

# EHRlichiosis and BABESIOSIS ON THE ISLAND CURAÇAO

## DIAGNOSTICS AND THERAPIES

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## Abstract

Canine monocytic ehrlichiosis, caused by the rickettsia *Ehrlichia canis* is the most known tick-borne disease and is a global problem. Canine babesiosis is a emerging vector-borne disease caused by intraerythrocytic protozoa of the genus *Babesia*. This article considers a three months during research of 71 dogs on the island Curaçao suspected with clinical signs of ehrlichiosis and/or babesiosis. These dogs were shelter dogs or dogs brought to veterinary clinics. The diagnosis of canine babesiosis and ehrlichiosis by clinical signs, complete blood count analyzed by the IDEXX QBC Vet Autoread, Snap® 4 Dx® test, stained bloodsmears, PCR test and RLB analysis methods is reviewed, together with the treatment of these diseases. The dogs exhibited anemia, thrombocytopenia, leucopenia, pancytopenia. Anorexia, lethargy, weight loss, conjunctivitis, severe bleeding tendencies, fever, splenomegaly and pale mucosae were also seen.

In this reasearch all the dogs had clinical signs suspecting them of having ehrlichiosis and/or babesiosis.

Fourty-seven dogs were tested for hematological abnormalities of which 77% had thrombocytopenia, 75% anemia, 52% eosinophilia 15% neutropenia and 13% leucopenia. Out of twenty-seven dogs, 89% of them were positive on the Snap® 4 Dx® test for *E. canis*. Morulae detection showed that in 64 studied dogs, fourteen had Ehrlichia in the buffy coat smears and three had Babesia in bloodsmears. Polymerase chain reaction and reverse line blot testings of hundred-five whole bloodsamples, including 29 bloodsamples after therapy, revealed *Ehrlichia canis*, *Ehrlichia ovina*, *Anaplasma*, *Babesia* and *Theileria*. The prevalence of the *Rhipicephalus sanguineus* ticks, *Ehrlichia canis* and *Babesia vogeli* were respectively 100%, 7,6% and 14,3%.

A treatment with antibiotic doxycycline (5-10 mg/kg/day) was given for three weeks.

If the dog had still clinical symptoms and the platelet count was still low, an additional treatment was given with Imidocard dipropionate 6,6 mg/kg (Imizol®) or two injections given with a 14-day interval or prednisolone-acetate 0,5-4 mg/kg/day (Predisolone®).

All owners were requested to come back after therapy to do a clinical, hematological check-up and a PCR test and RLB analysis. This was performed on 32 out of 71 dogs (45%). Six dogs had additional treatment with doxycycline and one of the six dogs received also prednisolone.

## Introduction

### *Ehrlichiosis*

Canine monocytic ehrlichiosis is a tickborne bacterial infection caused by *Ehrlichia canis*, a gram negative obligate intracellular parasite of monocytes (Shaw *et al.*, 2005; Jongejan, 2001). It is transmitted by the tick *Rhipicephalus sanguineus*, or the brown dog tick, worldwide, but being endemic in (sub-)tropical areas (Overgaauw *et al.*, 2008).

The incubation period time is 8-20 days. Canine monocytic ehrlichiosis can be divided in a acute, subclinical and chronic stage (Stich *et al.*, 2008). The acute phase occurs after 2 to 3 weeks after infection and lasting up for 1 to 4 weeks when most dogs recover by giving adequate therapy. In this phase it can lead to non-specific mild symptoms to severe life-threatening symptoms. Depression, anorexia, weight loss, splenomegaly, petechiae, ecchymoses, epistaxis and lymphadenopathy, thrombocytopenia can result (Shaw *et al.*, 2005).

Dogs in the subclinical phase can become persistent carriers of *E. canis* for months or years. These infected dogs can become healthy again or if the immune system is unable to eliminate the *Ehrlichia* parasite the chronic phase will commence. Polyuria, polydipsia, lameness, neurological disorders, ophthalmic disorders, anorexia, oedema, anemia and other blood disorders can result. The prognosis of chronic ehrlichiosis is guarded. As a result of bleedings and/or secondary infections it can be fatal (Shaw *et al.*, 2005).

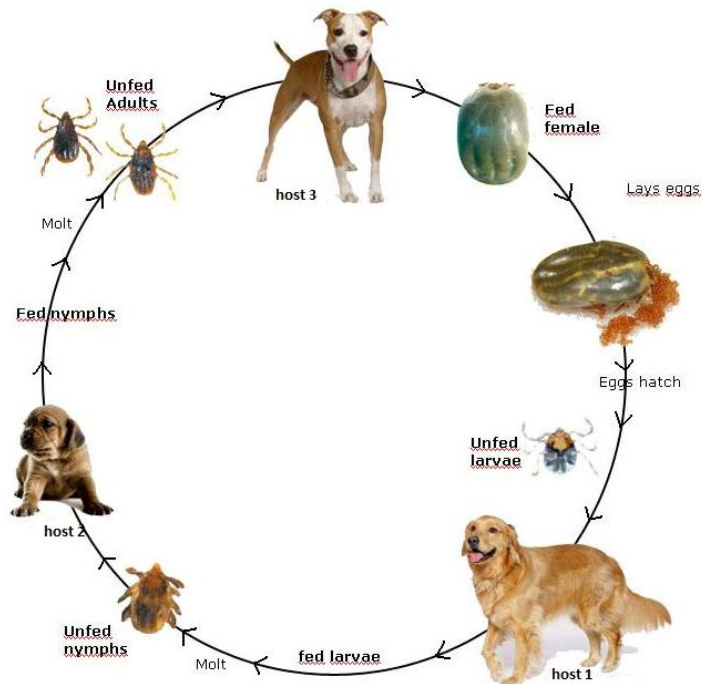
Diagnosis is most commonly achieved by clinical signs and by serologic testing the blood for the presence of antibodies against the parasite *Ehrlichia*. It is an easy and fast method for in the clinic. However the test can be falsely negative in the acute stage because the immune system did not have the time to make antibodies against the infection (Waner *et al.*, 2001; Bélanger *et al.* 2002). In addition a hematology test can show abnormalities in the numbers of erythrocytes, white blood cells and platelets (Tiley *et al.*, 2007). Another way to diagnose Ehrlichiosis in dogs is by looking under the microscope at a stained blood smear of the buffy coat for the presence of morulae in monocytes and/or lymphocytes (Mylonakis *et al.*, 2003). A diagnosis can also be made by a bone marrow aspiration blood smear or a PCR test (Nelson *et al.*, 2003; Tiley *et al.*, 2007).

Treatment that commonly is given to infected dogs is doxycycline (10 mg/kg/day) for up to 3-4 weeks. Clinical improvement can be expected within 24-48 hours by dogs in the acute phase (Neer *et al.* 2002; Overgaauw *et al.*, 2008). The effectiveness of doxycycline seems to be in the subclinical phase less effective than in the acute phase. Imidocarp dipropionate can then also be used or when the level of platelets is so low that the condition is life threatening (Blaauw, 2008). Supportive care can be essential in the form of intravenous fluids given to dehydrated animals and blood transfusion to severely anemic dogs.

*Rhipicephalus sanguineus* is very host specific, because it primarily feeds on dogs. These ticks will attach almost anywhere on a dog, but they are commonly attached on those areas where it is not easily to groom. They are mostly found on the head, neck, in the ears, between the toes and in the axillae. Beside dogs these ticks will also be found on wild- and domesticated animals and humans. In (sub-)tropical areas the tick is active all year round (Estrada-Peña *et al.*, 2004; Dantas-Torres, 2008). The tick is also prevalent in warm houses and dog kennels because *R. sanguineus* is endophilic (Estrada-Peña *et al.*, 2004; Shaw *et al.*, 2005).

There are four active developmental stages in the life cycle of *R. sanguineus*; egg, larvae, nymphs and adults. These ticks need three hosts in one cycle. Every developmental stage feeds once and molt in a sheltered area. The life cycle of *R. Sanguineus* is presented in figure 1. Female adults feed on the host for 5-21 days. When the engorged female is fertilised by a male, she falls off and find a sheltered area near the host to lay her eggs. This is in advantage for the larvae, because after these eggs hatch they can find the host for further development. The mean oviposition period takes 16-18 days where the female lays around 4000 eggs. Both larvae and nimphs feed for 3-10 days on a host. If the environmental temperature is between 20°C to 30°C the life cycle can be completed in 63-91 days (Dantas-Torres, 2008).

The tick can transmit a disease through transstadial and transovarial passage. *E. canis* is transmitted transstadial (through successive life stages).



**Figure 1:** Life cycle of *Rhipicephalus sanguineus*  
Made by A.G. de Mooij

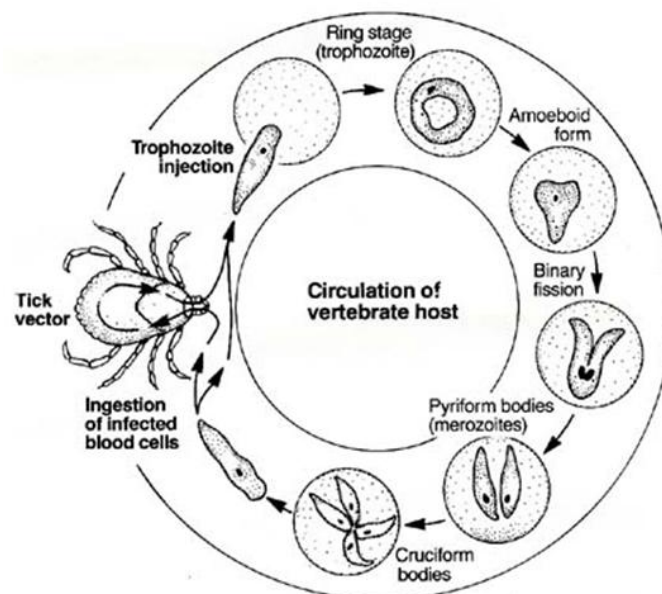
*R. sanguineus* can transmit through transstadial and transovarial passage pathogens. *E. canis* is being transmitted transstadial (Dantas-Torres, 2008).

Beside *E. canis*, *R. sanguineus* can also transmit the pathogens *Babesia canis*, *Rickettsia conorii*, *Dipetalonema dracunculoides* and maybe also *Leishmania infantum*.

## ***Babesiosis***

Babesiosis is a tickborne infection caused by an intraerythrocytic protozoa of the genus *Babesia*. Babesiosis, also called piroplasmosis, is prevalent in domesticated dogs and cats, wild canidae, wild felidae and humans. *Babesia canis* and *Babesia gibsoni* are commonly known organisms to infect dogs. *B. gibsoni* is particularly prevalent throughout Mid-, East-Africa, North-America, Europe and Australia. *B. canis* is found worldwide and three subspecies have been identified. The most pathogen is *B. canis rossii* prevalent in Africa with *Haemaphysalis leachi* as vector. *B. canis canis* has *Dermacentor reticulatus* tick as vector and is found in South-, Mid-, and East-Europe. Throughout North- and South-Africa, North-, East- and South-Africa, Asia and North- and Central Australia the pathogen *B. canis vogeli* is commonly found, which is transmitted by the tick *Rhipicephalus sanguineus* (Nelson *et al.*, 2003; Tilley *et al.*, 2007; De Lange *et al.*, 2005; Yabsley *et al.*, 2008).

Figure 2 reflects the life cycle of *Babesia spp.* An infected tick releases *Babesia spp.* trophozoites into the blood, thereby infecting the erythrocytes where the parasite asexual multiplies. After merozoites hatched out an erythrocyte they come into the circulation where they infect other erythrocytes. Naïve adult ticks become infected with *Babesia spp.* when they ingest a blood meal on the dog (Jongejan, 2001; Overgaauw *et al.*, 2008). Besides transovarial transmission *Babesiosis* is also transmitted transstadial, where the parasite is passed from larvae to nymph to adult stage (De Lange *et al.*, 2005).



**Figure 2:** Life cycle of *Babesia spp.* (Gardiner C.H. *et al.*, 1998)

Dogs can be infected with every stage (larvae, nymph and adult), whereby the female adult plays the biggest part. The parasite will be transmitted after a bloodmeal of 2 to 3 days (Jongejan, 2001; Overgaauw *et al.*, 2008).

Canine babesiosis has a wide variation of clinical signs, ranging from mild anemia to multiple organ failure and death (Shaw *et al.*, 2005). Clinical symptoms can occur peracute, acute or chronic and depend on immunity, age of the dog (peracute form in pups, acute form in young dogs), type of *Babesia* and the degree of contamination. An important cause of the infection is hemolysis which will result in hemoglobinuria, hemoglobinemia, bilirubinemia, bilirubinuria and metabolic acidosis.



Dogs present with the peracute form, especially caused by *B. canis rossi*, is characterized by pale mucous membrane, depression, hemoglobinuria with later neurological symptoms, hypovolemic shock, tachycardia, weak pulse, tachypnoe and death within 1 to 2 days. Dogs in this stage can have fever but are mostly hypotherm. Dogs in the acute form can have fever (41°C), anemia, hepatosplenomegaly, petechiae, ecchymoses, metabolic acidosis, vomiting and sometimes hemoglobinuria with icterus. Dogs typically present with the chronic form of babesiosis will show fever, weight loss, exhaustion, anemia and intermittent anorexia. Carriers will not show any symptoms. Babesiosis can be complicated with an co-infection of *Ehrlichia* spp. en *Bartonella* spp. (Tilley, 2007; Overgaauw *et al.*, 2008; Nelson *et al.*, 2003; Shaw *et al.*, 2005).

Babesiosis is classically, beside clinical symptoms, diagnosed with a stained blood smear demonstrating trophozoites and/or merozoites in erythrocytes (Tilley, 2007; Skotarczak *et al.*, 2007; De lange *et al.*, 2005; Overgaauw *et al.*, 2008; Vet Med Lab, 2007; Shaw *et al.*, 2005). In addition an hematology test can show abnormalities in erythrocytes, platelets, proteins and white blood cells. (Tilley, 2007; Nelson *et al.*, 2003). Other diagnostic test for babesiosis can be made by urinalysis, PCR test and immunofluorescence test (Tilley, 2007; Nelson *et al.*, 2003; Overgaauw *et al.*, 2008; Vet Med Lab, 2007)

Medical treatment that is given to infected dogs is Imidocarb dipropionaat (6,6 mg/kg SC or IM) with the second injection 2 weeks later. Improvement in symptoms will be seen within 1-2 weeks. Hypovolemic animals should get intravenous fluid and very anemic dogs an blood transfusion (Tilley, 2007; De lange *et al.*, 2005; Overgaauw *et al.*, 2008).

Preventive treatment include beside antiparasitic treatment also vaccination with vaccins like Pirodog® en Nobivac Piro®. Vaccination will be given twice with an interval of 3-6 weeks that repeatedly must be given around 6 months. The vaccin will not prevent the infection with *Babesia* spp. but will reduce the severity of the symptoms (Tilley, 2007; De lange *et al.*, 2005; Overgaauw *et al.*, 2008).

## Aim of the study

This three months during study took place on the island Curaçao in the Caribbean Sea. On this island tick-borne diseases and their vectors are a major problem amongst dogs, particularly Canine monocytic ehrlichiosis what is also locally called “Karpattenziekte”.

Recently there has been done scientific researches on Curaçao about the prevalence of the tick *Rhipicephalus sanguineus*, of *Ehrlichia canis* and of *Babesia canis vogeli* on dogs brought to the veterinary clinic of Parera. Beside that there has also been made a start in the prevalence of ticks on eccentric animals. Only the results of the first research are known. In that research a total of sixty dogs had clinical signs suspecting them of having Ehrlichiosis. Fifty dogs were eventually diagnosed with a *E. canis* infection. From forty-two dogs had 76 % hematological abnormalities. Out of fifty-two dogs, 67 % of them were positive on the Snap® 3 Dx® test for *E. canis* (Krogt, 2010).

Also on the island Aruba an similar research was performed. In this study the prevalence of *E. canis* in 100 shelter dogs was investigated which showed that 58% of the dogs were positive on the Snap® 3 Dx® test for *E. canis* and 14% of these dogs were positive for *E. canis* by an PCR test (Straaten, 2008).

The aim of this study is to assess the clinical symptoms caused by Ehrlichiosis and Babesiosis, to get a better view about diagnostics and therapies that local vets practiced and the predispose factors of *E. canis* and *Babesia vogeli* infections on the island Curaçao. This study will include dogs brought to the veterinary clinics and shelter dogs with clinical signs of Ehrlichiosis. Diagnostic research, in the form of blood smears, will also be done on pups from the shelter. This because pups are mostly in the acute fase of an *E. canis* infection so the possibility to detect morulae monocytes and/or lymphocytes is higher.

This research is also for great importance for the Netherlands, since through import of dogs from Curaçao *E. canis* and *B. vogeli* infection can be brought over to the Netherlands. In a research of Blaauw appear that dogs infected with Ehrlichiosis were imported from the island (Blaauw, 2008).

The ticks collected from the dogs and the DNA made out of blood taken from the dogs will be examined at the University of Utrecht to look for pathogens. Thus far the main pathogens that has been found are *E. canis* and *B. vogeli*.

Hopefully to find, with this research information, is a better effective way to eradicate ticks and tick-borne diseases and to improve the health situation of the dogs on the island.



## Materials and methods

### *Study population*

Dogs with symptoms suspecting them of having canine monocytic ehrlichiosis or babesiosis and not yet been treated were included in the study. Most dogs were presented at 6 different cooperating veterinary clinics throughout the island and some were from the animalshelter. A total of 71 dogs were included in this research during 9 weeks between september 2010 and december 2010. Each dog fulfilled at least one of the following three criteria: 1) clinical symptoms of ehrlichiosis or babesiosis; 2) platelet count  $<175 \cdot 10^9/\mu\text{L}$ ; 3) positive on the Snap® 4 Dx® test for *E. canis*.

### *Questionnaires and ticks collection*

Randomly a couple of questions were consulted with owners that came with their pet to the clinics about ehrlichiosis and babesiosis, appendix 1. The general information, like the owners name, age of the dog and breed were acquired from the computer. From the owners of the 71 dogs an anamnesis was consulted and the clinical findings of these dogs were analysed and noted, appendix 2. The dogs were examined for the location of the ticks and the average amount of ticks. A maximum of ten living ticks were collected by hand in labeled sealed cups. These ticks were identified by breed, development stage and on sex if it was an adult. The cups and questionnaires were labeled with an code like in appendix 3. See also the research article of my colleague Lisa Joeglal (Joeglal, 2012). The collected ticks were send to Utrecht Centre for Tick-Borne Diseases in an envelop. There took further research place with a PCR test and Reverse Line Blot analysis to identify the pathogens that these ticks might carry.

### *Blood collection and diagnostic procedures*

Blood samples of 3 ml were collected from the vena cephalica or vena jugularis in 4,5 ml EDTA vacutainers. These vacutainers were labeled with the code of the dog or pup and the date of collection and stored in a refrigerator of 4 °C.

To confirm the suspicion of ehrlichiosis in some cases a Snap® 4 Dx® test and/or hematologic evaluation was performed. Because of financial limits a complete blood count (CBC) analyzed by the IDEXX QBC Vet Autoread was not performed in all 71 dogs. Only if the owners of the dogs were prepared to pay for the CBC test it was accomplished. This is also considered for performing the Snap® 4 Dx® test for *E. canis*. See appendix 4 for the protocol of the Snap® 4 Dx® test. Beside the clinical symptoms, the CBC test and the Snap® 4 Dx® test a peripheral blood smear was made of all 71 dogs. See also appendix 5 for the protocol of making an stained blood smear. At a magnification of 1000x with oil-immersion, the diff quick stained buffy coat smears were screened for morulae in monocytes and/or lymphocytes indicative for *Ehrlichia canis*. Beside making smears of the buffy coat there has also been made smears of the part just beneath the buffy coat for detecting merozoites in erythrocytes indicative for *Babesia* spp. Of every dog two blood smears were made of the buffy coat and two of the part just beneath the buffy coat for extra control. These were labeled with the code of the dog or pup and with an E for *Ehrlichia* or B for *Babesiosis*.

The genomic DNA was extracted from 200 µl of whole blood by using the NucleoSpin® Tissue kit. See appendix 6 for the protocol of DNA extraction. The DNA extractions were sent to Utrecht Centre for Tick-Borne Diseases for further research on the prevalence of ehrlichiose and babesiose with a PCR test and Reverse Line Blot analysis.

### ***Treatment and follow-up***

After the diagnosis a specific treatment with the antibiotic doxycycline (5-10 mg/kg/day) was given for up to 1-3 weeks. The owners were asked to come back after three weeks for a check up. Again a anamnese and physical examination was performed and 3 ml of blood was collected from the vena cephalica or vena jugularis in a 4,5 ml EDTA vacutainer. See also appendix 7 for the follow-up protocol. A complete blood count (CBC) analyzed by the IDEXX QBC Vet Autoread was performed to look if the heamatological abnormalities were changed to normal. If there were still clinical symptoms of ehrlichiosis and/or babesiosis and the platelet count was still low a additional treatment was given to the dog. This could either be lmidocard dipropionate 6,6 mg/kg (Imizol<sup>®</sup>) or two injections given with a 14-day interval or prednisolone-acetate 0,5-4 mg/kg/day (Predisolone<sup>®</sup>). In some cases supportive care was essential in the form of intravenous fluid and/or blood transfusion. Again DNA extractions were made and sent to Utrecht Centre for Tick-Borne Diseases to determine if the parasites were eradicated.

## Results

### Clinical data

This study included 31 female and 40 male dogs with body weights ranging from 1,1 kg to 57,2 kg. The age of these dogs ranged from 4 months to 11 years. The study population consisted of mixed-breed dogs (n=43), Chihuahuas (n=4), Sheperds (n=3), Minipincher (n=1), Dobberman (n=1), Jack Russel (n=1), Cane Rott (n=1), Poedels (n=2), Maltesers (n=2), Rodegian Ridgebacks (n=3), American Bulldog (n=1), Labradors (n=2), Bordeaux Dogs (n=2), Husky (n=1), Filo Brasileiro (n=1), Rottweiler (n=1), Boxer (n=1) and a Welsh Corgi (n=1).

Out of the anamnesis came out that the historical abnormalities included anorexia (n=29), lethargy (n=36), weight loss (n=19) and bleedings/epistaxis (n=27). Both historical and physical abnormalities are summarized in table 1.

**Table 1:** Symptoms of the participated dogs

Symptoms	Numbers of dogs
Anorexia	29
Lethargy	36
Weight loss	19
Conjunctivitis	16
Bleedings/epistaxis	27
Petechiae/ecchymoses	12
Dyspnoea	1
Fever ( $\geq 39,5$ °C)	24
Splenomegaly	17
Pale mucosae	32
Large lymphnodes	41

During this study one dog died shortly after arriving at the clinic, two were euthanized and in another case a dog died with not ehrlichiosis or babesiosis as the underlying cause.

### Laboratory abnormalities

A complete blood count analyzed by the IDEXX QBC Vet Autoread was performed on 47 dogs of the in total 71 dogs. Dogs were diagnosed with anemia if the hematocrit is lower than 37%, thrombocytopenia if the platelet count is lower than  $175 \cdot 10^9 /L$ , leucopenia if the white blood cell count (WBC) is lower than  $6,0 \cdot 10^9 /L$ , neutropenia if the amount of neutrophil granulocytes is lower than  $2,8 \cdot 10^9 /L$  and eosinopenia if the number of eosinophil granulocytes is lower than  $0,5 \cdot 10^9 /L$ .

Hematological abnormalities included anemia in 36 dogs tested in 47 dogs total (77%), neutropenia in 2 dogs tested in 13 dogs total (15%), eosinophilia in 11 dogs tested in 21 dogs total (52%), thrombocytopenia in 37 dogs tested in 47 dogs total (79%), leucopenia in 6 dogs tested in 47 dogs total (13%). Nonregenerative anemia was diagnosed in 11 dogs tested in 18 dogs total (61%) based upon a reticulocyte production index of  $<2$ . See also table 2.

**Table 2:** Percentages of hematological abnormalities before follow up

Hematological abnormalities	Percentages
Anemia	75
Neutropenia	15
Eosinophilia	52
Thrombocytopenia	77
Leucopenia	13
Nonregenerative anemia	61

### Cytopathology

There were made 64 blood smears of the in total 71 dogs participated in the research.

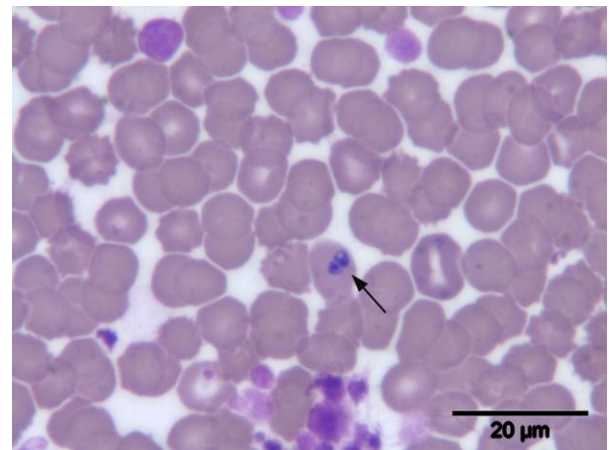
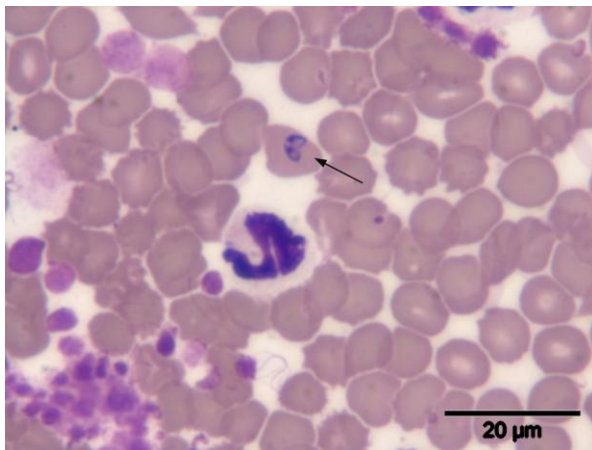
In 14 of the 64 dogs (21,9%) monocytic *Ehrlichia canis* morulae were found in the buffy-coat smears (table 3). Of these 14 dogs, 3 of them were pups from the animal shelter.

In erythrocytes *Babesia spp.* merozoites were visualized in samples obtained from 3 dogs of the 64 dogs (4,7%). Of these 3 dogs, 1 was a pup from the animal shelter.

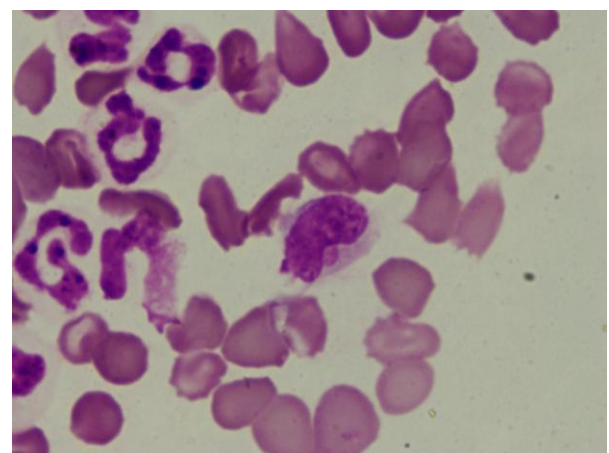
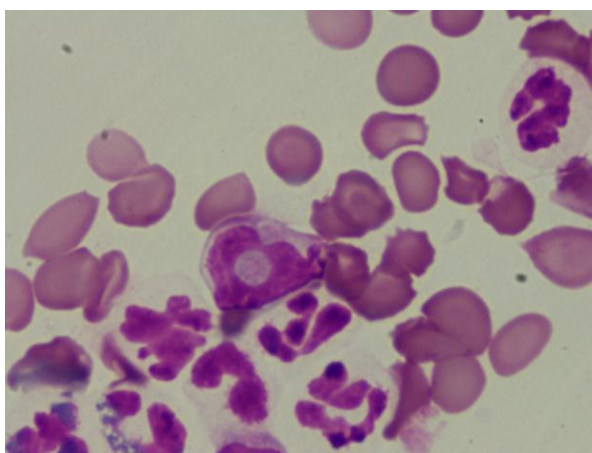
**Table3:** Blood smear results for Ehrlichia and Babesia

	<b>Ehrlichia</b>	<b>Babesia</b>
<b>Total blood smears</b>	64	64
<b>Total negative</b>	50	61
<b>Total positive</b>	14	3
<b>% negative</b>	78,1	95,3
<b>% positive</b>	21,9	4,7

Microscopic pictures of positive blood smears with *Ehrlichia canis* morulae and *Babesia canis* merozoites are in figure 3,4 and 5,6 respectively.



**Figure 3 and 4:** Two microscopic pictures with *Babesia canis* merozoites in erythrocytes.



**Figure 5 and 6:** Two microscopic pictures with *Ehrlichia canis* morulae in monocytes.

## Serology

A Snap® 4 Dx® test for *Ehrlichia canis* was performed on 71 dogs. Of these dogs were twenty-four tested positive (89%), the rest had negative results. Two dogs were also positive for heartworm (*Dirofilaria immitis*), 8 dog were also positive for Anaplasmosis (*Anaplasma phagocytophylium*) and one dog was also positive for Lyme Disease (*Borrelia Burgdorferi*). See also table 4 for the positive results.

**Table 4:** Positive results from Snap® 4 Dx® test on 27 dogs

	Positive results
<i>E. canis</i>	24
<i>D. immitis</i>	2
<i>A.phagocytophylium</i>	8
<i>B. burgdorferi</i>	1

## Polymerase chain reaction and Reverse Line Blot

The extracted DNA from whole blood used by the Nucleospin® Tissue kit were further tested at the Utrecht Centre for Tick-Borne Diseases with a polymerase chain reaction. This resulted in amplification of *Ehrlichia spp.*, *Babesia spp.*, *Anaplasma spp.* and *Theileria spp.*The PCR products were then brought on the reverse line blot for detection of specific pathogenes. In total there were 105 DNA samples collected of which 29 were from the same dogs after a three week during therapy. From the in total 105 DNA bloodsamples were 22 positive for ehrlichiosis or babesiosis (21%), of which 7,6% for *E.canis* and 14,3% for *B. vogeli*. One of the 105 bloodsamples was positive on E/A catch all and there was also found one co-infection. See also table 5 and 6 for the results (Luijten, 2011). Before therapy the prevalence of *E.canis* and *B.vogli* were respectively 10,5% and 14,5%.

Research on the ticks (n=82) did not reveal any positive results with the RLB(Luijten, 2011). (table 5)

**Table 5:** Prevalence of tickborn pathogenes

	Total DNA bloodsamples	Before therapy	After therapy	Ticks
<i>E. canis</i>	7,6% (8/105)	10,5% (8/76)	-	-
<i>B. vogeli</i>	14,3% (15/105)	14,5% (11/76)	13,8% (4/29)	-
<i>E/A catch all</i>	1% (1/105)	1% (1/76)	-	-
<i>T/B catch-all</i>	-	-	-	-
<i>E. canis + B. vogeli</i>	1% (1/105)	1% (1/76)	-	-

**Table 6:** Positive bloodsamples with RLB (black= first sample and red= sample after therapy)

Samples	RLB result
D=dog	
D1~	<i>E/A catch-all, E. canis</i>
D2~	-
D2~	-
D3*	-
D4*	-
D5*	<i>E/A catch-all</i>
D6*	-
D6*	-
D7°	-

D7°	-
D8°	-
D8°	-
D9~	-
D10*	-
D11^	-
D12*	-
D13 <sup>x</sup>	-
D14 <sup>x</sup>	-
D15~	-
D16~	<i>E/A catch-all, E. canis</i>
D17^	-
D17^	-
D18*	<i>B catch-all1, B catch-all2, T/B catch-all</i>
D18*	-
D19*	<i>E/A catch-all, E. canis</i>
D20*	-
D20*	-
D21*	-
D22*	-
D22*	-
D23*	-
D24*	-
D25*	-
D25*	-
D26^	<i>E/A catch-all, E. canis</i>
D27*	-
D28*	-
D29*	-
D30 <sup>A</sup>	<i>E/A catch-all, E. canis</i>
D31 <sup>A</sup>	<i>E/A catch-all, E. canis, T/B catch-all, B catch-all1, B. vogeli</i>
D32*	-
D33*	-
D34*	-
D34*	-
D35*	-
D35*	-
D36*	-
D37*	<i>B catch-all1, B catch-all2, T/B catch-all</i>
D37*	-
D38*	<i>T/B catch-all, B catch-all1, B. vogeli</i>
D38*	<i>T/B catch-all, B catch-all1, B. vogeli</i>
D39*	-
D39*	-
D40 <sup>A</sup>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
D40 <sup>A</sup>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
D41 <sup>A</sup>	-
D42 <sup>A</sup>	-
D43 <sup>A</sup>	<i>T/B catch-all, B catch-all1, B. vogeli</i> <i>T/B catch-all, B catch-all1, B. vogeli</i>



<b>D43<sup>A</sup></b>	
<b>D44*</b>	-
<b>D45*</b>	-
<b>D46<sup>A</sup></b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>D46<sup>A</sup></b>	-
<b>D47<sup>A</sup></b>	-
<b>D47<sup>A</sup></b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>D48*</b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>D49*</b>	-
<b>D50*</b>	<i>E/A catch-all, E. canis</i>
<b>D50*</b>	-
<b>D51<sup>^</sup></b>	-
<b>D52*</b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>D53*</b>	-
<b>D53*</b>	-
<b>D54*</b>	-
<b>D54*</b>	-
<b>D55*</b>	-
<b>D55*</b>	-
<b>D56*</b>	-
<b>D56*</b>	-
<b>D57*</b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>D58<sup>°</sup></b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>D59*</b>	<i>E/A catch-all, E. canis</i>
<b>D59*</b>	-
<b>D60*</b>	-
<b>D60*</b>	-
<b>D61*</b>	-
<b>D62*</b>	-
<b>D63*</b>	-
<b>D63*</b>	-
<b>D64*</b>	-
<b>D64*</b>	-
<b>D65<sup>A</sup></b>	-
<b>D66*</b>	-
<b>D66*</b>	-
<b>D67*</b>	-
<b>D68*</b>	-
<b>D69*</b>	-
<b>D69*</b>	-
<b>D70<sup>°</sup></b>	-
<b>D71~</b>	-
<b>P1<sup>A</sup></b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>P2<sup>A</sup></b>	-
<b>P3<sup>A</sup></b>	-
<b>P4<sup>A</sup></b>	<i>T/B catch-all, B catch-all1, B. vogeli</i>
<b>P5<sup>A</sup></b>	-

## Treatment

Of the 71 studied dogs all were immediately treated with the antibiotic doxycycline hyclate (5-10 mg/kg/day PO) for up to three weeks.

## Follow up

For the check up 3 to 5 weeks after starting with the treatment 32 of the 71 owners (45%) came back with their dog. All these dogs had no clinical signs, except for two dogs who still had pale mucosae.

Bloodsamples were taken from 23 dogs to analyse it for hematological changes. Eosinophilia was detected in 14 dogs (61%), thrombocytopenia in 4 dogs (17%), anemia in 5 dogs (22%) and low hemaglobine in 4 dogs (17%). Of the 23 dogs, 5 dogs recovered completely based on these bloodresults.

In total there were 105 DNA samples collected of which 29 were from the same dogs after a three week during therapy with doxycycline. Four of the bloodsamples were tested positive (13,8%), all on *B. vogeli*. Three of the four were positive before the therapy. One of the samples became after the therapy negative for *B. vogeli* (sample D46<sup>A</sup>) (Luijten, 2011). See also table 6.

Treatment was continued for 6 dogs, because these were still suffering from anemia and/or thrombocytopenia. In one case an Imidocarb dipropionate (6,6 mg/kg, subcutaneously) injections were given in combination with atropine-sulfate (0,03-0,1 mg/kg) to prevent possible side effects, 14 days apart. In four cases Dexamethasone was given per injection (2mg/ml, intravenous) and in one case it was given in a tablet form (0,5 mg/kg/day, per os). One of the 6 dogs received beside the additional 3 weeks treatment with doxycycline, also prednisolone-acetate (5mg/kg/day).

## Discussion

### Clinical data

Some clinical findings of the dogs participated within this research were not completely reliable. This because these dogs have been under anesthesia for an operation. Anesthetic drugs can constrict vessels in the periphery what can cause pale mucosae. This could be a misinterpretation of anemia. Of these dogs the clinical findings were not complete. Still these dogs participated in this research because these were bleeding more during the operation indicating of thrombocytopenia and a candidate for the research on ehrlichiosis and babesiosis.

### Laboratory abnormalities

A complete blood count (CBC) analyzed by the IDEXX QBC Vet Autoread was performed on 47 dogs of the in total 71 dogs. Not all dogs were analyzed for their CBC, because of financial limits.

### Cytopathology

Only 64 bloodsmears were made of the in total 71 dogs participated in this research. This because sometimes the bloodsamples were already thrown away after the complete blood count analysis in the clinic and other times there was not enough blood to make also bloodsmears. Mostly little dogs like chihuahuas were difficult to draw blood from.

Thirty minutes was the maximum time for looking under the microscope to find monocytic *Ehrlichia canis* morulae and/or erythrocytes *Babesia spp.* merozoites per bloodsmear. It is not excluded that this was not enough time to look and to find these parasites, because it is difficult to find them even working with stained bloodsmears. Of the 64 bloodsmears, 14 were positive with *Ehrlichia canis* (21,9%) and 3 for *Babesia canis* (4,7%). The percentages could be much higher if the looking-time was longer, the experience in finding these pathogens was higher and working with a better microscope. In buffy-coat and under the buffy-coat cytopathology, respectively *Ehrlichia* morulae and *Babesia* merozoites were detected in only 14 and 3 cases of the in total 64 dogs, thus lowering its diagnostic value to detect these pathogenes by making bloodsmears. Future studies are required to determine if spleen samples and bone marrow aspiration are more suitable for detecting ehrlichiosis en babesiosis by cytopathology. For ehrlichiosis the low parasitemia could be due to the bacteria remaining longer in the spleen than in the blood, so there is a higher number of morulae in this organ (Harrus, 1999; Faria et al., 2010). Further it is better to make a division between acute, subacute and chronic patients. This because the detection by bloodsmears for both pathogenes are more sensitive in the acute phase of the disease (Mylonakis *et al.*, 2003; Irwin, 2009). In our study we studied naturally infected dogs that could be in any of the three phases of the disease.

Also further diagnostic research with pups are of value, because pups are mostly in the acute fase of an *E. canis* infection so the possibility to detect morulae monocytes and/or lymphocytes is higher. During this study only 5 pups were participating, because during the research no more pups with clinical signs of ehrlichiosis and/or babesiosis were there. This number is not enough to draw conclusions out of these results.

### Serology

Of the in total 27 dogs who were also tested by an Snap® 4 Dx® test where 8 dogs positive for on *A. phagocytophilum* (29,6%). The vector for this pathogen (*Ixodes spp*) has not been detected on Curaçao. The question was if this is because of a crossreaction with *A. platys* or that *R. sanguineus* could be a vector for the transmission of *A. phagocytophilum*. All the ticks of these 8 dogs were with the PCR test and the RLB analysis not tested positive on *A. phagocytophilum*, so a crossreaction seems to be the most logical explanation.

The result of an Snap® 4 Dx® test of one dog was besides *Ehrlichia* and *Anaplasma* also positive for *Borrelia Burgdorferi*. The vector *Ixodes spp* is not yet found on Curaçao so also this could be a crossreaction or *R. sanguineus* can play a part as a vector for the transmission of *Borrelia Burgdorferi*. A second Snap® 4 Dx® test was made but this resulted in a negative result for Lyme. Maybe because the second Snap test was over its expired date. More blood of the dog was needed for further research, but this was at the time not possible because the owner was on vacation.

Ticks were not collected from this dog so a PCR test and a RLB analysis could not explain if a crossreaction was a factor or that the ticks of this dog played a part in transmitting *Borrelia Burgdorferi*.

The high serological result for *E. canis* (88,8%) does not resemble the results of *E. canis* in the ticks (0%). This could be because that the titers for *E. canis* are high for a very long period, up to 16 months, however the pathogene is already eliminated out of the body (Neer *et al.*, 2002). The labtechniques, like DNA-extraction, could also play a role that all of the ticks were tested negative for *E. canis*.

#### **DNA extraction**

The extracted DNA from whole blood used by the Nucleospin® Tissue kit was made by the procedures, but sterility management and the travel conditions to Utrecht could play a part in inconsequent results out of the PCR and RLB.

#### **Polymerase chain reaction (PCR) and reverse line blot (RLB)**

Research on all the ticks (82) did not reveal any positive results on *E. canis* or any other pathogen with the RLB. This could be because the ticks were free of pathogens. Another explanation is that these ticks were tested on an old mebrane what gave less and inconsequent results. Also the *Ehrlichia* primers were not working well.

Because all the dogs were suspecting of having *E. canis*, based on clinical signs, blood results and/or a positive *E. canis* Snap® 4 Dx® test, there were a relatively low *E. canis* positive bloodsamples (7,6%). A negative bloodsample will not mean that the dog is not infected. The relatively low *E. canis* positive bloodsamples might be caused by troubles with obtaining, conserving or transporting the test materials or with the PCR and RLB tests. Possibly the DNA-extractions were not done correctly or not stable enough for transport from Curaçao to the Netherlands, but on the other hand the DNA extractions of the ticks were done in Utrecht following a well-known procedure and also gave less than expected for *E. canis* positive results. Another anomalie may suggest that the PCR test and RLB analysis have false negative results as a result of low parasitemia, what also is reported by Harrus *et al.* (2004). Another explanation for the low *E. canis* positive bloodsamples is that the clinical symptoms of both ehrlichiosis and babesiosis are very similar. Also a co-infection was detected with *E. canis* and *B. vogeli* (sample D31<sup>A</sup>), what often occure (Schoeman, 2009). The presence of another on Curaçao yet unidentified pathogen susceptible for treatment with doxycyclin is another possible scenario.

A sensitiver way to detect *E. canis* is to make stained bloodsmears from the spleen, lymph node or from a bonemarrow aspiration (Faria *et al.*, 2010; Harrus, 1999; Mylonakis *et al.*, 2003). Future studies are required to determine if tissues like bone marrow, lymph nodes, spleen and lung are more suitable then bloodsamples for PCR testing and RLB analysis.

### **Treatment**

Of the 71 studied dogs, 27 dogs were treated with Doxycycline (5-10 mg/kg/day PO) for up to three weeks. Depending in which stage the dog is in, this therapy can cure the patiënt.

Doxycycline (6 mg/kg/day, twice daily, PO) gives 100% clearance of *E.canis* if given for two weeks in the acute phase (Breitschwerdt *et al.*, 1998). In the subclinical phase doxycycline (10 mg/kg/day) gives 75% clearance of *E. canis* after six weeks (Harrus *et al.*, 1998). Dogs within the chronic phase if treated with doxycycline ( 5 mg/kg/day, twice daily) gives 100% clearance of *E. canis* after three weeks (Eddlestone *et al.*, 2009). So the treatment for the dogs in the subclinical phase is not always sufficient.

In one case an Imidocarb dipropionate (6,6 mg/kg, SC) injections were given in combination with atropine-sulfate (0,03-0,1 mg/kg) to prevent possible side effects, 14 days apart. This gives no clearance of *E. canis* but it does for *Babesia spp.* (Eddlestone *et al.*, 2009).

### **Follow up**

Three to five weeks after starting with the treatment 32 of the 71 owners (45%) came back with their dog. This is a low number, especially if 6 dogs needed prolonged and/or additional treatment. This means that dogs that did not return to the clinic for a check-up could still be infected. So in the future the owners should be more warned of the fact that their dog could still be infected, especially when in the subclinical phase, and that a check-up based on clinical examination and a blood analysis is necessary to exlude or to confirm it.

## Conclusions

Based on this study, patients with canine chronic ehrlichiosis caused by *Ehrlichia canis* and/or babesiosis caused by *Babesia canis* were associated with anorexia, lethargy, pale mucosae, bleedings/epistaxis, large lymph nodes, fever, splenomegaly, conjunctivitis and petechiae/ecchymoses.

Hematological abnormalities often found are thrombocytopenia (77%), anemia (75%) and eosinophilia (52%). These abnormalities are not pathognomonic for ehrlichiosis, so a definitive diagnosis can not be made only by these results.

Cytopathology takes a lot of time and experience to find monocytic *Ehrlichia canis* morulae found in the buffy-coat smears and *Babesia spp.* merozoites in erythrocytes. Only 21,9% positive results for finding *E. canis* and 4,6% for finding *B. spp.* in bloodsmears. This concludes that the sensitivity is very low and that this diagnostic procedure can not be used alone to make a definitive diagnosis for ehrlichiosis or babesiosis. Future studies are required to determine if spleen samples and bone marrow aspiration are more suitable for detecting ehrlichiosis and babesiosis by cytopathology. Further it is better to make a division between acute, subacute and chronic patients. This because the detection by bloodsmears for both pathogens are more sensitive in the acute phase of the disease. More research should be done to conclude if cytopathology is more useful for pups to diagnose ehrlichiosis, because pups are mostly in the acute phase of an *E. canis* infection so the possibility to detect morulae monocytes and/or lymphocytes is higher. In this research only 5 pups were participating, because no more were there during the research.

The sensitivity of the Snap® 4 Dx® test is 96.2%, meaning that there are dogs tested false negative. Results of whole blood PCR and RLB seemed to be less sensitive as expected by reflecting the results of the cytopathology and serology. Future studies are required to determine if tissues like bone marrow, lymph nodes, spleen and lung are more suitable than whole blood samples for PCR testing and RLB analysis. Although the PCR test and the RLB analysis are highly sensitive and specific, it should not be the only diagnostic test. A definitive diagnosis for canine ehrlichiosis or babesiosis should be done by more than one test, combined with clinical signs, symptoms, complete blood count, and/or an Snap® 4 Dx® test and making stained bloodsmears.

This is the first research that confirmed the presence of *B. vogeli* in the blood of dogs from Curaçao by bloodsmears and PCR and RLB. Before this intraerythrocytic protozoa has not been detected diagnostically on the island. However it was already discovered on Aruba with a prevalence of 12,8% (Moreta, 2009). *B. vogeli* plays an important part because it has similar symptoms like ehrlichiosis. Also a co-infection of *E. canis* and *B. vogeli* was confirmed.

Dogs diagnosed with ehrlichiosis were treated with doxycycline hyclate (5-10 mg/kg/day, PO) for a period of 3 weeks. It is important that the patient returns after the treatment for a check-up, because some dogs can not completely clear *E. canis* from its blood or it was an infection with *Babesia spp.* Treatment then needs to be prolonged or changed. During this research 45% came back for a check-up. This number can go higher if the owners are well posted about this by beginning the treatment.



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## Appendices

### Appendix I

#### Vragenlijst diereigenaar

Kliniek: \_\_\_\_\_

Datum: \_\_\_\_\_

Naam eigenaar: \_\_\_\_\_

Telefoonnummer: \_\_\_\_\_

Adres: \_\_\_\_\_

Naam hond: \_\_\_\_\_

Geslacht: M / V

Leeftijd: \_\_\_\_\_

Ras: \_\_\_\_\_

Vachttype: Lang harig / Medium-lang harig / Kort harig

Vachtkleur: Donker/ Licht / Gevlekt

Reden van bezoek: \_\_\_\_\_

Bent u bekend met de Karpattenziekte? Ja / Nee

Lijdt uw hond hieraan of heeft uw hond hieraan geleden? Ja / Nee

Indien Ja:

- Hoe lang is dit geleden? \_\_\_\_\_
- Wat is u opgevallen aan de hond waardoor u naar de dierenarts bent gegaan? \_\_\_\_\_
- Wat merkt u nu nog aan uw hond? \_\_\_\_\_
- Hoe is de diagnose gesteld? \_\_\_\_\_
- Wat voor behandeling is ingesteld? \_\_\_\_\_
- Heeft u het idee of de behandeling effectief was? \_\_\_\_\_

Bent u bekend met Babesiose? Ja / Nee

Lijdt uw hond hieraan of heeft uw hond hieraan geleden? Ja / Nee

Indien Ja:

- Hoe lang is dit geleden? \_\_\_\_\_
- Wat is u opgevallen aan de hond waardoor u naar de dierenarts bent gegaan? \_\_\_\_\_
- Wat merkt u nu nog aan uw hond? \_\_\_\_\_
- Hoe is de diagnose gesteld? \_\_\_\_\_
- Wat voor behandeling is ingesteld? \_\_\_\_\_
- Heeft u het idee of de behandeling effectief was? \_\_\_\_\_

Heeft u nog meer dieren zo ja, wat voor en hoeveel?

Hebben deze dieren ooit Karpattenziekte gehad? Ja/ Nee

Doet u aan tekenbestrijding? Ja / Nee

Waar?:

- Dierenarts       (Dieren)Winkel

Welk middel?:

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> Frontline druppels | <input type="checkbox"/> Poeder              | <input type="checkbox"/> Preventic halsband |
| <input type="checkbox"/> Frontline spray    | <input type="checkbox"/> Ovitrol             | <input type="checkbox"/> Shampoo            |
| <input type="checkbox"/> Paramite           | <input type="checkbox"/> Ivomectine injectie | <input type="checkbox"/> Anders: _____      |

Hoe vaak?: \_\_\_\_\_

Bent u tevreden met de producten? Ja / Nee

Zo nee, waarom niet? \_\_\_\_\_

Verwijdert u de teken van uw hond? Ja / Nee

Hoe vaak doet u dit? \_\_\_\_\_

Kunt u een inschatting geven van het aantal teken per keer? \_\_\_\_\_

Bestrijdt u ook teken in de omgeving (pest control)? Ja / Nee

Zo ja, waar, waarmee en hoe? \_\_\_\_\_

Leeft en slaapt u hond binnenshuis of buitenshuis?

Binnen / Binnen + Buiten / Buiten / Kennel / Ketting / Los

Indien buiten, op welk type grond? Zand / Steentjes / Gras / Beton

Heeft u uw hond wel eens meegenomen naar het buitenland? Ja / Nee

Zo ja, waar naar toe? \_\_\_\_\_

**Bedankt voor uw medewerking!**

## Appendix II

### Gegevens hond, teken en bloed

Naam kliniek: \_\_\_\_\_

Datum bezoek: \_\_\_\_\_

Naam eigenaar \_\_\_\_\_

Naam hond + gewicht: \_\_\_\_\_

Geslacht hond: \_\_\_\_\_

Leeftijd: \_\_\_\_\_

Ras: \_\_\_\_\_

- Algemene indruk hond: \_\_\_\_\_  
\_\_\_\_\_

- Bevindingen na onderzoek:

Symptomen	Wel/niet aanwezig
Anorexie	
Lethargisch	
Gewichtsverlies	
Conjunctivitis	
Vergrote lymfeknopen	
Bloedingen/epistaxis	
Petechiën/ ecchymoses	
Dyspnoe	
Koorts ( $\geq 39,5$ °C)	
Splenomegalie	
Bleke slijmvliezen	
Overig	

+ = wel aanwezig

- = niet aanwezig

- Aantal teken: 0 / <10 / 10-30 / 30-50 / >50  
globale locatie:  
\_\_\_\_\_

- Uitslag Snap® 4Dx® test *E. canis* :  Positief  Negatief



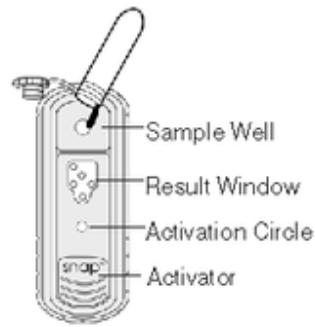
- Uitslag Algemeen Bloed Onderzoek:

	Resultaten
Hematocriet	
Hemoglobine	
WBC	
Granulocyten	
% granulocyten	
Neutrofielen	
Eosinofielen	
Trombocyten	
Reticulocyten	
Lymfocyten/monocyten	
% lymfocyten/monocyten	
MCHC (hemoglobine)	

- Eventueel ingestelde therapie tegen Karpattenziekte/Babesiose inclusief dosering:

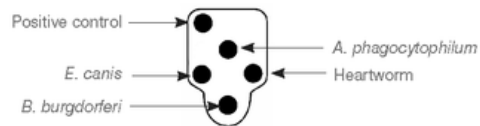
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Figuur 1: SNAP-test instrument

**Positieve resultaten:**



**Negatieve resultaten:**



**Ongeldige resultaten:**

- **Achtergrond:** Indien het monster voorbij de activeercirkel is gestroomd kan er een achtergrondkleur ontstaan. Een beetje is normaal, maar indien het de testresultaat onduidelijk maakt, moet de test worden herhaald.
- **Geen kleurontwikkeling:** Indien positieve controle geen kleur ontwikkelt dan test herhalen

- In serum, plasma of vol bloed detectie van:
  - *Dirofilaria immitis* antigeen
  - *Anaplasma phagocytophilum* antistoffen
  - *Borrelia burgdorferi* antistoffen
  - *Ehrlichia canis* antistoffen
- Serum, plasma of ontstold vol bloed (EDTA, heparine) gebruiken, hetzij vers of **maximaal 1 week oud indien bij 2°-8°C bewaard. Voor langere bewaring serum en plasma invriezen bij een temperatuur van -20°C of lager waarna het opnieuw moet worden gecentrifugeerd voor gebruik.**

## Appendix V

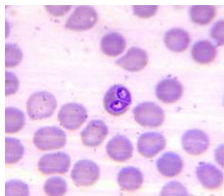
### Protocol bloeduitstrijkje + Diff Quick kleuring

#### Benodigdheden voor een bloeduitstrijkje:

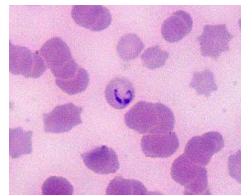
- 5ml. spuit + naald passend bij het dier
- Volbloed in een 4,5 ml. EDTA buisje
- 2x objectglazen
- 1x dekglasje
- Binoculaire lichtmicroscop
- Pincet of handschoenen
- Laboratorium jas
- Stopwatch
- Kraanwater
- Erlenmeyer
- Diff-Quick kleuring

#### Serum voor detectie Babesiose:

1. Bloedafname van v. saphena of v. jugularis in een EDTA buisje van 4,5 ml.
2. Volbloed circa 30 minuten laten staan, waardoor zich een buffy coat vormt
3. Prepareerglasje coderen met code van hond/pup en een B van Babesiose
4. Met een pipet bloed afnemen van net onder de buffy coat
5. De inhoud van de pipet op een schoon, ontvet objectglas pipetteren in een druppel
6. Plaats een objectglas voor de druppel in een hoek van 30-40°
7. Raak met het objectglas de druppel aan zodat de bloeddruppel zich verspreid over het oppervlak en maak daarna een vloeiende beweging naar links zodat een vlamvormig bloeduitstrijkje ontstaat
8. Het objectglas met het preparaat circa 15 min. drogen aan de lucht
9. Bloeduitstrijkje kleuren:
  - a. 5x 1 sec. in groen potje dopen met pincet of handschoenen  
(Fixatie vloeistof; triphenylmethaan in 100% methanol)
  - b. 5x 1 sec. in rood potje dopen met pincet of handschoenen  
(Eosinofiele kleuring; xantheen kleurstof in natrium azide 0.01%)
  - c. 5x 1 sec. in blauw potje dopen met pincet of handschoenen  
(Basofiele kleuring; thiazine kleurstof in natrium azide 0.01%)
10. Gekleurde bloeduitstrijkje spoelen met kraanwater uit een erlenmeyer
11. Het objectglas met het preparaat circa 15 min. drogen aan de lucht
12. Dekglasje op het bloeduitstrijkje
13. Minimaal 20 min. bekijken onder een binoculaire lichtmicroscop bij een vergroting van 1000x. Het bloeduitstrijkje bekijken van boven naar beneden en van links naar rechts



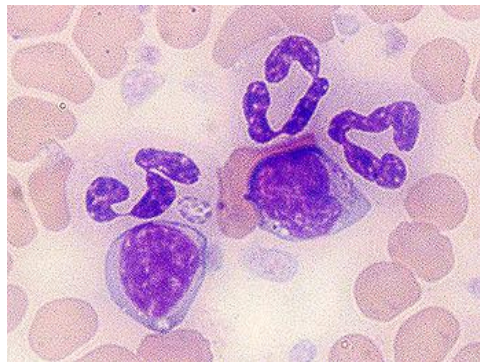
Twee *Babesia* sp. trophozoten in een bloeduitstrijkje van een geïnfecteerde hond.



*Babesia caballi* in een erythrocyt van een paard.

### Buffy coat voor detectie Ehrlichia canis:

1. Bloedafname van v. saphena of v. jugularis in een EDTA buisje van 4,5 ml.
2. Bloed circa 30 minuten laten staan, waardoor zich een buffy coat vormt
3. Prepareerglaasje coderen met code hond/pup en een E van Ehrlichia canis
4. Met een pipet op 13 µl de buffy coat opzuigen
5. De inhoud van de pipet op een schoon, ontvet objectglas pipetteren in een druppel
6. Plaats een objectglas voor de druppel in een hoek van 30-40°
7. Raak met het objectglas de druppel aan zodat de bloeddruppel zich verspreid over het oppervlak en maak daarna een vloeiende beweging naar links zodat een vlamvormig bloeditrijkje ontstaat
8. Het objectglas met het preparaat circa 15 min. drogen aan de lucht
9. Buffy coat uitstrijkje kleuren:
  - a. 5x 1 sec. in groen potje dopen met pincet of handschoenen  
(Fixatie vloeistof; triphenylmethaan in 100% methanol)
  - b. 5x 1 sec. in rood potje dopen met pincet of handschoenen  
(Eosinofiele kleuring; xanthene kleurstof in natrium azide 0.01%)
  - c. 5x 1 sec. in blauw potje dopen met pincet of handschoenen  
(Basofiele kleuring; thiazine kleurstof in natrium azide 0.01%)
10. Gekleurde buffy coat uitstrijkje spoelen met kraanwater uit een erlenmeyer
11. Het objectglas met het preparaat circa 15 min. drogen aan de lucht
12. Dekglasje op het bloeditrijkje
13. Minimaal 20 min. bekijken onder een binoculaire lichtmicroscop bij een vergroting van 1000x. Het bloeditrijkje bekijken van boven naar beneden en van links naar rechts



Buffy coat uitstrijkje van een hond met Ehrlichiosis.  
Er zijn onder andere drie segmentkernige neutrofielen te zien, waarvan één een grijs braamachtig morula bevat.

#### Resultaat van de kleuring:

erythrocyten	roze tot geelrood
trombocyten	violet met paarse granula
neutrofielen	kernen blauw, cytoplasma roze, granula violet
eosinofielen	kernen blauw, cytoplasma blauw, granula rood
basofielen	kernen paars tot donkerblauw, granula violet
monocyten	kernen violet, cytoplasma lichtblauw
Bacteriën	blauw

## Appendix VI

### DNA-extractie doen op de bloedmonsters in het laboratorium

1. Benodigheden:
  - Laboratoriumjas
  - Handschoenen
  - Veiligheidsbril
  - NucleoSpin® Tissue kit voor 250 preps
  - 96-100% ethanol
  - 1,5ml microcentrifuge epjes (voor in eppendorf centrifuge)
  - Rekje voor microcentrifuge epjes
  - 1000µl en 100µl pipet met disposable punten
  - Afvalbak voor pipetpunten
  - Afvalbak voor vloeibare stoffen
  - Maatbekers 10ml en 200 ml
  - Eppendorf centrifuge 5415 D
  - Vortex mixer
  - Hitteblok tot minimaal 70 graden Celsius
  - Koelkast +4 graden Celsius
  - Vriezer ten minste -18 graden Celsius
  - Transport koelbox (+4 graden Celsius)
  - Bloedmonsters
  - Thermometer
  - Labjournaal
  - Transportdoosje (10x10)
  - Watervaste markers (zwart + rood)
2. Trek een laboratoriumjas aan
3. Pak het labjournaal en houdt deze bij.
4. Zet de NucleoSpin® Tissue kit klaar op een lege en schone tafel in het laboratorium.
5. Controleer of de B3 buffer, de B5 buffer (in de kit) en Proteinase K (in de vriezer in het laboratorium) bereid zijn.
  - Zo ja: controleer de datum op de buffers
    - Buffer B3 is tot 1 jaar houdbaar na bereidingsdatum op kamertemperatuur (18-25 graden Celsius)
    - Buffer B5 is tot 1 jaar houdbaar na bereidingsdatum op kamertemperatuur
    - Proteinase K is tot 6 maanden houdbaar in de vriezer bij -4 graden Celsius
    - Wanneer al het bovenstaande nog houdbaar is ga verder met stap 3. Zo nee, overleg met begeleider om bij te bestellen.
  - Zo nee: de buffers moeten eerst klaargemaakt worden. De benodigde stoffen voor de buffers zitten in de kit. Deze moeten eerst nog geactiveerd worden.
    - *Buffer B3 bereiden*
      - Zet een veiligheidsbril op en doe handschoenen aan (in verband met guanidine hydrochloride in buffer 1: irriterend voor de ogen de huid en bij inslikken)
      - Haal buffer B1 en buffer B2 uit de kit en zet ze op tafel.
      - op een sticker ‘‘buffer B3 + datum van vandaag’’ en plak deze sticker op een leeg potje uit de kit
      - Open de potjes met buffer B1, buffer B2 en het lege potje uit de kit
      - Giet voorzichtig de totale inhoud van buffer B1 en buffer B2 in het potje ‘‘buffer B3’’, draai de dop erop en mix de oplossing goed. Buffer B3 is klaar.

- *Buffer B5 bereiden:*
    - Haal de twee potjes met ‘‘Wash buffer B5 concentrate’’ uit de NucleoSpin® Tissue kit.
    - Pak de 200 ml maatbeker en vul deze met 160 ml ethanol.
    - Voeg de ethanol toe aan een potje de ‘‘Wash buffer B5 concentrate’’.
    - Zet een kruisje rechts onder op het label om aan te geven dat de ethanol is toegevoegd. Schrijf ook de datum van bereiding op.
    - Voeg op een zelfde wijze bij het andere ‘‘Wash buffer B5 concentrate’’ potje 160 ml ethanol toe en bewerk het label op een zelfde wijze.
  - *Proteinase K bereiden:*
    - Haal de twee flesjes proteinase K uit de NucleoSpin® Tissue kit.
    - Haal de proteinase buffer PB uit de verpakking.
    - Pak de 10 ml maatbeker en vul deze met 3,35 ml Proteinase buffer PB
    - Zet de bereidingsdatum op het label
    - Berg het flesje met de proteinase K oplossing op in de vriezer op -4 graden Celsius
6. Warmtebad
    - Open het deksel en controleer of het waterbad voldoende gevuld is tot ca. 5 cm boven het rooster.
    - Plaats een rekje in het warmtebad en plaats hierin buffer BE.
    - Sluit het deksel.
    - Zet het warmtebad aan en stel in op 70 graden Celsius.
  7. Het bloedmonster moet gelyseerd worden. Dit wordt gedaan met behulp van proteinase K welke de eiwitten in het bloedmonster lyseert.
    - Gebruik de pipetten om gedurende de volgende stappen oplossingen bij elkaar toe te voegen. Zie bijlage 1 voor welke pipet nodig is voor welke hoeveelheid.
    - Zwenk het EDTA buisje met volbloed 5x
    - Pak de administratie-ordner en schrijf op het overzichtsblad welke plek elke DNA-extractie in het transportdoosje gaat krijgen + de nieuwe batchcode (A t/m ...). Voeg deze batchcode aan de diercode toe op het overzichtsblad.
    - Zet voor elk bloedmonster een 1,5 ml microcentrifuge epje klaar en schrijf met een watervaste stift
    - Pipeteer 200µl bloed in een 1,5 ml microcentrifuge epje
    - Haal de proteinase K uit de vriezer en voeg hiervan 25 µl aan het bloed in het epje toe.
    - Zet de proteinase K weer terug in de vriezer.
    - Voeg 200 µl Buffer B3 toe.
    - Vortex de oplossing 20 seconden.
    - Plaats het epje in het rekje en laat 5 minuten bij kamertemperatuur staan.
    - Vortex 10 seconden
    - Controleer of het warmtebad 70 graden Celsius is met de thermometer.
    - Zo nee, wacht even. Zo ja, plaats het monster in het warmtebad.
    - Haal de monsters na 20 minuten uit het waterbad.
  8. Veranderen van DNA binding condities (zorgen dat DNA onoplosbaar wordt)
    - Voeg 210 µl ethanol toe
    - Vortex het monster 10 seconden
  9. Het DNA binden aan desilicamembraan van de NucleoSpin® Tissue kolom
    - Neem een NucleoSpin® Tissue kolom geplaatst in een opvangbuisje uit de NucleoSpin®kit.
    - Pipeteer het monster uit het 1,5 ml epje in de NucleoSpin® Tissue kolom.
    - Zet de centrifuge aan en stel in op 2 minuten en 11,000 x g met de draaiknoppen.

- Plaats het monster in de centrifuge. Is er een even aantal monsters zorg dan dat ze gelijk verdeeld zijn in de centrifuge. Is er een oneven aantal monsters zorg dan dat je een leeg monster vult met water met hetzelfde gewicht om een gelijke verdeling te kunnen krijgen.
  - Sluit het deksel en druk op start
  - Haal de monsters uit de centrifuge. Controleer of de vloeistof volledig door de matrix is gegaan (alle vloeistof moet onderin het buisje zitten).
    - Wanneer bij één of meerdere monsters de vloeistof niet volledig door de matrix is gegaan dan moet gecentrifugeerd worden 2 minuten op 14,000 x *g*. Deze stap moet herhaald worden tot wel alle vloeistof door de matrix heen is gegaan.
  - Til de NucleoSpin® Tissue kolom uit het opvangbuisje. Gooi van elk monster het opvangbuisje in de afvalbak voor vloeistoffen.
  - Plaats een nieuw opvangbuisje onder elk monster.
10. Het wassen van het silicamembraan
- Eerste wasstap:
    - Voeg 500 µl van Buffer BW toe
    - Centrifugeer 1 minuut op 11,000 x *g*
    - Til de NucleoSpin® Tissue kolom uit het opvangbuisje.
    - Giet het doorgezeefde materiaal in het opvangbuisje in de afvalbak voor vloeistoffen.
    - plaats de NucleoSpin® Tissue kolom terug in het opvangbuisje
  - Tweede wasstap
    - Voeg 600 µl buffer B5 in de NucleoSpin® Tissue kolom
    - Centrifugeer 1 minuut op 11,000 x *g*
    - Giet het doorgezeefde materiaal in het opvangbuisje in de afvalbak voor vloeistoffen.
    - Plaats de NucleoSpin® Tissue kolom terug in het opvangbuisje
11. Het overige ethanol verwijderen van het silica membraan
- Centrifugeer de kolom voor 1 minuut op 11,000 x *g*
12. Uitwassen van hoge kwaliteit DNA
- Plaats de NucleoSpin® Tissue kolom in een 1,5 ml microcentrifuge epje
  - Haal buffer BE uit het warmtebad van 70 graden Celsius
  - Voeg 100 µl voorverwarmde buffer BE toe aan het monster
  - Laat het monster staan bij kamertemperatuur gedurende 1 minuut
  - Centrifugeer 1 minuut op 11,000 x *g*
13. Plaats het monster in een transportdoosje op de juiste plek
14. Plaats het transportdoosje in een koelbox
15. Rij naar de bloedbank en plaats transportdoosje met plattegrond in -80 graden Celsius vriezer, zie bijlage 7.



## Appendix VII

### Follow-up na ingestelde behandeling

- Veranderingen verschijnselen dier:

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- Gebruikte therapie en therapietrouw:

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- Effecten therapie volgens eigenaar: \_\_\_\_\_

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- 3 ml bloed afnemen voor Algemeen Bloed onderzoek en de rest op laten halen of opsturen naar dierenkliniek Parera.

	Resultaten
Hematocriet	
Hemoglobine	
WBC	
Granulocyten	
% granulocyten	
Neutrofielen	
Eosinofielen	
Trombocyten	
Reticulocyten	
Lymfocyten/monocyten	
% lymfocyten/monocyten	
MCHC (hemoglobine)	