Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.286

CASE REPORT

First Clinical Evidence with One-Year Monitoring of *Babesia Gibsoni* Mono-Infection in Two Dogs from Serbia

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ARTICLE HISTORY (24-581)

Received:	September 22, 2024
Revised:	October 24, 2024
Accepted:	October 26, 2024
Published online:	December 5, 2024

Key words:
Canine babesiosis
Immune-mediated hemolytic anemia
Parasitemia
PCR
Thrombocytopenia

ABSTRACT

In Serbia, *Babesia gibsoni* in dogs is less common than *Babesia canis*. Although two clinical cases were reported a decade ago, no additional clinical reports have since been published. Recently, a co-infection of *B. gibsoni* and *B. canis* was documented in Austria following a dog's trip to Serbia. The objectives of this study were to present comprehensive clinicopathological results of two clinical cases of *B. gibsoni* mono-infection in dogs in Serbia. Two male dogs: an 11-year-old Shih Tzu, and a 2-year-old Pit Bull Terrier, presented with clinical signs related to babesiosis with a history of biting by another dog. Both dogs had regenerative anemia, thrombocytopenia, and monocytosis while Shih Tzu had immune-mediated hemolytic anemia (IMHA). *B. gibsoni* mono-infection was confirmed by PCR testing. Both dogs were treated with a single dose of imidocarb-dipropionate, and a combination of metronidazole, clindamycin, and doxycycline (MCD protocol). The Shih Tzu also received prednisolone for three weeks. Following the MCD protocol, the Pitt Bull recovered, although thrombocytopenia persisted for nine months. In contrast, Shih Tzu's clinical condition worsened. The prednisolone treatment was discontinued, and azithromycin and atovaquone were introduced, leading to recovery after another three weeks of treatment. Long-term clinical and PCR monitoring revealed that the Pit Bull Terrier exhibited a more favorable response and a lower frequency of relapses compared to Shih Tzu. The findings suggest that *B. gibsoni* has become a clinically significant pathogen in Serbia. The MCD protocol appears effective for treating acute *B. gibsoni* infection in dogs, but further investigation is required to evaluate its efficacy in eliminating the parasite.

To Cite This Article: Milošević S, Božović Ilić A, Magaš V, Sukara R, Tomanović S, Radaković M, Spariosu K, Kovačević Filipović M and Francuski Andrić J, 2024. First clinical evidence with one-year monitoring of *Babesia gibsoni* mono-infection in two dogs from Serbia. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2024.286

INTRODUCTION

Babesia gibsoni infection in dogs is often asymptomatic in Europe (Karasova *et al*., 2022); however, it is emerging as a significant pathogen (Liu *et al.*, 2023). Compared to *B. canis*, *B. gibsoni* is generally considered less pathogenic. The disease typically follows a chronic course, varying from a subclinical form to multiorgan failure and death (Karasova *et al*., 2022). The acute form is characterized by pyrexia, lethargy, icterus, regenerative hemolytic anemia, thrombocytopenia, and splenomegaly (Bajer *et al.*, 2022; Karasova *et al*., 2022). Clinical presentation differs depending on the strains involved and their specific virulence, as well as factors influencing the host's immune response, including age, individual immune status, and other concurrent infections or diseases (Baneth, 2018). Dogs with asymptomatic infections may exhibit clinical signs if their immunity decreases due to various triggers. Infected dogs cannot permanently eliminate the parasites from the bodies. Currently, there are no sufficiently effective treatments available, and some strains show resistance to existing drugs (Baneth, 2018; Liu *et al*., 2022). Asymptomatic and chronic carriers significantly contribute to the parasite's spread and transmission, since tick vectors, such as *Haemaphysalis longicornis, Haemaphysalis bispinosa, and Rhipicephalus sanguineus* do not transmit *B. gibsoni* vertically *(Baneth, 2018; Strobl et al.,* 2021*).* This underlines the importance of

asymptomatic and chronic carriers who significantly contribute to the parasite's spread and transmission. Moreover, transmission can occur through infected dog bites, blood transfusion, or transplacental infection (Karasova *et al*., 2022).

Previously, two clinical cases of *B. gibsoni* in Serbia were identified as part of a larger study that retrospectively analyzed species of *Babesia* in 60 cases treated for canine babesiosis (Davitkov *et al*., 2015). However, detailed clinicopathological findings of these two cases were not fully reported. Since then, *B. gibsoni* has only been detected in asymptomatic outdoor dogs (Kovačević Filipović *et al*., 2018). Despite a recent report of a clinical case of *B. gibsoni* in Austria following travel to Serbia (Strobl *et al*., 2021), no clinicopathological reports on *B. gibsoni* infection in dogs from Serbia currently exist.

Our study aimed to describe the first clinicopathological findings with *B. gibsoni* mono-infection in two dogs over one year of monitoring, including clinical signs, hematological changes, polymerase chain reaction (PCR) tests, and therapeutic approach.

MATERIALS AND METHODS

Case 1 (Shih Tzu): An eleven-year-old male Shih Tzu was presented at the Teaching Hospital, Faculty of Veterinary Medicine, University of Belgrade (FVMUB), Serbia, in February 2022, showing clinical signs of decreased appetite, lethargy, fever, pale mucous membranes, tachycardia, and dyspnea. Four years ago, before the clinical signs appeared, the owners moved from the United States to Serbia. The dog had no known history of chronic illness or tick bites, and his routine vaccinations and treatments for intestinal and external parasites were up to date. However, the owner reported that the dog had previously tested positive for *Ehrlichia canis* antibodies on a point-of-care test (Bionote, Korea) and was on doxycycline treatment at 10 mg/kg per os (PO) prescribed for three weeks. Furthermore, another dog bit him two weeks before his hospital visit.

Case 2 (Pit Bull Terrier): In April 2022, a two-year-old male Pit Bull Terrier, regularly vaccinated and treated for intestinal and ectoparasites, without a history of tick bites, was referred to the Teaching Hospital, FVMUB, Serbia, with clinical signs of fever, decreased appetite, lethargy, pale mucous membranes, tachycardia, and dyspnea. The owner also reported a history of being bitten by another dog. The dog had no known history of chronic illness either.

Hematological and biochemical analyses: Venous blood was collected in standard vacutainer tubes and analyzed within 30 minutes. Using the ProCyte Dx hematology analyzer (Idexx, United States), anemia was defined as Hct<37.3% (reference interval (RI) 37.3-61.7%), thrombocytopenia as a platelet count below 148×10^9/L (RI 148-484×10^9/L) and neutropenia as a neutrophil count of <2.95×10^9/L (RI 2.95-11.64×10^9/L). Pancytopenia was characterized as the simultaneous occurrence of anemia, neutropenia, and thrombocytopenia. Blood smears were prepared to examine the presence of *Babesia* spp. merozoite. The saline agglutination test (SAT) involved mixing one drop of EDTA blood with four drops of saline at room temperature. The presence of erythrocyte agglutination was observed microscopically. Routine serum biochemical profiles were performed using the Mindray BS-240 (China).

Urine analyses: Urine was collected by cystocentesis. The complete urinalysis included examination of color, turbidity, urine specific gravity (measured with a refractometer), dipstick analyses (pH, protein, glucose, ketones, blood, bilirubin), and urine sediment examination. The protein-creatinine ratio (UPC) was used to quantify proteinuria.

Treatment protocols for *B. gibsoni* infection: The MCD protocol included a combination of three antibiotics: metronidazole (15 mg/kg PO twice daily), clindamycin (25 mg/kg PO twice daily), and doxycycline (5 mg/kg PO once daily) for 30 days. The AA protocol included Atovaquone (13.3 mg/kg PO twice daily) and Azithromycin (10 mg/kg PO once daily) for 10 days (Almendros *et al*., 2020). Additionally, Imidocarb-dipropionate (6.6 mg/kg IM) was administered twice, 14 days apart. An immunosuppressive dose of prednisolone (2 mg/kg PO) was given for 7 days.

DNA extraction: Total DNA was isolated from 200 µL of whole blood using the Gene Jet Genomic DNA Purification Kit (Thermo Scientific), following the manufacturer's protocol, and stored at -20 °C before PCR analysis.

Conventional PCR: Initial detection of *Babesia* spp. was conducted using the following primers: BJ1 (5´-GTC TTG TAA TTG GAA TGA TGG-3´) and BN2 (5´-TAG TTT ATG GTT AGG ACT ACG-3´), which amplified the fragment of *18S rRNA* unique to several *Babesia* species (Casati *et al*., 2006). The tested sample gave a positive signal. Subsequently, the presence of *Babesia gibsoni* was confirmed using *B. gibsoni*-specific primers: Gib599F (5´-CTC GGC TAC TTG CCT TGT C -3´) and Gib1270R (5´-GCC GAA ACT GAA ATA ACG GC-3´) (Inokuma *et al*., 2004). To exclude other tick-borne pathogens in co-infection, the following PCR assays were performed: *16S rDNA* amplification for members of the family Anaplasmataceae (*Ehrlichia, Anaplasma, Neorickettsia, Neoehrlichia,* and *Wolbachia*), primers EHR16SD/EHR16SR (Parola *et al*., 2000), and primers HepF_for/HepR_rev that amplify the 666-bp fragment of the *18S ssrRNA* gene to exclude the presence of *Hepatozoon* spp. (Inokuma *et al*., 2002). The presence of hemotropic mycoplasmas in a Pit Bull Terrier was detected using commercial identification kits according to the manufacturer's instructions (BIORON GmbH, Römerberg, Germany) for *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* via quantitative polymerase chain reaction. Since a Shih Tzu was presented to the FVMUB while undergoing doxycycline therapy, which was also used to treat hemotropic hemoplasmosis, the dog was not tested for their presence by PCR.

Sequencing: Bidirectional Sanger sequencing of the *18S rRNA* fragment (460bp) obtained using BJ1 and BN2 primers was performed, and the consensus sequences were

compared with sequences in Gen Bank using BLAST and deposited in GenBank under the accession numbers: PQ114141 (Shih Tzu), PQ114142 (Pit Bull).

Statistical analysis: Hematology results for each dog and multiple line graphs (MedCalc® v14.8.1) presented the analyzed points.

RESULTS

Case 1 (Shih Tzu): On presentation, the CBC showed mild non-hemolytic, normocytic normochromic regenerative anemia, severe thrombocytopenia, and monocytosis without changes in the total white blood cell count (WBC), lymphocytes, and neutrophils (Figures 1 and 2). Significant reticulocytosis and nucleated red blood cells (nRBC) were also present. *Babesia* spp. merozoite was not found microscopically, and PCR analyses were requested for the presence of *Babesia* spp., *E. canis*, *Anaplasma phagocytophilum*, and *Hepatozoon canis*. Routine biochemistry analyses were within the reference interval (RI) (data not shown). Until the PCR result was obtained, the dog continued to receive the doxycycline therapy, previously prescribed before presentation to the FVM teaching hospital. The clinical condition worsened on the third day after presentation, and pancytopenia occurred (Figure 1. A-J, Figure 2. A-F). A diagnosis of immune-mediated hemolytic anemia (IMHA) was established based on the positive SAT, erythrocyte ghosts, and spherocytosis on the blood smear (Garden *et al*., 2019). Additionally, *Babesia* piroplasm forms were found on the blood smear (Figure 3), and *B. gibsoni* mono-infection was confirmed by PCR analysis. To treat *B. gibsoni* infection and IMHA the dog received a combination of three different antibiotics (MCD treatment protocol), imidocarb-dipropionate, and prednisolone. The dog started to respond well to MCD protocol, and a follow-up CBC on the second day of treatment revealed mild regenerative anemia and thrombocytopenia with no changes in WBC count (Figures 1 and 2). *Babesia* piroplasm forms were not found on the blood smear. However, after seven days of therapy, the dog became lethargic, stopped drinking and eating, and developed severe thrombocytopenia alongside persisting mild regenerative anemia (Figure 1. A-C, H). Again, parasitemia occurred, and a repeated PCR analysis confirmed the presence of *B. gibsoni*. Prednisolone was gradually discontinued, and the AA protocol was applied for the next 10 days. Hematological analyses were repeated 7 and 14 days after the start of AA therapy. After 14 days of treatment, the dog showed no CBC changes, except for reticulocytosis without anemia. After three weeks of treatment, the dog fully recovered, and CBC (Figure 1. A-J) and biochemical assays (data not shown) were unchanged. After two and five months following the acute *B. gibsoni* infection, the health check showed hematological (Figure 1. A-J) and biochemical testing results (data not shown) within RI. However, urine analyses showed the presence of proteinuria (UPC = 0.6). The staging and treatment of chronic kidney disease (CKD) were determined based on the 2023 International Renal Interest Society (IRIS) guidelines and recommendations. Because there was no azotemia, no visible kidney structure changes on ultrasound and radiology, and no changes in blood pressure, the dog was graded as stage 1. A kidney biopsy was declined, and a clinical renal diet was prescribed, along with a recommendation for monthly check-ups. After a year, the dog started to show clinical signs of apathy, difficulty breathing, and easy fatigue once again. The dog had not undergone regular check-ups during this period. A CBC revealed mild regenerative anemia, with the presence of nRBC. The total count of thrombocytes and leukogram was within RI (Figure 1. H, Figure 2. A-F). An ultrasound examination showed a significantly enlarged spleen. PCR confirmed the presence of *B. gibsoni* for the third time. The dog passed away a few days later, and the owners declined an autopsy.

Case 2 (Pit Bull Terrier): A CBC revealed severe normocytic normochromic regenerative anemia, with no changes in WBC and other leukocytes, and severe thrombocytopenia at presentation (Figures 1 and 2). There was significant reticulocytosis and an increase in nRBC. The blood smear showed *Babesia* piroplasm forms without the presence of spherocytosis, and the SAT was negative. PCR analyses were requested for *Babesia* spp., *E. canis*, *A. phagocytophilum*, *H. canis*, and *B. gibsoni* mono-infection was confirmed. The dog received the MCD protocol and responded positively to the therapy. After three weeks, the dog recovered completely and tested negative on PCR for *B. gibsoni* 30 days after the treatment. The dog showed no clinical signs two months after the treatment ended. Thrombocytopenia was the only hematological alteration, with increased mean platelet volume (Figure 1. H, I). A blood smear revealed the presence of macroplatelets, without *Babesia* piroplasm forms. The PCR results were negative for the presence of *B. gibsoni*. The dog remained clinically healthy eight months after receiving the MCD, but thrombocytopenia persisted, and PCR revealed the presence of *B. gibsoni* (Figure 1. H-J). The same protocol was applied for the second time, and six months later (1.5 years after acute infection with *B. gibsoni*), the dog was clinically healthy, and no hematological alterations were seen during the control examination (Figures 1 and 2). Urine analyses and UPC were within RI.

DISCUSSION

These two cases highlight three specific points that are important to note. First, since the microscopic examination of stained blood smears has relatively low sensitivity and is insufficient to determine the exact *Babesia* species, molecular analysis, characterized by a high level of sensitivity and specificity, is crucial for identifying the causative agent and administering appropriate therapy. Using species-specific and genus-specific primers followed by sequencing, the infection with *B. gibsoni* was unambiguously confirmed in both cases. The obtained sequences are mutually identical and showed 100% identity with *B. gibsoni* sequences previously deposited in GenBank.

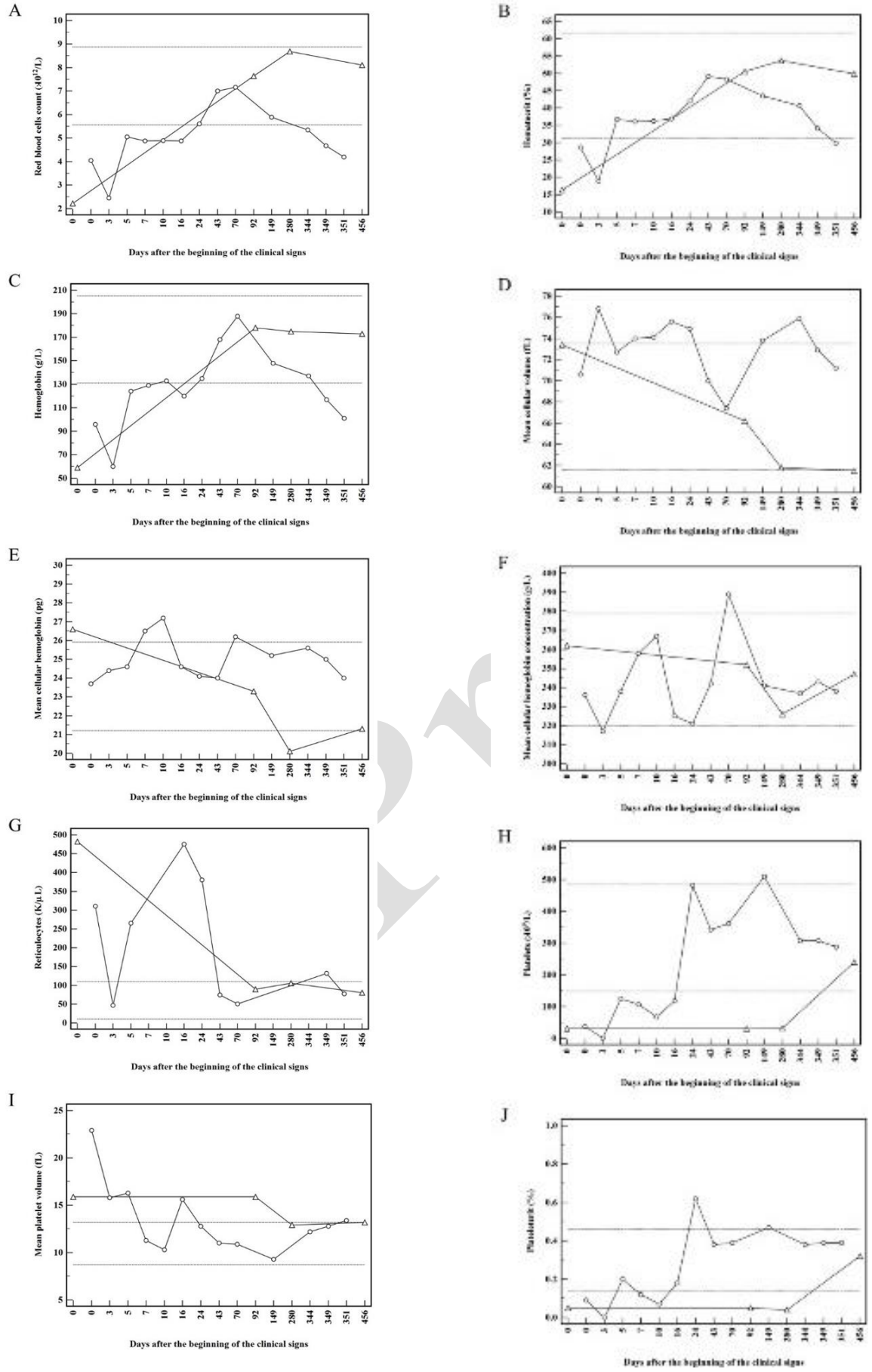

Fig. 1: Hematology results for (A) red blood cell count, (B) hematocrit, (C) hemoglobin concentration, (D) mean cellular volume, (E) mean cellular hemoglobin, (F) mean cellular hemoglobin concentration, (G) reticulocytes, (H) platelets, (I) mean platelet volume, (J) plateletcrit, for the Shih Tzu (Case 1, lines connected with circles) and the Pit Bull Terrier (Case 2, lines connected with triangles). The horizontal axis indicates the days after the presentation of the clinical signs. Multiple line graph test, MedCalc® v14.8.1.

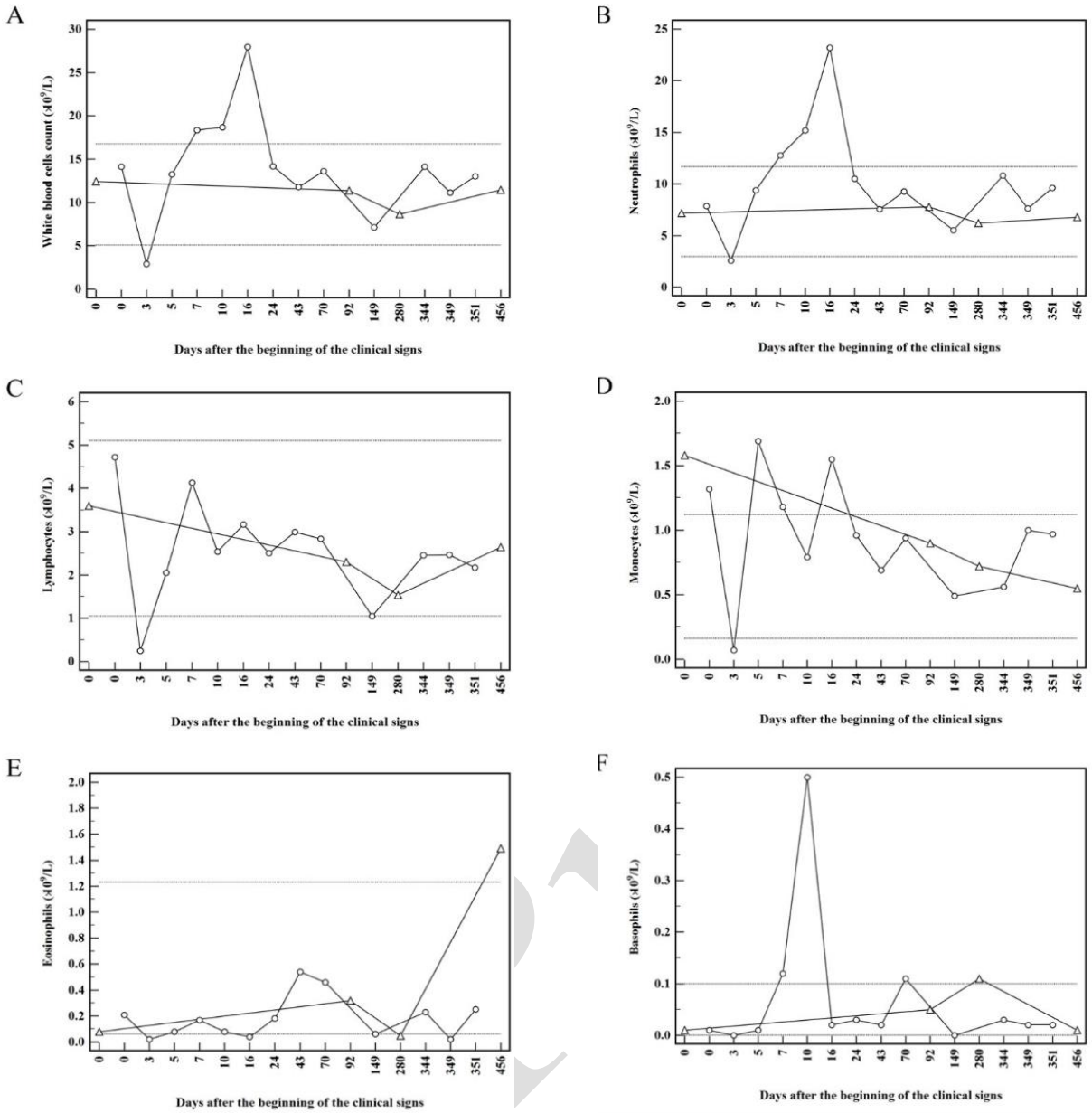

Fig. 2: Hematology results for (A) white blood cell count, (B) neutrophils, (C) lymphocytes, (D) monocytes, (E) eosinophils, (F) basophils, for the Shih Tzu (Case 1, lines connected with circles) and the Pit Bull Terrier (Case 2, lines connected with triangles). The horizontal axis indicates the days after the beginning of the clinical signs. Multiple line graph test, MedCalc® v14.8.

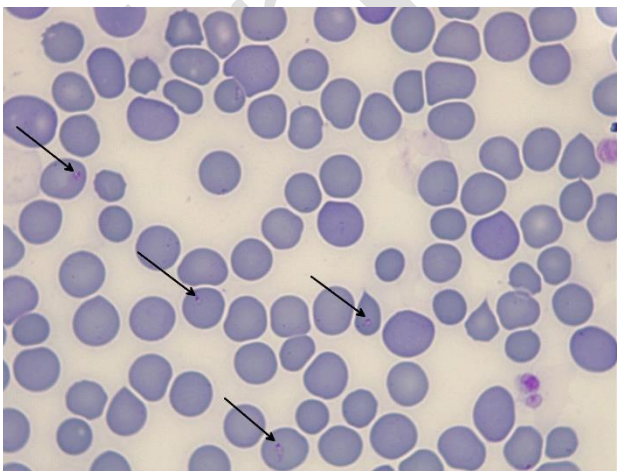

Fig. 3: A Diff-Quick stained peripheral blood smear. The black arrows represent *Babesia* piroplasm forms that were confirmed by PCR to be *B. gibsoni.*

The second point concerns the increased risk of infection with *B. gibsoni* in Serbia. A consensus statement on the epidemiological situation and expected frequency of canine vector-borne diseases in Serbia classifies the risk of infection with *B. gibsoni* as intermediate (Potkonjak *et al*., 2020). The existence of asymptomatically infected outdoor dogs in Serbia over the past decade (Kovačević Filipović *et al.*, 2018) is likely the cause of the rising clinical cases of *B. gibsoni*. The report by Strobl *et al.* (2018) on acute *B. gibsoni* infection in a dog that had been to Serbia, along with these two clinical cases, demonstrates that the risk of infection is probably higher now. In addition, these clinical cases suggest that, apart from *B. canis*, the most common *Babesia* species in Serbia, *B. gibsoni* should also be considered when hemolytic anemia and severe thrombocytopenia occur. Interestingly, these dogs did not have a history of tick bites. Tomanović *et al.* (2013) documented the presence of

Haemaphysalis concinna and *Haemaphysalis punctata* in Serbia, with *R. sanguineus* being the most abundant tick that could carry *B. gibsoni* in the region (Davitkov *et al*., 2016). However, to the best of the authors' knowledge, there is no evidence of *Haemaphysalis longicornis or Haemaphysalis bispinosa in Serbia*. This suggests that the transmission was most likely due to the bite of an infected dog.

The third point concerns the clinicopathological findings and therapeutic approach with PCR monitoring. Currently, AA is the primary treatment choice for *B. gibsoni* infection in most countries (Almendros *et al*., 2020). Although AA leads to clinical improvement, it has been associated with elevated recurrence rates, possibly due to resistance caused by mutations in the *B. gibsoni cytb* gene (Almendros *et al.*, 2020). The concurrent administration of various drug combinations, such as clindamycin, diminazene, and imidocarb; clindamycin and diminazene; metronidazole, doxycycline, and enrofloxacin; and metronidazole, clindamycin, and doxycycline, has resulted in a significant reduction in infection recurrence and an increase in the elimination of parasitemia (Almendros *et al.*, 2020). Due to limited drug availability for the AA protocol and its economic impact in Serbia, the clinical decision for these two clinical cases was to use the MCD protocol as the primary treatment choice.

Although both dogs developed acute infection with *B. gibsoni*, their clinical presentations and responses to therapy differed. The Shih Tzu, who initially had regenerative IMHA, positively responded to the MCD protocol for the first seven days. However, due to the worsening clinical signs, the AA treatment was subsequently implemented. A limitation of this study was our inability to determine the *B. gibsoni* mutation, so we cannot be certain why the Shih Tzu didn't respond well to the MCD treatment. It is unclear if the strain was resistant to the drugs or if corticosteroid administration was the issue. Prednisolone could have reduced the immune system's ability to fight *B. gibsoni* infection, leading to an increase in the number of parasites in the blood and worsening clinical conditions. In such cases, it may be necessary to modify the previously effective treatment for the dog. In contrast to the Shih Tzu, the Pit Bull Terrier, who had more severe anemia and visible *Babesia* piroplasm forms on the blood smear at presentation, responded well to the MCD protocol. The Pit Bull Terrier's age and lack of prior vector-borne infections likely contributed to its faster recovery and a longer period without a relapse of the disease. Furthermore, another intriguing distinction among these two cases is the pattern of thrombocytes and basophils. After applying both therapeutic protocols, the Shih Tzu did not have thrombocytopenia, except when pancytopenia occurred. Platelets returned to physiological limits nine days after the application of the AA therapy protocol. In contrast, severe thrombocytopenia with macroplatelets persisted in the Pit Bull Terrier even nine months after the onset of clinical signs. The question remains whether AA improves thrombocytopenia by lowering the inflammatory response, or if the Pit Bull Terrier had a more severe reaction to infection, resulting in extended platelet involvement in the inflammatory response. Moderate basophilia associated with neutrophilia was only noted in the Shih Tzu and was probably related to immune response during parasite infection and IMHA (Held and Mochizuki, 2023). However, knowledge of the clinical importance of increased basophil number is limited (Held and Mochizuki, 2023). The Shih Tzu developed proteinuria, in contrast to the Pit Bull Terrier. This is most likely the outcome of a type III hypersensitivity reaction caused by prolonged *B. gibsoni* and previously *E. canis* infection.

Currently, in monitoring *B. gibsoni* infection, two repeated PCR tests on days 60 and 90 are the standard protocol after therapy, as PCR can produce false-positive results due to the residual DNA that can be amplified (Almendros *et al.*, 2020). Almendros *et al.* (2020) found that around 90% of the dogs, based on this guideline, tested negative by day 60, which was also noted in this study. However, no data is available for day 30 to evaluate earlier conversion or the possibility of false positives. On day 30, the Pit Bull Terrier tested negative by PCR and showed no initial clinical signs, although thrombocytopenia persisted. The question arises whether this result could be an early false negative. Since the Pit Bull tested negative 60 days after therapy, we can assume that the dog developed an earlier PCR conversion. Moreover, nine months later, the Pit Bull Terrier tested positive when clinical signs reappeared, suggesting that positive PCR findings coincide with worsening patient conditions. Dogs recovering from an acute infection could have persistent parasitemia for at least 38 months (Karasova *et al*., 2022). In the chronic form, parasitemia can persist for years, highlighting the need for more clinical studies to create a consensus on monitoring and treatment of *B. gibsoni* infection.

Conclusions: *B. gibsoni* has become a clinically significant canine pathogen in Serbia. Based on these two clinical cases, the MCD protocol, which is accessible and affordable in this country, can be used to treat acute *B. gibsoni* infection in dogs. However, the prognosis of the disease could be worse in older dogs and those that develop immune-mediated anemia. Further investigations should assess the effectiveness of the MCD protocol in eliminating *B. gibsoni.* Additionally, the immunosuppressive effect of prednisolone should be considered when administering it to dogs infected with *B. gibsoni*.

Animal Welfare Statement: Dog owners signed informed consent that the residual blood samples and the obtained results could be used for scientific purposes. The Ethical Committee at the Faculty of Veterinary Medicine approved this research, and permission was acquired from the Ministry of Agriculture, Forestry and Water Management, Republic of Serbia (permission number: 001327728202414841002000323022).

Declaration of Competing Interests: The authors report no competing interest.

Funding Sources: The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (contract numbers: 451-03-66/2024-03/200143 and 451-03-66/2024-03/200015).

Authors' Contribution: JFA designed the study; SM and AIB collected the samples; SM, AIB, and VM performed clinical examination; RS, MR, KS, and JFA performed the analyses; RS, ST, MKF, and JFA interpreted the data; SM and JFA wrote the manuscript; ST and MKF corrected and edited the manuscript. All authors have read and approved the manuscript.

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