Frequency of *Trypanosoma cruzi* parasitemia among infected blood donors with a potential association between parasite lineage and transfusion transmission

David A. Leiby,[1] Megan L. Nguyen,[1] Melanie C. Proctor,[1] Rebecca L. Townsend,[2] and Susan L. Stramer[2]*

BACKGROUND: *Trypanosoma cruzi* is endemic to the Americas where it demonstrates multiple lineages over a vast geographic range (i.e., United States to Argentina). These lineages possess divergent geographic and biologic characteristics, including variations in disease manifestations. Herein, we report the frequency of parasitemia among seropositive US blood donors and the potential association between parasite lineage and transfusion transmission.

STUDY DESIGN AND METHODS: Blood donors identified as *T. cruzi* seropositive during screening were enrolled in follow-up studies, including hemoculture testing and a risk factor questionnaire. Positive hemocultures were expanded to obtain sufficient parasites for molecular lineage determination and analysis. Country of birth, obtained from the questionnaire, was used to predict parasite lineage in the absence of demonstrable parasitemia for infected donors.

RESULTS: Eighteen (6.8%) of 263 seropositive donors were hemoculture positive. Among the 17 hemocultures expanded for lineage determination, TcV was identified more frequently (n = 12), compared to TcI (n = 2), TcII (n = 1), and TcVI (n = 2). When presumptive parasite lineages were compared to hemoculture results, only two of 157 (1.3%) TcI versus 13 of 38 (34.2%) TcII/TcV/TcVI non-US donors were parasitemic; three of 44 (6.8%) US donors were TcV or TcVI.

CONCLUSIONS: Based on lineage determination for donors with parasitemia; hemoculture positivity associated with presumptive parasite lineage; and implicated donors from US, Canadian, and Spanish transfusion cases, donors from Southern South America are significantly more likely to have parasitemia and transmit infection to blood recipients (TcII, TcV, or TcVI vs. TcI). Thus, parasite lineage may be associated with risk of transfusion-transmitted *T. cruzi*.

Trypanosoma cruzi, the etiologic agent of Chagas disease, is naturally transmitted to humans after contact with the feces of an infected triatomine insect vector. *T. cruzi* and a variety of competent vectors are endemic to the Americas, specifically portions of Central America, South America, and southern North America including Mexico, where it is estimated that 8 to 10 million people are infected with the parasite.[1] After a short-lived, relatively mild acute disease phase, most infected persons enter a lifelong indeterminate chronic phase characterized by elevated serologic titers and low-level intermittent parasitemia. Among those chronically infected, 20% to 30% will develop clinical manifestations of Chagas disease, including cardiac and gastrointestinal complications.[2,3]

In addition to vectorial transmission, *T. cruzi* is also transmitted to humans congenitally, orally, through organ transplant, and by blood transfusion. For several

ABBREVIATIONS: ARC = American Red Cross; CFS = Chagas follow-up study; COB = country of birth; DTU(s) = discrete typing unit(s); LIT = liver infusion tryptose; RR = repeat reactive.

From the [1]Transmissible Diseases Department, American Red Cross Holland Laboratory, Rockville, Maryland; and the [2]Scientific Support Office, American Red Cross, Gaithersburg, Maryland

Address reprint requests to: David A. Leiby, Division of Emerging and Transfusion Transmitted Diseases, FDA/CBER/OBRR, 10903 New Hampshire Avenue, Silver Spring, MD 20993; e-mail: david.leiby@fda.hhs.gov.

**Present address:* Food and Drug Administration, Silver Spring, Maryland.

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nonendemic countries, including the United States, transfusion-transmitted *T. cruzi* emerged as a blood safety concern during the past 30 years due to the increased emigration of *T. cruzi*-infected persons from endemic areas of Latin America.[4] Based largely on documented US transfusion cases,[5-9] seroprevalence studies that demonstrated significant rates of infection among selected blood donor populations,[10-12] and projected transmission rates in the United States of 12% to 20% derived from historical South American data,[13-16] licensed blood donor screening for *T. cruzi* was implemented in January 2007. While blood screening results validated previous seroprevalence estimates, recipient tracing studies of prior transfused components from seropositive donors (i.e., lookback) suggested that in contrast to South American data, transfusion transmission in the United States occurred relatively infrequently. Indeed, despite testing 259 recipients of blood products from seropositive donors, only two recipients (0.8%) who received blood products from the same donor became infected via blood transfusion.[17,18]

Explanations provided for this apparent discrepancy in transmission rates include a parasite predilection for platelet (PLT) products,[19] chronologically remote infection among seropositive donors, and asymptomatic infections with low to intermittent parasitemia, but none of these provide a clear and compelling answer. While most transmission cases have implicated PLT products, a recently published case from Belgium indicates that red blood cells (RBCs) may also play a role, albeit limited.[20] The observation that Spain has reported higher relative rates of transfusion transmission than the United States suggests the need to examine alternative explanations.[21] Latin American emigration to Spain primarily involves persons from South America as opposed to the Central American and Mexican immigrants predominantly seen in the United States. Moreover, transfusion cases in the United States and Canada have predominantly implicated infected donors from the Southern Cone of South America.[22] Recent evidence suggests that Chagas disease manifestations, which have been observed to vary geographically, may in part be predicated on the infecting strain or lineage of the *T. cruzi* parasite.[23-25] Thus, our working hypothesis was that parasite lineage associated with South American isolates may be a contributing factor to the likelihood of transfusion transmission. To determine if the donor's country of origin and concomitant strain of parasite influence rates of transmission, we tested seropositive donors by hemoculture to determine those persons most likely to be parasitemic and capable of transmitting *T. cruzi* by blood transfusion in comparison to their country of birth (COB) or residence. To further validate any strain differences, parasite isolates were typed molecularly to confirm their lineage and likely historical geographic association.

MATERIALS AND METHODS

Study population

During the period from January 29, 2007, to August 31, 2011, more than 21 million blood donations from the American Red Cross (ARC) and its contract partners were screened for *T. cruzi* antibodies. All index donations were screened serologically using the following testing algorithm. Index plasma donations were screened by enzyme-linked immunosorbent assay (ELISA) for *T. cruzi* antibodies using the Food and Drug Administration–licensed *T. cruzi* test system (Ortho Clinical Diagnostics) and if found to have a signal to cutoff ratio of more than 1.00 were interpreted as initially reactive and retested in duplicate. If reactive in one or both duplicate retests, the sample was interpreted as repeat reactive (RR) and subsequently tested using a research-based radioimmunoprecipitation assay (RIPA; Quest Diagnostics). Samples demonstrating reactivity with glycoproteins at 72 and 90 kDa on RIPA were considered confirmed positive for *T. cruzi* antibodies and the associated donor invited to join the Chagas follow-up study (CFS); the goal of the CFS was to further characterize the donors' infection status. At the time of CFS enrollment, study subjects were administered informed consent, completed an ARC risk factor questionnaire, and provided two 10-mL clot tubes for serologic testing by the Ortho ELISA and RIPA, two 6-mL EDTA tubes for real-time polymerase chain reaction (PCR) testing, and three 10-mL heparin tubes for hemoculture testing, the focus of this study. All donors found RR for *T. cruzi* antibodies were informed of their test results, given information about the test's meaning, deferred permanently from donating blood, and if appropriate, provided contact information for medical follow-up. The study was reviewed and approved by the ARC Institutional Review Board (IRB) before initiation.

Hemoculture and parasite expansion

The three heparin tubes obtained from each enrolled CFS donor were mixed by inversion, pooled into a sterile 50-mL conical tube, centrifuged at $2800 \times g$ (4°C) for 30 minutes and the plasma was removed by pipette leaving the buffy coat and RBC layers intact. Twenty-five milliliters of liver infusion tryptose (LIT) medium was added to the conical tube, mixed by inversion, centrifuged at $2800 \times g$ (4°C) for 30 minutes, and the supernatant was again removed by pipette. The remaining buffy coat layer and RBC pellet (top and bottom layers) were removed by pipette and added to three separate 15-mL conical tubes containing 6 mL of LIT medium. The tubes were sealed, mixed by inversion, and stored in a 27°C incubator. Wet mount slide preparations (10-μL) from each hemoculture tube were observed by microscopy (40×) for motile parasites beginning at 6 weeks of culture and every 2 weeks thereafter until positive or until 16 weeks if they remained

negative. Hemocultures were maintained at 27°C throughout and mixed by inversion biweekly.

For parasite expansion, a 50-μL sample was removed from each positive hemoculture, added to a 25-cm^2 cell culture flask containing LIT medium, and observed weekly for parasite replication. Upon strong initial growth, the culture was transferred to liver digest neutralized tryptose medium and expanded in 75-cm^2 cell culture flasks to obtain sufficient quantities of parasites for molecular lineage determination.

Lineage determination by PCR

For lineage determination, a 1-mL aliquot was removed from the expanded parasite cultures maintained in liver digest neutralized tryptose, mixed with an equal volume of GE (6 mol/L guanidine HCl, 0.2 mol/L EDTA) lysis buffer, vortexed, and stored for at least 24 hours at room temperature. Before DNA extraction, the sample was boiled for 10 minutes and cooled to room temperature. DNA was extracted from 200 μL of each sample using a DNA blood mini kit (QIAamp, Qiagen) as per the manufacturer's instructions. Ten microliters of extracted parasite DNA was run in six separate reactions using primers p3/p6, p7/p8, p9/p10,[26] D71/D72, TC/TC1/TC2, and V1/V2[27] with 25 μL of DNA polymerase (AmpliTaq Gold 2×, Applied Biosystems) for a final volume of 50 μL. Amplified products were separated by electrophoresis and visualized under ultraviolet light, and lineage was determined as described by Brisse and colleagues.[26]

A no-template control, negative extraction control, and a spiked positive (*T. cruzi* parasites) extraction control were included in each run. Negative controls were obtained from ARC Research Blood Program under IRB approval; research donors had no risk factors for Chagas (e.g., birth, travel, or residence in an endemic country). Positive controls were obtained by spiking negative control blood with serial dilutions of culture derived *T. cruzi* trypomastigotes.

Questionnaire data

At the time of enrollment in the CFS, study subjects were requested to fill out a risk factor questionnaire. The questionnaire asked them to provide their sex; pregnancy status (if applicable); age; city of birth, state of birth, and COB; if they have any children; ethnicity; current country of residence; any previous residences in other countries (≥ 1 year); if they have lived in a house with dirt floors or walls or a thatched roof or in a rural area; or if they have received a blood transfusion, had ever been bitten by a kissing bug (i.e., reduviid bug), or had been previously infected with *Leishmania* sp. Additionally, study subjects were queried about the residence(s) of their mother and maternal grandmother. Health questions were asked about the donor and donor's mother and family including swelling of feet or ankles, difficulty breathing while laying down, heart irregularities, trouble swallowing, intestinal issues, and if they have ever been to the doctor for these issues. If a questionnaire was incomplete, further information was obtained by contacting the donor via telephone or through the blood donor records.

TABLE 1. Hemoculture testing results with sex and age comparisons

Test result	Number (%) n = 263	Sex		Age (years)		
		Male	Female	Mean	Median	Range
Positive	18 (6.8)	13	5	48	47	22-71
Negative	245 (93.2)	106	139	43	42	16-83

Statistical analysis

Statistical differences in age and sex for hemoculture-positive donors, and parasite lineage and hemoculture positivity were determined using chi-square statistics. The frequencies of observed parasite lineages were compared using a one-sided binomial exact test. A p value of less than 0.05 was considered significant (R v 3.3.0, R Foundation for Statistical Computing).

RESULTS

A total of 22,250,104 blood donations were tested for *T. cruzi* antibodies of which 2933 (0.01%) were RR. RR samples were subsequently tested by RIPA; 717 (24.4%) were confirmed positive for an overall seropositive rate of one in 31,032. All confirmed seropositive donors (n = 717) were invited to participate in the CFS described previously and 289 (40%) donors completed enrollment. Herein we report only data associated with hemoculture testing, subsequent lineage determinations, and the risk factor questionnaire.

As indicated in Table 1, 263 of the 289 (91%) seropositive donors enrolled in the CFS were tested by hemoculture for *T. cruzi* parasites. Eighteen (6.8%) of 263 donors were observed to be hemoculture positive, with 17 of 18 identified as positive on the first follow-up sample; one hemoculture-positive donor was identified on the second follow-up sample. While the majority of hemoculture-positive donors were male (13 of 18, 72%), no significant differences were observed for sex comparisons (Table 1). Similarly, donors with positive hemocultures were slightly older than those with negative hemocultures (mean of 48 years vs. 43 years), but this difference was not significant.

Parasite cultures for 17 of the 18 hemocultures were successfully expanded for subsequent lineage determination and analysis. Results for lineage determinations and associated donor demographic data are listed in Table 2. TcV represented the predominant parasite lineage (12 of 17, 71%), which was observed at a significantly ($p < 0.0001$)

TABLE 2. Index collection date, parasite lineage determination, and donor demographic information associated with positive hemocultures

Isolate	Index collection date	Parasite lineage	City/COB	Age (years)	Sex	Ethnicity
1	Feb 20, 2007	TcVI	Baysvillage, OH	40	Male	Caucasian
2	Feb 22, 2007	TcV	Cochabamba, Bolivia	52	Male	Hispanic
3	Mar 7, 2007	TcVI	Neuland, Paraguay	45	Male	Caucasian
4	Mar 24, 2007	TcI	Manta, Colombia	60	Male	Hispanic
5	May 3, 2007	TcV	Las Heras, Argentina	71	Male	Hispanic
6	Feb 18, 2008	TcV	Cochabamba, Bolivia	39	Male	Hispanic
7	Mar 7, 2008	TcV	Santa Cruz, Bolivia	42	Female	Hispanic
8	Jul 24, 2008	TcV	Cochabamba, Bolivia	68	Female	Hispanic
9	Sep 10, 2008	TcV	Washington, DC	29	Male	Hispanic
10	Sep 24, 2008	TcV	Santiago, Chile	60	Male	Hispanic
11	Nov 8, 2008	TcII	Santa Cruz, Bolivia	54	Male	Hispanic
12	Nov 16, 2008	TcV	Bolivia	49	Female	Hispanic
13	Feb 26, 2009	TcI	San Miguel, El Salvador	45	Male	Hispanic
14	Mar 1, 2009	TcV	Cochabamba, Bolivia	22	Male	Hispanic
15	Jun 28, 2009	TcV	La Paz, Bolivia	44	Female	Hispanic
16	Aug 15, 2009	TcV	Philadelphia, PA	32	Male	Hispanic
17	Jun 9, 2011	TcV	Concepcion, Chile	48	Male	Hispanic

TABLE 3. Comparison of hemoculture results with predicted *T. cruzi* lineage based on COB

Predicted lineage based on COB	Hemoculture Results		
	Number (%)	Negative	Positive (%)
TcI	157 (59.7)	155	2 (1.3)
TcII, TcV, and TcVI	38 (14.4)	25	13 (34.2)*
United States	45 (17.1)	42	3 (6.7)
Other or unknown	23 (8.7)	23	0
Totals	263	245	18† (6.8)

* Significantly different compared to donors with a predicted TcI lineage ($p < 0.0001$).

† COB (number positive) for 18 hemoculture positives = Bolivia (8), United States (3), Argentina (2), Chile (2), Colombia (1), El Salvador (1), and Paraguay (1).

higher frequency when compared to TcI (n = 2), TcII (n = 1), and TcVI (n = 2). In addition, parasite isolates obtained from a donor–recipient pair in a previously published transfusion case[8] were investigated to determine lineage type and related demographics; both typed as TcV. Overall, for each parasite lineage determination, the observed lineage agreed directly with the predicted parasite lineage based on the donor's known COB with the exception of the isolates obtained from US-born donors (n = 3) and the infected transfusion recipient (also US born) mentioned above.

To better understand the impact of parasite lineage on the likelihood of parasitemia in seropositive blood donors (n = 263), we compared the predicted parasite lineage based on the donor's COB with observed hemoculture results (Table 3). Based on a predicted parasite lineage of TcI, found primarily north of the Amazon Basin including Central America and Mexico, we observed only two of 157 (1.3%) donors with positive hemocultues. In contrast, for those donors with a predicted parasite lineage of TcII, TcV, and TcVI, found primarily in association with the Southern Cone of South America, a significantly ($p < 0.0001$) greater number of donors, 13 of 38 (34.2%), were observed to be positive. Of interest, three of 44 (6.8%) donors born in the United States were positive by hemoculture, but all three isolates typed as TcV or TcVI, isolates associated only with the Southern Cone of South America. For 8.4% of donors (n = 22) we could not predict the *T. cruzi* lineage since their COB was not known; all 22 were negative by hemoculture.

DISCUSSION

The endemic range of *T. cruzi* stretches from the southern United States to Patagonia in Argentina. Along this vast geographic area the parasite is associated with a complex zoonotic disease in humans and animals, implicates over 70 genera of mammals as reservoir hosts, and is transmitted by a variety of hematophagous triatomine insect species. It also has been recognized that over this extended geographic range *T. cruzi* demonstrates diversity within its genome, leading to multiple genotypes and phenotypes.[25] Beginning in 2000, six discrete typing units (DTUs) were designated for *T. cruzi* that were subsequently renamed in 2009 and now correspond to TcI to TcVI.[26,28] These DTUs demonstrate divergent geographic and biologic characteristics that include variations in disease manifestations,[23,29] which may have important implications for transfusion transmission of *T. cruzi*.

TcI is the most abundant and widely distributed *T. cruzi* DTU in the Americas, causing both sylvatic and domestic infections. Although the sylvatic form of TcI stretches from the southern United States to northern Chile and Argentina, domestic infections that produce cardiomyopathy in humans are almost exclusively limited to Mexico, Central America, and portions of northern South America. There have been relatively few reports of human

infection caused by TcI south of the Amazon Basin.[25] TcII, TcV, and TcVI are all localized to southern South America (i.e., the Southern Cone), often with overlapping and indistinguishable geographic ranges. TcII is predominantly found in central and southern regions of South America where it causes cardiac disease manifestations in humans, occasionally with concomitant megaesophagus and megacolon, but sylvatic infections are rare. Likewise, TcV and TcVI occur in similar areas of southern South America and are also associated with cardiomyopathy and mega syndromes in humans; however, they are virtually unknown as sylvatic isolates. The remaining two DTUs, TcIII and TcIV, are almost exclusively linked to sylvatic infections and have minimal impact on the current discussion.[25]

In this study, 18 (6.8%) of 263 seropositive donors tested by hemoculture were demonstrably parasitemic. By definition, for transfusion transmission to occur, peripheral blood collected from donors must contain viable parasites. Among the 18 parasitemic donors, 13 were born in countries associated with southern South America, three were US born, and one each was born in Colombia and El Salvador. When typed molecularly, 15 of the 17 isolates (12 from South America and three from the United States) were identified as TcII, TcV, or TcVI, DTUs associated with the Southern Cone of South America. The remaining two isolates that typed as TcI, the parasite lineage generally found north of the Amazon basin, were consistent with the donors' birth in Colombia and El Salvador. Isolates associated a donor–recipient pair from a published transfusion transmission both typed as TcV.[8]

These data suggest that donors from southern South America (i.e., those typed as TcII, TcV, or TcVI) are more likely to be parasitemic than donors from north of the Amazon basin (i.e., TcI type). To extend this observation, we analyzed all donors who were tested by hemoculture (positives and negatives) to determine the frequency of parasitemia in association with predicted parasite lineage based on donor COB. Among the 261 donors tested by hemoculture for whom COB was known, 157 (60.2%) were from countries associated with TcI. While this group represents the vast majority of tested donors, only two of 157 (1.3%) were hemoculture positive. In contrast, while only 38 donors (14.6%) were from southern South American countries associated with TcII, TcV, and TcVI, 13 of 38 (34.2%) were hemoculture positive. Additionally, we tested 44 donors born in the United States and 22 donors whose COB was not known; three (6.8%) and zero were hemoculture positive, respectively. As already mentioned, the three isolates obtained from the US-born donors typed as TcV or TcVI.

Taken together, the data presented in this study suggest that cases of transfusion-transmitted Chagas are most likely to be associated with donors from southern South America infected with the TcII, TcV, and TcVI lineage of *T. cruzi*. Historical studies from South America suggested transmission rates in the range of 12% to 20%,[13-16] with rates as high as 46.7% from Bolivia.[30] This observation is supported by a review of the seven cases of transfusion-transmitted *T. cruzi* reported in the United States and Canada.[5-9,22,31] An implicated donor was identified in six of the seven reported cases and for five of these cases the implicated donor was born (and likely infected) in a country from southern South America (i.e., Bolivia, Chile, and Paraguay). Moreover, *T. cruzi* isolates obtained from the donor and recipient pair associated with the 1999 Miami transfusion case[8] both typed as TcV in this study. The only positive lookback investigation since licensed blood donor screening was initiated in the United States involved a donor who was originally from Argentina; two recipients were infected via separate donations.[18]

Similarly, there have been at least six cases of transfusion-transmitted Chagas reported in Spain.[32-36] Estimates from 2008 indicate that more than 2 million Latin American immigrants were residing in Spain, compared to 40 million in the United States, suggesting that six cases of transfusion-transmitted Chagas in Spain is disproportionate to the number of recognized immigrants.[21] For Spain, the most frequent country of origin for Latin American immigrants was Ecuador (25%), Colombia (16%), Bolivia (14%), Argentina (8%), Peru (7%), and Brazil (6.6%).[21] Indeed, among the donors implicated in the six Spanish cases, five were originally from Bolivia and one was from Brazil. Similarly, the recent transmission case in Belgium implicated a donor who originally was born and resided in an endemic rural area in the extreme southern portion of Brazil.[20] In contrast, transfusion transmission is relatively rare in Mexico despite seroprevalence ranging between 0.1 and 1.28% in several serosurveys.[37] Thus the relative absence of transfusion-transmitted *T. cruzi* cases and successful lookback investigations in the United States may be related in part to donor demographics; specifically, the majority of at-risk Latin American donors in the United States are from Mexico and Central America (especially El Salvador) thereby harboring TcI lineage *T. cruzi* that appears to be associated with infrequent parasitemia and an associated lower risk of transfusion transmission.

Of note, three hemoculture-positive donors were born in the United States, two of whom typed as TcV, while one typed as TcVI. For the two infected with the TcV DTU, the most likely explanation was congenital transmission. In both cases the donors identified themselves as Hispanic and their mothers were born in South America. An explanation for the donor infected with the TcVI isolate is more problematic. This donor was born in Ohio, but likely became infected as a youth during a Boy Scout camping trip in Texas, thus suggesting that he may have been infected with a sylvatic isolate. There has been

limited typing of sylvatic isolates in the United States; thus this explanation would require additional study. Based on our laboratory experience this isolate appears unique and replicated in our culture systems at a rate that far exceeded any other isolate in our possession.

Several limitations are inherent to this study. First, detection of parasitemia by hemoculture is limited in part by the sensitivity of the assay compared to PCR.[38] However, unlike PCR, hemoculture is 100% specific and detects only live parasites, not dead parasites or DNA fragments. Sensitivity of hemoculture was enhanced by using 30 mL of patient blood per assay and was repeated three times using separate blood draws with hemocultures examined over a 16-week period allowing for parasite amplification to visibly detectable levels. It could be argued that TcI isolates may not grow as well in hemoculture as other isolates and thereby are underrepresented, but historical literature suggests that they adapt well to culture conditions. Further, the preponderance of transfusion cases in North America and in Spain associated with isolates presumably from southern South America would support the possibility of variable transmission rates by lineage unrelated to ability to grow in vitro. Second, the actual location where study subjects became infected is difficult to ascertain precisely. However, most infected persons in endemic countries are thought to be infected in childhood. Thus, one can assume that barring extensive travel, most persons became infected at or near the location of their birth in an endemic country. Responses to questionnaires regarding location (city, country) of their birth were used as the presumed locations of initial infection. Finally, for infected donors with negative hemoculture results, the likely *T. cruzi* lineage was based on the donor's COB and studies mapping the geographic distribution of known parasite lineages. While not precise, distinct geographic distributions have been described for parasites identified as TcI, while those associated with TcII, TcV, and TcVI have overlapping, but clearly geographically distinct, distributions from TcI. Other DTUs, TcIII and TcIV, are linked almost exclusively with sylvatic infections and have little consequence for human infections. Thus, one can reasonably assign predicted parasite lineages for infected, but hemoculture-negative, donors when differentiating between TcI and southern South American DTUs (i.e., TcII, TcV, and TcVI).

In conclusion, compared to South America and Spain, the rate of transfusion-transmitted *T. cruzi* in the United States is relatively low. The recognition that *T. cruzi* lineages may impact the frequency of infected donors presenting as parasitemic, thereby suggesting an association with transfusion transmission is a key finding. Thus, a correlation between transmission risk and parasite lineages associated with southern South America may explain the disparity in observed transmission rates. Additional analyses of *T. cruzi* lineages associated with donors with parasitemia and transfusion cases going forward will be critical to further define this potential transmission risk.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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