In-clinic laboratory diagnosis of canine babesiosis (Babesia canis canis) for veterinary practitioners in Central Europe

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Key words
Dog, babesiosis, haematological abnormalities, climate conditions

Summary
Objective: Haematological changes in dogs and climatic conditions favourable for the vector may assist in the quick in-house diagnosis of canine babesiosis. Material and methods: Blood samples from 358 dogs suspected to have canine babesiosis were evaluated. The diagnosis was confirmed in 113 dogs by detection of Babesia canis by microscopic examination of a stained blood smear using the concentration line technique. Results: Thrombocytopenia was present in all 113 dogs. Red blood cell count, packed cell volume and haemoglobin values were below the reference range in 62.8%, 61.1% and 46.0% of affected dogs, respectively. An increased reticulocyte count was apparent in five Babesia canis-positive dogs. Leukopenia, lymphopenia, neutropenia and monocytosis were present in 54.9%, 47.8%, 30.4% and 6.5% of the dogs, respectively. Evaluating haematological parameters by CART-analysis revealed a predictive model (accuracy = 93.5%) for canine babesiosis, when using the leucocyte, thrombocyte, and reticulocyte count. Climatic conditions present at the most probable time of Babesia canis-infection accounted for biseasonal occurrence. Changes of climatic factors during the year influence the vector activity and in conclusion should highlight babesiosis in the ranking of differentials for veterinarians. Conclusion: The results demonstrate that a tentative diagnosis of canine babesiosis can be made based on typical haematological changes. The results recorded match well with the seasonality of the tick vector and were confirmed here by the month of sample submission.

Schlüsselwörter
Hund, Babesiose, hämatologische Veränderungen, Klimadaten

Zusammenfassung

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Labordiagnose der kaninen Babesiose (Babesia canis canis) in der tierärztlichen Praxis
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Introduction

Canine babesiosis has become evident in central Europe over the last three decades and has long been endemic in the Mediterranean area and Eastern Europe. Introduction of infected ticks to Central Europe by dogs imported from Southern European countries was first documented in 1975 (7). In Central Europe, babesiosis is caused by the large, piriform-shaped Babesia canis canis. It is an intracellular protozoan parasite of erythrocytes and is transmitted by the hard tick Dermacentor reticulatus (4). This vector tick tolerates low temperatures and low humidity, although warm and humid conditions are preferred for development and reproduction (23). Activity is reduced or ceases during cold or hot periods. Reactivation in spring appears to start immediately after a few moderately warm days (12). In southern Europe Babesia canis vogeli, leading to a less symptomatic disease than infection by Babesia canis canis, is transmitted by Rhipicephalus sanguineus and the highly pathogenic Babesia canis rossi is transmitted by Haemaphysalis leachi in the southern region of Africa (14).

An important factor in the pathogenesis of canine babesiosis, apart from the mechanical and toxic damage induced by the parasite, is the immune-mediated response of the affected dog. Antibodies, directed against the antigens and other components of the membranes of the infected and uninfected erythrocytes, are produced. As a consequence, destruction of the erythrocytes and an intravascular and extravascular haemolysis occurs, which induces fever, anaemia, hypoxia, and haemoglobinuria (2). The incubation time in canine babesiosis is reported to range from 4 to 21 days (2), most likely 7 to 14 days (12). After this period most dogs that are presented to a veterinary practitioner show unspecific initial clinical signs like fever, anorexia and depression. In this acute phase of the disease half of the affected dogs have dark-coloured urine (15). In chronic babesiosis dogs may show intermittent fever, weight loss, renal failure, paresis and seizures (13). Unspecific clinical signs (fever, anaemia, petechial bleeding) in dogs may prompt veterinary practitioners to take blood samples for haematological examination.

The most common laboratory findings in canine babesiosis are initially a mild normocytic, normochromic non-regenerative anaemia and a severe thrombocytopenia (19, 22). The anaemia becomes macrocytic and regenerative after a couple of days (6). This regeneration is proportional to the severity of the anaemia (5). Leukocyte disorders may include leukocytosis, neutrophilia, monocytes or leukopenia, neutropenia with left shift and lymphocytosis (24). A promptly applicable method for the diagnosis of canine babesiosis is the microscopic evaluation of a blood smear. As erythrocytes parasitized by Babesia spp. are less dense, these erythrocytes are mostly found in the periphery of the blood smear and in the “feather-edge” at the end of the blood film (9).

Another method of diagnosis is the molecular analysis (PCR) of blood samples for Babesia canis. Serological tests are not useful in the diagnosis of acute canine babesiosis, firstly because the seroconversion appears approximately 1–2 weeks after infection, and secondly because the seroprevalence in endemic areas is high (2, 8).

The aim of the present study was to evaluate whether haematological changes in dogs suffering from acute babesiosis together with data of local climate conditions favourable for the vector tick, may serve as major decision parameters for veterinary practitioners and indicate whether further specific examinations for the diagnosis of canine babesiosis are necessary.

Materials and methods

Patients and investigations

From January 2005 to December 2008 a complete blood count (CELL-DYN 3500 with Veterinary Package Software, Abbott Laboratories, IL, USA) was performed in 358 dogs with clinical signs suspicious for canine babesiosis, as requested by veterinary practitioners. Infrequently reported histories revealed an assumed geographical area of infection in eastern Austria and western Hungary. Symptoms of the dogs commonly revealed tick infestation and clinical signs such as fever, anaemia, depression and dark coloured urine. Submitting veterinarians frequently proposed granulocytic anaemia as a differential diagnosis. Samples from animals included in the study were examined for Anaplasma phagocytophilum microscopically; however, no positive cases were identified.

The examined dogs (163 males, 26 castrated males, 71 females, 77 neutered females and 21 dogs with not reported gender history) were 9 weeks to 17 years old and belonged to 87 breeds.

An absolute reticulocyte count was performed, only when requested by the clinical veterinarian, in 154 dogs (81 negative dogs, 73 positive dogs) by microscopic evaluation as mentioned by Tvedten (21). A microscopic 100-cell differential was performed on stained blood smears (Hemacolor®, Merck, Vienna, Austria). The presence of Babesia canis was confirmed by microscopic examination of stained blood smears by the concentration line technique, which is a modified blood smear. Instead of the “feather-edge”, the blood film ends with a straight line. The parasitized erythrocytes are found in larger numbers in this line; examination of this area facilitates the detection of Babesia-infected erythrocytes (Fig. 1 and 2).

Fig. 1 Preparation of a blood smear using the concentration line technique.

Abb. 1 Anfertigung eines Blutausstrichs mit besonderer Technik zur Parasitenanreicherung
Haematological parameters such as white blood cell count (WBC), leukogram, red blood cell count (RBC), haemoglobin (HGB) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), thrombocyte count and absolute reticulocyte count were evaluated. In accordance with Tvedten (21) the anaemia was classified as mild (PCV: 0.30–0.37 l/l), moderate (PCV: 0.20–0.29 l/l), severe (PCV: 0.13–0.19 l/l) and very severe (PCV: < 0.13 l/l). The degree of regeneration was classified as mentioned by Tvedten (21); none: up to 60 000 reticulocytes/μl, slight: up to 150 000/μl, moderate: up to 300 000/μl, marked: more than 500 000/μl. Thrombocytopenia was classified as slight (150–100 × 10⁹/l), mild (99–50 × 10⁹/l), moderate (49–25 × 10⁹/l) and severe (< 25 × 10⁹/l) (5).

For each case of babesiosis, climate data (mean daily air temperature, relative humidity, precipitation) recorded during the most probable incubation time were analysed. Climate data were measured at a meteorological station (Adcon, A730MD) located near Vienna (Raasdorf, operated by the Institute of Agronomy and Plant Breeding, BOKU – University of Natural Resources and Applied Life Sciences, Vienna).

**Statistical analysis**

For statistical analysis (SPSS v. 14; SPSS Inc., Chicago, US) a classification and regression tree model (CART) was calculated. The CART analysis consisted of a two step procedure: firstly a training analysis from one part of the randomized split data sample, and secondly a test analysis of the remaining data to test the goodness of fit.

Independent variables in the first CART consisted of packed cell volume (PCV), haemoglobin, mean corpuscular volume (MCV) and temperature, relative humidity, and precipitation. The CART analysis showed that the haemoglobin concentration (HGB) concentration, packed cell volume (PCV) and temperature were the most important factors in the model.

**Table 1**  Haematological parameters in Babesia canis-positive and -negative dogs.

<table>
<thead>
<tr>
<th>Parameter (reference values)</th>
<th>Percentage of Babesia-negative and -positive samples compared to reference values</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>below</td>
<td>within</td>
</tr>
<tr>
<td>thrombocytes (150–500 × 10⁹/l)</td>
<td>38.4</td>
<td>100</td>
</tr>
<tr>
<td>RBC (5.5–8.0 × 10¹²/l)</td>
<td>24.1</td>
<td>62.8</td>
</tr>
<tr>
<td>PCV (0.37–0.50 l/l)</td>
<td>31.8</td>
<td>61.1</td>
</tr>
<tr>
<td>HGB (120–180 g/l)</td>
<td>23.7</td>
<td>46.0</td>
</tr>
<tr>
<td>MCV (60–77 fl)</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>MCHC (31–35%)</td>
<td>3.3</td>
<td>3.5</td>
</tr>
<tr>
<td>reticulocytes (&gt; 60 × 10⁹/l)</td>
<td>55.5</td>
<td>93.1</td>
</tr>
<tr>
<td>WBC (6–12 × 10⁹/l)</td>
<td>4.1</td>
<td>54.9</td>
</tr>
<tr>
<td>neutrophils (3.3–9.0 × 10⁹/l)</td>
<td>4.1</td>
<td>30.4</td>
</tr>
<tr>
<td>lymphocytes (1.0–3.6 × 10⁹/l)</td>
<td>25.9</td>
<td>47.8</td>
</tr>
<tr>
<td>monocytes (&lt; 1.0 × 10⁹/l)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>eosinophils (&lt; 0.5 × 10⁹/l)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Mann-Whitney U-test
Fig. 3
Haematological results of *Babesia canis*-positive and -negative dogs. a) Thrombocyte count (reference range [RR]: 150–500 × 10^9/l); b) reticulocyte count (RR: > 60 × 10^9/l); c) white blood cell count (RR 6.0–12.0 × 10^9/l).

Abb. 3
Hämatologische Werte bei *Babesia canis*-positiven und -negativen Hunden. a) Thrombozyten (Referenzbereich [RB]: 150–500 × 10^9/l); b) Retikulozyten (RB: > 60 × 10^9/l); c) Leukozyten (RB 6,0–12,0 × 10^9/l)
Fig. 4  CART of the test analysis including white blood cell count, thrombocyte count, and reticulocyte count.

Abb. 4  CART der Test-Analyse unter Berücksichtigung von Leukozyten-, Thrombozyten- und Retikulozytenzahl
mean corpuscular haemoglobin concentration (MCHC), erythrocyte-, reticulocyte-, thrombocyte-, and white blood cell count (WBC). For the second CART climatic parameters as mentioned above were added as independent variables. The CART was specified as maximal five tree branches and a minimum of 10 cases per superior node for further split. Pruning was used to avoid overfitted results.

Haematological and climatic parameters of both groups were compared by a Mann-Whitney U-test (Tables 1 and 2), age and gender distributions by a chi-square test.

Results

In 113 of the 358 examined dogs (31.6%) large piriform or amoeboïd, sometimes paired inclusion bodies (Babesia canis) were demonstrated by microscopic evaluation of the blood smear. Haematological results are displayed in Table 1 and Figure 3. In accordance with the classification mentioned above, a severe thrombocytopenia was found in 63 dogs (55.8%). The lowest value was $5 \times 10^9/l$. A moderate thrombocytopenia was seen in 34 dogs (30.1%), a mild thrombocytopenia in 13 dogs (11.5%). A slight thrombocytopenia was found in only 3 dogs (2.6%) with the highest value being $147 \times 10^9/l$. The anaemia was classified as mild in 44 dogs (63.8%), moderate in 22 (31.9%), severe in 2 (2.9%) and very severe in 1 dog (1.4%). According to the classification mentioned above, analysis of the absolute reticulocyte counts showed the following results: 68 positive dogs (93.2%) had no regeneration and in 5 dogs (6.8%) a slight regeneration was noticed.

The results of the CART-analysis (WBC, thrombocyte, reticulocyte) are shown in Figure 4 revealing a prediction accuracy of 92.7% in the training analysis (standard error: 0.018) and 93.5% in the test analysis (standard error: 0.021). If leukocyte count is $\leq 7.25 \times 10^9/l$ and thrombocyte count is $\leq 55.0 \times 10^9/l$, or if a dog has a leukocyte count $> 7.25 \times 10^9/l$, a thrombocyte count $\leq 58.0 \times 10^9/l$ and a reticulocyte count $\leq 61.6 \times 10^9/l$, then the dog is to be expected to have a 93.5% likelihood of suffering from babesiosis. The normalised significance of the independent variables revealed that the thrombocyte count (100%) is the most relevant parameter followed by WBC (60.9%) and reticulocyte count (33.2%). Other haematological parameters were excluded as they did not assist in the calculation of the predictive value.

The majority (80.5%) of the Babesia canis-positive dogs were diagnosed in April to May and September to November (Fig. 5) with a mean air temperature range of 8.8 °C to 13.7 °C in spring and 8.1°C to 18.3°C in autumn. Comparing relative humidity and mean air temperature in both groups did not result in a significant difference, precipitation in Babesia canis-positive cases was significantly lower (Table 2). Adding climatic parameters to the CART analysis reduced the p-value (61.3%) and they were also less relevant than haematological parameters when comparing the normalised significance.

Table 2 Climate parameter Positive (mean ± SD) range Negative (mean ± SD) range p-value

<table>
<thead>
<tr>
<th>Climate parameter</th>
<th>Positive</th>
<th>Negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean air temperature (°C)</td>
<td>11.50 ± 4.85</td>
<td>12.58 ± 6.04</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>–2.2 to +27.5</td>
<td>3.2 to +23.3</td>
<td></td>
</tr>
<tr>
<td>relative humidity (%)</td>
<td>73.56 ± 9.17</td>
<td>71.89 ± 8.67</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>49–89</td>
<td>49–95</td>
<td></td>
</tr>
<tr>
<td>precipitation (mm/week)</td>
<td>4.82 ± 8.62</td>
<td>9.69 ± 15.28</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0–38</td>
<td>0–99</td>
<td></td>
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</table>

Fig. 5 Biseasonal occurrence of canine babesiosis.

Abb. 5 Bisaisonales Auftreten der kaninen Babesiose
Discussion

Canine babesiosis is a world-wide protozoal infectious disease. The importance of this tick-borne disease has gained rising attention in the last few years. As clinical signs of acute canine babesiosis are often unspecific, veterinarians usually request haematological analysis. The main haematological abnormality found in our study was a thrombocytopenia, in most cases it was a pronounced thrombocytopenia. This result is in accordance with other studies (16, 19). The mechanism of the thrombocytopenia is not yet clear. In a French study, the bone marrow of affected dogs revealed normal to high numbers of megakaryocytes and the authors came to the conclusion that the thrombocytopenia found in infections with *Babesia canis* is of peripheral origin (15). Possible reasons for this peripheral thrombocytopenia are an immune-mediated destruction of the platelets and an increased consumption (5).

Anaemia was present in approximately 60% of the dogs in the present study. Most of the affected dogs showed a mild to moderate anaemia. This result is in accordance to the results of a similar study (5). In Central Europe, the major babesia strain found is *Babesia canis canis* (4). This strain seems to induce a mild to moderate anaemia in most clinical cases. The percentage and the severity of anaemia in dogs affected by *Babesia canis canis* increases when the onset of clinical signs occurred more than 24 hours previously (6). The MCV was within the reference range (normocytic) in most of our dogs, which is in accordance with an experimental study (22). MCHC values above the reference range were measured in approximately half of our dogs. A possible reason for increased MCHC may be intravascular haemolysis caused by *Babesia canis*. However, a more common explanation for increased MCHC is a preanalytic one, for example inadequately cooled samples during transport to the laboratory (haemolytic serum). As dogs had normal MCHC values even in the phase of highest parasitaemia (22), the high percentage of increased MCHC in our retrospective study may be due to preanalytic reasons.

Bone marrow normally needs 3–4 days to flush out enough red blood cells into the peripheral blood circulation to compensate anaemia. As dogs in an endemic area are presented to the veterinarian mostly within a few days after clinical symptoms start, most of them do not show any or only a slight regeneration, as in this study. The lack of regeneration in almost all of our dogs (93.1%) is accordingly due to the acute stage of the infection (5, 22).

Leukopenia was the most common finding in the WBC count. In the acute stage of babesiosis, as in other infections, there is often a decrease in leukocytes as a result of a severe consumption, sepsis or possibly a sequestration in the spleen. Leukopenia is well described in early babesiosis. The duration of the leukopenic phase varies between 4 and 28 days (22). Guelfi et al. (6) demonstrated that the level of leukopenia varies with the onset of clinical symptoms. In most cases the leukopenia was consistent with a neutropenia and a lymphopenia. While the prevalence of neutropenia was practically identical, the prevalence of lymphopenia was higher than that described in an experimental infectious study (22). In contrast to the experimental infectious study mentioned above we could not detect eosinophilia in the affected dogs.

Relating to the age and gender distribution there was no significant difference (p = 0.90 and p = 0.97, respectively) between the *Babesia canis*-positive and -negative dogs. Similar results were published in other studies on vector-borne diseases (10, 11).

The results of the CART analysis show that a combination of the appropriate parameters leads to a high predictive value for the presence of canine babesiosis. When including WBC, thrombocytes, and reticulocytes, the CART analysis, already used for decision making in vector-borne disease (17), gives the highest possible predictive value (93.5%) for the discrimination between *Babesia canis*-positive and -negative dogs. In contrast the CART analysis including climate parameters produced a low p-value (61.3%), because veterinarians were partially aware of the seasonal occurrence of babesiosis leading to a biased high rate sampling from April to May and September to November. A high number of blood samples was also submitted in June and July when mean air temperature was > 18 °C and less appropriate for the vector and therefore the probability for the occurrence of canine babesiosis was low (Fig. 5).

As only the adult *D. reticulatus* suck blood from dogs, the seasonal appearance of canine babesiosis in dogs is related to the activity of the adult stage of *D. reticulatus* (2). Seasonal tick activity seems to be strongly related to climate conditions (20), of which changes are easy to observe and measure. In agreement with other authors the typical biseasonal occurrence of canine babesiosis was confirmed by the month of sample submission (12, 14). Analysis of seasonal occurrence of canine babesiosis resulted in two critical periods. On one hand this should lead to major attention of veterinary practitioners in spring when temperature rises above 8 °C and in September when temperature drops below 18 °C. On the other hand single unexpected time frames especially during winter with appropriate climate conditions for the vector should also make the veterinarians aware of the possible occurrence of canine babesiosis (Fig. 5). Comparing both groups in this study, precipitation seemed to be a relevant factor (Table 2) for the activity of the vector and therefore for the occurrence of *Babesia canis* positive cases while relative humidity was not. Nevertheless the knowledge of the climate-dependent occurrence of canine babesiosis is an essential tool when deciding whether to carry out further tests for the specific detection of the pathogen. It is also an important fact, when deciding to apply preventive measures such as vaccination or acaricids (3).

Regarding the sensitivity of *Babesia* detection, PCR is reported to reveal higher sensitivities than the blood smear technique (1). The specificity of the PCR depends on the primers used (4), while detecting *Babesia canis* in blood smears from infected dogs depends on the quality of the smear as well as on the experience of the examiner (18). In cases with low parasitemia or an arguable result of the blood smear examination PCR may be an alternative way of detection, although sensitivity does not reach 100%, costs for the dog’s owners are unequally higher, and until now a rapid in-house...
Conclusion for practice
The main clinicopathological findings in Babesia canis-positive dogs were a moderate to severe thrombocytopenia, a mild to moderate anaemia with almost no signs of regeneration, and a leukopenia with neutropenia and lymphopenia. This study demonstrates a decision guideline with an accuracy of 93.5% on the basis of the results of white blood cell, thrombocyte- and reticulocyte count, which should lead to further testing (blood smear with concentration line) to confirm the tentative diagnosis of canine babesiosis. Climate conditions should be taken into consideration when ranking canine babesiosis in a list of differentials in cases with appropriate clinical signs.

testing by PCR is still not provided. In our study we cannot rule out that single cases with low parasitemia may have been missed, unlikely changing the main results of this study.

Conflict of interest
None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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