Evidence of meaningful levels of *Trypanosoma cruzi* in platelet concentrates from seropositive blood donors

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BACKGROUND: According to the reported cases of transfusion-acquired *Trypanosoma cruzi* infection, the risk of *T. cruzi* transfusion transmission appears to be higher with platelet (PLT) products than with other blood components. The aim of this study was to investigate by quantitative real-time polymerase chain reaction (qPCR) the parasitic load detected in leukoreduced plasma and PLT concentrates collected by apheresis from seropositive *T. cruzi* blood donors and compare them with peripheral whole blood (WB).

STUDY DESIGN AND METHODS: During 2011 to 2013, a prospective study was carried out in a group of blood donors originating from Chagas-endemic areas but who are now living on the island of Majorca, Spain. Leukoreduced plasma and PLT concentrates were collected by apheresis from seropositive blood donors with detectable parasitemias in peripheral WB.

RESULTS: Seropositivity was found in 23 of 1201 donors studied (1.9%), and *T. cruzi* DNA with less than 1 parasite equivalent/mL was detected in peripheral WB in 60.86% (14 of 23) of these. The study in blood components obtained by apheresis from these donors showed that *T. cruzi* DNA with a mean ± SD parasitic load of 5.33 ± 6.12 parasite equivalents/mL was detected in 100% of the PLT concentrate samples. Parasite DNA was undetectable in the extract taken from plasma collected from donors with a positive qPCR in peripheral WB.

CONCLUSION: The higher parasitic load found in PLT concentrates compared to plasma and peripheral WB would explain the higher transfusion transmission risk of Chagas disease associated with PLT transfusions described in the reported cases of transfusion-acquired *T. cruzi* infection.

As a result of globalization and immigration, emerging blood-borne pathogens such as *Trypanosoma cruzi,* the causal agent of Chagas disease, are gaining importance in nonendemic countries.[1-3] The 20 cases associated with transfusion transmission of *T. cruzi* reported in North America and Spain between 1987 and 2011 were all related to platelet (PLT) concentrates or whole blood (WB), some of which had even been leukoreduced and irradiated.[4] In these cases, transfusion transmission was confirmed using hemoculture, polymerase chain reaction (PCR), or serology to study the presence of parasites in blood from transfused patients. The retrospective study of the blood component or WB donors involved in these donations was in all cases carried out by serology and, additionally in some cases, using PCR to search for parasite DNA. No

ABBREVIATIONS: BIBB = Balearic Islands Blood Bank; Cq = quantification cycle; GEB = guanidine-EDTA-whole blood; qPCR = quantitative real-time polymerase chain reaction; WB = whole blood.

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parasite cultures or PCR DNA amplifications were performed in the units that had already been transfused.[5-8]

As *T. cruzi* is present in the human bloodstream as a trypomastigote, the extracellular form of *T. cruzi*, experimental assays have suggested that as *T. cruzi* adheres to leukoreduction filter fibers, such filters could be used as a means of reducing this organism in infected blood.[9,10] However, the efficacy of leukoreduction to remove parasites from red blood cell (RBC) units has not been studied and it is not known whether this method completely eliminates the risk of transfusion transmission.

Even though all blood components are potentially infectious, there is no empiric evidence in the literature that RBCs or plasma units are implicated in the transfusion transmission of *T. cruzi*. Most of the reported cases have been associated with PLT transfusion.[5-8,11]

Over the past few decades, innovative devices, new technologies, and new techniques have been developed in an attempt to decrease the risk of pathogen transfusion transmission, as well as to reduce adverse reactions to transfusion, without altering the quality of blood components. One of these techniques is the leukoreduction of blood products using centrifugation, elutriation, or filtration, based on size, density, or stickiness of the white blood cells (WBCs). The generally accepted clinical benefits include prevention of febrile reactions, alloimmunization to HLAs, and transmission of infectious agents, as well as pathogens associated with WBCs, such as cytomegalovirus or Epstein-Bar virus.[12]

During 2011 through 2013, a prospective study was carried out in blood donors who, although living on the Island of Majorca, originated from Chagas-endemic areas. *T. cruzi* serologic screening was performed to identify positive donors and determine the seropositivity in our geographical area and to enable their inclusion in a qualitative and quantitative parasite study. The parasitic load in PLTs and plasma concentrated obtained by apheresis was studied by quantitative real-time PCR (qPCR) and compared with the parasitemia in peripheral WB.

The aim of this study was to investigate the parasite loads present in WB and other blood components, such as plasma and PLTs, from seropositive *T. cruzi* blood donors and determine whether or not there are any differences in parasitemia levels that could explain which component presents the greatest risk of transmitting pathogens through transfusion.

MATERIALS AND METHODS

Blood donors

During 2011 through 2013, a total of 1201 blood donors who originated from Chagas-endemic areas, but are now living in the Balearic Islands, were tested for *T. cruzi*–specific antibodies in the Balearic Islands Blood Bank (BIBB). All donors with positive serology who had not been previously treated for the parasite, and consented to be included in the study, were analyzed for parasite load in peripheral WB and blood components, plasma, and PLTs obtained by apheresis. All of them were permanently rejected as donors for patient transfusions.

Ethics statement

This study was approved by the ethical committees of the participating institutions, the Balearic Island Ethic Committee and the Research Ethics Committee of the University of Barcelona. Written informed consent was obtained from all donors for the screening and apheresis study.

In accordance with the Spanish and European regulations, our standard operating procedure for donors who were seropositive for *T. cruzi* by the screening test at the blood bank involved permanent deferral for these individuals. Additionally, donors were immediately referred to the Infectious Disease Unit at the University Hospital in Majorca for follow-up and treatment. Treatment was never delayed by participation in this study.

Screening study

Five milliliters of serum was obtained for serologic study and 10 mL of peripheral WB was collected and immediately mixed with an equal volume of 6 mol/L guanidine hydrochloride-0.2 mol/L EDTA buffer (Sigma, St Louis MO) for molecular study. The mixture guanidine-EDTA-WB (GEB) was stored at 4°C until the DNA purification. GEB samples were not boiled.[13]

Apheresis study

Cell separators and procedures

Fenwal Amicus Version 3.11. Fenwal Amicus (Fresenius Kabi, Bad Homburg, Germany) is an apheresis device capable of dual- and single-needle plateletpheresis. The PLT-rich plasma is separated from the RBCs and WBCs in a bell-shaped chamber with two compartments. After the elutriation process, PLT-rich plasma is pumped to the collection chamber as so-called dry PLTs, that is, high-concentration plasma-reduced PLTs. At the end of the process, the PLTs are manually resuspended by shaking and adding PLT-poor plasma and then transferred to the storage bags with additive solution (AS) at a ratio of approximately 35% plasma and 65% AS.

Two Amicus devices, Software Version 3.11, set code REF R4R2337, were used for the study. All procedures used a WB:ACD ratio of 11:1 (i.e., 1 part ACD per 11 parts WB) and citrate infusion flow of 1.35 mL/kg/min. The maximum inlet and return rates were 110 and 120 mL/min, respectively.

Trima Accel Version 5.22. Trima Accel (Terumo BCT, Lakewood, CO) is a single-needle, continuous-separation apheresis device. Trima Accel Version 5 has a single-stage

separation channel with all of the blood components flowing in one direction. The blood components separate into layers over the entire circumference according to specific gravity. PLTs are harvested from the component layer between RBCs and plasma, leukoreduced with the process-controlled leukoreduction system, and collected in the final storage container.

Three Trima Accel machines, Version 5.22, Set Code REF 80420, were used for the study. All procedures used a WB:ACD ratio of 11:1 (i.e., 1 part ACD per 10 parts WB) and maximum citrate infusion flow of 1.1 mL/min/L total blood volume. The maximum inlet and return rates were 100 and 140 mL/min, respectively. The PLT concentrates were collected in small amounts of plasma and diluted in AS at the end of the procedure to maintain a ratio of approximately 35% plasma and 65% AS.

Haemonetics MCS+. The Haemonetics MCS+ system (Haemonetics Corp., Braintree, MA) uses intermittent-flow centrifugation to collect plasma, PLTs, WBCs, or RBCs. The grenade-shaped plasmapheresis rotation bowl is used for plasma collection. WB enters the bowl through the stationary tube and forms vertical layers of plasma and RBCs. As the bowl fills, packed cells accumulating on the outer wall displace the plasma inward, pushing it out through the effluent tube. When the bowl is full of RBCs, it must be stopped and emptied before the process can be repeated.

Two Haemonetics MCS+ apheresis machines, Set Code REF 792 P, PLT-poor plasma, were used for the study. All procedures used a WB:ACD ratio of 12:1 (i.e., 1 part ACD per 11 parts WB). The maximum inlet and return rates were 100 and 150 mL/min, respectively.

Preparations of blood components

Plateletpheresis and PLT-plasmapheresis donations were performed on selected donors using Trima and Amicus automatic cell separators. The mean PLT unit volume collected in the plateletpheresis procedures was 300 mL. PLT components containing 3.5×10^{11} were suspended in approximately 35% plasma and 65% PLT AS (PAS IIIM; Terumo BCT). The mean volume of plasma unit collected in the PLT-plasmapheresis procedures was 300 mL. All PLT and plasma components collected were leukoreduced by in-process leukoreduction (leukoreduction system chamber for Trima and elutriation for Amicus).

Plasmapheresis donations were carried out using the Haemonetics MCS+. The mean plasma volume collected was 600 mL, and leukoreduction was performed using a leukoreduction plasma filter (Plasmaflex Plas 4, Maco-Pharma, Mouvaux, France) at the end of the collection procedure.

A total of eight apheresis procedures were performed from which six plasma and six leukoreduced PLT concentrates were obtained. Three blood donors with detectable parasitemia in peripheral WB consented to donate blood components by apheresis to study the parasite load. Each blood donor included in the study gave blood components in different donations. The first underwent one plateletpheresis procedure; the second one plasmapheresis and two PLT-plasmapheresis procedures; and the third one plasmapheresis, one plateletpheresis, and two PLT-plasmapheresis procedures. Within the apheresis samples, ten milliliters of GEB was obtained to determine the basal parasitic load (Table 2).

Samples were collected in the BIBB and sent, no more than 2 days after the donation, to the Parasitology Laboratory of the Universitat de Barcelona for molecular analysis. The samples of plasma, PLTs, and GEB were sent at −20°C, at room temperature and 4°C, respectively, and once received were immediately processed for DNA extraction.

Serologic study

The serologic study for detection of specific *T. cruzi* antibodies was performed by an enzyme-linked immunosorbent assay (ELISA) test for qualitative detection of total antibodies to *T. cruzi* in human serum or plasma (Bio-ELISA Chagas, Biokit, Werfen Group, Barcelona, Spain) with recombinant antigen of *T. cruzi* and another commercially available ELISA test system (Ortho Clinical Diagnostics, Raritan, NJ) with antigen of a total extract of epimastigotes from *T. cruzi* in BIBB. Confirmation was performed in the Parasitology Laboratory of the Universitat de Barcelona by a third diagnostic method using Western blot with antigen of a total extract of epimastigotes from the *T. cruzi* Maracay strain.[14]

Molecular study

DNA extraction

For each sample, DNA was extracted in triplicate (Table 2) from different samples: 200 μL of GEB from all seropositive donors (screening sample), 200 μL of GEB at the moment of apheresis (basal sample), 200 μL of PLT concentrate, and 200 μL of plasma concentrate (the last two obtained by apheresis). The extraction was performed with a PCR template preparation kit (High Pure, Roche, Mannheim, Germany) and eluted in 200 μL of elution buffer according to the manufacturer's instructions. The eluate was stored at −20°C for qPCR analysis. To build the standard curve for quantification of parasitic loads, DNA from a culture of epimastigotes of *T. cruzi* (Maracay strain, 1×10^5 parasite/mL) was extracted in the same way as previously reported.

qPCR assay

Five microliters of extracted DNA was amplified in triplicate by qPCR (Table 2) in a qPCR instrument (LightCycler 480, Roche) device. The primers, probes, and conditions of

TABLE 1. Results of *T. cruzi* serologic screening of blood donors from Chagas-endemic areas living on the Island of Majorca and presence of *T. cruzi* DNA in peripheral WB by qPCR in seropositive blood donors studied

	Argentina	Ecuador	Bolivia	Venezuela	Total
Donors	649	362	106	84	1201
Percentage of donors*	1.42	0.78	0.24	0.19	0.66
Seropositive donors†	4 (0.61)	1 (0.27)	17 (16.03)	1 (1.19)	23 (1.91)
Presence of *T. cruzi* DNA†	3 (75)	0 (0)	10 (58.8)	1 (100)	14 (60.86)

* Percentage of Latin American donors with respect to the total donor population.
† Data are reported as number (%).

the technique were as described by Piron and coworkers[15] with some modifications. Briefly, the following were used, primers Cruzi 1 and Cruzi 2 and probe Cruzi 3, which was labeled with 6-carboxyfluorescein and minor groove binder. The final concentrations in the PCR mixture were as follows: 1× LightCycler 480 Probes Master (Roche), 750 nmol/L each *T. cruzi* primer, and 250 nmol/L for the *T. cruzi* probe in a 20-μL reaction volume. Detection of the RNase P human gene (0.3× RNase P detection reagents, Life Technologies, Austin, TX) was included as an internal control of amplification in multiplex qPCR, and a nontemplate sample and DNA from seronegative human blood were included in each run as a negative control.

A sample was considered valid when the internal control was efficiently amplified and was considered positive when the quantification cycle (Cq) was less than or equal to 40 and if at least three of the nine replicates studied for each sample were amplified.[16] A standard curve was constructed with 1-in-10 serial dilutions of total DNA extracted from Maracay strain (1×10^5- 0.1 parasite equivalents/mL). The parasitic load of every sample was calculated using the LightCycler 480 software through the second derivative maximum method.

RESULTS

Screening study

From a total of 1201 blood donors coming from Latin America, 23 were *T. cruzi* seropositive by the three serologic tests ,which corresponded to 1.91% of prevalence with respect to the total donor population. The Bolivian population with 16.03% (17 of 106; 95% confidence interval [CI], 10.1-24.2) seropositivity had the highest prevalence (Table 1).

Parasite DNA was detected in peripheral WB by qPCR in 14 of the 23 seropositive donors, which corresponds to 60.86% (95% CI, 40.7-77.9) positivity in chronic patients (Table 1). The mean ± SD Cq in these screening samples (GEB) was 34.35 ± 2.87, which implies a parasitic load of 0.39 ± 0.2 parasite equivalents/mL.

Apheresis blood components study

The qPCR study of the PLTs indicated that all samples amplified with 100% positivity in all the extractions (18 of 18) and 100% positivity in all replicates performed (54 of 54), with a significant mean ± SD parasitemia of 5.33 ± 6.12 parasite equivalents/mL. In contrast, parasitemia was undetectable in all extract from plasma samples with 0% positivity in all the extractions (0 of 18) for parasite DNA, with 0% of amplification in all replicates (0 of 54). The peripheral WB (basal sample) obtained on the day of plasma and PLT donation by apheresis showed that all samples were positive with a mean ± SD parasitemia of 0.42 ± 0.32 parasite equivalents/mL (Table 2), although not all the replicates amplified in five extractions (59 of 72 replicates amplified by qPCR, 81.9%; 95% CI, 71.3-89.2).

DISCUSSION

Blood transfusion is the second most common mechanism for the transmission of *T. cruzi* in endemic areas and the first in nonendemic areas. The transfusion transmission risk of Chagas disease is thought to depend on multiple factors, including the level of parasitemia in the donor, the type of component transfused, and perhaps the strain of the parasite.[17,18] In Spain, of the reported cases of *T. cruzi* transfusion transmission, all the patients were transfused with PLT concentrates and all the donors implicated in the donation originated from Chagas-endemic areas,[4] three of the four donors from Bolivia and one from Brazil.

The serologic screening performed in our study in the Balearic Islands showed that 1.91% of the blood donors coming from Chagas-endemic areas were seropositive. The largest Latin American population living in the Balearic Islands originates from Argentina and is the Latin American group that donates blood most often; however, Bolivian donors have the highest positive detection rate for *T. cruzi* at 16.03%. These results are consistent with the 2007 study performed in Catalonia, Spain, by Piron and colleagues,[19] in which the seroprevalence of donors studied was 0.62%, and the highest positive detection rate for *T. cruzi* (10.2%) was in Bolivian blood donors.

The detection of *T. cruzi* by molecular methods, such as by PCR in blood samples, has high specificity and sensitivity. This technique has been successfully used in the diagnosis of acute chagasic disease for more than 15 years;[20] however, due to low parasitemia, the sensitivity and reproducibility of the technique decreases in chronic patients. The sensitivities reported in this group of

TABLE 2. Peripheral WB and apheresis blood components from *T. cruzi*–seropositive blood donors and quantification results of parasitic load obtained by qPCR

Sample	Total of DNA extractions by sample*	Total of qPCR replicates†	Positives replicates amplified	Positives samples	Mean Cq‡	Quantification of parasitic load	
						Mean§	Range§
GEB (n = 8)	24	72	59/72	8/8 (100%)	35.0 ± 2.93	0.42 ± 0.32	0.04-0.93
Plasma (n = 6)	18	54	0/54	0/6 (0%)	>40	Nondetectable	Nondetectable
PLTs (n = 6)	18	54	54/54	6/6 (100%)	30.7 ± 2.42	5.33 ± 6.12	0.73-16.73

* Three extractions of DNA of each sample were performed.
† Three replicates of each DNA extracted were amplified by qPCR.
‡ Mean ± SD of all replicates. Value of qPCR expressed in Cq.
§ Expressed in parasites equivalent/mL.

patients are between 20 and 60% and depend on the conditions under which the technique is performed.[15,21] In our study using a quantitative, validated, highly sensitive real-time PCR we found 60.86% (95% CI, 40.7-77.9) positivity in 23 seropositive blood donors living in the Balearic Islands. This percentage is slightly higher than the 41% found in 2007 by Piron and coworkers[15] in seropositive blood donors in Catalonia and similar to the 56.5% found in 2011 by Schijman and colleagues,[21] using qPCR in chronic patients from endemic areas.

Low and oscillating parasitemias are described in chronic chagasic patients[15,21] and are consistent with the results found in peripheral WB in our pool of seropositive donors, a group in which parasitemias were lower than 1 parasite equivalent/mL. In these samples it was observed that not all replicates amplified according to the low or nonexistence of parasites. Consequently, it is strongly recommended that replicates of DNA extractions and qPCR of WB are performed when a diagnosis is made to increase the possibility of finding positives results, especially when working with small volumes of samples.

Our extract from plasma results did not show any positive amplifications of the parasite DNA in any of the donor samples (0/6). By contrast, in the leukoreduced PLT concentrates, we found parasitic load values of 5.33 ± 6.12 parasite equivalents/mL, with a parasitic load range of 0.72 to 16.73 parasite equivalents/mL, more than five times greater than the parasitic load of the peripheral WB. The qPCR results were positive in all samples (6/6) and, interestingly, 100% of qPCR replicates of all samples studied (54/54) were positives. It is possible that this percentage would decrease if no previous screening for the presence of parasite in peripheral WB was performed to select the studied individuals, as in the 2009 study carried out by Dzib and colleagues, [22] using a conventional PCR of minicircle kDNA in blood components leukoreduced by centrifugation from 21 units of seropositive donors, which found 50% positivity in PLT concentrates. Results published in 2005 by Coronado and coworkers[23] indicate that the circulating DNA belongs to living parasites and not to circulating DNA from the remains of dead parasites, because this is rapidly degraded. It would be interesting to study parasite viability to discriminate between viable and nonviable parasites.

Leukoreduction is considered by some authors to be a good method for reducing the *T. cruzi* transfusion transmission risk, but more detailed studies will be needed to confirm this theory. A report published by Hernández-Becerril and colleagues,[24] in 2005, studied 70 PLT and RBC components leukoreduced from seropositive blood donors by conventional PCR and hemoculture. However, no parasitemia was detected by either of the methods; this factor could be attributed to the leukoreduction. Similarly, in 2006 Cardo and Asher[9] reported a filtration assay study in which *T. cruzi* trypomastigotes spiked in plasma remained in the leukoreduction filters as a result of adherence. In 1995, Moraes-Souza and coworkers,[10] using leukoreduction filters in spiked human blood, showed that this process reduced the number of *T. cruzi*. By contrast, in our study, leukoreduction was not entirely effective at eliminating the parasitic load; in fact, the parasite loads detected in PLTs obtained by apheresis were higher than the parasite load in peripheral WB. A study performed by Dzib and coworkers[22] in 2009 reported that leukoreduction by centrifugation did not eliminate *T. cruzi* from infected blood units. In fact, they found parasite DNA in RBCs, buffy coat, and PLT concentrates.

Certainly, the risk of *T. cruzi* transmission per infected blood product transfused is estimated to be from 10% to 25%, with PLT products having a much higher risk than RBCs, frozen plasma, or cryoprecipitated products.[4,9,25] In fact, there have been no reported cases of transfusion transmission through blood components that were frozen and stored at −20°C or RBCs, even though a report published by Martin and coworkers[26] in 2014 observed that *T. cruzi* in spiked human blood and in infected culture cells was able to survive long periods of storage at +4°C and −80°C, suggesting that *T. cruzi*–infected blood or tissues stored under these conditions are potentially infectious.

The reason why we detected parasite DNA in PLTs, and not in plasma concentrates obtaining by apheresis from the same donor who was PCR positive in peripheral blood, could be related to the method that apheresis machines use to process WB. Basically, apheresis machines use centrifugation and filtration, the first as a method of

separating and concentrating the selected blood component, through centrifugation that uses differences in specific gravity or density to separate and isolate blood components, and the second, filtration as a method for reducing the WBC count in PLT concentrates (some machines also use elutriation as an alternative to filtration). Filtration takes advantage of differences in particle size to separate and, as has already been stated, centrifugation is based on the specific gravity of blood cells. The trypomastigote form of *T. cruzi* has a size of 16 to 20 μm[18] and a specific gravity between PLTs and WBCs.[27,28] According to these data, it is reasonable to think that, since PLTs are concentrated along with WBCs throughout the apheresis procedure, trypomastigotes are concentrated along with WBCs in the PLT fraction rather than in the plasma or RBC fraction. Therefore, the PLT fraction initially collected by apheresis contains WBCs, although it is leukoreduced during the procedure by elutriation or after the procedure by filtration. However, it is important to note that, despite leukoreduction (i.e., $\leq 1 \times 10^6$/L WBCs) the level of residual WBCs in leukoreduced PLT concentrates could be up to 1×10^6 per unit according to our national standards. Accordingly, if a considerable number of WBCs are still present in leukoreduced PLT products, it is logical to think that parasites can also remain.

Using laboratory techniques, in seropositive *T. cruzi* blood donors we have seen that PLTs are the component with the main parasitic load; therefore, it could explain what is observed in clinical cases of transfusion transmission of Chagas disease reported in the literature, in which PLT concentrates are the major component implicated in transmission.[4-9,29] It would be challenging to investigate the role of new pathogen inactivation technologies, in reducing the parasite load in blood components, for example, PLT concentrates, obtained from seropositive *T. cruzi* donors, and to study whether they are effective in these samples, because so far they have only been studied experimentally in blood components spiked with *T. cruzi*. [30,31]

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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