Tick-borne pathogens in ticks collected from dogs in the Tickbusters survey in 2013

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Summary

Ticks are of great veterinary and medical importance; they can transmit a greater variety of diseases than any other group of arthropods. In the Netherlands the pathogen causing most human disease cases is *Borrelia burgdorferi* s.l., but also other pathogens are present in *I. ricinus* ticks, like *Rickettsia, Babesia* and *Ehrlichia* species.

Over a period of 8 months in 2013 a total of 1724 ticks originating from dogs were submitted to UCTD. From these 400 ticks were selected (287 *I. ricinus*, 72 *I. hexagonus*, 40 *D. reticulatus*, 6 *R. sanguineus*, 2 *R. turanicus*) for pathogen screening. The ticks were screened by polymerase chain reaction (PCR) and reverse line blot (RLB) hybridization for the presence of *Ehrlichia, Anaplasma, Babesia, Theileria, Borrelia* and *Rickettsia* species.

Of the *I. ricinus* ticks 6.4% (18/280) was infected with *Ehrlichia/Anaplasma* spp., 1.8% (5/280) with *Babesia* spp., 1.8% (5/280) with *Theileria spp*., 9.6% (27/280) with *Borrelia* spp., and 8.9% (25/280) with *Rickettsia* spp. (all *Rickettsia* spp. were specified as *R. helvetica*).

Of the *D. reticulatus* ticks 2.5% (1/40) was infected with *Babesia* spp. and 1.4% (1/72) of *I. hexagonus* ticks with *B. burgdorferi* sensu lato (subspecies sensu stricto).

The pathogen *B. afzelii* was present in 17 of the 280 *I. ricinus* ticks (6.1%), which is quite a high prevalence for one of the subspecies of *B. burgdorferi* sensu lato. It was also found that *I. ricinus* ticks contained *T. equi* (1.1%). It needs to be confirmed if *I. ricinus* plays a role in addition to *D.reticulatus* ticks as vectors of equine piroplasmosis.

It can be concluded that there are different zoonotic pathogens present in the indigenous tick population in the Netherlands. Continued surveillance is relevant in order to stay up to date on the current pathogen prevalence status and to keep track of possible exotic tick species entering into the Netherlands.

Introduction

Ticks are the primary arthropod vectors of disease agents affecting livestock, humans and companion animals globally, which make them of great medical and veterinary importance[1]. Ticks can be vectors of protozoa, bacteria, viruses and fungi[2]. Also a reaction in response to the tick bite itself can have consequences. Ticks secrete a complex mixture of bio-active compounds, primarily proteins, during the blood meal[3,4]. These compounds can induce a range of immuno-mediated reactions.

Systematics. Ticks are classified in the class Arachnida, subclass Acari, order Parasitiformes, and suborder Ixodida[5]. There are around 900 different species, which can be divided into three families. The majority belongs to two families, the Argasidae (soft ticks) and the Ixodidae (hard ticks)[6,7]. Of these, 28 species are vectors for pathogens known to cause human disease[8]. The family Ixodidae includes the genera *Ixodes, Rhipicephalus, Dermacentor* and several others[6].

Situation Netherlands. In the Netherlands several species of ticks are indigenous and infest companion animals, production animals and wildlife, whereas humans are accidental hosts[9]. Dogs and cats are mainly infested with *Ixodes ricinus*. The species *I.hexagonus* is mainly being detected on hedgehogs, and occasionally on dogs and cats. *I.ricinus* is not host-specific[10].

Also *Dermacentor reticulatus* was found on dogs that never left the country and on several vegetation sites. Several exotic ticks were found on dogs which were imported into the Netherlands or by dogs traveling to and from endemic areas, including *Rhipicephalus sanguineus* and *Rhipicephalus turanicus*[10,11].

I.ricinus can transmit Lyme borreliosis (*Borrelia burgdorferi* s.l.), Babesiosis, several forms of rickettsioses and anaplasmoses, and tick-borne encephalitis virus to humans[1]. A high prevalence of *R. helvetica* is present in *I. ricinus* ticks in the Netherlands. *D. reticulatus* is known as a vector of *Rickettsia slovaka, Coxiella burnetti*, *Babesia caballi* and *Babesia canis*[1,12]. *I. hexagonus* can act as a vector of *A. phagocytophilum,* Borreliosis and Rickettsioses[1,12].

Life cycle. All ticks have three life stages; larva, nymph and adult. However the life cycles varies according to the family of the tick[13]. The life cycles of hard-bodied ticks can differ in the number of times they change hosts during the three feeding stages, and if juvenile ticks molt on or off their host.[8] Most ixodid species, like *Ixodes ricinus*, feed three times on three different hosts; which is called a three-host tick, see Figure 1. They feed to completion as larvae, drop off their host to the ground, and molt into nymphs. Nymphs attach to another host animal, feed to completion, and fall of. After molting into adults, each female attaches to a host, mates, completes the blood meal, and drops to the ground, where she will lay eggs and die[8]. Generally adult males feed only briefly and sparingly and some do not feed at all.

The life cycle of ixodid ticks is usually completed in 2-3 years, but it may take from 6 months to 6 years, depending on environmental conditions[8]. Each species has their own optimal environmental conditions and biotopes that determine the geographic distribution of the ticks. The *Ixodes ricinus* tick in Europe favors woods and forests with high relative humidity and will not be found in dry places[8].

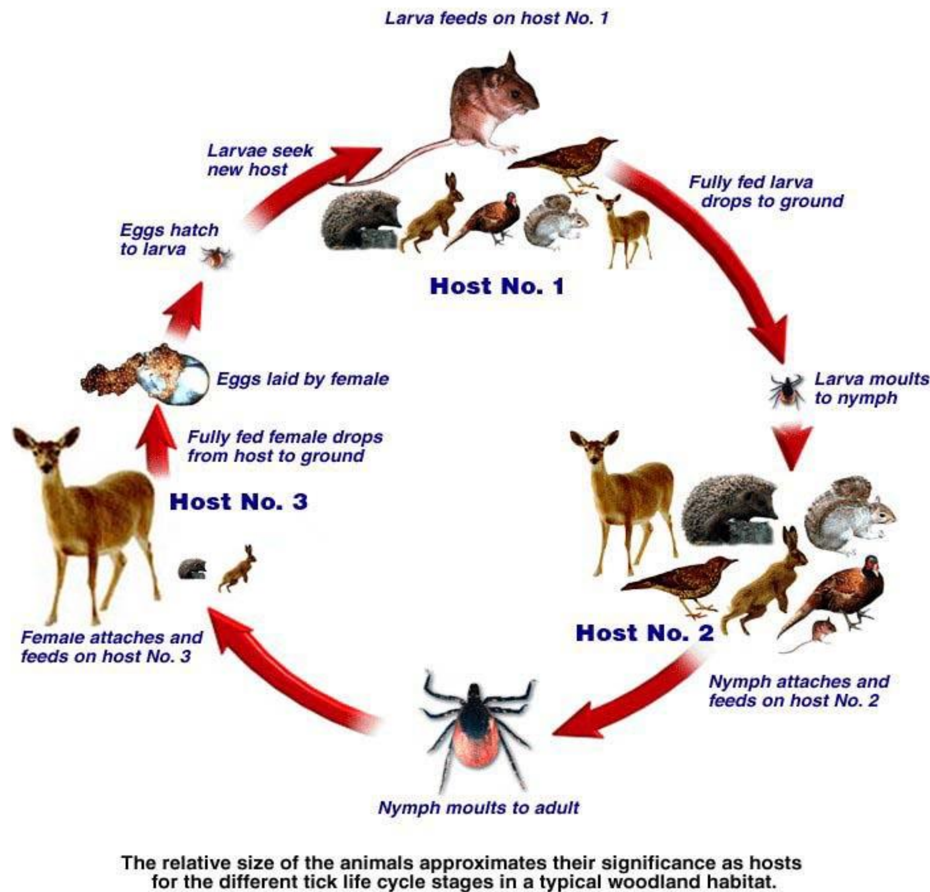

Figure 1: The life cycle of the *I. ricinus* tick[14].

Tick-borne pathogens

During this research the ticks were screened for the presence of *Anaplasma* spp., *Theileria* spp., *Borrelia* spp., *Babesia* spp. and *Ehrlichia* spp.

Babesiosis. Protozoa of the genus *Babesia* are intracellular parasites of red blood cells. The parasite destroys erythrocytes and this may be fatal in immune-compromised (elderly and HIV-positive) individuals. Within 1-3 weeks after infection, clinical symptoms may occur. Symptoms can be fever, weakness, fatigue, anorexia, gastro-intestinal symptoms, myalgia, arthralgia, respiratory symptoms and headaches[15]. Also neurological symptoms can occur and in severe cases it can result in renal failure and myocardial infarction. The severity of the disease is dependent of host factors, and the species and virulence of the parasite involved. The different species belonging to the genus *Babesia* are known to infect a large variety of animals and some of them are zoonotic. Human infections are in-frequent, but rising in prevalence. In Europe, most clinical cases of human babesiosis are caused by *Babesia divergens*. The known vector of *B. divergens* is *I.ricinus,* and is also a possible vector for *B. capreoli, B. venatorum (EU1)* and *B. microti*[15]. Each lifecycle stage is a competent vector for the transmission of the pathogen. *B. divergens, B. venatorum* and *B. microti* are present in the Netherlands and were detected in ticks originating from Dutch dogs[12,16]. In Europe *B. microti* is known to cause a less acute illness than *B. divergens*. The prevalence of *B. microti* in rodents is high[15].

Canine babesiosis is caused by the pathogen *Babesia canis,* and increasingly by *Babesia gibsoni.* In Europe *B. canis* is moderately pathogenic. Both parasites cause progressive haemolytic anaemia. The more severe disease caused by *B. rossi* can involve hypoxic, hypotensive shock with disseminated intravascular coagulation, systemic inflammatory response syndrome and multiple organ dysfunction syndrome[17].

Theileriosis. Like Babesiosis, Theileriosis is a protozoan disease and is primarily causing disease in cattle. Different *Theileria* species play a role, which are being transmitted by different tick species. *Theileria* sporozoites first invade host leucocytes unlike *Babesia,* which parasitizes first erythrocytes. *T. parva* and *T. annulata* are the two species which affect cattle and are the most economically important[2].

Babesia and *Theileria* genera are the etiologic agents of piroplasmosis, which are haematic tick-borne infections which can lead to malaria-like symptoms[18]. These agents can infect a variety of domestic and wild animals and also humans. In Europe the involved *Babesia* species are *B. divergens, B. venatorum* and *B. microti.* The *Theileria* species involved is *T. equi*[18]. Equine piroplasmosis is caused by *B. caballi* and/or *T. equi* and poses a threat to the horse industry. Possible vector ticks for both pathogens are *Dermacentor, Hyalomma* and *Rhipicephalus*[19].

Anaplasmosis. The agent *Anaplasma phagocytophilum* is a bacterium, which can infect many different mammalian species and infects neutrophils, causing neutropenia and thrombocytopenia. The most characteristic signs of infection in mammals are high fever and loss of appetite [20]. In humans the disease is called human granulocytic anaplasmosis (HGA). Symptoms can appear 10 days to 3 weeks after a tick bite[15]. *A. phagocytophilum* has been found in nearly all European countries and studies indicate that migratory birds play an important role in spreading the tick and its infectious agents[20]. In Europe *I. ricinus* is the only known vector for *A. phagocytophilum.* Different mammals are suspected to be a reservoir (sheep, goats, birds and roe). Dogs can also be a reservoir for the agent in the more urban environments[20]. The prevalence of the pathogen in Europe ranges from moderate to high, but the median prevalence of the pathogen in *I.ricinus* ticks is around 3%, but only a few acute cases have been described[15]. Although it is present in the ticks in the Netherlands, only one human case has been reported[21].

Ehrlichiosis. Like *Anaplasma, Ehrlichia* is an intracellular bacterium. The difference between members of the genera *Ehrlichia* and *Anaplasma* is their cellular tropism. The pathogen infects reticuloendothelial cells, including macrophages, with a preference for monocytes[2]. *Echrlichia chaffeensis*, causing human monocytotropic ehrlichiosis (HME), is an emerging zoonosis. It causes symptoms within several days to 2 weeks. The commencement is abrupt and symptoms of fever, chills, headache, myalgia, and arthralgia occur and can lead to multi-organ failure[21]. The pathology of HME involves, leukopenia, thrombocytopenia, and anemia[2]. The main vector is *Amblyomma americanum* , which is causing disease in de U.S.[2]

Ehrlichiosis emerges in many parts of the world and an infection with *E. canis* is often fatal to dogs. *Ehrlichia canis* parasitizes monocytes, granulocytes and platelets. This causes thrombocytopenia, which results in bleeding diathesis and immunological destruction of platelets. When the infection becomes chronic, it can lead to irreversible bone marrow destruction[17]. *E. canis* is responsible for disease in tropical and temperate areas in the world and the

distribution has expanded with the distribution of *R. sanguineus.* Ehrlichiosis is more severe in certain dog breeds and in younger animals. However, coinfection, immune status and strain variation could all play a role[17]. In Europe ehrlichiosis is acquired by dogs travelling to Mediterranean areas[1].

Candidatus Neoehrlichia mikurensis. A novel candidate species in the family of *Anaplasmataceae,* called *Candidatus Neoehrlichia mikurensis (N. mikurensis),* is detected in several tick species and rodents in different parts of the world[22]. In 2010 the first human cases were reported. The symptoms were non-specific and in compliance with any other ordinary inflammatory reaction. Almost all the cases described occurred in immunocompromised people. Recent research showed that in 1999 *N. mikurensis* was already present in the Netherlands and that the current Dutch tick population is infected with *N. mikurensis,* with the main vector being the *I. ricinus* species[21]. Remarkably no human and animal cases have been described in the Netherlands. According to research the overall presence of *N. mikurensis* is 7% in questing nymphs and adult ticks. Transmission in ticks appears to be horizontally and not vertically[21].

Lyme borreliosis (LB). LB is caused by Gram-negative spirochetes of the *B. burgdorferi* sensu latu group. Worldwide there are different genospecies causing *Borreliosis* with differences in disease induction. In Europe, several of the at least 18 genospecies of the *B. burgdorferi* s.l. complex are pathogenic to humans: *B. afzelii, B. garinii, B. burgdorferi* sensu stricto, *B. bavariensis* and *B. spielmanii*[23] . In Europe the main vector is the *I. ricinus* tick. Nymphs are being considered to be main vector in transferring lyme borreliosis, because they generate the greatest number of tick bites in many areas and they stay often longer attached to the host, because they are more difficult to detect[14]. LB primarily affects humans and some domestic animals, whereas natural reservoir hosts, which are rodents, do not appear to develop disease[14]. Risk of infection can be reduced by immediate removal of the attached tick. *Borrelia* spirochetes stay inactive in an infected tick until the tick starts feeding. Next they multiply within the tick midgut, and migrate to their salivary glands. This can take 24-48 hours, which is enough time for prevention of transmission by removing the tick[15]. A human infection is characterized by a multisystem, multistage inflammatory infection. Often an expanding red skin lesion, called erythema migrans, is being observed, caused by cutaneous infection at the site of the tick bite. This lesion appears around 7-14 days after the bite. When staying untreated, it can result in severe disease symptoms months or years after infections, affecting skin, CNS, heart and joints. [15] In dogs it can cause non-erosive polyarthropathy and central and peripheral neurological signs[17].

Rickettsiosis. Rickettsiae are Gram-negative, intracellular bacteria, which invade white blood cells[2]. Rickettsiae are usually transmitted via arthropod vectors and can transmit *Rickettsiae* between humans, animals and from animals to humans[15]. *Ixodidae* are the main vectors of the spotted fever syndrome in humans, which is caused by almost 20 different *Rickettsia* species, of which half of them circulate in Europe. The clinical symptoms of spotted fever rickettsiosis depend on the rickettsial species involved, but usually begin 4-10 days after a tick bite. Clinical signs are febrile illness, headache, malaise, muscle pain, rash, local lymphadenopathy and a characteristic inoculation eschar/ crust at the site of the bite. Also leukopenia, anemia and thrombocytopenia can be

encountered during laboratory testing[15]. In Europe, emerging tick-borne rickettsiosis caused by *R. helvetica, R. massiliae, R. sibirica mongolitimonae* and *R. slovaca* have been identified, but epidemiological data are scarce. *R. helvetica* is suspected of causing acute perimyocarditis, unexplained febrile illness and sarcoidosis[4]. In Europe the vector for *R. helvetica* in Europe is *I. ricinus.*

Dutch ticks are known to have a high prevalence of *R. helvetica* , but no endemic cases in humans have been observed[4]. The disease responds well to antibiotic treatment. Any delay in treatment can have severe consequences and can even have a fatal outcome[15].

Materials and Methods

Tick collection. The Tickbusters project started in 2005. Veterinarians were requested to submit ticks from their patients to UCTD. Also animal owners could submit ticks from their pets to the center. Information packages were sent to veterinary clinics, that wanted to participate in the study. The packages contained collection tubes and forms on which information about the tick could be written. The form contained questions about the host species on which the tick was found, the location where the tick was found, the date of collection, and whether the pet on which the tick was found had traveled out of the Netherlands prior to finding the tick. Each year UCTD received more ticks. From January 2013 until August 2013, around 3500 ticks were sent to the UCTD and around 1500 ticks originated from dogs.

Each tick was identified to species level, with stage and sex, with the help of a microscope. Each tick received a unique number before it was emerged in 70% ethanol and stored in cabinets. The information of every received tick is stored in an electronic database and the veterinary clinic receives feedback after every submission.

DNA extraction. The DNA was extracted from 400 ticks. The extraction was done by using the Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) following the protocol of the UCTD for DNA extraction (see Annex 1). When the protocol was completely finished, the result was 100 µl DNA originating from 1 tick. Every tube with DNA gets correctly labeled and stored in a freezer at -20 °C.

Polymerase chain reaction (PCR) amplification. After DNA extraction a PCR was performed on every tick sample. With PCR it is possible to amplify a specific DNA fragment a million times in vitro. The PCR technique is very sensitive. An advantage of the high sensitivity level is that it is possible to detect pathogens even with the presence of a very small amount of DNA material. A disadvantage of the high sensitivity level is that the technique is really sensitive for contamination, which can result in false positive samples.

The used master mix for the PCR existed of the following substances:

- 5x Phire reaction buffer
- 10 mM dNTPs
- F primer (20 pmol/ul)
- R primer (20 pmol/ul)
- 2U/ul Phire Hot Start II DNA polymerase
- PCR grade H_2O
- DNA sample of interest

To the master mix were added forward-primers and reverse-primers of the different pathogens of interest. These primers were added in excess. For the sequence of the used primers, see Table 1.

Primer*	Sequence	Tm (°C)
RLB-F2	5'- GAC ACA GGG AGG TAG TGA CAA G	57.9
RLB-R2	5'- Biotin-CTA AGA ATT TCA CCT CTG ACA GT	53.7
Ehr-F	5'- GGA ATT CAG AGT TGG ATC MTG GYT CAG	61.0
Ehr-R	5'- Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT	69.5
Bor-F	5'- ACC ATA GAC TCT TAT TAC TTT GAC CA	?
Bor-R	5'- Biotin-GAG AGT AGG TTA TGC AGG G	?

Table 1: RLB-F2/RLB-R2 for Babesia/Theileria, Ehr-F/Ehr-R for Anaplasma/Ehrlichia and Bor-F/Bor-R for Borrelia. Symbols used to indicate degenerate positions: M= A+C, Y = C+T

Three different master mixes were used, which resulted in three different PCR products for every DNA sample. The PCR products were used for the detection of *Borrelia, Theileria, Babesia, Anaplasma and Ehrlichia* species by reverse line blot (RLB) hybridization. *Rickettsia* PCR conditions were similar to the PCR conditions for the amplification of *Theileria, Babesia, Anaplasma* and *Ehrlichia* species. With every master mix there was a negative and positive control, to check if the PCR was performed correctly.

After adding the DNA sample to the master mix, the sample was centrifuged. Then it was placed in a thermocycler, which performed the PCR. The PCR reaction existed of different steps, which were repeated till 40 times. For the used protocol see Table 2 and 3. The amplification occurred step by step. First the temperature rose to denaturize the DNA. The hydrogen bridges were broken between the DNA strings, which caused the DNA helix to split. Double stranded DNA became single stranded DNA. In the next step the temperature dropped and the primers attached to the complementary base pairs of the single stranded DNA. This resulted in double stranded DNA. Next the temperature rose again. DNA polymerase used the double stranded DNA of the previous step to start a further synthesis of the complementary DNA-string. All these steps were repeated several times. For the used protocol, see Annex 2.

1 cycle	30 sec	98°C	Initial denaturation of DNA and activation of the polymerase
10 cycles	5 sec 5 sec 7 sec	98°C 67°C-57°C 72°C	Touchdown: 1°C lower per cycle.
40 cycles	5 sec 5 sec 7 sec	98°C 57°C 72°C	
1 cycle	1 min	72°C	Final extension

Table 2: Thermocycler program for Ehrlichia/Anaplasma and Babesia/Theileria touchdown PCR.

1 cycle	30 sec	98°C	Initial denaturation of DNA and activation of the polymerase
10 cycles	5 sec 5 sec 7 sec	98°C 60°C-50°C 72°C	Touchdown: 1°C lower per cycle.
40 cycles	5 sec 5 sec 7 sec	98°C 57°C 72°C	
1 cycle	1 min	72°C	Final extension

Table 3: Thermocycler program for Borrelia touchdown PCR.

Agarose gel electrophoresis. To determine if the PCR was successfully performed, a gel electrophoresis was completed. With gel electrophoresis the presence of DNA can be detected in the PCR samples. A negative result of the gel indicates that the PCR has to be repeated, or else the RLB will not be successful.

First the agarose gel was being prepared. For the exact steps, see Annex 3. To the mixture of the gel ethidiumbromide was added, which had the property to bind to the DNA-fragments. The gel was placed into a tank, which is filled with a salt solution that conducts the electricity. For the performance of the gel electrophoresis the PCR products were mixed with a 6x Loading Dye. This gave the PCR products a blue color and made the migration of the DNA fragments visible. Also the Loading Dye caused a higher density of the PCR products in comparison with the used buffer, which caused a better settlement of the PCR product into the pre-cast wells in the agarose gel (see Figure 2).

When the PCR products were loaded in the wells, an electric current was applied to create an electric field on the gel. The phosphate backbone of the DNA molecule is negatively charged and migrates in the direction of the positive charged electrode. Smaller fragments migrate faster than larger fragments. The gel ran for 30 to 45 minutes.
When the electrophoresis was finished, the gel was read under UV-light in the UV-illuminator (see Figure 3). The bounded DNA-fragments could be visualized under UV-light, because of the binding to the ethidiumbromide. The size of the fragments were being compared with a reference, the DNA-Ladder. The DNA ladder was also placed into one of the wells, at the same time as the PCR products. If there was a positive result, the size of a DNA fragment of a certain pathogen could be compared with a known quantity of base pairs of that particular pathogen. A gel electrophoresis was successful when the positive control had a positive result and the negative control had a negative result.

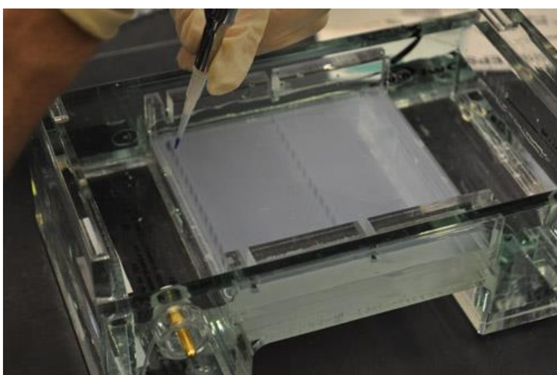

Figure 2: loading a DNA sample into a pre-cast well in the gel. [26]

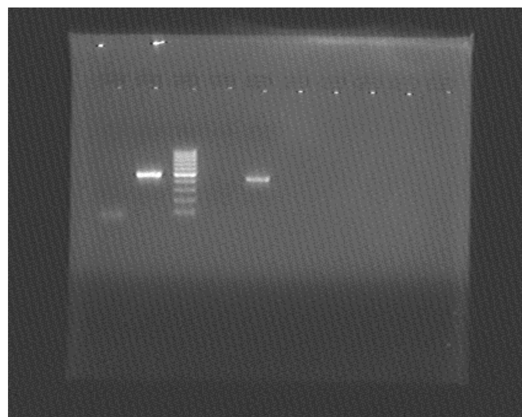

Figure 3: visualization of gel electrophoresis under UV-light.

Reverse Line Blot (RLB). After a successful electrophoresis, the RLB hybridization was performed. To detect specific pathogens a special membrane was used, which was designed specifically for this study (see Annex 6). On this membrane species-specific oligonucleotides were placed and were covalently bounded to the membrane. These nucleotides exist of short pieces of DNA and function as a primer. With the help of a miniblotter each species-specific oligonucleotide was placed in a straight line on the membrane. The resulted is 43 lines of species-specific nucleotides. The PCR-products were placed onto the membrane with the help of a miniblotter, in lines that were opposite to the direction of the lines of de oligonucleotides. When an oligo-nucleotide of a specific pathogen crossed with a PCR product that contained the same DNA, the oligonucleotide would bind to the DNA (hybridization, see Figure 4). The nucleotide acted as a primer. With the help of DNA-polymerase the oligonucleotide got extended and the complementary DNA-string replicated. After a thorough washing process, the unbounded PCR-products were removed from the membrane and the bounded PCR products were made visible with the help of chemiluminescence (see Figure 5).

Luminescence was possible because a biotin label was attached to the bounded PCR products. After the addition of streptavidine, the membrane was incubated with ECL, a peroxidase substrate. The combination with ECL caused a light producing reaction, which could be captured on an X-ray film. On the places where oligonucleotides were bound to PCR products, dark spots arose on the film. Such a spot, with the help of a raster, could be connected to a certain sample and a specific pathogen. For the list of the RLB probes included in this study, see Annex 4.

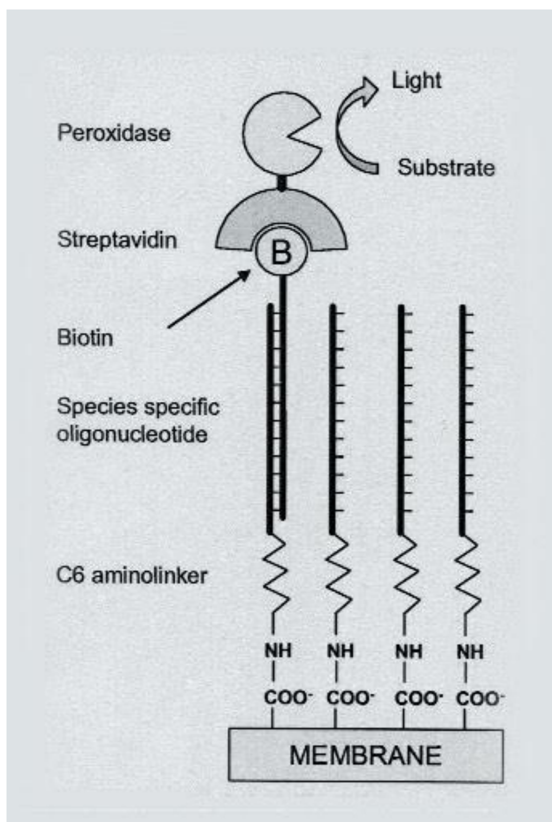

Figure 4: Schematic principle of the hybridization principle. [27]

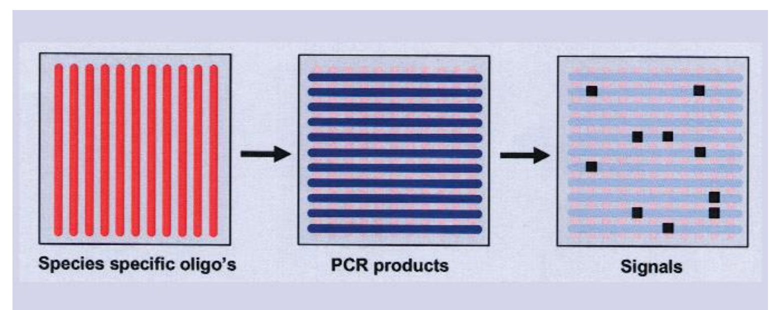

Figure 5: Schematic representation of the RLB assay. [27]

Results

Submitted ticks

From January 2013 until the end of august 2013, a total of 1720 ticks removed from dogs were submitted. The distribution of the ticks by species, stage and sex is summarized in Table 4. The number of ticks per submission ranged between 1 and 68 (the latter consisted of *Ixodes* nymphs). The *R. sanguineus* and *R. turanicus* ticks originated from dogs with a foreign history. All the *D. reticulatis* originated from dogs that had no foreign history, which means the ticks came from Dutch vegetation. From the 1604 adult ticks a not random selection was carried out for pathogen detection. This selection was based on the location where the dog probably got invested with the tick(s) (and with no history of travelling abroad, except for the *Rhipicephalus* ticks). With the selection of *I. ricinus* ticks, the goal was to select from every province an equal amount of ticks, to create a complete representation of the pathogens present in the whole country. For the provinces, which did not submit 25 ticks, a larger number was chosen for the provinces, which had a large number of submissions. For the origin of the selected *I. ricinus* ticks, see Figure 6.

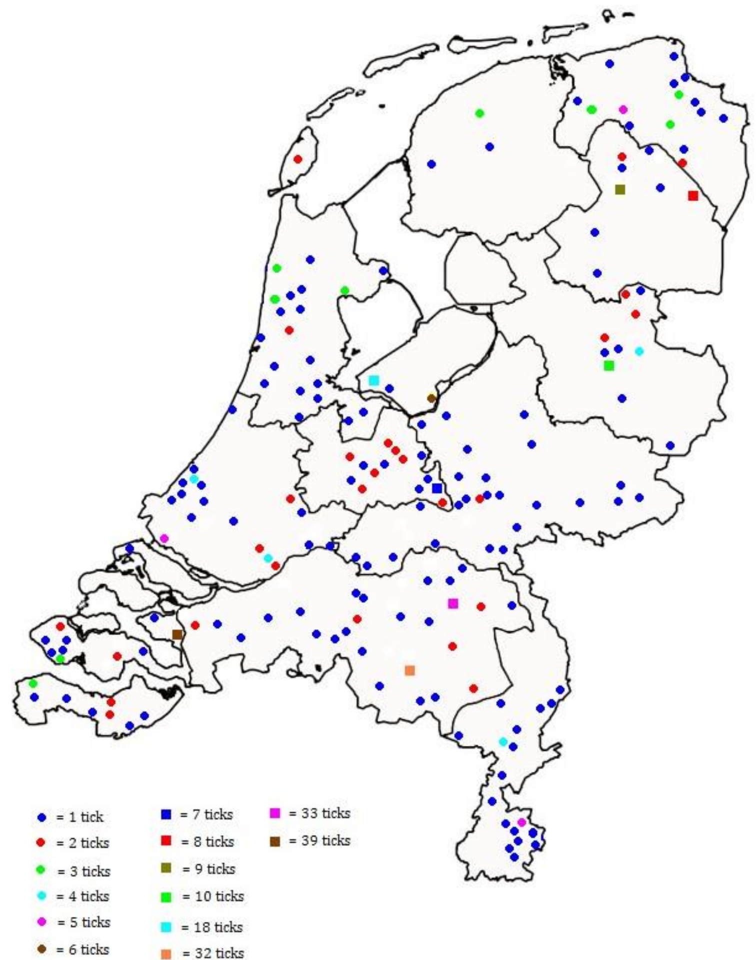

Figure 6: Origin of the 400 submitted ticks used for pathogen detection.

	Stage					
	Adult females	Adult males	Nymphs	Larvae	Undetermined	Total
D. reticulatus	58	29	0	0	0	87
Hy. lusitanicum	1	0	0	0	0	1
I.hexagonus	112	1	69	0	0	182
I.ricinus	1147	244	2	0	0	1393
Ixodes spp.	0	0	35	1	0	36
R. sanguineus	4	2	0	0	0	6
R. turanicus	2	2	0	0	0	4
Rhipicephalus spp.	1	1	0	0	0	2
Undetermined	N/A	N/A	N/A	N/A	13	13
Total	1325	279	106	1	13	1724

Table 4: Ticks originating from dogs submitted from January 2013 till August 2013. Thirteen ticks could not be identified as they were damaged. N/A = not available.

Pathogens detection

A total of 400 ticks; 280 *I. ricinus*, 72 *I. hexagonus,* 40 *D. reticulatus,* 6 *R. sanguineus* and 2 *R. turanicus* were screened by PCR/ RLB in this research for the presence of *Borrelia-*, *Babesia-*, *Theileria-*, *Anaplasma-* and *Ehrlichia-* species (see Table 5).

Thirty *I. ricinus* ticks were positive for *Ehrlichia/Anaplasma* spp. Of which 1 was positive for *Anaplasma marginale,* 6 *for A. phagocytophilum* and 9 for *Neoehrlichia mikurensis.* Two ticks were contaminated with two different species of *A. phagocytophilum*. The two *R. turanicus* ticks and one *D. reticulatus* tick were positive for *Ehrlichia/ Anaplasma spp*, without any differentiation in species.

5 *I. ricinus* ticks were positive for *Babesia spp*, of which 1 was positive for *B. divergens*, 1 for *B. microti*, 1 for *B. venatorum (EU1)* and 2 without further differentiation. One tick had a positive *Theileria/Babesia* catch-all and was positive for *B. canis*. One *D. reticulatus* tick was positive for *Babesia* spp., with no further differentiation in species.

In 4 *I. ricinus* ticks *Theileria* spp. were discovered; in 2 ticks it was specified as *T.equi* and 1 tick was positive for two species, *T. equi* and *T. equi-like*. One tick did not show any further specification for the exact species (only positive for the *Theileria/ Babesia* spp. and the *Theileria* spp.).

Spirochetes of *Borrelia burgdorferi* sensu *lato* were discovered in 27 *I. ricinus* ticks. From which 4 were pointed out to be *B. garinii*, 17 *B. afzelii* and 5 *B. valaisiana*. One *I. ricinus* tick was contaminated with more than one *Borrelia spp.* Three *B. burgdorferi sensu lato* were positive without any other specification on the exact species. One tick was positive for *B. afzelii*, but had no *B.burgdorferi* sensu lato positive signal. One *I.hexagonus* was positive for *Borrelia burgdorferi* sensu lato, which was further specified as *Borrelia burgdorferi* sensu stricto.

Rickettsia spp. was discovered in 17 *I.ricinus* ticks and they were specified by the RLB as *R. helvetica.* Seven *I. ricinus* ticks were contaminated with *R. helvetica* , but had no positive signal for *Rickettsia spp.* For 12 *I. ricinus* ticks who had a positive signal for *Rickettsia* spp. and *R. helvetica,* the RLB also showed a positive signal for *Ehrlichia/ Anaplasma* spp.*,* without specific signal for any of these subspecies. In one tick only *R. helvetica* was discovered with as well a positive signal for *Ehrlichia/Anaplasma* spp. One *D. reticulatus* tick was positive for *Rickettsia* sp.*/raoultii.* The two *R. turanicus* ticks were also positive for *Rickettsia spp.*

	I.ricinus	*I.hexagonus*	*D.reticulatus*	*R. sanguineus*	*R. turanicus*
Total	280	72	40	6	2
Ehrlichia / Anaplasma spp.	30 (10.71%)	-	1	-	2 (100%)
A. marginale	1 (0.36 %)	-	-	-	-
A. phagocytophilum	6 (2.14%)	-	-	-	-
Neoehrlichia mikurensis	9 (3.21%)	-	-	-	-
Theileria / Babesia spp.	10 (3.57%)	-	-	-	-
Babesia spp.	5 (1.79%)	-	1 (2.5%)	-	-
Babesia divergens	1 (0.36%	-	-	-	-
Babesia microti	1 (0.36%)	-	-	-	-
Babesia canis 2	1 (0.36%)	-	-	-	-
Babesia venatorum (=Babesia sp.(EU 1))	1 (0.36%)	-	-	-	-
Theileria spp.	1 (0.36%)	-	-	-	-
Theileria equi	3 (1.07%)	-	-	-	-
Theileria equi-like	1 (0.36%)	-	-	-	-
Borrelia burgdorferi sensu lato	27 (9.64%)	1 (1.39%)	-	-	-
Borrelia burgdorferi sensu stricto	-	1 (1.39%)	-	-	-
Borrelia garinii	4 (1.43%)	-	-	-	-
Borrelia afzelii	17 (6.07%)	-	-	-	-
Borrelia valaisiana	5 (1.79%)	-	-	-	-
Rickettsia spp.	17 (6.07 %)	-	-	-	2 (100%)
Rickettsia helvetica	25 (8.93%)	-	-	-	-
Rickettsia sp. (DnS14) / raoultii	-	-	1 (2.5%)	-	-

Table 5: Pathogens detected in submitted adult ticks originating from dogs by RLB screening

Discussion

During the selection process of the specific ticks for RLB screening, it was found that not all the selected ticks from the electronic database were still in storage. Apparently these ticks were used for other purposes within UCTD. This resulted in an adjusted selection, which resulted in a less representative sample size from all provinces.

Among the submitted ticks, a *Hyalomma lusitanicum* tick was sent to UCTD. The occurrence of this tick species is restricted to the Mediterranean area[24]. The dog, from which the submitted tick originated, had according to the information of the owner no history of travelling abroad. There are no records of this tick being found in Dutch vegetation. Probably this is an occasional finding, which may be explained by contact with dogs that did go to *H. lusitanicum* endemic areas and transferred the tick to the dog in question.

The *R. turanicus* and *R. sanguineus* ticks screened in this research were from dogs with a foreign history. This makes the detected pathogens in these ticks not representative for the pathogens present in the Netherlands. They were included in the screening out of interest for their pathogen status and possible threat for infection of indigenous species. The two *R. turanicus* were both positive for *Ehrlichia/Anaplasma* spp. and for *Rickettsia* spp., without any further specification. *R. turanicus* is known to harbor *Rickettsia massiliae,* which is known to be an human pathogen[12]. In order to be certain, which pathogen is involved, further research is needed. Because both ticks were positive for *Rickettsia* spp., this resulted in a prevalence of 100% in this species. It should be mentioned that the status of only two screened ticks, which were found on the same dog, does not represent the true infection status of this species. It is important to stay aware of exotic ticks spreading towards the Netherlands and their pathogen status.

In this study, the infection rate of *I. ricinus* ticks with *R. helvetica* of 9% is lower than in comparison with previous studies, which were 13.4%[4] and 21.5%[12]. The difference in host and geographical area can be a possible explanation. There are no reported human cases with *R. helvetica* infection in the Netherlands, despite the high level of infection in Dutch ticks, which would suggest that it is underdiagnosed or it is not that easily transmitted to humans.

Of the 16 *Ehrlichia/Anaplasma* spp. positive ticks, 12 were also positive for *Rickettsia* spp. and *R. helvetica*. It appeared that there was a cross-reaction with DNA material from *Rickettsia* spp. This means that there were false positive *Ehrlichia/Anaplasma* spp. signals, which resulted in a higher prevalence than in reality. Also 4 ticks were positive for *Ehrlichia/Anaplasma* spp. without any further specification. In this case it is not certain if this is an *Ehrlichia/ Anaplasma* species or a *Rickettsia* species. To be sure which pathogens this exactly are, further research is needed. Seven ticks, which were positive for *R. helvetica,* had no positive signal for *Rickettsia* spp. This raises the question if the *Rickettsia* catch-all is sensitive enough.

The *N. mikurensis* prevalence in this study was 3.2%. Former research showed a prevalence in the Netherlands of 16% (varying from 1% to 16% depending on study area) in 2009 and 8% in 2010[21]. The lower prevalence in this study could be correlated with the area where the ticks originated from.

Three *I. ricinus* ticks were contaminated with *T. equi* and the dogs from which the ticks originated had no history of travelling abroad. In 2012 research already showed that two horses were infected with *T. equi* in the Netherlands[19]. They suspected that with the establishment of *D. reticulatus* populations and unrestricted importation of horses from piroplasmosis endemic areas, as horses being the primary reservoir for *T. equi*, the number of cases were likely to increase. [19] In this publication *I. ricinus* was not mentioned as a possible vector. However, in Italy, research showed that the *I. ricinus* tick may be a vector for *T. equi*[18]. It seems that *T. equi* is spreading in the Netherlands and that *I. ricinus* is one of the vectors. To be sure what the exact prevalence is in the Dutch *I. ricinus* population, more research is needed.

B. afzelii appeared to be the predominant subspecies of *B. burgdorferi* sensu lato. The prevalence of *B. afzelii* is in this study is quite high (6.1%) in comparison with other findings (around 2%[4,12,25]). Although looking at the overall prevalence of *B. burgdorferi* s.l., the prevalence is not that much higher. This could mean that the predominant subspecies of *B. burgdorferi* s.l. is changing, but not the overall infection rate of *I. ricinus* ticks.

Comparing the pathogen prevalence in Dutch ticks this research with published data elsewhere, there are some differences. These could be explained by difference in study design, with regards to host, life stages of the vector and region of origin, which makes results difficult to compare and can cause variation in pathogen prevalence.

Conclusion

This study confirmed that dogs in the Netherlands in 2013 were primarily invested by *I. ricinus* ticks (80.8%), followed by *I. hexagonus* (10.6%) and *D. reticulatus* (5.1%). The exotic ticks *R. sanguineus, R. turanicus* and *H. Lusitanicum* were also discovered in the Netherlands. This shows that these ticks, together with the possible pathogens they harbor, are being introduced into the Netherlands by animals traveling abroad.

Of the *I. ricinus* species, 6.4% (18/280) was infected with *Ehrlichia/Anaplasma* spp., 1.8% (5/280) with *Babesia* spp., 1.8% (5/280) with *Theileria spp*., 9.6% (27/280) with *Borrelia* spp., and 8.9% (25/280) with *Rickettsia* spp. (all *Rickettsia* spp. were specified as *R. helvetica*).

Of the *D. reticulatus* ticks 2.5% (1/40) was infected with *Babesia* spp and 1.4% of *I. hexagonus* ticks with *B. burgdorferi* sensu lato (subspecies sensu stricto) (1/72).

This research showed that ticks indigenous to the Netherlands harbor pathogens. Which can be dangerous for animals and humans. Humans should be aware of the danger and take precautions when entering tick-infested areas. As long as a tick is quickly removed, the chance of the transmission of pathogens is relatively small.

It can be concluded that there are different zoonotic pathogens present in the indigenous tick population in the Netherlands. Proper surveillance is important in order to keep track of the current pathogen prevalence status and possible exotic tick species entering the Netherlands. Finally, it is crucial to remain aware of emerging health risks, which are of medical and veterinary relevance.

Acknowledgements

The time has come to end this report and to look back at the great time I had at the UCTD.

I would like to thank Prof. Dr. F. Jongejan for giving me the opportunity to gain a lot of knowledge and practical skills, which will be useful for the rest of my career. It was my first encounter with performing scientific research and it showed me that there are more worlds to explore as a veterinarian. Also I would like to thank Michiel Wijnveld and Laura Berger for being so helpful and teaching me all the necessary laboratory techniques, which were a fundamental part of my research.

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Annexes

Annex 1: Protocol UCTD DNA extraction from ticks

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

DNA extraction from ticks

UCTD/13/001/2

17 Oct 2013

DNA EXTRACTION FROM TICKS

Room	
Number of samples	
Sample description	

Wear gloves and use filter pipet tips

Water bath ID	
Sonification bath ID	
TissueLyser LT ID	
Heating block ID	
Centrifuge ID	

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with sodium hypochloride.	
2	Turn on a water bath at 56°C.	
3	Take the proteinase K solution from the freezer and store at 4°C.	
4	Wash the ticks in a sonofication bath with demineralized water for up to 30 seconds.	
5	Put the ticks, with cleaned forceps, in 1.5ml tubes with 70% ethanol and vortex for several seconds.	
6	Wash the forceps in 70% ethanol followed by washing in demineralized water after each tick.	
7	Take the ticks from the tubes and let it dry on a clean tissue paper and place the dried ticks in a sterile 2ml tube with 180µl T1 lysis buffer.	
8	Freeze the samples at -80°C for 15 minutes.	
9	Add a 5 or 7mm (depending on tick size) metal bead to the frozen samples.	
10	Disrupt the ticks in the TissueLyser LT at 50 oscillations per second for 3 minutes.	
11	Briefly spin down the tubes. **1000x g maximum!**	
12	Add 25µl proteinase K and vortex.	
13	Prelyse the samples at 56°C in a water bath for 3 hours and vortex every hour.	
14	**During the incubation**; empty and clean the sonification bath.	
15	**During the last incubation hour**; turn on the heating block at 70°C and preheat the BE buffer.	
16	Briefly spin down the tubes. **1000x g maximum!**	
17	Add 200µl B3 buffer and vortex.	

18	Incubate the tubes at 70°C for 15 minutes.	
19	Briefly spin down the tubes. **1000x g maximum!**	
20	Add 210µl 96% ethanol, vortex and briefly spin down the tubes. **1000x g maximum!**	
21	Transfer the supernatant to new sterile 1.5ml tubes. (Tick parts are allowed to be transferred.)	
22	Centrifuge the tubes at 11,000x g for 2 minutes.	
23	Transfer the supernatant to spin columns. Avoid pipetting tick parts, as it can block the spin column.	
24	Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
25	Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
26	Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
27	Centrifuge the columns at 11,000x g for 1 minute.	
28	Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly.**	
29	Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.	
30	Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.	
31	Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.	
32	Turn off all equipment and clean working space with sodium hypochloride.	

DNA extraction done:

by ______________________ on ________________ ______________________________

Signature

Comments:

Annex 2: Protocol UCTD PCR RLB procedure

PCR RLB PROCEDURE

Rooms		
Number of samples		
Sample description		

Workstation 1 ID	
Workstation 2 ID	
PCR machine ID	

Wear (green) gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

Primers:	*Anaplasma Ehrlichia*	*Babesia Theileria*	*Borrelia*	*Rickettsia*	Other:

Reagent	1x	Number of samples + 10%	
PCR grade H_2O	15.875µl		
5x Phire reaction buffer	5.0µl		
10mM dNTPs	0.5µl		
Forward primer (20pmol/µl)	0.5µl		
Reverse primer (20pmol/µl)	0.5µl		
2U/µl Phire Hot Start II DNA polymerase	0.125µl		

		Done
1	Put DNA samples a (few) day(s) before the PCR at 4°C.	
2	Turn on the DNA workstations in the clean room and the dirty room.	
3	Clean workspace in both DNA workstations with sodium hypochloride.	
4	Label the PCR and Eppendorf tubes and put them in the DNA workstation in the clean room	
5	Turn on the UV-light in both DNA workstations for 20 minutes.	
6	**During the UV-light**; thaw the PCR reagents at room temperature, except the polymerase.	

7	Prepare the PCR mix in the Eppendorf tube(s). Multiply the reagent volumes by the number of samples plus 10% of the number of samples: 40 DNA samples + 1 PCR control = 41 + 10% = 45 samples.	
8	Pipet the master mix gently up and down to mix well.	
9	Pipet 22,5µl master mix to each PCR tube and add the leftover mix to an additional tube which will be the negative PCR control.	
10	Close the PCR tubes and remove them from the workstation, clean the workspace with sodium hypochloride and turn on the UV-light for 20 minutes.	
11	Take the closed PCR tubes to the dirty room and place them in the workstation.	
12	Vortex the DNA samples, spin them down briefly at 11,000x g and place them in the workstation.	
13	Add 2.5µl DNA sample to the corresponding PCR tube.	
14	Add 2.5µl of the positive control (, corresponding to the PCR to be performed,) to the positive PCR control tube.	
15	Vortex and spin down briefly.	
16	Clean the workstation with sodium hypochloide and turn on the UV-light for 20 minutes.	
17	Run the corresponding PCR program.	
18	Store the PCR products at 4°C for use within the next few days or store at -20°C for long term preservation.	
19	Turn off both DNA workstations after the UV-light is switched off.	

PCR done:

by ______________________ on ______________ ______________________________

Signature

Comments:

Annex 3: Protocol UCTD Agarose gel electrophoresis procedure

AGAROSE GEL ELECTROPHORESIS PROCEDURE

Room	
Number of samples	
Sample description	

Microwave ID	
Electrophoresis unit ID	
Gel-dock system ID	

Wear blue gloves and use non-filter pipet tips

Ethidiumbromide is carcinogenic!

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	For a large gel: add 2.25g agarose to an Erlenmeyer and add 150ml 1x TAE buffer. For small gel: add 0.563g agarose to an Erlenmeyer and add 37.5ml 1x TAE buffer.	
2	Heat the solution in a microwave until the agarose is fully dissolved.	
3	Let the agarose cool down until about 60°C, add 2.5µl ethidiumbromide (10mg/ml) and mix by swirling.	
4	**During the cooling down of the agarose solution**; prepare the gel tray by putting the rubber sides on the edges of the tray and placing the comb(s).	
5	Pour the agarose solution onto the tray. Air bubbles can be removed with a pipet tip.	
6	Leave the agarose solution until it has solidified into a gel.	
7	Remove the rubber sides and the comb(s) and place the gel with the tray in the electrophoresis unit.	
8	If needed, fill up the 1x TAE level in the electrophoresis unit until it fully covers the gel.	
9	Pipette 1µl 6x loading buffer in a well of a 96-wells plate for each sample.	
10	Add 5µl PCR product to the loading buffer, mix by pipetting up and down and load onto the gel.	
11	Pipette 5µl DNA marker and load onto the gel.	
12	Run the gel for 30-45 minutes and check the gel using the gel-dock system (LabWorks program).	
13	Discard the gel in the bio-waste bin and clean workspace.	

Gel electrophoresis done:

by ______________________ on ________________ ________________________________

Signature

Annex 4: Protocol UCTD Reverse line blot hybridization procedure

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

UCTD/13/006/3

28 Oct 2013

Reverse Line Blot Hybridization

REVERSE LINE BLOT HYBRIDIZATION PROCEDURE

Room	
Number of samples	
Sample description	

Heating block ID	
Water bath ID	
Hybridization oven ID	
Shaker ID	
Membrane ID	
Blotter ID	

Wear gloves and use non-filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with 70% ethanol.	
2	Turn on a heating block at 100°C.	
3	Turn on the hybridization oven at 42°C en preheat 50ml 2x SSPE/0.5% SDS solution.	
4	Turn on the water bath at 50°C en preheat the bottle with 2x SSPE/0.5% SDS solution.	
5	Combine and dilute the PCR products per DNA sample in a 1.5ml tube. Take 10µl of every PCR produc and add 2x SSPE/0.1% SDS to a final volume of 160µl. (10µl *Anaplasma/Ehrlichia* PCR + 10µl *Babesia/Theileria* PCR + 140µl 2x SSPE/0.1% SDS.)	
6	Take 10µl of the RLB positive controls and add 150µl 2x SSPE/0.1% SDS to a 1.5ml tube,	
7	Denature the diluted PCR samples and controls at 100°C for 10 minutes.	
8	**During the denaturation step**; wash the membrane at room temperature with 2X 2SSPE/0.1% SDS fo 5 minutes under gentle shaking and fill a bucket with ice.	
9	Immediately transfer the samples in order on ice after the denaturation.	
10	Prepare the miniblotter by placing the membrane on the lanes, with the line pattern of the membrane perpendicular to the lanes of the blotter. Place de support cushion on the membrane followed by the other half of the blotter. Turn the blotter right-side up without moving the membrane and turn the screws hand-tight,	
11	Remove residual fluid in the slots by aspiration.	
12	Briefly spin down the tubes at 4°C and place them back on ice in order.	
13	Fill the slots with the samples (150µl) and fill the first, last and other empty slots with 2x SSPE/0.1% SDS. **Avoid air bubbles.**	
14	Hybridize the blotter at 42°C for 60 minutes in the hybridization oven without shaking.	
15	Remove the samples by aspiration.	

16	Dissemble the blotter and remove the membrane from the blotter.		
17	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS at 50°C for 10 minutes under gentle shaking.		
18	**During the washing step**; clean the blotter and the support cushion.		
19	Incubate the membrane with 50ml 2x SSPE/0.5% SDS + 5µl streptavidin at 42°C for 30 minutes in the hybridization oven under gentle shaking. **Discard the streptavidin solution in a tube and into the bio-waste bin. Do not pour it in the sink.**		
20	**During the streptavidin hybridization**; change the water bath temperature to 42°C and preheat the bottle with 2x SSPE/0.5% SDS solution. Keep the lid open.		
21	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS solution at 42°C for 10 minutes under gentle shaking.		
22	Change the water bath temperature to 80°C and preheat the bottle with 1% SDS solution.		
23	Wash the membrane **twice** with 2x SSPE at room temperature for 5 minutes, under gentle shaking.		
24	**During the washing step**; prepare the foil and film cassette and check if the developing machine is on (5th floor).		
25	Add 10ml ECL (5ml ECL1 + 5ml ECL2) to the membrane and gently shake by hand until the whole membrane is covered. **Discard the ECL in a tube and into the bio-waste bin. Do not pour it in the sink.**		
26	Cover the membrane in foil and place it in the film cassette. **Avoid air bubbles.**		
27	Go to the dark room and expose a film to the membrane for 10 minutes.		
28	Develop the film with the developing machine.		
29	Strip the membrane or store it in a seal bag with 20mM EDTA at 4°C until stripping.		
30	Turn off all equipment and clean workspace.		

RLB hybridization done:

by ______________________ on ________________ ______________________________

Signature

Comments:

Annex 5: Protocol UCTD Membrane stripping procedure

MEMBRANE STRIPPING PROCEDURE

Room	

Water bath ID	
Shaker ID	
Membrane ID	

Wear gloves

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Turn on the water bath at 80°C and preheat the bottle with 1% SDS.	
2	Wash the membrane **twice** with preheated 1% SDS at 80°C for 30 minutes under gentle shaking.	
3	Wash the membrane with 20mM EDTA at room temperature for 15 minutes under gentle shaking.	
4	Store the membrane in a seal bag with 20mM EDTA at 4°C.	

Membrane stripping done:

by ______________________ on ________________ ________________________________

Signature

Comments:

Annex 6 Probes used for the membranes for the RLB

Membrane 3 UCTD

Lane	Name	Probe sequence (5' → 3')
1	*Ehrlichia / Anaplasma catch-all*	GGG GGA AAG ATT TAT CGC TA
2	*Anaplasma centrale*	TCG AAC GGA CCA TAC GC
3	*Anaplasma marginale*	GAC CGT ATA CGC AGC TTG
4	*Anaplasma phagocytophilum 1*	TTG CTA TAA AGA ATA ATT AGT GG
5	*Anaplasma phagocytophilum 3*	TTG CTA TGA AGA ATA ATT AGT GG
6	*Anaplasma phagocytophilum 5*	TTG CTA TAA AGA ATA GTT AGT GG
7	*Anaplasma phagocytophilum 7*	TTG CTA TAG AGA ATA GTT AGT GG
8	*Anaplasma bovis*	GTA GCT TGC TAT GRG AAC A
9	*Anaplasma platys*	GTC GTA GCT TGC TAT GAT A
10	*Ehrlichia canis*	CTC TGG CTA TAG GAA ATT GTT AGT GG
11	*Ehrlichia chaffeensis*	ACC TTT TGG TTA TAA ATA ATT GTT
12	*Ehrlichia ruminantium*	AGT ATC TGT TAG TGG CAG
13	*Ehrlichia sp. Omatjenne*	CGG ATT TTT ATC ATA GCT TGC
14	*Neoehrlichia mikurensis*	CGA ACG AAT TGT ARY TRT AGT TTA CT
15	*Theileria / Babesia catch-all*	TAA TGG TTA ATA GGA RCR GTT G
16	*Babesia catch-all 1*	ATT AGA GTG TTT CAA GCA GAC
17	*Babesia catch-all 2*	ACT AGA GTG TTT CAA ACA GGC
18	*Babesia felis*	TTA TGC GTT TTC CGA CTG GC
19	*Babesia divergens*	ACT RAT GTC GAG ATT GCA C
20	*Babesia microti*	GRC TTG GCA TCW TCT GGA
21	*Babesia bigemina*	CGT TTT TTC CCT TTT GTT GG
22	*Babesia bovis*	CAG GTT TCG CCT GTA TAA TTG AG
23	*Babesia rossi*	CGG TTT GTT GCC TTT GTG
24	*Babesia canis*	TGC GTT GAC CGT TTG AC
25	*Babesia canis 2*	TGG TTG GTT ATT TCG TTT TCG
26	*Babesia vogeli*	AGC GTG TTC GAG TTT GCC
27	*Babesia major*	TCC GAC TTT GGT TGG TGT
28	*Babesia caballi*	GTG TTT ATC GCA GAC TTT TGT
29	*Babesia caballi catch-all 2*	GCT TGA TTT TCG CTT CGC TT
30	*Theileria catch-all*	ATT AGA GTG CTC AAA GCA GGC
31	*Theileria equi*	TTC GTT GAC TGC GYT TGG
32	*Theileria equi-like*	TTC GTT GTG GCT TAG TTG GG
33	*Borrelia burgdorferi sensu lato*	CTT TGA CCA TAT TTT TAT CTT CCA
34	*Borrelia burgdorferi sensu stricto*	AAC ACC AAT ATT TAA AAA ACA TAA
35	*Borrelia garinii*	AAC ATG AAC ATC TAA AAA CAT AAA
36	*Borrelia afzelii*	AAC ATT TAA AAA ATA AAT TCA AGG
37	*Borrelia valaisiana*	CAT TAA AAA AAT ATA AAA AAT AAA TTT AAG G
38	*Rickettsia catch-all*	TTT AGA AAT AAA AGC TAA TAC CG
39	*Rickettsia conorii*	CTT GCT CCA GTT AGT TAG T
40	*Rickettsia helvetica*	GCT AAT ACC ATA TAT TCT CTA TG
41	*Rickettsia massiliae*	TGG GGC TTG CTC TAA TTA GT
42	*Rickettsia sp. (DnS14) / raoultii*	CTA ATA CCG CAT ATT CTC TAC G
43	*Theileria catch-all*	ATT AGA GTG CTC AAA GCA GGC

Membrane 4 UCTD

Lane	Name	Probe sequence (5' → 3')
1	*Ehrlichia / Anaplasma catch-all*	GGG GGA AAG ATT TAT CGC TA
2	*Anaplasma centrale*	TCG AAC GGA CCA TAC GC
3	*Anaplasma marginale*	GAC CGT ATA CGC AGC TTG
4	*Anaplasma phagocytophilum 1*	TTG CTA TAA AGA ATA ATT AGT GG
5	*Anaplasma phagocytophilum 3*	TTG CTA TGA AGA ATA ATT AGT GG
6	*Anaplasma phagocytophilum 5*	TTG CTA TAA AGA ATA GTT AGT GG
7	*Anaplasma phagocytophilum 7*	TTG CTA TAG AGA ATA GTT AGT GG
8	*Anaplasma bovis*	GTA GCT TGC TAT GRG AAC A
9	*Anaplasma platys*	GTC GTA GCT TGC TAT GAT A
10	*Ehrlichia canis*	CTC TGG CTA TAG GAA ATT GTT AGT GG
11	*Ehrlichia chaffeensis*	ACC TTT TGG TTA TAA ATA ATT GTT
12	*Ehrlichia ruminantium*	AGT ATC TGT TAG TGG CAG
13	*Ehrlichia sp. Omatjenne*	CGG ATT TTT ATC ATA GCT TGC
14	*Neoehrlichia mikurensis*	CGA ACG AAT TGT ARY TRT AGT TTA CT
15	*Theileria / Babesia catch-all*	TAA TGG TTA ATA GGA RCR GTT G
16	*Babesia catch-all 1*	ATT AGA GTG TTT CAA GCA GAC
17	*Babesia catch-all 2*	ACT AGA GTG TTT CAA ACA GGC
18	*Babesia felis*	TTA TGC GTT TTC CGA CTG GC
19	*Babesia divergens*	ACT RAT GTC GAG ATT GCA C
20	*Babesia microti*	GRC TTG GCA TCW TCT GGA
21	*Babesia bigemina*	CGT TTT TTC CCT TTT GTT GG
22	*Babesia bovis*	CAG GTT TCG CCT GTA TAA TTG AG
23	*Babesia rossi*	CGG TTT GTT GCC TTT GTG
24	*Babesia canis*	TGC GTT GAC CGT TTG AC
25	*Babesia canis 2*	TGG TTG GTT ATT TCG TTT TCG
26	*Babesia vogeli*	AGC GTG TTC GAG TTT GCC
27	*Babesia major*	TCC GAC TTT GGT TGG TGT
28	*Babesia caballi*	GTG TTT ATC GCA GAC TTT TGT
29	*Babesia caballi catch-all 2*	GCT TGA TTT TCG CTT CGC TT
30	*Babesia venatorum (=Babesia sp.(EU 1))*	CGA TTT CGC TTT TGG GAT T
31	*Theileria equi*	TTC GTT GAC TGC GYT TGG
32	*Theileria equi-like*	TTC GTT GTG GCT TAG TTG GG
33	*Borrelia burgdorferi sensu lato*	CTT TGA CCA TAT TTT TAT CTT CCA
34	*Borrelia burgdorferi sensu stricto*	AAC ACC AAT ATT TAA AAA ACA TAA
35	*Borrelia garinii*	AAC ATG AAC ATC TAA AAA CAT AAA
36	*Borrelia afzelii*	AAC ATT TAA AAA ATA AAT TCA AGG
37	*Borrelia valaisiana*	CAT TAA AAA AAT ATA AAA AAT AAA TTT AAG G
38	*Rickettsia catch-all*	TTT AGA AAT AAA AGC TAA TAC CG
39	*Rickettsia conorii*	CTT GCT CCA GTT AGT TAG T
40	*Rickettsia helvetica*	GCT AAT ACC ATA TAT TCT CTA TG
41	*Rickettsia massiliae*	TGG GGC TTG CTC TAA TTA GT
42	*Rickettsia sp. (DnS14) / raoultii*	CTA ATA CCG CAT ATT CTC TAC G
43	*Babesia venatorum (=Babesia sp.(EU 1))*	CGA TTT CGC TTT TGG GAT T