The Effects of Blood Storage Time, Glycerol, and Temperature on *Babesia Microti* Infected Blood Samples

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Abstract

Human babesiosis is an emerging tick-borne disease caused by the protozoan *Babesia microti*. Once infected, a person may experience malaria-like symptoms that range from asymptomatic to severe and potentially life threatening. Patients are normally diagnosed by a blood smear examination or through the use of polymerase chain reaction (PCR). Often for research purposes, fresh blood is processed and its components are kept in freezers at differing temperatures. This study was conducted in order to assess the effects of storage conditions such as glycerol, storage time and temperature on *B. microti* infected human blood. A multiplex qPCR assay was utilized to target two different genes: 18S *rDNA* and gapdh. It was concluded that glycerol, blood storage time and temperature hinder the ability to detect *B. microti*. Storage conditions that deviate from normal standard laboratory conditions result in Ct values above the control value.

Introduction

People who partake in outdoor activities or live in wooded areas are aware of the dangers of the tick-transmitted Lyme disease, but many aren't aware of the numerous other tick-borne diseases or that they can be transmitted through blood transfusions. Researchers have presently identified 16 tick-borne diseases in the United States. Lyme disease is the most commonly reported tick-borne illness, with more than 30,000 cases reported each year (cdc.gov). One tick-borne illness in particular, human babesiosis, is commonly mistaken for Lyme disease yet causes similar symptoms to malaria. Unfortunately, human babesiosis has been overlooked in the medical field within the past several years, only becoming a nationally notifiable condition in January of 2011 (cdc.gov). Human babesiosis is difficult to work with; those who have the disease are usually asymptomatic and unaware that they are infected. As of today, there are no FDA-approved screen tests for *Babesia* in blood (Bloch, et al., 2014) which would help prevent the occurrence of infection through blood transfusion.

Human babesiosis or *B. microti* infection is caused by microscopic parasites known as *Babesia microti*. They are commonly transmitted by *Ixodes Scapularis* "deer" ticks which can be found in wooded, grassy areas of the Northeast and Western U.S. Once a nymphal tick attaches itself to a host, thousands of sporozoites are released. The pathogen will then enter the bloodstream, and reside in an erythrocyte, an oxygen carrying red blood cell (Wormser, et al., 2006). As they mature into merozoites, these parasites will begin to destroy the cell and begin the cycle once again (Na, et al., 2014). Besides a tick-bite, *B. microti* can be transmitted through blood transfusion. The number of cases of transfusion-transmitted babesiosis has been increasing (Krause, et al., 2012) due to insufficient technology and a lack of understanding of the disease. In most cases, people who are already infected yet decide to donate blood are asymptomatic, making transmission difficult to prevent. The third and least common way of transmission is through birthing (Bednarska, et al., 2015), where infection is transmitted from the mother to her infant. Once infected, a patient may experience a combination of malaria and flu-like symptoms such as fever, headache, muscle pains, nausea and fatigue (Vannier, et al., 1998). Infection severity is dependent on the patient's health.

In 2015, Young, et al., developed the first laboratory based blood donor screening program for babesiosis. Donor screening was performed on 1783 donors using real-time PCR.

The application of the technology was successful in quantifying parasitemia as well as categorizing patients by severity of infection. Blood screening programs such as these could maximize the detection of infected blood donations, and bring more awareness to blood centers and hospitals; however, molecular tools such as qPCR need improvements in sensitivity and specificity to be consistent and effective. With the right technology and application, this could potentially eliminate the occurrence of transfused transmissions.

Quantitative polymerase chain reaction (qPCR) or real-time polymerase chain reaction has been used in multiple studies dealing with tick-borne diseases for decades. The difference between qPCR and regular PCR is in the qualitative and quantitative effectiveness to detect gene expression through cDNA. The problem lies in its accuracy and application. PCR has been used to confirm the results of certain studies after other laboratory tests have been performed, yet has the potential to do more on its own. Tonnetti, et al., assessed the use of PCR with the use of riboflavin (RB) and ultraviolet (UV) light to kill the bacteria in blood samples (2012). In previous studies such as those conducted by Tonnetti in 2012, scientists were successful in reducing the levels of pathogens in apheresis plasma and platelet units. These results concluded that RB and UV light were able to decrease the parasite amount in whole blood units, with the use of PCR to determine parasitemia levels.

Because *B. microti* may remain in the body even after treatment, it might not always be detected in a typical antibody-based screening such as blood smears. High performance molecular tools such as qPCR may be useful in determining more than just the presence of these pathogens. It can determine the amount of parasites per specific blood unit, which allows researchers to characterize parasite activity. The degree to which a DNA sample is amplified, otherwise known as sensitivity, plays a crucial role in determining how factors such as storage conditions affect parasitemia. The development of an assay could be used as a clinical diagnostic tool in the near future, which would be used with complimentary standard antibody assays and blood smears. Researchers have come a long way in diagnosing and treating babesiosis, but many questions are yet to be answered. How long can parasite DNA stay in a red blood cell? Can *B. microti* hide in our bodies once they enter the bloodstream? The purpose of this study was to improve laboratory measures and determine what factors may influence measured parasitemia.

Methodology

Real-time qPCR primers and probe were specifically designed to target the 18S rRNA gene which contains a sequence specific for *B. microti*. In order to assess the primer and probe specificity, a multiplex real-time PCR assay was performed to simultaneously target B. microti *18S* and gapdh, a human housekeeping gene. These probes were tagged with FAM and VIC, respectively. DNA was extracted from samples A, B, C and D on two separate days using a QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). DNA extraction and assays were repeated a second time the following week. The assay was carried out on the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher, Waltham, MA).

DNA extraction and Storage Conditions

Yale School of Public Health received an infected patient's fresh whole blood in August 2016. Approximately 1.5 ml of whole blood was aliquoted into four, 400 µl samples that were each assigned to a storage condition. The remaining whole blood was stored in a freezer. The first sample, A, was shaken gently for 15 seconds and immediately placed in a -80˚C freezer for three hours. This provided ample time for the sample to completely solidify before thawing. Next, the sample was placed on a heating block at 30˚C and stood for fifteen minutes which gradually liquefied the blood once again. The freezing and thawing cycle of sample A was repeated three more times before DNA extraction. The second sample, B, was vortexed for 15 seconds before being stored at 4˚C. After approximately 120 hours, sample B was removed from the refrigerator and left to come to room temperature prior to DNA extraction. Next, 500 µl of glycerol was pipetted into the third sample, C, which resulted in total volume greater than samples A, B or D. Sample C was vortexed for 30 seconds to ensure a homogenous mixture then frozen at -80˚C for 24 hours before DNA extraction. The fourth sample, D, served as the positive control. It was not assigned to a storage condition and its DNA was extracted 24 hours after being frozen at -80˚C. All DNA samples were washed with 50 µl of elution buffer and extracted at room temperature using the QIAamp DNA Blood Mini kit as outlined by Wang, et al. (2015).

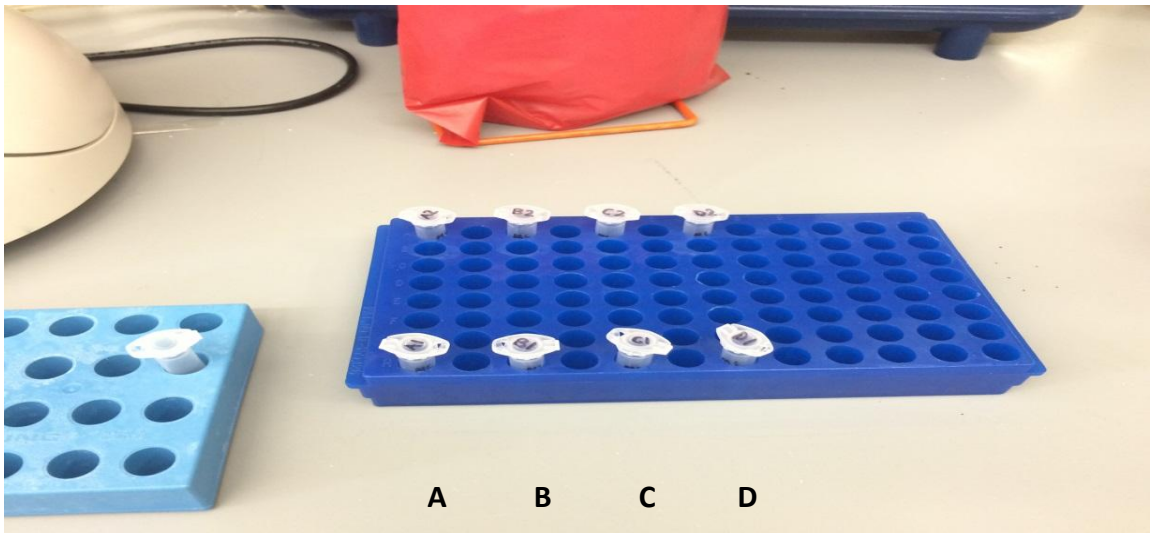

Figure 1: The four storage condition samples are shown above with a duplicate.
A1: Freeze thaw cycles
B1: Stored at 4˚ C for 120 hours
C1: Added glycerol
D1: (positive control). Original samples were duplicated and labeled as A2, B2, C2 and D2 in order to run a multiplex qPCR.

Dilutions and B. microti qPCR assay

In order to amplify both the *18S* and gapdh housekeeping gene in the same reaction, a multiplex qPCR was performed. GAPDH is present in all human cells and was used in order to confirm a successful qPCR reaction by comparing its gene expression to that of the 18S rDNA. The reaction contained 15 µl of 1 x TaqMan Fast Universal PCR MasterMix with 1.8 µl of forward primers and reverse primers and 1.8 µl of probe which would bind to *B. microti*, and 0.1333 µl of forward and reverse primers and 0.04 µl of probe which would bind to the human housekeeping gene, gapdh. Nuclease free water served as the negative control of the experiment, and all reagents were vortexed after being removed from the fridge. A qPCR standard curve was created by preparing 10-fold dilutions for the fresh blood samples as well as five additional blood samples, grouped as N14, that had been stored in a freezer for a longer period of time. The qPCR plate was sealed by gently placing an adhesive seal on the top of the plate. The seal was carefully rubbed to ensure all wells were covered completely. The plate was spun in a plate spinner for 20

seconds before being placed in the PCR machine. The 7500 Fast PCR instrument was set to the following temperatures and time intervals: 95˚ C for the first 20 seconds of the reaction, followed by 40 cycles of denaturing for 95˚ C for three seconds, and annealing at 60˚C for 30 seconds (Wang, et al. 2015). A sample was considered positive if the 18S rDNA target sequence produced a Ct value less than or equal to 38.0. On the contrary, a sample is negative if the 18S rDNA gene was not found by the PCR, but the housekeeping gene produced a Ct mean less than or equal to 40.

Statistical Analysis

A one-tailed student's t-test was used to determine the statistical significance between the storage condition samples A, B, and C, and the control sample D. Each standard deviation was calculated using the day 1 and day 2 Mean Ct values of the *B. microti 18S* gene. The standard deviation values were then used to calculate the t and p values of samples A, B, C and D with a DF of 2. The degrees of freedom (DF) used to calculate the p-value from the T-test was 2. A p-value greater than 0.10 was considered statistically significant.

Results

B. microti qPCR assay

A multiplex qPCR assay was run on two separate occasions. The quantity of any sample can be determined using a standard curve after preparing serial dilutions. Ct values are inversely proportional to the amount of nucleic acid present in the sample, and were produced as a result of the concentration of the human gene, gapdh, and the *B. microti* gene present in the samples. A Ct value is determined by the number of cycles that have passed before a fluorescent signal is released after a target gene was found. In the tables below, day 1 and day 2 Ct values are the mean of two different values. The average Ct was taken of both days for a final Mean Ct value of each storage condition for both gapdh and *18S*.

		GAPDH		
Sample	Condition	Day 1 Ct Mean (mg-min/L)	Day 2 Ct Mean (mg-min/L)	Mean Ct
A	Freeze-Thaw	40	23.73	31.87
B	4 C for 120 hours	29.29	24.5	26.9
C	Glycerol	29.87	24.41	27.14
D	Control	40	24.52	32.36

Table 1: Above are the Ct values of the housekeeping gene, gapdh, produced after completion of the qPCR reaction.

		18S		
Sample	Condition	Day 1 Ct Mean (mg-min/L)	Day 2 Ct Mean (mg-min/L)	Mean Ct
A	Freeze-Thaw	40	36.88	38.44
B	4 C for 120 hours	36.81	36.75	36.78
C	Glycerol	40	40	40
D	Control	25.54	40	32.77

Table 2: Above are the Ct values of the *B. microti 18S* gene produced after completion of the qPCR reaction.

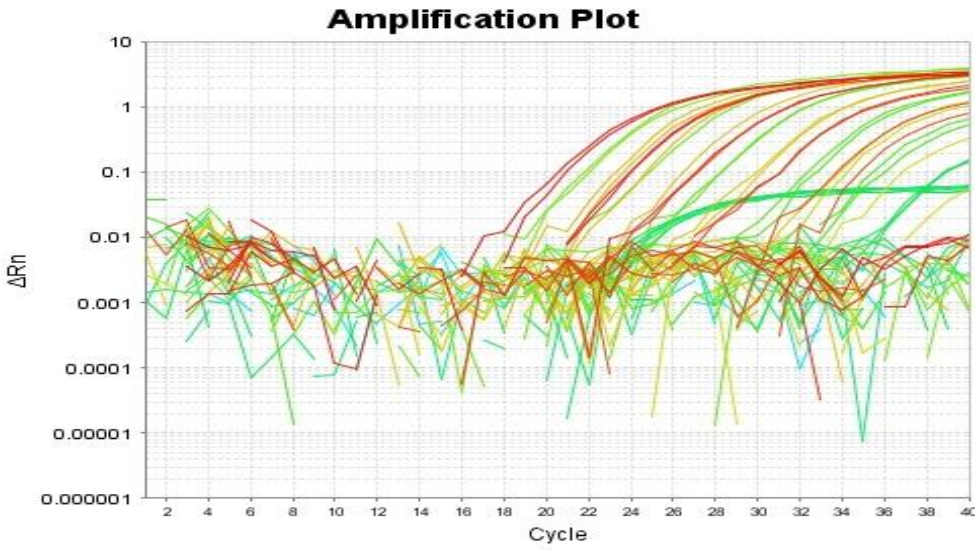

Figure 2: Shown above is the multiplex qPCR curve of the amplified B. microti *18 S rDNA* from day 2. ΔRn represents the magnitude of the fluorescent signal from the dye FAM and is plotted against the number of cycles. Amplification curves further to the right represent a higher Ct value.

Storage Conditions

Sample D: Positive Control

Each tested storage condition resulted in an 18S Ct value greater than the control Ct value. The positive control sample, D, produced a mean Ct value of 32.77 (Table 2). The qPCR assay successfully detected high levels of *Babesia microti* in the sample and produced the lowest Ct value of all four samples, which was expected.

Sample A: Multiple freeze thaw cycles

The mean Ct for the freeze thaw sample, A, was 38.44 (Table 2). A sample is considered *B. microti* positive if it produces a Ct value of less than or equal to 38.0 (Wang, et al. 2015). The number of cycles it took for a fluorescence to cross the threshold exceeded the control Ct value of 32.77.

Sample B: Fridge storage at 4˚ C for 140 hours

Sample B produced a mean Ct value of 36.78 (Table 2), which was also greater than the control Ct value of 32.77. The lengthened storage time of this blood sample in a refrigerator set to 4˚ C also negatively affected the number of detectable parasites. In comparison to multiple freeze thaw cycles, storing infected blood in a refrigerator produced a lower Ct value, thus this condition imposed less harm to the red blood cell and the existing *Babesia* parasites.

Sample C: Addition of glycerol

Sample C produced a Ct of 40 which exceeds the control Ct value of 32.77 (Table 2). As seen in Table 1, sample C produced a Ct value of 27.14 for the human gene gapdh. This sample is considered negative because no 18S rDNA was detected, yet gapdh was amplified with a Ct of less than or equal to 40 (Wang, et al. 2015). The addition of glycerol made it very difficult to detect any parasites. Compared to the other three samples, sample C produced the highest Ct value.

Statistical Significance

Condition for *18S*	Standard Deviation	T-Value	P-Value
A - Freeze-Thaw	2.206	0.645	0.293
B - 4°C for 120 hours	0.04243	0.5529	0.318
C - Glycerol	0	1.00	0.211
D - Control	10.22		

Table 3: A one-tailed student's t-test was used to determine the statistical significance between storage condition samples A, B, and C, and the control sample D of the *18S* gene. A p-value ≤ 0.1 was considered significant.

The *18S* Mean Ct values of days 1 and 2 were used to computate the standard deviation values shown in the table above. According to Table 2, control sample D had the greatest difference in Mean Ct, a value of 14.46, between days 1 and 2. This resulted in the control sample having the greatest standard deviation, a value of 10.22, among all four samples (Table 3). Storage condition sample B had a Mean Ct difference of 0.06 bewteen day 1 and 2 which resulted in a standard deviation value of 0.04243. On the contrary, storage condition sample B had a difference in Mean Ct value, a value of 0.06, between days 1 and 2. This resulted in sample B having the smallest standard deviation, a value of 0.04243, among all four samples (Table 3).

As seen in table 3, the p-values of samples A, B, and C were each greater than 0.1. Generally, a smaller standard deviation between two variables allows for comparable data, and a p-value that is ≤ 0.1 rejects the null hypothesis. Therefore, samples A, B and C produced p-values that failed to reject the null hypothesis, and the *18S* Mean Ct values produced were insignificant.

Discussion

The addition of glycerol to the *B. microti* infected human blood sample, prolonged storage time, and repeated freezing and thawing hindered the qPCR machine's ability to detect *B. microti* 18S rDNA. The sensitivity of the qPCR assay in this study can be compared to (Wang, et al. 2015), although slight alterations were made during optimization. As observed in this study, the

addition of glycerol to *B. microti* infected blood was deemed as the worst storage condition for replicating a control Ct. On the contrary, storing the sample in a fridge at 4˚ C for 120 hours had the least impact on parasite detectability.

The conditions of sample A and B resulted in a similar outcome: shearing of *B. microti* DNA. During the heating portion of the freeze thaw cycles for sample A, red blood cell membranes were completely ripped apart. This lead to the release of *Babesia microti* and an increase in the ratio between *18S* gene and the human gene gapdh during DNA extraction. However, *Babesia* parasites may have been denatured which would result in a decrease in parasitemia. The denaturing of *B. microti* parasites may be due to increased exposure to extreme temperatures, thus making it more difficult for the forward and reverse primers and probe to bind during the qPCR reaction. The 4-cycle increase between the control Mean Ct and the condition sample B Ct values may have resulted from alterations during assay optimization.

The purpose of blood preservation is to supply blood components such as platelets and plasma for patients requiring a blood transfusion. Preservation methods such as the addition of glycerol to human blood samples before being stored in a refrigerator or freezer protects the red blood cells and its contents from temperature damage (Nagai, et al., 1998). In this study, the addition of glycerol to sample C decreased the volume of blood used for DNA extraction, thus interfering with the ability to detect *B. microti*. Additionally, the Ct values produced for both the *18S rDNA* and gapdh gene for sample C may have resulted from *18S* relation to gapdh activity. Glycerol may facilitate amplification by enhancing the hydrophobic interactions between protein domains and raising the thermal transition temperature of proteins (Demeke, et al., 1992) The presence of glycerol may have lowered the strand separation temperature of human DNA (Nagai, et al., 1998) in sample C which could facilitate the amplification gapdh. Furthermore, this facilitation would alter the gapdh to *18S* gene ratio. It should be noted that glycerol may also act as a buffer around gapdh during freeze thaw cycle.

The p-values for storage samples A, B and C were significantly greater than a p-value of 0.1. The large p-values are a result of the large standard deviation between the the control sample's Mean Ct values. Therefore, it cannot be said with ninety percent confidence that the results of this experiment were significant. Changes in optimization prior to running the qPCR greatly contributes deviation between the *18S* Mean Ct values between days 1 and 2. These experimental

methods and results investigated how different storage conditions affect *B. microti* and gapdh gene expression and their ability to be amplified. Researchers who wish to pursue this investigating should prioritize qPCR optimization. This study raises the question, *how long can B. microti remain in a red blood cell*? Future studies should explore the role of glycerol furthur, how it protects human DNA when it is added to blood samples before freezing or thawing as well as other storage conditions such as the mixing of infected human blood and anticoagulant.

Conclusion

The goal of this study was to determine how multiple freeze thaw cycles, the addition of glycerol, and prolonged storage time of *B. microti* infected human blood samples affect parasitemia by performing a multiplex quantitative polymerase chain reaction. The storage conditions tested in this study represent inconsistencies that might occur in laboratory storage. Mishandling of samples or storing them incorrectly are common mishaps that can happen to any scientist, regardless of his or her expertise. The results of this study were surprising and raise new questions that can only be answered by performing multiple trials. Furthermore, the methods developed in this study provide a template for researchers investigating the effects of storage on human blood. The multiplex qPCR is a powerful tool and should be utilized to its full potential in order to improve human babesiosis diagnosis. If optimization is improved, the qPCR assay may produce more accurate Ct values. The number of cases of transfusion-transmitted babesiosis can be significantly reduced by developing current diagnostic tools and methods, improving laboratory measures and promoting public health.

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