Published in final edited form as:

Transfusion. 2014 March ; 54(3): 585–591. doi:10.1111/trf.12357.

***Babesia*: Impact of Cold Storage on the Survival and the Viability of Parasites in Blood Bags**

Jeny R. Cursino-Santos, Andy Alhassan, Manpreet Singh, and Cheryl A. Lobo*

Department of Blood-Borne Parasites Lindsley Kimball Research Institute, New York Blood Center, New York, NY 100 065

Abstract

BACKGROUND—*Babesia* represents one of the major infectious threats to the blood supply since clinically silent infections in humans are common and these can be life-threatening in certain recipients. It is important to understand the effect of blood storage conditions on the viability of babesia as this will impact the occurrence and severity of transfusion transmitted babesiosis.

STUDY DESIGN AND METHODS—*Babesia divergens* was introduced into blood bags containing leukoreduced RBC and stored at 4°C for 0–31 days. Samples were withdrawn for assessment of the presence, morphology and viability of parasites. Blood smears were made immediately on removal from blood bags at different time intervals and evaluated blood film microscopy. RBCs withdrawn from the bags were also cultured for 8 days using conditions optimal for parasite reproduction and growth to allow assessment of parasite viability.

RESULTS—After 24 h of storage at 4°C, there was a substantial reduction of parasitemia in the blood-bags, which was maintained throughout storage. This decrease was accompanied by a change in morphology of parasites, with the number of altered parasites increasing through the period of storage. However, viability was maintained through 31 days of cold storage with a lag in achieving exponential growth seen in the parasites subjected to longer periods of refrigeration.

CONCLUSION—Refrigeration of *B. divergens* leads to an alteration of parasite morphology and a decrease in parasite numbers. However, there are sufficient parasites that are robust enough to survive 31 days of storage at 4°C and yield high end-point parasitemia.

Babesiosis is a tick-borne zoonotic disease endemic in the north-east and Midwest US[1]. There are multiple species of *Babesia* that invade and infect the red blood cells of various animal species, however, only a few of them have been shown to be zoonotic. Primarily among these are *B. microti*, a rodent parasite that is responsible for most of the infection in the US; *B. divergens*, a cattle parasite seen primarily in Europe [2], although *B. divergens* like variants have been found in the US [3]; and *B. duncani*, a few cases of which have been identified in California [4,5].

Besides their natural route of transmission through tick bites, the spread of *Babesia* is becoming increasingly common and problematic through blood transfusions. In fact, babesiosis has become the most frequent transfusion-transmitted infection with approximately 162 cases reported since 1980 and 12 associated fatalities in the period 2005–2008 [6–9]. These figures probably undercount the actual number of transfusion-associated cases [6]. This is because the disease is clinically silent in most healthy adults who are the bulk of blood donors. In the absence of a licensed test, current safeguards against babesia

*Corresponding Author cloblo@nybloodcenter.org Tel: 212-570-3415 Fax: 212-570-3126.

Conflict of Interest: The authors declare no conflict of interest

remain a questionnaire relating to past history of the infection and have not proven to be effective in protecting against the parasite [10].

In order that the parasite be successfully transmitted by transfusion, it has to survive at 4°C, under the same conditions used for storing donor blood [11]. A recent paper on *P. falciparum*, the malaria parasite showed that the parasite remains detectable in blood smears upto 28 days, although after 14 days it is no longer viable [12]. As this question has not been reliably investigated for babesia, we performed a similar study, using *B. divergens*, which is currently the only babesial pathogen capable of causing disease in man that can be cultured in human RBCs *in vitro*. *B. divergens*, however, has been shown to be a transfusion threat only in Europe and not in the US.

MATERIALS AND METHODS

Parasite Propagation

Blood stages cultures of the *B. divergens* (Bd Rouen 1986 strain) were maintained *in vitro* in human A + RBCs using RPMI 1640 (Invitrogen) medium supplemented with 10% human serum and 0.24% (w/v) sodium bicarbonate solution (Invitrogen). Cells were cultured at 37 °C in a 90% CO₂, 5% nitrogen, and 5% oxygen, as previously described [13]. Blood smears were made from cultures and stained with Giemsa to calculate parasitemia (number infected RBC out of every 100 RBCs).

Preparation of *B. divergens* infected Blood Bags

Leuko-reduced RBC units were obtained from the New York Blood Center. Units were used on the same day that they were drawn. Parasite cultures were centrifuged, parasitemia determined by Giemsa staining and required aliquots of infected RBC were added to each bag to obtain desired parasitemia. The zero time (ZT) sample which served as the control for the refrigerated stored samples, was withdrawn immediately after thoroughly mixing and then the bags were stored at 4 °C for various time periods.

Determination of zero-time (ZT) parasitemia from various storage-time samples

After removal from 4°C, bags were thoroughly mixed on a shaker for 30 minutes. 5 mL of zero time (ZT) aliquots from the 7 storage time points (day 0, 1, 3, 10, 17, 24 and 31) were withdrawn. The bags were then returned to 4°C after withdrawal of each aliquot. Smears were immediately made from each ZT aliquot, stained with Giemsa and parasitemia enumerated.

Culturing and testing of viability of aliquots from blood bags (assessing parasitemia for days 1–8 following culture)

To determine the viability of parasites in the 5 mL aliquots drawn from the blood bags at the 7 storage time-points, the blood was centrifuged, washed thrice with RPMI-1640 and put *in vitro* culture using standard conditions (see parasite propagation). Cultures were maintained for 9 days or until a parasitemia of 50% was reached, whichever was earlier. Smears were made and Giemsa stained to analyze growth of the parasites on a daily basis (upto day 9) **until** parasite cultures crashed (>50% parasitemia).

RESULTS

Morphology of parasites changes upon storage at 4°C

One of the striking effects of refrigeration on *Babesia* parasites was a distinct change in parasite morphology. *Babesia* parasites maintained at 37°C, appear normal (Figure 1, panel

A). Parasites replicate by binary fission and in normal cultures, a variety of these forms can be seen, in 1 (ring), 2 (“figure 8” stage) or 4 celled (Maltese cross) stages (Figure 1A). Upon storage at 4°C, parasites appear to undergo a condensation or shrinking effect, forming crisis forms and can be seen to occupy smaller volumes of the RBC as compared to non-stressed parasites (Figure 1, panel B). The number of these “condensed” parasites as well as the degree of condensation of these parasites increases steadily with days of cold stress with the maximum numbers of these abnormal parasites seen when parasites are withdrawn from the blood bags on days 24 to 31. However, very small numbers of morphologically normal parasites are also present at this time (day 31 has almost none) and thus it is the ratio of normal to abnormal parasites that decreases through the period of refrigeration. It is not clear what the fate of these abnormal parasites would be when they are put into culture at 37°C, with respect to viability and time needed to propagate.

Parasitemia decreases upon storage at 4°C

For the purpose of parasite enumeration, all stained organisms (normal and abnormal morphology) within the RBC were counted to obtain parasitemia at different time periods. This was done as it is not possible, by mere examination of the Giemsa stained smear, to predict the viability and potential reproductive abilities of the altered parasites. Parasitemias on day 0 (ZT) of all storage time-points were assessed to determine the effect of cold temperature on the survival of *B. divergens* and the impact of length of storage at 4°C on the survival of the parasites.

Parasitemia at zero-time (ZT) in all 4°C storage time-points—Parasites were introduced into bags to obtain a final parasitemia of ~0.25–0.27% (Table 1, 0 storage time-point at ZT), as this level represents an average typical parasitemia in blood donors. Parasitemia represents the number of infected RBCs out of every 100 RBCs. We measured ZT parasitemia for the various time periods of storage by taking an aliquot from the bag at the specified time (days 1, 3, 10, 17, 24, 31) as soon as the bag is taken out from the refrigerator and mixed thoroughly (ZT for all bags). After just one day of storage at 4°C, ZT parasitemia dropped by ~30–40% and this drop was maintained through the prolonged period of cold storage (0.25% versus **0.15%** in bag 1 and 0.27% versus 0.19% in bag 2, see Table 1). However, the decrease in parasitemia was not severely impacted by the length of storage at 4°C, up to day 24 of blood bag storage (0.15% versus 0.15% in bag 1 and 0.19% versus 0.21% in bag 2, Table 1). Thus, there was no significant difference between the decrease in parasitemia as measured by ZT parasitemia on day 1 or day 24 of storage at 4°C. This was unexpected and indicated that the impact of sub-optimal temperatures on *Babesia* is immediate and does not increase substantially over time. However, the read-out of this effect is based on Giemsa stained smears and while differences resulting in severely deformed and disintegrating parasites (Figure 1) can be seen, we cannot assess the effect of these changes on the survival or viability of the parasite, so all stained parasites were included in the tally, irrespective of morphology. On day 31, however, there was a further decrease in parasitemia in both bags averaging a 50–60% decrease from control smears (Table 1, parasitemia 0.12% in bag 1 on day 31 and 0.11% in bag 2 on day 31).

Viability of parasites is affected by storage at 4°C

While total numbers of parasites remained relatively constant from day 1 through day 24 of cold storage, we probed for effects of the stress on the parasites that would impact their viability by comparing the ability of parasites, stored at 4°C for the different time periods (1, 3, 10, 17, 24 and 31 days) and parasites not exposed to 4°C (0 storage time-point) to reproduce when put into *in vitro* culture.

Control Cultures—The time between one cell division and the next in the asexual erythrocytic *B. divergens* is approximately 8h. Thus, control parasites in the blood bag, which were not subjected to low temperature stress, reproduced normally through successive intra-erythrocytic cycles and by day 6, showed a typical parasitemia of over 50%.

Parasitemia on Days 1–2 of culture in all 4°C storage time-points—Parasitemia in RBCs stored for days 1, 3, 10 and 17 showed typical progression of asexual multiplication and growth after being put into culture. Thus, parasitemia rose steadily, although final levels were a little lower than those found in the control flasks on all days of culture. However, we found that in the case of day 24 and 31 storage time-points, parasitemia on days 1 and 2 following introduction into culture resulted in a decrease in parasitemia as compared to that sample's zero time (ZT) parasitemia. For example, day 24 storage time-point samples showed a parasitemia of ~0.15% at ZT, which dropped to 0.13 % on Day 1 and 0.11% on day 2. Again, for the day 31 storage time-point samples, we record ZT parasitemia of around 0.12% which drops to 0.08% on day 1. **We hypothesize that this is due to** the disintegration and removal of the severely cold-damaged parasites which were enumerated at Day 0, but are no longer around to contribute to parasitemia on days 1 and 2 of culture. Thus, day 1–2 parasitemias after being put into culture may represent a better parameter to measure the initial effect of refrigeration on these parasites. Figure 2 shows this difference in early culture parasitemia among aliquots withdrawn after different periods of storage at 4°C.

Parasitemia on Day 3–8 of culture in all 4°C storage time-points—The assessment of parasitemia from RBCs stored for various lengths of time at 4°C allowed us to separate them into two groups. The first is parasitized RBCs stored upto day 10 storage time-point at 4°C, where we see, that while the parasitemia was lower at ZT, the parasites recovered efficiently on subsequent days in culture to produce parasitemias similar to the control flasks by day 3 in culture (parasitemia averaging 2.5% in bag 1 and 1.4% in bag 2). These parasites, as did the control, reached parasitemia over 50%, by day 6 in culture. The second group encompasses those parasites recovered from the blood bags after 17 or more days of cold storage. These parasites experienced a lag in multiplication rates and this was seen in the time taken by these parasites to reach 50% parasitemia once put into culture. Thus, aliquots withdrawn at day 17 of storage time, took 7 days to reach ~50% parasitemia while parasites subjected to 24 days of cold storage required 8 days to achieve 50% parasitemia. Parasites recovered after 31 days of storage had not reached 50% parasitemia levels on day 8, the end-point of our study. Thus, although ZT parasitemia were similar from day 1 to day 24 of cold storage, the effect of the low temperatures did impact the viability of *B. divergens*. This difference in viability may be a reflection of the increased amounts of condensed parasites seen in the aliquots withdrawn from the blood bags at later time points. Thus, *in vitro* culture is a needed analytical tool to probe these differences in the parasites.

DISCUSSION

From a blood safety perspective, transfusion-transmitted infections involving *Babesia* have become increasingly problematic, with progressively more reported each year [14]. In the period from 2005 to 2010, 3.6% (11/307) of transfusion-related fatalities reported to the FDA were due to TTB [15]. This risk of transmission of *Babesia* via transfusion is intimately linked to its capacity to survive blood collection and blood storage procedures. In this study, the focus was on determining the impact of cold storage (4°C) on the viability of the parasite that was introduced into bags at donor-typical levels of parasitemia (**Lobo et al, unpublished observations**). As blood bags are stored for more than 31 days, we followed parasite viability by assessing blood samples at weekly intervals during the period of storage.

Systematic laboratory screening of donor blood in the form of FDA-licensed serological and nucleic acid testing (NAT) assays is available for many pathogens like blood-borne viruses to prevent their spread by transfusion [16]. Unfortunately, the lack of comparable, sensitive screens available for protozoan parasites like *Babesia*, has resulted in the current complete dependence on a donor response questionnaire to safeguard the nation's blood supply [10]. This safe-guard, as we can see from the increasing numbers of TTB cases, is not efficient in ensuring the safety of the 5 million or so recipients that receive blood every year. In the absence of a suitable test, small changes to blood storage conditions may have an impact on TTB incidence. Storage of the infected blood for just 24 h resulted in a precipitous drop in the number of parasites in the unit, which augurs well for transfusions involving recipients with functioning immune systems as we hypothesize that the reduced parasite numbers will result in a sub-clinical episode if any, of babesiosis. Unfortunately, our finding that *B. divergens* can survive and replicate after 31 days of storage at 4 °C, raises other concerns for transfusion safety. Although parasite levels decreased from day 0 through day 31 of storage, the parasite needed very little time (2–3 days) to reach exponential growth. This rapid burst of multiplication resulting in parasitemia over 50% is what makes *Babesia* a particular concern for transfusion recipients that suffer from immune-deficiencies or asplenic recipients. The latter are a special concern because the majority of this group is sickle cell and thalassemic patients that require repeated transfusions, which heightens the overall risk of acquiring the parasite [9]. The high parasitemia found *in vitro* culture is not an artifact but reflects what happens *in vivo*, in the absence of effective immune responses.

A detailed look into the reduction in numbers of *Babesia* parasites that survived refrigeration reveal that while the numbers decreased through cold storage, there still remain parasites capable of transmitting infection probably in part due to the vast numbers of RBCs involved in a single transfusion event. We chose a modest starting parasitemia as this would represent a typical parasitemia in donors who would have to be devoid of any clinical symptoms if they felt well enough to donate blood (**Lobo et al, unpublished observations**). This 0.25% parasitemia represents a number of 1.25×10^8 parasites per mL of blood or 3.75×10^{10} parasites totally in the blood unit, assuming an average of 5×10^{10} RBCs per mL of packed blood and 300 mL of packed RBCs/unit. Even when the parasitemia drops by large percentages, for example ZT parasitemia on day 31 of storage showed a 50% drop in parasitemia, there still remains $\sim 10^{10}$ parasites in the blood unit, which would be capable of causing illness and/or death in certain recipients. The change in parasite morphology through the extended period of storage at 4°C, while **significant, does not appear to result in a total loss of infectivity**, although parasitemias found in the latter half of the storage period could be resulting from the few morphologically normal parasites that are still present. It would take additional studies to clearly determine the fate of these condensed parasites in contributing to future rounds of asexual multiplication.

This study is the first systematic analysis of the effect of refrigeration temperatures on the presence and persistence of *B. divergens*. An earlier study focused on *B. microti* with significant differences in methodology [17]. The parasite source in that study was hamster RBCs infected with *B. microti* and as *B. microti* cannot be cultured successfully long term *in vitro*, the end-point assay for viability was a xenodiagnostic assay where aliquots of infected blood were inoculated into hamsters to look for the ability to multiply and infect hamster RBCs. The sensitivity afforded by *in vitro* culture of *Babesia* should be superior to that of hamster inoculation. Stability of hamster red cells versus human red cells over the 30 day period is an additional confounder to results obtained with that study. Another difference related to conditions of storage of the infected blood as in the earlier study, which was in tubes and not blood bags, thus gaseous exchange which is optimal in blood bags was not facilitated in the tubes, which could have impacted the results. However, the conclusions from that study are on the whole in agreement with ours, that infectivity of *Babesia*

remained through 21 days of storage at 4°C, albeit, dropping to 25% on day 21[17]. This is also corroborated by a case of TTB involving a blood unit stored for 35 days [18]. A similar study with *P. falciparum*, the malaria parasite revealed that parasites can be detected through day 28 of cold storage; however, after day 14, the viability of the parasite is in question as no asexual multiplication was observed when the parasites were put into in vitro culture [12]. Unlike *Plasmodium*, *Babesia divergens* robustly maintained its ability to divide and this resistance to cold shock elevates its threat to the blood supply. From our study, it becomes clear that while the infectivity of *B. divergens* persists through the shelf-life of stored blood, adding to the problem of preventing TTB, there is a significant drop in parasite levels resulting from just one day of storage. This may have implications for the use of stored blood in transfusions in areas endemic for babesia.

Acknowledgments

Funding for this study was provided by a grant from the NIH to CAL: RO1-HL105694

REFERENCES

1. Leiby DA. Babesiosis and blood transfusion: flying under the radar. *Vox Sang.* 2006; 90:157–165. [PubMed: 16507014]
2. Gray JS. Identity of the causal agents of human babesiosis in Europe. *Int J Med Microbiol.* 2006; 296(Suppl 40):131–136. [PubMed: 16524772]
3. Herwaldt B, Persing DH, Precigout EA, Goff WL, Mathiesen DA, et al. A fatal case of babesiosis in Missouri: identification of another piroplasm that infects humans. *Ann Intern Med.* 1996; 124:643–650. [PubMed: 8607592]
4. Conrad PA, Kjemtrup AM, Carreno RA, Thomford J, Wainwright K. Description of *Babesia duncani* n.sp. (Apicomplexa: Babesiidae) from humans and its differentiation from other piroplasms. *Int J Parasitol.* 2006; 36:779–789. [PubMed: 16725142]
5. Bloch EM, Herwaldt BL, Leiby DA, Shaieb A, Herron RM, et al. The third described case of transfusion-transmitted *Babesia duncani*. *Transfusion.* 2012; 52:1517–1522. [PubMed: 22168221]
6. Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D, et al. Transfusion-associated babesiosis in the United States: a description of cases. *Ann Intern Med.* 2011; 155:509–519. [PubMed: 21893613]
7. Tonnetti L, Eder AF, Dy B, Kennedy J, Pisciotto P, et al. Transfusion-transmitted *Babesia microti* identified through hemovigilance. *Transfusion.* 2009
8. Johnson ST, Van Tassell ER, Tonnetti L, Cable RG, Berardi VP, et al. *Babesia microti* real-time polymerase chain reaction testing of Connecticut blood donors: potential implications for screening algorithms. *Transfusion.* 2013
9. Asad S, Sweeney J, Mermel LA. Transfusion-transmitted babesiosis in Rhode Island. *Transfusion.* 2009; 49:2564–2573. [PubMed: 19761547]
10. Leiby DA. Transfusion-associated babesiosis: shouldn't we be ticked off? *Ann Intern Med.* 2011; 155:556–557. [PubMed: 21893616]
11. Leiby DA. Transfusion-transmitted *Babesia* spp.: bull's-eye on *Babesia microti*. *Clin Microbiol Rev.* 2011; 24:14–28. [PubMed: 21233506]
12. Chattopadhyay R, Majam VF, Kumar S. Survival of *Plasmodium falciparum* in human blood during refrigeration. *Transfusion.* 2011; 51:630–635. [PubMed: 20849405]
13. Gorenflot A, Brasseur P, Precigout E, L'Hostis M, Marchand A, et al. Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man. *Parasitol Res.* 1991; 77:3–12. [PubMed: 1994368]
14. Babesiosis surveillance - 18 States, 2011. *MMWR Morb Mortal Wkly Rep.* 2012; 61:505–509. [PubMed: 22785341]
15. Cushing M, Shaz B. Transfusion-transmitted babesiosis: achieving successful mitigation while balancing cost and donor loss. *Transfusion.* 2012; 52:1404–1407. [PubMed: 22780893]

16. Lindholm PF, Annen K, Ramsey G. Approaches to minimize infection risk in blood banking and transfusion practice. *Infect Disord Drug Targets*. 2011; 11:45–56. [PubMed: 21303341]
17. Eberhard ML, Walker EM, Steurer FJ. Survival and infectivity of *Babesia* in blood maintained at 25 C and 2–4 C. *J Parasitol*. 1995; 81:790–792. [PubMed: 7472878]
18. Mintz ED, Anderson JF, Cable RG, Hadler JL. Transfusion-transmitted babesiosis: a case report from a new endemic area. *Transfusion*. 1991; 31:365–368. [PubMed: 2021001]

Fig 1.

Morphology of *B. divergens* parasites as seen by Giemsa staining of smears.

Panel A: grown without cold stress under normal condition at 37°C. From left to right, ring, 2 celled figure 8, 4 celled Maltese cross and multiply invaded RBC.

Panel B: smeared from bag after storage at 4°C for 1–31 days.

Fig. 2.

Growth curves of parasites in the early period of culture showing a distinct lag in growth as time of storage at 4°C increases. Zero time-Flask, no cold storage (ZT); Culture started from bag stored for 1 day(1d); 3 days(3d); 10 days(10d), 17 days(17d), 24 days(24d) and 31 days (31d)

Panel A: Bag1; **Panel B:** Bag2

Table 1

Monitoring of parasite presence (ZT) and growth (time in culture) at seven storage time-points when aliquots of blood were collected directly from the bags and left in culture for noted times, through day 8.

Storage time point at 4°C (days)		Parasitemia (%)															
		Time in culture at 37 °C (days)															
		BAG1								BAG2							
		**ZT	1	2	3	4	5	6-7	8	**ZT	1	2	3	4	5	6-7	8
*0		0.25	0.45	0.78	3	8	39	61	Crash	0.27	0.70	1.39	2	5	25	57	Crash
1		0.15	0.28	0.64	2	5	27	67	Crash	0.19	0.43	0.91	1	5	18	55	Crash
3		0.18	0.45	1.43	3	7	28	63	Crash	0.22	0.35	0.76	1	4	18	58	Crash
10		0.16	0.47	0.50	2	5	27	67	Crash	0.21	0.38	0.68	1	2	10	59	Crash
17		0.16	0.40	0.48	2	3	13	55	Crash	0.21	0.24	0.37	0.71	2	8	42	Crash
24		0.15	0.13	0.11	0.58	0.91	5	35	Crash	0.21	0.18	0.09	0.28	1.05	1.25	20	45
31		0.12	0.08	0.09	0.52	0.52	0.63	5	11	0.11	0.08	0.12	0.28	0.38	1.34	3	7

Crash: Further growth of parasites cannot be supported without addition of fresh RBC.

* Control: Sample collected immediately after *B. divergens* was inoculated into blood bag and not subjected to cold stress.

** Zero Time (ZT) of all Storage Time-Points before culturing.