

***Ehrlichia canis* infections on the island of Aruba**



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Abstract

During a ten week period dogs in Aruba were examined for *Ehrlichia canis* infections. These were dogs that came to the veterinary clinic as well as neglected dogs in the kennel of the veterinary services. The clinical signs were described, ticks were removed, a blood sample was taken and the dog owners filled in a questionnaire. This way a database of the dog population infected with *Ehrlichia canis* was made and the problem of canine ehrlichiosis on the island of Aruba was surveyed. Most common clinical sign was pale mucous membranes, but lethargy, emaciation and fever were also often seen. The clinical signs are however non-specific and in the participating dogs there seems to be no relation between the clinical signs and being actually infected. The hematocrit was determined for 87 dogs in the veterinary clinic. Sixty-three of these dogs turned out to be anemic (72%). In the kennel of the veterinary 31 of the 32 dogs (97%) turned out to be anemic. The Snap 4Dx test was performed on 59 dogs in the veterinary clinic, with a positive test result for *Ehrlichia canis* for 36 dogs (61%). For heartworm (*Dirofilaria immitis*), *Anaplasma phagocytophilum* and *Borrelia Burgdorferi* this was respectively 27%, 27% and 0%. An examination of the buffycoat smears was performed. In dogs from the veterinary clinic, in 11 of 86 (13%) morulae were found. In the dogs from the kennel of the veterinary services this was 6 of 32 (19%). The high prevalence of *E.canis* and heartworm form a potential risk of importing these diseases into the Netherlands. More research is required on the tick-borne diseases on Aruba and on the ticks that were sent to the Netherlands.

Introduction

Ehrlichia canis is an obligate intracellular bacterium that belongs to the family of Rickettsiaceae and lives in so called morulae in the monocytes and macrophages of a host. The bacteria causes in dogs a systemic disease known as 'monocytic ehrlichiosis' or 'canine ehrlichiosis'. After an incubation period that lasts up to 3 weeks, *E.canis* infection causes an acute, subclinical or chronic phase of the disease. The disease is in the acute phase characterized by a vasculitis that leads to all kinds of symptoms like fever, mild thrombocytopenia, petechial haemorrhages, leukopenia, anemia, lethargy, anorexia, generalised lymphadenopathy, serous to mucopurulent nose- and eye discharge and sometimes dyspnea. The acute phase, that can last for 2-4 weeks, is followed by a subclinical phase that can last for months until years. The blood values do stay low (thrombocytopenia and anemia) but the clinical symptoms are minimal. A minority of the dogs will develop a severe form of the disease later on, when the immune system did not succeed to overcome the *E.canis* infection. This chronic phase is characterized by e.g. depression, pancytopenia, persistent bonemarrow depression, along with bleedings, pale mucous membranes, neurological disturbances and emaciation. This phase often leads to death. [1,2]

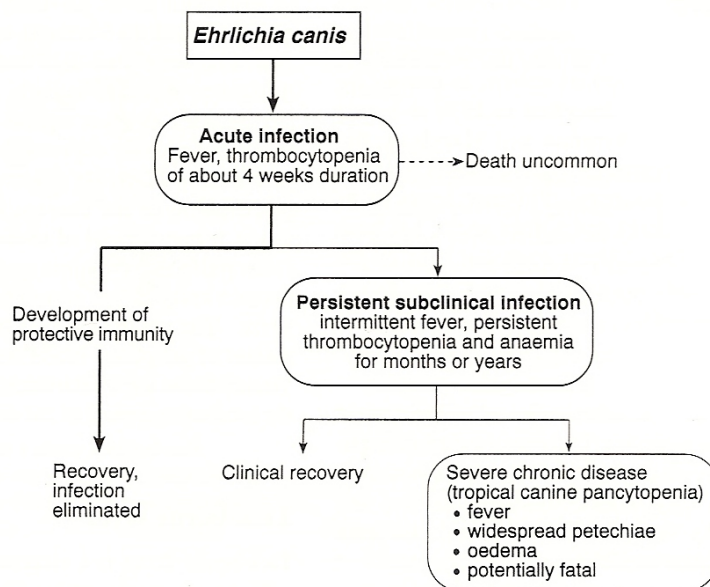
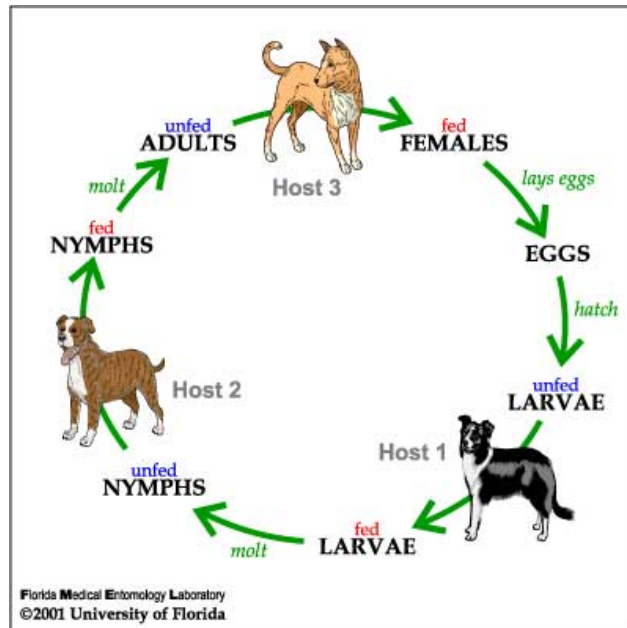


Figure 1. Possible outcomes of an infection with *Ehrlichia canis*
Source: Veterinary Microbiology and Microbial Disease [2]

Ehrlichia canis persists by passage via ticks on dogs. *E.canis* is not transmitted transovarially in ticks, so non-exposed ticks have to feed on infected dogs in the acute phase to continue the disease. [1] The tick *Rhipicephalus sanguineus* (also called the 'brown dog tick') is the vector of *E.canis*. This tick belongs to the family of Ixodidae, genus *Rhipicephalus*. *R. sanguineus* exists worldwide in warm humid areas, mainly between 35 degrees southern latitude and 50 degrees north latitude. [3] The primary host of *R. sanguineus* is the dog, but the tick can also be found on other animals and humans. [3] The tick is capable of completing a full cycle indoors. [4]

Rhipicephalus sanguineus is a 3-host tick. [4] The lifecycle contains 4 stages: eggs, larvae, nymphs and adults. [3] Dogs are the hosts of all development stages. Adults and nymphs attach to the ears, neck and shoulder, larvae attach mainly to the belly and flanks. Hosts other than dogs are usually only infested when there are dogs present to maintain the population of ticks. [4] Adult females stay on the host for a period of 5-12 days. After this period she lets go and falls off to process the blood meal and lay eggs in a sheltered place usually high above the ground and near places where the host sleeps. Oviposition is preceded by a pre-oviposition period of 3-14 days. Oviposition takes about 16-

18 days and an average of 4000 eggs is laid. The best temperature for oviposition is between 20 and 30 degrees Celsius. After the female laid her eggs she dies. The incubation period of the eggs is 6-23 days, little larvae come out of the eggs and immediately start to find a host. The blood meal of larvae takes 3-10 days, after this meal they fall from the host and develop to the nymph stage. This development takes about 15 days. The nymphs feed for 3-11 days and then again fall off and develop to the adult stage, this takes 9-47 days. Under ideal circumstances the cycle takes 63-91 days. [3] The development of the non-parasitic periods in the cycle are influenced by climate conditions. The cycle is inhibited at temperatures lower than 18 degrees Celsius. The humidity is also a factor, because when this drops below 50% a limitation of the continuity of the cycle occurs. [8]



In a research by G. van der Straten, ticks were collected from 100 neglected dogs. All of these ticks were found to belong to the species *R.sanguineus*. In the same research there was also blood collected from the 100 dogs to detect *E.canis* antibodies and the bacterium *E.canis* itself. Via the Snap® 3Dx® *E.canis* test, 58% of the dogs turned out to be positive for *E.canis* antibodies. In 14% of the dogs *E.canis* was detected in blood via PCR. [5] In a research by V. Moreta in the first consultation 22 (47%) of the 47 bloodsamples (taken from dogs suspected of ehrlichiosis in the veterinary clinic) were tested positive for *E.canis* with the Snap test. Of these 47 dogs, only 1 turned out to be positive for *E.canis* by PCR. This was probably due to a too low amount of blood that was tested. [7] These preceding researches that took place on Aruba showed that the Snap test and PCR on blood from the dogs were no ideal methods to detect *E.canis* in blood of dogs. In this research, therefore, the Snap® 4Dx® test was used along with a microscopic test where buffycoat smears were scanned for morulae. The microscopic technique was not used in any of the preceding researches on Aruba. The article of Mylonakis et al. demonstrates that this method has a higher sensitivity than the method with fullblood smears. [9] *R. sanguineus* ticks are not endemic in the Netherlands, but still *E.canis* infections have been reported in the Netherlands. In a research by F. Blaauw in 2009 it turned out that in the period from september 2006 until august 2008, all *R.sanguineus* ticks that were send to Utrecht Centre for Tick-borne Diseases originated from dog owners who had taken their dogs with them on holiday, imported their dogs or had emigrated. Of the sent-in *R.sanguineus* ticks, 6 of the 16 were positive for the 'Ehrlichia catch all' PCR signal. [6] Because Aruba is an island where many people are emigrating to from the Netherlands, where Dutch people like to go on holiday and where dogs are imported from to the Netherlands, it was relevant to perform further research to the prevalence of *E.canis* infections on Aruba.

The goal of this research project was to make a database of the dog population infected with *Ehrlichia canis*. This way the problem of canine ehrlichiosis on the island of Aruba can be surveyed. The conditions of the dogs were examined through a questionnaire, a clinical examination of the dogs and additional blood examination. This research is divided in two parts: part 1 concerns the dogs presented in the veterinary clinic, part 2 concerns the dogs in the kennel of the veterinary services of Aruba. Eventually a relation between the clinical features and present antibodies and morulae in the blood is made.

Materials and methods

PART 1 Research of the dogs presented in the veterinary clinic

For a period of 2,5 months the Veterinary Clinic of Aruba in Wayaca participated in this research project. A total of 128 dogs were examined in this period. The pet owners were notified about the research project by a information note that was placed at the frontdesk. At random dog owners that came with their dogs for a consult were selected. Then it was up to the owners to decide whether they wanted to participate in the project or not. If the owner agreed to participate in the research, the owner and the dog were taken to a separate room. First they were asked the questions that were in the questionnaire (see appendix 1). The results of the clinical examination of the dogs were listed on the questionnaire form. Decreased appetite was a symptom that the dogowner declared. Skin problems were very diverse, so this was noted as one group. This skin problems included alopecia, shells, pustulae and itch. The clinical signs were noted to use it as an indication for an *E. canis* infection. If the clinical signs contained one or more of those for ehrlichiosis, this indicated that further blood examination was needed. When during the consultation more clinical signs came up, these were also added to the list. Then ticks were collected with a maximum of ten ticks. When there were found more than ten ticks on one dog, this was registrated on the questionnaire form with an estimation of how many ticks were remaining on the dog. The ticks were collected in plastic tubes with a maximum of 3 ticks per tube. The tubes were collected in a paper envelope per dog. These envelopes were stored in a cardboard box at room temperature and eventually sent to the Utrecht Centre for Tickborne Diseases in the Netherlands by a transport company. Finally, blood was taken from the dog when possible. Approximately 1 ml of blood was taken en put in EDTA vacutainer tubes. If blood was already taken by the veterinarian before the owner was questioned, this blood was used. This blood was first used to determine the hematocrit. This was done by transferring some of the blood from the EDTA tube to a heparin capillary. This capillary was centrifuged for 60 seconds at 6060 rpm. After spinning the RBC percentage was read (a hematocrit of 40% or less was considered to be anemic, for puppies this was 38% or less) . The remaining EDTA blood was stored at 4 degrees Celsius for a maximum of 2 days.

Within 2 days after the bloodsample was taken, the blood was examined in the laboratory of the veterinary service of Aruba. Here the hematocrit was determined again in twofold. First the EDTA vacutainers were placed on a mixer for about 5 minutes to homogenize the blood sample and allowing the temperature of the blood to get to about room temperature. Then two plain capillary tubes (containing no heparin) per blood sample were filled and spinned in a hematocrit centrifuge for one minute and again the RBC percentage was read. Then smears were made of the buffycoat and they were examined under the microscope for morulae. To detect morulae in peripheral blood of the dogs, the already centrifuged capillaries were used. First, the buffycoat was separated by making an incision in the capillary with a diamondpen at the intersection of red blood cells and the buffycoat. At the incision the capillary was broken in two and the buffycoat was put on 2 microscope glasses, so there were 2 slides per dog. These slides were dried and then coloured using a DiffQuick colouring procedure and then dried again. Subsequently the slides were looked at under the microscope, by a 100x magnification with a oil immersion lens. At least 200 white blood cells per slide were scanned for morulae. A dog was called positive for this test when one or more morulae were detected in the smears of the buffycoat . For 59 bloodsamples there were Snap® 4Dx® tests available, so on 59 bloodsamples these tests were performed in addition to the hematocrit tests and the microscopical examination of the bloodsmears. The test was used conform the enclosed instructions (see appendix 2).

The results of the 3 hematocrit tests in total, the outcome of the cytological examination of the buffycoat smears and the outcome of the Snap[®] 4Dx[®] test, if available, were also written down on the questionnaire form of the corresponding dog.

PART 2 Research of the dogs in the kennel of the veterinary service

The kennel of the veterinary services of Aruba is a kennel where people can place their unwanted dogs. The background of these dogs is therefore unknown, but most of the dogs that are in this kennel are neglected and did not receive any veterinary care. Because there were found very few morulae in the blood of the dogs presented in the clinic, there was decided to also include some dogs from the veterinary service kennel in the research, hoping this would give better results.

For 2 weeks dogs in the kennel were examined. A total of 40 dogs was examined in this period. Here again ticks and blood samples were collected. For these dog another registration form was used (see appendix 3). The ticks and blood samples were processed in the same way as described above for the dogs that were presented in the veterinary clinic. Except for then serological testing, since there were no more Snap[®] 4Dx[®] tests available.

Results

PART 1 Research of the dogs presented in the veterinary clinic

Clinical symptoms

Findings which only appeared once are not mentioned. Of the 115 examined dogs, 63 dogs did not show any abnormalities.

Table 1:

Symptoms displayed by the examined dogs in the veterinary clinic

Symptoms	Number of dogs
Pale mucous membranes	36
Lethargy	25
Fever	19
Emaciation	18
Appetite↓	15
Diarrhea	9
Lymphadenomegaly	7
Skin problems	6
Conjunctivitis	4
Epistaxis	4
Petechial haemorrhages / haematomas	4
Vomiting	3
Coughing	3
Dyspnea	2
Tachypnea	2

Ticks

In the veterinary clinic 117 dogs were checked for ticks. The amount of collected ticks varied from 0 until a maximum of 10. All of the collected ticks were found to belong to the species *R. sanguineus*. The number of ticks found on a dog was categorized into one of five categories: 0 for no ticks on the dog and 5 for dogs with more than 100 ticks.

Table 2:

Tick infestation on dogs in the veterinary clinic

Category	Number of ticks on the dog	Number of dogs
0	0	44
1	1-24	52
2	25-49	12
3	50-74	4
4	75-99	0
5	≥100	5

Hematocrit

The hematocrit was determined for 87 dogs. Considering the average of the three hematocrit tests per dog, 63 dogs turned out to be anemic (72%).

Serological testing

On 59 of the blood samples taken from dogs that were presented in the veterinary clinic a Snap® 4Dx® test was performed. Of the 59 dogs, 16 were tested negative for all of the included pathogens. So 43 dogs were tested positive for one or more pathogens.

Table 3:
Outcome of the Snap® 4Dx® tests per pathogen

Pathogen	Number of positive tested dogs	Percentage of total
E. canis	36	61%
Heartworm	16	27%
A. phagocytophilum	16	27%
B. burgdorferi	0	0%
Negative for all 4	16	27%

Table 4:
Outcome combinations of the Snap® 4Dx® tests

Outcome	Number of dogs	Percentage of total
E. canis alone	17	29%
A. phagocytophilum alone	4	7%
Heartworm alone	2	3%
E.canis & heartworm	8	14%
E.canis & A.phagocytophilum	6	10%
A. phagocytophilum & heartworm	1	2%
E.canis & heartworm & A.phagocytophilum	5	8%
Negative for all four	16	27%
Total	59	100%

Buffy coat smears

Eighty-six smears were made from peripheral blood of the dogs. Of these 86 smears 71 tested negative (83%), 11 tested positive (13%) and 4 were suspected (5%). Positive means that morulae were detected in the bloodsmears of the buffycoat.

Table 5:

Correlation with serological testing

Buffy coat smear test	<i>E. canis</i> snap test	Number of dogs
Positive	Positive	6
Positive	Negative	2
Positive	NA	3
Negative	Positive	28
Negative	Negative	19
Negative	NA	24
Suspected	Pos	2
Suspected	Neg	1
Suspected	NA	1

PART 2 Research of the dogs in the kennel of the veterinary service

Clinical signs

Findings which only appeared once are not mentioned in the table.

Table 6:

Symptoms displayed by the examined dogs in the kennel of the veterinary service

Symptoms	Number of dogs
Pale mucous membranes	34
Lymphadenomegaly	26
Emaciation	18
Fever	13
Conjunctivitis	8
Skin problems	7
Lethargy	5
Dehydration	3
Diarrhea	2

Ticks

Forty dogs we checked for ticks. The amount of collected ticks varied from 0 until a maximum of 10. All of the collected ticks were found to belong to the species *R. sanguineus*. The number of ticks found on a dog was categorized into one of five categories: 0 for no ticks on the dog and 5 for dogs with more than 100 ticks.

Table 7:

Tick infestation on dogs in the kennel of the veterinary services

Category	Number of ticks on the dog	Number of dogs	
0	0	2	
1	1-24	10	
2	25-49	9	
3	50-74	4	
4	75-99	1	
5	≥100	14	

Hematocrit

For 32 of the 40 examined dogs, the Ht was determined in twofold in the laboratory of the veterinary services. Considering the average of the two hematocrit tests per dog, 31 of the 32 dogs (97%) turned out to be anemic.

Buffycot smears

Of the 40 examined dogs, 32 was taken blood from. Of these 32 dogs, 6 (19%) were positive for morulae. Twenty-four (75%) were tested negative, 2 smears (6%) failed (1 due to edema, 1 due to failed technique).

Discussion

Clinical symptoms

When looking at the clinical signs of the dogs and the outcome of their snaptest and microscopic findings, there seems to be no relation at all. Some dogs exhibiting clinical symptoms of ehrlichiosis were tested negative for the snaptest and/or microscopic test for morulae. The other way around, some of the dogs tested positive for these tests had no clinical symptoms at all or symptoms not according to those of ehrlichiosis. It is however remarkable that symptoms of canine ehrlichiosis score very high as the patients were selected randomly.

Ticks

All of the collected ticks were of the species of *R.sanguineus*. This result was also found in the researches of G. van der Straten and V.Moreta. [5,7] Thus so far this is the only species of ticks identified on Aruba.

Serological testing

Of the tested dogs, 61% was positive for *E.canis*. In the researches by G. van der Straten and V. Moreta this was 58% resp. 47%. The result found in this research seems to be relatively higher than the percentages found before. This, because the dogs were selected at random this time so one would expect that a lower percentage would be found than in the research by Moreta where dogs were examined that were suspected of ehrlichiosis. Of the tested dogs, 27% was positive for heartworm. G. van der Straten found 24% of the dogs to be positive. V. Moreta found that 17% was positive. So again, a higher percentage was found this time. This might be because of a higher prevalence of heartworm in the dog population on Aruba or because of failing prevention methods. It is also possible that current diagnostic methods for heartworm are not adequate, since a many of these dogs were not already diagnosed for heartworm by the veterinary clinic itself.

Sixteen dogs (27%) tested positive for antibodies reactive with *Anaplasma phagocytophilum* in the Snap® 4Dx®, but because *Ixodes* spp. have not been reported in Aruba, these serologic reactions were likely due to cross-reaction with *A.platys*. This problem is also reported in an article by Yabsley et.al. where dogs were also tested with the Snap® 4Dx®. [11] Or it might suggest that *R.sanguineus* can play a role in the transmission of *A.phagocytophilum*. Further research about this phenomenon is needed.

Morulae testing

E. canis is characterized by intracytoplasmic inclusions (morulae) which it produces in circulating monocytes, lymphocytes and rarely neutrophils. [12] However, in this research the morulae were mostly found in the neutrophils and in only one dog in a monocyte. But in a research by Mylonakis et. al. morulae were more often detected in lymphocytes than in monocytes. [9] This might suggest that *E. canis* morulae in neutrophils are not that rare or that the morulae that were detected in this research were in fact not *E.canis* morulae. In a research by E. Elias in 1992 inclusion bodies were seen in 220 (88%) cases, of which only 9 (4%) cases exhibited morulae. Thus inclusions other than morulae may play an important role in the diagnosis of canine ehrlichiosis in enzootic areas. [13] This points out that experience with their morphology and careful observation is very important to avoid a surplus of false positive diagnoses.

In the research by Mylonakis et. al. at 1000 OIF's (oil immersion fields), the number of morulae in buffycoat smears ranged from 1 to 7. The sensitivity of this test was 66% and the time to screen 1000 OIF's ranged from 50-60 minutes. [9] In this research at least 200 white blood cells per slide were scanned for morulae. This took about 10 minutes. So to avoid a surplus of false negative result it

would be better to search for morulae for a longer period of time. In this research however there was not enough time to achieve this.

The benefits of morulae testing seem to be a relatively high sensitivity and the fact that it is an easy procedure to use in the veterinary clinic. The demonstration of morulae or clinical manifestations may precede seroconversion in the acute phase. [9] So a substantial number of missing diagnoses would be expected by applying serology alone. An important disadvantage is that it takes a lot of time to search for the morulae so the screening may exceed the time allowances in a busy practice.

Further research of the collected material in Utrecht Centre for Tick-borne Diseases

The ticks were sent to the UCTD in the Netherlands, because further research on these ticks had to be performed. The goal was to isolate *E.canis* from these ticks and so determine how many of the ticks really are infected with *E.canis*. In Utrecht there is a real-time PCR available to detect *E.canis* in the ticks that are sent to the UCTD. Unfortunately this could not be performed during this research, so the outcome of these test are not yet available. Isolation of *E.canis* by feeding the living ticks on membranes unfortunately also failed. This, because most of the ticks died during transport. *E.canis* could also be isolated by grinding a batch of ticks and making a dilution of this. Then this material can be inoculated on a cell line and *E.canis* can replicate. But again, there were not enough ticks that could be used for this procedure.

Conclusion

Canine ehrlichiosis is a serious problem threatening the dog population on the island of Aruba. The symptoms of the disease are non-specific. Pale mucous membranes is the most common symptom, but lethargy, emaciation and fever are also often seen. Anemia is often found (72% in dogs in the veterinary clinic, 97% in dogs in the kennel of the veterinary service). All of the collected ticks were of the species of *R.sanguineus*. The Snap 4Dx test was performed on 59 dogs in the veterinary clinic, with a positive test result for *Ehrlichia canis* for 36 dogs (61%). For heartworm, *Anaplasma phagocytophilum* and *Borrelia Burgdorferi* this was respectively 27%, 27% and 0%. A cytological examination of the buffycoat smears was performed. In 11 of the 86 (13%) dogs from the veterinary clinic, morulae were found. In the dogs from the kennel of the veterinary service this was 6 of the 32 (19%). More research is needed for the apparent prevalence of *Anaplasma phagocytophilum*. Furthermore, research is needed for the ticks that were sent to the Utrecht Centre for Tickborne Diseases to know more about the prevalence of *E.canis* in the collected ticks.

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References

- [1] Nelson, R.W., Couto, C.G., Small animal internal medicine. 3rd ed., 2003; 1267-1270
- [2] Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J., Leonard, F.C., Veterinary microbiology and microbial disease. 2002; 203-207
- [3] DANTAS-TORRES, F. (2008) The brown dog tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) from taxonomy to control. *Veterinary Parasitology* **152** (3-5), pp. 173-185.
- [4] ESTRADA-PENA, A., BOUATTOUR, A., CAMICAS, J.L., WALKER, A.R. (2004) Ticks of domestic animals in the mediterranean region. *Rhipicephalus sanguineus* In: A guide to identification of species. Univerity of Zaragoza, first edition. Atlanta B.V., Houten, The Netherlands, pp. 124-127.
- [5] Straten van der, G. A survey of ticks, *Ehrlichia canis* and current control methods on dogs on the island of Aruba. Research report Utrecht Centre of Tick-borne diseases, 2008
- [6] Blaauw, F. *Het voorkomen van Ehrlichia canis en Rhipicephalus sanguineus in Europa en Nederland*. Research report Utrecht Centre of Tick-borne diseases, 2008
- [7] Moreta, V. *Examination of the Diagnostic and Therapeutic Effectivity among Dogs Suspected of an Ehrlichia canis infection on the Island of Aruba*. Research report Utrecht Centre of Tick-borne diseases, 2009
- [8] SILVEIRA, J.A.G., PASSOS, L.M.F., RIBEIRO, M.F.B. (2009) Population dynamics of *Rhipicephalus sanguineus* (Latrielle, 1806) in Belo Horizonte, Minas Gerais state, Brazil. *Veterinary Parasitology* **161**, pp. 270-275.
- [9] MYLONAKIS, M.E. et al. (2003) Evaluation of cytology in the diagnosis of acute canine monocytic ehrlichiosis (*Ehrlichia canis*): a comparison between five methods. *Veterinary Microbiology* **91**, pp. 197-204
- [10] Blaauw, F. Pilotstudie naar de transmissie van *Ehrlichia canis* met behulp van in vitro membraanvoeding en capillairvoeding en voorkomen van *Ehrlichia canis* en *Rhipicephalus* in Europa en Nederland. Faculteit diergeneeskunde Utrecht, 2008
- [11] YABSLEY, M.J. et al. (2007) Prevalence of *Ehrlichia canis*, *Anaplasma platys*, *Babesia canis vogeli*, *Hepatozoon canis*, *Bartonella vinsonii berkhoffii*, and *Rickettsia* spp. in dogs from Grenada. *Veterinary Parasitology* **151**, pp. 279-285
- [12] HILDEBRANDT, P.K. et al. (1974) Ultrastructure of *Ehrlichia canis*. *Infect. Immun.* **7**, pp 265-271
- [13] ELIAS, E. (1992) Diagnosis of ehrlichiosis from the presence of inclusion bodies or morulae of *E. canis*. *Journal of Small Animal Practice* **33**, pp. 540-543

Appendix 1

Questionnaire dogowners

Vragenlijst

Datum: _____

Volgnummer: _____

Naam hond: : _____

DVM hond: _____

Naam eigenaar: _____

DVM eigenaar: _____

Adres eigenaar: : _____

Geslacht: _____

Leeftijd: _____

Ras: _____

Vachttype: _____

Vachtkleur: _____

-
- Doet u aan tekenbestrijding op de hond?
 - Wat doet u precies?
 - Met welke middelen?
 - Hoe vaak?
 - Sinds wanneer?
 - Bent u tevreden met de producten?
 - Bestrijdt u ook teken in de omgeving (pest control)?
 - Wat doet u precies?
 - Waarmee?
 - Hoe vaak?
 - Sinds wanneer?
 - Bent u tevreden met de producten?
 - Verwijdert u teken van uw hond?
 - Hoe vaak doet u dit?
 - Kunt u een inschatting geven van het aantal teken per keer?

- Indien u meerdere middelen heeft gebruikt, merkte u verschil?
- Leeft en slaapt uw hond binnenshuis of buitenshuis?
 - Hoe ziet uw tuin eruit?(indien buitenshuis)
 - Afgesloten/niet afgesloten (hek/muren)
 - Zand en aarde/bestraat/grind
- In welk gebied woont u? (Knoek of stad, welke regio)
- Laat u de hond ook uit buiten de tuin?
 - Waar laat u de hond uit (gebied)?
- Heeft u uw hond wel eens meegenomen naar het buitenland?

- Bevindingen lichamelijk onderzoek

- Teken verwijderd?
 - hoeveel?
 - schatting hoeveel overgebleven?
 - aant adult
 - aant man/vrouw
- Bloed afgenomen?
 - Ht?
- Snaptest
- Uitslag uitstrijkje buffycoat

Appendix 2

Instructions IDEXX Snap® 4Dx® test



Canine SNAP® 4Dx*

In-vitro diagnostic for the detection of *Dirofilaria immitis* (HW) antigen, antibody to *Anaplasma phagocytophilum* (AP), antibody to *Borrelia burgdorferi* (LY) and antibody to *Ehrlichia canis* (EC) in canine serum, plasma or whole blood.

Precautions and Warnings

- All wastes should be properly decontaminated prior to disposal.
- Do not mix components from kits with different lot numbers.
- Do not use a SNAP device that has been activated prior to the addition of sample.

Storage

- Store at 2°C–8°C until expiration date.
- SNAP devices and reagents can be stored at room temperature (15°C–25°C) for 90 days or until the expiration date, whichever occurs first.
- After SNAP devices and reagents are removed from 2°C–8°C for more than 24 hours, the expiration date is 90 days or the printed expiration date, whichever occurs first.
- If the 90-day expiration date occurs prior to the printed expiration date, record the new date in the space provided on the kit.

Kit Components

Each kit contains one 8-mL bottle of anti-HW/AP/LY/EC:HRPO conjugate and one reagent rack; as well as 5, 15 or 30; transfer pipettes, sample tubes and SNAP devices. Each SNAP device contains 0.4 mL of wash solution and 0.6 mL of substrate solution.

Sample Information

- Samples must be at room temperature (15°C–25°C) before beginning the test procedure.
- Serum, plasma or anti-coagulated whole blood (e.g., EDTA, heparin), either fresh or stored at 2°C–8°C for up to one week, can be used.
- For longer storage, serum or plasma can be frozen (-20°C or colder) and then re-centrifuged before use.
- Hemolyzed or lipemic samples will not affect test results.

Test Procedure

1. If stored in a refrigerator, allow all components to equilibrate at room temperature (15°C–25°C) for 30 minutes. **Do not heat.**

2. Using the pipette provided, dispense 3 drops of sample into a new sample tube.

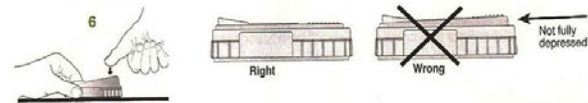
3. Holding the bottle vertical, add 4 drops of conjugate to the sample tube.

4. Cap the sample tube and mix it thoroughly by inverting it 3–5 times.

5. Place the device on a horizontal surface. Add the entire contents of the sample tube to the sample well, being careful not to splash the contents outside of the sample well.

The sample will flow across the result window, reaching the activation circle in 30–60 seconds. Some sample may remain in the sample well.

6. When color **FIRST** appears in the activation circle, push the activator firmly until it is flush with the device body



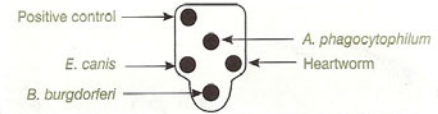
NOTE: Some samples may not flow to the activation circle within 60 seconds, and, therefore, the circle may not turn color. In this case, press the activator after the sample has flowed across the result window

7. Read test result at eight minutes.

Interpreting the Test Results

Positive Results

Any color development in the sample spots indicates the presence of heartworm antigen, *A. phagocytophilum* antibody, *B. burgdorferi* antibody or *E. canis* antibody in the sample.



NOTE: Initial research data suggests the SNAP 4Dx *Anaplasma phagocytophilum* spot could cross react with *Anaplasma platys*. In studies involving dogs infected with a laboratory strain of *A. platys*, the SNAP 4Dx was reactive with serum from 10 out of 10 infected animals.

Negative Results

Only positive control spot develops color.



Invalid Results

- Background**—If the sample is allowed to flow past the activation circle, background color may result. Some background color is normal. However, if colored background obscures test result, repeat the test.
- No Color Development**—If positive control does not develop color, repeat the test.

IDEXX SNAPshot Dx* Analyzer

Test results can also be read using the SNAPshot Dx analyzer. A complete description of how to enter patient data and read test results using the SNAPshot Dx analyzer can be found in the SNAPshot Dx analyzer user guide.

Comparison Test	Sample Size					Sample Type	Relative Sensitivity and Specificity 95% Confidence Limit	Kappa Statistic
	SNAP 4Dx Test	Reference Test	+/+	-/+	-/-			
HTVM ^{1,2}	118	1	0	236	355	Serum	Sens., 99.2% (95% CL 94.6%–100%) Spec., 100% (95% CL 98%–100%)	0.99
<i>A. phagocytophilum</i> ³	217	2	0	236	455	Serum	Sens., 99.1% (95% CL 96.5%–100%) Spec., 100% (95% CL 98%–100%)	0.99
<i>B. burgdorferi</i> ³	166	2	0	236	404	Serum	Sens., 99.8% (95% CL 95.4%–99.9%) Spec., 100% (95% CL 98%–100%)	0.99
<i>E. canis</i> ⁴	100	4	0	236	340	Serum	Sens., 96.2% (95% CL 90.1%–98.8%) Spec., 100% (95% CL 98%–100%)	0.97

Reference ¹Necropsy and ²PetChek ³*A. phagocytophilum* IFA / Western Blot ⁴*E. canis* IFA / Western Blot ⁵*B. burgdorferi* IFA / Western Blot
Sensitivity and Specificity are based on visual interpretation of SNAP results

USA/Canada 1-800-248-2483 • Europe 00800 1234 3399 • Australia 1-800-655-978
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Covered by U.S. Patent Nos.: 5,627,026; 5,726,010; 5,728,013; 5,750,333; 6,007,969; 6,204,252; 6,305,402; 6,475,492; 6,660,274; 6,719,963; 6,740,744; 6,923,363; 6,984,855; 7,063,848; 7,067,372; 7,407,770; 7,458,321; 7,445,788 and/or 7,446,191. Other U.S. and/or foreign patents issued or pending.

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Appendix 3

Form streetdogs

Onderzoek deel 2: Straathonden _____ **volgnummer:** _____

Datum: _____

Ras: _____

Geslacht: _____

Leeftijd: _____

Vachttype: _____

Kleur: _____

- Bevindingen lichamelijk onderzoek

- Teken verwijderd?
 - Hoeveel?
 - Schatting aantal overgebleven
 - Aant. adult
 - Aant. man/vrouw

- Bloed afgenomen?

- Ht lab 1: % Ht lab 2: %

- Uitslag uitstrijkje buffycoat: