VISCERAL LEISHMANIASIS IN URBAN AREAS: THE ISSUE OF CROSS REACTIVITY AMONG SEROLOGICAL TESTS

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By

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ABSTRACT

INTRODUCTION: Urbanization of *Trypanosoma cruzi* and *Leishmania infantum* have increased in Latin America. This has result in an increased risk of infections to a large population. There is mandatory blood screening for *T. cruzi* infection, but there is none for *L. infantum*.

MANUSCRIPT 1: Evaluation of Leishmania infantum infection in humans using commercial Trypanosoma cruzi assay: the importance of assessing cross-reaction

BACKGROUND: *L. infantum* and *T. cruzi* share many common epitopes, with cross-reaction between serological assays.

OBJECTIVES: To evaluate the sensitivity of a commercial *T. cruzi* ELISA assay for diagnosing *L. infantum* infection.

METHODS: A total of 573 subjects from an endemic area of visceral leishmaniasis in Brazil were studied. Of those, 350 were symptomatic visceral leishmania patients (Symp VL); 87 were recovered VL individuals (Rec VL), and 96 were healthy household contacts of a VL case (HHC). Leishmania infection was estimated by a commercial *T. cruzi* (CHAGAS III) assay and soluble Leishmania antigens (SLA) ELISA, PCR and skin delayed type hypersensitivity responses (DTH).

RESULTS: The sensitivity of the *T. cruzi* assay to ascertain Symp VL was 76% and its specificity was 91.7%. There was a positive correlation of both the

T. cruzi and SLA ELISA assays with the level of anti-Leishmania antibodies (r=0.487, p<0.0001). The response was higher for Symp VL in both the *T. cruzi* (OR 39.1; p<0.0001) and the SLA (OR=41.5; p<0.0001) relative to HHC assays. There was an inverse relationship between presence of antibodies and DTH response: 88.2% of people who were DTH positive were *T. cruzi* negative and 53.9% who were DTH negative were *T. cruzi* positive (p<0.0001).

CONCLUSIONS: The commercial *T. cruzi* assay (CHAGAS III) is sensitive for detecting *L. infantum* infection. The positivity of the test correlated with the level of anti-Leishmania antibodies.

MANUSCRIPT 2: SEROLOGICAL SURVEY OF LEISHMANIA INFECTION IN BLOOD DONORS FROM AN ENDEMIC AREA OF VISCERAL LEISHMANIASIS IN BRAZIL

BACKGROUND. Asymptomatic Leishmania infection has increased in urban areas of Brazil, which could pose a risk of Leishmania transmission through blood transfusion or organ transplantation.

OBJECTIVES: To determine the prevalence of *Leishmania infantum* infections in blood donors of an endemic area for visceral leishmaniasis in Brazil.

METHODS. A cross-sectional study was performed, with weekly recruitment of volunteers from February 2014 to January 2015. A total of 1556 participants who were donating blood in Natal, Brazil were recruited. Participants

answered a questionnaire related to exposure to Leishmania. Results of their blood screening were analyzed. Blood samples were tested for Leishmania infection using a soluble Leishmania antigens (SLA) ELISA assay. *Likelihood Ratio Chi-Square* test evaluated the association between categorical variables. Simple and multiple regression models evaluated the effect of covariates and factors on Leishmania response. The results of the SLA ELISA were log transformed and ratio obtained, considering the cutoff value for each plate.

RESULTS: A total of 51,463 people donated blood to public blood banks in 2014 and of those, 1554 (3%) were recruited. The majority of donors were males (82.4%) and married (51.8%). The literacy rate was high with 68% of the participants having completed high school. The mean body mass index for males and females were, respectively, 27.96 and 27.99 kg/m^2. The seroprevalence of *T. cruzi* and Leishmania were 0.19% and 2.5%. Leishmania was isolated from one of the samples cultured (600).

CONCLUSIONS. There is risk of Leishmania transmission through the blood supply. Close monitoring of blood recipients is warranted, principally in those with some type of immunosuppression. *T. cruzi* ELISA assay can identify a subgroup of *L. infantum* contaminated blood, but does not exclude all positive samples.

BIOGRAPHICAL SKETCH

Selma M.B. Jeronimo received a Bachelor in Biology from the Federal University of Rio Grande do Norte, in Natal, Brazil; she also earned her Doctor of Medicine from the same institution. She is currently a Professor of Biochemistry and the Director of the Institute of Tropical Medicine, from the Federal University of Rio Grande do Norte. She is interested in improving strategies for treatment and eventual control of neglected tropical diseases in her home state. The implementation of the Institute of Tropical Medicine is one of the means to reach these goals.

Dedicated to my beloved mother, Dulcimar Bezerra de Medeiros, *in memorian*

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Evaluation of *Leishmania infantum* infection in humans using commercial *Trypanosoma cruzi* assay: the importance of assessing cross-reaction

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CONFLICT OF INTEREST NOTIFICATION PAGE

There are no conflicts of interest to report.

ABSTRACT

MANUSCRIPT 1: Evaluation of *Leishmania infantum* infection in humans using commercial *Trypanosoma cruzi* assay: the importance of assessing cross-reaction

BACKGROUND: Asymptomatic *L. infantum* infection has increased in urban areas of Brazil and there could be a risk of its transmission through the blood supply. We hypothesized *T. cruzi* screening would identify a subset of the Leishmania positive samples because of cross reactivity.

OBJECTIVES: To evaluate the sensitivity of a commercial *T. cruzi* ELISA assay for diagnosing *L. infantum* infection.

METHODS: A total of 573 subjects residing in the state of Rio Grande do Norte, Northeast Brazil were studied. Of those, 350 were symptomatic visceral leishmaniasis patients (Symp VL); 87 were recovered VL (Rec VL), and 96 were healthy household contacts of a VL case (HHC). We reviewed their clinical status and analyzed their blood for Leishmania infection by using both a commercial *T. cruzi* (CHAGAS III) assay and soluble Leishmania antigens (SLA) ELISA assays. Quantitative PCR was used to estimate the load of Leishmania in the blood. Rec VL and HHC subjects were tested for their skin delayed type hypersensitivity responses (DTH). The McNemar´s test assessed the null hypothesis that there was no association between the assays. The

sensitivity and specificity of the *T. cruzi* assay in identifying Leishmania infection were determined.

RESULTS: Of the Symp VL, 98.6%(345) were positive by SLA ELISA and 76.0% (266) were positive by the *T. cruzi* assay; whereas for the Rec VL, the positivity of the SLA and *T. cruzi* assays were, respectively, 81.6% (71) and 16.1% (14), and for the HHC, they were, respectively, 60.4% (58) and 8.3% (8). In all three groups, a significant association between the results of the *T. cruzi* and SLA assays was observed. The sensitivity of the *T. cruzi* assay to ascertain Symp VL was 76% and its specificity was 91.7%.There was a positive correlation of both the *T. cruzi* and SLA ELISA assays with the level of anti-Leishmania antibodies ($r=0.487$, $p<0.0001$). The response was higher for Symp VL in both the *T. cruzi* (OR39.1; $p<0.0001$) and the SLA (OR=41.5; $p<0.0001$) assays relative to HHC assays. There was an inverse relationship between presence of antibodies and DTH response: 88.2% of people who were DTH positive were *T. cruzi* negative and 53.9% who were DTH negative were *T. cruzi* positive ($p<0.0001$).

CONCLUSIONS: The commercial *T. cruzi* assay (CHAGAS III) is sensitive for detecting a *L. infantum* infection. The positivity of the test correlated with the level of anti-Leishmania antibodies. These findings indicate the need for better assays to rule out dual infection and to ascertain the correct diagnosis of either *L. infantum* or *T. cruzi* infections.

INTRODUCTION

Visceral Leishmaniasis (VL) is a vector-borne illness that in Latin America and Europe is an anthropozoonosis, commonly caused by *Leishmania infantum,* a Trypanosomatidae.(Deane, 1958; Mott et al., 1990) In Latin America, this pathogen is transmitted by the byte of the sand fly vector *Lutzomyia longipalpis*. Transmission of Leishmania by organ transplants or blood transfusions has also been documented.(Mestra et al., 2011; Dey and Singh, 2006; Antinori et al., 2008)

Prior to the mid 1980's, VL in Brazil was found as sporadic cases, mostly in rural areas, where the burden of Leishmania infection was less in humans and more in animal populations, such as foxes, dogs and rodents.(Badaro et al., 1983; Badaro et al., 1986; Evans et al., 1992) However, with increased urbanization the proportion of humans and dogs infected with Leishmania in densely populated areas has increased.(Marzochi et al., 1994; Lima et al., 2012; Costa et al., 1990; Jeronimo et al., 1994) VL has also reemerged in Europe, mostly with the HIV epidemic, (Villanueva et al., 2000; Alvar et al., 1996; Gramiccia et al., 2004) but also among immunocompetent people.(Millan et al., 2014; Calderaro et al., 2014; Ehehalt et al., 2014)

Chagas Disease is caused by *Trypanosoma cruzi*, another Trypanosomatidae, and is endemic in 21 Latin American countries with over 6 million people infected.(Otani et al., 2009; Coura, 1988; 1991; Mott et al., 1990) Transmission of this pathogen to humans usually occurs by contact with feces of the reduvidae bugs when they take a blood meal. Infections through

blood and organ transplants have also been reported.(Coura and Borges-Pereira, 2010) More recently in Brazil, outbreaks of acute Chagas were reported by oral infection, through the contamination of juices, such as açai and sugar cane.(Souza-Lima et al., 2013; Coura, 2006)

The severity of Chagas Disease, and the risk of *T. cruzi* transmission through the blood supply, led to mandatory blood screening for *T. cruzi* in most Latin American countries beginning in the mid 1970's. However, there is no serological assay to screen for *L. infantum* infections. Serological assays developed for either Leishmania or Trypanosoma genus have to estimate the potential for cross-reaction, since these pathogens share many common antigens.(Vega Benedetti et al., 2013; Sabino et al., 2010; Otani et al., 2009; Houghton et al., 2009) However, for blood screening purposes, there is a need for the test to be highly sensitivity (Kirchhoff et al., 2006; Otani et al., 2009) and, as result, false positive results occur, mostly in geographic areas where the two pathogens overlap. A radioimmune precipitation assay (RIPA) is the gold standard for the ascertainment of *T. cruzi* infection.(Shah et al., 2010) However, this assay is not available in Latin America.

Because of the increased burden of asymptomatic Leishmania infection in urban areas of Brazil, there could be an increased risk of transmission of Leishmania through the blood supply. Since in Brazil all blood units are tested for *T. cruzi* infection, we hypothesized that this risk is lower than expected, because the screening for *T. cruzi* would likely identify a proportion of Leishmania-contaminated blood. We determined the value of a commercially

available *T. cruzi* assay to identify Leishmania infection by testing samples of people with symptomatic visceral leishmaniasis (Symp VL), recovered visceral leishmaniasis (Rec VL) and healthy household contacts (HHC) of a VL case. We added the latter group, because they were at increased risk of Leishmania infection and at risk of developing VL for living .(Evans 1992, Jeronimo, 2004)

METHODS

Study Population

A total of 573 subjects residing in the State of Rio Grande do Norte, Northeast Brazil, were studied. This area is endemic for both VL and Chagas Disease.(Jeronimo et al., 1994; da Camara et al., 2013) Of the samples screened, 350 were from Symp VL patients in which the diagnosis had been confirmed by clinical and parasitological findings, 87 were Rec VL subjects, who had also clinical and parasitological confirmation of disease, and 96 were their HHC. The last two groups were part of a cohort of people studied by our group.(Jeronimo et al., 2004; Jeronimo et al., 2007)

T. cruzi ELISA

Serum samples were screened for anti-*T. cruzi* antibodies using the Chagas III ELISA Test (BIOS, Chile). The test was carried out in an ELISA plate coated with whole extracts of *T. cruzi* epimastigotes. To each well, a total of 200-µl diluent provided by manufacturer was added, followed by 20 µl of sera. Plates were sealed and incubated for 1 hour at 37^{o} C. We then followed the manufacturer's protocol. The presence of antibodies was estimated using positive and negative sera provided by the manufacturer. Samples which OD

reading was 1 standard deviation above the cut off value was grouped as indeterminate and were not considered in the analysis.

Leishmania ELISA

Anti-Leishmania antibodies were detected by ELISA using a soluble Leishmania antigen (SLA), prepared from a local Leishmania isolate as previously described.(Braz et al., 2002) Briefly, plates were coated with 500 ng of SLA, blocked and incubated with diluted sera. Anti-SLA antibodies were detected with a secondary anti-human IgG (Promega, Wisconsin, USA).

Leishmania quantitative PCR

Quantitative PCR was used to estimate the burden of Leishmania in the blood. Genomic DNA was extracted from leukocytes using a standard protocol (Grimberg et al., 1989) as previously described.(Macedo-Silva et al., 2014) All reactions were conducted on a 7500 Real Time PCR system from Applied Biosystems in a 10 µl reaction mixture, using the Mag1 chromosomal, and/or kDNA7 kinetoplast DNA primers and probes.(Weirather et al., 2011) A standard curve was generated from DNA extracted from a known number of cultured *L. infantum* promastigotes ranging from 10^6 to 10^{-3} promastigotes per well. The data were analyzed with SDS v1.3.1 software.

Leishmanin skin test

The delayed skin-test hypersensitivity response (Delayed Type Hypersensitivity) to Leishmania antigens was performed using 25 µg of *Leishmania amazonensis* proteins (Centro de Produção e Pesquisa de

Imunobiológicos, Secretaria de Saúde, Paraná, Brazil), which were injected intradermally. The size of induration was assessed after 48–72 hours and was measured in two perpendicular directions.(Sokal, 1975) An induration equal or greater than 5mm was considered positive.(Gouvea et al., 2007)

Statistical analysis

Chi-square likelihood ratio test was used to assess homogeneity of the distribution of categorical variables in independent groups. The McNemar´s test assessed the hypothesis that there was no association between the serological assays. Tables of frequency were constructed using two- and three-way analysis. A linear logistical regression model was used to evaluate the association between the tests and explanatory variables as groups (SympVL, RecVL and HHC), considering sex and age. The ANOVA test was used to compare the mean age between independent groups.

Ethical considerations

The protocol was reviewed and approved by the Universidade Federal do Rio Grande do Norte Ethical Committee (CEP-UFRN). The certificate of approval is 12675013.7.0000.5537. All subjects provided written informed consent.

RESULTS

1. Assessment of serological assays for *L. infantum* and *T. cruzi* among people with confirmed VL (current or recovered) and their HHC

The demographics of the groups are shown in Table 1. Of the 573 people studied, 368 were males (64.2%) and the distribution of sex across the

three groups did not differ significantly p=0.202. The average age differed significantly among groups (p=0.002). The Rec VL were younger than the Symp VL and their HHC with a mean (±SD) age of 14.67±15.45 years. The recovered VL had mean 3.6± 3.3 years after treatment, varying in the range of 0.2-13.0 years.

Table 1. Sex and age distribution of the study population by group

	Groups				
Variables	Symp VL n = 388	Rec VL n = 87	HHC n = 98	Total n = 573	p
Sex-Male n (%)	271 (69.8)	51 (58.6)	46 (46.9)	368 (64.2)	0.202(1)
Age (mean±sd)	21.2±18.4	14.6±15.4	23.7±19.2	20.6±18.3	**0.002**(2)

(1) Chi-Square = 18.791, 2 df p=0.202 (2)ANOVA F Test F(2, 570)=6.086; **p=0.002.**

Table 2 shows that 76% (n=266) of the Symp VL were positive in the *T. cruzi* assay, whereas 16.1% (n=14) and 8.3% (n=8), respectively, of the Rec VL and HHC were positive (p<0.0001). Thus, the sensitivity of *T. cruzi* assay in diagnosing SympVL was 76% and the specificity was 91.7%.

Table 2. Results of the *T. cruzi* assay among people exposed to *L. infantum* with distinct clinical outcomes

Results *T. cruzi*	Groups							
	Symp VL N (%)		Rec VL N (%)		HHC N (%)		Total* N (%)	
Positive	266	(76.0)	14	(16.1)	8	(8.3)	288	(54.0)
Negative	84	(24.0)	73	(83.9)	88	(91.7)	245	(46.0)
Total	350	(100.0)	87	(100.0)	96	(100.0)	533	(100.0)

Chi-Square Likelihood Ratio = 217.826, 2 df, p<0.0001. The results of the tests are heterogeneous among the phenotypes. * Samples which had indeterminate results were not considered, i.e., optic density close to the cut off value.

2. Analysis of Serological assays for Trypanosomatidae

A total of 98.6% (n=345) of the Symp VL were positive in the SLA ELISA, whereas 81.6% (n=71) and 60.4% (n=58), respectively, of Rec VL and HHC were positive (Table 3). The positive concordance between the two tests was greatest for the Symp VL group, with 74.9% of the 266 Symp VL positive in both methods, whereas the positive concordance for the Rec VL and their HHC were, respectively, 13.8% and 6.3%, p<0.0001.

Figure 2 shows that there is a positive correlation of the two tests (Pearson correlation=0.487, p<0.0001, n=573) with an increase in the level of anti-leishmania antibodies, *i.e*, there is more concordance between the two tests the higher the anti-Leishmania antibodies. Symp VL (green dots) present the higher concordance, followed by recovered VL (blue dots).

Table 3. Cross tabulation of the results of the *T. cruzi* and the SLA ELISA assays.

Groups	*T. cruzi* ELISA Assay	Soluble Leishmania Antigens ELISA Assay					
		Positive	n (%T)	Negative	n (%T)	Total	n (%)
Symp VL	Positive	262	74.9	4	1.1	266	76.0
	Negative	83	23.7	1	0.3	84	24.0
	Total	345	98.6	5	1.4	350	100.0
Rec VL	Positive	12	13.8	2	2.3	14	16.1
	Negative	59	67.8	14	16.1	73	83.9
	Total	71	81.6	16	18.4	87	100
HHC	Positive	6	6.3	2	2.1	8	8.3
	Negative	52	54.2	36	37.5	88	91.7
	Total	58	60.4	38	39.6	96	100.0

McNemar Test p<0.0001.Graycells show discordance between the two tests.

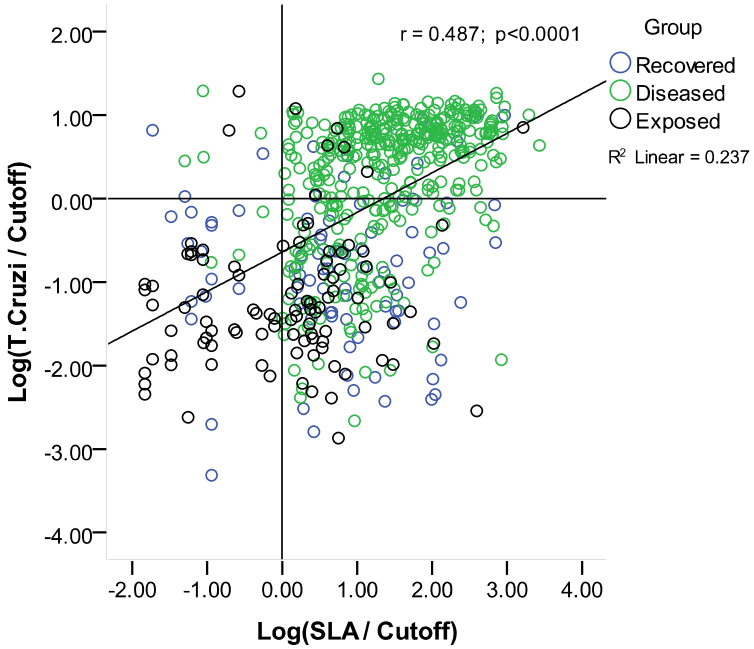

Figure 2. Results of the log of the(SLA ELISA/Cutoff) (X axis) versus the log of the(*T. Cruzi*/Cutoff)assay (Y axis). Values greater than 0 indicate that the assay is positive.

Relative values were used because of variation in the cut off value. Green dots= Symp VL; blue dots =Rec VL and black dots=HHC.

There was no difference in the positivity of the SLA-ELISA when considering gender (p=0.202), but there was a difference in the *T. cruzi* ELISA, with more males positive (p<0.0001) (Table 4). Using a logistic regression model and taking into account gender and the three groups, *i.e*, Symp VL, RecVL and HHC, it was observed that the risk of ascertaining the status as Symp VL was 32.8 times greater when the *T. cruzi* ELISA was positive (p<0.001) (Table 5).

Table 4. Results of serological assays for *T. cruzi* and *L. infantum* by sex

Serological ELISA Assay	Results	Sex				Total	
		Male N (%)		Female N (%)		N (%)	
Soluble Leishmania Antigens (SLA)	Positive	334	90.8	179	87.3	513	89.5
	Negative	34	9.2	26	12.7	60	10.5
	Total	368	100.0	205	100.0	573	100.0
T. cruzi							
	Positive	204	60.0	84	43.5	288	533
	Negative	136	40.0	109	56.5	245	46.0
	Total	340	100.0	193	100.0	54.0	100.0

SLA, (Chi-Square = 1.627, 1 df p=0.202); *T.cruzi*, (Chi-Square = 13.467, 1 df p<0.0001)

Table 5. Regression model considering the results of the two serological tests (SLA and *T. cruzi*) and groups.

Covariate	p	Odds Ratio Exp(β_1)	95% Confidence Interval	
			Lower Bound	Upper Bound
Symp VL/HHC	<0.001	32.8	15.2	70.6
Rec VL/HHC	0.134	2.0	0.8	5.1
Sex (Male)	0.089	1.4	0.9	2.3

Model: $\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1$Recovered VL + $\beta_2$symptomatic VL + β_3Male + error

Table 6 shows the logistic regression model, which took into account the log of the odds of the serological assays (SLA and *T. cruzi,* respectively) and as explanatory variables, the phenotype (Symp VL, Rec VL or HHC) and age (years). For the *T. cruzi* assay the positivity was higher for Symp VL (OR 39.1, CI 17.9-85.5, p<0.001) than for Rec VL (OR 2.2; p=0.053). The positivity of the assays increased with each additional year of the subject's age (p=0.006). Similar results were observed in the SLA assay, but with a decrease in the

positivity with age (OR=0.977; p=0.004). The odds ratio of SLA for Symp VL was 41.5 (CI 16.6-103.6).

Table 6. Parameter estimates of logistical models with the serological assay results as response and group status and age as predictors

Assay	Source	S.E.	p	Odds Ratio Exp(β)	95% Confidence Interval	
					Lower	Upper
T. cruzi	Symp VL/HHC	0.40	**<0.001**	39.14	17.91	85.51
	Rec VL/HHC	0.48	0.053	2.53	0.99	6.48
	Age (Years)	0.01	**0.006**	1.02	1.01	1.03
SLA	Symp VL/HHC	0.47	**<0.001**	41.55	16.65	103.65
	Rec VL/HHC	0.36	**0.016**	2.35	1.17	4.71
	Age (Years)	0.01	**0.004**	0.98	0.96	0.99

Model: $log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1$Recovered + β_2Symptomatic VL + β_3Age + error Household contact is the reference.

3. Anti-Leishmania antibodies and DTH responses

The mean (±SD) concentration of anti-leishmania antibodies [log(SLA/Cutoff)]for Symp VL, Rec VL and HHC were1.39 ± 0.83, 0.75 ± 1.11 and 0.02 ± 1.05, respectively. The DTH response was significantly different among the groups, with a greater response among the Rec VL (66.2%) than HHC (50.8%) or Symp VL (0.8%), (p<0.0001). As expected, the quantity of Leishmania DNA was higher in the Symp VL (Table 8).

Table 7 – Distribution of DTH response among groups

Results	Symp VL n (%)		Rec VL n (%)		HHC n (%)		Total n (%)	
Positive	1	(0.8)	45	(66.2)	30	(50.8)	76	(29.7)
Negative	128	(99.2)	23	(33.8)	29	(49.2)	180	(70.3)
Total	129	(100.0)	68	(100.0)	59	(100.0)	256	(100.0)

Chi-Square Likelihood Ratio = 130.88, 2 df, p<0.0001.

Table 8. Quantification of Leishmania DNA in the blood

Groups	PARASITEMIA Mean ± SEM	
	kDNA-7 (parasite⁄80ng DNA)	MAG-1 (parasite⁄80ng DNA)
Symp VL	329.8 ± 122.7	212.1 ± 88.5
Rec VL	48 ± 0.24	38 ± 18.0
HHC	13.1 ± 9.5	6.0 ± 5.8

SEM: Standard error of mean

DISCUSSION

Several studies have addressed the issue of cross reactivity between serological assays for Leishmania and *T. cruzi*.(Carvalho et al., 1987; Sabino et al., 2010; Otani et al., 2009). Caballero et. al. reviewed five commercially available ELISAs for *T. cruzi*, and Leishmania spp were the most common pathogens to cross react.(Caballero et al., 2007) In this work, we used one of the assays tested by Caballero (Chagas III) and examined individuals living in a region endemic for both Chagas disease and VL who were either Symp VL, Rec VL or exposed HHC. The *T. cruzi* ELISA assay was very sensitive in detecting Leishmania infection, but its concordance was greater with an increasing level of anti-Leishmania antibodies.

Although there have been available assays for the diagnosis of VL,(Matlashewski et al., 2013; Khanal et al., 2010; Chappuis et al., 2006; Braz et al., 2002) there is no gold standard. Parasitological confirmation by bone marrow or spleen aspirations is still widely used in most endemic countries to

confirm VL. In addition, in Latin America VL used to be a rural disease with sporadic cases presenting with prominent cachexia and high mortality (Badaró 1986, Evans et al, 1992). VL has emerged now as a disease of urban areas, (Costa et al., 1990; Jeronimo et al., 1994; Marzochi et al., 1994; Silva et al., 2001), concomitant with transmission to previously non-endemic areas in the south of Brazil and north of Argentina. (Salomon et al., 2008) Thus, there is a large population of potential Latin American blood donors who are at risk of Leishmania infection. But, based on previous studies (Caballero et al., 2007; Sabino et al., 2010) and the universal screening for *T. cruzi* in Brazil and other Latin American countries, we hypothesized that the risk of Leishmania transmission could actually be lower than expected since the *T. cruzi* ELISA assay could detect some of the Leishmania-contaminated blood. We observed that the *T. cruzi* ELISA assay had a sensitivity of 76% and specificity of 91.7% for identifying symptomatic VL, but the sensitivity was lower with lower levels of anti-Leishmania antibodies.

RIPA is the confirmatory test used in the United States for *T. cruzi* infection.(Shah et al., 2010) However, this assay is not available in Latin America, where there is a great need of a very specific assay if the first ELISA screening is positive. Recombinant proteins are being tested and might be an easier assay than immunofluorescence (Caballero et al., 2007) or RIPA,(Shah et al., 2010). There is high cross reactivity in secondary immunofluorescent assays, and no availability of RIPA in most endemic area and recombinant antigens might be an option.(Duthie et al, manuscript in preparation)

Although this study was not primarily an epidemiological one, we observed there was no gender difference in the level of antibodies detected by SLA ELISA, but for *T. cruzi*. The discordance for gender results could be due to the type of antibodies in accordance to disease status. In addition, there was an age difference between the two assays, that might be because the SympVL people were on average older than the Rec VL. Indeed, we have seen an increase in the age of VL in Brazil.(Nascimento et al, 2010) As we have previously reported, Leishmania infection tends to occur in the vicinity of the household, and Leishmania transmission by the sandfly vector is usually after sunset, when most families are at home with potentially equal exposure.(Jeronimo et al., 2004; Macedo-Silva et al., 2014)

The discordance between the two assays was greater for the Rec VL and HHC. A potential explanation for this discordance could be the IgG isotype switch from IgG1 to IgG2 that occurs after cure or self-resolution of Leishmania infection.(Bhattacharyya et al., 2014) We also have previously observed that during recovery from VL, there is an inverse correlation between the level of antibodies and the DTH response to Leishmania.(Miles et al., 2005), which reflects the switch in IgG type and development of a protective Th1 response.

Both qPCR assays were able to detect Leishmania DNA in the blood of all three groups studied. These two assays are very specific for *L. infantum* infection.(Weirather et al., 2011) However, the results of the tests depend on the quantity of DNA. The sensitivity of any molecular method technique to

diagnosis a pathogen improves with the amount of DNA.(Loonen et al., 2013) We had the limitation that most samples tested for culture or DNA were extracted from a small volume of blood (4-7 ml) and from a single blood draw. Therefore, we might have missed identifying parasites. However, quantitative PCR, if standardized, would be a more sensitive and specific assay, and easier to implement in most endemic areas, than culture.

CONCLUSIONS

The commercial *T. cruzi* assay (CHAGAS III) is sensitive for detecting *L. infantum* infection. The positivity of the test correlated with the level of anti-Leishmania antibodies. Similarly, the SLA ELISA assay could also detect occult *T. cruzi* infection. These findings indicate the need for better assays to rule out dual infection and to ascertain the correct diagnosis of either *L. infantum* or *T. cruzi* infections. Standardized qPCR might be an option that could be implemented to distinguish infection by either pathogen.

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CHAPTER 2

Serological Survey of Leishmania Infection in Blood Donors from an Endemic Area of Visceral Leishmaniasis in Brazil

TITLE PAGE

SEROLOGICAL SURVEY OF LEISHMANIA INFECTION IN BLOOD DONORS FROM AN ENDEMIC AREA OF VISCERAL LEISHMANIASIS IN BRAZIL

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CONFLICT OF INTEREST NOTIFICATION PAGE

There are no conflicts of interest to report.

ABSTRACT

MANUSCRIPT 2: Serological survey of Leishmania infection in blood donors from an endemic area of visceral leishmaniasis in Brazil

BACKGROUND. Asymptomatic Leishmania infection has increased in urban areas of Brazil, which pose a risk of Leishmania transmission through blood or organ transplant.

OBJECTIVES: to determine the prevalence of Leishmania infantum and *Trypanosoma cruzi* infections in blood donors of an endemic area for visceral leishmaniasis in Brazil.

METHODS. A cross sectional study was performed, with weekly recruitment, of volunteers, from February 2014 to January 2015. A total of 1556 participants who were donating blood in Natal, Brazil, were recruited. Participants answered a questionnaire related to exposure to Leishmania. Results of their blood screening were analyzed. Blood samples were tested for Leishmania infection using a soluble Leishmania antigens (SLA) ELISA assay. Descriptive statistics and graphs were used to describe distribution of continuous variables. *Likelihood Ratio Chi-Square* test evaluated the association between categorical variables. Simple and multiple regression models to evaluate the effect of covariates and factors on Leishmania response. The results of the SLA ELISA were log transformed.

RESULTS: A total of 51,463 people donated blood to public blood banks in 2014 and of these, 1554 (3%) were recruited into the study over a 12-month period. The majority of donors were males (82.4%) and married (51.8%). The mean (± sd) age of the participants was 35.6 ± 9.9, ranging between 16.1 to 69.6 years. The literacy rate was high with 68% of the participants had completed high school. The mean (± standard deviation) body mass index for males and females were, respectively, 27.92 ± 3.96 and 27.99 ± 4.38. The prevalence of *T. cruzi* and Leishmania were 0.19% and 2.5 %. Leishmania was isolated from one of the samples cultured (600).

CONCLUSIONS. There is risk of Leishmania transmission through the blood supply. It is warranted close monitoring of blood recipients in VL endemic areas, principally in those with some type of immunosuppression. *T. cruzi* ELISA assay can identify a subgroup of *L. infantum* contaminated blood, but does not exclude all Leishmania positive samples.

INTRODUCTION

Leishmania infantum is the major etiological agent for visceral leishmaniasis (VL) in Latin America and Europe. (Garnham, 1971; Belo et al., 2014) Changes in in the epidemiology of VL have occurred in the last 30 years with migratory movements, environmental changes, urbanization, global warming and HIV pandemic. (Daher et al., 2009; Desjeux, 2001a; Mott et al., 1990; Alvar and Jimenez, 1994; Nascimento et al., 2011; Fernandez-Guerrero et al., 1987) In Brazil, *L. infantum* is transmitted to vertebrates through the bite of female *Lutzomyia longipalpis*. Dogs are presumed to be the main reservoir of Leishmania, but the role of humans as reservoir has not been carefully considered in Europe or Latin America.(Ready, 2008; Deane, 1961)

Attempts to control human and canine VL have been aimed to culling of dogs, after the recognition of human disease, in a particular neighborhood. However, this has not proved to be effective in Brazil.(Courtenay et al., 2002; Costa et al., 2013; Nunes et al., 2008). Control measures are only launched when there are reports of human VL. Furthermore, there is a large variation in Leishmania incubation period in humans, *i.e.*, from infection to disease, varying from several months to years after infection, (Badaro et al., 1986a; Evans et al., 1992; Jeronimo et al., 2000). Therefore, the epidemiological milieu at the time that controls measures are implemented may differ from the time when Leishmania infection occurred. Moreover, the removal of infected dogs is sporadic and the burden of asymptomatic infections in either dogs or humans has not been taken into account.

Transmission of leishmania is usually thought to occur through the female sand fly vectors. However, vertical transmission in dogs, in the United States, (Boggiatto et al., 2011), and cases reports in humans, raises awareness about other routes of infection. There is an increased risk of Leishmania infection in household contact of a VL case (Evans et al, 1992). Infection through needle sharing was also a mode of Leishmania transmission in Spain, India and Latin America (Mestra et al., 2011; Dey and Singh, 2006; Le et al., 1999). In Europe, VL reemerged in HIV-infected/injection drug users who shared needles.(Alvar et al., 1989; Villanueva et al., 2000; Desjeux et al., 2001) These observations reinforce the need to better estimate alternative means of Leishmania transmission.

VL in Brazil used to be a disease of poverty and cases were sporadic occurring in rural areas of Northeast Brazil. (Deane, 1958) The risk factors for disease development included malnutrition, coinfections and immunosuppressive disorders. (Badaro et al., 1986c; Cerf et al., 1987; Desjeux, 2001b) The ratio of Leishmania infection to VL development was high then, (Badaro et al., 1986b), but lower in endemic areas in Europe. (Moral et al., 2002) Nevertheless, in Brazil, with improvement in public health care and in the economy that occurred in mid-1990's, decreased some of these risk factors for VL. (Maciel et al., 2008; Andrade et al., 2009; Lima et al., 1992; Lima Maciel et al., 2014)

Although asymptomatic *L. infantum* infection has increased in Brazil, with autochthonous of VL in all geographic regions of the country (Lima et al.,

2012; Gouvea et al., 2007) there has not been an increase in the incidence of symptomatic VL. (Maia-Elkhoury et al., 2008) In a preliminary study conducted in Natal,(Monteiro et al, 2015, submitted), we showed presence of viable Leishmania in blood collected from apparently health donors. Those samples had been discarded because of positivity for *T. cruzi* or because of insufficient volume. This finding led us to perform a serological survey of *L. infantum* to estimate the prevalence of infection by this pathogen in an endemic area for VL in Brazil. We also aimed to determine whether there was seasonality in Leishmania infection rate.

METHODS

Study Design and population

A cross sectional study was performed to estimate the prevalence of occult Leishmania infection in blood donors from Natal, state of Rio Grande do Norte, Brazil, an area endemic for VL.(Jeronimo et al., 1994; Jeronimo et al., 2004) Participants who were blood donors from the two main public blood banks in Natal were asked to participate into this study. The sample size was calculated based on prevalence of asymptomatic *L. infantum* infection as varying from 5-20%, 95% CI. However, as the prevalence in January 2014, was lower than estimated, we recalculated the sample based on a prevalence of 2%. A total of 1546 people were recruited over a 12 month period, from February 2014 through January 2015, with a mean weekly recruitment of 30 participants. We had permission of the donors to obtain the results of their

laboratory tests performed as part of the blood screening. People with hematocrit less than 40 or history of surgery in the past 12 months were excluded from donation. Participants were recruited into our study after being interviewed by the blood bank staff and after having had the hematocrit determined. Samples were collected at the end of their blood donation. Blood was collected in vacutainer tubes (Becton Dickinson) containing EDTA as anticoagulant (2 tubes) and no anticoagulant (1 tube).

Blood screening panel used in Natal, Blood bank.

All blood collected in Brazilian blood banks are screened by ELISA for anti-HIV, anti-HCV, anti-HBc, anti-HTLV1 and anti-*T. cruzi*. VDRL was determined by the flocculation method. In October 2014, molecular methods started to be implemented (NAT-HCV and NAT-HIV by PCR). However, we only used the serological results, since samples recruited in the first 8 months of the study were not screened using molecular methods. Natal is not endemic for malaria, so malaria screening is not in place in the blood bank. People with recent history of travelling to endemic areas for malaria in Brazil are usually excluded from donation.

Screening for Leishmania infection

Leishmania culture and speciation

Buffy coats were prepared from 4 ml of fresh blood by centrifugation at 1000 x g for 15 min. Twenty µl of buffy coat overlying erythrocytes was

inoculated into each of two tubes containing NNN blood agar or Schneider's medium. Capped tubes were cultured at 26°C, and samples were examined for growth every 3 days for up to 6 weeks. *Leishmania* isolates were typed by PCR, genotyping for ITS (Kuhls et al., 2005), following revised protocol.(Macedo-Silva et al., 2014)

Leishmania quantitative PCR

Quantitative PCR was used to estimate the Leishmania loads. Total DNA was extracted from 4-6 ml ml of blood.(Grimberg et al., 1989) All reactions were conducted on a 7500 Real Time PCR system from Applied Biosystems in a 10 µl reaction mixture, using Mag1 chromosomal, and kDNA7 kinetoplast DNA primers and probes.(Weirather et al., 2011) A standard curve was generated from DNA extracted from a known number of cultured *L. infantum* promastigotas, local isolate, ranging from 10^6 to 10^{-3} promastigotes per well. The cycle threshold (C_ts) was analyzed with SDS v1.3.1 software, with the threshold for fluorescence detection set manually at 0.2 for all reactions.

ELISA to detect anti-Leishmania antibodies

Anti-Leishmania antibodies were detected by ELISA using a soluble Leishmania antigen (SLA), a soluble lysate of promastigotes prepared from a local Leishmania isolate as previously described.(Braz et al., 2002) Briefly, plates were coated with 500 ng of SLA. They were blocked and incubated with

diluted blood. Anti-Leishmania antibodies were detected with a secondary anti-human IgG (Promega, Wisconsin, USA).

Rainfall data

The 3-year time series of rainfall data was obtained from the State of Rio Grande do Norte Agriculture Secretariat. The station is located in Natal, latitude -5.91 and longitude -35.2.

Statistical Analysis

Descriptive statistics and graphs were used to describe distribution of continuous variables. ANOVA and t-student tests were used to compare the means among independent groups. Two by two tables and the *Likelihood Ratio Chi-Square* test evaluated the association between categorical variables. Simple and multiple regression models evaluated the association of covariates and SLA response. The results of the SLA ELISA were log transformed and the cutoff value for each plate was considered. Microsoft Excel and SPSS (www.spss.com) were used for data analysis.

Ethical considerations.

The protocol was reviewed and approved by the Universidade Federal do Rio Grande do Norte Ethical Committee (CEP-UFRN, certificate of approval Nº12675013.7.0000.5537). All participants signed both the blood bank and our research consent form.

RESULTS

1. Characteristics of the blood donors

A total of 51,463 blood samples were screened in public blood banks of the state of Rio Grande do Norte, Brazil, between January 2014 to January 2015. Of those, 1544 (3%) were recruited into our studies and the majority of were males (82.4%) and married (51.8%). Of those, 45.1% had completed high school and 23% had a college degree or were college students, and only 0.3% were illiterate. They earned (82.4%) less than 3 Brazilian monthly minimal wages (approximate U$800), and 98.8%resided in the state of Rio Grande do Norte and had lived in the same municipality for over 5 years (87.5%). The majority (77%) had donated blood more than once. There was no difference in the BMI between male and females (p>0.05), but only 24.8% of the population had normal BMI, Figure 1. BMI increased with the age (Table 1), as expected.

Table 1. Statistics for BMI by age groups

Age Group	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
<= 28 years	360	27.11	4.02	26.69	27.52	18.93	48.45
28 --| 34 years	355	27.81	4.14	27.38	28.25	17.72	40.04
34 --| 42 years	395	28.19	3.80	27.81	28.56	19.93	42.94
> 42 years	385	28.57	4.06	28.17	28.98	18.42	46.61
Total	1495	27.94	4.04	27.73	28.14	17.72	48.45

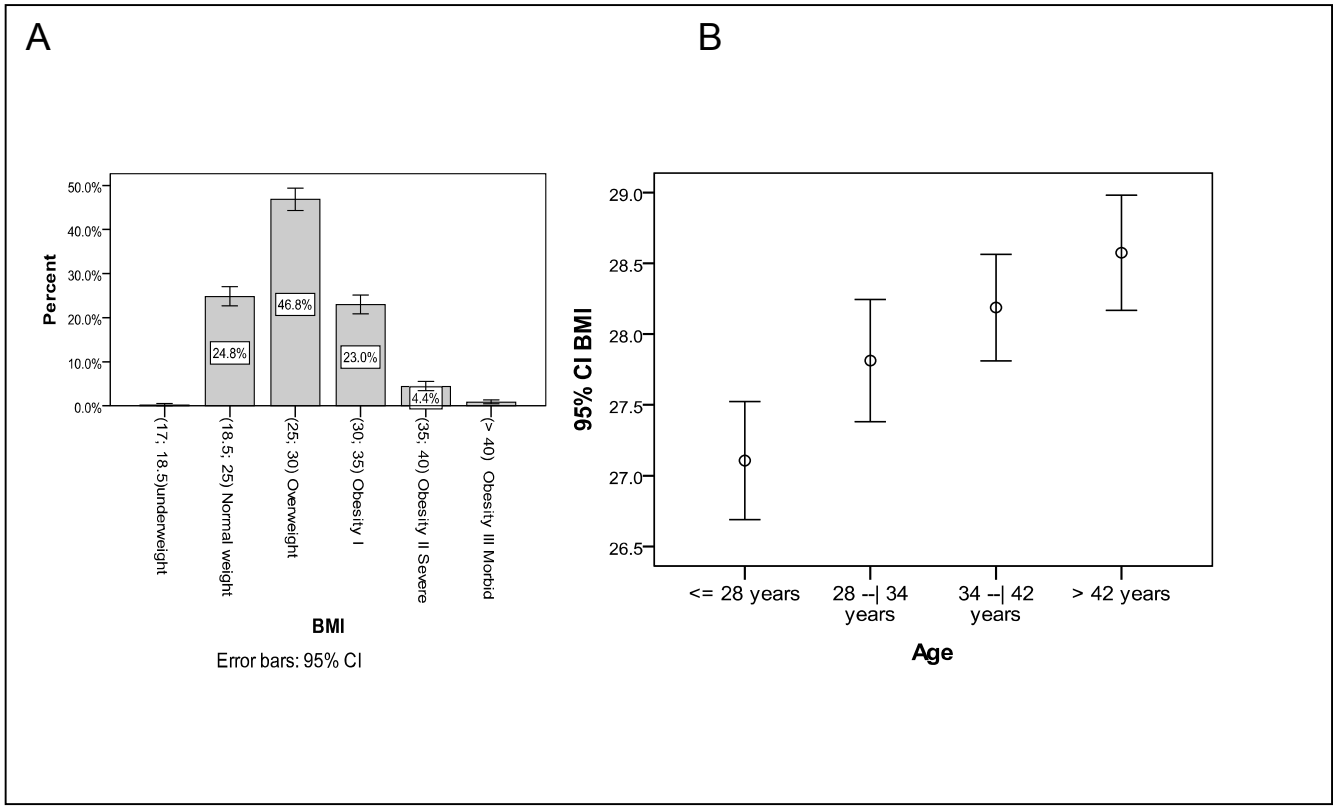

Figure 1. Distribution of the body mass index by category (A) and age group (B).

2. Results of the blood bank screening

The results of the blood screening assays for the entire overall population who donated blood in the state of Rio Grande do Norte and our sample populations are shown in Table 2. For any positive pathogen results, a second blood sample was requested, by the blood bank, and confirmatory assays were performed. We had no access to the second sample or to the confirmatory test results. Anti-HBc was the most prevalent positive serology in

both the general blood donor population (82.7 per 10,000) and our study sample (90.8 per 10,000, 95%CI[43.4; 138.1]), Table 2.; whereas anti-*T. cruzi* was 15.3/10,000 for the general population and 19.5 per 10,000 people in our sampling, 95%CI[0.0; 41.4].

Table 2. Results of the blood screening. The ratio infection rate is presented per 10,000 with the 95% confidence interval.

ScreeningTests	Population			Sample			95% Confidence Interval(2)	
	Positive	N	per(1) 10000	Positive	n	per 10000	Lower	Upper
Anti-HbC	426	51463	82.7	14	1542	90.8	43.4	138.1
VDRL	206	51463	40.0	4	1541	26.0	0.6	51.4
Anti HIV I/II (REC)	88	51463	17.1	3	1541	19.5	0.0	41.5
Anti T. cruzi	79	51463	15.3	3	1542	19.5	0.0	41.4
Anti HTLV I/II	69	51463	13.4	1	1542	6.5	0.0	19.2
Anti HCV	68	51463	13.2	2	1542	13.0	0.0	30.9
Anti HIV I/II (PS)	66	51463	12.8	0	1542	0.0	0.0	0.0
HbsAg	63	51463	12.2	5	1542	32.4	4.0	60.8

(1) Note that these values are within the 95% CI. (2) 95% CI for sample prevalence by 10,000.

3. Prevalence of Leishmania infection in blood donors from Natal,Brazil

The prevalence of Leishmania infection estimated by SLA ELISA was 2.5%, with 2.7% in males and in1.5% in females ($\chi^2(1)= 1.674$; p=0.196), Table 3. The quantitative results of the anti-leishmania antibodies, either positive or negative, are shown in Figure 2. Of those who were positive, the mean estimate of Leishmania by kDNA was 8.5 parasites/80 ng of total DNA (±7.5).

Table 3. Results of the anti-leishmania antibodies in blood donors

SLA ELISA	Sex				Total	
	Male n(%)		Female n (%)		n (%)	
Negative	1238	97.3	267	98.5	1505	97.5
Positive	35	2.7	4	1.5	39	2.5
Total	1273	100.0	271	100.0	1544	100.0

$\chi^2(1)= 1.674$; p=0.196

We log transformed the results of the SLA ELISA, after dividing its absorbance by the cut off value of the test. Figure 3 shows the distribution of the logSLA ratio, a value greater than 0 indicates that the test was positive. There was no difference in infection rate between sex (p=0.963).

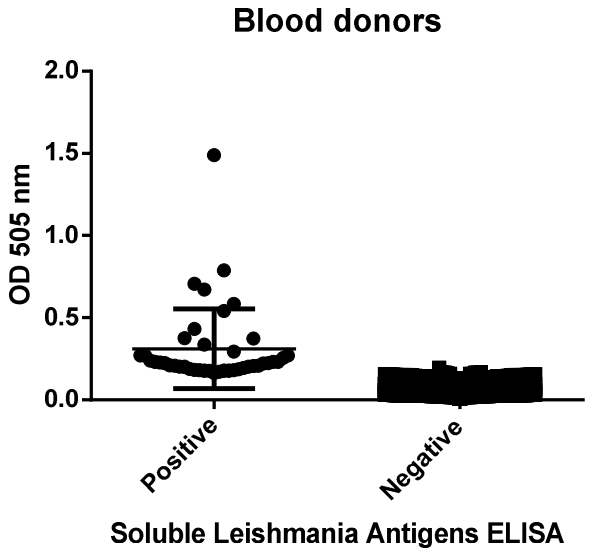

Figure 2. Quantitative results of the SLA ELISA, stratified by positive positive and negative values.

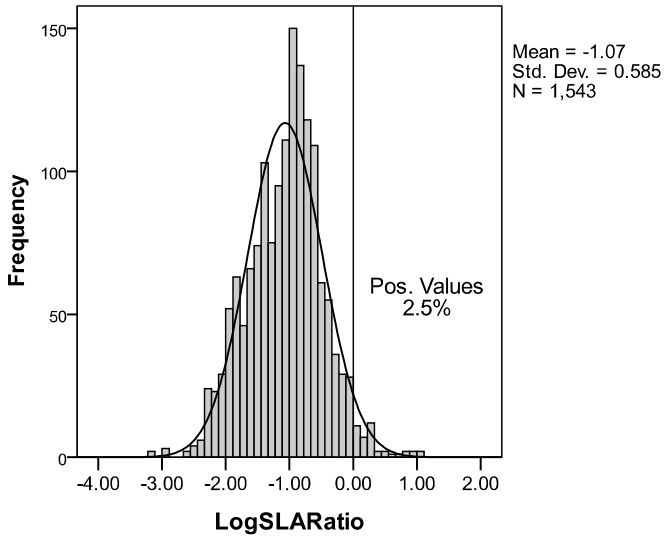

Figure 3. Frequency Distribution of LogSLARatio

The concordance of SLA and *T. cruzi* was high, with 97.3% (1500) negative for both assays (Table 4). The prevalence of *T. cruzi* was 0.19% (3/1542). One sample was both *T. cruzi* and SLA positive. Of the samples cultured (n=600), one was positive and the pathogen isolated was typed as *L. infantum*. This blood sample was also positive for anti-Leishmania antibody.

Table 4. Cross tabulation of SLA ELISA with *T. cruzi* ELISA

Anti *T. cruzi*	SLA				Total N (%)	
	Positive N (%)		Negative N (%)			
Negative	39	(2.5)	1500	(97.3)	1539	(99.8)
Positive	1	(0.1)	2	(0.1)	3	(0.2)
Total	40	(2.6)	1502	(97.4)	1542	(100.0)

McNamar Test, p<0.0001

3. Seasonal variation of Leishmania infection

Figure 3A shows the quarterly rainfall in Rio Grande do Norte, and Figure 3B shows the LogSLA ratio with the 95% confidence interval for the Leishmania seroprevalence by quarter over the span of a year. There is a

noticeable intra-annual variation, with an increase in Leishmania seropositivity between the months of April-September (quarters 2 and 3). We used a linear model considering the LogSLA ratio as the response and the quarters as predictor. A significant variation of LogSLA ratio was observed depending on quarter ($F(3, 1549) = 14.1$, $p<0.001$) with increased prevalence of Leishmania infection between April-June ($\beta=0.299$; $p<0.0001$) and for July-September when October-December was used as reference, Table 5.

Table 5. Parameter estimates of Leishmania infection along the year, using a linear model considering the Log SLA ratio as the Dependent Variable

Parameter	B	Std. Error	t	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	-1.291	.030	-43.278	<0.0001	-1.350	-1.233
[Quarter=1]	0.083	.044	1.881	0.0600	-0.004	0.170
[Quarter=2]	0.409	.039	10.387	**<0.0001**	0.332	0.486
[Quarter=3]	0.299	.041	7.372	**<0.0001**	0.220	0.379
[Quarter=4]	reference	.	.	.	.	.

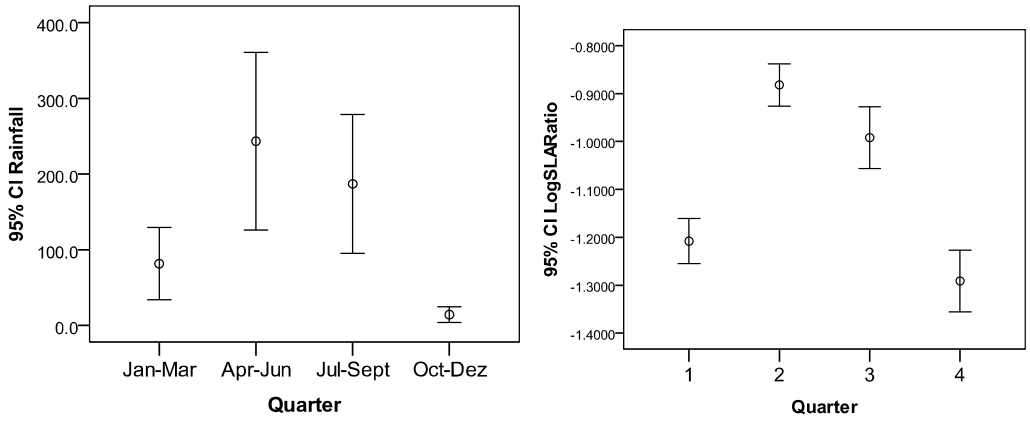

Figure 4 – Rainfall per quarter for the state of Rio Grande between January 2012 to February 2015 (A) and the Log of the SLA/Ratio, between December 2013 to January 2015, with 95% confidence interval (B).

The temporal relation of rainfall index with Leishmania seropositivity detected over calendar year 2014 is shown in Figure 4. There is a lag between detection of Leishmania infection and the increase in rainfall.

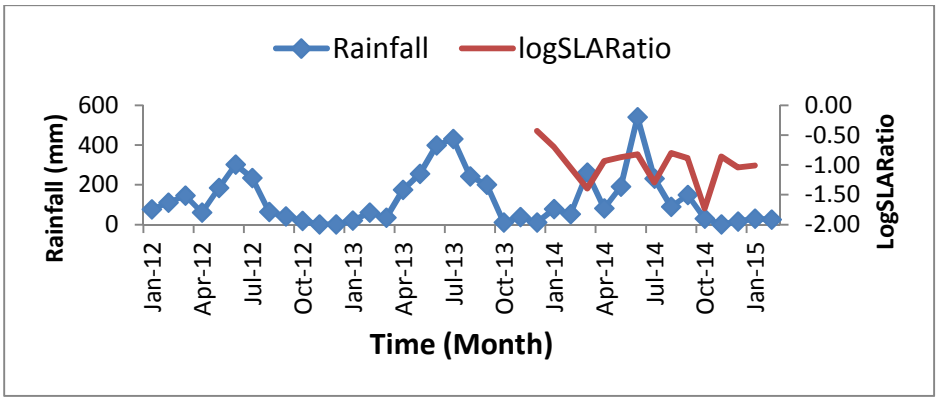

Figure 5. Temporal variation in rainfall from January 2012 to February 2015 and the LogSLARatio from December2013 to January 2015.

4. Risk factors for Leishmania infection

Several putative risk factors for Leishmania infection, including history of VL in the family, presence of dogs in the house or in neighborhood, history of Chagas disease in the family, presence of dogs, but they were not associated with Leishmania seropositivity. The only factor associated was history of travel outside of their municipality with an odds ratio of a positive SLA of 2.2 (P = 0.021) (Table 6).

Table 6. Logistic Regression Models of Potential risk factors for leishmania infection

Risk/n of response	SLA		Significance		
	Positive n (%)	Negative n (%)	OR	SE	p
History of travel (n=1537)	25 (65.8)	696 (46.4)	2.22	0.035	**0.021**
History of VL in the family (n=1548)	-	19 (1.3)	-	-	0.998
History of Chagas disease in the family (n=1534)	1 (2.6)	42 (2.8)	0.94	1.03	0.948
Presence of dogs in the household (n=1548)	20 (52.6)	688 (44.7)	1.37	0.33	0.334
Presence of dogs in the neighborhood (n=1530)	27 (71.1)	989 (66.3)	1.25	0.36	0.540
Resided in the same house Chagas Case (n=1042)	-	26 (2.6)	-	-	0.998
Reduvidae bug in the residence (n=1133)	2 (5.6)	60 (5.5)	1.02	0.74	0.982
Alcohol intake (n=1529)	8 (21.1)	526 (35.3)	0.49	0.40	0.075

DISCUSSION

The safety of the blood supply is a challenge everywhere. This task becomes more complex, with the emergence or re-emergence of pathogens, which warrant a need for setting up new techniques for blood

screening.(Salunkhe et al., 2015; Leparc, 2015) Introduction of molecular techniques to ascertain presence of pathogen, in addition to antibody responses, has been an important improvement in safety.(Leparc, 2015) Policies regarding the exclusion of certain groups as a result of the HIV pandemic have been questioned.(Brailsford et al., 2015) However, the ease of travelling and migratory movements nowadays, with changes in what used to be traditional endemic areas, signal the need of continuous surveillance and implementation of new screening methods for blood products. We aimed to estimate the burden of Leishmania infection in blood donors residing in an endemic area for VL in Brazil.(Mott et al., 1990; Gratz, 1999; Nascimento et al., 2008) We found that the prevalence of Leishmania infection was 2.5% based on the antibody response, with one sample blood sample of 600 tested that had viable Leishmania confirmed by growth of leishmania in culture.

Malnutrition used to be an important health problem in Brazil, increasing the risk of developing symptomatic infectious diseases, (Badaro et al., 1986c; Harrison et al., 1986; Cerf et al., 1987; Lima et al., 1992). Several studies show the association of malnutrition with development of VL.(Harrison et al., 1986; Cerf et al., 1987) However, more recently Brazil has gone through a transitional phase in which obesity has become a public health issue (Strufaldi et al., 2008; Hallal and Irwin, Jr., 2012), and the burden is greater in people from a lower socioeconomic stratum.(Lyra et al., 2012) Our data reflects this transition in nutrition status, in which 75% of our study participants were in the group of overweight (46.8%) or obesity 1 (23.0%) or Obesity 2 (4.4%).

The majority of our donors were males, reflecting the overall pattern of blood donation in Brazil, where males more often donate blood. About one third of donors came to the blood bank to help a friend or a relative who needed blood. This is a common practice in Brazil, but there is also encouragement from the health sector for this practice, aiming to keep an adequate blood supply level. There is no compensation for blood donation in Brazil. This policy was started in early 1970's to decrease the likelihood of underprivileged people donating blood multiple times because of the compensation. (Andrade et al., 2009)

The overall prevalence of *T. cruzi* infection in the donors was 0.15%, with 40% of the cases positive from the Natal area. Half of those positive cases were low-reactive (data not shown). Low levels of antibodies are a common finding in many endemic areas for Chagas disease and the significance of this is not entirely understood.(Remesar et al., 2009; Remesar et al., 2015). This could reflect either decrease in the burden of infection or an evolving one. In either situation, close follow-up is needed. About half of the samples that tested positive for Chagas came from the Natal area, which is not endemic for Chagas, but for *L. infantum*.(Jeronimo et al., 2004; Lima et al., 2012) However, since there has been migration from the rural areas endemic for Chagas disease to Natal, we could not exclude that some of these positive samples would have dual infection. Natal area is highly endemic for VL and in focal areas where there has been either human or canine VL the rate of asymptomatic infection can be as high as 40%. (Jeronimo et al, 2004; Lima et

al, 2012) At any rate, there is a need of a better confirmatory assay for both *L. infantum* and *T. cruzi*. Careful monitoring of cardiac function is warranted. Ribeiro et al, found electrocardiographic (ECG) abnormalities more frequently in *T. cruzi* seropositive than in seronegative blood donors. The ECG abnormalities could help the recognition of seropositive cases, who warrant long-term careful follow-up.(Nascimento et al., 2012; Ribeiro et al., 2013)

We first determined the sample size with an estimate based on a previous study around Natal. (Lima et al, 2012). However, because of the low detection rate observed in the first two months of the study, we increased the sample size. This shows the importance of close monitoring of cross-sectional studies of vector-born diseases.

Leishmania transmission depends on vector density, which is influenced by environmental factors. Several studies have addressed the seasonality of VL, but there is not to our knowledge studies showing the seasonality of infection with *Leishmania infantum*. Leishmania infection tends to occur in focal areas, and the presence of canine leishmaniasis influences the level of infection in humans.

We collected blood samples, weekly, aiming to identify any potential variability in infection along the year. Studies on El Niño have shown implications in diseases such as malaria, (Gagnon et al., 2002), and leishmaniasis (Chaves et al., 2014; Franke et al., 2002). We observed a discrete lag between detection of Leishmania infection and increase in rainfall. However, we had only monitored for 12 months, not long enough to confirm

this trend. More observations are needed, with surveillance of greater than a 5 year cycle, to accurately determine the pattern of Leishmania transmission. Finally, the fact that we successfully cultured Leishmania from one blood donor, and having obtained it from a small sample of blood, confirms that there is a true risk of Leishmania transmission through the blood supply.

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