areas in Mexico.

In this work we estimated the prevalence of infection by *T. cruzi* in blood donors living in urban, suburban and rural conditions from a non-endemic area, i.e. the Puebla state (Mexico), with almost 5 millions of inhabitants, us-

ing two serological tests, i.e. enzyme linked immunosorbent assay (ELISA) and indirect haemagglutination (IHA), as suggested by World Health Organization (WHO 1991), employing an autochthonous antigen attributed to the genetic group *T. cruzi* I (Momen 1999).

POPULATION AND METHODS

Study area - The study was carried out in the Puebla state, which is located in the Southeast region of Mexico (Fig. 1), in a valley up to 1,800 m above the sea level with a warm climate (average annual temperature of 22ºC). The population is composed of around 4,579,810 inhabitants; 60% of them live in rural and suburban conditions, which represents appropriate ecological and socioeconomic conditions for the transmission of Chagas disease.

Studied population - Healthy blood donors, from blood banks of the clinics and hospitals of Instituto Mexicano del Seguro Social (IMSS), Puebla, Mexico, were selected. The criteria included residents of Puebla, aged between 18-65, > 50 kg, health clinically and seronegative to Hepatitis B Virus (HBC), Hepatitis Virus (HCV), *Brucella abortus*, (BrA) Hepatitis B surface antigen (HBsAg), Venereal Disease Research Laboratory (VDRL) and Human Immunodeficiency Virus (HIV) and without history of immunization, transplantation, menstruation, pregnancy or lactation, according to the Technical Norm for Banks of Blood protocol (TNBB). Detection of anti-*T. cruzi* antibodies by the immunoenzymatic assay ELISA and IHA tests were performed in the Laboratorio de Parasitología, Centro de Investigación Biomédica de Oriente, IMSS, Puebla, México.

In the design of the statistic sample, a stratified model was elaborated. The population was random-stratified considering as total population 4,579,810 inhabitants, distributed in seven regions, denominated Geostatistic Basic Areas (GBA) (National Institute of Geography Information, INEGI), that include the 217 municipalities of the state (Fig. 1). This model represent the urban, suburban and rural populations of the state, according the socioeconomical, political and cultural levels. The sample size was 2,140 individuals, stratified according the population density in each GBA (Table). The sample was determined with an estimated prevalence for *T. cruzi* antibodies based on data obtained from the NSS, the desired level of precision for national estimates, with a confidence level of 95% and a variation coefficient of 0.3, which corresponded to a minimal expected prevalence of 0.01 (Velasco et al. 1992). The level of precision was allowed to decrease for the different regions. The rural area was characterized by the lacked of sanitary services and presence of poor housing inhabit and coexistence with domestic and peridomestic animals. The suburban population was identified by the poor-housing situated in peripheral area of big cities and lacked of sanitary services and finally the urban population included the individuals, living in an area with total sanitary services.

Serum samples - The sanguine samples from blood donors, were collected of peripherical vein in Vacutainer system, in each selected blood banks of the clinics, hospitals, and Banco Central de Sangre, Hospital de Especialidades, Centro Médico Nacional Manuel Ávila Camacho, IMSS, Puebla. The serum was separated by centrifugation (1,200 g for 10 min), it was alicuoted in eppendorf tubes and it was frozen at -4ºC and transported to Laboratorio de Parasitología, Centro de Investigación Biomédica de Oriente, IMSS, Puebla where it were stored in freezing (-20º C) until use.
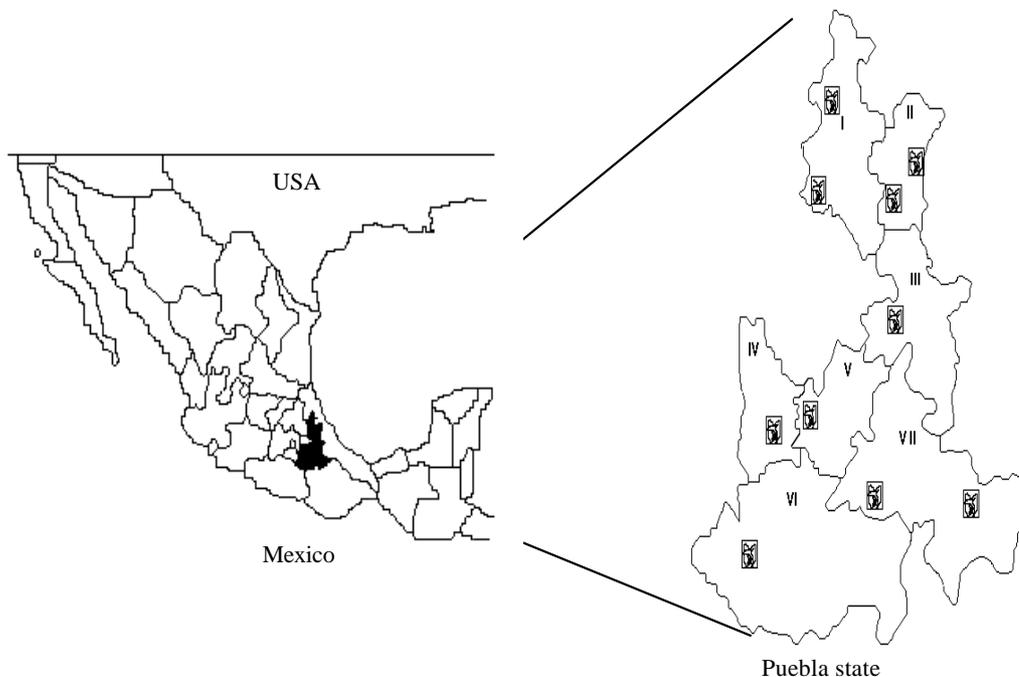

Fig. 1: geographical situation of Puebla state, Mexico, and origin of blood donors considering the GBA (Geostatistics Basic Areas by the INEGI). GAB I: Huauchinango; GAB II: Teziutlán; GAB III: Ciudad Serdán; GAB IV: Cholula; GAB V: Puebla; GAB VI: Izúcar de Matamoros; GAB VII: Tehuacán.

T. cruzi antigen characterization - *T. cruzi* parasites, employed as autochthonous antigen, were obtained from a *Triatoma barberi* specimen collected in the area locality, Puebla, Mexico. The isolated parasites were characterized by Multilocus Enzyme Electrophoresis (MLEE), RAPDs and Biodeme like *TBAR/ME/1997/RyC-V1 (T. cruzi I)*.

T. cruzi autochthonous antigen preparation - Total antigen from the Puebla strain RyC-V1 were obtained as previously reported (Pérez-Fuentes et al. 1998). In brief, the parasites (epimastigotes) were cultured and propagated in a liver infusion tryptose medium, supplemented with 10% fetal calf serum while growing at logarithmic phase was harvested, then sonicated in the presence of protease inhibitors, and spun down to 10,000 g x 30 min at 4ºC. The supernatant was used as a crude antigenic extract whose protein concentrations, quantified by the method of Lowry were adjusted to 1 mg/ml and stored at -70ºC.

Serological characterization - Technique of ELISA - Briefly, polystyrene plaques (Dynatech) were sensitized with the crude antigen extract of epimastigotes at a concentration of 100 μg/ml in carbonates buffer pH 9.5 and blocked with bovine fetal serum to 1% in PBS-Tween 20 (0.01%). The blood donors and controls sera were diluted 1:100 and incubated by 1 h, washed with PBS. It was employed conjugated human anti-IgG coupled to radish peroxidase, the colorimetric reaction was developed with orto-phenilen-diamino and peroxide of hydrogen. The reaction was stopped and it was reading at 490 nm in ELISA's reader.

Technique of IHA - The sensitization of the blood red cells was required for this assay. Ram erythrocytes was used at a concentration of 2.5% in buffer of phosphate pH 7.2, they were mixed with tannic acid at a dilution of 1:60,000 and they were sensitized with autochthonous antigen from *T. cruzi* at 37ºC for 20 min, they were washed themselves with PBS pH 7.2 and adjusted at a concentration of 0.2 mg/ml. The problem sera at dilutions 1:8, 1:32 and 1:64 were incubated by 2 h to room temperature. The identification of the antibodies was determined by the agglutination presence in the bottom of the wells of the plate. It was considered the title of 1:8 as infection and 1:32 as confirmatory. All samples were analyzed by triplicate and repeated twice.

Positive and negative controls were included in each test. A positive result was defined as titer > 1:32 for the IHA test and > 0.200 for the ELISA. In both assays we employed the autochthonous *T. cruzi* antigens. Soluble epimastigote antigen was prepared as previously described.

RESULTS

A total of 2,140 random blood samples of the seven areas of the Puebla were evaluated for anti *T. cruzi* antibodies using ELISA and IHA tests. All subjects were > 18 years of age, weight > 50 kg, clinically healthy, and were residents from rural, suburban and urban areas in Puebla. The average age of the individuals was 32 years old ranging from 19 to 50 years with a female:male ratio of 1:3. All donors were seronegative for HBC, HCV, (HBsAg), BrA, VDRL and HIV, and all of them fulfilled criteria to Technical Norm of Bank of Blood. Due to the lack of serologic "gold standard" for the diagnostic of Chagas disease, the sera employed in the evaluation were characterized by both matched IHA and ELISA tests. In each case, we tested for antibodies against *T. cruzi* using epimastigotes of autochthonous antigens. Of the 2,140 serum samples analyzed, 194 (9.1%) showed antibodies against *T. cruzi* with ELISA and 182 (8.5%) for IHA techniques, respectively. However, we considered as carriers of *T. cruzi* antibodies, the 166 (7.7%) individuals (127 men and 39 women, mean age 36.8 ± 16.4), that were seropositive to both assays, i.e. ELISA and IHA, according to WHO recommendations (1991).

Then, the samples were divided (Fig. 2) considering the distribution of seropositive blood donors for *T. cruzi* antibodies by both ELISA and IHA tests and according to the stratification in seven district regions proposed by the INEGI.

Table illustrates the distribution of seropositive samples, including positive, negative and discordant results, by studied areas. Prevalence rates were different between urban, suburban and rural origins for patients

TABLE

Distribution of IHA and ELISA test seropositive blood donors according to their geographical origin and total samples

GBA [a]	Total population [b]	Sample [c]	IHA	ELISA	(+) [d]	(-) [e]	Discordant [f]	Mean age [g]
I	609,670	285	16	18	15	266	4	42±15
II	446,840	208	14	1	14	187	7	37±14
III	346,335	162	11	15	10	146	6	38±17
IV	700,804	328	21	18	18	307	3	33±18
V	1,360,557	636	86	84	80	546	10	36±19
VI	370,077	173	6	5	4	166	3	35±16
VII	744,827	348	28	33	25	312	11	37±16
Total	4,525,110	2,140	182	194	166	1930	44	

a: Puebla state is divided in seven Geostatistics Basic Areas (GBA) in order to make economical and geographic analysis; *b:* total population from each GBA; *c:* representative randomized sample from each GBA; *d:* true positives using both assays; *e:* true negatives using both assays; *f:* assay results differing between the two serological analysis; *g:* mean age of seropositives from each GBA; IHA: indirect hemagglutination. The positive samples were at 1:32 dilution; ELISA test: the cut value was 0.044 ± 0.009 O.D. All cases were using the same dilution 1:100.

tested by IHA (Fisher's exact test with correction, $p = 10^{-5}$), by ELISA ($p = 2.4 \times 10^{-4}$) and by both tests ($p = 10^{-4}$). In addition, comparison were also significant between urban and suburban plus rural when plotted together by IHA ($p = 10^{-5}$), by ELISA ($p = 10^{-5}$) and by both tests ($p = 10^{-6}$).
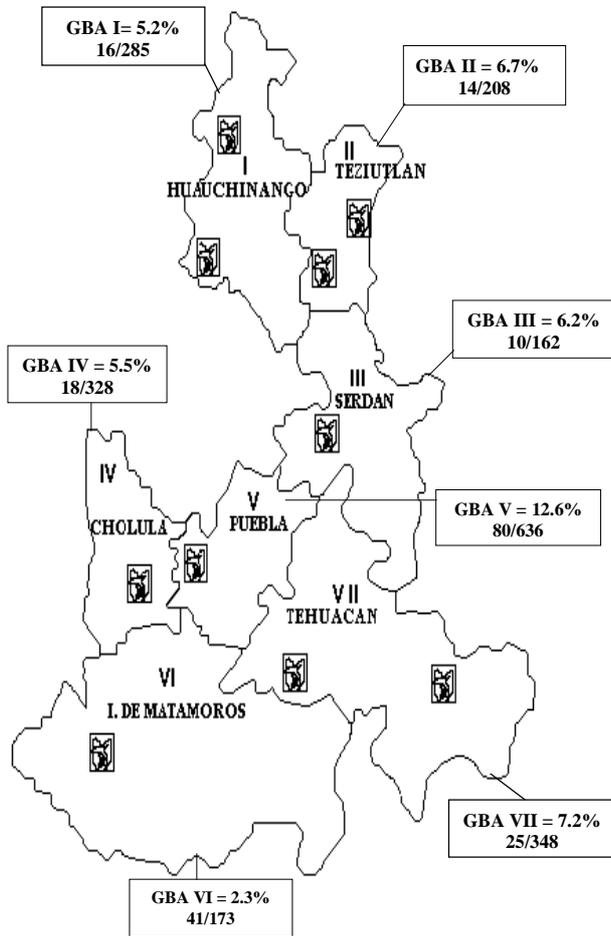

Fig. 2: distribution of blood donors seropositives to antibodies against *Trypanosoma cruzi* by both IHA and ELISA tests. The results show the absolute values and % of seropositives according to the population density in each Geostatistcs Basic Areas (GBA).

DISCUSSION

While in many Latin American countries, Chagas serological tests are mandatory for all blood donors, in Chile (Ministerio da Saúde 1993) and in Mexico (Guzmán-Bracho et al. 1998) this measure is applied only in endemic areas. Furthermore, screening in Mexico is not routinely done in all endemic states (Schmunis 1999b).

The data presented in this work are relevant because they clearly establish the seroprevalence of *T. cruzi* antibodies in blood donors, and provide information on the prevalence of the infection in Mexico, where the information regarding the importance of the transmission of Chagas disease is either limited or under estimated.

This study shows that the seroprevalence found in blood donors of a non-endemic area of Mexico (7.7%), i.e. the Puebla, is relatively high, and comparable to those found in endemic states of others countries. These findings strongly contrast with the NSS in Mexico carried out in 1987, and published in 1992, which showed a low prevalence of 1.6%. These differences may be in part attributed to the fact that this last study was performed with statistics specifically designed for the characterization of urban transmissible diseases, such as Aids, hepatitis, but not for rural transmissible diseases such as Chagas disease (Velasco et al. 1992). Besides, our findings (7.7%) contrast with the more recent study carried out by the official SSa, that showed only 1.5% of national prevalence and specifically 1.8% from Puebla (Guzmán-Bracho et al. 1998). The discrepancy could be explained in part by the serologic tests employed (we used both ELISA and IHA assays), strain of the parasite used in the antigen preparation (autochthonous antigen), and mainly, by the size (random sample) and characteristic of analyzed population. Thus, in this work was performed in 64,969 blood donors, where approximately 850,000 donations are done yearly, including samples from 18 of the 32 Mexican states, and the study did not rely on any specific statistics.

The antibody based immunoassay play a relevant role as diagnostic tool because of their high sensitivities. In the particular case of Chagas disease, no serologic "gold" standard exists, since detection of *T. cruzi*-specific antibodies depends on many factors. Nevertheless, IFA is the most commonly used serologic test for Chagas disease and, as result, is widely accepted as the gold standard (Ferreira & Moraes de Avila 1995). Recent reports show that in-house IFA and in-house ELISA were highly concordant (Oelemann et al. 1998). Like is suggested by Organización Panamericana de la Salud (OPS), IHA is a screening technique while ELISA or IFA is for confirmatory diagnosis with a higher specificity than IHA. In this work, in total agreement with the OPS recommendations that mandatory serology must be implemented in all countries where *T. cruzi* is endemic by using at least two serological tests (OPAS 1998), we used two independent techniques, i.e. in-house IHA and in-house ELISA with the same antigen, the last assay presented a higher specificity to Chagas disease detection (Pérez-Fuentes et al. 1998).

However, few countries use more than one test for blood donor screening (Schmunis 1999). A study carried out in 1998 in Cuernavaca (town of Morelos, Mexico) showed a seropositivity in blood donors of 17%, using a commercial ELISA kit in consecutive blood samples from 318 donors (Rangel et al. 1998).

Our findings can be explained in part by the kind of antigen we have used in serologic tests, further studies have suggested that the specificity of different methods depends on the selection of an adequate antigen (Mendes et al. 1997), considering that *T. cruzi* is polymorphic, and different parasite strains circulate in different areas (Dias 1992). Then, genetic characterization of *T. cruzi* parasites have shown that Mexican stocks belonging to *T. cruzi* I and are closely related to each other (Boseno et al. 2002). In Mexico, most studies have been performed using *T.*

cruzi antigens extracted from strains originating from other geographical origins, mainly South America, that show different genotype (Bucio et al. 1999).

Previously, we have shown that assays utilizing commercial antigens from other countries are lesser sensitive than using *T. cruzi* authocthonous antigen in the diagnostic of Chagas diseases (Pérez-Fuentes et al. 1998). Recently this finding was demonstrated by others authors (Sánchez et al. 2001).

The specificity of our analysis was to use autochthonous antigens from local *T. cruzi* strains isolated in the same area and characterized as *T. cruzi* I, could be naturally reflect locally adapted host immune response against *T. cruzi* natural clones circulating in the area.

In Mexico, the infection by *T. cruzi* is mainly silent. Thus, like in most countries of Latin America, Chagas disease has become an urban disease, due to the migration of people from endemic areas to the cities (Moraes-Souza 1999). In this work, although all of individuals were resident of the region in study, the prevalence rate for *T. cruzi* antibodies found in the blood donors of urban origin, i.e. Puebla (V Area), were twice higher than those of rural regions, where the identification of *T. barberi*, the insect vector and the conditions for natural infection and transmission exists (unpublished data, Pérez-Fuentes R et al.). This could be explained by the migration of infected people from rural to urban areas as Puebla, capital city, due to the poor socioeconomic as in many Latin American countries. This could be suggest that urban transmission is now overcoming the classical rural transmission.

Many recent reports (Guzmán-Bracho et al. 1998, Dumontiel 1999, Schmunis 1999) show the need for a comprehensive evaluation of the prevalence and distribution of Chagas disease in Mexico. In the present study, the identification of substantial prevalence of seropositive blood donors to *T. cruzi* suggests the existence of a high risk of contamination by blood transfusion. As others have proposed, the routine programs of serologic screening with immunological techniques with high sensibility and specificity definition, i.e. *T. cruzi* antigens extracted from local strains, are urgently needed and applied. This should be done in all areas, including those that are considered as non-endemic.

The last years, fortunately the situation has improved in Mexico. The ministry of Health has approved a law about screening for anti-*T.cruzi* antibodies in the whole territory. Also, epidemiological surveillance and vector control programs have started to inform regulation (Guzmán-Bracho 2001).

Our results confirm that blood transfusion is an important parameter in Chagas disease transmission in Mexico, besides the classical vectorial transmission. Vector control therefore must be completed by adequate screening measures in all Mexican transfusion centers.

ACKNOWLEDGEMENTS

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