ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2007, 14, 287-290

LONG TERM MONITORING OF *BABESIA MICROTI* INFECTION IN BALB/C MICE USING NESTED PCR

Renata Welc-Falęciak, Anna Bajer, Małgorzata Bednarska, Anna Paziewska, Edward Siński

Department of Parasitology, Institute of Zoology, Faculty of Biology, University of Warsaw, Poland

Welc-Falęciak R, Bajer A, Bednarska M, Paziewska A, Siński E: Long term monitoring of *Babesia microti* infection in BALB/c mice using nested PCR. *Ann Agric Environ Med* 2007, **14**, 287-290.

Abstract: In this study we report the usefulness of nested PCR for screening of the persistent *B. microti* infections in rodent hosts. Female BALB/c mice were inoculated with 100 µl of donor blood infected with *B. microti*. Infections were detected using microscopic examination of Giemsa-stained blood smears. To determine whether *B. microti* DNA was present in blood and/or spleen tissue, nested PCR was performed targeting a specific fragment of the gene encoding the 18S rRNA. Blood was sampled every 10 days post-infection (dpi) until day 30, after which mice were sampled every 30 days until the end of experiment at 360 dpi. The most extensive parasitaemia (39% of infected erythrocytes) was observed at 10 dpi. Between 20-60 dpi, less then 1% of infected erythrocytes were detected in blood smears, and from 90 dpi onwards, infected erythrocytes were no longer observed. *B. microti* DNA was successfully amplified from the blood of mice from 10 dpi until 180 dpi, as well as from spleens of infected mice at 10 and 20 dpi. The presented results show that nested PCR is the method of choice for monitoring infections of *B. microti* in the blood of rodent hosts, and could therefore be a tool for environmental monitoring of naturally infected rodents which are the predominant source of infection for tick vectors.

Address for correspondence: Edward Siński, Department of Parasitology, Institute of Zoology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland.
E-mail: esinski@biol.uw.edu.pl

Key words: ***Babesia microti*, BALB/c mice, infection, PCR.**

INTRODUCTION

Babesia microti, a tick-transmitted intraerythrocytic parasite of the genus *Babesia* (Apicomplexa, Piroplasmida), is a common parasite of wild rodents, which are potential reservoir hosts for human babesiosis, often lethal for immunocompromised individuals. Most cases of human babesiosis caused by *B. microti* occur in North America [7], but in Europe, infections with *B. microti* in humans are being increasingly recognized as a health problem [6, 10]. Although *B. microti* may be accidentally transmitted by blood transfusion [14], however, the normal means of transmission is via infected *Ixodes* ticks [4, 24]. It is well known that free-living small rodents play an important role as reservoir hosts, and the presence of *B. microti* in various rodent species has been documented in North America [22, 3], Europe [1, 5, 17] and Asia [21, 23]. The specific diagnosis of babesiosis is made initially by microscopic identification of the organism in Giemsa- or Wright-stained blood smears [2], but it is important to note that the prevalence of *B. microti* infections in rodents assessed by PCR is much higher than that using Giemsa-stained blood smears [18, 25]. Generally, our recent microscopy-based epizootiologic studies in wild rodents revealed that the vast majority of *B. microti* infections occur with very low parasitaemia, thus suggesting that these infections are in the chronic and persistent phase [1, 12]. A similar conclusion was drawn from the earlier study conducted on *Peromyscus leucopus* by Spielman *et al.* [19]. The questions, however, remain: how long does the infection of *B. microti* persist in blood and spleen tissues of infected rodent hosts? How sensitive and comparable is nested PCR with microscopy?

Received: 16 August 2007
Accepted: 30 October 2007

Thus, the aim of this study was to detect the duration of persistent infection of *B. microti* in BALB/c mice, and to evaluate the sensitivity and usefulness of nested PCR for detection of these piroplasms, in both blood and spleens of mice between 10 days and 12 months post-infection, the time during which infected rodents might be a reservoir of infection for the tick vectors.

MATERIAL AND METHODS

Parasites and experimental animals. *B. microti* strain King's 67, originally obtained from Dr. S. Randolph (Oxford University) and maintained by weekly blood passage in BALB/c mice, was used in this study. The mice were housed under conventional animal house conditions with access to food and water *ad libitum*. Thirty six female BALB/c mice aged 12 weeks were inoculated with 100 µl of donor blood by intraperitoneal injection. Three mice were left uninfected as a control group. At each time point in the experiment, 3 animals were killed with an overdose of ether inhalation and their spleens collected, weighed to a precision of ± 0.001 g, frozen and stored separately. Initially, mice were sacrificed every 10 days post infection (dpi) until day 30, after which they were sampled every 30 days until the end of experiment at 360 days.

Blood samples and microscopic examination. Blood collected by heart puncture was used to prepare thin smears on microscope slides, and the remainder pipetted into 0.001M EDTA and stored at 4°C. Blood smears were air-dried, fixed in absolute methanol, and stained with Giemsa, prior to examination at ×1000 magnification using a bright-field microscope (Nikon). A total of 200 fields were scanned, and the number of red blood cells (RBC) infected with *B. microti* recorded. Parasitemia was expressed as the number of infected cells/1000 RBC.

Detection of *B. microti* DNA by nested PCR. Genomic DNA was prepared either from 200 µl of blood in 0.001 M EDTA, or from 20 mg of spleen tissue using either whole-blood (Genomic Mini AX Blood, A&A Biotechnology) or tissue DNA extraction kits (Genomic Mini AX Tissue, A&A Biotechnology) as appropriate. The purified DNA was used as a template for nested PCR using the outer primers CRYPTORN (5'-GAATGATCCTTCCGCAGGTTCACCTAC-3') and CRYPTOFL (5'-AACCTGGTTGATCCTGCCAGTAGTCAT-3') and the inner primers Bab1 and Bab4, targeting a specific fragment of the gene encoding the small-subunit ribosomal RNA (18S rRNA). The conditions for nested PCR were as described previously [13]. Briefly, PCRs commenced at 94°C for 1 min, followed by 94°C for 1 min, 58°C for 1 min and 72°C for 2 min (35 cycles), and 72°C for 7 min. PCR products of 238 bp were separated using electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Statistical analysis. Parasitaemia in blood smears was calculated as the percent of infected RBC. Statistical evaluation was supported by SPSS v. 14 software. Quantitive data (parasite abundance and spleen weight) were analysed using one-way ANOVA with dpi entered as a factor. Analysis of correlation between parasite abundance and spleen weight was conducted using the Spearman rank correlation test. The comparison of detection methods was performed using Fisher's Exact Test.

RESULTS

Monitoring of *B. microti* infection using microscopy. All 36 experimentally inoculated mice became infected with *B. microti*. The course of infection, as revealed by examination of blood smears, is shown in Figure 1. The highest parasitaemia (39% of erythrocytes) was observed at 10 dpi. Between 20-60 dpi, few parasites were detected in blood smears (≤1% of erythrocytes), and from 90 dpi onwards, no infected erythrocytes were observed until the experiment ended at 360 dpi (main effect of dpi on *B. microti* infection intensity: $F_{11,35} = 94.3$, $p<0.001$) (Fig. 1, Tab. 1).

Monitoring of *B. microti* infection using nested PCR. *B. microti* DNA was successfully amplified from the blood of infected mice from 10 dpi (the earliest animals sampled) until 180 dpi (Fig. 3, Tab. 1). The comparison of sensitivity between microscopy and nested PCR of blood samples revealed a significant correlation between the two methods (comparability = 72%) (Fisher's Exact Test: $p = 0.0008$). However, the use of nested PCR detected 28% more positive samples. *B. microti* DNA was successfully amplified from spleens of infected mice at 10 and 20 dpi (Fig. 3, Tab. 1). The comparison of sensitivity between nested PCR on blood samples and nested PCR on spleen samples revealed

Table 1. Comparison of sensitivity between microscopy and nested PCR on blood and spleen samples ([+] - positive, [-] – negative).

Dpi	Microscopy (No. positive/ No. tested)	Nested PCR (No. positive/No. tested)	
		Spleen	Blood
10	+ (3/3)	+ (3/3)	+ (3/3)
20	+ (3/3)	+ (3/3)	+ (3/3)
30	+ (2/3)	-	+ (3/3)
60	+ (2/3)	-	+ (3/3)
90	-	-	+ (2/3)
120	-	-	+ (2/3)
150	-	-	+ (3/3)
180	-	-	+ (1/3)
210	-	-	-
240	-	-	-
315	-	-	-
360	-	-	-

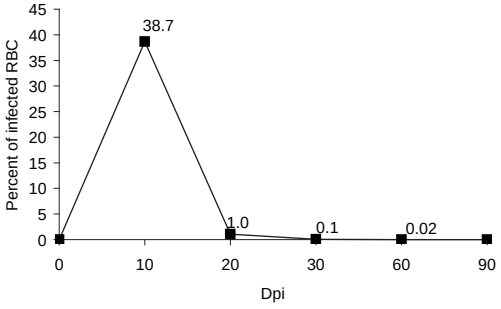

Figure 1. Course of *B. microti* infection on the basis of Giemsa-stained blood smears.

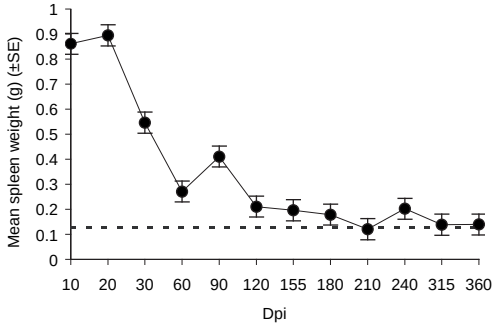

Figure 2. Changes of mean spleen mass in infected mice depending on dpi (horizontal, dotted line – mean spleen mass of control group of mice).

significant association of these two methods (comparability = 61%) (Fisher's Exact Test: p = 0.0240). However, nested PCR of blood samples detected 39% more positive samples.

Monitoring of spleen mass. Mean spleen mass in infected mice changed in a manner dependent on the duration of infection (Fig. 2; main effect of dpi on spleen mass: $F_{11,35}$= 42.5 p<0.001). Between days 10-20 p.i., spleens weighed up to 8 times more than those of uninfected control mice (Fig. 2). Between 20-60 dpi, spleen mass declined significantly, but remained elevated until the end of the experiment (experimental mice = 0.139 g ± SE, control mice = 0.128 g ± SE on 360 dpi) (Fig. 2). For the group of 10 mice with a determined intensity of *B. microti* infection, a Spearman rank correlation analysis was performed between mean spleen mass and the intensity of parasite infection (No. of infected cells/1000 erythrocytes). The analysis revealed a strong positive correlation between infection intensity and mean spleen weight (r^2= 0.787, p=0.007). Additionally, the results of nested PCR from spleen samples were associated with mean spleen mass (main effect of PCR result on spleen weight: $F_{1,35}$= 114.0 p<0.001). The mean spleen mass in the group of nested-PCR-positive mice was 0.878 g ± 0.054 g in comparison to 0.247 g ± 0.024 g in nested-PCR-negative mice.

DISCUSSION

In this study we have demonstrated the value of nested PCR for the detection and monitoring of the persistent phase of *B. microti* infection in BALB/c mice. *B. microti* infections in laboratory mice have generally been believed to last 4-6 weeks [8, 11]. However, in the present experiment, *B. microti* DNA was amplified from mice blood up to 180 dpi, despite very low or undetectable parasitemia observed from 30 dpi (<1%). Microscopic examination of *Babesia* parasites in stained blood smears revealed that infection ended within 2 months. Much higher sensitivity of nested-PCR methods revealed that infection persisted for the next 4 months.

Splenomegaly following infection persisted for even longer, until the end of the experiment at 360 dpi. However, *Babesia* DNA in spleens of infected mice was only detected until day 20 following infection. In this early period of infection, *Babesia* schizonts [9] are probably detectable in the spleen, where schizogony occurs in lymphocytes. BALB/c mice are moderately sensitive to *B. microti* infection [16], and thus constitute a reasonable model for studying the pattern of parasite infection. Our results support previous ideas concerning the existence of persistent

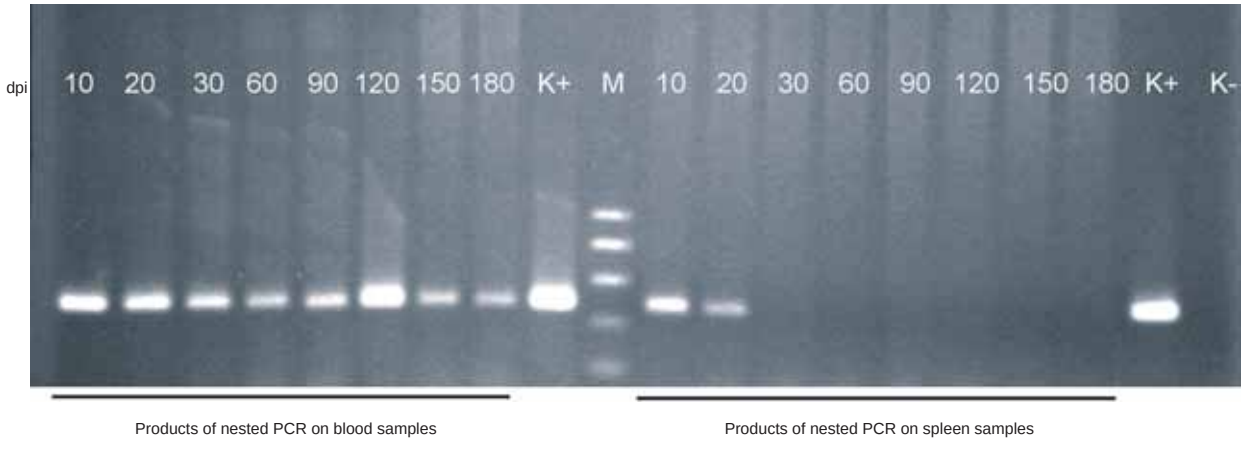

Figure 3. PCR products of small subunit rDNA of *B. microti* electrophoresed in 2% agarose gel and stained by ethidium bromide (dpi – days post infection; M – size marker [100, 200, 300, 400, 500 bp]; K+ – positive control; K- – negative control).

infections in reservoir hosts [7, 19]. In naturally infected hosts, the great majority of recognized infections are of a very low intensity, suggesting a long chronic phase in these hosts [12, 19]. Taking into account the presented results and the fact that the average life span of wild rodents is restricted to 3-4 months [15], it seems that once acquired, infections may persist until the end of the rodent life.

In the present experiment, two detection methods were compared. Nested PCR of blood had a higher sensitivity of *B. microti* detection than either the visual inspection of Giemsa stained blood smears or the nested PCR of spleens. The latter was the least sensitive method, giving positive results only during the acute phase of the infection. Splenomegaly is clearly associated with *B. microti* infection in mice, lasting even longer than the chronic phase of infection.

The real significance of long-lasting persistent infection in reservoir hosts remains unclear. In laboratory experiments, the probability of vectors becoming infected after feeding on chronic carriers was very low [4]. However, in the natural environment, the majority of rodent hosts seem to be in this chronic carrier state, but feeding sometimes high numbers of ticks (n=50-150, unpublished data), thus probably may serve as the source of infection for tick vectors. If this is the case, persistent infections may play a significant role in the epidemiology of *B. microti* infections, also in humans.

Acknowledgements

The study was supported by the Ministry of Science and Higher Education (previously State Committee for Scientific Research), Grant No. N303 029 31/0865. The experiment was conducted in accordance with the guidelines of the Local Ethical Committee (No. 575/2006).

REFERENCES

1. Bajer A, Pawelczyk A, Behnke JM, Gilbert FS, Sinski E: Factors affecting the component community structure of haemoparasites in bank voles (*Clethrionomys glareolus*) from the Mazury Lake District region of Poland. *Parasitology* 2001, **122**, 43-54.

2. Böse R, Jorgensen WK, Dalgliesh RJ, Friedhoff KT, de Vos AJ: Current state and future trends in the diagnosis of babesiosis. *Vet Parasitol* 1995, **57**, 61-74.

3. Burkot TR, Schneider BS, Pieniazek NJ, Happ CM, Rutherford JS, Slemenda SB, Hoffmeister E, Maupin GO, Zeidner NS: *Babesia microti* and *Borrelia bissettii* transmission by *Ixodes spinipalpis* ticks among prairie voles, *Microtus ochrogaster*, in Colorado. *Parasitology* 2000, **121**, 595-599.

4. Gray J, von Stedingk LV, Gurtelschmid M, Granstrom M: Transmission studies of *Babesia microti* in *Ixodes ricinus* ticks and gerbils. *J Clin Microbiol* 2002, **40**, 1259-1263.

5. Healing TD: Infections with blood parasites in the small British rodents *Apodemus sylvaticus*, *Clethrionomys glareolus* and *Microtus agrestis*. *Parasitology* 1981, **83**, 179-189.

6. Hildebrandt A, Hunfeld KP, Baier M, Krumbholz A, Sachse S, Lorenzen T, Kiehntopf M, Fricke HJ, Straube E: First confirmed autochthonous case of human *Babesia microti* infection in Europe. *Eur J Clin Microbiol Infect Dis* 2007, **8**, 595-601.

7. Homer MJ, Aguilar-Delfin I, Telford III SR, Krause PJ, Persing DH: Babesiosis. *Clin Microbiol Rev* 2000, **13**, 451-469.

8. Konopka E, Sinski E: Experimental infection of mice with *Babesia microti*: characterization of parasitemia. *Wiad Parazytol* 1996, **42**, 395-406.

9. Mehlhorn H, Schein E: Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitol Res* 1998, **84**, 467-475.

10. Mitrović S, Kranjcić-Zec I, Arsić-Arsenijević V, Dzamić A, Radonjić I: Human babesiosis – recent discoveries. *Med Pregl* 2004, **57**, 349-353.

11. Pastusiak K, Konopka E, Doligalska M, Sinski E: *Babesia microti*: parasitaemia and antibody responses to primary and challenge infections in BALB/c mice. *Folia Parasitol* 2003, **50**, 237-239.

12. Pawelczyk A, Bajer A, Behnke JM, Gilbert FS, Sinski E: Factors affecting the component community structure of haemoparasites in common voles (*Microtus arvalis*) from the Mazury Lake District region of Poland. *Parasitol Res* 2004, **92**, 270-284.

13. Presing DH, Mathiensen D, Marshal WF, Telford SR, Spielman A, Thomford JW, Conrad PA: Detection of *Babesia microti* by ploymerase chain reaction. *J Clin Microbiol* 1992, **30**, 2097-2103.

14. Popovsky MA: Transfusion-transmitted babesiosis. *Transfusion* 1991, **31**, 296-298.

15. Pucek Z, Ryszkowski L, Zejda J: Estimation of average length of life in bank vole *Clethrionomys glareolus* (Schreber, 1780). **In:** Petrusewicz K, Ryszkowski P (Eds): *Energy flow through small mammals populations*. Warsaw 1970, PWN, 187-201.

16. Ruebush MJ, Hanson WL: Susceptibility of five strains of mice to *Babesia microti* of human origin. *J Parasitol* 1979, **65**, 430-433.

17. Sebek Z, Rosicky B, Sixl W: The occurrence of babesiasis affecting small terrestrial mammals and the importance of this zoonosis in Europe. *Folia Parasitol* 1977, **24**, 221-228.

18. Sinski E, Bajer A, Welc R, Pawelczyk A, Ogrzewalska M, Bhenke JM: *Babesia microti*: prevalence in wild rodents and *Ixodes ricinus* ticks from the Mazury Lakes District of North-Eastern Poland. *Int J Med Microbiol* 2006, **296**, 137-143.

19. Spielman A, Etkind P, Piesman J, Ruebush TK 2nd, Juranek DD, Jacobs MS: Reservoir hosts of human babesiosis on Nantucket Island. *Am J Trop Med Hyg* 1981, **30**, 560-565.

20. Stowell PCh, Gelfand JA, Shepard JAO, Kratz A: Case 17-2007: a 25-year-old woman with relapsing fevers and recent onset of dyspnea. *N Eng J Med* 2007, **356**, 2313-2319.

21. Tsuji M, Wei Q, Zamoto A, Morita C, Arai S, Shiota T, Fujimagari M, Itagaki A, Fujita H, Ishihara C: Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasite. *J Clin Microbiol* 2001, **39**, 4316-4322.

22. Watkins RA, Moshier SE, O'Dell WD, Pinter AJ: Splenomegaly and reticulocytosis caused by *Babesia microti* infections in natural populations of the montane vole, *Microtus montanus*. *J Protozool* 1991, **38**, 573-576.

23. Wei Q, Tsuji M, Zamoto A, Kohsaki M, Matsui T, Shiota T, Telford SR 3rd, Ishihara C: Human babesiosis in Japan: isolation of *Babesia microti*-like parasites from an asymptomatic transfusion donor and from a rodent from an area where babesiosis is endemic. *J Clin Microbiol* 2001, **39**, 2178-2183.

24. Wójcik-Falta A, Cisak E, Chmielewska-Badora J, Zwoliński J, Buczek A, Dutkiewicz J: Prevalence of *Babesia microti* in *Ixodes ricinus* ticks from Lublin region (Eastern Poland). *Ann Agric Environ Med* 2006, **13**, 319-322.

25. Zamoto A, Tsuji M, Kawabuchi T, Wei Q, Asakawa M, Ishihara C: U.S.-type *Babesia microti* isolated from small wild mammals in Eastern Hokkaido, Japan. *J Vet Med Sci* 2004, **66**, 919-926.