The transmission dynamics of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks

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Abstract

Silicone membranes have been used in this study to feed *Rhipicephalus sanguineus* ticks in an *in vitro* feeding system. The objective was to obtain high attachment rates so that the transmission dynamics of *Ehrlichia canis* could be studied. In the acquisition feedings the ticks were fed with blood infected with *E. canis*. The transmission feedings following the acquisition feedings were used to determine if *E. canis* transmission to uninfected blood could be established. The results of this study show that *E. canis* can be transmitted within 17 hours. Further research is needed to determine the minimum time required for transmission.

The same *in vitro* feeding system was used to study *Babesia canis* and *Babesia vogeli* transmission using *Dermacentor reticulatus* and *R. sanguineus* ticks respectively. Transmission of *B. vogeli* could not be established. *B. canis* transmission could however be established within 24 hours *in vitro*, but only after pre-feeding the ticks on rabbits before entering them in the *in vitro* feeding system.

During the *E. canis in vitro* feeding trials another *Rickettsia* spp. was transmitted. This *Rickettsia* spp. appeared already present in the ticks that were used for the *in vitro* feeding trials. Despite the use of several Rickettsia probes the exact species could not yet be determined.

Introduction

Brief history of *in vitro* artificial membrane feeding
For over 50 years researchers have been developing ways to feed ixodid ticks *in vitro* on artificial membranes. One of the first records dates back to 1956, when *Boophilus microplus* larvae were cultivated on the membrane of an embryonated hen egg.[10,14] In 1975 researchers were able to feed >50% of *B. microplus* larvae to repletion on enriched tissue culture medium through thin slices of cattle skin.[8,10] Eight years later researchers first started to use silicone membranes to feed the hard tick *Ixodes holocyclus*.[18] It took another eight years to feed the ixodid tick *Rhipicephalus appendiculatus* to repletion on a glue-impregnated Baudruche membrane.[10,20] But it was not until 1993 that silicone membranes successfully became in use, which led to significant improvement of *in vitro* feeding of hard ticks.[5,10] In two years researchers managed to complete the entire life-cycle of *Amblyomma hebraeum* on a silicone membrane. *A. hebraeum* was chosen as a model for the ixodid tick because of the broad range in feeding duration, hypostome length and weight increase between and within the instars. Their natural variety in morphological and physiological characteristics made the *in vitro* behavior applicable to other hard ticks.[10,11] In 2004 a Swiss research group designed silicone membranes that could mimic the elasticity of the skin. This allowed ticks to detach from the membrane without causing leakage inside the feeding unit, therefore creating better conditions for feeding ticks to repletion. The experimental setting used served as a base for years of further research.[9]

Applying and improving *in vitro* feeding techniques
Today the *in vitro* feeding techniques have been adapted to accommodate several species of ixodid ticks including *R. appendiculatus*, *Rhipicephalus evertsi evertsi* and *Dermacentor reticulatus*.[12] However, the *in vitro* feeding of *Rhipicephalus sanguineus* ticks in the past has proven to be difficult and the attachment rates remained low.[1,12] Therefore, the main objective of this study was to improve the experimental settings for *in vitro* feeding in such a way that *R. sanguineus* ticks could also be fed *in vitro* on blood infected with *Ehrlichia canis* to study the transmission dynamics. Furthermore *in vitro* transmission feedings were performed using *R. sanguineus* and *D. reticulatus* ticks infected with *Babesia vogeli* and *Babesia canis* respectively. In conclusion, an *in vitro* serum feeding was carried out using fetal bovine serum, again with *R. sanguineus* ticks.

The ticks and their pathogens
The *R. sanguineus* tick [Figure 1], or brown dog tick, is a widely distributed ixodid tick that feeds primarily on dogs. It is a three-host tick that is a known vector of a variety of pathogens including *E. canis* and *B. vogeli*.[3,13] The *D. reticulatus* tick [Figure 2] is also a three-host tick and is a known vector of *B. canis*.[19]

Figure 1: *Rhipicephalus sanguineus* ticks
Nymph, larva, female and male tick.

Figure 2: *Dermacentor reticulatus* ticks
Female and male tick.

Ehrlichiosis
In dogs, the Rickettsia *E. canis* causes canine monocytic ehrlichiosis (CME). During an infection, the bacteria invade the canine monocytes where they replicate. After an incubation period of 8-20 days the first symptoms can be witnessed. Although the clinical signs vary greatly, lethargy, anorexia, pyrexia, lymphadenomegaly, splenomegaly and hemorrhagic diathesis are amongst the more common ones being observed. If left treated insufficiently, the disease can become subclinical, chronic or even fatal.[6] The disease, like his vector, is slowly advancing and is becoming one of the most important tick-borne diseases in dogs.[1,12]

Babesiosis
Canine babesiosis is caused by *B. canis*, *B. vogeli* and *B. rossi*.[7] Al three Babesia species can cause babesiosis, but the severity of the clinical manifestations they induce vary between them.[17] Symptoms like anorexia, pale mucus membranes, icterus, pyrexia and splenic and hepatic enlargement can be found due to the haemolysis these parasites induce.[7] Because vectors of this disease are spread worldwide, the disease itself can also be found around the world giving it global significance.[17]

Materials and methods

Ticks
In feeding 1 *R. sanguineus* nymphs were used. The nymphs originated from adults collected in France that were allowed to feed on rabbits and produce eggs. In feeding 2-5, 8-9 and 11-13 adult *R. sanguineus* ticks were used that also originated from France. In feeding 6 adult *R. sanguineus* ticks were used that originated from South Africa and were infected there with *B. vogeli*. In feeding 7 adult *D. reticulatus* ticks were used that originated from South Africa and were infected with *B. canis*. In feeding 8-9, besides the adult *R. sanguineus* ticks, adult *D. reticulatus* ticks were used that originated from the Netherlands. In feeding 10 the adult *R. sanguineus* ticks infected with *B. vogeli* and adult *D. reticulatus* ticks infected with *B. canis* from South Africa were used after pre-feeding on rabbits.

Over the entire experimental period the following numbers of ticks were used:
128 uninfected *R. sanguineus* nymphs;
284 uninfected *R. sanguineus* adults (145♂, 139♀);
20 uninfected *D. reticulatus* adults (15♂, 5♀);
88 *B. vogeli* infected *R. sanguineus* adults (41♂, 47♀);
37 *B. canis* infected *D. reticulatus* adults (17♂, 20♀).

All ticks were stored in an incubator at 20°C and about 90% relative humidity.

Blood
Bovine blood was used to feed the ticks *in vitro*. At the start of every feeding fresh blood was collected from two heifers, alternating the cows per week. The heifers were stalled at the department of farm animal health in a small herd. Blood was collected in a sterile bottle using a sterile needle and catheter. The acquired amount of blood varied per feeding ranging from 150 ml to 500 ml. To prevent clotting, the blood was defibrinated manually by stirring it for 20 minutes with a sterile pipette. After defibrinating the blood, D (+) glucose was added in the laboratory in a dosage of 2 grams per liter and the blood was stored at 4°C. In case of *Babesia* spp. transmission feedings and the serum feeding, Gibco® gentamicin 50 mg/ml (15750-037, Invitrogen™) was added to the blood to prevent bacterial growth at a dosage of 5 µl per 10 ml blood.

Membranes
Silicone membranes were used in the *in vitro* feeding experiments. To prepare the membranes, first a layer of kitchen plastic film was spread over a glass sheet of 40x30 cm and fixed with adhesive tape. Eight 70x120 mm lens cleaning papers (EK1546027T, Tiffen®) were then spread out evenly on top of the plastic film and were fixed with adhesive tape as well. In order to create the proper characteristics of the membrane the following quantities and materials were used: 15 grams E4 silicone (Elastocil®, Wacker), 4.5 grams DC 200 silicone oil (85411, Sigma-Aldrich®), 2.9 grams 15% Hexane (34859, Sigma-Aldrich®) and a drop (± 0.05 grams) of colour paste FL RAL 9010 (Elastocil®, Wacker) to enhance membrane visibility. After mixing the ingredients, the silicone mixture was spread evenly over the lens cleaning papers using a 80 mm wide silicone scraper. In order to create membranes of proper thickness (70-110 µm) a clot of silicone mixture was spread out by moving the scraper several times in both vertical directions, up and down. After spreading out the mixture, the membranes were left to polymerize for at least 24 hours at room temperature. After polymerization the thickness was checked using micro calipers. Suitable membranes were impregnated with dog odor to make them more attractive to the ticks.

Feeding units
The feeding units were made of Plexiglas units (custom made by WSV Kunststoffen BV) that were 26 mm in diameter, 2 mm wall thickness and 45 mm high. Each unit had an acrylic glass ring fixed around it at 4 mm from the bottom. This allowed the unit to be placed in blood filled six-well plates without the bottom of the unit touching the bottom of the well. The bottom of the Plexiglas units was glued to the prepared silicone membranes using E4 silicone. The excess glue on the inside was removed with a small paintbrush and the units were then left to dry for at least three hours. One silicone membrane could be used to glue a maximum of four Plexiglas units. After drying the feeding units were separated using a № 12 scalpel (0104, Schwann-Morton®) and the excess membrane on the outside was removed from the feeding unit using a pair of scissors. The kitchen plastic film was carefully peeled of the membrane using blunt tweezers. To check the feeding unit for leaks, they were placed in a Petri dish with 70% percent ethanol for 20 minutes. The feeding units that did not leak were used for *in vitro* feedings. To make the membranes even more attractive a small amount of animal hair was cut into small pieces and placed directly on top of the membrane. For *R. sanguineus* ticks dog hair was used, for *D. reticulatus* ticks bovine hair was used. To keep the ticks inside the unit organza fabric was wrapped around a perforated stopper and placed on top of the Plexiglas unit.

In vitro feedings
Three types of feedings have been performed: acquisition feedings, transmission feedings and a serum feeding.
The acquisition feedings were used to infect *R. sanguineus* nymphs and adults with *E. canis*. To do so 1-2 ml of an *E. canis* cell culture (CDC strain no.251 in DH82 cells; Ivory Coast strain no.33 in DH82 cells in feeding 12 unit 5-8) was mixed with 11.4-10.4 ml bovine blood and distributed to the four outer wells of a sterile six-well cell culture plate (657160, Greiner Bio-One).
In transmission feedings 12.4 ml of clean bovine blood was distributed to the four outer wells of a sterile six-well cell culture plate.
In the serum feeding 6.2 ml of clean bovine blood was distributed to the first two wells of a sterile six-well cell culture plate. The second two wells held a mixture of 3.1 ml clean bovine blood and 3.1 ml Gibco® Foetal Bovine Serum (10106-169, Invitrogen™), the final two wells were filled with 6.2 ml Foetal Bovine Serum.
The *in vitro* feedings took place at 37°C in a thermostat-controlled water bath with an aquarium with a triangular metal cap inside the bath. A nearly 100% relative humidity was maintained inside the aquarium. This created ideal environmental conditions for the ticks. The entire setting was covered by a thick cloth to keep the ticks in the dark 24 hours/day, this to prevent disturbing the ticks during day-time. When the blood in the six-well plate reached 37°C the ticks were transferred from the incubator to the feeding units and placed inside the six-well plate. The plate was then put inside the inner aquarium were it could float in a 37°C solution. The feeding units were checked daily for attachment and mortality, removing all dead ticks from the units before continuing the feeding. Blood was changed daily in feedings 6-7 and 11, twice a day in feedings 1-4, 8-10 and 12 and three times a day in feedings 5 and 13. The blood samples were collected when the blood was refreshed and stored at -20°C.
When ending the feeding any large blood clots and other debris were removed from the ticks using two blunt tweezers. The ticks that were selected for a second feeding or were used in the serum feeding were returned to the incubator. Ticks that were selected for DNA extraction were cleaned again thoroughly in the sonification bath and then put on alcohol.

DNA extraction

Blood samples and ticks from the *in vitro* feeding experiments were tested for infections with *E. canis*, *B. vogeli* or *B. canis*. The DNA from the blood samples was extracted using NucleoSpin® Blood kits (740951.250, Macherey-Nagel) following manufacturers manual. The DNA from the ticks was extracted by first cutting the ticks into smaller pieces using a sterile № 10 scalpel (0483, Schreiber® instrumente) and then grinding the pieces in 2.0 ml tubes using a T10 Basic ULTRA-TURRAX® (3420000, IKA®). The DNA was then extracted using NucleoSpin® Tissue kits (740952.250, Macherey-Nagel) following manufacturers manual. The 100 µl DNA samples were stored at -20°C.

Polymerase Chain Reaction amplification[22]

After extraction the DNA samples were used in a polymerase chain reaction. Three different genus-specific primer sets were used, one set for each pathogen. The DNA of the ticks and blood samples infected with *E. canis* was amplified using *Ehrlichia/Anaplasma* primers. In feedings 4-5 and 8-9 *Rickettsia* primers were used beside the *Ehrlichia/Anaplasma* primers. The DNA of the ticks and blood samples infected with *Babesia* spp. was amplified using *Babesia/Theileria* primers. The reverse primers contained a Biotin label on the 5' end to be used in the Reverse Line blot hybridization. [Table 1: primers and their sequence]

Table 1: primers and their sequence

Pathogen	Primers	Sequence
Ehrlichia	Ehr-F	5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG
	Ehr-R	5'-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT
Rickettsia	Rick-F1	5'-GAA CGC TAT CGG TAT GCT TAA CAC A
	Rick-R2	5'-Biotin-CAT CAC TCA CTC GCT ATT GCT GGA
Babesia	RLB-F2	5'-GAC ACA GGG AGG TAG TGA CAA G
	RLB-R2	5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT

To start a PCR the following mixture was added to 2.5 µl of each DNA sample: 15.875 µl H_2O; 5.0 µl 5x Phire reaction buffer; 0.5 µl 10 mM dNTPs; 0.5 µl Foreward primer; 0.5 µl Reverse primer and 0.125 µl 2U/µl Phire® Hot Start II DNA polymerase (F-122L, Fermentas). The Arktik™ Thermal Cycler PCR machine (TCA 0096, Thermo scientific) was then set to amplify the selected DNA fragment by first increasing the temperature to 98°C to denature the DNA. The two strands separated and the temperature dropped to allow the primers to bind to the DNA strands. The temperature increased again to 72°C to allow the DNA polymerase to synthesize double strands of DNA. In the first 10 cycles the annealing temperature dropped 1°C every cycle creating a touchdown PCR program. Besides the ticks and blood samples a positive and negative test control was also taken along to see if the PCR was successfully.
[Table 2: thermo cycler program for PCR]

Table 2: thermo cycler program PCR

№ Cycles	Time	Temperature
1 cycle	30 sec	98°C
10 cycles	05 sec	98°C
	05 sec	67→57°C
	07 sec	72°C
50 cycles	05 sec	98°C
	05 sec	57°C
	07 sec	72°C
1 cycle	60 sec	72°C

Agarose gel electrophoresis[23]

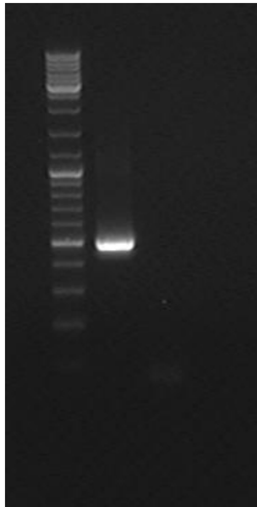

Figure 3

To check the PCR a gel was run on the positive and negative test control. A 1.5% gel was prepared using the UCTD protocol. The samples were mixed with a 6x DNA loading dye (R0611, Fermentas) and loaded into the sample wells with a reference 100bp DNA ladder (GeneRuler™, SM0331, Fermentas). The gel was left to run for 30-45 minutes. The negative charge of the DNA caused it to run toward the positive side of the gel. Larger DNA fragments migrate slower than smaller DNA fragments causing separation of the fragments by size. The loading dye provided coloring of the samples so you could see the progress of the samples running through the gel. After sufficient migration the gel was observed under an UV-illuminator (AutoChemie™ system, UVP BioImaging Systems). A successful PCR was visible as only one fragment of around 500bp size in the positive sample and none in the negative sample with the DNA ladder as a reference of fragment size. [Figure 3]

Reverse Line Blot hybridization[21]

To detect the pathogens in the tick and blood samples reverse line blot hybridization was used. The RLB was executed following the protocol developed by UCTD. A miniblotter (Miniblotter MN45, ISOGEN) was used together with a 45 μm Biodyne C membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA). The membrane contained covalently bonded oligonucleotides using a C6-aminolinker on the 5' end of the probes. These oligonucleotides (probes) can bind specific DNA fragments that are unique to certain pathogens. The amplified DNA samples were first diluted in a buffer and then denaturized on a heating block. The samples were cooled on ice to keep the DNA denaturized before applying onto the membrane using the blotter. [Figure 4]

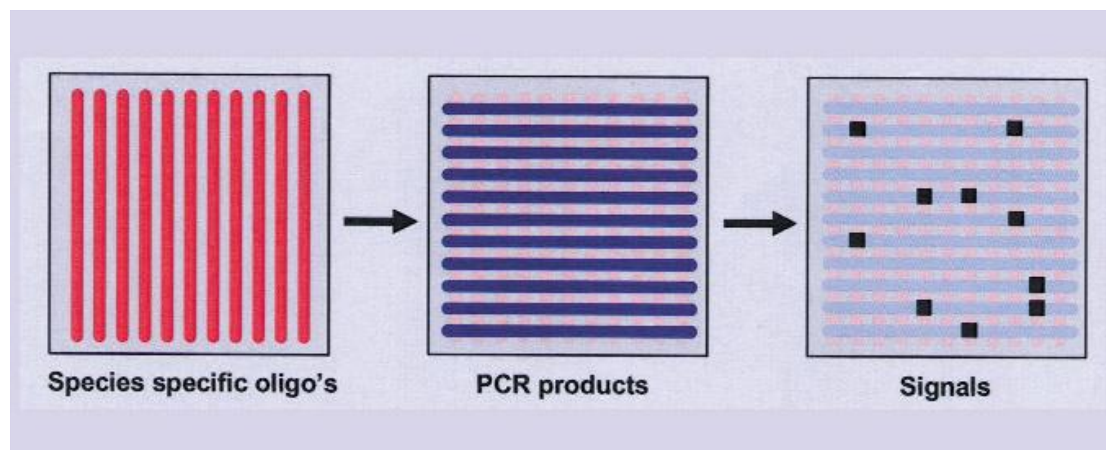

Figure 4: schematic representation of a RLB assay

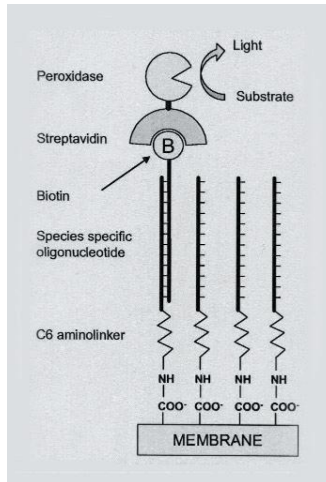

Figure 5: RLB principle

The DNA samples were then left to hybridize with the probes on the membrane for 60 minutes at 42°C. A series of washing steps followed to ensure only the DNA complex that bonded to the probes remained. To demonstrate the probe-DNA complex, a Straptavidin–Peroxidase conjugate was used as shown in figure 5. By adding Amersham ECL detection reagents (RPN2105, GE Healthcare Life Science) as a substrate for chemical luminescence and exposing Amersham Hyperfilm™ ECL (28-9068-37, GE Healthcare Life Science) to the membrane for 10 minutes and developing it, the results were made visible. Figure 5 shows a schematic representation of the Reverse Line Blot principle [Figure 5].

<u>Protocols</u>

Protocols used in the *in vitro* feedings: appendix I.
Protocols used in the tick and blood sample analysis: appendix II.

Results

F1: acquisition feeding *Ehrlichia canis*

In F1 a total of 128 *R. sanguineus* nymphs were distributed over 4 feeding units and placed on 10.4 ml bovine blood infected with 2.0 ml *E. canis* cell culture. The blood was warmed to 37°C before it was mixed with the 37°C *E. canis* cell culture. The nymphs used were pre-fed for three days on rabbits before entering the feeding.

After 24 hours only 11 nymphs attached to the membranes and after 48 hours all but one had detached again from the membrane and mortality became very high in one unit. After 72 hours mortality became high in all four units reaching up to 60% in one unit. All live nymphs were put on alcohol to be tested for an *E. canis* infection. Only 4 out of 40 nymphs tested (vaguely) positive. [Figure 6]

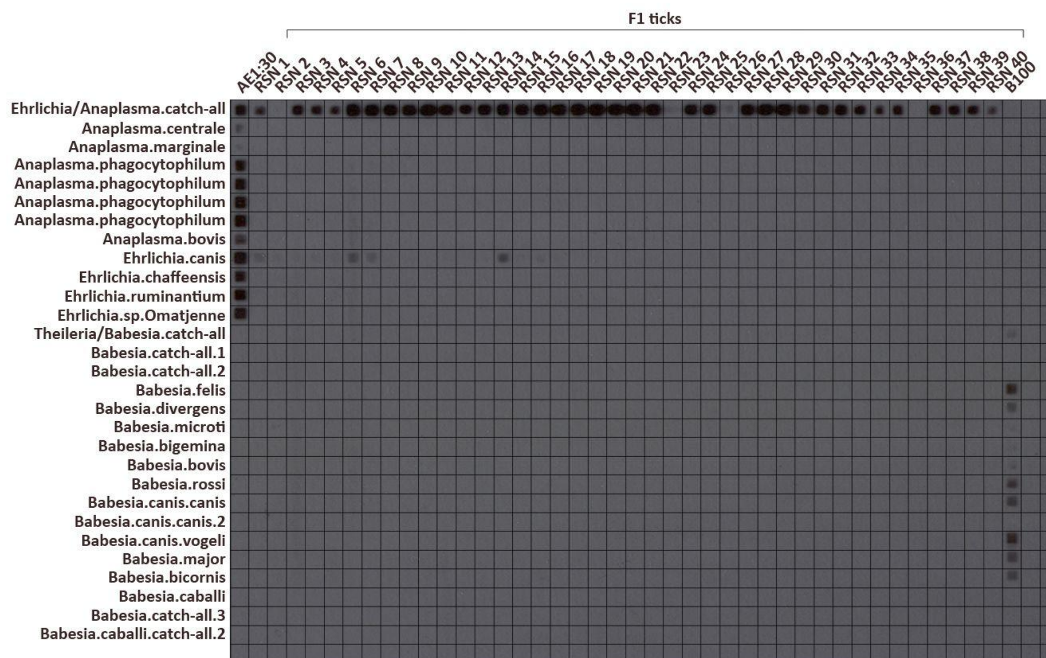

Figure 6: RLB F1

F2: acquisition feeding *Ehrlichia canis*

In F2 adult *R. sanguineus* ticks were put on 10.4 ml bovine blood infected with 2.0 ml *E. canis* cell culture. The blood was warmed to 37°C before it was mixed with the 37°C *E. canis* cell culture. On day 1 a total of 40 ticks were distributed over four feeding units. Blood was changed twice daily. After 36 hours all live ticks from unit 1 were transferred to a spare unit with some tick feces as an extra attractant because of leakage of the unit. After 90 hours all live male ticks were returned to the incubator for 3 days till being reused in F3. All female and dead male ticks were put on alcohol and tested for an *E. canis* infection. After blotting, 15 out of 22 ticks tested positive.

[Table 3: overview F2][Figure 7: RLB F2]

Table 3: overview F2

Time	Unit	Attached			Unattached			Mortality			Total		
t=24	U1	6			3			1		1♀	10	5♂	5♀
	U2	8			1			1		1♀	10	5♂	5♀
	U3	9			1			0			10	5♂	5♀
	U4	6			4			0			10	5♂	5♀
	Mean	7,3			2,3			0,5			10,0		
	%	72,5			22,5			5,0			100		
t=36	U1	6			3			0			9	5♂	4♀
	U2	8	5♂	3♀	1		1♀	0			9	5♂	4♀
	U3	8	4♂	4♀	2	1♂	1♀	0			10	5♂	5♀
	U4	6	3♂	3♀	4	2♂	2♀	0			10	5♂	5♀
	Mean	7,0			2,5			0,0			9,5		
	%	73,7			26,3			0,0			100		
t=60	U1	8	4♂	4♀	1	1♂		0			9	5♂	4♀
	U2	8	5♂	3♀	1		1♀	0			9	5♂	4♀
	U3	8	5♂	3♀	2		2♀	0			10	5♂	5♀
	U4	8	4♂	4♀	1	1♂		1		1♀	10	5♂	5♀
	Mean	8,0			1,3			0,3			9,5		
	%	84,2			13,2			2,6			100		
t=90	U1	9	5♂	4♀	0			0			9	5♂	4♀
	U2	8	4♂	4♀	0			1	1♂		9	5♂	4♀
	U3	7	4♂	3♀	2		2♀	1	1♂		10	5♂	5♀
	U4	7	4♂	3♀	0			2	1♂	1♀	9	5♂	4♀
	Mean	7,8			0,5			1,0			9,3		
	%	83,8			5,4			10,8			100		

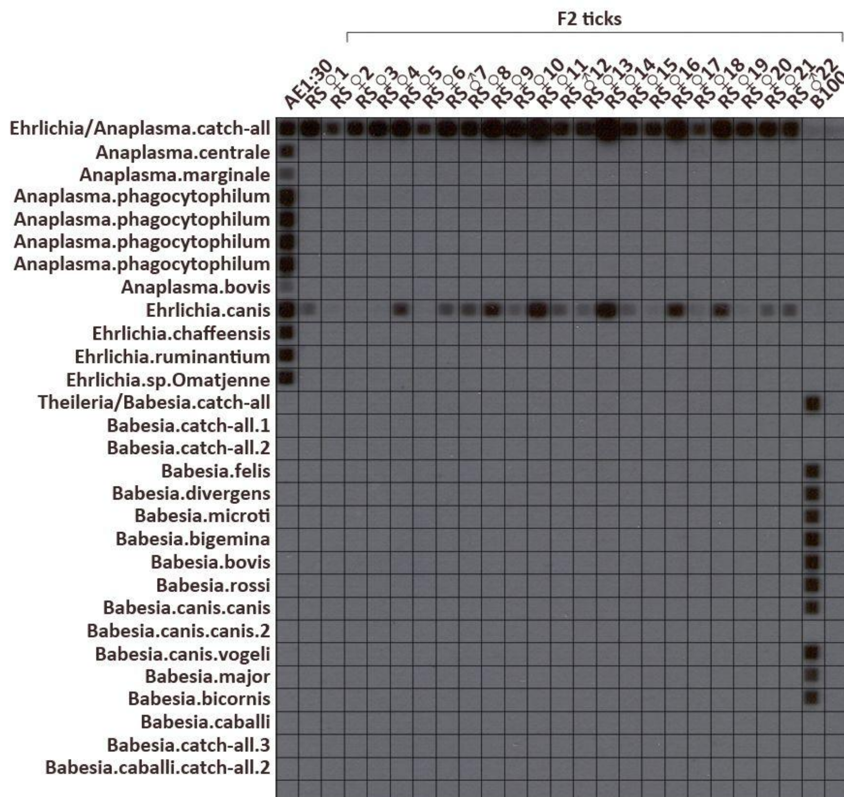

Figure 7: RLB F2

F3: transmission feeding *Ehrlichia canis*

In F3 the adult male *R. sanguineus* ticks from F2 were used together with uninfected female *R. sanguineus* ticks to determine if transmission of *E. canis* to blood could be established. On day 1 16 males and 14 females were distributed over three feeding units. A fourth feeding unit was used as a control unit and therefore held no ticks. Every time blood samples were taken, the blood was changed as well. After 89 hours in feeding all of the ticks were put on alcohol and tested for an *E. canis* infection. Only the first 40 blood samples were tested for an *E. canis* infection. After blotting, 13 out of 30 ticks tested (vaguely) positive. The blood samples showed transmission within 17 hours. Control unit 4 also showed a positive hit on *E. canis*. [Table 4: overview F3][Figure 8: RLB F3]

Table 4: overview F3

Time	Unit	Attached			Unattached			Mortality			Total		
t=17	U1	7	6♂	1♀	3		3♀	0			10	6♂	4♀
	U2	7	4♂	3♀	2	1♂	1♀	1		1♀	10	5♂	5♀
	U3	7	5♂	2♀	3		3♀	0			10	5♂	5♀
	Mean	7,0			2,7			0,3			10,0		
	%	70,0			26,7			3,3			100		
t=41	U1	7	5♂	2♀	3	1♂	2♀	0			10	6♂	4♀
	U2	5	3♂	2♀	3	2♂	1♀	1		1♀	9	5♂	4♀
	U3	6	5♂	1♀	4		4♀	0			10	5♂	5♀
	Mean	6,0			3,3			0,3			9,7		
	%	62,1			34,5			3,4			100		
t=65	U1	8	6♂	2♀	2		2♀	0			10	6♂	4♀
	U2	5	4♂	1♀	3	1♂	2♀	0			8	5♂	3♀
	U3	6	5♂	1♀	4		4♀	0			10	5♂	5♀
	Mean	6,3			3,0			0,0			9,3		
	&	67,9			32,1			0,0			100		
t=89	U1	8	6♂	2♀	2		2♀	0			10	6♂	4♀
	U2	2	2♂		5	2♂	3♀	1	1♂		8	5♂	3♀
	U3	6	5♂	1♀	4		4♀	0			10	5♂	5♀
	Mean	5,3			3,7			0,3			9,3		
	%	57,1			39,3			3,6			100		

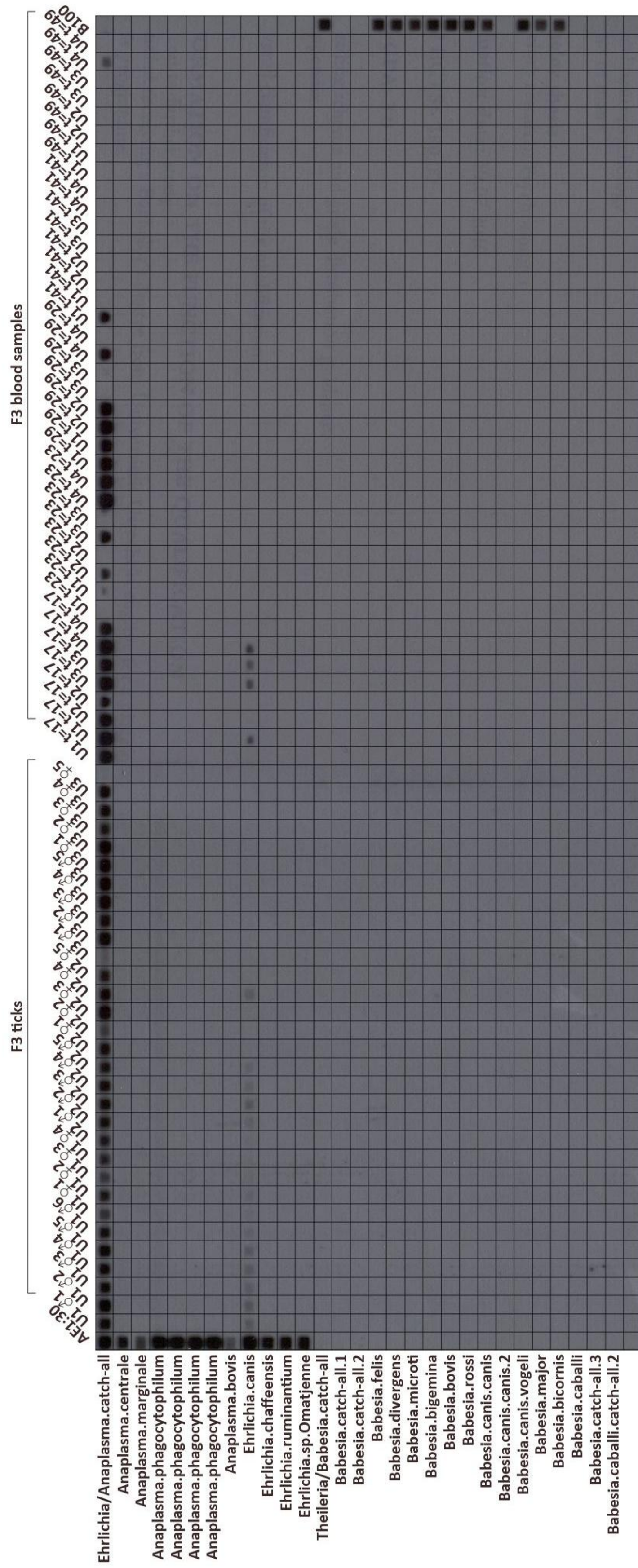

Figure 8: RLB F3

F4: acquisition feeding *Ehrlichia canis*

In F4 adult *R. sanguineus* ticks were put on 10.4 ml bovine blood infected with 2.0 ml *E. canis* cell culture. The blood was warmed to 37°C before it was mixed with the 37°C *E. canis* cell culture. On day 1 a total of 40 ticks were distributed over four feeding units. Blood was changed twice daily and after 92 hours the live male ticks that had been attached were returned to the incubator for 4 days till being reused in F5. The female ticks that were attached or showed signs of blood intake were tested for an *E. canis* infection and for infection with another Rickettsia species. One of the female ticks showed a vaguely positive signal for *E. canis*. All females showed clear Rickettsia catch-all signals with cross reactions with *R. massiliae* and *R. raoultii*. [Table 5: overview F4][Figure 9: RLB F4 and F5]

Table 5: overview F4

Time	**Unit**	**Attached**			**Unattached**			**Mortality**			**Total**		
t=16	U1	1		1♀	9	5♂	4♀	0			10	5♂	5♀
	U2	5			5			0			10	5♂	5♀
	U3	0			10	5♂	5♀	0			10	5♂	5♀
	U4	4	4♂		6	1♂	5♀	0			10	5♂	5♀
	Mean	2,5			7,5			0,0	0		10,0		
	%	25,0			75,0			0,0			100		
t=40	U1	2		2♀	7	4♂	3♀	1	1♂		10	5♂	5♀
	U2	6	5♂	1♀	3		3♀	1		1♀	10	5♂	5♀
	U3	3	2♂	1♀	7	3♂	4♀	0			10	5♂	5♀
	U4	6	4♂	2♀	3	1♂	2♀	1		1♀	10	5♂	5♀
	Mean	4,3			5,0			0,8			10,0		
	%	42,5			50,0			7,5			100		
t=64	U1	5	2♂	3♀	3	1♂	2♀	1	1♂		9	4♂	5♀
	U2	6	5♂	1♀	3		3♀	0			9	5♂	4♀
	U3	1		1♀	8	4♂	4♀	1	1♂		10	5♂	5♀
	U4	6	4♂	2♀	2	1♂	1♀	1		1♀	9	5♂	4♀
	Mean	4,5			4,0			0,8			9,3		
	%	48,6			43,2			8,1			99,9		
t=92	U1	5	2♂	3♀	3	1♂	2♀	0			8	3♂	5♀
	U2	7	4♂	3♀	1		1♀	1	1♂		9	5♂	4♀
	U3	1		1♀	8	4♂	4♀	0			9	4♂	5♀
	U4	7	4♂	3♀	1	1♂		0			8	5♂	3♀
	Mean	5,0			3,3			0,3			8,5		
	%	58,8			38,2			2,9			99,9		

F5: transmission feeding *Ehrlichia canis*

In F5 the male *R. sanguineus* ticks from F4 were used to determine if transmission of *E. canis* to blood could be established. On day 1 the male ticks were distributed over unit 1 and unit 2 and 5 uninfected female ticks were added to each unit. After six hours unit 2 appeared to be leaking and all the males were transferred to unit 1. The female ticks in unit 2 were excluded from further experiment. Every time blood samples were taken, the blood was changed as well. After 48 hours in feeding all males were put on alcohol and tested for an *E. canis* infection and for infection with another Rickettsia species. One of the male ticks showed a positive signal for *E. canis*. All males showed clear Rickettsia catch-all signals with cross reactions with *R. massiliae* and *R. raoultii*. The blood samples tested negative for *E. canis* but positive for the other *Rickettsia* spp. after 12 hours.

[Table 6: overview F5][Figure 9: RLB F4 and F5]

Table 6: overview F5

Time	Unit	Attached	Unattached	Mortality	Total
t=6	U1	0	9 4♂ 5♀	0	9 4♂ 5♀
	U2	0	10 5♂ 5♀	0	10 5♂ 5♀
	Mean	0,0	9,5	0,0	9,5
	%	0,0	100	0,0	100
t=12	U1	9 6♂ 3♀	5 3♂ 2♀	0	14 9♂ 5♀
	%	64,3	35,7	0,0	100
t=24	U1	12 8♂ 4♀	2 1♂ 1♀	0	14 9♂ 5♀
	%	85,7	14,3	0,0	100
t=48	U1	7 4♂ 3♀	7 5♂ 2♀	0	14 9♂ 5♀
	%	50,0	50,0	0,0	100

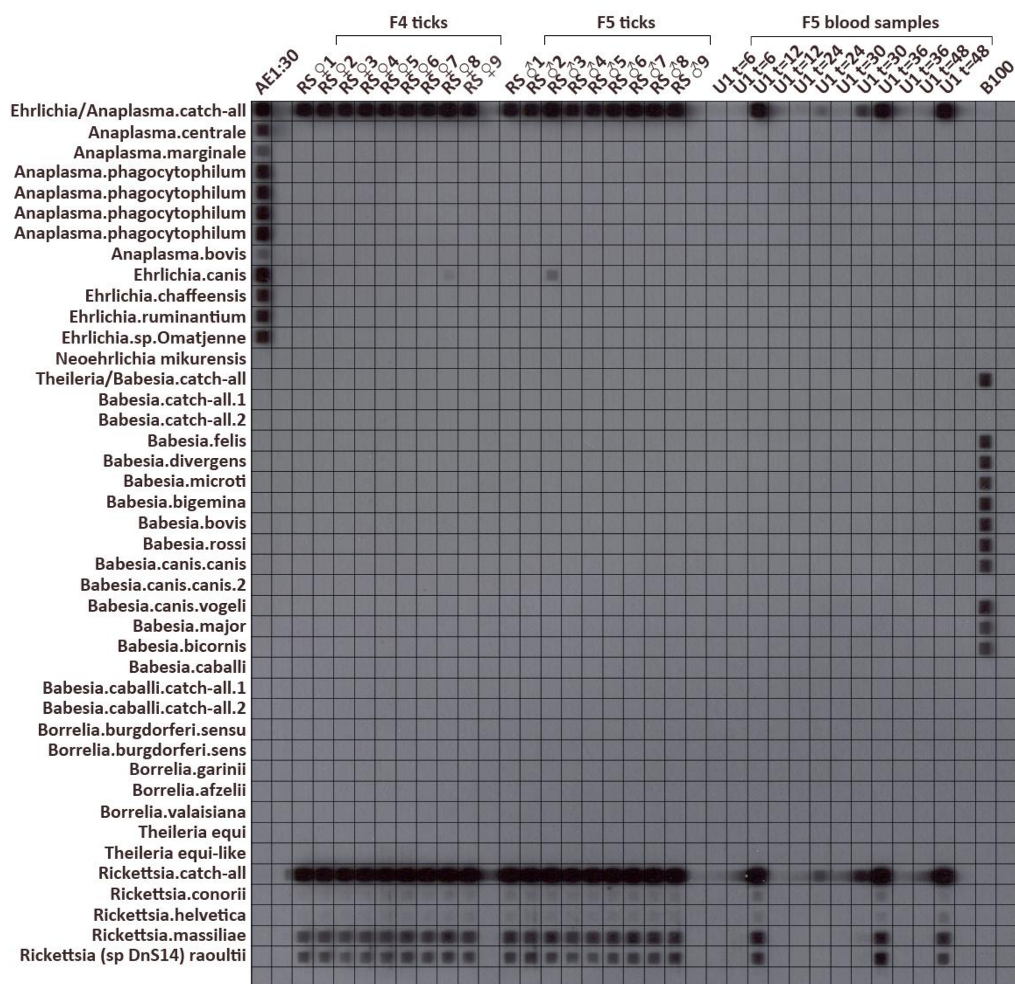

Figure 9: RLB F4 and F5

F6: transmission feeding *Babesia vogeli*

In F6 adult *R. sanguineus* infected with *B. vogeli* were used to determine if transmission to blood could be established.
On day 1 eight units with a total of 80 ticks (10 per unit: 5♂, 5♀) were entered into the feeding on clean bovine blood. After 24 hours the first 4 units showed no attachment at all and were excluded from the experiment. Both units 5 and 8 each showed only one male tick attached. Unit 6 and 7 had no attachments but were kept in the experiment to see if they would establish attachment at a later time. After 84 hours only one attachment remained in unit 8 and the mortality rate in all units started to increase drastically. Therefore all unattached ticks were eliminated from further experiment after 84 hours.
The male tick in unit 8 was attached from 24 to at least 102 hours.
Blood was initially changed daily, and after 84 hours changed every time blood samples were taken. After F6 was terminated, both tick and blood samples were tested for a *B. vogeli* infection. The tick appeared to be uninfected with *B. vogeli* and therefore could not have established a transmission to the blood. However, on t=84 and t=102 two positive Babesia catch-all signals were witnessed in the blood samples. [Table 7: overview F6][Figure 10: RLB F6 and F7]

Table 7: overview F6

Time	**Unit**	**Attached**	**Unattached**	**Mortality**	**Total**
t=54	U5	1 1♂	9 4♂ 5♀	0	10 5♂ 5♀
	U6	0	10 5♂ 5♀	0	10 5♂ 5♀
	U7	0	10 5♂ 5♀	0	10 5♂ 5♀
	U8	1 1♂	9 4♂ 5♀	0	10 5♂ 5♀
	Mean	0,5	9,5	0,0	10,0
	%	5,0	95,0	0,0	100
t=84	U5	0	7	3	10 5♂ 5♀
	U6	0	2	8	10 5♂ 5♀
	U7	0	8	2	10 5♂ 5♀
	U8	1 1♂	9 4♂ 5♀	0	10 5♂ 5♀
	Mean	0,3	6,5	3,3	10,0
	%	2,5	65,0	32,5	100
t=102	U8	1 1♂	0	0	1 1♂
	%	100	0,0	0,0	100
t=126	U8	0	1 1♂	0	1 1♂
	%	0,0	100	0,0	100

F7: transmission feeding *Babesia canis*

In F7 adult *D. reticulatus* infected with *B. canis* were used to determine if transmission to blood could be established. On day 1 three units were entered with a total of 26 ticks. Every time blood samples were taken, the blood was changed as well. After F7 was terminated, all ticks that were attached or showed signs of blood intake were tested for a *B. canis* infection. Even though the ticks themselves were highly infected with *B. canis*, there was no transmission of *B. canis* demonstrable. Furthermore, the ticks showed positive signal on the Babesia divergens probe and a weak signal on the Babesia major probe.

[Table 8: overview F7][Figure 10: RLB F6 and F7]

Table 8: overview F7

Time	Unit	Attached			Unattached			Mortality			Total		
t=18	U1	7	4♂	3♀	3	1♂	2♀	0			10	5♂	5♀
	U2	5	3♂	2♀	4	1♂	3♀	1	1♂		10	5♂	5♀
	U3	2	1♂	1♀	3	2♂	1♀	1		1♀	6	3♂	3♀
	Mean	4,7			3,3			0,7			8,7		
	%	53,8			38,5			7,7			100		
t=42	U1	9	4♂	5♀	1	1♂		0			10	5♂	5♀
	U2	4	2♂	2♀	4	1♂	3♀	1	1♂		9	4♂	5♀
	U3	1	1♂		4	2♂	2♀	0			5	3♂	2♀
	Mean	4,7			3,0			0,3			8,0		
	%	58,3			37,5			4,2			100		
t=66	U1	7	3♂	4♀	3	2♂	1♀	0			10	5♂	5♀
	U2	2	1♂	1♀	4	2♂	2♀	2		2♀	8	3♂	5♀
	U3	1	1♂		3	2♂	1♀	1		1♀	5	3♂	2♀
	Mean	3,3			3,3			1,0			7,7		
	%	43,5			43,5			13,0			100		
t=90	U1	5	2♂	3♀	5	3♂	2♀	0			10	5♂	5♀
	U2	5	3♂	2♀	0			1		1♀	6	3♂	3♀
	U3	1	1♂		2	1♂	1♀	1	1♂		4	3♂	1♀
	Mean	3,7			2,3			0,7			6,7		
	%	55,0			35,0			10,0			100		
t=108	U1	7	3♂	4♀	3	2♂	1♀	0			10	5♂	5♀
	U2	3	2♂	1♀	2	1♂	1♀	0			5	3♂	2♀
	U3	2	2♂		1		1♀	0			3	2♂	1♀
	Mean	4,0			2,0			0,0			6,0		
	%	66,7			33,3			0,0			100		

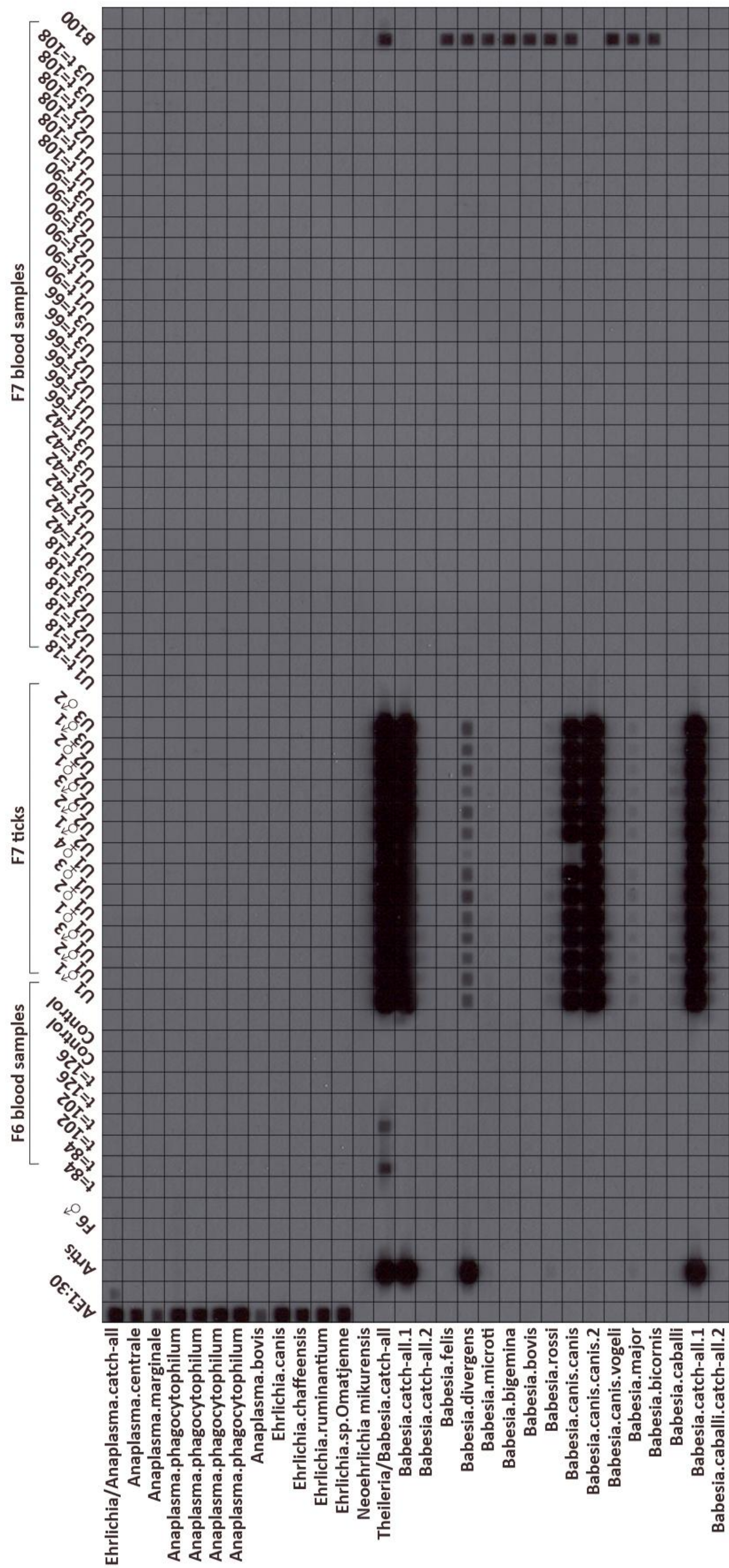

Figure 10: RLB F6 and F7

F8: acquisition feeding *Ehrlichia canis*

In F8 adult *R. sanguineus* and *D. reticulatus* ticks were put on 11.4 ml bovine blood infected with 1.0 ml *E. canis* cell culture in order to infect the ticks with *E. canis*. The *E. canis* cell culture was stored at 4°C and mixed with the bovine blood before warming to 37°C. On day 1 20 *R. sanguineus* ticks were put in unit 1 and unit 2, 20 *D. reticulatus* ticks were put in unit 3 and unit 4. Unit 1 and 3 contained 5♂ and 5♀ ticks, unit 2 and 4 contained 10♂ ticks. A fifth control well was filled with blood to check for contaminations. The ticks were put on uninfected blood. After eight hours the units were examined to detect if attachment was apparent and the *E. canis* cell culture was added to the newly offered blood except for the control well. Once a day the offered blood was sampled to see if the working methods were adequate and twice a day the blood was changed to prevent bacterial growth.

After 72 hours in feeding and 64 hours on infected blood, the live male ticks were returned to the incubator for 4 days till being reused in F9. The female ticks that were attached or showed signs of blood intake were tested for an *E. canis* infection and for infection with another Rickettsia species. None of the female ticks became infected with *E. canis*. All females did show clear Rickettsia catch-all signals with the *R. sanguineus* ticks showing cross reactions with *R. massiliae* and *R. raoultii* and all but one *D. reticulatus* ticks showing a *R. raoultii* infection. Transmission of the other *Rickettsia* spp. was visible in the blood samples after 24 hours. The blood samples also showed a vague Borrelia valaisiana signal.

[Table 9: overview F8][Figure 11: RLB F8 and F9]

Table 9: overview F8

Time	Unit	Attached	Unattached	Mortality	Total
t=8	U1	3	7	0	10 5♂ 5♀
	U2	8 8♂	2 2♂	0	10 10♂
	U3	3 2♂ 1♀	7 3♂ 4♀	0	10 5♂ 5♀
	U4	6 6♂	4 4♂	0	10 10♂
	Mean	5,0	5,0	0,0	10,0
	%	50,0	50,0	0,0	100
t=24	U1	6 2♂ 4♀	4 3♂ 1♀	0	10 5♂ 5♀
	U2	9 9♂	1 1♂	0	10 10♂
	U3	7 4♂ 3♀	3 1♂ 2♀	0	10 5♂ 5♀
	U4	9 9♂	1 1♂	0	10 10♂
	Mean	7,8	2,3	0,0	10,0
	%	77,5	22,5	0,0	100
t=48	U1	10 5♂ 5♀	0	0	10 5♂ 5♀
	U2	9 9♂	1 1♂	0	10 10♂
	U3	6 4♂ 2♀	4 1♂ 3♀	0	10 5♂ 5♀
	U4	9 9♂	1 1♂	0	10 10♂
	Mean	8,5	1,5	0,0	10,0
	%	85,0	15,0	0,0	100
t=72	U1	10 5♂ 5♀	0	0	10 5♂ 5♀
	U2	8 8♂	1 1♂	1 1♂	10 10♂
	U3	6 4♂ 2♀	4 1♂ 3♀	0	10 5♂ 5♀
	U4	7 7♂	3 3♂	0	10 10♂
	Mean	7,8	2,0	0,3	10,0
	%	77,5	20,0	2,5	100

F9: transmission feeding *Ehrlichia canis*

In F9 the adult male *R. sanguineus* and *D. reticulatus* ticks from F8 were used to determine if transmission of *E. canis* to blood could be established. On day 1 13 male *R. sanguineus* ticks were put in unit 1 and 15 male *D. reticulatus* ticks in unit 2. Every time blood samples were taken, the blood was changed as well. After 72 hours in feeding the attached males were put on alcohol and tested for an *E. canis* infection and for infection with another Rickettsia species. None of the male ticks became infected with *E. canis*. All males showed clear Rickettsia catch-all signals with the *R. sanguineus* ticks showing cross reactions with *R. massiliae* and *R. raoultii* and the *D. reticulatus* ticks showing a *R. raoultii* infection. Transmission of the other *Rickettsia* spp. was visible in the blood samples after 8 hours. The blood samples also showed a weak positive B. canis signal from t=24 to t=56. [Table 10: overview F9][Figure 11: RLB F8 and F9]

Table 10: overview F9

Time	Unit	Attached	Unattached	Mortality	Total
t=8	U1	8 8♂	5 5♂	0	13 13♂
	U2	8 8♂	7 7♂	0	15 15♂
	Mean	8,0	6,0	0,0	14,0
	%	57,1	42,9	0,0	100
t=24	U1	9 9♂	4 4♂	0	13 13♂
	U2	7 7♂	7 7♂	1 1♂	15 15♂
	Mean	8,0	5,5	0,5	14,0
	%	57,1	39,3	3,6	100
t=48	U1	9 9♂	2 2♂	2 2♂	13 13♂
	U2	6 6♂	8 8♂	0	14 14♂
	Mean	7,5	5,0	1,0	13,5
	%	55,6	37,0	7,4	100
t=72	U1	8 8♂	1 1♂	2 2♂	11 11♂
	U2	7 7♂	7 7♂	0	14 14♂
	Mean	7,5	4,0	1,0	12,5
	%	60,0	32,0	8,0	100

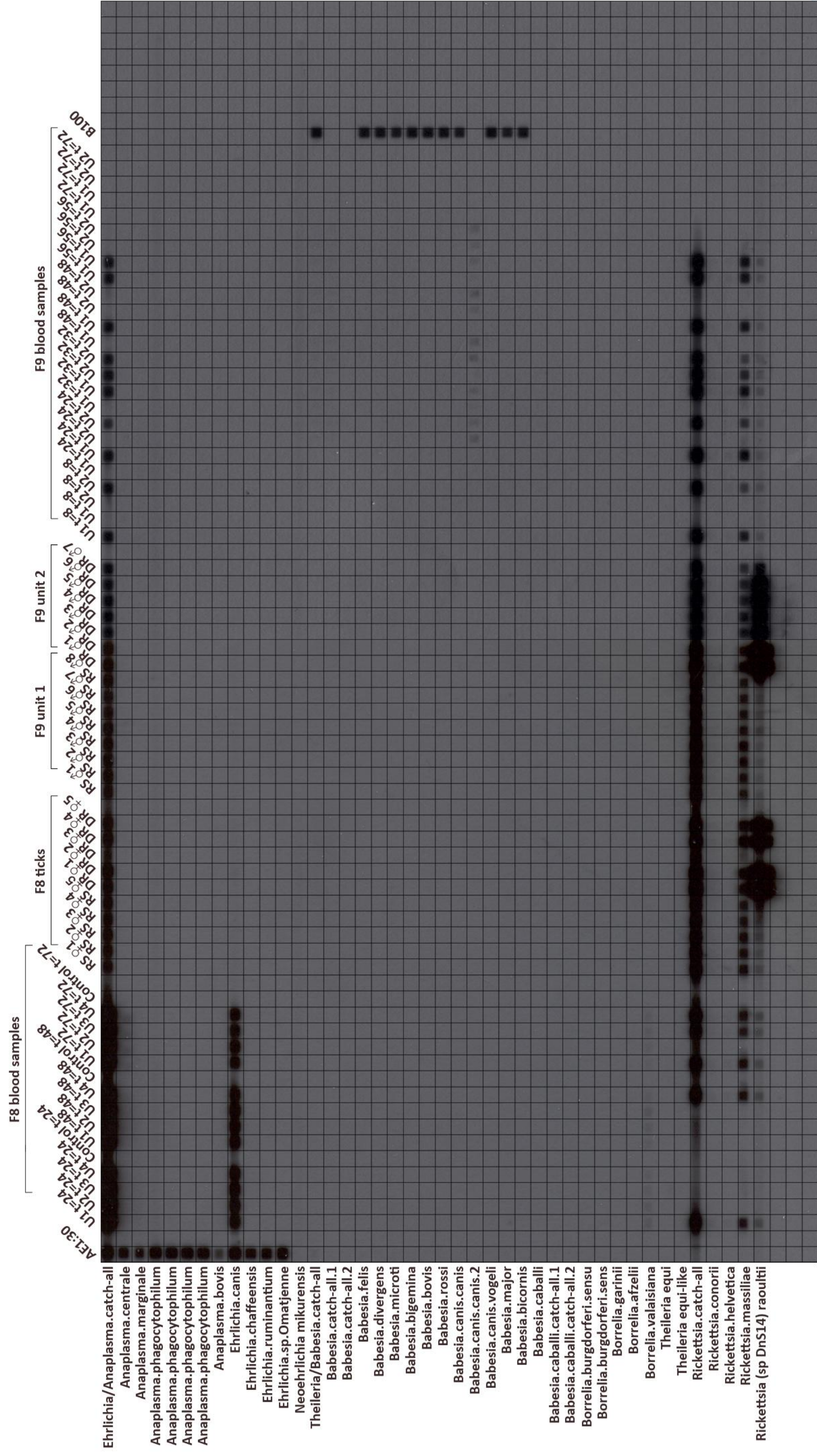

Figure 11: RLB F8 and F9

F10: transmission feeding *Babesia vogeli* and *Babesia canis*

In F10 adult *R. sanguineus* and *D. reticulatus* ticks infected with *B. vogeli* and *B. canis* respectively were used to determine if transmission to blood could be established. The ticks were pre-fed on rabbits before entering F10. On day 1 eight *R. sanguineus* ticks were put in unit 1 and 11 *D. reticulatus* ticks in unit 2. Every time blood samples were taken, the blood was changed as well. After 72 hours in feeding the attached ticks were put on alcohol and tested for infection. The *R. sanguineus* ticks should have been infected with *B. vogeli*, but the blot showed only one *B. vogeli* infected tick. The other two *R. sanguineus* ticks were infected with *B. canis*. Of the seven *D. reticulatus* ticks five ticks were indeed infected with *B. canis* and after 24 hours transmission to bovine blood was visible. There were strong positive signals on the Babesia canis probes and also weaker positive signals for the Babesia divergens probe. [Table 11: overview F10][Figure 12: RLB F10]

Table 11: overview F10

Time	**Unit**	**Attached**			**Unattached**			**Mortality**		**Total**		
t=8	U1	0			8	1♂	7♀	0		8	1♂	7♀
	U2	5	3♂	2♀	6	1♂	5♀	0		11	4♂	7♀
	Mean	2,5			7,0			0,0		9,5		
	%	26,3			73,7			0,0		100		
t=24	U1	2		2♀	6	1♂	5♀	0		8	1♂	7♀
	U2	10	4♂	6♀	1		1♀	0		11	4♂	7♀
	Mean	6,0			3,5			0,0		9,5		
	%	63,2			36,8			0,0		100		
t=48	U1	2		2♀	6	1♂	5♀	0		8	1♂	7♀
	U2	9	3♂	6♀	2	1♂	1♀	0		11	4♂	7♀
	Mean	5,5			4,0			0,0		9,5		
	%	57,9			42,1			0,0		100		
t=72	U1	3	1♂	2♀	5		5♀	0		8	1♂	7♀
	U2	7	1♂	6♀	3	2♂	1♀	1	1♂	11	4♂	7♀
	Mean	5,0			4,0			0,5		9,5		
	%	52,6			42,1			5,3		100		

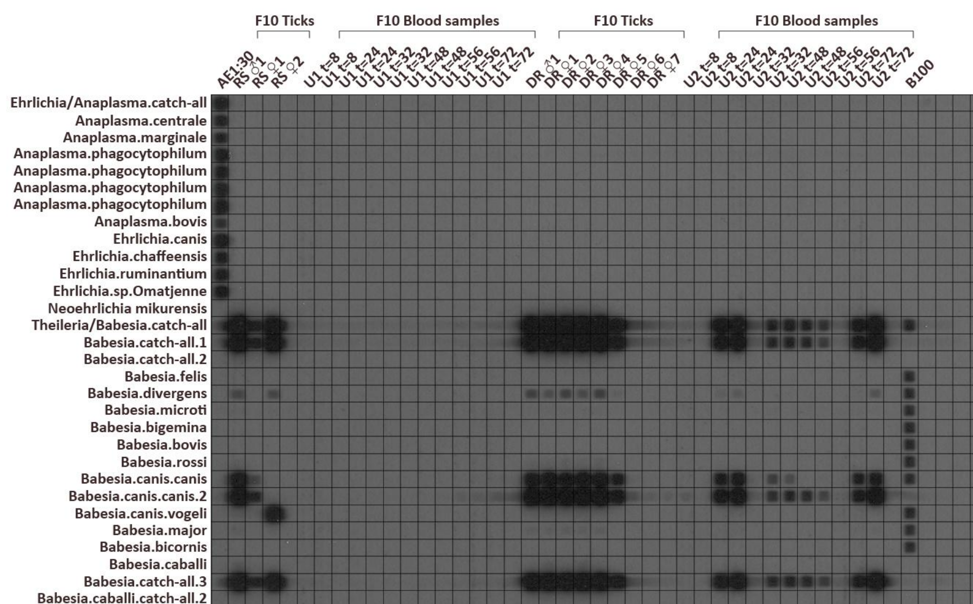

Figure 12: RLB F10

F11: serum feeding

In F11 adult *R. sanguineus* ticks were used to test if ticks would feed on fetal bovine serum. On day 1 60 *R. sanguineus* ticks (30♂, 30♀) were evenly distributed over six feeding units. The ticks in unit 1 and 2 were allowed to feed on uninfected bovine blood. The ticks in unit 3 and 4 were allowed to feed on a mixture of uninfected bovine blood and fetal bovine serum. The ticks in unit 5 and 6 were allowed to feed on fetal bovine serum. Every 24 hours blood and serum was changed and the units were examined to detect if attachment was apparent. After 24 hours in feeding there was a leakage in unit 5 and the unit was excluded from further experiment. After 62 hours in feeding, all live ticks were returned to the incubator.

It appears that the ticks were not inclined to feed on pure bovine serum. The ticks in the other units did reached high attachment rates, whether on pure bovine blood or on the mixture. [Table 12: overview F11]

Table 12: overview F11

Time	**Unit**	**Attached**			**Unattached**			**Mortality**			**Total**		
t=24	U1	2	1♂	1♀	7	3♂	4♀	1	1♂		10	5♂	5♀
	U2	5	3♂	2♀	5	2♂	3♀	0			10	5♂	5♀
	U3	6	3♂	3♀	4	2♂	2♀	0			10	5♂	5♀
	U4	5	3♂	2♀	5	2♂	3♀	0			10	5♂	5♀
	U5	0			0			10	5♂	5♀	10	5♂	5♀
	U6	4	3♂	1♀	5	1♂	4♀	1	1♂		10	5♂	5♀
	Mean	3,7			4,3			2,0			10,0		
	%	36,7			43,3			20,0			100		
t=48	U1	6	3♂	3♀	3	1♂	2♀	0			9	4♂	5♀
	U2	3	2♂	1♀	7	3♂	4♀	0			10	5♂	5♀
	U3	4	3♂	1♀	6	2♂	4♀	0			10	5♂	5♀
	U4	6	3♂	3♀	4	2♂	2♀	0			10	5♂	5♀
	U6	1		1♀	8	4♂	4♀	0			9	4♂	5♀
	Mean	4,0			5,6			0,0			9,6		
	%	41,7			58,3			0,0			100		
t=62	U1	6	3♂	3♀	3	1♂	2♀	0			9	4♂	5♀
	U2	6	4♂	2♀	4	1♂	3♀	0			10	5♂	5♀
	U3	8	4♂	4♀	2	1♂	1♀	0			10	5♂	5♀
	U4	8	5♂	3♀	2		2♀	0			10	5♂	5♀
	U6	0			9	4♂	5♀	0			9	4♂	5♀
	Mean	5,6			4,0			0,0			9,6		
	%	58,3			41,7			0,0			100		

F12: acquisition feeding *Ehrlichia canis*
In F12 adult *R. sanguineus* ticks were put on 11.4 ml bovine blood infected with 1.0 ml *E. canis* cell culture in order to infect the ticks with *E. canis*. The *E. canis* cell culture was stored at 4°C and mixed with the bovine blood before warming to 37°C.
On day 1 80 *R. sanguineus* ticks (40♂, 40♀) were evenly distributed over 8 units. The ticks were put on uninfected blood. After 12 hours the units were examined to detect if attachment was apparent and the *E. canis* cell culture was added to the newly offered blood. Units 1-4 were given the CDC strain no.251; units 5-8 were given the Ivory coast strain no.33. Twice a day the blood was changed to prevent bacterial growth. After 24 hours, there was a leakage in unit 6 and the unit was excluded from further experiment. After 72 hours in feeding and 60 hours on infected blood, the live male ticks were returned to the incubator for 4 days till being reused in F13. The female ticks that showed clear signs of blood intake were tested for an *E. canis* infection. None of the female ticks became infected with *E. canis*. Six female ticks did show Rickettsia catch-all signals with weak cross reactions with *R. massiliae* and *R. raoultii*. The offered blood in units 1, 5, 7 and 8 at t=24 and of units 5, 7 and 8 at t=48 and t=72 also showed no signs of *E. canis* present. The offered blood in the control wells at t=48 did show signs of *E. canis* present. The blood samples did have a vague positive hit on the Borrelia valaisiana probe. [Table 13: overview F12][Figure 13: RLB F12 and F13]

Table 13: overview F12

Time	Unit	Attached			Unattached			Mortality			Total		
t=24	U1	5	4♂	1♀	5	1♂	4♀	0			10	5♂	5♀
	U2	9	5♂	4♀	1		1♀	0			10	5♂	5♀
	U3	5	4♂	1♀	4	1♂	3♀	1		1♀	10	5♂	5♀
	U4	7	4♂	3♀	3	1♂	2♀	0			10	5♂	5♀
	U5	6	3♂	3♀	4	2♂	2♀	0			10	5♂	5♀
	U6	0			0			10	5♂	5♀	10	5♂	5♀
	U7	5	3♂	2♀	5	2♂	3♀	0			10	5♂	5♀
	U8	7	5♂	2♀	3		3♀	0			10	5♂	5♀
	Mean	5,5			3,1			1,4			10,0		
	%	55,0			31,3			13,8			100		
t=48	U1	5	4♂	1♀	5	1♂	4♀	0			10	5♂	5♀
	U2	8	5♂	3♀	2		2♀	0			10	5♂	5♀
	U3	7	4♂	3♀	2	1♂	1♀	0			9	5♂	4♀
	U4	5	4♂	1♀	5	1♂	4♀	0			10	5♂	5♀
	U5	8	4♂	4♀	2	1♂	1♀	0			10	5♂	5♀
	U7	6	3♂	3♀	4	2♂	2♀	0			10	5♂	5♀
	U8	2	1♂	1♀	6	2♂	4♀	2	2♂		10	5♂	5♀
	Mean	5,9			3,7			0,3			9,9		
	%	59,4			37,7			2,9			100		
t=72	U1	5	3♂	2♀	3	2♂	1♀	2		2♀	10	5♂	5♀
	U2	7	4♂	3♀	3	1♂	2♀	0			10	5♂	5♀
	U3	7	4♂	3♀	2	1♂	1♀	0			9	5♂	4♀
	U4	7	4♂	3♀	3	1♂	3♀	0			10	4♂	5♀
	U5	6	2♂	4♀	4	3♂	1♀	0			10	5♂	5♀
	U7	6	3♂	3♀	4	2♂	2♀	0			10	5♂	5♀
	U8	4	2♂	2♀	4	1♂	3♀	0			8	3♂	5♀
	Mean	6,0			3,3			0,3			9,6		
	%	62,7			34,3			3,0			100		

F13: transmission feeding *Ehrlichia canis*

In F13 the adult male *R. sanguineus* ticks from F12 were used to determine if transmission of *E. canis* to blood could be established. On day 1 11 male *R. sanguineus* ticks that had been feeding on blood infected with CDC strain no.251 were put in unit 1 and 12 male *R. sanguineus* ticks that had been feeding on blood infected with Ivory coast strain no.33 in unit 2. Every time blood samples were taken, the blood was changed as well. After 72 hours in feeding the attached males were put on alcohol and tested for an *E. canis* infection. Both unit 1 and 2 lost one attached male during the termination of the experiment. None of the male ticks became infected with *E. canis*. All but two ticks did show Rickettsia catch-all signals with weak cross reactions with *R. massiliae* and *R. raoultii*. Two blood samples from unit 2 did show signs of *E. canis* infection at t=48 and t=72. [Table 14: overview F13][Figure 13: RLB F12 and F13]

Table 14: overview F13

Time	Unit	Attached		Unattached		Mortality		Total	
t=6	U1	6	6♂	5	5♂	0		11	11♂
	U2	10	10♂	2	2♂	0		12	12♂
	Mean	8,0		3,5		0,0		11,5	
	%	69,6		30,4		0,0		100	
t=24	U1	11	11♂	0		0		11	11♂
	U2	7	7♂	5	5♂	0		12	12♂
	Mean	9,0		2,5		0,0		11,5	
	%	78,3		21,7		0,0		100	
t=48	U1	11	11♂	0		0		11	11♂
	U2	7	7♂	4	4♂	1	1♂	12	12♂
	Mean	9,0		2,0		0,5		11,5	
	%	78,3		17,4		4,3		100	
t=72	U1	10	10♂	1	1♂	0		11	11♂
	U2	7	7♂	3	3♂	1	1♂	11	11♂
	Mean	8,5		2,0		0,5		11,0	
	%	77,3		18,2		4,5		100	

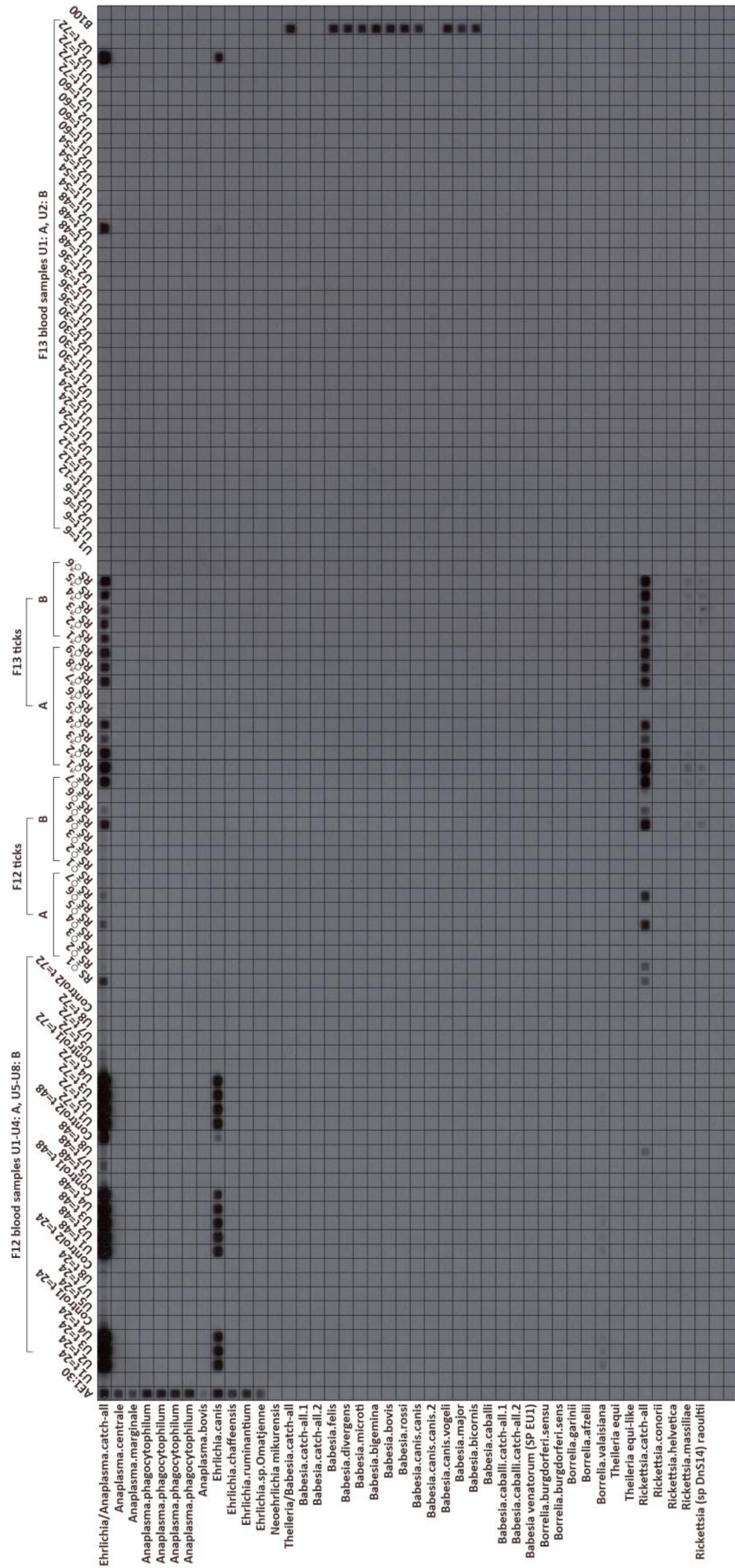

Figure 13: RLB F12 and F13

Discussion

The first *in vitro* feeding was carried out with pre-fed *R. sanguineus* nymphs. The goal was to feed the nymphs *in vitro* to repletion whilst infecting them with *E. canis*. After the feeding the nymphs would then be returned to the incubator to allow them to molt into adults before using them in a transmission feeding.

Unfortunately the attachment rate was very low and the mortality rate increased drastically within 72 hours reaching up to 60%. Thereafter, the experiment was terminated. There are several reasons imaginable that could explain the disappointing outcome of the feeding. *R. sanguineus* ticks have a strong dog host preference. To stimulate feeding on a silicone membrane the membranes are impregnated with dog odor before using them. In this feeding however, the prepared membranes were all found to be leaking and therefore feeding units were used that were assembled a few weeks earlier. The time between the impregnation and feeding could have been long enough to reduce the dog smell of the membranes, rendering them unappealing to the ticks.

Furthermore after 24 hours in feeding the relative humidity inside the aquarium dropped to 77% because the solution had evaporated too strongly and the temperature had increased to 40°C. The solution was replenished and the thermostat of the outer water bath was adjusted. The conditions recovered but no improvement in attachment was seen.

After 48 hours a decrease in activity was visible in 2 of 4 feeding units and 24 hours later large numbers of ticks were found dead in all units. The reason for this could have been the pre-fed condition of the nymphs. The pre-feeding increased their metabolism but the ticks were not allowed to feed to repletion on the rabbits. This in combination with the high temperature inside the aquarium could have been acting against the nymphs, exhausting them and possibly starving them to death.

However, in the short period that the few nymphs were attached to the membrane some acquisition of *E. canis* had been established. The strong positive hit on the Ehrlichia/Anaplasma catch-all probe however, isn't explained by the much weaker E. canis signal present. The reason why almost all the nymphs tested positive for the catch-all probe but not the E. canis probe has been suggested to be caused by a cross reaction of this unspecific probe with another *Rickettsia* spp. Ticks are known to carry a wide variety of Rickettsiae with them.[15] New membranes had to be made with specific Rickettsia probes to rule out other explanations. In *in vitro* feeding 4 and 5 the new membranes were able to show a *Rickettsia* spp. present in the ticks. After 12 hours transmission of this *Rickettsia* spp. was visible and remained visible throughout the entire feeding. The new probes could not however determine which exact *Rickettsia* spp. was concerned. To this end, further sequence analysis is required. Another *Rickettsia* spp. transmission was witnessed in *in vitro* feeding 8 and 9.

The second *in vitro* feeding was performed with adult *R. sanguineus* ticks, thereby overcoming the difficulties in handling much smaller nymphs and having to pre-feed them. The goal was to infect the ticks with *E. canis*, using the females as a control method to see if acquisition was accomplished and using the males to see if transmission could be established. This model was chosen keeping in mind that male ticks tend to feed multiple times under natural circumstances and are capable of doing this without having to copulate between meals, making them more suitable for a multiple *in vitro* feeding trials.[2]

After 36 hours small leakages were visible on the attachment sites of the first three units. The strong clustering of the ticks in these units could have caused the membranes to start sagging and becoming semi permeable to the blood on these sites forming blood clots around the ticks. The ticks of unit 1 were transferred to a spare unit; the ticks in the other two units appeared to had died already. Leakages were also visible in *in vitro* feedings 3 and 8-13.

After 90 hours in feeding all units were cleaned out and all of the ticks were washed in the sonification bath to rid them of the blood clots. After washing the ticks in the two unchanged, leaking units they appeared to have survived the blood clots. All ticks survived washing in the sonification bath, but to keep the ticks in the best condition possible a decision was made not to wash ticks in the sonification bath when they were needed for other *in vitro* feedings. The fact that the spiraculae are located on the abdomen could have made it easier for the ticks to sustain themselves inside the blood clots. To prevent small leakages of the membranes a minimal thickness of 90 µm could be needed.

Almost 70% of the female and dead male ticks were tested positive for *E. canis* so that the assumption made in *in vitro* feeding 1 was made plausible. Under the right conditions ticks can acquire *E. canis* by means of *in vitro* feeding.

The third *in vitro* feeding was carried out with the male ticks from feeding 2 combined with uninfected females to mimic natural circumstances as much as possible. The goal here was to see if the ticks could also transmit *E. canis in vitro*. This was the first time a control well filled with blood but without ticks was added to see if the methods applied were adequate.

After 41 hours the blood supporting unit 2 turned black. This was also seen in *in vitro* feedings 4, 5, 8-9, 12 and 13. The most plausible explanation for this is bacterial growth because no bacteriostatic is added to the blood. All units were sterilized before entering a feeding. The blood however was collected as sterile as possible but not completely germfree. Combining this unsterile blood with a 37°C temperature in the *in vitro* feedings and you create perfect conditions for bacterial growth. To overcome this problem absolute sterile collection of blood is needed. In this study it was not attainable to do so. All units with black blood were however submerged in a sterile saline to limit bacterial growth as much as possible before being transferred to fresh blood. The saline rids the units from any attached blood. Sterilization is not possible whilst in feeding because the mouthparts of the ticks should not come in contact with any sterilizing agent. This procedure was repeated in all the *in vitro* feedings every time the blood turned black.

When looking at the results it is apparent that unit 2 had a declining attachment rate. This could have been caused by the bacterial growth in the blood making it less appealing to the ticks. Unit 2 was also the unit with the only mortality. Besides the quality of the blood, there could have been a problem with the quality of the unit or the ticks as well. No obvious differences were visible between the units which tend to another reason for the low attachment rate. Given the fact that all ticks came from the same batch of ticks and the high initial attachment rate is it most likely that the ticks detached because of the poor quality of blood. This was also seen in *in vitro* feeding 5. When looking at unit 1, where the blood turned black at a later time, the reason that the ticks did not detach could be the anchoring of the mouthparts with cement. When ixodid ticks feed they anchor themselves by using attachment cement secreted by the salivary glands. The longer they feed the more cement the ticks produce, thereby impeding themselves from immediate detachment.[4] This was also seen in *in vitro* feedings 4, 8, 9 and 12.

When looking at the results of *in vitro* feeding 3, 43% of the ticks are infected with *E. canis*. What is remarkable is the fact that the first two females in unit 1 and the fourth female in unit 2 also show a positive hit for *E. canis*. The female ticks used were not infected with *E. canis* before entering the *in vitro* feeding. The most likely explanation for this is the so called co-feeding of ticks, where ticks can infect each other without infecting the host during feeding.[16]

Further, transmission of *E. canis* is visible in the blood samples after 17 hours. All blood samples have been taken in duplicatee; this is demonstrated in the results. However only one positive *E. canis* hit is seen in the blood supporting unit 1. When taking the blood samples the blood is first pipetted up and down to mix the blood inside the well. When mixed, another sterile pipette is used to take the blood samples. This is done in order to prevent misinterpretation by taking non-homogenous samples. Despite this method of sampling the results remained non-uniform. A reason for this could be contamination of the blood whilst pipetting up and down. The smallest amount of contamination with *E. canis* infected blood can cause a positive result on a blot through PCR amplification. This could explain the positive hit in unit 4. This unit should have been negative, but shows one positive result on t=17. The real question remains where the contamination came from. It appears to have come from unit 3, but when you compare the blood samples with the tick results none of the ticks in unit 3 had been infected with *E. canis*. Somewhere in the sampling must have been an error. A possible explanation is the mixing up of samples. When the blood in the six-well cell culture plate is being sampled, the feeding units have already been transferred to fresh blood. Before transferring the units it is vital to make sure that the unit numbers have been written on the corresponding wells in the plate. If this is forgotten, it is usually impossible to recollect which unit went in which well. Given the *E. canis* infection in the ticks of unit 2, it could be that the blood samples corresponding with unit 3 belong to the ticks from unit 2 or even unit 1. The non-duplicatee results are also found in *in vitro* feedings 5, 6, 9 and 10. Contamination and mixing up of samples are also seen in *in vitro* feeding 6, 12 and 13.

The fourth and fifth *in vitro* feedings were performed with adult *R. sanguineus* ticks following the same design as *in vitro* feeding 2 and 3. The attachment rate remained low in feeding 4 resulting in only one *E. canis* infected female and male tick. No visible *E. canis* transmission could therefore be established.

After six hours in feeding 5 the membrane in unit 2 appeared to be leaking severely. The cause of this could be a gluing problem. If the silicone membrane is not glued onto the units perfectly, the ethanol can partly dissolve the glue leaving parts of the membrane weakened. Even the slightest pressure can then cause membrane leakage. This was also seen in feedings 11 and 12.

After 48 hours the attachment rate had declined to 50%. The unit was wet and sticky on the inside as if there was condensation. This could have been the reason for the disappointing attachments rate. The same was seen in unit 3 of *in vitro* feeding 8.

The sixth *in vitro* feeding was carried out with adult *R. sanguineus* ticks infected with *B. vogeli* as adults in the previous generation. The experiment started out with eight units but there was almost no attachment at all. The reason for this could lie in unattractiveness of the feeding units. The units were however assembled in exact the same matter as before. Another explanation is the use of different ticks. Although *R. sanguineus* ticks were used, they came from a different source than the ticks used in previous feedings. Perhaps the ticks used weren’t eager enough to feed and therefore unwilling to feed on a silicone

membrane. This was also seen in *in vitro* feeding 10 where the same batch of *R. sanguineus* ticks were used and did not show a high attachment rate compared to the *D. reticulatus* ticks used.

The seventh *in vitro* feeding was performed with adult *D. reticulatus* ticks infected with *B. canis* as adults in the previous generation. Although the attachment rates were significant and the ticks were all infected with *B. canis*, no transmission was established. The reason for this was suggested to lie in the time needed for the pathogen to multiply inside the tick. The ticks are kept at 20°C in the incubator and bacterial multiplication is usually at its optimum around 37°C temperature. So whilst being in feeding, the *B. canis* first had to multiply inside the ticks before any significant transmission could be established. It is very well possible that the *in vitro* feeding did not last long enough for this to happen. To overcome this problem the ticks in *in vitro* feeding 10 were pre-fed on rabbits before entering the *in vitro* feeding. The pre-feeding indeed led to *B. canis* transmission.

Besides *B. canis* the *D. reticulatus* ticks also demonstrated a positive hit on the Babesia divergens probe and a weaker hit on the Babesia major probe. The signals are however weaker than the Babesia catch-all signal. The reason for this probably lies in cross reactions of *B. canis* with the other two Babesia probes. The higher the analogy of the probe, the more obvious the cross reaction signal is seen in the results. The same phenomenon is witnessed in *in vitro* feeding 10.

The eighth and ninth *in vitro* feedings were carried out with adult *R. sanguineus* ticks. *D. reticulatus* ticks were added to the feeding trial to see if the attachment rates would improve and *E. canis* acquisition and transmission could be established.

An important change was made in feeding 8 by storing the *E. canis* cell culture at 4°C for the entire feeding. This was done to see if bacterial contamination could be managed in the cell culture during the *in vitro* feeding experiment. Although attachment rates were high in feeding 8 none of the ticks got infected with *E. canis*. The reason for this could be that the storing under chilled conditions damaged the *E. canis* or prevented sufficient multiplication inside the ticks. This was also seen in *in vitro* feeding 12.

The blood samples of feeding 8 showed besides a Rickettsia signal coming from the ticks a very weak Borrelia valaisiana signal. Cross reaction was not possible in this case due to the fact that no Borrelia species were used in the feeding. What could be a possibility is that the Biodyne C membrane used was not stripped adequately before using it in this *in vitro* feeding. Another possibility is contamination of the membrane by the miniblotter. Both membrane and blotter are used repeatedly by different people. Adequate rinsing of membrane and blotter after every use is vital to prevent contamination for the next user. The blood samples of feeding 9 show a very weak B. canis signal that is probably also caused by membrane or blotter contamination. In feeding 12 the same is seen in the blood samples with a very weak Borrelia valaisiana signal.

The tenth *in vitro* feeding was performed with adult *R. sanguineus* and *D. reticulatus* ticks. The *R. sanguineus* ticks did not establish *Babesia* spp. transmission whilst the *D. reticulatus* ticks did. The reason for this is that only two females had been attached during most of the feeding trial. Of these two female ticks only one was infected with *B. vogeli*, the other female was infected lightly with *B. canis*. Two ticks are probably not enough to induce transmission. The reason the *R. sanguineus* ticks were not all infected with *B. vogeli* remains unclear.

The eleventh *in vitro* feeding was carried out with adult *R. sanguineus* ticks. The goal was to see if we could let ticks feed on fetal serum. The main advantage of this is the availability of sterile serum compared to unsterile blood and no use of live animals. Unfortunately one of the two units on serum developed membrane leakage and was excluded and therefore made it difficult to make assumptions based on one serum unit. More *in vitro* feeding experiments are needed to see if ticks are able to feed on serum or blood-serum mixtures and if pathogen transmission can be realized doing so. It is also possible that a mixture with *E. canis* culture medium could work.

The twelfth and thirteenth *in vitro* feedings were performed with adult *R. sanguineus* ticks. Two strains of *E. canis* were used to see if a difference in transmission could be witnessed. After testing it appeared that the new *E. canis* strain, Ivory coast no.33, did not contain *E. canis* at all. No positive signal was seen on the blot. The reason for this could have been an infection in the cell culture with another bacterium. Under the microscope an intracellular infection was seen, but not typical of *E. canis*. Further research is needed to determine what kind of bacterium infected the cell culture used.

Conclusion

Acquisition and transmission of *E. canis* in an *in vitro* feeding system using adult *R. sanguineus* ticks is possible. The environmental conditions in the *in vitro* feeding system have to be optimal in order to get the desired attachment rates together with ticks being in excellent condition. High attachment over a prolonged period of time is needed to ensure proper *E. canis* uptake. Ideal is to keep the ticks on a viable *E. canis* source for at least three days. The quality of the blood offered also needs to be guarded by using as sterile blood as possible. The possibility of using sterile fetal bovine serum instead of bovine blood needs further research. The *E. canis* offered can derive from cell cultures that need to be stored at 37°C and is ideally mixed with blood at that same temperature.

Transmission of *E. canis* is possible within a short period of time after attachment of an infected tick. The exact time required for transmission still needs further research but the results implicate that less than 17 hours is needed for transmission.

Transmission of *B. canis* in an *in vitro* feeding system using adult *D. reticulatus* ticks is possible as well. *D. reticulatus* ticks are more inclined to feed *in vitro* even under suboptimal conditions. Nevertheless are the environmental conditions important to ensure optimal results. *B. canis* transmission differs in *E. canis* transmission in that the protozoa need substantial time to multiply inside the ticks before being able to cause contagion. At least 24 hours at 37°C and pre-feeding on rabbits was needed in our study to realize transmission.

Despite disappointing results regarding *E. canis* transmission, distinct Rickettsial transmission has been witnessed in some of the *in vitro* feedings. It was beyond the reach of this study to determine which species of Rickettsia infected the *R. sanguineus* ticks and whether or not this *Rickettsia* spp. could be of veterinary importance.

Recommendations

To successfully feed *R. sanguineus* ticks *in vitro* the following should be kept in mind:

- Always use ticks in the best condition possible.
- Use only non-chemical attractants when feeding ticks *in vitro* such as species-own tick feces instead of odor-solutions.
- Keep the environmental conditions stable with a high relative humidity at 37°C.
- Light-dark regimes are not necessary when you keep the ticks in the dark.
- Collect blood as sterile as possible. Distributing the collected blood in smaller sterile falcon tubes helps maintaining a low bacterial growth by limiting possible contamination. Add gentamicin to the blood if possible.
- Never warm more blood than is needed and use immediately in the six-wells plates.
- Submerge the units in a sterile saline when there are blood clots attached. This is best done when the blood is refreshed.
- When using *E. canis* it is important to keep the cell cultures at 37°C at all times. Do not store it at 4°C or mix it with cold blood from the refrigerator.
- When performing acquisition and transmission feedings it is important to keep in mind which pathogen you are working with. It is usually required to incubate the tick at 37°C for a few days before you can establish transmission. Pre-feeding of ticks can help to reduce the time needed for acquisition and transmission *in vitro*.

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Appendix I

Materials & methods

1. Ticks: *preconditioning adult Ixodes ricinus to enhance attachment*

- 3 – 6 month post ecdysis ticks are used: obtained from a laboratory rearing at the University of Neuchâtel. Before placing them in the feeding chamber, they must be preconditioned for at **least one week**, better **3 weeks**, at 20 to 23°C and 85 to 98 % relative humidity, with 10 to 16 h light per day.

  ! Important: it is critical to avoid low temperatures (i.e. < 14°C) in autumn and wintertime to prevent ticks going into diapause.

2. Blood: *defibrinated blood collection and preparation*

2.1 Blood collection

- Blood is collected weekly from an abattoir, defibrinated manually, by stirring rapidly for twenty minutes with a big spoon to collect the clot which will form attached to the spoon. Blood is poured into 1 litre sterilized bottles and supplemented immediately with 2g/l glucose and stored at 4°C (KUHNERT *ET AL.* 1995, KUHNERT 1996).

2.2 Preparation of blood for feeding

- All blood preparation is carried out in a sterile hood (Scan USE-2000-120). Gentamycine and ATP are added to the blood just before the blood is exchanged in the wells. ATP must be applied freshly in order to act as attachment and/or feeding stimulus before being metabolised,

  A 5 µl aliquot of a Gentamycine solution (Sigma, Germany, 10 mg/ml in sterile deionised water) is added to 10 ml of blood to achieve a final concentration of 5 µg/ml blood.

  A 100 µl aliquot of ATP (Fluka, Switzerland) solution (0.1 molar in NaCl 0.9%), sterile filtered (at 0.2 µm) is added to 10 ml of blood to achieve a final concentration of 10^{-3} molar in the blood.
- The well plates are then covered with the well lid and warmed to 37°C in the water bath prior to adding the feeding units.

  ! Important: in all experiments, blood must be exchanged twice daily at 12 hour intervals (max interval 14 h) in each well.
- During an experiment, the membrane surface facing the blood is rinsed with sterile saline (9 g NaCl pa, Fluka, in demineralised water) before placing the feeding unit in a fresh well (with ticks still attached).
- Fungal infections under the membrane are treated daily with Nystatin solution (Sigma, Germany, 10,000 units/ml DPBS) for 10 min during the blood exchange when the daily evaluation of ticks is made.

- The amount of blood required for each well is 3.1 ml. For calculated examples for a whole experiment see spread sheet 'blood calc' in file 'IVF Ir method'.

3. Blood treatments: *compound preparation for adding to the blood*

The blood treatments are: control (nothing added), dimethyl sulfoxide as placebo (DMSO, Fluka, Switzerland) at 2.5 µl/ml blood, the reference agent fipronil (Pestanal, Riedel de Haën, Germany) and the test compounds made up at 0.001, 0.01, 0.1, 1, 10 µg in DMSO/ml blood. Four feeding units are used for each treatment concentration.

Worked example: 15 ml of blood is required for 4 feeding units, i.e. 3.1 ml/feeding unit. 37.5 µl of DMSO stock solution (containing 4 mg test product/ml) is added to give 10 µg test compound/ml blood (see spread sheet 'prod conc's' in file 'IVF Ir method').

4. Membrane preparation: *silicone membrane preparation for the tick feeding unit*

Ixodes ricinus is fed on bovine blood through a silicone membrane reinforced by Kodak® lens cleaning paper, similar to that already developed in this laboratory (Kuhnert *et al.* 1995, Guerin *et al.* 2000). This membrane is modified to increase the attachment rate of *I. ricinus* by rendering the silicone soft such that it mimics the elasticity of skin. This ensures closure of tick penetration sites on the membrane to prevent bleeding. A silicone glue is selected with a low shore hardness A (expressed in degrees) - a measure of the indentation hardness of soft materials.

4.1 Silicone preparation for the membranes

The silicone glue RTV-1 Elastosil E4, (Wacker-Chemie GmbH, München Germany) with a very low shore hardness A of about 16° is used. Mixing silicone oil (30% DC 200, ~ 10 mPa.s, Fluka, Switzerland) to the silicone glue further increases softness and reduces 'frog grip' - the sticky nature of the resulting silicone surface. 15 % Hexane is added to render the glue more fluid for application to the matrix.

Note: the mixing should be done under dry conditions (as a low relative humidity as possible) to reduce the polymeristion of the silicone to a minimum.

- **Worked example**

  Quantitites for a smaller amount (i.e. a quarter) given in brackets

  60 g *(15 g)* Wacker silicone E4

  0.6 g *(0.15 g)* Wacker FL colour paste (1 % of the silicone)

  18 g *(4.5 g)* FLUKA DC 200 silicone oil (30 % of the silicone)

  11.7 g *(2.9 g)* Hexane (technical quality, 15 % extra weight)

- Kodak lens cleaning paper (70 x 120 mm), a non-woven tissue made of regenerated cellulose rayon (Eastman Kodak, Rochester, NY) is used as the matrix. The lens paper is placed on a layer of kitchen plastic film (30 cm wide) which has been laid on a glass sheet, making sure that there is a 30 mm working space between each lens paper. The lens

paper is held down with sticky tape. The silicone mixture is spread evenly over the lens paper using a 80 mm wide scraper made from a sheet of silicone (3mm thick).

- Membranes are left to polymerize for 12 h at room conditions, or to accelerate polymerisation, for 4 to 6 h in 80 to 90 % humidity at 25°C.
- The thickness of every membrane is measured using micro callipers, and only those between 70 to 110 µm are used.

5. Feeding Units: *preparation*

See figures at back of manual

- The feeding units are made of Plexiglas® tubing (26 mm i.d., 2 mm wall thickness, 45 mm high) with a ring made of acrylic glass fixed around each tube to limit the depth (4 mm) to which the unit sinks into the blood in the wells (Fig 1). The feeding membrane is attached to the angled (1 deg) lower end of the tube (Fig 3) using silicone glue (Wacker Elastosil E4) and left to dry (min. 3 h). See Fig. 3 for a construction drawing of an acrylic feeding unit **at the end of the manual.**
- To improve the attachment rate of the ticks to the membrane, a piece of glass fibre mosquito netting (1.4 mm mesh, 25 mm diameter) is cut out by using a 25 mm cutting tool. The netting is glued to the membrane in the feeding unit with silicone glue (WACKER Elastosil E4) and left to dry (Fig. 2).
- Following this, the membranes are cut flush with the outer wall of the feeding unit using scissors and the feeding units are checked for leaks by sitting them in Petri dishes with 70% ethanol for 20 min.

  ! <u>Important</u>: it is critical that the ethanol does not enter the feeding unit.
- Check for any holes in the membrane under a stereo microscope and repair any small holes using Wacker E41silicone diluted with 40% toluene with a fine paint brush. Strictly avoid applying thick drops of silicone.
- A plastic tile spacer (2 mm thick tile spacer, size of the 4 arms adjusted to the 26 mm diameter of the feeding unit) is placed on the membrane to create additional borders where ticks prefer to attach (Fig 1).

6. Attachment stimuli: *preparation*

White or light coloured bovid hair is shaven from a non treated animal. The colour of the hair is important in order to see the ticks in the feeding units, but unimportant for the extract (below). Hair is cut into 4 to 7 mm pieces and kept frozen (-20°C) in a jar for adding to the feeding unit and for preparing the cow hair extract.

Preparation of cow hair extract

Hair (50 g) is cut off a young light-coloured cow on one side and collected in a beaker. The hair is extracted in three successive 20 minute steps to increase the yield:

- add 250 ml of dichlormethane (DCM, Merck, extra pure grade), leave for 20 minutes then remove the solution (about 100 ml) and replace it with a fresh 100 ml of DCM. Leave this for a further 20 minutes, then remove the DCM solution (about 100 ml). Repeat this extraction with 100 ml DCM one more time.

- The three 100 ml extracts are combined. Either the extract is centrifuged at 3000 rpm for 20 minutes and the supernatant is removed or the extract is filtered (Macherey & Nagel glass fiber filter MN GF-2, 0.5-µm pores, Düren, Germany). This is then concentrated by roto-evaporation to about 100 ml and stored in a freezer at -80°C. The amount of material of low volatility per unit volume (henceforth indicated as the 'low volatile mass', LVM) is estimated by evaporating 1 ml of extract on a glass slide and weighing after 30 min at room temperature. The stock solution is adjusted to 100 mg LVM/ml.
- Prepare a working solution of 7 mg LVM/ml by diluting the stock with DCM. The working solution is kept at -20°C prior to application on to the feeding membrane.

  (If the lipid extraction is carried out using methanol or hexane the evaporation time lasts a minimum of 30 minutes).

7. Attachment: *application of attachment stimuli and placing ticks in feeding units*

- 75 µl of bovid hair extract (0.5 mg LVM lipids extracted from freshly shaven bovid hair with DCM applied in 75 µl DCM per feeding unit) is applied to the membrane with a micropipette. The feeding units are placed for 15 to 30 min on a metal grid placed on top of a hot plate at 40°C to evaporate the solvent (DCM).
- The feeding units are placed in six-well cell culture plates (COSTAR, 34.8 mm diameter) with 3.1 ml of the test blood and warmed to 37°C using a thermostat-controlled water bath (740 mm long x 540 mm deep x 215 mm high) with a sloping Perspex hood to keep the air above the feeding units near 100% R.H. A warm plate may also be used but stable temperature in the blood must be assured and high humidity around the feeding units must be maintained.

  ! Important: the bath must subject to a 16:8 h light: dark cycle, this is critical for attachment.
- The six-well plates with the feeding units sit on a metal support submerged 15 mm below the water surface in the water bath.
- Ten female and five male *I. ricinus* ticks are put into each feeding unit with soft forceps, covered with a 1 cm layer of cow hair, cut to a length of 4 to 7 mm, and the ensemble held down with a brass grid (25 mm diam., 3 mm mesh, 0.55 mm wire). Each feeding unit is closed with a perforated stopper (0.5 mm Sefar plastic mesh, Fig. 1).

  ! Important: placing the ticks in the feeding units must be carried out towards the end of the 16:8 h light: dark cycle to encourage attachment.

8. Recording data on compounds tested

- Four feeding units are used for each compound at each dose level.
- The ticks are evaluated once a day to count the number of living and dead ticks attached to the membrane as well as the unattached living and dead ticks. All dead ticks are removed from the feeding units. Knock down observations are also made.
- If a large amount of tick faeces accumulates this can be removed by gently tapping the feeding unit upside down, being careful not to dislodge any of the ticks. Sometimes faeces get stuck, especially to mating ticks and need to be removed. This is done by dislodging the faeces with a pair of forceps, being careful not to dislodge the feeding ticks.
- The feeding experiments are complete after nine days, or earlier , depending on the experimental protocol.

9. Statistical analysis

Survival curves are calculated from the numbers of dead ticks recorded per day over the different doses of each treatment using the Kaplan-Meier Statistics (KLEINBAUM 1995) with Peto test of the survdiff algorithm in S-plus (V6.2 build 6713).

10. Relevant references

Detailed accounts of this hard tick feeding assay have already been published (KRÖBER AND GUERIN 2007a and 2007b). See section 12 (Literature) of this manual

11. List of materials and suppliers

Chemicals, catalogue numbers and suppliers

NaCl (Fluka 71380, pa , > 99.5 % (AT)), for saline at 9 g/l (http://www.sigmaaldrich.com)

Glucose (D(+)-Glucose Monohydrate, Fluka 49159, >99 % (HPLC)

ATP (Fluka 02060, > 95.0 % (HPLC)), 10^{-3} mmolar in the blood

Gentamycine solution (Sigma G1272, sterile filtered 10 mg/ml), 5 µg/ml blood or Gentamycine sulfate (Sigma G3632)

DMSO (dimethyl sulfoxide, Fluka 41650, > 99.0 % (GC)), 2.5 µl/ml blood as solvent for test products

Fipronil (Riedel de Haen, Pestanal 46451, > 97.5 % (HPLC)), reference acaricide

Nystatin solution (SIGMA N-1348, 100 units/ml) for treatment of fungi, 10 min daily when necessary (http://www.sigmaaldrich.com)

Hexane (technical grade)

Toluene (Merck, supraSolv, No 1.08389.1000)

Dichlormethane (DCM, Merck, SupraSolv, No 1.06054.1000)

Silicone oil DC200 ~10 mPa.s 250 ml FLUKA No. 85411

Feeding units, catalogue numbers and suppliers

Tubes Plexiglas ® XT clear 29070 (http://www.roehm.de/en/plexiglas.html)

External diameter 30 mm, internal 26 mm, wall 2 mm (for corpus)

External diameter 40 mm, internal 30 mm, wall 5 mm (for ring)

ACRIFIX® 106, glue for ring around the feeding unit, from Plexiglas or Rhoem

Stopper (PE-Caps), 26 mm with 15 mm hole, PET netting glued with hot glue (BOSCH)

Polyester Netting Sefar, Switzerland, PET 1000 18-180W PW (www.sefar.com)

Glass fiber mosquito netting, grey, HSB Phifer Inc. Tuscaloosa, AL, USA (www.phifer.com), Art-No 257251, on membrane

Tile spacer, 2 mm cross, white plastic, Germany

Filters

Glass fiber filter MN GF-2, 0.5-µm pore (Macherey & Nagel, Germany) or other.

Membranes, catalogue numbers and suppliers

Kitchen roll of PE cling film (Tangan No11, house brand from Migros Switzerland)

Silicone oil DC 200, ~10 mPa.s, Fluka 85411, Switzerland,

Wacker ELASTOSIL® E4 RTV-1 Silicone Rubber (http://www.wacker.com/internet/noc/Products/ProductsAZ)
Wacker ELASTOSIL® COLOR PASTE FL white RAL

Wacker ELASTOSIL® E41 RTV-1 Silicone Rubber (contains Toluene, used for repairing small holes in the membrane or the sealing between the acrylic glass and membrane.

Ticks

Ixodes ricinus from lab rearing at Neuchâtel (www.unine.ch) 10 females + 5 males per feeding unit.

Illustrations

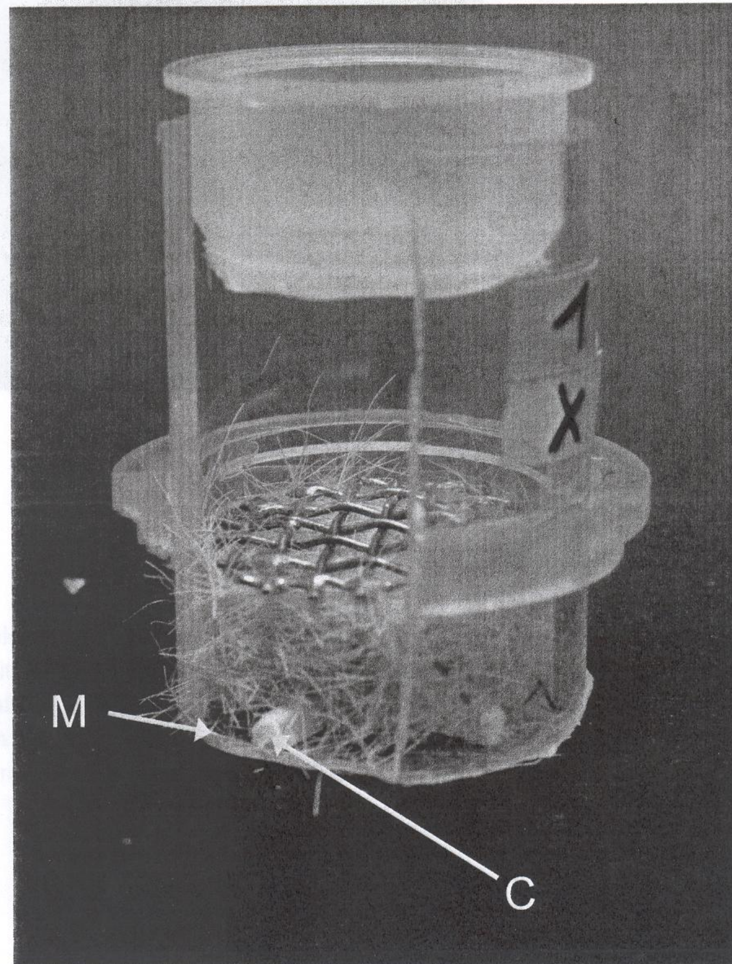

Figure 1

Cut out view of the *in vitro* feeding unit for *Ixodes ricinus* made from an acrylic glass tube (45 mm high x 30 mm o.d., 2 mm thick wall). Part of the plastic cross (**C**) placed on the membrane (**M**) is visible, and the layer of cow hair placed on the membrane is held lightly down with a brass grid. The ring around the unit assures that a layer of 2 mm of blood lies under the membrane when placed in the well. A perforated plastic stopper is inserted on top.

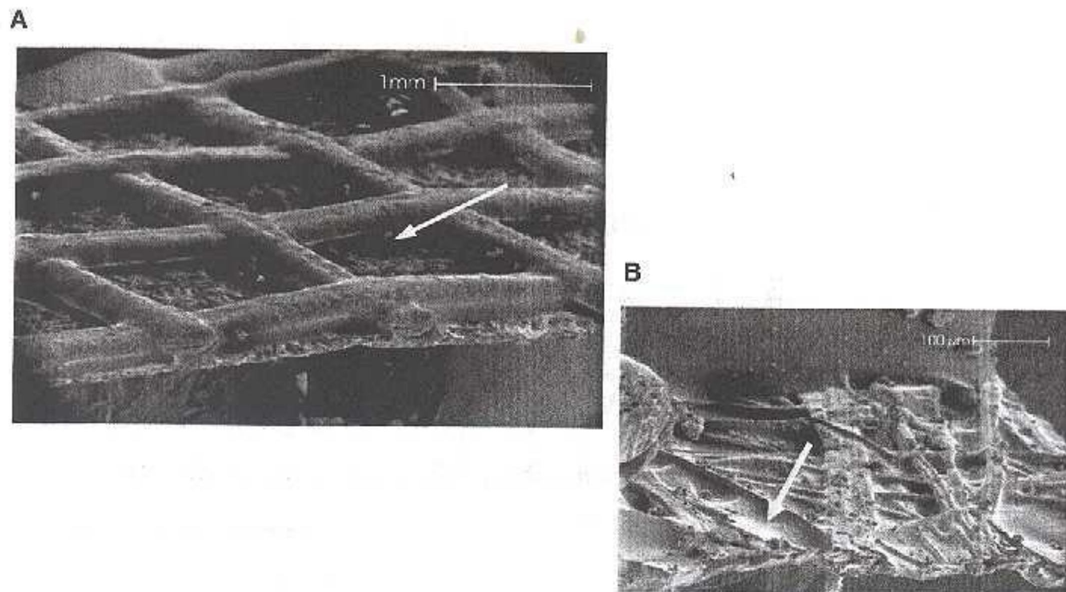

Figure 2

(**A**) Scanning micrograph of a feeding membrane with mosquito netting glued on to it. Only a minimum quantity of glue was used to attach the netting to the membrane so as to leave cavities (**arrow**) which allow the ticks to obtain a perch with their mouthparts in the membrane. (**B**) The spaces between the cellulose fibres of the lens cleaning paper are only partly filled with silicone providing small regions where the membrane is even thinner (**arrow**) than the thickness of the paper.

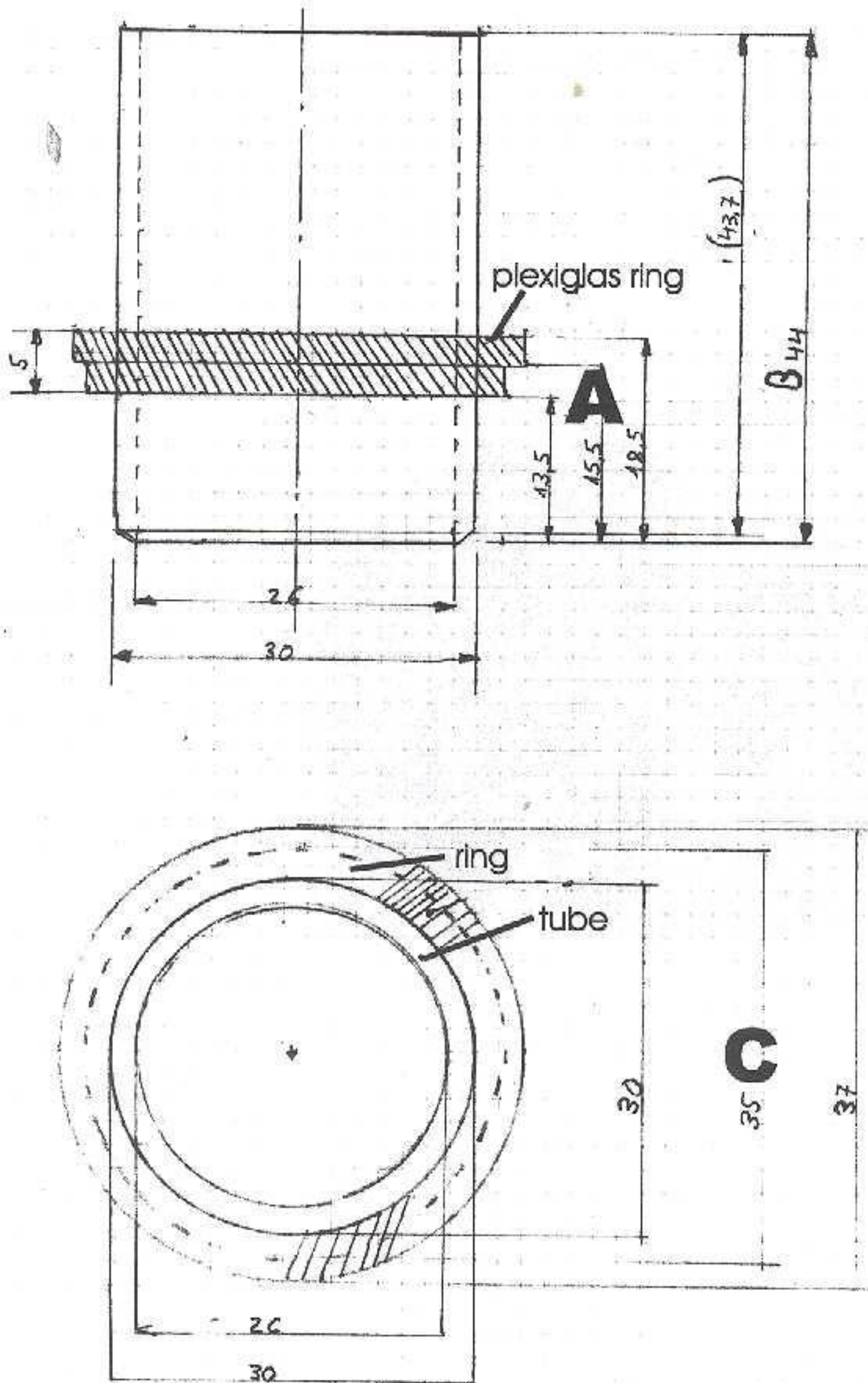

Figure 3

Construction drawing of the feeding unit made from acrylic glass tubing, scale 2 : 1 in mm; measure (**A**) should have ≤ 0.1 mm tolerance to ensure an equalized layer of blood under the membrane. Measure (**C**) should be checked for easy fitting without too much play in the well.

Appendix II

Teken determineren

Om te beginnen, **denk aan je eigen veiligheid**. Tussen de ingezonden teken zitten nog veel teken die in leven zijn. Zorg dan ook dat je goed oplet dat ze niet aan de wandel gaan.
Pas op dat sommige teken eitjes hebben kunnen leggen in de buisjes en dat hier mogelijk larven uit gekomen zijn. Tref je larven aan of vertrouw je het niet, laat dan het buisje dicht zitten en gooi dit in de gele bak weg. Let met eitjes er op dat deze niet verspreid raken, voorkom zoveel mogelijk dat eitjes met de teek mee uit het buisje gaan en dat ze hier door overal op je werk plek terecht komen. Er mogen wel enkele eitjes met het vrouwtje mee het nieuwe buisje in. Vul het oude buisje, waar de overige eitjes in zitten met 70% alcohol, draai de dop er weer op en gooi het geheel in de gele bak. Mochten er eitjes op tafel terecht gekomen zijn, dan kan je de tafel desinfecteren met 70% alcohol.

1. Haal de teek uit het verzendmateriaal en bekijk hem onder de microscoop. Hier voor kan je gebruikmaken van een object glaasje waar klei opgeplakt is. Hier in kan je de teek makkelijk vast pinnen waardoor je de teek goed van alle kanten kan bekijken.
2. Determineer de teek. Om onderscheid te maken is het belangrijk om naar de volgende zaken te kijken:
    - De vorm van het schild.
    - De genitale opening en dan met name de positie hier van.
    - De sporen op de basis van het eerste poten paar.
      (Zie bijlage I voor informatie over hoe je enkele van de veel voorkomende teken in Nederland kan herkennen.)
3. Wanneer je bepaald hebt welke soort teek het is dan doe je deze in oude buisje als deze nog redelijk schoon is en anders in een nieuw buisje. Teken, afkomstig van dezelfde gastheer, van het zelfde geslacht (♀/♂) en zelfde stadia (nimf/larve) mogen bij elkaar in 1 buisje. (Dus als er 3 ♀, 1 ♂, 2nimfen en 1 larve ingezonden zijn die van bijvoorbeeld 1 hond afkomen heb je uit eindelijk 4 buisjes.)
   Bij larven en nimfen is het niet mogelijk om te bepalen bij welke ondersoort ze horen, deze worden dan ook opgeschreven als bv. *Ixodes sp.* 1 x larve.
4. Geef elke buis een uniek nummer en schrijf op het formulier de datum van determinatie, het nummer van het buisje en wat er in het buisje zit. Mochten je meerdere buisjes hebben, dan graag meerdere formulieren in vullen waarbij je een deel van het originele formulier over neemt zo dat duidelijk is dat deze bij elkaar horen en schrijf op elke formulier weer de datum, nummer van het buisje en wat er in zit. (Deze formulieren worden naast dat het in de database gezet wordt, ook opgeslagen.)
5. Vul de buisjes aan met 70% alcohol zo dat de teken ruim onder staan. Vervolgens kunnen ze in de lade gedaan worden waarin ze opgeslagen worden.

Teken die stuk gegaan zijn, of erg beschimmeld zijn worden niet opgeslagen. Deze mag je in de gele bak weg doen. Probeer deze teken wel te determineren, eventueel alleen het hoofdgeslacht (b.v. *Ixodes*) en vul het formulier wel aan met deze informatie. Deze teken krijgen tevens een nummer, alleen hoef je deze niet op het buisje te plakken maar op het formulier zelf. Indien er meerdere teken zijn, waarvan er 1 niet opgeslagen wordt dan schrijf je op het formulier wat er in het buisje zit en dat je 1 teek weg gegooid hebt. (bv. Datum, buis nummer, *I. ricinus* 2x♀ en 1x *I.ricinus* ♀ weggegooid.)

Mocht je twijfelen over de teek vraag dan altijd de hulp van Frans Jongejan of iemand anders. Mocht er niemand zijn die je verder kan helpen, zet dan het buisje apart zodat er later alsnog naar gekeken kan worden.

Let er ook op dat je geen dubbele buisnummers gebruikt.

Wanneer je klaar bent met determineren, zorg dan dat je de plek schoon en opgeruimd achter laat. Desinfecteer je werkplek met 70% alcohol.

DNA isolatie uit de teek

In de T1 en B3 buffer kan neerslag gevormd zijn als deze lang niet gebruikt zijn. Verwarm deze buffers in een waterbad tussen de 50-70^0C tot de neerslag opgelost is.
Zet tevens een waterbad aan op 56^0C en een hitteblok op 70^0C. Voorverwarm de BE buffer op 70^0C.
Voor je begint, verwijder de blender delen en was deze in een buis met gedemineraliseerd water, vervolgens in 70% alcohol en hierna weer in gedemineraliseerd water. Droog de onderdelen en zet de blender weer in elkaar. Vul tevens het sonificatie bad met gedemineraliseerd water.

1. Was de teek in het sonificatie bad gedurende 20-30 seconden, controleer eventueel onder de microscoop of de teek schoon getrild is.
2. Stop vervolgens de individuele teek in een epje welke ruim wordt aangevuld met 70% alcohol en vortex de teek gedurende 5 tot 10 seconden.
3. Was de pincet eerst in 70% alcohol en vervolgens in gedemineraliseerd water.
4. Haal de teek uit het epje, het epje kan vervolgens gesloten in de gele bak weg gegooid worden, en laat deze enkele tellen drogen op een tissue of filter papier.
5. Wanneer de teek droog is, plaats deze dan op een rond filter papier en snij hem voorzichtig met het scalpel mesje in 2, of als het een grotere teek is in 4, stukjes. Neem voor elke teek een schone plek op het filter.
6. Doe deze stukjes teek in een 2 ml epje en voeg hier 180µl T1 buffer aan toe en label dit epje correct. (Schrijf het nummer van het buisje op, met toevoeging van een letter als er meerdere teken in het buisje zaten en schrijf ook de datum van de DNA isolatie op.)
   a. Blender de teek tot er nog maar zeer kleine stukjes over zijn.
   b. Verwijder de blender onderdelen en was deze in gedemineraliseerd water, vervolgens in 70% alcohol en hierna weer in gedemineraliseerd water.
   c. Droog de onder delen en zet de blender weer in elkaar.
   Was de pincet, net zoals bij stap b, na de teek uit het sonificatie bad gehaald te hebben en na het in het epje doen van de gesneden tekenstukjes.
   (Ververs om de 5 teken de twee buizen met gedemineraliseerd water en de 70% alcohol)
7. Voeg 25µl proteinase K toe en vortex. (Proteinase K ligt in de vriezer.)
8. Incubeer vervolgens 1 tot 3 uur bij 56^0C. Vortex elk uur. De duur van deze incubatie is afhankelijk van hoe snel de teek afgebroken wordt, maar duurt meestal 3 uur.
   a. Als het hitte blok nog niet aan staat op 70^0C, doe dit dan voor het laatste uur incuberen en voorverwarm de BE buffer.
9. Voeg 200µl B3 buffer toe.
10. Incubeer vervolgens 10 tot 15 minuten bij 70^0C. Draai de epjes vervolgens kort af in de centrifuge zodat de epjes weer vrij zijn van condensvorming.
11. Voeg 210µl 100% alcohol toe en vortex.
12. Centrifugeer 2 minuten op 11.000 x g.
13. Breng het supernatant over naar een nucleospin kolom en centrifugeer 1 minuut op 11,000 x g . Verwijder de doorgelopen vloeistof. (Voorkom dat stukjes teek, mee genomen worden op de kolom. Label de kolom correct.)
14. Voeg 500µl BW buffer toe en centrifugeer 1 minuut op 11.000 x g. Verwijder de doorgelopen vloeistof.
15. Voeg 600µl B5 buffer toe en centrifugeer 1 minuut op 11.000 x g. Verwijder de doorgelopen vloeistof.
16. Centrifugeer vervolgens nog een keer 1 minuut op 11.000 x g.
17. Plaats de colom in een nieuwe, steriele, 1,5ml epje. Label dit epje correct. (TeekID + Datum.)
18. Pipetteer vervolgens 100µl voorverwarmde BE buffer direct op het membraan en incubeer gedurende 1 minuut.
19. Centrifugeer 1 minuut op 8.000 x g en vervolgens nog 1 minuut op 11.000 x g.
20. Bewaar het verkregen DNA monster bij -20.

Leeg na afloop het sonificatie bad en desinfecteer het bad met 70% alcohol.

DNA isolatie uit bloed

In de B3 buffer kan neerslag gevormd zijn als deze lang niet gebruikt zijn. Verwarm deze buffer in een waterbad tussen de 50-70^{0}C tot de neerslag opgelost is.
Zet tevens een hitteblok op 70^{0}C. Voorverwarm de BE buffer op 70^{0}C.

1. Pipetteer tot 200µl bloed en 25µl Proteinase K in een 1,5ml epje. (Proteinase K ligt in de vriezer.)
2. Voeg 200µl B3 buffer toe en vortex goed (10 – 20s)
3. Incubeer de monsters 5 minuten bij kamertemperatuur.
4. Incubeer vervolgens 15 minuten bij 70 op het hitteblok. (De monsters moeten nu bruinig kleuren, is dit niet het geval of wordt er met oud bloed gewerkt dan kan de incubatie verlengd worden tot 30 minuten. Vortex enkele keren goed.)
5. Draai de monsters kort af.
6. Voeg 210µl 96-100% ethanol toe.
7. Vortex de monsters.
8. Breng de monsters over op correct gelabelde Nucleospin kolommen
9. Centrifugeer 1 minuut op 11.000 x g en verwijder de doorgelopen vloeistof.
10. Voeg 500µl BW buffer toe en centrifugeer 1 minuut op 11.000 x g. Verwijder de doorgelopen vloeistof.
11. Voeg 600µl B5 buffer toe en centrifugeer 1 minuut op 11.000 x g. Verwijder de doorgelopen vloeistof.
12. Centrifugeer vervolgens nog een keer 1 minuut op 11.000 x g.
13. Plaats de colom in een nieuwe, steriele, 1,5ml epje. Label dit epje correct. (bloedID + Datum.)
14. Pipetteer vervolgens 100µl voorverwarmde BE buffer direct op het membraan en incubeer gedurende 1 minuut.
15. Centrifugeer 1 minuut op 8.000 x g en vervolgens nog 1 minuut op 11.000 x g .
16. Bewaar het verkregen DNA monster bij -20.

PCR using Phire Hot Start II DNA polymerase

Master Mix for 1 reaction:

5.0 µl	5x Phire reaction buffer
0.5 µl	10 mM dMTPs (2,5 µl 2 mM solution)
0.5 µl	F primer (20 pmol/µl)
0.5 µl	R primer (20 pmol/µl)
0.125 µl	2U/µl Phire Hot Start II DNA polymerase
15.875 µl	H_2O (or 13.875 if using 2mM dNTP solution)
y µl (usually 2,5 µl)	cDNA or DNA

Final volume: 25 µl (22.5 µl mix + 2.5 µl PCR product)

PCR program:

Initial denaturation	98°C	30s
Denaturation	98°C	7s
Annealing	Lowest T_m+3°C	5s
Extransion	72°C	10-15s/1 kb
Final extension	72°C	1 min.
Hold at 4°C/room temperature		∞

Agarose gel electroforese

1. Om 1 liter 1x TEA te verkrijgen moet de een deel van de 50x TEA stockoplossing verdund worden (20ml 50x TAE aanvullen tot 1000 met Milli-Q water)
2. Weeg vervolgens 1,125 gram agarose af en voeg hier 75ml 1x TEA aan toe en verwarm vervolgens de oplossing in een magnetron tot dat de agarose gesmolten is.
3. Laat de oplossing afkoelen tot ongeveer 60 ^{O}C en voeg 2,5µl van de (10mg/ml) Ethidiumbromide oplossing toe. **LET OP! Ethidiumbromide is carcinogeen! Draag handschoenen!**
4. Plak van de electroforese tray beide kanten af met tape, zodat de gel niet kan gaan lekken, en plaats de kam.
5. Giet voorzichtig de gel. (Eventuele luchtbellen kunnen verwijderd worden met een pipet punt.)
6. Wanneer de gel gestold is kan de kam voorzichtig verwijderd worden en de tray in het electroforese apparaat gezet worden.
7. Vul indien nodig de 1x TAE niveau aan tot de volledige gel onder een klein laagje buffer staat.

PCR product voorbereiden op de electroforese
Pipetteer 1µl 6x loadingbuffer in een 0,2ml epje of in een welletje van een 96 wells plaat. Voeg hier 5 µl PCR product aan toe.
Pipetteer vervolgens voorzichtig het PCR product in een slotje. De loadingbuffer bevat een hogere dichtheid dan de TEA buffer, hierdoor zal je PCR product + loadingbuffer naar onderen zakken in het slotje. Echter, wanneer te vlug gepipetteerd wordt bestaat de kans dat het slotje "overstroomt" en je PCR product niet in het slotje blijft.

Pipetteer vervolgens 5 µl van de DNA ladder aansluitend of voorafgaand aan je PCR monsters. (1 per "rij" slotjes die gebruikt worden.)

Run vervolgens de gel gedurende 30-45minuten.

Vervolgens kan de gel, indien de producten vergenoeg door de gel heen gemigreerd zijn, bekeken worden onder UV licht en tevens kan er een foto van de gel gemaakt worden. (De DNA ladder bevalt kleurstoffen welke als referentie dienen tijdens het electroforeren. Aan de hand van deze kleur fracties kan er bepaald worden of er lang genoeg geëlectroforeert is. Wanneer de fragmenten erg langzaam migreren door de gel, kan het zijn dat de buffer te vaak gebruikt is en dient deze vervangen te worden. Giet de oude buffer in het afvalvat waar duidelijk "EtBr Waste" opgeschreven staat.)

Reverse Line Blot (RLB) hybridisatie

Controleer of een PCR beschikbaar is, en schrijf je in voor de tijd dat de PCR gebruikt zal worden. (Tijd begin, tijd eind – Naam. Bij een overnacht PCR kan er O/N geschreven worden als eind tijd.)

1. Combineer en verdun de verkregen PCR producten in een 1,5 epje. Neem van elk PCR product 10 µl en vul dit aan tot een totaal volume van 160 µl met 2x SSPE/0,1% SDS. (B.V. 10 µl Anaplasma/Ehrlichia PCR + 10 µl Babesia/Theileria PCR + 140 µl 2x SSPE/0,1%SDS. De verdunning van de PCR producten kan ook de dag voor de RLB gedaan worden, de verdunde monsters kunnen vervolgens bewaard worden in de koude kamer.)
2. Denatureer de verdunde PCR producten gedurende 10 minuten bij 100°C op een heating block en koel vervolgens de epjes meteen op ijs. Centrifugeer, short spin, de epjes kort nadat ze zijn afgekoeld.
3. Incubeer tijdens de denaturatie van de PCR producten het RLB membraan gedurende 5 minuten in ±100ml 2x SSPE/0,1%SDS bij kamertemperatuur onder zacht schudden.
4. Plaats het membraan op een ondersteunend kussen in de miniblotter, met de sloten van de miniblotter haaks op de aangebrachte probes op het membraan.
5. Verwijder de buffer uit de sloten van de miniblotter met behulp van vacuüm.
6. Pipetteer vervolgens de PCR verdunde producten in de sloten. (De sloten kunnen 150 µl bevatten dus je houd 10 µl van de 160 µl over. Deze overmaat is zodat de gehele slot gevuld kan worden ZONDER luchtbellen. Wanneer luchtbellen ontstaan, zuig met behulp van de pipet het monster weer op en pipetteer opnieuw tot dat er geen luchtbellen meer in de slot aanwezig zijn.)
7. Laat de PCR producten gedurende 60 minuten hybridiseren bij 42°C in de stoof zonder te schudden.
8. Zet alvast 30ml 2x SSPE/0,5%SDS in een tube in de stoof bij 42°C om op temperatuur te komen.
9. Verwijder de monsters met behulp van vacuüm.
10. Was het membraan 2x met ±100ml voorverwarmde 2x SSPE/0,5% SDS gedurende 10 minuten bij 50°C onder rustig schudden in het waterbad.
11. Incubeer het membraan met 30 ml 2x SSPE/0,5% SDS + 5 µl streptavidine gedurende 30 minuten bij 42 in de stoof onder rustig schudden. Zet het waterbad op alvast op 42°C met de 2x SSPE/0,5%SDS zodat beide op de juiste temperatuur komen.
12. Was het membraan 2x met ±100ml 2x SSPE/0,5% SDS gedurende 10 minuten bij 42°C onder rustig schudden.
13. Was het membraan 2x met ±100ml 2x SSPE gedurende 5 minuten bij kamertemperatuur onder rustig schudden.
14. Verwijder de 2x SSPE.
15. Spreid 10ml ECL (5ml ECL1 + 5ml ECL2, koude kamer) over het membraan door met de hand de bak heen en weer te bewegen tot het volledige membraan bedekt is met ECL.
16. Plaats het membraan tussen 2 overhead sheets of tussen keuken folie, voorkom luchtbellen.
17. Plaats het membraan in de foto cassette.
18. Ga naar de donkere kamer op de 5^{e} verdieping en plaats de x-ray film op het membraan. (Markeer hoeken zodat het uiteindelijk makkelijker oriënteren is.)
19. Belicht de x-ray gedurende 10 minuten.
20. Ontwikkel de foto met behulp van het ontwikkelingsapparaat.

RLB membraan strippen

Plaats de 1% SDS oplossing in het waterbad en laat beide opwarmen tot 80°C.

1. Was de gebruikte membraan 2x met 1% SDS oplossing gedurende 30 minuten bij 80°C onder rustig schudden.
2. Wanneer er gedurende langere tijd geen gebruik gemaakt wordt van het membraan volgt nog 1x wassen van het membraan met 20mM EDTA oplossing gedurende 15 minuten bij kamertemperatuur.
3. Berg het membraan op in de sealbag en voeg ±2ml 20mM EDTA toe.