The utility of pathogen inactivation technology: a real-life example of *Leishmania infantum* inactivation in platelets from a donor with an asymptomatic infection

Teresa Jimenez-Marco[1], Cristina Riera[2], Roser Fisa[2], Enrique Girona-Llobera[1], Matilde Sedeño[1], Raymond P. Goodrich[3], Alba Pujol[2], Carmen Guillen[2], Josep Muncunill[1]

[1]Blood and Tissue Bank, Majorca, Balearic Islands; [2]Parasitology Laboratory, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain; [3]CaridianBCT Biotechnologies, Lakewood, Colorado, United States of America

Introduction

Visceral leishmaniasis is typically caused by *Leishmania infantum* (*L. infantum*) in the Mediterranean littoral. The majority of infections with *L. infantum* are asymptomatic and resolve spontaneously in individuals with normal immune systems. A minority progress to classic, full-blown visceral leishmaniasis, known in many areas as kala-azar[1,2]. *Leishmania* infection is most often transmitted to humans via the bite of the phlebotomine female sand fly, however transmission of *Leishmania* by transfusion has also been reported[3-13]. Populations at risk of clinically apparent disease caused by *L. infantum* after insect bites or transfusion include infants and immunosuppressed patients.

Most cases of transfusion-transmitted leishmaniasis have been reported in endemic areas and, because the infection is often asymptomatic in healthy individuals, it is difficult to calculate the transmission risk precisely. The existence of cryptic *Leishmania* infections, associated with intermittent low density circulation of the parasite [1,14], has been the main cause of transmission by blood.

In the Balearic Islands, our region, the prevalence of asymptomatic *L. infantum* infection in blood donors is high (11% of blood donors tested had a positive reaction to the delayed-type hypersensitivity test, 3.1% had specific antibodies and *L. infantum* DNA was detected in the blood of 5.9% of donors studied)[15] which is in agreement with other findings regarding asymptomatic individuals from the Mediterranean region[1,16,17].

Although some research studies have investigated *Leishmania* infection in blood donors[1,14-17], currently there is no suitable *Leishmania* donor screening test that meets Blood Bank requirements with respect to aspects such as speed, automation and standardisation.

At present, the only measure applied to prevent transmission-transfusion leishmaniasis is based on donor selection criteria. According to the "Guide to Preparation, Use and Quality Assurance of Blood Components" from the European Committee on Blood Transfusion, as well as the Spanish Regulation on Blood Donation, the strategy adopted in Europe is based on permanent deferral for donors who have had or currently have visceral leishmaniasis. In other countries, such as the USA, donors are deferred for a period of 1 year from the date of their last departure from an endemic country.

Since there is currently no adequate donor screening test for *Leishmania*, several methods have been used to inactivate *Leishmania* in blood products[18-20] and to eliminate the parasites through filtration using whole blood, fresh plasma and red blood cell (RBC) leucodepletion systems[15,21-23].

As an approach to reducing the risk of transfusion-related transmission in our area, we investigated the ability of pathogen inactivation technology using riboflavin and ultraviolet light to eliminate *L. infantum* in apheresis platelet units obtained from an asymptomatic infected blood donor.

This is the first report on the utility of pathogen inactivation technology in the elimination of a parasite from a blood component collected from an asymptomatic infected blood donor.

Case presentation

This study was conducted under a protocol approved by the Balearic Island Ethic Committee after written informed consent had been obtained from

the participating donor. The donor met the eligibility criteria defined by the Spanish Blood Donation Regulations and the Guide to the Preparation, Use and Quality Assurance of Blood Components by the European Committee on Blood Transfusion.

The 52-year old male blood donor, previously discovered to be asymptomatically infected by *L. infantum,* after *L. infantum* DNA had been detected in his peripheral blood*,* was enrolled in order to study the efficacy of pathogen reduction technology in eliminating the parasite from platelet concentrates collected by apheresis.

On separate occasions, the selected donor gave two apheresis platelet donations using the MCS Plus version LDL for single-dose platelets (Haemonetics Corp., Braintree, MA, USA) which uses a filtration technique for leucoreduction (Purecell LRF High Efficiency Leukocyte Reduction Filtration System for Platelets; PALL Corporation, NY, USA). The machine was programmed to obtain a platelet unit suspended in 100% plasma at a concentration of approximately $1{,}400x10^9$ cells/L and a planned yield of $3.5x10^{11}$ cells/unit. The platelet volume obtained was 350 mL with a platelet yield of $3.5x10^{11}$ cells/unit the first time, and $3.6x10^{11}$ cells/unit the second time.

After rest period of at least 2 hours after collection, the units were inactivated using riboflavin and ultraviolet light (MIRASOL Pathogen Reduction Technology System for Platelets and Plasma; CaridianBCT Biotechnologies, Lakewood, CO, USA) according to the manufacturer's instructions. The bag containing platelets was connected to the pathogen-reduction illumination/storage bag using a Terumo sterile tubing welder, and the contents of the original collection bag were drained into the illumination/storage bag. Following the transfer of the platelets, the collection bag was removed and discarded. The sterile riboflavin bag, containing 35 mL of riboflavin solution (500 µM), was connected to the illumination/storage bag containing the platelet product. After transferring the riboflavin and eliminating the air the riboflavin bag was disconnected. Each platelet product to be treated contained 210 mL of 83% platelet product in a 1-l citrate-plasticized, polyvinyl chloride (PVC) ELP bag with a surface area of 347 cm^2 per side. Each bag was labelled on one side, which resulted in a total surface area of 590 cm^2 through which light could pass to reach the contents of the bag. The Mirasol Pathogen Reduction Technology System illuminator delivers 6.2 J/cm^2 ultraviolet (UV) light (265-370 nm) in approximately 5-10 minutes. The total process results in irreversible photo-oxidative damage to nucleic acid, including nucleated blood cells and infectious contaminating agents.

The donor underwent a 2-year follow-up during which tests were carried out to determine whether parasite DNA was present in the peripheral blood before donating platelets by apheresis. In addition, western blotting of the donor's serum and real-time polymerase chain reaction (RT-PCR) analyses were performed on peripheral blood samples (after obtaining the peripheral blood mononuclear cell fraction as previously described[15]) on the day of the apheresis donation. RT-PCR was also performed on platelet units both before and after inactivation with the MIRASOL Pathogen Reduction Technology System. All PCR-positive samples were cultured.

Anti-*Leishmania* antibodies were tested by western blotting using a whole *L. infantum* antigen (MHOM/FR/78/LEM 75). We considered serum to be positive when immunoreactivity against the 14 and/or 16 kDa *L. infantum* antigen fraction was observed.

The presence of *Leishmania* DNA was analysed through the amplification of kinetoplast DNA sequences by RT-PCR as previously described by Mary *et al.* with some modifications[24,25]. Each amplification was performed in triplicate, in a 20 µL reaction mixture containing 1xiTaq supermix with Rox (Bio-Rad, Hercules, CA, USA), 15 pmol of direct primer (CTTTTCTGGTCCTCCGGGTAGG), 15 pmol of reverse primer (CCACCCGGCCCTATTTTACACCAA), 50 pmol of the labelled TaqMan probe (FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA) and 5 µL of sample DNA. Amplifications and detection were performed in an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) in two-step temperature (94 ºC and 55 ºC) cycling over 45 cycles. Positive controls (DNA from *L. infantum* MHOM/ES/04/BCN-61) and negative controls were included in each PCR analysis. RT-PCR was considered positive for *Leishmania* spp. when the threshold cycle (C_T) was <45. The C_T for a given sample is the first cycle of the PCR reaction in which fluorescence is detected above the baseline.

In vitro culture was performed using NNN

medium and Schneider's insect culture medium (Sigma) supplemented with 20% heat-inactivated foetal calf serum, 1% sterile human urine, and 25 µg/mL gentamicin solution. Two tubes of NNN and one flask with 20 mL of Schneider's medium were inoculated with 250 µL and 500 µL of the peripheral blood mononuclear cells, respectively. Cultures were maintained between 24 ºC and 26 ºC, examined twice a week, and subcultured every 2 weeks for 6 months before being considered negative.

Results

The *Leishmania*-specific antibodies were revealed by western blotting of the sera of this donor and showed the characteristic 16 kDa band. The presence of the parasite was evident in the blood of this donor by RT-PCR analyses. In fact, both western blotting and RT-PCR analyses on peripheral blood samples taken the day of apheresis donation were positive on two different occasions. The pre-inactivation platelet units were positive by RT-PCR at the level of 0.01 parasites/mL. After MIRASOL inactivation of platelet units, RT-PCR analyses were negative. All *in vitro* cultures of RT-PCR-positive samples were negative after 6 months of follow-up.

Discussion

The risk of transfusion-associated parasite infection is mainly determined by five factors: the prevalence of infection, the incidence of peripheral parasitaemia in donors, the survivability of parasites stored in blood, the minimal infectious dose and the immunocompetence of the recipient.

In our region, the epidemiology criteria were met fully: *L. infantum* DNA was detected in the blood 5.9% of blood donors studied[15]. An *in vitro* study has also demonstrated the survivability of *Leishmania* under blood bank storage conditions. In fact, the parasites can survive as intracellular forms in monocytes for 25 days in RBC fractions kept at 4 ºC, for 5 days in platelet fractions kept at 24 ºC, for 35 days in RBC fractions frozen with glycerol, and for 30 days in unprocessed whole blood left at 4 ºC[26]. In addition, the infectivity of blood contaminated by *Leishmania* and stored under blood bank conditions has been proven in animal studies[27,28].

To date, there have been 11 reports of transfusion-acquired leishmaniasis[3-13]. Of these, 10 were individual or two-case reports and, in the remaining report, 32 cases of kala-azar were described in 82 patients undergoing haemodialysis.

The donors were identified in only four out of the 11 reports on transfusion-transmitted leishmaniasis. However, it was not possible to demonstrate the presence of the parasite circulating either in the bloodstream of any of the donors or in transfused blood components[3,4,11,12]. All four donors were asymptomatic at the time of blood donation.

Certainly, asymptomatic *L. infantum* infected blood donors, with intermittent and low level parasitaemia, are an important source of transfusion-related transmission in endemic areas. *L. infantum* infected blood recipients include immunosuppressed and critically ill patients who are at risk of serious illness from even a small inoculum[26]. Currently there is no adequate donor screen for *Leishmania*. However, several methods have been used to prevent transfusion-acquired leishmaniasis based on either inactivating or eliminating *Leishmania* in blood products. Riboflavin and ultraviolet light technology achieved a 5 to 6 log reduction of *Leishmania* in platelet units deliberately contaminated with amastigote-laden macrophages[18], photochemical treatments with amotosalen plus ultraviolet light produced a 5.4 log reduction of *Leishmania* in platelet concentrates contaminated with *L. mexicana* metacyclic promastigotes[19], and no *Leishmania* growth was observed at any dilution (from 0 to 8 log) in RBC suspensions intentionally contaminated with *L. donovani infantum* promastigotes treated with thiazole orange in the presence or absence of light[20]. Eliminating the parasite by filtration using a leucodepletion system has also been reported as a method for decreasing the transfusion-transmission risk in endemic areas: PCR-positive RBC samples from donors with *L. infantum* infection became PCR-negative after leucodepletion[15]. All studies on pathogen inactivation technologies applied to the reduction of *Leishmania* risks are based on *in vitro* studies. Basically, *Leishmania*-infected monocytes and/or promastigotes were deliberately added to blood components collected from healthy, non-infected blood donors, i.e. either amastigote-laden macrophages produced by incubating normal donor whole blood with a dose of $1.0x10^8$ of *Leishmania* promastigotes or by directly inoculating $2x10^{10}$

promastigotes. The presence of the viable parasite post-inactivation was studied by culture methods[18-20].

Parasitological techniques, such as microscopic examination and/or culture of bone marrow and spleen aspirates, are the reference methods for confirming an active infection by *Leishmania* in patients. However, epidemiological studies on healthy individuals have shown that these methods seem to be unsuitable for detecting asymptomatic carriers. Other less aggressive diagnostic methods have been evaluated in the last few years. Several studies have shown that PCR analysis is a good alternative to traditional methods. An important advantage of PCR is that it can be performed on peripheral blood and it has high sensitivity; the only minor inconvenience being that a blood sample has to be taken from the subject being studied. In fact, PCR has been used to detect *L. infantum* DNA in the peripheral blood of patients[29,30] and asymptomatic carriers[1,14,15]. Additionally PCR testing can be considered as a true direct method for detecting the presence of the parasite, as previous studies showed that *Leishmania* nucleic acids were rapidly degraded following parasite death[31,32]. Another essential point is the low sensitivity of cultures compared to the high sensitivity of PCR when these methods are used for the detection of asymptomatic carriers[14,15]. This may be due to the low level of circulating parasites in asymptomatic individuals, which is sufficient to give a positive PCR result but not a positive culture. The size of the inoculum clearly influences the growth of *Leishmania*, whatever the culture medium used. Indeed, *L. infantum* do not grow from diluted "inocula" (about 10^4 parasites/mL)[33]. The low levels of parasitaemia in asymptomatic subjects, ranging from 0.001 parasites/mL to 1 parasite/mL[34], which give rise to a very small inoculum, are definitely insufficient to produce cell growth in culture media. Culture techniques do not, therefore, seem to be the best method for detecting asymptomatic carriers given the low level of circulating parasites found in these subjects.

As a result, we defined an asymptomatic carrier of *L. infantum* as an otherwise healthy individual but with parasite DNA detectable in peripheral blood, i.e. without any evidence of clinical visceral leishmaniasis.

From our pool of asymptomatic infected donors we chose a blood donor with *Leishmania* DNA detectable in peripheral blood on two occasions in 2 consecutive years. Follow-up at 24 months did not show any evidence of clinical visceral leishmaniasis in this donor.

Western blotting and RT-PCR analyses on the peripheral blood samples taken the day of the donation were positive on two different occasions. The pre-inactivation platelet units from the two donations were positive although the *in vitro* culture results were negative. This is not surprising given the small size of the "inoculum" (0.01 parasites/mL), far below the dose that is considered necessary to produce cell growth (over 10^4 parasites/mL)[33], as already mentioned above, and also much inferior to the dose used by other authors who investigated the elimination of the parasite applying pathogen inactivation technologies in *in vitro* studies[18,19]. However, RT-PCR analyses were negative on both occasions after MIRASOL inactivation of the platelet units.

The levels of parasite in the peripheral blood, and also in the pre-inactivation platelet fraction, were very low, 0.01 parasites/mL, on two separate occasions. This level of parasitaemia is in accordance with the reference values for *L. infantum* parasitaemia reported for asymptomatic subjects, which ranged from 0.001 parasites/mL to 1 parasite/mL, in contrast to those for visceral leishmaniasis patients at diagnosis, which ranged from 8 to 1,400,000 parasites/mL[34]. Low and episodic parasitaemia in asymptomatic *Leishmania*-infected individuals has been previously reported by other authors[1,14-17]. The parasitaemia in our donor seems to be low but persistent, since it has been present for at least 2 years.

Conclusion

In vitro studies on *Leishmania* inactivation have demonstrated good inactivation of high levels of parasite in blood components[18-20]. It is not, therefore, surprising that parasites present at a low concentration in platelet fractions were effectively inactivated using MIRASOL technology. In fact, it is probable that in real life the levels of parasites in blood and blood components collected from asymptomatic carriers, and which need to be inactivated, could be much lower than those used in *in vitro* spiking studies. The methods used to detect asymptomatic carriers should be more sensitive than culture, for example PCR.

MIRASOL pathogen reduction technology may be a useful approach to preventing transfusion-transmitted leishmaniasis, particularly in an "at risk" context. However, this needs to be confirmed by more numerous case series.

Keywords: donors, transfusion-transmitted disease, pathogen inactivation, *Leishmania infantum*.

Acknowledgement

The authors thank Martin Hadley-Adams for assisting with the English language and preparation of this manuscript. This work was supported by the National Plan of I+D+I 2008-2011 and ISC III - Subdirección General de Evaluación y Fomento de la Investigación (PI 10/00533).

Contributions

Teresa Jimenez-Marco and Cristina Riera equally contributed to this work.

Conflict of interest disclosure

Dr. Raymond P. Goodrich is employed by CaridianBCT Biotechnologies, the company that developed and sells the MIRASOL PRT. The remaining authors declare no conflict of interest.

References

1) le Fichoux Y, Quaranta JF, Aufeuvre JP, et al. Occurrence of Leishmania infantum parasitemia in asymptomatic blood donors living in an area of endemicity in Southern France. J Clin Microbiol 1999; **37**: 1953-7.
2) Control of the Leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases. In: WHO press. WHO Technical Report Series; 2010. Geneva, Switzerland; 2010; **949**: 1-201.
3) Chung HL, Chow KK, Lu JP. The first two cases of transfusion kala-azar. Chin Med J 1948; **66**: 325-6.
4) Andre R, Brumpt L, Dreytus B, et al. Leishmaniose Cutanee. Leishmaniose cutanee-ganglionnare et kala-azar transfusional. Trop Dis Bull 1958; **55**:379-81.
5) Kostman R, Barr M, Bengtson E, et al. Kala-azar transferred by exchange blood transfusion in two Swedish infants. In: Proceedings of the Seventh International Congress of Tropical Medicine and Malaria. World Health Organization: Geneva, Switzerland; 1963. p. 384.
6) Cohen C, Corazza F, Moll PD, Brasseur D. Leishmaniasis acquired in Belgium. Lancet 1991; **338**: 128.
7) Cummins D, Amin S, Halil O, et al. Visceral leishmaniasis after cardiac surgery. Arch Dis Child 1995; **72**: 235-6.
8) Singh S, Chaudhry VP, Wali JP. Transfusion-transmitted kala-azar in India. Transfusion 1996; **36**: 848-9.
9) Luz KG, da Silva VO, Gomes EM, et al. Prevalence of anti-Leishmania donovani antibody among Brazilian blood donors and multiply transfused hemodialysis patients. Am J Trop Med Hyg 1997; **57**: 168-71.
10) Mathur P, Samantaray JC. The first probable case of platelet-transmission- transmitted visceral leishmaniasis. Transfus Med 2004; **14**: 319-21.
11) Singh S. Transfusion transmitted leishmaniasis: a case report and review of literature. Indian J Med Microbiol 2006; **24**: 165-70.
12) Mpaka MA, Daniil Z, Kyriakou DS, Zakynthinos E. Septic shock due to visceral leishmaniasis, probably transmitted from blood transfusion. J Infect Dev Ctries 2009; **3**: 479-83.
13) Mestra L, Lopez L, Robledo SM, et al. Transfusion-transmitted visceral leishmaniasis caused by Leishmania (Leishmania) mexicana in an immunocompromised patient: a case report. Transfusion 2011; **51**: 1919-23.
14) Riera C, Fisa R, Udina M, et al. Detection of Leishmania infantum cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg 2004; **98**: 102-10.
15) Riera C, Fisa R, López-Chejade P, et al. Asymptomatic infection by Leishmania infantum in blood donors from the Balearic Islands (Spain). Transfusion 2008; **48**: 1383-9.
16) Scarlata F, Vitale F, Saporito L, et al. Asymptomatic Leishmania infantum/chagasi infection in blood donors of western Sicily. Trans R Soc Trop Med Hyg 2008; **102**: 394-6.
17) Biglio A, Bola C, Concialdi E, et al. Asymptomatic Leishmania infantum infection in an area of North-Western Italy (Piedmont region) where such infections are traditionally endemic. J Clin Microbiol 2010; **48**: 131-6.
18) Cardo LJ, Rentas FJ, Ketchum L, et al. Pathogen inactivation of Leishmania donovani infantum in plasma and platelet concentrate using riboflavin and ultraviolet light. Vox Sang 2006; **90**: 85-91.
19) Eastman RT, Barrett LK, Dupuis K, et al. Leishmania inactivation in human apheresis platelets by a psoralen (amotosalen HCL) and long-wavelength ultraviolet irradiation. Transfusion 2005; **45**: 1459-63.
20) Wagner SJ, Skripchenko A, Salata J, Cardo LJ. Photoinactivation of Leishmania donovani infantum in red cell suspensions by a flexible thiopyrylium sensitizer. Vox Sang 2006; **91**: 178-80.
21) Kyriakou DS, Alexandrakis MG, Passam FH, et al. Quick detection of Leishmania in peripheral blood by flow cytometry. Is prestorage leucodepletion necessary for leishmaniasis prevention in endemic areas? Transfus Med 2003; **13**: 59-62.
22) Cardo LJ, Salata J, Harman R, et al. Leukodepletion filter reduce Leishmania in blood products when used at collection or at the bedside. Transfusion 2006; **46**: 896-902.
23) Cardo LJ, Asher L. Transfusion medicine illustrated. Electron micrographic study of the removal of

Leishmania from blood products by leukodepletion filters. Transfusion 2006; **46**: 315-6.

24) Mary C, Faraut F, Lascombe L, Dumon H. Quantification of Leishmania infantum DNA by a real-time PCR assay with high sensitivity. J Clin Microbiol 2004; **42**: 5249-55.
25) Martin-Ezquerra, Fisa R, Riera C, et al. Role of Leishmania spp. infestation in nondiagnostic cutaneous granulomatous lesions: report of a series of patients from a Western Mediterranean area. Br J Dermatol 2009; **161**: 320-5.
26) Grogl M, Daugirda JL, Hoover DL, et al. Survivability and infectivity of viscerotropic Leishmania tropica from operation Desert Storm participants in human blood products maintained under blood bank conditions. Am J Trop Med Hyg 1993; **49**: 308-15.
27) de Freitas E, Melo MN, da Costa-Val AP, Michalick MS. Transmission of Leishmania infantum via blood transfusion in dogs: potential for infection and importance of clinical factors. Vet Parasitol 2006; **137**: 159-67.
28) Urs G, Oakley DA, Owens SD, Schantz P. Leishmania donovani transmission by packed RBC transfusion to anemic dogs in the United States. Transfusion 2002; **42**: 381-3.
29) Fisa R, Riera C, Ribera E, et al. A nested polymerase chain reaction for diagnosis and follow-up of human visceral leishmaniasis using blood samples. Trans R Soc Trop Med Hyg 2002; **96**: S1/191-S1/194.
30) Lachaud L, Dereure J, Chabbert E, et al. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with reference to AIDS patients. J Clin Microbiol 2000; **38**: 236-40.
31) Prina E, Roux E, Mattei D, Milon G. Leishmania DNA is rapidly degraded following parasite death: an analysis by microscopy and real-time PCR. Microbes Infect 2007; **9**: 1307-15.
32) de la Llave E, Lecoeur H, Besse A, et al. A combined luciferase imaging and reverse transcription polymerase chain reaction assay for the study of Leishmania amastigote burden and correlated mouse tissue transcript fluctuations. Cell Microbiol 2011; **13**: 81-91.
33) Lemesre JL, Darcy F, Kweider M, et al. Requirements of defined cultivation conditions for standard growth of Leishmania promastigotes in vitro. Acta Trop 1988; **45**: 99-108.
34) Mary C, Faraut F, Drogoul MP, et al. Reference values for Leishmania infantum parasitemia in different clinical presentations: quantitative polymerase chain reaction for therapeutic monitoring and patient follow-up. Am J Trop Med Hyg 2006; **75**: 858-63.

Arrived: 16 December 2011 - Revision accepted: 1 January 2012
Correspondence: Teresa Jimenez-Marco
Fundació Banc de Sang i Teixits de les Illes Balears
C/ Rosselló i Caçador, 20
07004 Palma de Mallorca-Balearic Islands, Spain
e-mail: matejimenez@hotmail.com