Distribution and survival of *Borrelia miyamotoi* in human blood components

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BACKGROUND: *Borrelia miyamotoi,* the agent of relapsing fever, is a tick-borne spirochete first isolated in Japan in 1994. Since then, the spirochete has been detected in ticks globally, generally in the same vectors as the Lyme disease agent. Human infection has been reported in Russia, Europe, Japan, and the United States, as influenza-like febrile illness. In addition, two cases of meningoencephalitis caused by *B. miyamotoi* have also been reported in immunocompromised patients. Here we evaluate the ability of the spirochete to survive in human blood components stored under standard blood bank conditions.

STUDY DESIGN AND METHODS: Freshly collected human whole blood was spiked with in vitro cultured *B. miyamotoi* or *B. miyamotoi*–infected mouse plasma and separated into red blood cells (RBCs), plasma, and platelets. Components were either injected into immunocompromised (SCID) or wild-type immunocompetent mice or cultured in vitro, right after separation and after storage at the appropriate conditions. Infection was monitored by microscopic observation, blood smears, and polymerase chain reaction.

RESULTS: In vivo, all the SCID mice challenged with the components before storage and the RBCs stored for up to 42 days developed the infection. Wild-type mice also developed the infection when injected with prestorage samples from all components, while a lower number of mice were infected by RBCs stored for 42 days. In vitro, spirochetes grew in all samples but frozen plasma.

CONCLUSIONS: This study demonstrated that *B. miyamotoi* can survive standard storage conditions of most human blood components, suggesting the possibility of transmission by blood transfusion.

The current approach to preventing transfusion-transmitted infections is composed of multiple strategies including pathogen surveillance, testing, and donor deferral. These strategies have greatly enhanced the overall safety of the blood supply. Nevertheless, new emerging pathogens represent a persistent threat that requires careful evaluation and monitoring. Experimental data establishing the transmissibility by blood transfusion of emerging pathogens are necessary to determine if the organisms could be transmitted by blood transfusion. *Borrelia miyamotoi* is a newly described spirochete transmitted by ticks.[1] Human infections are characterized by influenza-like illness with possible relapsing episodes of spirochetemia and fever, usually completely cleared by a single course of antibiotics.[2] However, not unlike other tick-borne pathogen caused infections, the disease can be severe in immunocompromised patients, who can develop meningoencephalitis.[3,4] The first step in establishing if a pathogen is at risk of being transmitted by blood transfusion is to determine its survival under standard blood storage conditions. A previous study has shown the capability of *B. miyamotoi* to survive in murine blood for up to 7 days at 4°C.[5] In this study we determine the presence of the spirochete in different human blood

ABBREVIATIONS: PRP = platelet-rich plasma; RT = room temperature; WB = whole blood.

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components after separation and its ability to survive standard storage blood bank conditions.

MATERIALS AND METHODS

Collection of whole blood

Blood was collected from volunteer donors by the Holland Laboratory Research Blood Program under an approved institutional review board protocol. For in vivo experiments, four 4.5-mL 3.2% sodium citrate tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) were collected from a single donor on the day of each experiment and kept at room temperature (RT) until spiking. For in vitro experiments, whole blood (WB) units were collected using CP2D/AS-3 blood bag with in-line leukoreduction filter (ATS-LPL, Haemonetics, Braintree, MA) on the day of each experiment.

Small-volume WB spiking and component preparation

WB tubes were inoculated with *B. miyamotoi*–infected mouse pooled plasma to a final concentration of 6×10^4 spirochetes/mL. Spiked tubes were centrifuged at $150 \times g$ for 10 minutes at RT and the PLT-rich plasma (PRP) was collected and pooled; 500 μL of buffy coat was then removed from the red blood cell (RBC) fraction and RBCs were pooled. The PRP was centrifuged at $400 \times g$ at RT for 10 minutes to separate plasma and platelets (PLTs). Approximately two-thirds of the plasma was removed and the remaining was used to dilute the PLT fraction. Aliquots of RBCs were stored at 4°C while aliquots of plasma were frozen at −20°C; PLT samples for the in vivo experiments were not stored.

Animal inoculation and blood collection

Two strains of mice were used for this study: CB17 SCID (Charles River Laboratories, Wilmington, MA) and *Peromyscus leucopus* (University of South Carolina, Columbia, SC). Spiked RBCs, plasma, and PLTs (200 μL) were injected intravenously, before storage, into groups of five animals. Aliquots of spiked RBCs were also injected after 21 and 42 days of storage at 4°C, while aliquots of spiked plasma were injected after 30 days of storage at −20°C. All animals were monitored for development of spirochetemia on Days 3, 7, and 30 postinoculation. In addition, mice of both strains injected with RBCs were also monitored on Day 20 and *P. leucopus* mice monitoring included additional observations on Day 9. Before blood collection, the animals were warmed under a heat lamp for 2.5 minutes. A drop of blood was collected by lateral tail vein nicking into a heparinized capillary tube and plasma was separated from RBCs in a capillary tube centrifuge. Spirochetemia was determined using a Petroff-Hauser counting chamber by counting the spirochetes in the plasma fraction under a 40× objective.

At the end of the study, mice were euthanized by cardiac puncture and the collected blood was used to prepare blood smears and for polymerase chain reaction (PCR) analysis. All mice procedures were performed using a protocol approved by the American Red Cross Animal Care and Use Committee. In conducting research using animals, the investigator(s) adhered to the Animal Welfare Act Regulation and other Federal statutes relating to animals and experiments involving animals and the principles set forth in the current version of the Guide for Care and Use of Laboratory Animals, National Research Council.

Microscopic observation of blood smears

Thin blood smears were prepared by spreading a drop of blood across a microscope slide and allowing it to air dry. For acridine orange stain, smears were fixed in 100% methanol for 1.5 minutes, stained with acridine orange for 2 minutes, rinsed in water, and allowed to air dry. HEMA stain was performed as directed by the manufacturer. Slides were observed under a 100× oil-immersion objective.

DNA extraction and glycerophosphodiester phosphodiesterase (GlpQ) real-time PCR

DNA extraction was performed from mouse blood collected by cardiac puncture. DNA was extracted from 200 μL of mouse RBCs using a high-molecular-weight DNA purification kit (Gentra Puregene Blood Core Kit B, Qiagen, Valencia, CA) with slight modification of the manufacturer's instructions. RBCs were diluted 1:1 in sterile phosphate-buffered saline and 300 μL was incubated in RBC lysis buffer for 1 hour. After rehydration with 100 μL of molecular-grade water, DNA was incubated overnight at RT. Real-Time PCR was performed on a real-time PCR system (ABI 7500, Applied Biosystems, Foster City, CA) running universal conditions. Sequences for the primers and probe were designed in applications-based primer design software (Primer Express, Version 3.0, Applied Biosystems) and synthesized by Life Technologies (Carlsbad, CA). The sequences are as follows: forward 5′-TGTTATAATGCACGACCCAGAAA-3′, reverse 5′-AGCTCGATTGGGAAATAATTGTG-3′, and probe 6FAM-TGACACAACCACAAATG-MGBNFQ. Each reaction was run in triplicate for 40 cycles using 2.5 μL of template DNA per replicate.

In vitro culture of *B. miyamotoi*

The cultures were initiated from cryopreserved spirochetes from infected mouse plasma and cultured in modified Kelly-Pettenkofer medium containing 50% of heat-inactivated human serum as described.[6] Cultures were maintained at 33°C with 6% CO_2 and diluted 1:25 in

TABLE 1. Number of mice infected versus the of number mice injected with each component before and after storage

Component	RBCs			Plasma		PLTs
Days of storage	0	21	42	0	30	0
Wild-type mice (infected/injected)*	5/5	4/5	2/5	2/5	0/5	3/5
Wild-type mice (infected/injected)†	4/5	5/5	3/5	3/5	0/5	3/5
SCID mice (infected/injected)*	5/5	5/5	5/5	5/5	0/5	5/5
SCID mice (infected/injected†	5/5	5/5	5/5	5/5	0/5	5/5

* Experiment 1.
† Experiment 2.

fresh medium when they reached the concentration of 10^7/mL.

WB unit spiking, component preparation, and leukoreduction

WB units were spiked with cultured *B. miyamotoi* spirochetes to a final concentration of 6×10^4 or 6×10^3/mL. To separate RBCs and PRP, spiked WB units were spun in a floor centrifuge (Sorvall RC3, Thermo Fisher Scientific, Waltham, MA) at 2280 × *g* (ACE 1.6×10^7) at 22°C with low stop break. Separated units were carefully removed from the centrifuge and placed in the plasma expresser and PRP was expressed into a satellite bag through the PLT filter to remove white blood cells. Immediately after PRP removal, the AS-3 was added to the collection bag containing the RBCs. The PRP bag was detached and centrifuged at 4204 × *g* (ACE 5.5×10^7) at 22°C, with low stop break to separate plasma and PLTs. Plasma was expressed into a new bag except for 50 mL, which was used to resuspend the PLTs. The RBCs were leukoreduced using a leukoreduction filtration system for RBCs (Leukotrap, Haemonetics), following the manufacturer's instructions. Approximately half of the RBCs were allowed to pass through the filter, while half were left in the original collection bag to compare leukoreduced and nonleukoreduced RBCs.

Spirochete detection in vitro

Five milliliter of each freshly separated component was immediately diluted with an equal volume of modified Kelly-Pettenkofer medium, distributed in 12-well plates, 2 mL/well, and incubated at 33°C. The rest of the components were stored at the appropriate conditions: plasma at −20°C, RBCs at 4°C, and PLTs at 22°C under agitation. Additional samples were collected from plasma at 30 days, from RBCs at 21 and 42 days, and from PLTs daily up to 5 days and cultured under the same conditions as the fresh samples. Plasma and PLT cultures were observed twice a week for spirochete detection by microscopic examination at 32× and 40× magnification under bright and dark fields. The RBCs were cultured undisturbed for 1 week to let the RBCs settle to the bottom of the well. One milliliter of the supernatant was then transferred to a new plate with 1 mL of medium and cultured for one more week before observation.

RESULTS

Table 1 shows the number of mice infected versus the number of mice injected for both the wild-type and the SCID mice experiments. In two independent experiments with CB17 SCID mice, *B. miyamotoi* spirochetes were detectable by blood smears in all 30 animals injected with RBCs from all storage conditions (no storage, 21 days, 42 days). Similarly, all the SCID mice (20 total) injected with the fresh plasma and PLTs derived from *B. miyamotoi*–infected WB were infected, while none of the 10 mice injected with plasma stored 30 days at −20°C showed signs of infection (Table 1). In the two experiments with *P. leucopus* mice, nine of 10 animals showed circulating spirochetes in the blood after injection with fresh RBCs and with RBCs stored 21 days at 4°C. RBC samples stored for 42 days at 4°C infected five of 10 mice injected (Table 1). Plasma and PLT samples inoculated before storage infected five of 10 and six of 10 challenged *P. leucopus,* respectively, and none of the wild-type mice were infected by the frozen plasma samples (Table 1). Samples of blood from SCID CB17 mice injected with RBCs were collected on Days 3, 7, 20, and 30 postinoculation (Fig. 1A), while the SCID CB17 mice injected with plasma and PLT samples were tested on Days 3, 7, and 30 postinoculation (Figs. 1B and 1C). SCID CB17 mice showed increasing levels of spirochetes ranging between 10^4 and 10^8/mL, with the highest titers recorded at the last observation (Fig. 1). Blood from *P. leucopus* mice injected with RBCs was collected on Days 3, 7, 9, 20, and 30 postinoculation (Fig. 2A) and on Days 3, 7, and 30 from mice injected with plasma and PLT samples (Figs. 2B and 2C). The spirochete titer in *P. leucopus* mice was markedly lower than that in the SCID mice, with the highest titer on the order of 10^5/mL (Fig. 2). Furthermore, CB17 SCID mice were unable to clear the infection by the last observation while, in the *P. leucopus* mice, no spirochetes were detectable by microscopic observation after Day 20 and by PCR after 30 days (no PCR analysis was performed before Day 30). During the last observation, mice were euthanized by cardiac puncture and the blood collected was used for preparation of blood smears and PCR analysis. Blood smears were stained with acridine orange (Fig. 3A) or HEMA (Fig. 3B) and used for qualitative observation of the spirochetes.

The bacteria concentration was determined by counting the spirochetes in the components used for the in vivo experiments, using a Petroff-Hausser counting chamber. In all four experiments, bacterial concentrations in plasma

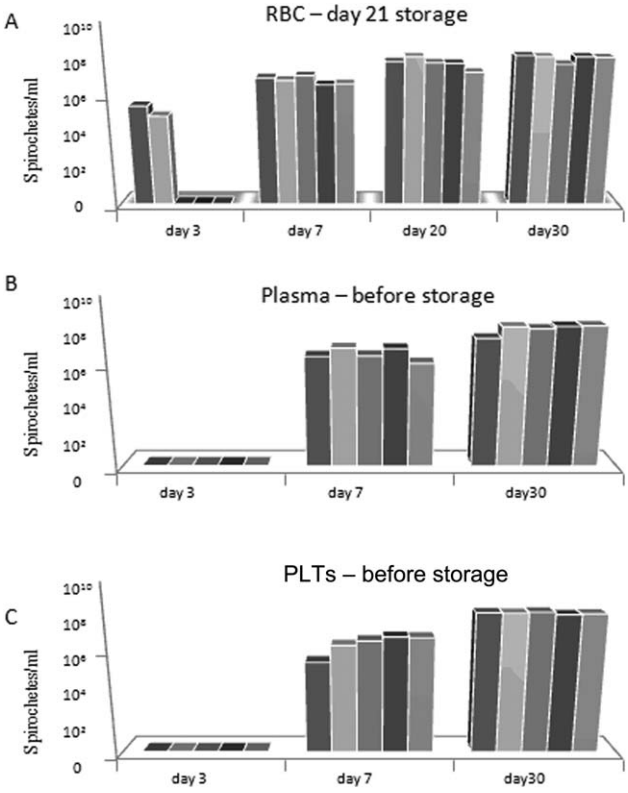

Fig. 1. Time course of spirochetemia in SCID CB17 mice: mice injected with *B. miyamotoi*–infected RBCs stored for 21 days at 4°C (A), *B. miyamotoi*–spiked plasma before storage (B), and *B. miyamotoi*–spiked PLTs before storage (C). Data shown in the figure are from one representative experiment and follow the spirochetima titers of five mice over time.

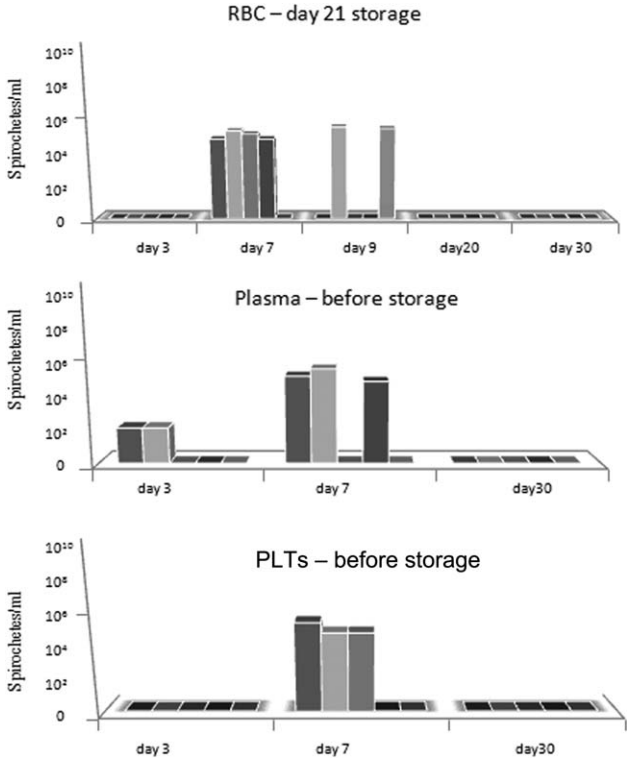

Fig. 2. Time course of spirochetemia in *P. leucopus* mice: mice injected with *B. miyamotoi*–spiked RBCs stored for 21 days at 4°C (A), *B. miyamotoi*–spiked plasma before storage (B), and *B. miyamotoi*–infected PLTs before storage (C). Data shown in the figure are from one representative experiment and follow the spirochetima titers of five mice over time.

and PLT fractions were similar. Concentrations of bacteria in the RBC components were not assessed because an accurate count was technically challenging due to the high concentration of the RBCs. However, a count could be estimated based on the total amount used to spike the WB and the spirochetes recovered in the plasma and PLT fractions. Based on this calculation, the majority of the bacteria were recovered in the RBC fraction (Table 2). Four in vitro experiments were performed to determine the survival of *B. miyamotoi* in leukoreduced plasma and PLTs and leukoreduced and nonleukoreduced RBCs, before and after storage (Table 3). In two experiments, the final concentration of spirochetes in the spiked WB units was 6×10^4, the same as that used in the in vivo experiment, while in two experiments, the WB units were spiked to a final concentration of 6×10^3 spirochetes/mL. In all experiments, motile spirochetes were visible by Day 7 in all Day 0 plasma and PLT samples as well as PLT samples collected daily up to Day 5. RBC samples were first monitored on Day 14 and mobile spirochetes were visible in all the Day 0 and Day 21 cultures, leukoreduced and nonleukoreduced. However, only the 42-day RBC cultures from WB units spiked with the higher concentration of spirochetes had detectable bacteria while no spirochetes were visible in the 42-day RBC cultures from WB units spiked with the lower concentration of spirochetes. Cultures of plasma samples stored at −20°C for 30 days were negative after 3 weeks of observation.

DISCUSSION

B. miyamotoi relapsing fever is a tick-borne spirochete, first isolated in Japan from *Ixodes persulcatus* in 1994.[1] Since then, the spirochete has been detected in ticks in Asia, Europe, and North America, generally in the same vectors as the Lyme disease agent, *Borrelia burgdorferi*.[2,7-22] In the United States, the prevalence of *B. miyamotoi* in ticks varies from 0% to 15.4% depending on the geographic area, and the infection can be carried by several species of ticks, including *Ixodes scapularis* in New England and New Jersey,[7,15,23] *Ixodes dentatus* in Michigan,[14] and *Ixodes pacificus* in California.[9] Although *B. miyamotoi* has been detected in birds and rabbits, the main reservoir of natural infection identified so far is the white-footed mouse, *P. leucopus*.[23] Testing of wild *P. leucopus* captured

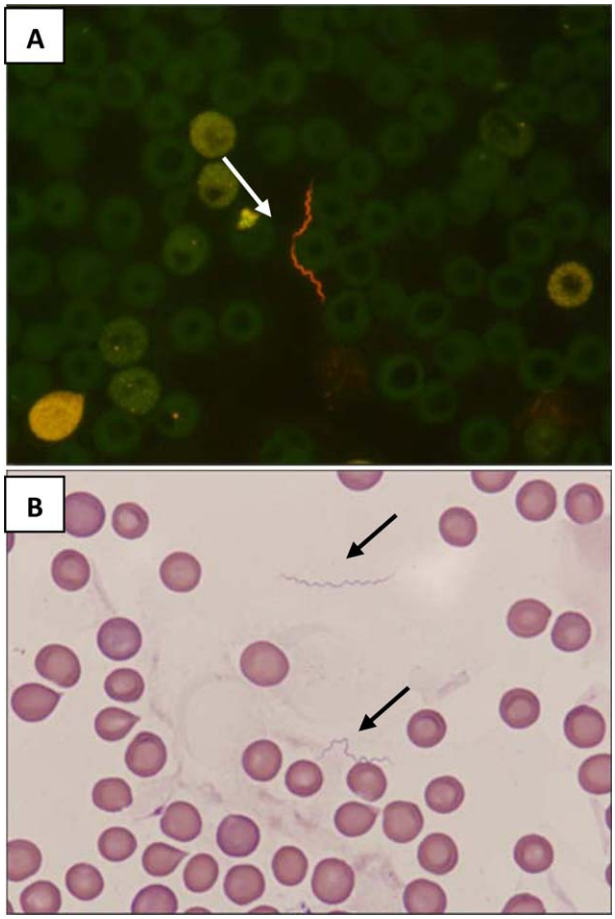

Fig. 3. *B. miyamotoi* spirochetes in blood smears: *B. miyamotoi*–infected SCID mouse blood smears stained with acridine orange (A) and HEMA (B) and observed under a 100× oil-immersion objective.

in southern Connecticut showed that infection with *B. burgdorferi* was twice as prevalent as *B. miyamotoi* in the animals, with a comparable duration of spirochetemia. Although *B. miyamotoi* density in the blood was fivefold higher than that of *B. burgdorferi*, the latter was more prevalent in the skin, where it produced a persistent higher-density infection.[23] It has been suggested that this observation could explain the lower prevalence of *B. miyamotoi* in ticks, as the opportunity for horizontal transmission for *B. miyamotoi* may be restricted to only the period of spirochetemia.[23] At the same time, the high titer of *B. miyamotoi* detected in mouse blood suggests the possibility of transmission by blood transfusion. The first human cases of *B. miyamotoi* were reported in Russia in 2011, in 46 patients hospitalized with flu-like symptoms;[2] other cases of human infection have been reported in the Netherlands,[3] the United States,[4,24-26] and Japan.[27] In two cases, immunocompromised patients developed meningoencephalitis,[3,4] and in a third one, the infection was initially described as human granulocytic anaplasmosis based on symptom manifestation.[26] In addition to clinical cases, a seroprevalence of 1% was detected in the serum of 584 healthy individuals living in areas endemic for Lyme disease between 1990 and 2010.[24] In the same study, serum samples from two other groups were tested: 277 patients from southern New England who were evaluated for suspected Lyme disease, and 14 patients from southern New York who were evaluated with a viral-like illness in late spring or summer. *B. miyamotoi* seroprevalence in the two groups was 3.2 and 21%, respectively. The data suggest the possibility that a portion of the human cases attributed to Lyme disease, especially in situations where the causative agent was not clearly determined, may have been caused by relapsing fever spirochetes, such as *B. miyamotoi*. Indeed, cross-reactivity between relapsing fever and Lyme disease antibodies in serologic tests based on whole cell enzyme-linked immunosorbent assay or indirect immunofluorescent assay has been previously reported.[28] Another possibility is dual infection as tick coinfection with multiple *Borrelia* species is not unusual. A study performed in New York in the fall of 2008 tested 286 ticks for multiple pathogens; this study detected at least one organism in 204 ticks (71%) and polymicrobial infection in 85 ticks (30%). *B. miyamotoi* was present in 2% of the tested ticks, all of which were coinfected with *B. burgdorferi*.[10]

TABLE 2. *B. miyamotoi* distribution in blood components

	Volume (mL)	Spirochete concentration (mL)
RBCs*	10	1×10^5
Plasma	5.9	1×10^4
PLTs	2.4	3×10^4

* Spirochete concentration estimated based on total number of spirochetes spiked in WB samples and the amount recovered in plasma and PLTs.

In a study published in 2014, Krause and colleagues[5] reported *B. miyamotoi* survival in mouse WB stored for up to 7 days at 4°C and its capability to be transmitted by blood transfusion in a mouse model. We started our study by investigating the survival and transmissibility by blood transfusion of the bacterium in human blood. We spiked small volumes of human WB with *B. miyamotoi*; separated the WB into RBCs, plasma, and PLT fractions; and tested the components for their ability to transmit the infection in mice. We utilized two mice strains in the study: an immunocompromised strain, which ensured sensitivity to the infection, and a wild-type strain to mimic an immunocompetent transfusion recipient. For the wild-type mice, we utilized a laboratory strain of *P. leucopus*, the most common reservoir of infection in the wild. After component separation, we estimated that the majority of the spirochetes were found in the RBC fractions, based on

TABLE 3. In vitro *B. miyamotoi* survival in blood components spiked with different concentration of spirochetes

Component	RBCs						Plasma		PLTs					
	LR*			Non-LR			LR		LR					
Days of storage	0	21	42	0	21	42	0	30	0	1	2	3	4	5
6×10^4 spirochetes /mL	+	+	+	+	+	+	+	−	+	+	+	+	+	+
6×10^3 spirochetes/mL	+	+	−	+	+	−	+	−	+	+	+	+	+	+

* Leukoreduced (LR) sample.

the difference between the total number of spirochetes inoculated in the WB and the amount recovered in plasma and PLTs. Furthermore, RBC fractions infected the highest number of wild-type mice and were still infectious after 42 days of storage at 4°C. However, a lower number of wild-type mice were infected from the RBC samples stored for 6 weeks, suggesting that a fraction of the bacteria did not survive the longer storage period. In all the in vivo experiments, the PLT fraction contained slightly more spirochetes than the plasma fraction; however, due to the different recovered volumes of the two components, mice injected with the PLT fraction received approximately three times more bacteria then the mice injected with the plasma fraction. The difference in spirochete load did not significantly affect the outcomes in the wild-type mice, with a comparable number of animals developing the infection in both groups. Freezing conditions killed the spirochetes since none of the frozen plasma samples were infectious. Near the conclusion of the in vivo study, conditions to culture the spirochetes in vitro were published and we were able to successfully establish a bacterial culture in our laboratory. With availability of higher number of spirochetes, we repeated the in vivo experiments in vitro, starting from WB units and preparing components using standard blood bank protocols. In addition to confirming the in vivo data, the in vitro study allowed us to test PLTs properly stored for 5 days and the effect of leukoreduction. For the in vitro study, we spiked the WB units to a final concentration of 6×10^4/mL, the same as that used for the in vivo experiments, and 6×10^3/mL, a concentration within the range of human infection, as determined by testing of human clinical samples.[29] Unsurprisingly, the spirochetes survived in stored PLTs and there was no detectable protection provided by filtration. Moreover, none of the cultures from frozen plasma tested positive, confirming the in vivo data. Interestingly, the survival of the bacteria in RBCs stored for 42 days was dependent of the initial load used to spike the WB unit, suggesting that the spirochetes may not survive for long periods of time at 4°C.

As expected, infection was more severe in immunocompromised mice, with all injected mice, except for the animals injected with frozen plasma, developing an infection with higher spirochetemia titers than the levels detected in *P. leucopus*. This observation is particularly relevant in light of the described cases of meningencephalitis in immunocompromised patients suffering from relapsing fever.[3,4] In contrast, data from immunocompetent mice demonstrate resistance and bacteria clearance. Taken together, the data suggest that if *B. miyamotoi* is transmitted by blood transfusion, it may pose a greater risk for severely immunocompromised patients.

As with many tick-borne pathogens that cause illnesses with symptoms similar to other common diseases, *B. miyamotoi* belongs to a category of infections often overlooked and misdiagnosed. However, with several tick-borne infections on the rise, seroprevalence of *B. miyamotoi* in humans and wildlife should be monitored and the infection should be included in the list of emerging pathogens that could represent a future threat to the safety of the blood supply.

In conclusion, the potential role of pathogen inactivation in protecting against emerging threats such as *B. miyamotoi* must be considered. Recently one pathogen inactivation technology was approved by the FDA for plasma and PLTs and more are in the pipeline, including systems for RBCs. Studies to determine if these techniques are effective against *B. miyamotoi* would provide additional value to the implementation of pathogen inactivation technology.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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