*ORIGINAL ARTICLE*

Annals of Agricultural and Environmental Medicine
www.aaem.pl



# Comparison of the detection efficiency of haemoparasite DNA in blood and faecal samples – the way to eco-epidemiological studies

**Anna Bajer**[1,A-D], **Dorota Dwużnik**[1,B,E-F], **Katarzyna Tołkacz**[1,A-B,E], **Mohammed Alsarraf**[1,B-C,E-F], **Ewa Julia Mierzejewska**[1,A-C,E-F]

[1] *University of Warsaw, Poland*

*A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of article*

Bajer A, Dwużnik D, Tołkacz K, Alsarraf M, Mierzejewska EJ. Comparison of the detection efficiency of haemoparasite DNA in blood and faecal samples – the way to eco-epidemiological studies. Ann Agric Environ Med. doi: 10.26444/aaem/109664

## Abstract

**Introduction and objective.** It is easier and non-invasive to obtain faecal samples compared with blood samples. Molecular techniques may enable detection of parasites even in tiny amounts of blood-containing faeces. We aimed to compare the sensitivity of detection of three Babesia species and Hepatozoon canis in blood and faecal samples, including samples derived from naturally infected hosts.

**Materials and method.** Three groups were involved: 1) Nine BALB/c mice infected with *Babesia microti* sampled during acute (n=3), post-acute (n=3) and chronic phases of infection (n=3); 2) Eight dogs with symptoms of babesiosis; 3) Six red foxes infected with *B. vulpes,* one fox infected with *B. canis*, four foxes infected with *H. canis*. Genomic DNA was extracted from blood and faeces by use of commercial kits and amplified with genus-specific primers in one-step or nested PCR reactions. Selected PCR products were sequenced.

**Results.** No positive results for faecal samples were obtained from *H. canis*-positive foxes in contrast to *Babesia* spp. infections. Positive results from PCRs were obtained for all BALB/c mice (100%), five dogs (62.5%) and four of seven foxes (57.1%). Successful sequencing was obtained for six selected murine samples (*B. microti*), four canine samples (*B. canis*) and for one fox sample (*B. vulpes*). The success of *B. microti* detection in murine faecal samples from acute, post-acute and chronic phases was identical (100%).

**Conclusions**. Detectability of *Babesia* spp. infections was lower in naturally infected dogs and foxes, compared to experimentally infected mice. Detection of DNA in faecal samples can be useful in the detection of *Babesia* infection in populations from which blood samples are hard to obtain, but due regard must be given to the possibility that prevalence of infection may be severely underestimated.

## Key words

*Babesia canis, Babesia microti, Babesia vulpes, Hepatozoon canis*, red fox, faecal samples, faeces

## Abbreviations

**DNA** – Deoxyribonucleic Acid, **EDTA** – Ethylene Diamine Tetra-acetic Acid, **PCR** – Polymerase Chain Reaction, **RBC** – Red Blood Cell, **RNA** – Ribonucleic Acids

## INTRODUCTION

It is much easier and far less traumatic, as well as entirely non-invasive, to obtain faecal samples from study subjects, compared to blood samples. Faecal samples from a range of animal species can be easily provided by animal owners and breeders, or can be found in typical wildlife environments by experienced staff. Such samples are often collected for use in studies of diet composition or for the detection of gastrointestinal parasites by their faecal transmission stages [1, 2]. Thus, the relatively simple availability of faecal samples is greatly advantageous over the more tedious and traumatic collection of blood samples.

Vector-borne blood parasites, including protozoa from the Apicomplexa phyllum, constitute a serious health risk worldwide. One of the major aims of eco-epidemiological studies is the identification of the wildlife reservoirs of these parasites. These studies are often restricted due to the limited access to blood or tissue samples from wild animals. However, there is growing evidence that faecal sample can be used for the identification of blood parasite infections in different host species [3–5]. Many apicomplexan parasites affect the intestinal blood circulation of their hosts, leading to haemorrhagic events resulting in some blood losses into the gut lumen and hence gut contents [6, 7]. Thus, the presence of some blood and haemoparasites may be expected in faecal samples during infection. Moreover, current sensitive detection techniques based on the specific amplification of parasite DNA may enable detection of parasites, even in tiny amounts of blood-containing faeces. Cell-free parasite DNA, released from destroyed infected RBC (iRBC), may also contribute to the successful detection of blood parasites in faecal samples [8].

Address for correspondence: Anna Bajer, University of Warsaw, Poland
E-mail: anabena@biol.uw.edu.pl

Received: 12.03.2019; accepted: 25.05.2019; *first published: 17.06.2019*

In recent years, several studies have been published reporting the successful detection of blood parasite/pathogen DNA in faecal samples. These involved ground-breaking studies on the evolutionary origins of malaria parasites (*Plasmodium* spp.) conducted in free-living African apes [9–12]. Other recent examples of successful detection of infections with important pathogens by analysis of faecal samples include HIV in chimpanzees [13], *Mycobacterium tuberculosis* in humans [14] and *Rickettsia* spp. in humans [4]. In addition to *Plasmodium* spp., faecal samples were recently used for the detection of *Trypanosoma brucei* infections in wild chimpanzees from Africa [3], and the detection of *Babesia* spp. in bat droppings in Hungary and the Netherlands [15].

However, despite these positive reports, to the best of the knowledge of the authors of the current study, there is still a lack of basic studies assessing the sensitivity, specificity and reliability of faecal assays for the detection of blood parasites, and comparing the results from this methodology to conventional estimates of infection prevalence based on blood samples. Thus the present study was initiated in to compare the efficiency of detection of three *Babesia* species and *Hepatozoon canis* in blood and faecal samples from different host species, including samples derived from naturally infected red foxes *Vulpes vulpes*.

## OBJECTIVES

The specific aims of the study were: 1) to assess the influence of phase of infection, during which parasitaemia levels can be expected to vary in intensity, on detectability of *Babesia microti* in experimentally infected mice; 2) to compare the detectability of *Babesia* spp. and *H. canis* in faecal samples by different PCR protocols: single-step and nested PCRs; 3) to compare detectability of parasite DNA in small and large volumes of faecal samples. On the basis of research hypothesis on the possibility of haemoparasite DNA detection in faecal samples, three predictions were made: 1) the success of detection should be higher during the acute phase of infection with high parasitaemia, than in the chronic phase of infection when there are very low levels of iRBC; 2) nested PCR protocols should be more sensitive than single-step PCRs in detection of parasites in faecal samples; 3) detection of parasite DNA should be more successful in larger volumes of faecal sample, increasing the probability of successful DNA extraction.

## MATERIALS AND METHOD

The origin of samples is provided in Table 1. Three groups of hosts were involved. The first group of samples was obtained from nine laboratory mice *Mus musculus* (adult females, BALB/c strain) infected with *B. microti* King's 67 strain maintained in the authors' laboratory [16]. The animals came from a breeding facility run by the animal house of the Faculty of Biology at the University of Warsaw (breeders' register number 012). Mice were transferred to the experimental unit of the animal house. Each cage contained a layer of standard sawdust, water and standard food pellets *ad libitum,* together with bedding material (paper tubes). Females (3–5 in cage) were kept at a constant temperature of +18 °C, and with a 12 (Day): 12 (Night) light-dark phase.

**Table 1.** Type and source of samples

| No. | Host species | Parasite species | Phase of infection | Blood sample | Faecal sample |
|---|---|---|---|---|---|
| N=3 | *Mus musculus* | *Babesia microti* | acute (6–7 dpi) | Yes | Yes |
| N=3 | *Mus musculus* | *Babesia microti* | post-acute (24–25 dpi) | Yes | Yes |
| N=3 | *Mus musculus* | *Babesia microti* | chronic (66- 68 dpi) | Yes | Yes |
| N=2 | *Canis familiaris* | uninfected | na | Yes | Yes |
| N=4 | *Canis familiaris* | *Babesia canis* | acute (babesiosis) | Yes | Yes |
| N=4 | *Canis familiaris* | *Babesia canis* | acute (babesiosis) | No | Yes |
| N=6 | *Vulpes vulpes* | *Babesia vulpes* | not known | Yes | Yes |
| N=1 | *Vulpes vulpes* | *Babesia canis* | not known | Yes | Yes |
| N=4 | *Vulpes vulpes* | *Hepatozoon canis* | not known | Yes | Yes |

N- number of samples; na – not applicable

To check the effect of parasitaemia on the success of DNA detection, three groups of three mice each were sampled at three time points:

1) during the acute phase following the first week of infection (on 6–7 days post infection, dpi) characterized by high parasitaemia up to 42–50% of iRBC (parasitaemia assessed, as described previously [16]);
2) during the recovery period characterized by low parasitaemia of less than 5% (2.5–4.1% on 24–25 dpi in this study);
3) during the latent/chronic phase (on 66 or 68 dpi) characterized by very low parasitaemia (0.001% of iRBC in this study), when reliable detection of infection is only possible by PCR methodology [16]. These three separate groups were selected to enable comparison of the success of detection in faeces during different phases of *B. microti* infection, characterized by different levels of parasitaemia.

The infection procedures for *B. microti* have been described in detail previously [16, 17]. On the day of sampling, mice were first transferred individually to new clean cages for the collection of faecal samples. Faecal pellets were collected into sterile Eppendorf tubes using sterile forceps. The tip of the tail was cut with sterile scissors. A drop of blood was collected on a glass slide and a thin blood smear was made to enable microscopic examination for the presence of parasitaemia. Autopsies were then carried out under terminal isoflurane anesthesia. Blood samples (about 200 µl) were taken directly from the heart into 0.001 M EDTA (anticoagulant) and kept at a temperature of +4 °C before DNA extraction.

The second group of samples was derived from eight dogs that had presented to the veterinary clinic in the town of Tłuszcz near Warsaw (Central Poland) in autumn 2017 because they showed clinical symptoms of babesiosis, i.e. apathy, anorexia, fever, jaundice. Both faecal and blood samples were obtained from four symptomatic dogs, while only faecal samples (faecal swabs) were obtained from another four dogs with babesiosis. Blood samples were collected into sterile EDTA-covered vials (5 ml) and faecal samples were collected from the rectum into sterile faecal tubes (10 ml). Blood and faeces from another two *Babesia*-negative dogs (as tested in this study) were treated as negative controls for all procedures (DNA extraction and amplification).

**Table 2.** Nucleotide sequences and annealing temperature of the primers used for PCRs

| Parasite genera | Gen | Primers | Primer sequence 5' 3' | Annealing temperature (°C) | Product size (bp) | Reference |
|---|---|---|---|---|---|---|
| *Babesia* | *18S rRNA* | Crypto F<br>Crypto R | AACCTGGTTGATCCTGCCAGTAGTCAT<br>GAATGATCCTTCCGCAGGTTCACCTAC | 59 | 1,200 | [18, 19] |
| | | BabGF<br>BabGR | GYYTTGTAATTGGAATGATGG<br>CCAAAGACTTTGATTTCTCTC | 59 | 559 | |
| *Babesia* | Mitochondrial *lsu4-lsu5* (*lsu*) | B-lsu-F<br>B-lsu-R2<br>Bmic-F | ACCTGTCAARTTCCTTCACTAAMTT<br>TCTTAACCCAACTCACGTACCA<br>TTGCGATAGTAATAGATTTACTGC | 60<br><br>62 | 150<br><br>230 | [22] |
| *Hepatozoon* | *18S rRNA* | HAM 1F<br>HPF 2R | GCCAGTAGTCATATGCTTGTC<br>GACTTCTCCTTCGTCTAAG | 56 | 1,700 | [21] |
| | | Hep F<br>Hep R | ATACATGAGCAAAATCTCAAC<br>CTTATTATTCCATGCTGCAG | 60 | 666 | [20] |

The third group of samples was obtained during our ongoing study on the role of red foxes as reservoir hosts of vector-borne blood parasites (Mierzejewska et al., unpublished). Fox carcasses were obtained from licensed hunters during the winter season of 2016/2017, and were first frozen at a temperature of -80 °C for two weeks before processing, to minimize the risk of contracting *Echinococcus multilocularis* infection during autopsies. Blood samples were obtained using sterile syringes, from the femoral vein, caudal vena cava or portal vein, placed into sterile vials (2 and 10 ml) and frozen at a temperature of -20 °C before DNA extraction. In the case of severe damages of the chest or heart, clotted blood was taken directly from the chest or abdominal cavity. Additionally, faecal samples were collected from the large intestine into sterile vials (20 or 50 ml) and frozen. Eleven infected foxes were selected for this study, including six foxes infected with *B. vulpes,* one infected with *B. canis* and four infected with *H. canis*.

Because the main aim of the present study was to evaluate the usefulness of faecal samples for detection of haemoparasites in wildlife, we compared two independent DNA extractions from fox faecal samples, using different amounts of faeces.

For the extraction of genomic DNA from all faecal samples the QIAamp PowerFaecal DNA kit (Qiagen, USA) was used. In this protocol, typical small amounts of faecal sample (up to 0.25 g) were used. For the additional extraction of genomic DNA from much larger amounts of faecal samples (5–10 g of faeces), the DNeasy PowerMax Soil kit (Qiagen, USA) we used. Faecal material from ten foxes was available for these double DNA extractions. Genomic DNA was extracted from murine, canine and fox blood samples using the DNeasy Blood & Tissue kit (Qiagen, USA) and stored at a temperature of -20 °C.

**Molecular detection.** Three procedures were selected for the PCR-based detection of parasites in blood and faecal samples. In the first procedure, a single-step PCR was applied, which is usually successful in the detection of parasite DNA in blood samples. Specific primers were used to amplify the *18S rRNA* gene fragment of *Babesia/Theileria* (559 bp) [18, 19] or *Hepatozoon* [20]. Primers, primer source and annealing temperatures are listed in Table 2. Reactions were performed in 1× PCR buffer, 1U DreamTaq polymerase, 1 µM of each primer and 2 µl of the extracted DNA sample. Negative controls were performed with 2 µl of sterile water, in the absence of template DNA.

The second procedure involved nested PCRs (Tab. 2). For the detection of *Babesia* spp. DNA, in the first step of a PCR, long fragments of 18S rDNA were amplified with apicomplexan 18S rRNA-specific primers: Crypto F and Crypto R [18, 19]. In the second step, the primers Bab GF/ Bab GR were used, as for the single-step PCR. To enhance detectability, secondary reactions were performed with different volumes of the first PCR product: 1 or 0.5 µl, or finally with 2 µl of a 1:9 dilution in sterile water. As positive controls, we used the genomic DNA of *B. canis* isolated from dog with canine babesiosis.

For the detection of *Hepatozoon* spp., DNA, in the first step of the PCR the full-length 18S rDNA was amplified with apicomplexan 18S rRNA-specific primers HAM 1F and HPF 2R [21]. In the second step the primers Hep F/ Hep R were used to amplify 666 bp fragment [20].

As DNA of blood parasites in faecal samples may be degenerated, we applied also the third PCR protocol to amplify the short fragment (150 or 230 bp depending on species) of *Babesia* spp. mitochondrial DNA, *lsu4-lsu5* [22]. For concurrent amplification of large (i.e. *B. canis*) and small babesiae DNA (i.e. *B. microti* or *B. vulpes*), three primers were used in this protocol (Table 2).

PCR reactions of each protocol were performed three times: samples positive two or three times were classified as positive; samples positive once or negative during three repetitions were classified as negative.

PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with Midori Green stain (Nippon Genetics, GmbH). Selected nested PCR products obtained from blood samples and the majority of PCR products obtained from faecal samples (selected products obtained from murine samples and all products obtained from canine and fox samples) were sequenced by a private company (Genomed S.A., Poland). DNA sequence alignments and analyses were conducted using MEGA v. 7.0. Consensus sequences were compared with sequences deposited in the GenBank database using BioEdit tool (http://www.ncbi.nlm.nih.gov/genbank/).

## RESULTS

**Detection of parasite DNA from blood samples.** All procedures were successful in amplifying parasite DNA from blood samples. *Babesia microti* was detected in nine experimentally infected mice; *B. canis* was detected in four dogs with babesiosis, while two control dogs yielded negative

PCR results. *Babesia vulpes* was detected in six foxes and *B. canis* in one fox. *Hepatozoon canis* DNA was detected in four foxes. Sequencing of selected PCR products confirmed *B. canis* infection in dogs, *B. vulpes, B. canis* and *H. canis* infections in red foxes (the authors' GenBank Accession Nos: MK862229-MK862234 for B. vulpes; MK872807 for B. canis, and MK872808-MK872810 for H. canis).

**Detection of parasite DNA from faecal samples.** The results of haemoparasite DNA detection in faecal samples are presented in Table 3.

***Hepatozoon canis*.** No positive results of any procedures were obtained for faecal samples from the *H. canis*-positive foxes.

***Babesia* spp.** Overall, positive results of PCRs (appearance of the product of correct size in at least two separate reactions of at least one procedure) were obtained for all nine experimentally infected mice (9/9=100%), five dogs (5/8=62.5%) and four of seven foxes (57.1%) and none of two *Babesia*-negative dogs. PCR products were successfully sequenced for all selected murine samples (6/6=100%, all sequences identical with the reference *B. microti* strain), for four canine samples (one nested PCR product and three short *lsu* products; total 4/8=50%) and for only one fox sample (Tab. 3).

Two representative sequences of *B. microti* King's 67 strain obtained from murine faecal samples were deposited in GenBank database under accession numbers MH553358 (18S rDNA) and MH614921 (*lsu*). Obtained sequences were identical with *B. microti* sequences obtained from blood samples.

In three dog samples, the nested PCR signal was of sufficiently good quality to enable sequencing, but finally only one nested PCR product was successfully sequenced and displayed the highest similarity (507/508 bp=99.8%) to several *B. canis* sequences obtained from a *Dermacentor reticulatus* tick, a golden jackal or a European wolf (KT272401, KY747491, KY359360, respectively). However, the same four dogs were positive by the *lsu* protocol, and in this case all three sequenced products enabled identification of *B. canis* (100% similarity with GenBank *Babesia canis canis* KC207822 and 98% similarity with *Babesia canis vogeli* KC207825). Two of these sequences were deposited in the GenBank database under Accession Nos. MH580883 and MH580884. *Babesia canis* sequences were identical with sequences obtained from blood samples.

Among samples derived from infected foxes, only one sample (fox No. 146) was consistently positive by both extraction protocols in all applied procedures, and in this case the sequences were obtained for both sequenced nested PCR products. These proved to be identical to *B. vulpes* (*B.* cf. *microti, B. annae;* reclassified as *B. vulpes* by Baneth et al. 2015 [23]) from red foxes from different European countries (KT233483, KT580785, KM115977) and an isolate from a dog (*Babesia* 'Spanish dog', AY457974). The sequence was deposited in the GenBank database under Accession No. MH553357, and was identical with the sequence obtained from the blood sample of this fox.

Short sequence of *lsu* also enabled identification of *B. vulpes* in a faecal sample from fox No. 146 (100% similarity with *Babesia* cf. *microti* KC207827 and 95% similarity with *B. microti* strain RILN871600), and this was also deposited in the GenBank under Accession No. MH614920.

The sequencing of nested PCR products from other fox samples was unsuccessful. Thus, the overall success of PCR detection of *Babesia* spp. in faecal samples of foxes based on sequencing results was much lower: 1/7=14.3%. No positive results were obtained for two control uninfected canine faecal samples.

**Comparison of PCR protocols for detection of parasite DNA in faeces.** The success of detection procedures could be compared only for samples from *Babesia* spp. positive hosts. The highest number of positive samples was obtained in the nested PCR, lower and similar in amplification of short *lsu*

**Table 3.** Detection of *Babesia* spp. in fecal samples using PCR/nested-PCR method

| N | Host species | Parasite species | Phase of infection | One-step PCR | | Nested PCR | Total | Sequencing results |
|---|---|---|---|---|---|---|---|---|
| | | | | lsu (150 or 230 bp) | GF/GR (559 bp) | 559 bp | PCR-pos. | (559 and/or 150/230 bp) |
| N=3 | *Mus musculus* | *Babesia microti* | acute | 3/3 | 3/3 | 3/3 | 3/3 | 3 × *Babesia microti* |
| N=3 | *Mus musculus* | *Babesia microti* | post-acute | 1/3 | 2/3 | 2/3 | 3/3 | 2 × *Babesia microti* |
| N=3 | *Mus musculus* | *Babesia microti* | chronic | 2/3 | 3/3 | 1/3 | 3/3 | 1 × *Babesia microti* |
| | | | | | | | **100%** | 6/6 *B. microti* |
| N=2 | *Canis familiaris* | uninfected | not applicable | 0/2 | 0/2 | 0/2 | 0/2 | not applicable |
| N=8 | *Canis familiaris* | *Babesia canis* | acute | 4/8 | 3/8 | 3/8 | 5/8 | 4 × *Babesia canis*<br>1 other sequence |
| | | | | | | | **62.5%** | |
| N=6 | *Vulpes vulpes* (small sample) | *Babesia vulpes* | not known | 1/6 | 1/6 | 3/6 | 3/6 | 1 × *Babesia vulpes**<br>2 × unsuccessful |
| N=1 | *Vulpes vulpes* (small sample) | *Babesia canis* | not known | 0/1 | 0/1 | 1/1 | 1/1 | unsuccessful |
| N=6 | *Vulpes vulpes* (large sample) | *Babesia vulpes* | not known | 0/6 | 0/6 | 2/6 | 2/6 | 1 × *Babesia vulpes**<br>1 × other sequence |
| | TOTAL | *B. vulpes/B. canis* | not known | 1/13 | 1/13 | 6/13 | 6/13 | |
| | N=13 | | | | | | **46.2%** | |
| | Total *Babesia* spp. positive by procedure | | | N=11 | N=12 | N=15 | | |

*-– PCR products from one fox (no. 146); pos.-positive; N-number of samples.

gen fragment or in the one-step PCR with Bab GF/Bab GR primers (Tab. 3).

**Comparison of detection of parasites between two DNA extraction protocols.** A higher number of positive samples was obtained for genomic DNA extracted from small amounts of faecal sample (n=4), in comparison to two positive samples obtained from genomic DNA extracted from the larger amounts of faecal sample (Tab. 3). However, the difference could not be statistically assessed due to the small number of compared samples.

**Comparison of detection of parasite DNA in faecal samples from different infection phases.** Overall, the success of *B. microti* detection in faecal samples from acute, post-acute and chronic phases was identical (100%) (Tab. 3). However, for two of three mice in the acute phase of infection, all detection procedures revealed positive results in each PCR run, as did only one sample from three mice in the chronic phase of infection, and none of the samples from the post-acute phase. The PCR signal obtained from one sample of the three mice in the chronic phase of infection was always weak, and not suitable for sequencing, in contrast to all other PCR products which were successfully sequenced.

The overall success of *Babesia* spp. detection was higher in canine samples derived from the acute phase of infection with *B. canis* (acute babesiosis) (4/8=50%) than in fox samples from unknown phases of infection with *Babesia* spp. (1/7=14.3%) (Tab. 3), but still twice lower than the overall success rate recorded for *B. microti* (9/9=100%).

## DISCUSSION

The presented study compared the detection success of three *Babesia* species and *H. canis* in blood and faecal samples of different host species, including samples derived from naturally infected red foxes. Detection of *H. canis* in faecal samples was unsuccessful; however, all three species of *Babesia* were detected successfully in faecal samples. Extraction of genomic DNA from small amounts of faecal material combined with nested PCR revealed the highest number of positive samples.

The key finding of this study is that *B. microti, B. vulpes* and *B. canis* DNA can be detected successfully in faeces of infected hosts, in contrast to *H. canis* DNA which cannot be detected. This difference may stem from many different reasons, including different localization of *H. canis* in a host (neutrophils, not RBC) or different pathogenicity of *H. canis* infections in comparison to *Babesia* spp. Although *H. canis* infections in dogs are often asymptomatic [24], no such data are available for foxes. Nevertheless, the very high prevalence of *H. canis* in fox populations in Central Europe [25–28], including Poland (up to 60%; Mierzejewska, unpublished) may support the low pathogenicity of this parasite for its natural hosts. On the other hand, *B. canis* infections constitute a serious health problem for dogs in Central Europe and Poland, contributing often to multi-organ complications and death [6, 29–31]. *Babesia vulpes* infections, however, are wide-spread among fox populations in Europe and the impact of this parasite, including its pathogenicity on fox health, has not been recognized to-date [32]. Thus the detectability of *Babesia* spp. in faeces may depend less on the actual species of pathogen being assessed, than on its pathological consequences for the host and the degree to which it is likely to cause some blood losses into the intestine and faeces.

The sensitivity of detection of *Babesia* spp. in faecal samples in the study differed between host species, and was lower in naturally infected hosts in comparison to experimentally infected mice. The BALB/c mice used for experimental infection originated from a laboratory-bred colony, and to some degree the success of detection of *B. microti* may be attributable to the lack of other infections with intestinal protozoan parasites, including other apicomplexans. As can be seen from sequencing results for some PCR products, there was some non-specific amplification of other protozoan DNA is samples from dogs and foxes, i.e. *Isospora, Dictyamoeba* or other organisms (not presented), likely confounding the attempts to specifically amplify *Babesia* DNA. The sensitivity of *T. brucei* detection in faecal samples has also been found to differ between experimentally and naturally infected hosts [3]. In experimentally infected mice, as in the current study, the detection success was 100%, but *T. brucei* DNA was identified in only three of 13 tested faecal samples from wild chimpanzees.

It is difficult to evaluate the usefulness of the detection of *Babesia* spp. DNA in faecal samples from naturally infected hosts (dogs, foxes). The success of detection varied regarding the sequencing results obtained: combined sensitivity and specificity was 14% in foxes and 50% in dogs. On the other hand, the sequences obtained were of very good quality, and not different from sequences obtained from blood samples, enabling species identification. Widely available detection techniques (commonly used commercial DNA extraction kits, PCR and nested PCR) were intentionally selected to test if this method can be widely used for monitoring. On the basis of the results obtained, it can concluded that this method can be of use in the monitoring of *Babesia* infection in populations from which blood samples are hard to obtain; but importantly, the estimation of prevalence can be largely biased and underestimated. In the study of Siregar et al. [5], several nested PCR protocols were evaluated for the detection of *Plasmodium* in macaque faeces. Although specificity of the implemented primers set was very high (100%), sensitivity varied between 23–29% for the cytochrome b gene, and 97% for mitochondrial small subunit rRNA (ssrRNA) gene fragment amplification [5]. Additionally, the same authors analyzed the sensitivity of methods used for the detection of *Plasmodium* DNA in faecal samples, as reported in other published papers, and again, depending on the PCR protocol and target, sensitivity/detectability differed profoundly from 20–30%, through 50–60% (both in the range of the current study), up to several studies reporting very high sensitivity of about/above 90% in faecal samples [5].

In the presented study, the first evaluation of detectability of *Babesia* spp. and *H. canis* in faecal samples was provided, and the results largely concur with those from similar studies on *Plasmodium*. To verify whether large amounts of faeces facilitate better detection of blood parasites or if detectability depends on the phase of infection, further quantitative studies on more samples from animals infected with identified haemoparasites are needed.

## CONCLUSIONS

The detectability of *Babesia* spp. infection differed between host species and was lower in naturally infected hosts in comparison to experimentally infected mice. Detection of DNA in faecal samples can be useful for monitoring of *Babesia* infection in populations from which blood samples are hard to obtain, but due regard must be given to the possibility that the prevalence of infection may be severely underestimated.

### Ethics approval and consent to participate

All of the procedures conducted on mice were approved by the First Ethics Committee for Animal Experimentation in Poland (Ethical License No. 635/2014), according to the principles governing experimental conditions and care of laboratory animals required by the European Union and the Polish Law on Animal Protection. The study was performed on the blood and faeces samples from dogs, with the permission of the dog owners; therefore, no Ethics License was required (as per Resolution No. 22/2006 of the National Commission for the Ethics of Experiments on Animals, 7th November 2006). The owners of dogs involved in this study were informed about the scientific aims of the study, provided blood and stool samples voluntarily, completed and signed required consent forms. Foxes were acquired by licensed hunters during legal hunting and samples were taken from killed animals. For this reason, there was also no need to obtain the consent of an Ethics Committee for this study.

### Competing interests

The authors declare that they have no competing interests.

### Acknowledgements

The authors express their thanks to the owners of the dogs for their kind cooperation. Professor Jerzy M. Behnke at the University of Nottingham, UK, undertook a critical review of the manuscript. The study was partially supported by the National Science Centre (NCN), grant Sonata Bis No. 2014/14/E/NZ7/00153, who had no role in the design of the study and collection of samples, analysis and interpretation of data and, or in the writing of the manuscript.

## REFERENCES

1. Kloch A, Bednarska M, Bajer A. Intestinal macro- and microparasites of wolves (*Canis lupus* L.) from north-eastern Poland recovered by coprological study. Ann Agric Environ Med. 2005; 12:237–245.
2. Paziewska A, Bednarska M, Nieweglowski H, Karbowiak G, Bajer A. Distribution of *Cryptosporidium* and *Giardia* spp. in selected species of protected and game mammals from North-Eastern Poland. Ann Agric Environ Med. 2007; 14:265–270.
3. Jirku M, Votypka J, Petrzelkova KJ, Jirku-Pomajbikova K, Kriegova E, Vodicka R, et al. Wild chimpanzees are infected by *Trypanosoma brucei*. Int J Parasitol Parasites Wildl. 2015; 4:277–282.
4. Keita AK, Fenollar F, Socolovschi C, Ratmanov P, Bassene H, Sokhna C, et. al. The detection of vector-borne-disease-related DNA in human stool paves the way to large epidemiological studies. Eur J Epidemiol. 2015; 30:1021–1026.
5. Siregar JE, Faust CL, Murdiyarso LS, Rosmanah L, Saepuloh U, Dobson AP, Iskandriati D. Non-invasive surveillance for *Plasmodium* in reservoir macaque species. Malar J. 2015; 14:404.
6. Zygner W. Arthropod-borne parasitoses which may threaten health of dogs travelling into Mediterranean area and Portugal. Part II. Babesiosis, theileriosis and hepatozoonosis. Życie Weterynaryjne 2006; 81:595–603.
7. Baric Rafaj R, Kules J, Selanec J, Vrkic N, Zovko V, Zupancic M, et al. Markers of coagulation activation, endothelial stimulation, and inflammation in dogs with babesiosis. J Vet Intern Med. 2013; 27:1172–1178.
8. Weerakoon KG, McManus DP. Cell-free DNA as a diagnostic tool for human parasitic infections. Trends Parasitol. 2016; 32:378–391.
9. Kaiser M, Lowa A, Ulrich M, Ellerbrok H, Goffe AS, Blasse A, et al. Wild chimpanzees infected with 5 *Plasmodium* species. Emerg Infect Dis. 2010; 16:1956–1959.
10. Liu W, Li Y, Learn GH, Rudicell RS, Robertson JD, Keele BF, et al. Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. Nature 2010; 467:420–425.
11. Jirku M, Pomajbikova K, Petrzelkova KJ, Huzova Z, Modry D, Lukes J. Detection of *Plasmodium* spp. in human faeces. Emerg Infect Dis. 2012; 18:634–636.
12. Liu W, Li Y, Shaw KS, Learn GH, Plenderleith LJ, Malenke JA, et al. African origin of the malaria parasite *Plasmodium vivax*. Nat Commun. 2014; 5:3346.
13. Keele BF, Van Heuverswyn F, Li Y, Bailes E, Takehisa J, Santiago ML, et al. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science 2006; 313:523–526.
14. El Khechine A, Henry M, Raoult D, Drancourt M. Detection of *Mycobacterium tuberculosis* complex organisms in the stools of patients with pulmonary tuberculosis. Microbiology 2009; 155:2384–2389.
15. Hornok S, Estok P, Kovats D, Flaisz B, Takacs N, Szoke K, et al. Screening of bat faeces for arthropod-borne apicomplexan protozoa: *Babesia canis* and *Besnoitia besnoiti*-like sequences from Chiroptera. Parasit Vectors 2015; 8:441.
16. Welc-Faleciak R, Bajer A, Bednarska M, Paziewska A, Sinski E, Long term monitoring of *Babesia microti* infection in BALB/c mice using nested PCR. Ann Agric Environ Med. 2007; 14:287–290.
17. Bednarska M, Bajer A, Drozdowska A, Mierzejewska EJ, Tolkacz K, Welc-Faleciak R. Vertical Transmission of *Babesia microti* in BALB/c Mice: Preliminary Report. PLoS One 2015; 10(9):e0137731.
18. Bonnet S, Jouglin M, L'Hostis M, Chauvin A. *Babesia* sp. EU1 from roe deer and transmission within *Ixodes ricinus*. Emerg Infect Dis. 2007; 13:1208–1210.
19. Bonnet S, Jouglin M, Malandrin L, Becker C, Agoulon A, L'Hostis M, Chauvin A. Transstadial and transovarial persistence of *Babesia divergens* DNA in *Ixodes ricinus* ticks fed on infected blood in a new skin-feeding technique. Parasitology 2007; 134:197–207.
20. Inokuma H, Okuda M, Ohno K, Shimoda K, Onishi T. Analysis of the 18S rRNA gene sequence of a *Hepatozoon* detected in two Japanese dogs. Vet Parasitol. 2002; 106:265–271.
21. Mitkova B, Hrazdilova K, D'Amico G, Duscher GG, Suchentrunk F, Forejtek P, et al. Eurasian golden jackal as host of canine vector-borne protists. Parasit Vectors 2017; 10:183.
22. Qurollo BA, Archer NR, Schreeg ME, Marr HS, Birkenheuer AJ, Haney KN, et al. Improved molecular detection of *Babesia* infections in animals using a novel quantitative real-time PCR diagnostic assay targeting mitochondrial DNA. Parasit Vectors 2017; 10:128.
23. Baneth G, Florin-Christensen M, Cardoso L, Schnittger L. Reclassification of *Theileria annae* as *Babesia vulpes* sp. nov. Parasit Vectors 2015; 8:207.
24. Baneth G, Mathew JS, Shkap V, Macintire DK, Barta JR, Ewing SA. Canine hepatozoonosis: two disease syndromes caused by separate *Hepatozoon* spp. Trends Parasitol. 2003; 19:27–31.
25. Hodzic A, Alic A, Fuehrer HP, Harl J, Wille-Piazzai W, Duscher GG. A molecular survey of vector-borne pathogens in red foxes (*Vulpes vulpes*) from Bosnia and Herzegovina. Parasit Vectors 2015; 8:88.
26. Hodzic A, Mrowietz N, Cezanne R, Bruckschwaiger P, Punz S, Habler VE, et al. Occurrence and diversity of arthropod-transmitted pathogens in red foxes (*Vulpes vulpes*) in western Austria, and possible vertical (transplacental) transmission of *Hepatozoon canis*. Parasitology 2018; 145:335–344.
27. Miterpakova M, Komjati-Nagyova M, Hurnikova Z, Vichova B. Retrospective molecular study on canine hepatozoonosis in Slovakia – Does infection risk for dogs really exist? Ticks Tick Borne Dis. 2017; 8:567–573.
28. Majlathova V, Hurnikova Z, Majlath I, Petko B. *Hepatozoon canis* infection in Slovakia: imported or autochthonous? Vector Borne Zoonotic Dis. 2007; 7:199–202.
29. Matijatko V, Torti M, Schetters TP. Canine babesiosis in Europe: how many diseases? Trends Parasitol. 2012; 28:99–105.
30. Kubelova M, Tkadlec E, Bednar M, Roubalova E, Siroky P. West-to-east differences of *Babesia canis* canis prevalence in *Dermacentor reticulatus* ticks in Slovakia. Vet Parasitol. 2011; 180:191–196.
31. Foldvari G, Hell E, Farkas R. *Babesia canis* canis in dogs from Hungary: detection by PCR and sequencing. Vet Parasitol. 2005; 127:221–226.
32. Koneval M, Miterpakova M, Hurnikova Z, Blanarova L, Vichova B. Neglected intravascular pathogens, *Babesia vulpes* and haemotropic *Mycoplasma* spp. in European red fox (*Vulpes vulpes*) population. Vet Parasitol. 2017; 243:176–182.

Ministry of Science and Higher Education
Republic of Poland

**Generation of the DOI (Digital Object Identifier)** – task financed under the agreement No. 618/P-DUN/2019 by the Minister of Science and Higher Education