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Article

Polymerase chain reaction and blood culture in blood donors screened by ELISA test for Chagas' disease

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The objective of this study was to evaluate, through blood culture and PCR, the results of the ELISA for Chagas' disease in the screening of blood donors in the public blood-supply network of the state of Paraná, Brazil, and to map the epidemiological profile of the donors with respect to their risk of infection by *Trypanosoma cruzi.* The negative and positive results of the ELISA were confirmed by blood culture and PCR for 190/191 individuals (99.5%). For one individual (0.5%), the ELISA was inconclusive, blood culture and IIF were negative, and IHA and PCR positive. Three individuals (1.6%) were positive for *T. cruzi* on all the tests. Donors were predominantly female, and natives of Paraná, of rural origin, had observed or been informed of the presence of the vector in the municipalities where they resided, had never received a blood transfusion, had donated blood 1 to 4 times, and reported no cases of Chagas' disease in their families. We concluded that PCR and blood culturing have excellent potential for confirming the results of the ELISA, and that candidate blood donors with negative or positive tests have a similar risk of infection by *T. cruzi,* indicating that the ELISA test is sufficiently safe for screening blood prior to use.

Uniterms: *Trypanosoma cruzi*/detection. ELISA/use for screening. Blood donors. Blood culture. PCR. Blood bank/screened donors.

O objetivo deste estudo foi avaliar, pela hemocultura e PCR, os resultados do teste ELISA utilizado para doença de Chagas na triagem de doadores de sangue na rede pública do Estado do Paraná, Brasil, e traçar o perfil epidemiológico dos doadores quanto ao risco de infecção pelo *Trypanosoma cruzi.* Os resultados negativos e positivos do ELISA foram confirmados pela hemocultura e PCR em 190/191 indivíduos (99,5%). Para um indivíduo (0,5%), o teste de ELISA foi inconclusivo, hemocultura e IFI foram negativas, HAI e PCR foram positivas. Três indivíduos (1,6%) foram positivos para *T. cruzi* em todos os testes. A maioria dos doadores era do sexo feminino, oriundos do Estado do Paraná, de origem rural, tinham observado ou foram informados da presença do vetor nos municípios onde residiam, nunca tinham recebido sangue, haviam doado sangue de 1 a 4 vezes e não relataram casos de doença de Chagas na família. Nós concluímos que a PCR e a hemocultura são excelentes testes para confirmar os resultados do ELISA e os candidatos a doadores de sangue com testes positivos e negativos apresentam risco semelhante de infecção pelo *T. cruzi*, reforçando o nível satisfatório de segurança do teste ELISA para liberar o sangue para o uso.

Unitermos: *Trypanosoma cruzi*/detecção. ELISA/uso para triagem. Sangue/doadores. Hemocultura. PCR. Banco de Sangue/triagem de doadores.

INTRODUCTION

The Ministry of Health of Brazil, through the National Health Monitoring Agency, approved resolution RDC No. 153 (Brasil, 2004) in June 2004. This resolution mandates that a highly sensitive immunoenzyme test for Chagas' disease must be used to screen blood bags prior to their release, in accordance with recommendations of the World Health Organization (WHO, 2002).

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The risk of transmission of *Trypanosoma cruzi* through transfusion of a single, 500-mL unit of whole blood ranges from 12% to 20% (WHO, 2002). In Brazil, the seroprevalence of Chagas' disease in the public blood-bank network is 0.6% whereas the seroprevalence rate in private blood banks is unknown (Silveira *et al.,* 2002). The serological tests (indirect immunofluorescence or IIF, indirect hemagglutination or IHA, and immunoenzyme or ELISA) used to diagnose Chagas' disease generally have sensitivity of 95% and specificity of 93 to 98%, indicating that no one test is sufficiently sensitive to totally eliminate the risk of transmission of *T. cruzi* through blood transfusion (Salles *et al.,* 1996; Oelemann *et al*., 1998; Saez-Alquezar *et al*., 1998).

Other techniques capable of verifying the presence of *T. cruzi* in infected patients include blood culture, xenodiagnosis, and PCR. Several investigators (Avila *et al.,* 1993; Wincker *et al.,* 1994; Gomes *et al.,* 1999; Castro *et al.,* 2002; Salomone *et al.,* 2003; Vera-Cruz *et al*., 2003; Batista *et al*., 2007) have reported that individuals with negative or inconclusive serology on the IIF and ELISA tests had positive results on xenodiagnosis, blood culture, or PCR. Some of these scholars (Castro *et al.,* 2002) did not rule out the possibility that the frequency of individuals who are serologically negative and have an active *T. cruzi* infection detected only by PCR, may be higher than previously thought. Other authors (Salomone *et al.,* 2003), having demonstrated the presence of *T. cruzi* by PCR in individuals that showed no serological evidence for Chagas' disease, believe that their findings challenge the present recommendations for Chagas' disease diagnosis, treatment, and monitoring of blood transfusions. Vera-Cruz *et al*. (2003), in a study comparing PCR with the commercially available ELISA test on samples collected in an endemic area in Mexico, found a higher positive rate using PCR than on ELISA. Based on these results, the authors raised the possibility that the ELISA method underestimates the prevalence of *T. cruzi* infections.

The genetic diversity of *T. cruzi* can also influence the performance of serological tests. Mice inoculated with parasites of the *T. cruzi* II group (referred to as Discrete Typing Units - DTU II by Zingales *et al*., 2009) showed rates of positivity with ELISA ranging from 83.3% to 93.3%, blood culture from 60.6% to 76.7%, and PCR 100%. For mice inoculated with parasites of the *T. cruzi* group I (DTU I by Zingales *et al*., 2009), the positivity of the ELISA and PCR were both 100%, whereas that of the blood culture ranged from 89.5% to 90.2% (Miyamoto *et al.,* 2006).

The Chagas' Disease Laboratory of the State University of Maringá, as part of its mission to improve the care of patients with this disease, established the program "Chagas' Disease Awareness through Comprehensive Education" (Araújo *et al.,* 2000). In order to expand this program and devise new action strategies, it is important to define the profile of the patients treated by this laboratory, including candidate blood donors.

Based on these considerations, the objective of this study was to confirm, by means of blood culture and PCR, the results of the ELISA for Chagas' disease obtained during the screening of candidate blood donors in the public blood-supply network of Paraná, and to map the epidemiological profile of the population studied, with respect to the risk of their being infected by *T. cruzi.*

MATERIALS AND METHODS

Population studied

From March 2007 through February 2008, a period in which the Maringá Regional Blood Donation Center (Hemocentro Regional de Maringá - HRM) in Paraná processed a total of 9358 candidate blood donors, 191 blood samples were collected from individuals of 16 municipalities belonging to the 15th Health Region of the State of Paraná (Figure 1). The collection routine of the HRM was maintained, and the sample was representative of the regional population. The criteria for inclusion of individuals in the study were the screening doctor's authorization to collect an additional volume of blood, and donor age, such that the same numbers of individuals for the age ranges 18 to 33, 34 to 49, and 50 to 65 years could be obtained. The sample size (n) was calculated using the formula $n = Nn_0 / (N+n_0)$, where $n_0 = p\ (1\text{-}p)\ (z_{0.05}/e)^2$, and N is the size of the population (considering the number of candidate blood donors of each age group predicted for the 12-month period). The p value refers to the occurrence of the event investigated, based on the mean of the preceding three years for Chagas' disease in the HRM (0.28); $z_{0.05}$ corresponds to the 95% percentage of the standard normal distribution, and to the maximum sampling error of 10% (0.1). The minimum sample size resulting from this calculation was 188, with a 90% confidence level.

Individuals who were clinically qualified to donate blood were informed of the purpose of the study, and signed a consent form approved by the Committee for Ethical Research on Human Beings of UEM. ELISA, blood culture, and PCR tests were done on the blood samples collected. Each prospective donor was interviewed by means of a specific questionnaire collecting data on items that could indicate a risk of infection by *T. cruzi*, including native state or country, rural or urban origins, original hou-

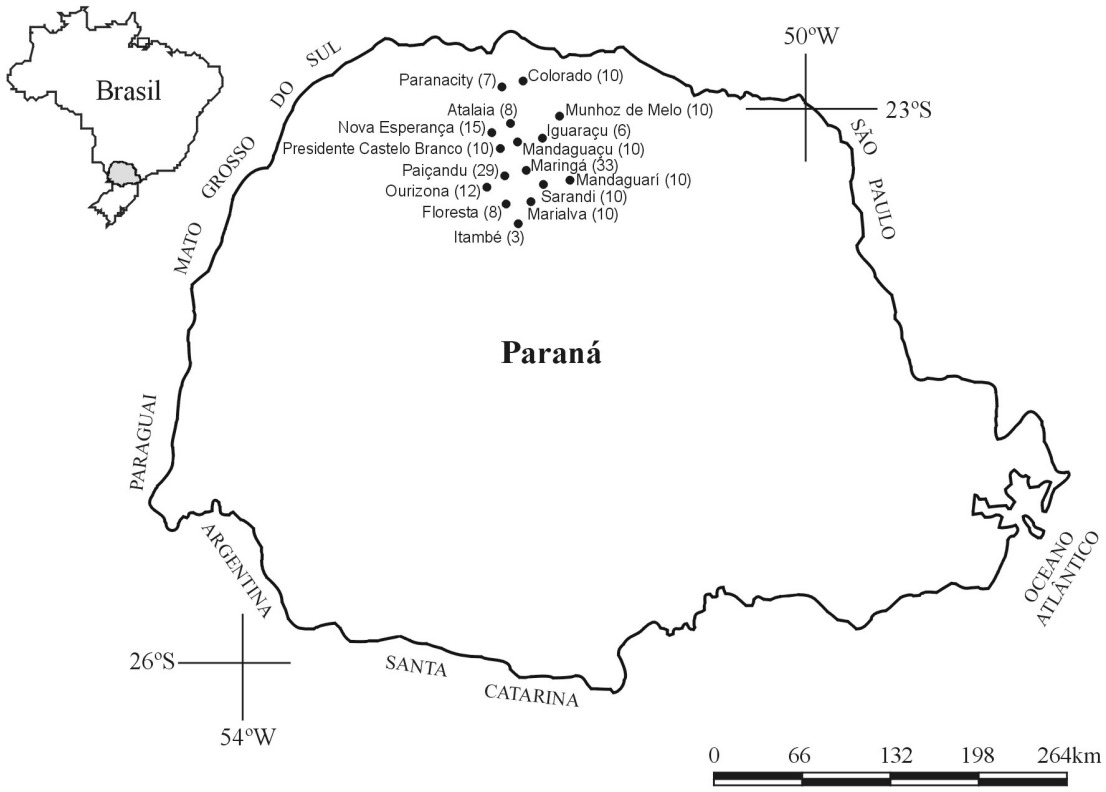

FIGURE 1 - Municipalities of origin of candidate blood donors studied, Paraná State, Brazil. Note: values in parentheses represent number of candidate blood donors from each municipality.

sing structure, presence of the vector in the municipality, present housing structure, rural laborer status, previous blood transfusion, previous blood donation, and presence of Chagas' disease in the family.

ELISA

The ELISA reaction to detect anti-*T. cruzi* IgG antibodies was performed with the ELISA III® Chagas Test diagnostic kit (BiosChile Ingenharia Genética S.A., Chile) made available by the Public Blood-Supply Network of Paraná, and applied according to the recommendations of the manufacturer. Whenever a sample was reactive or inconclusive in the initial test, the test was repeated in duplicate, using blood from the same sample.

Blood culture

To carry out this test, 30 mL of blood was collected in heparinized tubes. The blood cultures were then processed in LIT medium and incubated at 28 °C, according to Chiari *et al.* (1989) with modifications. The samples were homogenized once a week, and examined 30, 60, 90, and 120 days after processing.

PCR

For each individual, 10 mL of blood was added to an equal volume of 6 M Guanidine-HCl/0.2 M EDTA (Sigma Chemical Company, USA), pH 8.0. Using 200 µL of blood, the DNA was extracted with phenol-chloroform and precipitated in absolute ethanol in the presence of 100 mM sodium acetate (Gomes *et al.,* 1998). DNA was first resuspended in 20 µL of sterile deionized water. After 24 to 36 hours stored at 4 °C, the DNA was washed with 70% ethanol, precipitated, and resuspended in 10 µL of water.

The PCR was processed by mixing 2 µL of the DNA solution from each sample, together with 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 75 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP, Invitrogen Ltda.), 0.5 U of Taq DNA polymerase (Invitrogen), and 10 pmoles of each primer for 10 µL of reaction.

Primers 121 (5'AAATAATGTACGGG(T/G)GAGATGCATGA3') and 122 (5'GGTTCGATTGGGGTTGGTGTAATATA 3') were used as described by Wincker *et al.* (1994); these primers amplify fragments of a variable 330 bp region of the *T. cruzi* kDNA minicircle. The DNA was next amplified in an automatic thermocycler (MJ Re-

search, PTC-150) with denaturing at 95 °C for 1 min (with a longer initial step, for 5 min), annealing of the primers at 65 °C for 1 min, and extension at 72 °C for 1 min (with a 10-min final step) for a total of 35 cycles (Gomes *et al*., 1998). For the steps of DNA extraction and PCR, negative controls with blood samples from uninfected individuals from a non-endemic area, and positive controls of individuals with Chagas' disease were used. The products amplified by the PCR were observed by electrophoresis in 4% polyacrylamide gel and by silver staining (Santos, Pena, Epplen, 1993).

IIF and IHA

The tests described were carried out on those samples that were positive on ELISA, blood culture, or PCR tests. The IIF and IHA were executed with reagents manufactured by BioMérieux Brasil SA, according to the recommendations of the manufacturer. Anti-*T. cruzi* antibodies of the IgG class were investigated; a titer ≥ 40 was considered reactive for IIF. For the IHA, the qualitative test was carried out according to the positive and negative sera of the HRM and of the Laboratory for Teaching and Research on Clinical Analyses (Laboratório de Ensino e Pesquisa em Análises Clínicas - LEPAC) of UEM. The demonstrably positive cases were sent to the ACHEI program (Araújo *et al.,* 2000), to which the patients were referred for medical care, with etiological treatment and follow up every six months for at least five years.

RESULTS

Of the 191 candidate blood donors who underwent serological screening for Chagas' disease, 188 tested negative on ELISA. Two individuals were positive on the ELISA, IIF, and the IHA whereas 1 person had inconclusive results on the ELISA, testing negative on IIF and positive on IHA. On the blood cultures, 189 individuals presented negative, and 2 positive results. For the PCR, a total of 188 individuals tested negative and 3 positive (Table I).

The results of the ELISA were confirmed by blood culture and PCR for 190 individuals (99.5%). In one case (0.5%), the ELISA was inconclusive, blood culture was negative, and PCR positive. Of the three cases in which the ELISA was positive or inconclusive, two were shown to be positive by blood culture, and three by PCR (Table I).

Table II shows that the epidemiological profile of the population studied for risk of infection by *T. cruzi* was similar for subjects with negative (188) and positive (3) tests. The majority of the individuals investigated were natives of states where Chagas' disease is endemic and had lived in rural areas, both constituting risk factors associated with this disease. Also, the sample was predominantly female and the majority reported no history of Chagas' disease in the family. The three individuals with positive results on at least one of the tests were 48 years of age or older, presently residing in municipalities in Paraná (Colorado, Sarandi, and Floresta), were rural laborers, while two reported having had a case of Chagas' disease in the family, also considered a risk factor for Chagas' disease.

DISCUSSION

The HRM is a unit of intermediate complexity with macroregional responsibility, and provides support and assistance to the health network, supervises services, and performs serological examinations for all the collection units. It is a reference center for Northwestern Paraná, a region endemic for Chagas' disease. After the resolution of the Ministry of Health came into effect in 2004 (Brasil, 2004), the HRM began to use only the ELISA test to screen blood donors for Chagas' disease.

In the present study, the results of the ELISA were confirmed by blood culture and PCR in a high percentage of samples, affirming the use of ELISA as the sole test for screening prospective blood donors. Satisfactory results for different ELISA kits have been previously reported (Oelemann *et al.,* 1998; Leiby *et al*., 2000; Blejer, Saguier, Salamone, 2001; Gadelha *et al*., 2003; Pirard *et al*., 2005). However, the results

TABLE I - Results of tests performed on candidate blood donors at the Maringá Regional Blood Donation Center, Paraná, Brazil, from March 2007 through February 2008

	No. Individuals	ELISA	Blood culture	PCR	IIF	IHA	Positive (%)
	188	N	N	N	NT	NT	0.0
	2	P	P	P	P	P	1.1
	1	I	N	P	N	P	0.5
Total	191						1.6

No. = Number; % = Percentage; P = Positive; N = Negative; I = Inconclusive; NT = Not tested.

TABLE II - Epidemiological characteristics and positive tests for *Trypanosoma cruzi* among candidate blood donors at the Maringá Regional Blood Donation Center, Paraná, Brazil, from March 2007 through February 2008

Epidemiological characteristics	*n* (%)	Positive tests*
Gender		
Female	99 (51.8)	2†
Male	92 (48.2)	1‡
Native state or country		
Paraná	133 (69.7)	1†
São Paulo	31 (16.3)	1‡
Minas Gerais	8 (4.2)	1†
Mato Grosso do Sul	4 (2.1)	0
Mato Grosso	3 (1.6)	0
Alagoas	3 (1.6)	0
Bahia	2 (1.0)	0
Rio Grande do Sul	2 (1.0)	0
Pernambuco	2 (1.0)	0
Sergipe	1 (0.5)	0
Ceará	1 (0.5)	0
Japan	1 (0.5)	0
Area		
Rural	110 (57.6)	3†‡
Urban	81 (42.4)	0
Original housing structure		
Wood	130 (68.1)	1†
Concrete block with plaster	29 (15.2)	0
Wattle and daub	17 (8.9)	2†‡
Unplastered concrete block	0 (0.0)	0
Mixed	0 (0.0)	0
Not known	15 (7.8)	0
Presence of the vector in the municipality	109 (57.1)	2†
Yes	82 (42.9)	1‡
No		
Present housing		
Concrete block with plaster	163 (85.3)	3†‡
Wood	27 (14.2)	0
Unplastered concrete block	1 (0.5)	0
Wattle and daub	0 (0.0)	0
Mixed	0 (0.0)	0
Rural laborer		
Yes	128 (67.0)	3†‡
No	63 (33.0)	0
Blood transfusion		
No	177 (92.7)	3†‡
Yes	14 (7.3)	0
Previous blood donations		
1 to 4 times	71 (37.2)	1†
Never	54 (28.3)	2†‡
5 to 10	42 (22.0)	0
More than 10	24 (12.5)	0
Chagas' disease in the family		
No	169 (88.5)	1†
Yes	20 (10.5)	2†‡
Not known	2 (1.0)	0
Total	191 (100.0)	3†‡

n = Number; % = Percentage; * Candidate blood donors with positive results for *Trypanosoma cruzi* infection. † Elisa, Blood culture, PCR, IIF, and IHA positive. ‡ Elisa inconclusive, Blood culture and IIF negative, PCR and IHA positive.

presented in this study must be interpreted cautiously by considering the samples analyzed, the serological tests and reagents, the technical procedures, quality control, and the protocols for extraction and amplification of the DNA, and taking into account that the study area has a low prevalence of Chagas' disease. Although high levels of agreement, principally between serological and molecular tests, in diagnosing Chagas' disease have been reported (Avila *et al*., 1993; Britto *et al*., 1995; Junqueira, Chiari, Wincker, 1996; Gomes *et al*., 1999; Castro *et al*., 2002; Marcon *et al*., 2002; Salomone *et al*., 2003; Vera-Cruz *et al*., 2003; Coronado *et al*., 2006), positive results on xenodiagnosis, blood culture or PCR, in conjunction with negative or inconclusive serological tests, have also been demonstrated (Avila *et al*., 1993; Gomes *et al*., 1999; Castro *et al*., 2002; Marcon *et al*., 2002; Salomone *et al*., 2003; Vera-Cruz *et al*., 2003). As a result of this lack of agreement, Salomone *et al*. (2003) and Vera-Cruz *et al*. (2003) raised the question as to whether a single serological test to screen blood bags for release is safe, and whether the ELISA test underestimates the prevalence of *T. cruzi* infections.

In the present study, positivity for *T. cruzi* was 1.6% (3/191) for all the tests used. This positivity rate is very similar to that reported by the regional blood donation centers of Londrina/Paraná, Brazil (Oliveira-Marques *et al*., 2005) and Iguatu/Ceará, Brazil (Sobreira *et al*., 2001), reflecting the success of the program to eliminate *Triatoma infestans* and of the regular and rigorous screening of donors at blood banks in place since the 1980s (Franco-Paredes, Bottazzi, Hotez, 2009; Fernandes *et al*., 2009; Villela *et al*., 2009). Monteon *et al*. (2005) and Hernández-Becerril *et al*. (2005) also observed low seroprevalence (1.24% and 0.37%, respectively) in candidate blood donors in Mexico. However, the positivity rate observed in the present study is high compared to the data presented

by Schmunis and Cruz (2005), Sabino *et al*. (2003), Silva *et al*. (2007) and Araújo, Vianna, Berne (2008).

For two cases in which the ELISA was positive, the blood culture was also positive. In the one case which was inconclusive on ELISA, the blood culture was negative. These are very good results, because in the samples for which the ELISA was positive, the blood culture, a test known for its low sensitivity (Chiari *et al.*, 1989; Gomes *et al*., 1998; Castro *et al*., 2002), also showed a positive result. This good performance of the blood culture may be related to the level of parasitemia of the individuals at the time of donation, and to the quality of execution of this technique in our laboratory, given it is laborious and requires aseptic procedures and good-quality reagents.

As mentioned, the ELISA was inconclusive in one individual, yet even in this case the protocol proposed by the Ministry of Health enabled elimination of the sample collected, since it was positive for IHA, despite testing negative on the IIF. In addition, the PCR result was positive, indicating that the PCR technique could be a valid alternative if reference centers using this methodology are established. Other investigators have also shown that, in individuals with inconclusive serology, tests such as the PCR and the TESA-Blot can be used to confirm the results (Umezawa *et al*., 1996; Gomes *et al*., 1999; Marcon *et al*., 2002; Silveira-Lacerda *et al*., 2004; Batista *et al*., 2007; Araújo, Vianna, Berne, 2008). Although Duarte *et al*. (2006) observed sensitivity rates of 95.7 to 100% for IIF, IHA, and ELISA, and 1.2% for PCR, several investigators have reported good agreement of IIF and ELISA results with those of the PCR (Britto *et al*., 1995; Junqueira, Chiari, Wincker, 1996; Gomes *et al*., 1999; Hernández-Becerril *et al*., 2005; Coronado *et al*., 2006), in-line with the results of the present study.

Of the conventional serological tests used to diagnose Chagas' disease, the IHA has the lowest sensitivity (Oelemann *et al*., 1998; Leiby *et al*., 2000; Blejer, Saguier, Salamone, 2001; Gadelha *et al*., 2003; Pirard *et al*., 2005). However, in the present study, the IHA was able to confirm the diagnosis of one individual with an inconclusive ELISA and a negative IIF result. This result corroborates the concerns of several investigators (Blejer, Saguier, Salamone, 2001; Gadelha *et al*., 2003; Pirard *et al*., 2005; Araújo, Vianna, Berne, 2008) who have emphasized the importance of combining several effective tests when screening for Chagas' disease at the blood banks, thereby reducing false-negative or inconclusive results and cross-reactions (Saéz-Alquezar *et al.*, 1998).

In the present study, the inclusion of two other serological tests (IIF and IHA) was only required to confirm the diagnosis in 3 individuals with positive results on at least one of the tests applied (ELISA, blood culture or PCR), considering that the results of these three tests with different principles were concordant for most of the individuals investigated. As recommended by Luquetti (1990), confirmation of the diagnosis of individuals suspected of harboring *T. cruzi* infection screened in the blood-supply network and/or that have positive results on other tests, requires high sensitivity and specificity, made possible by combining two techniques (IIF and IHA).

According to Gontijo, Rocha, Oliveira (1996), risk factors for Chagas' disease include being born in a rural area, living in states endemic for Chagas' disease, and having a history of Chagas' disease in the family. Using these criteria, candidate blood donors in the present study all had a similar risk of infection by *T. cruzi*. Our results reinforce the effectiveness of serological screening and the good performance of the ELISA test, which whilst not perfect, provide a satisfactory level of safety for blood screening. Other evidence that demonstrated the discriminatory power of the ELISA was the three individuals with positive or inconclusive results on this test who, in addition to exhibiting risk factors associated with Chagas' disease, reported other important epidemiological characteristics such as presence of the vector in the municipalities of residence, and rural laborer status. With respect to age, all the individuals who were positive for infection were 48 years of age or older, a finding which may reflect the success of the Brazilian program to eliminate *T. infestans*, the principal domestic vector of *T. cruzi*.

Determining the profile of individuals living in areas of low prevalence, as achieved in the present study, yields important information that can be applied in areas where Chagas' disease is more prevalent and also in regions with active infection, thus allowing the groups at risk to be identified. Consequently, appropriate health education such as that carried out under the ACHEI Program (Araújo *et al*., 2000) can be developed. The ACHEI program has demonstrated, in practice, the benefits of psychological and psychosocial intervention in preventing aggravation of the disease, given that symptomatic patients were found to exhibit more physical and psychological stress, lower capacity for resistance (expressed as greater despair and emotional difficulties), and lower capacity for tenacity and innovation (Mota *et al*., 2006).

CONCLUSION

The results of this study allow us to conclude that blood culture and PCR have good potential for confirming results of the ELISA test. The data also indicate that the ELISA has desirable reliability for screening potential

blood donors at the HRM, since candidate blood donors with negative or positive tests showed a similar risk of infection by *T. cruzi*. However, the possibility of conflicting results from analyses performed in other blood-supply networks using different diagnostic kits cannot be ruled out. Taken together, these results demonstrate that the blood used in transfusions in Northwestern Paraná shows a satisfactory level of safety. Studies at multiple centers and including samples from regions with a high prevalence of Chagas' disease should be conducted, in order to confirm the observations of the present study.

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