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STUDY OF THE EPIDEMIOLOGY OF TICKS AND TICK-BORNE DISEASES IN CATTLE IN THE SOUTH OF THE IVORY COAST USING REVERSE LINE BLOT-PCR.

Thèse

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Par

Christine Rühle

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Acceptée sur proposition du jury:

Prof. B. Betschart, directeur de thèse
Dr L. Gern, co-directrice de thèse
Dr L. Achi, LANADA, Côte d'Ivoire
Dr J.-F. Graf, Basel
Prof. K. Pfister, Ludwig Maximilian Universität, München
Dr F. Stachurski, CIRAD, Madagascar

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FACULTE DES SCIENCES
Secrétariat-Décanat de la faculté
Rue Emile-Argand 11
CP 158
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Study of the epidemiology of ticks and tick-borne diseases in cattle in the South of the Ivory Coast using reverse line blot-PCR

Christine RUEHLE

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

Mme L. Gern (co-directrice de thèse), L. Achi (Abidjan),
MM. B. Betschart (directeur de thèse), K. Pfister (Münich D)
J.-F. Graf (Bâle) et F. Stachurski (Madagascar)

autorise l'impression de la présente thèse.

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Le doyen :
F. Kessler

Felix Kessler

Téléphone : +41 32 718 21 00 E-mail : secretariat.sciences@unine.ch www.unine.ch/sciences

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Summary

Livestock production throughout the world is confronted with serious economical losses caused by ticks and tick-borne diseases. In tropical countries, ticks are among the most important ectoparasites of the livestock. The presence of ticks has a great impact on the health of cattle as well as the productivity of livestock in general. Losses are caused by the damage inflicted by the tick infestation itself (as a result of severe bite wounds), but also by the vector role of ticks for a variety of disease causing parasites such as viruses, bacteria and protozoa. In essence, ticks and tick-borne diseases are a major impediment to the livestock production in the Ivory Coast and limit all measures taken to obtain a successful cattle industry. The problematic gained in importance by the importation of highly productive exotic breeds and cross-breeding of indigenous cattle breeds with more productive European breeds. Cattle breeds are known to vary in their resistance and sensibility towards ticks and tick-borne diseases.

Due to the great draught during the seventies, livestock production has been expanded from the Northern Savanna towards the Central and Southern regions, where no important food shortage occurs and conditions are favourable for most of the year. The South of the Ivory Coast is therefore gaining importance for cattle production.

The prevalence of ticks and tick-borne diseases has not been thoroughly investigated in the Ivory Coast yet. Previous studies reported heavy tick infestation and high calf mortality in the Northern part of the Ivory Coast and occurrence of ticks and Cowdriosis in the Center. In contrast, no study has assessed the tick species present in the Southern area or the prevalence of tick-borne diseases such as Cowdriosis, Anaplasmosis, Babesiosis and Theileriosis. Baseline data are needed in order to create an efficient tick control program.

The aim of the present study was to evaluate basic information on ticks and tick-borne diseases in cattle in the South of the Ivory Cost. The tick species present on cattle, their seasonal distribution, the parasites detected in the main vector species as well as the prevalence of the four major tick-borne diseases, Cowdriosis, Anaplasmosis, Babesiosis and Theileriosis in cattle were the subject of the present study. Four different herds were followed in a longitudinal study in close collaboration with the LANADA (Laboratoire Central Vétérinaire de Bingerville, Côte d'Ivoire,

Responsible person Dr L. Achi). It was the first time, molecular techniques, i.e. a RLB-PCR assay, were used to study the topic in the Ivory Coast.

The spectrum of tick species found on cattle in the South of the Ivory Coast was *Amblyomma variegatum*, *Rhipicephalus (Boophilus) decoloratus*, *R. (B.) geigyi*, *R. (B.) annulatus* and *R. lunulatus*. Only at one occasion a female *Hyalomma truncatum* tick was found on one of the investigated cattle. In accordance to other West African countries, *A. variegatum* was the most abundant tick species throughout the Ivory Coast. The favourable climatic conditions prevailing all year round permitted the presence of the major cattle tick species, i.e. *A. variegatum* and *Rhipicephalus (B.)* sp, throughout the year. More than one generation could be observed for both species and marked seasonal distribution patterns were absent.

Besides, various tick-borne parasites could be detected in cattle by RLB-PCR, namely *Anaplasma marginale*, *Ehrlichia sp*, *Babesia bovis*, *B. bigemina*, *Theileria mutans* and *T. velifera*, whereas neither *E. ruminantium*, *A. centrale* nor *E. bovis* could be detected. *T. mutans* and *T. velifera* were the most frequent parasites detected in cattle. No seasonal distribution could be shown for most haematoparasites, except for *T. mutans* and *T. velifera* where the prevalence did vary over the study period.

The spectrum of micro-organisms detected in the tick species *A. variegatum*, *R. (B.) geigyi* and *R. (B.) annulatus* by RLB-PCR did match well with the parasites present in cattle. *A. variegatum* was found to carry *T. mutans*, *T. velifera*, *B. bovis* and in one case *E. ruminantium*, *R. (B.) annulatus* was found to host *B. bovis*, *T. mutans* and *T. velifera*, whereas *R. (B.) geigyi* had the greatest spectrum, namely *A. marginale*, *Ehrlichia* sp, *B. bovis*, *B. bigemina*, *T. mutans* and *T. velifera*.

RLB-PCR and subsequent DNA sequence analyses did show the presence of three groups of « new » parasites in cattle, which have not been reported to exist in the Ivory Coast before, i.e. an *Anaplasma sp* (IC), an *Ehrlichia sp* (IC) and an *A. phagocytophilum*-like (IC). The new *Ehrlichia* (IC) and *A. phagocytophilum*-like (IC) were also present in *R. (B.) geigyi* and *R. (B.) annulatus*, respectively. The role of these parasites in the disease complex of tick-borne diseases in the Ivory Coast needs to be studied further.

Résumé

La production animale dans le monde entier est confrontée à des pertes économiques sérieuses provoquées par les tiques et les maladies qu'elles transmettent. Dans les pays tropicaux, les tiques sont les ectoparasites les plus importants du bétail et leur présence a un grand impact sur la santé du bétail ainsi que sur la productivité des élevages en général. Des pertes sont provoquées par les dommages infligés par l'infestation des tiques (en raison des blessures graves dues aux morsures), mais également par le rôle de vecteur des tiques pour différents parasites, tels que des virus, des bactéries et des protozoaires, causant des maladies. Les tiques et les maladies transmises par les tiques sont un frein important à la production animale en Côte d'Ivoire et limitent toutes les mesures prises pour obtenir une industrie bovine efficace. La problématique a gagné de l'importance avec l'importation de races exotiques fortement productives et avec le croisement des races indigènes avec des races européennes plus productives. Ces races de bétails sont connues pour avoir une résistance et une sensibilité différentes envers les tiques et les maladies qu'elles transmettent.

En raison de la grande sécheresse pendant les années soixante-dix, la production animale a été étendue de la savane au nord vers les régions centrales et du sud, où la nourriture manque rarement et les conditions y sont favorables pendant la majeure partie de l'année. Le Sud de la Côte d'Ivoire a donc gagné de l'importance pour la production de bétail. Jusqu'à maintenant, la prévalence des tiques et les maladies transmises par les tiques ont été peu étudiées en Côte d'Ivoire. Les études précédentes ont montrée une infestation importante de tiques et une mortalité élevée chez les veaux dans le nord de la Côte d'Ivoire, ainsi que la présence des tiques et de la cowdriose au centre du pays. En revanche, aucune étude n'a été effectuée sur les espèces de tiques dans le Sud, ni sur la prévalence des maladies transmises par les tiques telles que la cowdriose, l'anaplasmose, la babésiose et la theilériose. Des données de bases sont nécessaires afin de créer un programme de lutte efficace.

Le but de la présente étude est de mettre en place une information sur les tiques et les maladies transmises au bétail dans le sud de la Côte d'Ivoire. Ce travail a pour sujet l'étude des espèces de tiques présentes sur le bétail, de leur distribution saisonnière, des parasites détectés dans les principaux vecteurs, ainsi que la prédominance

des quatre principales maladies transmises par les tiques au bétail, la cowdriose, l'anaplasmose, la babésiose et la theilériose. Quatre troupeaux différents ont été suivis dans une étude longitudinale en collaboration étroite avec le LANADA (Laboratoire Central Vétérinaire de Bingerville, Côte d'Ivoire, responsable Dr L. Achi). C'était la première fois que des techniques moléculaires, comme la technique RLB-PCR, ont été utilisées pour étudier ce sujet en Côte d'Ivoire.

Les différentes espèces de tiques trouvées sur le bétail dans le Sud de la Côte d'Ivoire sont *Amblyomma variegatum*, *Rhipicephalus (Boophilus) decoloratus*, *R. (B.) geigyi*, *R. (B.) annulatus* et *R. lunulatus* et, une seule tique femelle de *Hyalomma truncatum*. Comme dans les autres pays africains occidentaux, *A. variegatum* est l'espèce de tiques la plus abondante dans l'ensemble de la Côte d'Ivoire. Les conditions climatiques du pays ont favorisé la présence des espèces principales de tiques, c.-à-d. *A. variegatum* and *Rhipicephalus (B.)* sp, tout au long de l'année. Plus d'une génération a été observée pour les deux espèces. Une variation saisonnière marquée était absente.

En outre, divers parasites transmis par les tiques ont pu être détectés dans le bétail par RLB-PCR, à savoir *Anaplasma marginale*, *Ehrlichia* sp, *Babesia bovis*, *B. bigemina*, *Theileria mutans* et *T. velifera*, tandis que *E. ruminantium*, *A. centrale* et *E. bovis* n'ont pas été détectés. *T. mutans* and *T. velifera* étaient les parasites les plus fréquents dans le bétail. Une distribution saisonnière des différents haematoparasites n'a pas pu être montrée pour la majorité de haematoparasites. Cependant, la prévalence de *T. mutans* et *T. velifera* a changé au cours de la période d'étude.

Les micro-organismes détectés dans *A. variegatum*, *R. (B.) geigyi* et *R. (B.) annulatus* par RLB-PCR sont identiques aux parasites présents dans le bétail. *A. variegatum* est infecté par *T. mutans*, *T. velifera*, *B. bovis* et dans un cas par *E. ruminantium*. *R. (B.) annulatus* est infecté par *B. bovis*, *T. mutans* et *T. velifera*, tandis que *R. (B.) geigyi* est porteur de la plus grande diversité de parasite, à savoir *A. marginale*, *Ehrlichia* sp, *B. bovis*, *B. bigemina*, *T. mutans* and *T. velifera*.

La RLB-PCR et l'analyse des séquences d'ADN ont montré la présence de trois groupes de "nouveaux" parasites dans le bétail, qui n'ont pas encore été répertoriés en Côte d'Ivoire, c.-à-d. une *Anaplasma* sp (IC), une *Ehrlichia* sp (IC) et une *A. phagocytophilum*-like (IC). Les nouvelles *Ehrlichia* (IC) et *A. phagocytophilum*-like (IC) étaient également présents dans *R. (B.) geigyi* et *R. (B.) annulatus*, respectivement. Le rôle de ces parasites dans l'ensemble des maladies transmises par les tiques en Côte d'Ivoire doit être étudié plus en avant.

Chapter 1

Introduction

1.1 Tick biology and tick-borne diseases

1.1.1 Tick biology

Ticks are a highly specialized group of obligate, blood sucking ectoparasitic arthropods that feed on mammals, birds and reptiles [19]. Belonging to the Arachnids, member of the subclass Acari, suborder Ixodida, they can be divided into three families, the *Nuttalliellidae*, the *Argasidae* (soft ticks) and the *Ixodidae* (hard ticks) [19] [23].
To date, 899 species have been described worldwide, namely 1 species for the *Nuttalliellidae*, 185 species for the *Argasidae* and 713 species for the *Ixodidae* (List of valid genus and species names of ticks as of February 2004 by SC Barker and A Murrell) [13].

All tick species of the *Ixodidae* have three mobile developmental stages, larva, nymph and adult, all taking blood meals on their host. In contrast, in the *Argasidae*, development is gradual, with multiple nymphal stages before reaching the adult form [23]. Another characteristic of the *Argasidae* is that females feed several times and oviposit after each blood meal, whereas females of the *Ixodidae* only feed and oviposit a single time.
The majority of hard ticks (*Ixodidae*) require three hosts to complete development. A typical example of such a triphasic life cycle is *Amblyomma variegatum*, where larvae, nymphs and adults each seek a different host (Annex B.1). Shortly, the larva hatched from the egg climbs on a host, becomes replete and drops to the ground to molt into the next stage. It climbs the second host as nymph, becomes replete and drops again to the ground to molt. Adults attach to a new host, feed and mate, the engorged female drops from the third host to lay eggs on the ground and dies [105]. In general, the life-cycle of 3-host ticks can be completed in less than a

year. However, climatic conditions and diapause may delay host seeking behaviour, development or onset of oviposition and extend the duration of the life cycle. Numerous variations in the basic tick life cycle occur. In some species, e.g. *Hyalomma anatolicum excavatum*, fed larvae remain on the host, molt and the unfed nymphs reattach. Only engorged nymphs detach from the host and drop to the ground to molt. These ticks are known as 2-host ticks (diphasic life cycle) [105]. Ticks of the subgenus *Rhipicephalus (Boophilus)* are examples of one-host ticks (monophasic), where larvae, nymphs and adults feed and molt on one and the same host, i.e. larvae attach to bovines and engorged females drop off the host some weeks later [105] (Annex B.2). These feeding habits are of significance in disease transmission and control of ticks.

The species of ticks of veterinary importance are mainly to be found in the family *Ixodidae* comprising 12 genus. In Africa, ticks of the genera of *Amblyomma*, *Rhipicephalus* (including the subgenus *Rhipicephalus (Boophilus))* and *Hyalomma* are known to be a problem for cattle production:

Amblyomma spp 142 species of *Amblyomma* are described worldwide today [13]. In Africa, several species occur, among these *A. hebraeum*, *A. variegatum*, *A. pomposum*, *A. gemma* and *A. lepidum*, all known to transmit heartwater, an important African disease of cattle, sheep and goats [130]. The two most important species in Africa are *A. variegatum* and *A. hebraeum* [130]. *A. variegatum* is one of the most common and widely distributed ticks on livestock in Africa, whereas the distribution of *A. hebraeum* is restricted to South Eastern Africa [123]. The less important *A. lepidum* is restricted to the Eastern regions [123].
A. variegatum is a three-host tick which is adapted to a wide variety of climates. In regions with a single annual rain season, the species is known to have a seasonal occurrence pattern, with adults being more abundant during the wet season, and larvae and nymphs during the dry season [110]. In West Africa, *A. variegatum* is one of the most harmful tick species [107]. Along with the fact that it is a main vector for heartwater, its bites cause serious wounds, especially in the region of the udder, often reducing milk production. During feeding, the tick causes severe blood loss and increases the spread of acute bovine dermatophilosis. It is difficult to be removed from the animals due to the long mouthparts which allow anchoring well the ticks.

Rhipicephalus spp To date, 79 species of *Rhipicephalus* are reported to occur worldwide including the new subgenus *Rhipicephalus (Boophilus)* sp [13]. Adults of most species parasitize wild and domestic artiodactyles or carnivores, while immatures feed mostly on smaller mammals [123]. The life cycle is normally a three-host cycle, but depending on the climatic conditions also two-host cycles do occur.

Among the diverse *Rhipicephalus* species, *R. sanguineus* is the most widespread tick. Though it can be found on cattle, the species is nevertheless specialised on dogs and not involved in transmitting diseases to cattle [123].
The former genus *Boophilus* has now been placed in a subgenus of *Rhipicephalus* sp and named *Rhipicephalus (Boophilus)* sp [13], but the name *Boophilus* is still in use. The subgenus comprises species that are among the most important tick species in the world. Each of the species of the sub-genus *Rhipicephalus (B.)* has a one-host life cycle that can be completed in a short time and results in heavy tick burden, especially under conditions of high temperature and humidity [123]. *Rhipicephalus (B.)* ticks are difficult and expensive to control and acaricide resistance is widespread [130].
Five *Rhipicephalus (B.)* sp have been described, namely *R. (B.) annulatus*, *R. (B.) decoloratus*, *R. (B.) geigyi*, *R. (B.) kohlsi* and *R. (B.) microplus* of which *R. (B.) annulatus*, *R. (B.) decoloratus*, *R. (B.) geigyi* and *R. (B.) microplus* can be found in Africa [2].
The most important tick is *R. (B.) microplus*, a species which was not reported so far in West Africa [35]. In the humid West African zones, *R. (B.) annulatus* mixes with or is totally replaced by *R. (B.) geigyi*. *R. (B.) decoloratus* does occur together with these two species in West Africa, but is also widely distributed in regions with savanna and temperate climate throughout Africa south of the Sahara [35]. In the Ivory Coast, all species except *R. (B.) microplus* have been reported [2] [81].

Hyalomma spp 25 species of *Hyalomma* are described to date. In principle, a 3-host tick, several important species undergo 2-host cycles as an adaptation to harsh environment [130]. The diverse *Hyalomma* species are well adapted to dry zones including desert climates. The tick bite can cause deep wounds, secondary infections, tick toxicosis or tick paralyses [130]. In Africa, *Hyalomma a. anatolicum* and *H. d. detritum* occur only in North Africa and are thought to be absent from West Africa [123]. In contrast, *H. m. rufipes* is widely distributed in Africa and adapted to a wide range of climatic regions. The other species known to occur in dry habitats in West Africa is *H. truncatum* [123].

The geographic distribution of the various tick species is depending on climatic factors, such as temperature and humidity, as well as the availability of suitable hosts. Many species have adapted to the seasonal variations in their habitats. In the tropics this is usually to overcome the adverse effect of the prolongated dry seasons [23], [123]. The presence of ticks is a hassle to their hosts. Especially one-host ticks can be present in very large numbers. Tick bites may inflict necrosis and open wounds to the animals which can get infected by myiasis and bacteria. In addition, since ticks do feed for a long period and do concentrate the blood ingested, an important amount of blood is absorbed from the animal [23]. Next to this, tick

species play an important role in disease transmission. Ticks get infected or transmit pathogens to the host during feeding. The ingested pathogen can be passed on from stage to stage (exp. *Theileria* sp in *R. appendiculatus*) or some pathogens can also be passed on transovarially (exp *Babesia bovis* in *R. (Boophilus)* sp) [23].
Ticks transmit various pathogens causing diseases in cattle and other host species (Table 1.1). For instance, several *Amblyomma* species are known vectors for heartwater [130], i.e. they transmit *Ehrlichia ruminantium* during feeding. *Rhipicephalus (B.)* species are vectors for *Babesia bovis*, *B. bigemina* and *Anaplasma marginale* [123], though the capacities in transmitting these pathogens may vary among the species [23]. For instance among the typical species of West Africa, *R. (B.) annulatus* is a major vector of *B. bigemina*, *B. bovis*, and *A. marginale*, *R. (B.) decoloratus* is an efficient vector of *B. bigemina* and *A. marginale*, but does not transmit *B. bovis* and the vector capacity of *R. (B.) geigyi* is only poorly known [123]. *Rhipicephalus* ticks transmit a variety of viruses (exp. Nairobi sheep disease and West Nile) and pathogens, such as *Theileria parva*, *T. taurotragi*, *E. bovis*, *B. canis* and *Rickettsia conorii* [130]. The species being responsible for transmitting *T. parva* which causes East Coast Fever in cattle, *R. appendiculatus*, is not known to occur in West Africa [123]. In general, little is known about the capacity for transmitting tick-borne diseases to cattle for the *Rhipicephalus* species occurring in West Africa, such as *R. senegalensis*, *R. guilhoni* and *R. musamae*. In Africa, the known vectors for *T. annulata* which causes tropical theileriosis in cattle (*Hyalomma a. anatolicum*, *H. d. detritum*) occur only in North Africa and are thought to be absent from West Africa [123].

Table 1.1: A selection of important African cattle tick species and pathogens transmitted [123] [23].

Vector species	Bovine pathogen[a]	Other pathogen
AMBLYOMMA		
A. variegatum	*E. ruminantium*, *E. bovis*, *T. mutans*, *T. velifera*	*T. ovis*, *R. conorii*, *R. africae*
A. hebraeum	*E. ruminantium*, *T. mutans*	*R. conorii*, *R. africae*
RHIPICEPHALUS		
R. appendiculatus	*T. parva*, *T. taurotragi*, *E. bovis*	*R. conorii*
R. lunulatus	poorly known	
R. e. evertsi	*A. marginale*	*B. ovis*, *B. caballi*, *T. separata*, *T. equi*
R. (Boophilus) sp		
R. (B.) microplus	*B. bovis*, *B. bigemina*, *A. marginale*, *Borrelia theileri*	
R. (B.) annulatus	*B. bovis*, *B. bigemina*, *A. marginale*	
R. (B.) decoloratus	*B. bigemina A. marginale*, *Bo. theileri*	
R. (B.) geigyi	poorly known	
HYALOMMA		
H. a. anatolicum	*T. annulata*, *E. bovis*, *Trypanosoma theileri*	*T. lestoquardi*, *T. equi*, *B. caballi*
H. d. detritum	*T. annulata*	*T. equi*
H. truncatum	*E. bovis*	*B. caballi*, *R. conorii*
H. m. rufipes	*A. marginale*, *B. occultans*	*R. conorii*

[a] Abbreviations for pathogens: A: Anaplasma, E: Ehrlichia, B: Babesia, T: Theileria, R: Rickettsia, Bo: Borrelia, Tr: Trypanosoma.

1.1.2 Tick-borne diseases

The parasites transmitted by tick vectors causing diseases in cattle can be divided into two major groups, i.e. into organisms of bacterial origin (order *Rickettsiales*) and organisms of protozoan origin (order *Piroplasmida*).

Rickettsiales

All of these procaryotes share some common features. They are all rod-shaped, coccoid, often pleomorphic gram-negative bacteria. They are obligate parasites and multiply only inside host cells. The targeted cells are reticuloendothelial and vascular endothelial cells or erythrocytes of vertebrates (host) and arthropods (vector) [43].
The intracellular bacteria were previously placed in taxa based upon morphological, ecological, epidemiological and clinical characteristics [33]. With the accessibility of molecular tools, the organisms belonging to the order *Rickettsiales* have been reclassified after extensive 16S rRNA and groESL operon gene analyses [33]. In this work we follow the new classification by Dumler et al [33]:
In the order *Rickettsiales*, four distinct genetic groups represent the genus *Anaplasma*, *Ehrlichia*, *Wolbachia* and *Neorickettsia* [33].
The *Anaplasma* group covers the species *A. marginale*, *A. centrale*, *A. phagocytophilum* (which is now regarded as synonymous to *E. equi* and the *Ehrlichia* causing Human granulocytic ehrlichiosis (HGE)), *E. bovis* and *E. platys*, whereas the group *Ehrlichia* covers the species *E. ruminantium* (former *Cowdria ruminantium*), *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. ovis* and *E. muris*.
Neither the *Wolbachia* nor the *Neorickettsia* (including *E. sennetsu*, *E. risticii*) group causes diseases in cattle [33].
The here listed bacteria are the cause of diverse diseases in many different host species. Herewith, only the diseases affecting cattle are discussed in more details.

Anaplasmosis The causative agents of bovine anaplasmosis are *A. marginale* and *A. centrale*, with *A. marginale* being the more lethal form. Anaplasmosis is a global disease present in most tropical, subtropical and many temperate countries, including the USA [27]. *Ixodid* ticks, such as *Ixodes* and *Rhipicephalus (Boophilus)*, act as vectors [61]. However, dipters such as tabanid flies are suspected to mechanically transmit *Anaplasma* sp [130]. The rickettsial pathogens invade the erythrocytes of their hosts, causing anaemia, weight loss, abortion and death [27].
Anaplasmosis is a severe disease of cattle, reaching over 50 % of morbidity, especially in the case of highly susceptible breeds, such as crossbred or milk breeds. If left untreated, most of the sick animals will die. Local breeds normally develop diseases of lesser severity and calves less than one year old have mild or no symptoms

[23]. All cattle recovering from clinical symptoms, remain chronically infected for at least several years [23].

Ehrlichiosis With the new classification of the *Rickettsiales*, the diseases previously described as ehrlichiosis are now also belonging to the anaplasmosis. However, veterinarian prefer still to call them ehrlichiosis instead of anaplasmosis in order to avoid confusion with infections caused by *A. marginale*. This is why ehrlichiosis has been conserved as title of this section.
E. phagocytophilum, renamed as *Anaplasma phagocytophilum* [33], is the causative agent of bovine ehrlichiosis in Europe. The disease is generally called tick-borne fever or pasture fever and transmitted by ticks of the genus *Ixodes* [67] [68]. The infection has now also been identified in India and in South Africa [128]. The bacteria attacks the granulocytes of the host. The clinical signs are anaemia, fever, anorexia, apathy and decreased milk production [94].
Another tropical bovine ehrlichiosis is caused by *E. bovis*. Its clinical signs can be similar to heartwater (see below). However, bovine ehrlichiosis is probably of minor importance in Africa [23] or maybe underestimated.

Cowdriosis Cowdriosis, also known as heartwater, is a disease of ruminants caused by the rickettsial parasite *E. ruminantium*, previously known as *C. ruminantium* [33]. Beside cattle, sheep and goats get infected and show more severe symptoms than cattle. *E. ruminantium* can be transmitted by several members of the genus *Amblyomma*, but the main vectors are *A. variegatum* and *A. hebraeum* [91]. Following the distribution of these vectors, the disease occurs in most sub-Saharan Africa and some Caribbean islands [62]. *E. ruminantium* attacks the vascular endothelial cells and neutrophils of its host, causing fever, nervous signs (oedema of the brain), hydropericardium and the outcome is frequently fatal [23].
Different severity degrees of the illness exist, suggesting the existence of various strains with different pathogenicity [128] [9]. The degree of illness goes from death within a few hours to inapparent infection [23]. Very young animals show age-related resistance, which may be reinforced by maternal antibodies. This is valuable for calves less than 3 weeks of age, whereas animals of 3–18 months are highly susceptible to the disease [23].
Infection does persist in recovered animals, though pathogens can not be found in the peripheral blood at all times and they do have their highest concentration in the capillaries of the brain and kidneys [105].

Protozoa

In the protozoan group, several pathogens belonging to the *Apicomplexa* (Sporozoa) are responsible for causing diseases in cattle. The pathogens, known as piroplasms

due to their pear-shaped appearance within the host cell [54], are all tick-borne, multiplying asexually (schizogony) in the cells of their vertebrate host, whereas they undergo a sexual gametogenesis in their tick vector. The organisms are obligate parasites, attacking the erythrocytes and/or lymphocytes of the host and being spread by the tick vector during feeding [43]. All piroplasms can be placed into the families, *Babesiidae* and *Theileriidae*, composed each of several species of *Babesia* and *Theileria*.

Babesiosis *Babesia* species are known to attack a wide range of hosts. However, the bovine babesiosis are of the greatest importance causing severe disease and economical damage [23].
Bovine babesiosis can be found in livestock worldwide [22]. Relevant for cattle are *B. bovis*, *B. bigemina* in the tropical and subtropical regions and *B. divergens* and *B. major* mainly in European countries [23]. All are transmitted by *Ixodid* ticks [54]. The clinical manifestations are anaemia, fever and death.
Babesiosis causes severe diseases especially in exotic cattle breeds. Without treatment, the mortality rate is of 30 to 60 % for *B. bigemina* and of 70 to 80 % for *B. bovis* [23]. In general, the tropical and subtropical traditional local breeds of Africa are less sensible to babesiosis due to their adaptation to the climatic and nutritional conditions and their capacity to acquire immunity. Although, they do get sick when the immune response is weakened due to stress or bad nutritional status, especially during the dry season [23].
Young calves up to 9 months old are known to show natural resistance and do not manifest the disease [23].
Animals recovering after acute or primary infections, generally stay infected with the parasite showing low parasitemia levels, no clinical illness and serve as a reservoir for transmission. This carrier state is microscopically undetectable [22]. In the case of *B. bovis*, the infection can persist in the cattle for 1-2 years, whereas *B. bigemina* usually disappears after 6-12 months [23].

Theileriosis Theileriosis is a widespread disease of wild and domestic animals (mostly Artiodactyla) caused by the genus *Theileria* [25]. Several species are known to infect cattle, but only *T. annulata* and *T. parva* are causing severe diseases in African cattle and are therefore of major economical importance [30]. *T. mutans* and *T. velifera* are regarded as provoking only mild symptoms [23]. Though, there are reports of pathogenic strains of *T. mutans* which might nevertheless be involved in causing severe diseases [130] [87].
All species first invade host lymphoid cells and later affect also the erythrocytes causing anaemia and sometimes even death [23].
The main distribution of *T. annulata* is North Africa, Mediterranean Europe, Near

and Middle East, India and Central Asia where it is transmitted by *Hyalomma* species. On the other side, *T. parva* is distributed in most countries of eastern and central sub-Saharan Africa as well as in the South [25] and is transmitted by *Rhipicephalus* species [23]. Both, *T. mutans* and *T. velifera* are more widely distributed and follow the distribution of their vector species *Amblyomma* sp. As in the case of other tick-borne diseases, calves of local breeds do have natural resistance against severe disease and animals recovering from illness do stay chronically infected.

1.1.3 Other diseases

Two other diseases relevant to cattle which are not transmitted by ticks are worth mentioning, one, Dermatophilosis, because it is closely associated with tick infestation, and the second, Trypanosomosis, because it is another important protozoan caused disease in Africa.

Dermatophilosis Dermatophilosis is an epidermal disease (dermatitis) mainly known in Tropical countries. The causative agent is a bacteria, *Dermatophilus congolensis*, producing severe dermatitis of livestock, often accompanied by the formation of necrosis [128]. In severe cases, animals get skinny, weak and the infection can even lead to death [128]. Dermatophilosis leads to important economical losses due to loss in meat, milk, wool, leather and death. Severe clinical cases are generally associated with tick infestation, mainly *A. variegatum*, but also *R. (B.) microplus* [128]. *D. congolensis* can not infect the intact skin, it profits from the lesions inflicted by arthropod bites.

Trypanosomosis Trypanosomosis is a vector-borne parasitic disease of African cattle (called Nagana in cattle). It is caused by protozoa belonging to the genus *Trypanosoma* and transmitted by tsetse flies which are found in all Sub-Saharan Africa [130]. Biting flies such as stomoxids and tabanids are also believed to be involved in transmitting *Trypanosoma*. Further, the tick *Hyalomma a. anatolicum* was found infected with *T. theileri* and successful transmission of parasites to calves could be shown [83]. In the Ivory Coast, *A. variegatum*, *B. geigyi* and *R. sanguineus* were also found to be infected by *T. theileri*, but it is not known, if these species actually can transmit the trypanosomes [46].
The disease presents a major obstacle to livestock production in the concerned areas. Typically, trypanosomosis is a wasting disease in which there is a slow progressive loss of condition accompanied by increasing anaemia (the main sign of the disease) and weakness to the point of extreme emaciation, collapse and death often due to heart failure. *T. congolense* or *T. vivax* are the main species involved in cattle diseases [130], whereas *T. theileri* is relatively non-pathogenic [23].

1.1.4 Tick control measurements

Today's battle for tick control aims mainly to reduce the effects of the diverse tick species to cattle and to reduce the losses in livestock production [23]. Acaricides have for a long period been the major tool to control ticks and tick-borne diseases in many parts of the world. Even to date, the use of various acaricides to kill or eliminate ticks on their cattle host is the most widely used control method to reduce the damage caused by ticks and the diseases they transmit [23]. The emergence of acaricide resistance in tick populations, the increasing costs for new acaricides and the demand for less toxic methods, have forced to look for alternatives in the approach of tick control such as biological control (bacteria, entomopathogenic nematodes, parasitoids or natural predators (exp oxpecker)) [102], agronomical control (homogenisation of herbacean surface or burning of pastures to eliminate natural hiding places for ticks, use of repulsive plants), rotation of pastures to minimize the exposure of the host to ticks and infections (normally combined with acaricide treatment), elevation of tick-resistant cattle breeds (crossbreeds between indigenous breeds and more productive exotic breeds), immunisation of animals by vaccines and use of anti-tick vaccines [23].
Detailed reviews of individual control methods are given in the section of tick control, in the Supplement 2004 of the Parasitology Journal [46][127][102].

Chemical control

In the first half of the century arsenic trioxide was the main acaricide used, but subsequently, other acaricides such as organochlorines, organophosphates, carbamates, amidines, pyrethroids have been put on the market [23].

The most widely used application methods today are dips or sprays. The dip tank is an efficient and convenient way to apply acaricide to a herd of livestock [130]. It involves a good infrastructure and maintenance and is therefore today often replaced by easier to use hand sprays, especially in small-scale farms. An alternative are pour-on formulations, which have the advantage of not requiring water or equipment for their application, but are expensive [79]. Once poured along the back line of a treated animal, the product spread and disperse over the hair and skin. Nevertheless, treatment of cattle with acaricides is combined with high costs and often considered too expensive by farm-owner of low-input regions in West Africa who continue to remove ticks manually [107].
An impediment to an extensive acaricide use is that all to date known acaricides will lead sooner or later to the emergence of resistant strains of ticks. Especially one-host ticks are particular sensitive to develop resistance to an acaricide [130]. However, before declaring a tick population resistant to a certain acaricide, it is necessary to check the correct application and concentration of the product.

It is very important to treat at a correct time schedule, using correct concentrations to minimalise chances that resistance to the product will occur, to keep costs low and to have the desired outcome of a treatment.

Integrated Approach

From today's point of view, a successful control of ticks and tick-borne diseases will need a multifactorial approach. Eradication programs for tick species have been (with a few exceptions) abandoned due to high costs and tick resistance and the aim today is to maintain endemic stability. Besides exploiting the ecology and seasonal distribution of tick species and to study the epidemiology of the diseases transmitted, the natural resistance of cattle breeds as well as immunization of sensible cattle breeds by means of vaccines are integrated to complement the application of acaricides.
Experience with other vector-borne diseases has shown that normally the most successful way to control vector-borne diseases is to control the vector. This was the key to develop a vaccine against *R. (B.) microplus*, TickGARD (Australia) and GAVAC (Cuba) [126]. These are the first commercial recombinant anti-parasite vaccines based on the Bm86 antigen of *R. (B.) microplus* [126]. However, in many countries laboratory equipments and financial means are not sufficient to provide secure application of vaccines [130]. Last but not least, the success of tick control also requires to inform and explain facts to the people involved.

1.2 The global importance of ticks and tick-borne diseases of domestic livestock

Ticks are regarded as the ectoparasites that have the biggest impact on livestock, especially cattle production throughout the world [130]. They are not only vectors for a wide spectrum of pathogenic organisms of protozoal, rickettsial, viral and nematode origin and therefore transmit various diseases to a range of different hosts, but also the tick infestation itself can affect the livestock and add to the economic loss [130]. Tick infestation can cause physical damage such as injuries due to tick bites, which are prone to myiasis and act as route of infection by bacteria and fungi, as well as screw-worms [130]. Furthermore, animals can suffer from a considerable loss of blood due to the feeding and some of the tick species can even cause paralysis or toxicosis while feeding [32]

Although, tick-borne diseases are a global problem, they are nevertheless most numerous and exert their greatest impact on livestock in the Tropical and Subtropical regions. With regard to bovine livestock, the major tick-borne diseases are: cow-

driosis (heartwater disease), anaplasmosis, theileriosis and babesiosis. All are economically damaging diseases not only causing severe losses every year by affecting the milk and meat production, inducing abortions and often causing fatalities, but also producing considerable costs due to control measures.

Over the last few years the significance of the tick-borne diseases has increased visibly, as many affected countries seek to improve their cattle production by introducing exotic cattle breeds which are highly susceptible to those diseases [82]. Reliable data describing world-wide prevalences of tick-borne diseases in general, or prevalences specific for Africa, are still not available. The same is true for the estimation of annual economical losses due to ticks and the diseases they inflict. In 1984 the Food and Agriculture Organization (FAO) estimated total annual losses due to ticks and tick-borne diseases in the world at roughly 7000 millions USD, assuming that the actual losses may in fact be much higher [120] [82]. In Tanzania only the total annual national loss due to tick-borne diseases was recently estimated at 364 millions USD [59].

Today, while losses due to other important cattle diseases are mostly under control, the diseases caused by ticks as well as the tick infestations themselves, are the main impediment to improve cattle production for local farmers in the affected countries. In many African countries, studies concerning tick-borne diseases are ongoing. However, it is very difficult to compare studies in order to obtain a global view of the distribution of tick-borne diseases in Africa. This is on one hand due to the fact that studies are often not published and on the other hand due to the great diversity of topics studied as well as the diversity of diagnostic assays used to study prevalences. Nevertheless, there exists maps predicting the possible distributions of tick-borne diseases, founded on the presence of vector tick species, climatic and environmental conditions [79].
To give an overview of the work done or going on in the many different African countries would be beyond the scope of this study. Hereinafter, the focus for presenting the distribution and prevalence of ticks and tick-borne diseases in more details will be put on West Africa, the region wherein the Ivory Coast is located.

1.3 Distribution of ticks and bovine tick-borne diseases

1.3.1 Current status in West Africa

West Africa covers an area of approximately 5 millions square km and is composed of the following countries : Benin, Burkina Faso, Ivory Coast, Gambia, Ghana, Guinea, Guinea-Bissau, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra

Figure 1.1: Map of West Africa. The countries where studies of ticks and/or tick-borne diseases were carried out are indicated with a blue dot.

Leone, Togo [129]. The western and southern borders of the region are formed by the Atlantic Ocean, whereas in the North the region is limited by the Sahara Desert. The northern section of West Africa is composed of the Sahelian belt. Beneath lies the Sudanian Savanna which gradually changes into the Guinean forest zone. Near the coast, tropical moist-forest is prevailing [89]. The climate varies greatly between the northern and southern areas. Rainfall decreases moving south to north in West Africa. The drier northern areas of West Africa have very marked seasons, i.e. the contrast between wet and dry periods is strong [48]. The climatic conditions prevailing in West Africa, i.e. high humidity for most of the year and tropical temperatures, favour the occurrence of ticks and tick-borne diseases in this area.

In several countries of West Africa studies have been undertaken to investigate the tick species present on cattle and to follow their distribution dynamics, namely in Ghana [63][122], Senegal [50] [51], Ivory Coast [60][2], Cameroon [110] [107], Burkina Faso [108] [107], Guinea [117], Gambia [24] [77], Mali, [115] and Nigeria [14] (Fig.1.1). The study of Morel [81] covered several countries of West Africa. Accordingly, the following species are present in the diverse countries such as Ghana, Senegal, Guinea, Mali, Gambia, Cameroon and the Ivory Coast : *A. variegatum*, several *Hyalomma* species like *H. truncatum*, *H. marginatum rufipes*, *H. impeltatum*, *R. (B.) decoloratus*, *R. (B.) geigyi*, *R. (B.) annulatus* and several *Rhipicephalus* sp species like *R. sanguineus*, *R. lunulatus*, *R. senegaiensis* and *R. evertsi evertsi*. However, not all the species listed can be found in all countries (Table 1.2).

In most of these countries, *A. variegatum* is stated to be the most abundant tick species [63] [81] [118] [14], though in the drier zones (northwards) the more draught

resistant *Hyalomma* species are predominant [50] [51] [81].

Ticks have adapted to their environment and often show seasonal distribution patterns. The distribution of *A. variegatum* was observed to vary with the respective environmental conditions. In the sahelian and soudanian zones, adult ticks are most abundant during the rainy season, whereas immature stages are most abundant during the dry season [81]. In contrast, adult ticks could be observed all year round in the guinean zone [81].
Generally, the second frequent species present in West Africa is *R. (B.) decoloratus* [81]. *Rhipicephalus (B.)* stages can normally be found throughout the year implicating the presence of more than one generation per year [84].

Though the presence of the diverse tick species in West Africa is well known, the prevalence of tick-borne diseases is only poorly understood. Moreover, most of the few studies investigating the presence of tick-borne diseases were undertaken by the observation of clinical symptoms and by simple blood smear examinations which lack specificity and sensitivity for low level parasitemia. A synthesis of the prevalences of tick-borne pathogens in cattle in West African countries are given in Table 1.3.

In the case of heartwater, seroprevalence studies have been made using two different ELISA assays to assess the prevalence of *E. ruminantium* in Ghana (PC-ELISA) [16] and the Ivory Coast (MAP1-B ELISA) [60]. Another study assessed the seroprevalence of *A. marginale*, *B. bovis* and *B. bigemina* in Gambia [77]. However, to our knowledge, no study did use new molecular diagnostic tools to examine the presence of haemoparasites in bovine blood in countries of West Africa.
So far, only two studies used molecular methods (PCR and RLB) to detect pathogens in ticks, namely to detect *E. ruminantium* in *A. variegatum* of Gambia by pCS20 PCR [36] and to analyse the presence of pathogens in adult *A. variegatum* by RLB in Guinea [118]. Whereas the PCR method allows only the detection of a single pathogen (*E. ruminantium*), the RLB method was designed for the simultaneous detection of many different tick-borne pathogens. In 1.6 to 15 % of the *A. variegatum* ticks of Gambia *E. ruminantium* could be detected by PCR [36] whereas no pathogen of the group *Anaplasma/Ehrlichia* was detected by RLB in *A. variegatum* of Guinea. The only pathogen detected by RLB in Guinea was *T. velifera* with a prevalence of 0.99% [118].

Table 1.2: A synthesis of tick species found on cattle in West African countries

Country	Tick species[a]	Ref.
Senegal	*A. variegatum, R. (B.). decoloratus, H. m. rufi pes, H. truncatum** *R. lunulatus, R. e. evertsi*, R. guilhoni, R. sulcatus, R. senegalensis*	[50] [51]
Ivory Coast	*A. variegatum*, R. (B.) annulatus, R. (B.) geigyi, R. (B.) decoloratus* *H. impeltatum, H. impressum, H. m. rufi pes, H. truncatum* *R. lunulatus, R. senegalensis, R. sanguineus, R. simpsoni, R. sulcatus, R. ziemanni*	[2] [60] (Achi)[b]
Ghana	*A. variegatum*, R. (B.) decoloratus, R. (B.) annulatus, R. (B.) geigyi* *H. m rufi pes, H. truncatum, R. senegalensis, R. e. evertsi, R. lunulatus*	[122] [63]
Guinea	*A. variegatum*, R. (B.) annulatus*, R. (B.) geigyi*, H. m. rufi pes, H. truncatum* *H. nitidum, R. turanicus, R. lunulatus, R. muhsamae, R. senegalensis, R. sulcatus*	[117]
Gambia	*A. variegatum*, R. (B.) geigyi*, R. (B.) decoloratus, R. senegalensis, H. m. rufi pes* *H. truncatum*	[24] [77]
Cameroon	*A. variegatum, R. (B.) decoloratus, H. nitidum, H. m. rufi pes, R. lunulatus, R. turanicus*	[110]
Mali	*A. variegatum*, R. (B.) annulatus, R. (B.) decoloratus, R. (B.) geigyi, H. dromedarii* *H. impeltatum, H. impressum, H. m. rufi pes, H. nitidum, H. truncatum* *R. suspidatus, R. e. evertsi, R. guilhoni, R. lunulatus, R. muhsamae, R. sanguineus* *R. senegalensis*	[115]

[a] *most frequent species (note: Different studies may have different results)
[b] unpublished data

Table 1.3: A synthesis of the prevalence of tick-borne parasites in cattle of West African countries.

Country	Pathogen	Prevalence (%)	Diagnostic method	Reference
Senegal	*A. marginale*	3 - 10.9[a]	Blood smears	[50], [51]
	E. bovis	0.5 - 6	"	
	T. mutans	6.3 - 13.3	"	
	T. velifera	0 - 0.3	"	
Ivory Coast	*E. ruminantium*	0	Brain smears	[60]
	A. marginale	0	Blood smears	[60]
	B. bovis	0.87	"	
	B. bigemina	0	"	
	E. ruminantium	31	ELISA	[60]
	E. ruminantium	no details	Brain smears	[Achi][b]
	B. bovis	0.12	Blood smears	[Achi][b]
	B. bigemina	0.04	"	
	Theileria sp	5.25	"	
Ghana	*E. ruminantium*	33	ELISA	[16]
	Anaplasma sp	60	Blood smears	[17]
	B. bigemina	61	"	
	T. mutans	97	"	
	T. velifera	87	"	
Guinea	*A. marginale*	5.6	Blood smears	[117]
	B. bovis	1.1	"	
	B. bigemina	21.1	"	
Gambia	*A. marginale*	3.2	Blood smears	[77]
	B. bovis	0.1	"	
	B. bigemina	0.9	"	
	A. marginale	29.6	ELISA	
	B. bovis	5.2	"	
	B. bigemina	44.7	"	
Benin	*A. marginale*	15.55	Blood smears	[88]
	B. bovis	31.11	"	
	B. bigemina	14.40	"	
	Theileria sp	57.77	"	
Cameroon	*A. marginale*	2.2	Blood smears	[84]
	B. bovis	47.3	"	
	B. bigemina	31.1	"	
Nigeria	*A. marginale*	no details	Blood smears	[3]
	B. bigemina	"	"	
	T. mutans	"	"	

[a]Prevalence varied with study sites
[b]unpublished data

1.3.2 Situation in the Ivory Coast

The Ivory Coast is situated in West Africa with a coastline on the Gulf of Guinea between Liberia on the west and Ghana on the east. It extends between the 4° and 10°N and the country covers about 330'000 square km [89]. Different vegetation zones cover the country from South to North (Fig. 1.2), but moist air flows over the whole country during most of the year. Only for short periods each year (3-5 months) the country is under the influence of continental air masses, with their low humidity and consequently lower rainfalls [48]. In the South the climate is characterised by two rainy seasons from March to July (main rainy season) and September to November, the second rainy season being much less marked than the first. Rainfall then becomes progressively less inland where finally the two separate rainy seasons of the coast merge into a single wet season extending from March to the end of October. The temperature variations in the South are less pronounced than in the North [48].

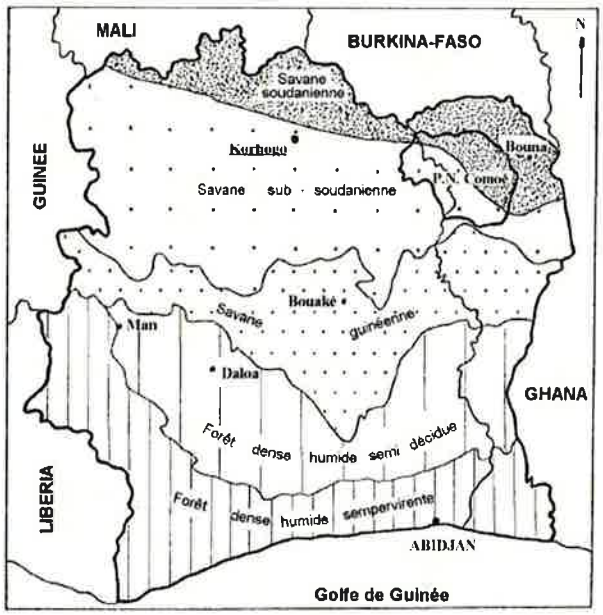

Figure 1.2: Map indicating the different vegetation zones of the Ivory Coast (Scale 1/5000000) (after Guillaumet et Adjanohoun, 1971; FAO [85])

The Ivory Coast is a very agriculturally oriented country and one of the largest producers for cocoa and coffee in the world. In the sector of livestock production, however, the country is strongly depending from other countries. The national livestock production of the Ivory Coast is covering only 46% of the need of meat and

hardly 10% of the need of fresh milk [1]. The Ivorian Government has founded the SODEPRA (Société de développement des Productions Animales) in 1970 in order to improve the effectiveness of the livestock production [130]. The political crisis of the country did impair the livestock production further. Today, ticks and tick-borne diseases are the main impediment to improve cattle production. However, only little is known about the prevalence of ticks and tick-borne diseases in the Ivory Coast.
The only important tick study including the whole country has been done 40 years ago (1967) by Aeschlimann [2] and its goal was to establish the spectrum and ecology of ticks which have been collected from different types of animals. During 3 years, a total of 9 gender and 38 species were collected and listed. Out of these the following species were collected from cattle: *A. variegatum*, *R. (B.) annulatus*, *R. (B.) geigyi*, *H. truncatum*, *H. rufipes*, *R. senegalensis* and *R. lunulatus*, whereas *R. (B.) decoloratus* was considered absent from the country. This is in contrast to the findings of Morel (1958), which reported *B. decoloratus* to be present in the Ivory Coast [81]. In general, Aeschlimann noticed that tick species adapted to arider regions are not present in the Ivory Coast due to its humid climatic conditions and that also species adapted to semi-arider regions have difficulties to survive and are therefore not very frequent [2]. *A. variegatum* was present in the whole country, showing a good adaptation to the variations in the temperature and humidity of the different regions of the Ivory Coast. In the central savanna region, *A. variegatum* manifested an annual cycle with adults infesting the host during the rainy season, whereas the main activity of nymphs was during the dry season [2]. However, in the forest, where climatic conditions are less pronounced, generation times tended to be shorter and the presence of adults and nymphs was overlapping [2] [81].
The *Rhipicephalus (Boophilus)* species were present the whole year round, generations were overlapping, i.e. adult, nymph and larva could be found at the same time on the animals [2] [81], indicating the existence of more than one generation per year. The presence of tick-borne pathogens was not assessed in the study.

More recently, the presence of *A. variegatum*, *R. (B.) geigyi*, *R. (B.) annulatus*, *R. (B.) decoloratus*, *H. rufipes H. marginatum rufipes*, *H. truncatum*, *R. sanguineus*, and *R. lunulatus* on cattle in the district of Toumodi, Central Ivory Coast [60] and in the North of the country was reported (Achi, unpublished data). However, the spectrum of tick species varied among the Northern and Central study sites.
In the Center, high proportions of animals were infested by *A. variegatum* adults during the rainy season (April to June) [60]. A smaller peak was found in October, at the end of the small rainy season. The nymphs followed the inverse trend, i.e. they were frequent during the dry season [60]. *R. (B.) annulatus* and *R. (B.) geigyi* showed several annual peaks [60].
In the North, adult and nymphal *A. variegatum* also showed inverse seasonal pattern, where adults were frequent during the rain season, and nymphs and larva during the

dry season. *R. (B.) geigyi* and *R. (B.) decoloratus* were present all year round (Achi, unpublished data).

In parallel, the impact of ticks, including aspects of tick-borne diseases, on N'Dama cattle in the Centre of the Ivory Coast were studied [60]. However, classic diagnostic techniques, i.e. Giemsa-stained blood smears analysed by microscopy, were used to estimate the presence of haemoparasites (Table 1.3). In 7 of 809 blood smears *B. bovis* could be detected. No other tick-borne pathogens were detected. This is probably an underestimation of the distribution and prevalence of tick-borne diseases due to the low sensitivity of the method.
Only in the case of Cowdriosis (prevalence of 30.9%) a serological test (ELISA) was used, which might also underestimate the prevalence, since it was developed for small ruminants and known to be less sensitive in the case of bovines [71] [78].

In a different study in the North of the country, almost all untreated cattle studied showed clinical signs of tick-borne diseases such as anaemia, apathy, fever, loss of weight and death (Achi, unpublished data). High mortality occurred especially in the case of untreated crossbred cattle. Within 4 months only 2 out of 15 animals survived. In the same study, the presence of *Theileria* sp, *Babesia* sp and *E. ruminantium* could be detected with the help of blood and brain smears (Table 1.3).

To date no study has assessed the presence of bovine pathogens in ticks and cattle of the Ivory Coast using PCR or RLB-PCR. *Ixodid* ticks such as *A. variegatum* and *H. truncatum* collected in the region of Abidjan and Korhogo are known to host rickettsial pathogens like *R. conorii* and *R. africae* [29]. Seven per cent of *A. variegatum* ticks collected around Abidjan and 2.6 % of ticks collected around Korhogo tested positive for such pathogens by PCR. Fourteen per cent of *H. truncatum* ticks were infected [29]. The DNA sequence targeted by the PCR was specific for the detection of rickettsial pathogens causing spotted fevers, i.e. *R. conorii*, *R. africae*, in human, but they might also be present in the blood of cattle [57]. In contrast, the PCR was not designed to detect pathogens of the group *Anaplasma/Ehrlichia* and therefore it is unknown, if also rickettsial pathogens of these types were present in the analysed ticks.
The cattle production was marked by changes over the past years. In order to increase the meat and milk production, farmers started crossbreeding by artificial insemination with sperm of exotic breeds, which is creating a new genetic situation. The main health impediment to efficient livestock production in the Ivory Coast is tick-borne diseases. They are a very important constraint to all trials to increase meat and milk production through artificial insemination of local breeds with more productive European cattle sperm. Studies have shown that local breeds are more resistant to tick infestations than crossbreeds (Louise Achi, unpublished data). Therefore, tick-borne diseases are likely to play a stronger role in those breeds, and crossbred cattle will thus require a different and perhaps even more intense tick control

in the same area.

For an effective control of tick infestation and for confining losses through tick-borne diseases, it is fundamental to know the local ecology of vector species and the local distribution of tick-borne diseases [130]. Though tick-borne diseases are known to be present [60] (Achi, unpublished data), the current situation regarding the real prevalence of tick-borne diseases in the Ivory Coast is not well known. This is especially true for the South where so far no data have been collected.
The South is gaining importance for cattle production. Besides the local village cattle, many crossbred cattle can be found in this region. They belong to the southern milk project (Projet laitier sud, Coopération Technique Belge, www.btcctb.org). The aim of the project is to assure self-sufficiency for milk and meat production in the region around Abidjan.

1.4 Diagnostic tools and associated problems for detection of tick-borne pathogens: an overview

Detection of parasites in the blood of bovines and in ticks themselves is very difficult. Traditional methods, which are still in use today, consist in studying blood smears for the presence of haemoparasites by microscopy. Fortunately, in the meantime a new generation of molecular diagnostic tools, such as PCR and DNA probes, has been developed. These methods show a higher sensitivity and specificity, especially in low level infections, and are facilitating today's studies on tick-borne diseases.

Identification of *Anaplasma* and *Ehrlichia* species is difficult because conventional bacteriological methods for cultivation and characterization can not be applied [67] [68]. Additionally, the use of morphological and serological methods is also limited due to morphological similarities and antigen cross-reactions between the species [67] [68].
One way to detect an infection with an *Ehrlichia* or *Anaplasma* organism was by means of post-mortem findings and microscopical detection of the organisms in brain endothelial cells, ex. *E. ruminantium* [25]. Further, microscopical detection of the organsism in Giemsa stained blood smears in the case of *A. marginale* [27] or identification of morula-containing granulocytes, *A. phagocytophilum* [41]. This is still a common method to confirm diseases in acute cases. For a long time, diagnosis by inoculation of tick homogenates into small ruminants and mice (*E. ruminantium*) [72] or inoculation of *A. marginale* infected erythrocytes into calves [41], has been the standard method for the determination of *Ehrlichia* or *Anaplasma* organisms. However, this method is an expensive, slow method and additionally shows low sensitivity.

Several serological tests have been developed, like the indirect fluorescent antibody test for *E. ruminantium*, complement fixation and card agglutination for *A. marginale* and more recently a range of enzyme-linked immunosorbent assay (ELISA) for both. Unfortunately, the ELISA using the major antigenic protein (MAP1) as target to detect *E. ruminantium* [121], demonstrated cross-reactions with the MAP1 homologues existing in other *Ehrlichia* and *Anaplasma* species and therefore showed false positive and false negative results. ELISA tests have also the tendency to detect old infections and therefore it can often not be distinguished, if an animal suffers from acute infection or if it already has recovered from an infection. It is for these reasons that routine application of serological tests is limited [72]. One of the most recent tests, MAP-1B indirect ELISA to detect *E. ruminantium* shows fewer cross-reactions and is satisfactory in sheep and goats, but not reliable for cattle [71]. In comparison, the polyclonal competitive ELISA (PC-ELISA) [114] was reported to have a reduced cross-reactivity and improved sensitivity compared to other ELISAs, but it still performed worse with bovine sera than with sheep sera [16].

The diagnosis of *Theileria* and *Babesia* species encounter the same problematic as seen before for *Anaplasma* and *Ehrlichia*. A routine diagnostic method is the identification of macroschizonts in Giemsa-stained lymph node biopsy smears for *T. annulata* [30] and in blood smears for *Babesia* [54]. This is an accurate method for acute infection, but in the carrier state the detection of piroplasms is very difficult and it is not possible to differentiate between pathogenic forms (like *T. annulata*) and non-pathogenic forms [30]. As for other pathogens, serological test like the indirect immunofluorescent antibody test (IFAT) and ELISA are in use to detect *Theileria* and *Babesia* species [20]. For the same reasons of cross-reactivity seen in *Anaplasma* and *Ehrlichia* species, these assays have limited specificities.

1.4.1 Polymerase chain reaction (PCR) and Reverse Line Blot (RLB) as diagnostic tools

PCR

Polymerase chain reaction (PCR) is a technique used for rapid DNA amplification from a specific DNA sequence [4]. Two sets of DNA oligonucleotides, chosen to flank the desired nucleotide sequence of the gene, are synthesized by chemical method. PCR starts with a double-stranded DNA and each cycle of reaction begins with a brief heatment to separate the strands. After strand separation, excess of primers are used to hybridize to complementary sequences in the two strands. DNA is then synthesized in the presence of DNA polymerase and the four deoxyribonucloeside triphosphates, starting from the two primers. The entire cycle is then

repeated again. Every cycle doubles the amount of DNA synthesized in the previous cycle. PCR is extremely sensitive; it can detect a single DNA molecule in a sample.

Ehrlichia ruminantium To date several PCR assays to detect *E. ruminantium* have been developed using MAP1, 16S rRNA or pCS20 gene sequences as targets [62] [72] [90] [91]. In a comparative study, the pCS20 sequence proved to be the most sensitive of all [8], and therefore most of the current PCR assays are based on this sequence. However, this comparison was based on the detection of *E. ruminantium* in *Amblyomma* ticks and needs to be treated with care in the case of ruminants, such as bovines and sheep, since there is no direct evidence that this sequence is also the best for infection in ruminants. Extensive investigations have been made for ticks of the species *Amblyomma* [72], [90], [91] where the pCS20-PCR assay showed a high specificity and high sensitivity [90], [91]. This seems to be a good tool to detect *E. ruminantium* in *Amblyomma* ticks, even in low level infections [91].
In addition to ticks, studies with the pCS20 gene were made in sheep, antelopes and to a lesser extend in cattle [62] [72]. The study on sheep and wild antelopes by Kock [62] did not give clear results and was not satisfactory. The same can be said for the goats investigated by Mahan [72]. In the attempt to detect *E. ruminantium* in the blood of cattle, the PCR only detected *E. ruminantium* in 3.3% of the 9-12 month old cattle, and in 26.7% of the 21-24 month old cattle [72]. The investigated cattle belonged to a farm, where the infection rate was high and most of the animals were believed to be almost certainly infected. This shows that the pCS20 PCR assay which is highly effective in detecting infection in ticks, appears to have a significantly lower sensibility to detect infections in ruminants [72]. Genotypic variability has been demonstrated for field sampled *E. ruminantium* [6]. Furthermore, multiple 16S genotypes could be found in parallel in the blood of cattle [7]. In light of this evidence, there is a need to evaluate further the situation of diagnostic methods of *E. ruminantium* in cattle to get a reliable diagnostic method. There is also a need to assess if the PCR assay of choice will be satisfactory for both cattle and sheep or if separate methods are preferable.

Anaplasma marginale To date, many different approaches have been developed to detect *A. marginale* in ticks and cattle [5] [39] [40] [64] [106] [111] [125]. One of the main problems is the close relation between *A. marginale* and *A. centrale*, which co-occur both in ticks and cattle. In ticks, studies have been made based on the 16S rRNA sequence. However, Wen et al [125] used primers specific for tick-borne *Ehrlichia* species and therefore the 16S rRNA based PCR assays in its current form detects any *Ehrlichia* species and needs to be followed by separate identification of the individual species [125]. A more effective method is the msp1-PCR which is more specific, and is successful in detecting *A. marginale* in ticks [111]. Different

approaches to develop a method to detect *A. marginale* in cattle and sheep have been evaluated. A sensitive and specific method is the msp1-PCR assay, which works for both cattle and sheep [64]. In the attempt to test their MSP5-cELISA, de Echaide et al [27] developed a nPCR based on the MSP5 gene sequence. It was sensitive and specific, but was not evaluated for its ability to detect early infections. Further, Gale et al [40] has found a PCR-ELISA which apparently detects minute levels of *A. marginale* DNA but they do not specify the sequence they used. The different methods should be compared to find a reliable detection method for *A. marginale* in cattle.

Anaplasma phagocytophilum Up to now, PCR-assays undertaken to detect *A. phagocytophilum*, used the 16S rRNA gene sequence as target. In fact, 16S rRNA based PCRs were used throughout Europe to determine the occurrence of *A. phagocytophilum* in ticks [68] [94] [95] [68] small mammals [67], deer and chamois [68], sheep [112] [41], dogs, horses and cattle [34]. Cows were used as a study object by Pusterla et al [94] [93]. In their approach, they tried two different assays of PCR, a nested PCR and a fluorescence TaqMan PCR, both based on the 16S rRNA gene. Both methods specifically detected *E. phagocytophilum*, *E. equi* and HGE, which are now classified as one species [33], and did not amplify any of the added *Rickettsia*, including *A. marginale*. In terms of sensitivity and specificity the two methods were identical. The fluorescence TaqMan PCR had an advantage in its shorter working time (shorter cycling time and absence of gel electrophoresis).

Babesia bovis Sensitive and specific PCR diagnostic assays exist for the detection of different *Babesia* species, including *B. bovis* [65] [22] [37], *B. bigemina* [38] [39], but no PCR specific for *B. divergens* has been developed [10]. Different PCR assays were developed to detect *B. bovis* in cattle, based on the apocytochrome b gene [37], the SSrRNA gene sequence [22] and on the variable length tandem repeats of the Bv80 and BvVA1 genes [65]. All are highly specific for *B. bovis*. However, Calder et al found that the region used first was highly variable among different strains [22]. After redesigning, primers are now specific for the four strains they tested. It remains to be determined whether the sequence of all *B. bovis* strains are constant in these regions.

Babesia bigemina Few investigations have been done on *B. bigemina*. The PCR assay to detect *B. bigemina* in cattle is based on the SpeI-AvaI fragment from pBbi16 [38] [39] and could be reproduced in the study of bovine piroplasms in Minorca [10]. Another attempt used the apocytochrome b gene, but in contrast to *B. bovis* [37], this PCR did have a low sensitivity compared to the complement fixation test [100].

Theileria annulata For several *Theileria* species sensitive and specific PCR diagnostic assays have been developed to detect the pathogens in their hosts [10]. These PCR assays include tests to detect *T. annulata* [30]. D'Oliveira et al used the gene encoding the 30K Da major merozoite surface antigen to detect *T. annulata* in the blood of infected cattle. The PCR was performed on blood samples from 4 different stocks, originating from Mauritania, Portugal, Spain and Turkey. For those strains the PCR assay was specific and detected *T. annulata* at low parasitaemias in carrier cattle. It remains to be tested for stocks from other geographical regions. To date the Tams1-based PCR from d'Oliveira et al was reproduced several times. It was used to detect bovine piroplasmes in Spanish cattle [10] [73]. Further, the Tams1 based PCR was successfully applied for detection of *T. annulata* in ticks [31] [58] and cattle [58]. Small ribosomal unit based PCRs are an alternative to the Tams1 gene, though being less specific. The SSU-rRNA was used to detect *T. annulata* in ticks [28] and cattle [99].

Theileria parva A PCR assay to detect *T. parva* in cattle was developed by Bishop et al [18]. It is based on the 67kDa sporozoite antigen, p67, which is conserved among *T. parva* strains. The PCR was able to detect parasites present at low parasitaemias as in carrier cattle. Further, an oligonucleotide based on the repetitive TPR1unit was designed, which specifically detects the *T. p. parva* - Marikebuni strain, which was used in the Kenyan vaccination trials and could not be detected with the p67 PCR [18]. However, the efficiency of PCR detecting *T. parva* in blood is not known and needs to be further evaluated. In ticks, the TPR1 region was used to amplify *T. parva* [124].

Table 1.4: A synthesis of some of the most frequently used sequences for PCR detection of individual tick-borne pathogens

Pathogen	Sequence	Reference (A selection)
E. ruminantium	MAP1, 16S rRNA, pCS20	[62] [72] [90] [91]
A. marginale	16S rRNA, MSP1, MSP5	[125][64][27]
A. phagocytophilum	16S rRNA	[68][94][112] [41][34]
B. bovis	Apocytochrome b, 16S rRNA, Bv80, BvVA1	[37][22][65]
B. bigemina	pBbi16, apocytochrome b gene	[38] [39][37]
T. annulata	Tams1, 16S rRNA	[30][58]
T. parva	p67, TPR1	[18][124]

Reverse Line Blot Hybridization

The Reverse Line Blot Hybridization (RLB) method is based on simultaneous PCR amplification of related species and subsequent identification of individual species by species-specific oligonucleotide probes which are covalently attached to a membrane. A single assay therefore allows the hybridization of multiple samples (up to 43) with multiple different oligonucleotide probes (up to 43). This facilitates enormously any large scale screening of samples. Another advantage is that new oligonucleotide probes can be designed and added to the assay at any time. Design of catch-all probes, which are specific for a whole group of pathogens allow the detection of new pathogen species due to possible subsequent DNA sequence analyses. Since the RLB combines PCR amplification followed by a hybridization step, the methods results in higher sensitivity than PCR alone.

Tick-borne diseases of livestock constitute a complex situation, where several different pathogens can be present in the ticks and/or animals at the same time. Chances are high to find several different pathogens simultaneously in the same sample. Although, many useful species-specific PCR assays have been developed to detect a particular tick-borne pathogen, it is very cumbersome to test samples for each pathogen individually. Therefore it is practical to have a diagnostic tool, which allows the simultaneous detection of a large number of pathogens.

The reverse line blot hybridization assay used to detect bovine *Theileria* and *Babesia* [49], uses one set of primers designed to detect the 18 small-subunit (SSU) rRNA spanning the V4 regions of all known *Theileria* and *Babesia* species. The sequences amplified by PCR are then hybridized to a membrane which contains species-specific oligonucleotides [49]. The RLB-PCR is a practical (only one PCR needed) and very sensitive method (detects 10^{-6} parasites of *T. annulata* in contrast to 10^{-3} by PCR).
The RLB-PCR method was also used to detect *Anaplasma* and *Ehrlichia* species in domestic ruminants and ticks [15]. The PCR amplifies the hypervariable V1 region of the 16S rRNA gene with a set of primers unique for species of the genera *Anaplasma* and *Ehrlichia*. As an universal test to detect all possible bovine tick-borne pathogens would be very useful, the RLB-PCR for *Babesia* and *Theileria* was combined with the RLB-PCR for *Anaplasma* and *Ehrlichia* [44]. The extended RLB-PCR was then applied to detect haemoparasites in Sicilian cattle [44], where it proved to be very useful.

The RLB had its first application for tick-borne pathogens (*Borrelia* spirochetes) [98] and *Ehrlichia* species [104]. In the meantime, the reverse line blot assay finds a wide use in diverse studies [103] [42] [106] [87] [118] [36] [101]. In Africa, to date the only study using the RLB for an epidemiological survey to detect haemoparasites in cattle was carried out in Uganda [87] and Sudan [101]. Further, the RLB

was applied to detect pathogens in ticks in Guinea [118] and Gambia [36]. Today, a RLB-PCR kit is now commercialised and available by Isogen (www.isogen-lifescience.com).

Molecular methods, such as PCR, RLB and DNA sequence analyses have brought new insights in the field of tick-borne diseases. Not only have they helped to clarify taxonomical questions of pathogens [33] and tick species [13], but novel pathogens or genotypes of already known parasites as well as their association with tick-borne diseases are continuously discovered. For example, in the study of different heartwater associated organisms by 16S rRNA gene analyses, not only four different genotypes of *E. ruminantium* were detected, but also a previously unknown *Ehrlichia* sp causing heartwater in sheep was discovered [9]. Further the different genotypes could be associated to different phenotypes [9]. A novel *Ehrlichia* sp closely related to *E. ruminantium* as well as a new *Anaplasma* sp closely related to *A. phagocytophilum* were found in dogs in South Africa [55]. The *Anaplasma* sp was also identified from goats in Mozambique and in sheep of South Africa which had signs of heartwater [55]. This is a foretaste for future detections in the field of tick-borne diseases.

1.5 Objectives

A longitudinal epidemiological survey of cattle was planned in the South of the Ivory Coast, to obtain baseline data on the tick species present as well as on the prevalences of tick-borne parasites in this area. A reverse line blot-PCR assay is validated in our laboratory in Neuchâtel, Switzerland, to be used for the detection of tick-borne parasites in ticks and blood samples. The aim of the present study is to identify the tick species present on cattle, to study their seasonal variation and to analyse the parasites they host. In parallel, the distribution of tick-borne cattle parasites in this area will be studied. The data are needed in order to be able to better understand the local situation in the South of the Ivory Coast and to adapt the control measures against ticks and tick-borne parasites to improve cattle breeding in this area.

1.5.1 Objectives of the project

- Optimize the molecular methods, PCR and RLB, in order to obtain epidemiological data on parasites in ticks collected from cattle as well as on parasites in blood of bovines in the Ivory Coast.
- To determine the genus and species of ticks found on cattle in the Southern forested zone of the Ivory Coast and to evaluate their seasonal variation.

- To determine the dominant tick species.
- To determine the prevalence and distribution of tick-borne parasites causing diseases such as Babesiosis, Theileriosis, Anaplasmosis and Cowdriosis in cattle using the molecular techniques (RLB-PCR) in parallel to traditional microscopical and clinical criteria.
- To analyse tick species of veterinary importance collected from cattle for the presence of parasites relevant for cattle by RLB-PCR

Chapter 2

Material and Methods

2.1 Design of the Field study

2.1.1 Study area

The study was carried out on four different sites all situated in the radius of about 100 km around Abidjan (5°10'0"N/3°56'0"W) , Ivory Coast. All sites were within the Southern Forest Zone of the Ivory Coast, which is characterised by two wet seasons, a major one lasting from March to July and a minor one from October to November.
The South of the Ivory Coast is very humid, with temperatures varying from 25° to 30° C, and the mean annual rainfall lies between 1400 to 2400 mm. This leads to a relative humidity which is generally above 80 %. The area is a flat or gently undulating plain or plateau and mostly covered by a dense rainforest, which is intersected with cultures and fallows in contrast to the drier North, where the land becomes savanna and sparse woodland.

In this area, many crossbred cattle belonging to farms participating in the southern milk project (« Projet laitier sud » ; Projet d'appui au développement de la production laitière dans le sud de la Côte d'Ivoire, Coopération Technique Belge) can be found next to the local village cattle.

The following farms were selected for the study :

- Farm Mesics (Me) Ivoire in Sikensi. Responsible person Tanoh Beugré. In the following Me will be used as abbreviation for this farm.
- Farm Calys (Ca) in Songon-Kassamblé in the district of Dabou. Responsible person M Diakité. In the following Ca will be used as abbreviation for this farm.

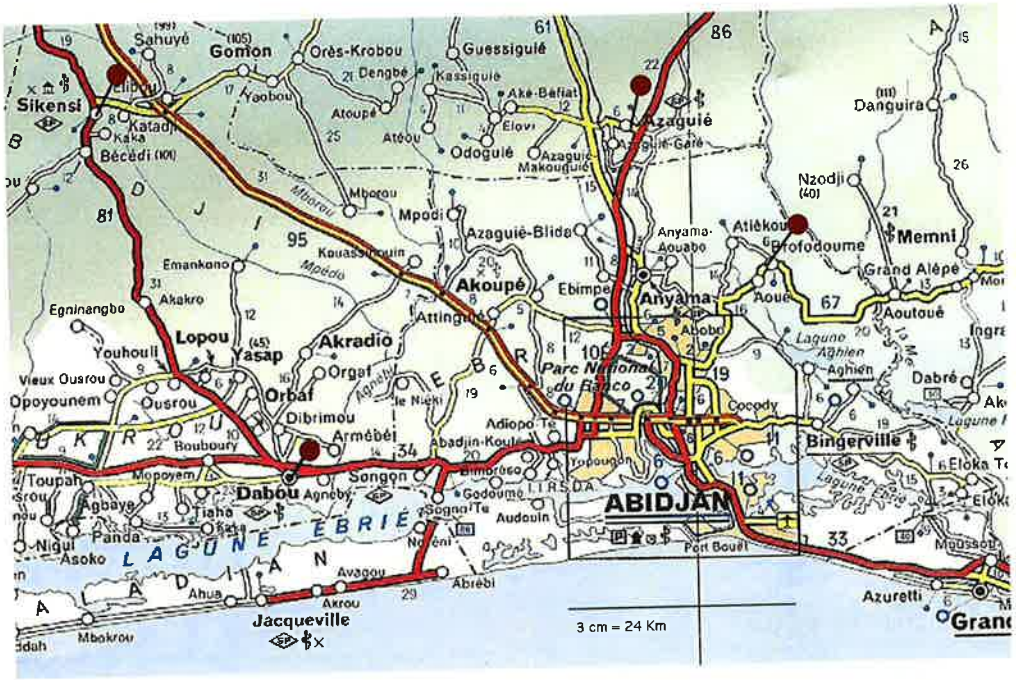

Figure 2.1: Map of the region around Abidjan showing the study sites. The location of each farm is indicated with a cayenne-coloured point. Farms are located at Sikensi, Dabou, Brofodoumé and Azaguié, respectively. Scale of the map: 1/800 000 (National map no. 747 of Michelin).

- Farm of M Atsain in Brofodoumé (Br). Responsible person M Atsain. In the following Br will be used as abbreviation for this farm.
- The milk farm of Azaguié (Az). Responsible person M Koffi-Kouadio. In the following Az will be used as abbreviation for this farm.

Two of the four farms were located Northwest of Abidjan, Sikensi and Dabou, and the other two Northeast of Abidjan, i.e. Brofodoumé and Azaguié. The exact locations are indicated on the map (Fig. 2.1).

2.1.2 Characteristics of the farms

The inclusion criteria for the selected farms were on one hand the willingness of the owner to participate in the study and on the other hand the breeds of bovines represented on the farm. The farms chosen held either animals which were crossbred cattle held under condition typical for crossbreds or animals representing local breeds belonging to village herds practising more traditional farming.

The farm Mesics Ivoire in Sikensi was rearing local Zebu breeds, practising traditional farming and animals were treated on a rather irregular rhythm with acaricides. The farm Calys in Songon-Kassamblé in the district of Dabou, was rearing local N'Dama breeds and animals were treated at a regular rhythm with acaricides. The farm of M Atsain in Brofodoumé was rearing crosses of N'Dama and Zebu, practising traditional farming and animals were treated upon availability of acaricides on an irregular rhythm with acaricides. The milk farm of Azaguié, was rearing crossbred cattle which are crossings of the local race N'Dama inseminated with sperm of European breeds such as Abondance, Holstein or Montbéliard. This is a modern governmental farm treating their animals on a regular rhythm with acaricides. Figures 2.2 to 2.5 are showing the different study sites with their respective cattle breeds.

2.1.3 Study design

A longitudinal study with repeated samplings of tick and blood material was performed on a selection of cattle for all of the study sites over the time period of one year, from November 2005 to October 2006. For each farm, 15 animals were chosen at random, eartagged and numbered to be included into the study. However, during the study period, some of the animals had to be replaced (27), because animals became to dangerous to be handled (17), were gestating (3), were sold (5) or died (2). Thirty-three animals participated from beginning to the end of the study without being replaced.

In total 60 cows were followed on a bimonthly basis. Each farm was visited twice a month. At the first visit of each month, both blood and tick samples were collected, whereas at the second visit only tick samples were collected. Cattle were allowed to graze in the open plains and were only kept in their enclosures the day of the sampling. Only in Azaguié six of the animals followed were calves younger than one year which did not or rarely go to the pasture. All animals were treated with acaricides during the whole study period using a hand-spraying method. The frequency of the treatment was normally once or twice per month. Farmers were not allowed to treat the animals the same week that the sampling took place.

The acaricide treatment was not standardized for the four farms. Each farmer treated after his own judgment and different products were used during the study course. However, at the beginning of the study, all animals were treated with alphacypermethrin (Dominex). The acaricides used during the study course were alphacypermethrin (Dominex), deltamethrin (Delta-Tick, Deltavet) and amitraz (Tick-Net 100, Antitic, Abotik). The name of the product utilised is noted in parenthesis.

Figure 2.2: The study site of Sikensi showing local Zebu cattle.

Figure 2.3: The study site of Dabou showing N'Dama cattle.

Figure 2.4: The study site of Brofodoumé showing N'Dama x Zebu cattle.

Figure 2.5: The study site of Azaguié showing crossbred cattle.

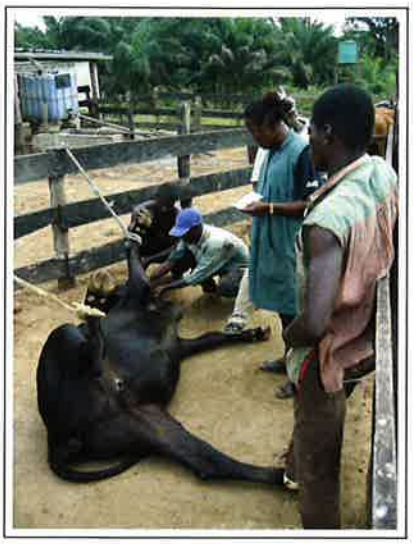

Figure 2.6: Example of the tick sampling on cattle in Azaguié

2.1.4 Sampling of the field material

Collection and identification of ticks:

Ticks were collected on one side of the cattle body. To facilitate the handling of the bovines during the sampling procedure, individual animals were fixed on the ground by helpers (Fig. 2.6). Ticks were afterwards collected by stroking the coat by hand to make sure also nymphal and larval stages were spotted. Gestating cows were not laid down, but kept instead upright in a corridor while they were searched for the presence of ticks.

The body of the cattle was divided into eight sectors (perianal area, groin and udder, abdomen, axillae, dewlap, head and ears, tail, legs and hooves) for the collection of ticks. For each of these sectors, the sampled ticks were determined to genus level and then counted separately for the respective stages (adult female, adult male, nymph and larva) of each identified genus. The ticks from one animal were then stored in one plastic tube containing 70% ethanol.

Once in the laboratory, the ticks collected during the field trips were determined to species level using a binocular (40x). For the determination of the diverse species, the guide to identification of species [123] was used. Shortly, to distinguish the different *Rhipicephalus (Boophilus)* species the following criteria were used: absence (*B. annulatus*) or presence (*R. (B.) geigyi*, *R. (B.) decoloratus*, *R. (B.) microplus*) of caudal appendage in males, the shape of the genital aperture in females and for both the number of hypostomal teeth (in 3+3 or 4+4 columns). *R. (B.) decoloratus* is the

only species with only 3 ranges of hypostomal teeth.
If these criterias were not sufficient, the classification key was applied in more details (presence/absence of setae etc.). In the case of failure to classify the correct species (due to missing parts for instance), they were classified only to genus level. Due to difficulties in identifying female *Rhipicephalus* only the male were determined to species level. When only one species was found on the animal, females were classified together with the identified males. Most of the work to determine the different tick species was done by Dr Louise Achi, Laboratoire National d'Appui au Développement Agricole (LANADA), Bingerville, Ivory Coast.

Collection and analyses of the blood samples in the field:

Blood samples were collected from each individual cattle by piercing the ear with the help of a needle. The blood was then collected into several hematocrite (anticoagulating) capillaries. The volume of two capillaries was applied onto one spot on the FTA cards (Whatman International Ltd). A further capillary was collected to determine the hematocrite level of each cattle. At the same time thin and thick blood smears were prepared for later microscopic analysis for the presence of blood-borne pathogens.
The hematocrite capillaries were centrifuged upon arrival in the laboratory to determine the hematocrite level for each bovine. Blood smears were fixed with 100% methanol and then stained with Giemsa colorant. Afterwards they were examined by microscopy (100x) for the presence of blood-borne parasites, such as *Trypanosoma* sp, *Babesia* sp, *Theileria* sp, *Anaplasma* sp and *Ehrlichia* sp by the veterinary technicians of the LANADA, Bingerville, Ivory Coast.

Clinical signs:

At every visit a form was filled out together with the farmer in order to note the overall health status of the animals. Any clinical signs including death or other specific events were noted. The acaricide used, its time of application and the dosage used was noted as well.

2.2 Analyses of the field samples

2.2.1 Extraction of DNA for PCR:

Blood stored on FTA cards was washed following the PCR downstream protocol of Whatman International Ltd (`www.whatman.com`) and directly used for PCR. Shortly a 1.2 mm disc was punched from the sample spot on the FTA card and put in a PCR tube. The disc was washed three times in the FTA purification reagent,

followed by washing twice with TE^{-1} (10mM Tris-HCL, 0.1mM EDTA, pH 8.0). The disc was then dried for 10 min at 56°C or 1 hour at room temperature and then directly used for PCR.

DNA of ticks was prepared by digestion with proteinase K followed by a phenol-chloroform extraction for subsequent PCR. The protocol of Wen et al [125] was modified as follows: Ticks were washed once in 70 % ethanol, twice in distilled water and afterwards let to dry on individual paper discs. The ticks were then grounded to a fine powder in liquid nitrogen using a mortar and pestle or eppendorf and plastic spreader depending on the size of the tick species. The powder was directly suspended in the freshly prepared extraction buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA and 1% SDS). For one *Riphilcephalus (B.)* tick (adult, nymph), one nymphal *Amblyomma* or half of an adult *Amblyomma* tick, 160 µl of TE, 20 µl of 10 % SDS and 5 µl of proteinase K (20 mg/ml) (Qiagen Switzerland) were added and the mixture was incubated over night at 55° C. The samples were then incubated for 10 min in a heating block and the supernatant was extracted twice, once with phenol-chloroform and once with chloroform following a classical protocol [11]: An equal volume of phenol/chloroform/isoamyl alcohol was added to the supernatant in a 1.5 ml microcentrifuge tube and mixed thoroughly by inversing the tube. Samples were then centrifuge for 10 min at maximum speed. Afterwards the top (aqueous) phase was carefully removed and transferred to a new tube. An equivalent volume of chloroform was added to the supernatant and mixed thoroughly by inversing the tube. The sample was centrifuged for 10 min at maximum speed. The aqueous phase was transferred to a new tube. 1/10 volume of 3 M sodium acetate and 2 volumes of 100 % ethanol (ice-cold) were added and the solution was mixed by flicking the tube several times with a finger and place in crushed ice for 15 to 20 min. Afterwards the mixture was centrifuged again for 10 min at maximum speed. The supernatant was removed and 1 ml 70% ethanol (room-temperature) was added. The tube was inverted several times, then centrifuged for 5 min at maximum speed. The supernatant was removed and the DNA pellet was left to dry for 1 hour, resuspended in 25 µl TE and stored at -20°C.

Purity and quantity of the DNA sample obtained was measured by photospectrometry. The absorption of a sample is measured at different wavelengths. The ratio of absorbance at 260 nm and 280nm can be used as indicator of nucleic acid purity. A 1-cm-pathlength spectrophotometer cuvette was used. The DNA solution was considered to be pure, if the ratio of 260/280 nm was of 1.8 to 1.9. Concentration can be calculated using the following formula : c(pmolµg/ml) = A260/0.020 [11].

2.2.2 PCR

Primers:

For the PCR amplification of *Anaplasma* and *Ehrlichia* one set of primers was used to amplify a 492-498 bp fragment of the 16S rRNA gene spanning the V1 region. The forward primer 16S8FE (5'-GGAATTCAGAGTTGGATCMTGGYTCAG-3') was previously described by Schouls et al [104] and the reverse primer B-GA1B-new (5'biotin-CGGGATCCCGAGTTTGCCGGGACTTYTTCT-3') by Bekker et al [15] and was labeled with a biotin molecule at the 5' site.

For the PCR amplification of *Theileria* and *Babesia* a set of primers was used to amplify a 429-430 bp fragment of the 18S rRNA spanning the V4 region. The forward primer, RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3') and the reverse primer, RLB-R2 (biotin-5'-CTAAGAATTTCACCTCTGACAGT-3'), were previously described by Georges et al [44]. The reverse primer was labeled with a biotin molecule at the 5' site.
Primers were obtained from Microsynth Switzerland.

PCR reaction:

Reaction conditions in a 50 µl volume were as follows: 10 x PCR buffer (Qiagen, Switzerland), 2.5 mM $MgCl_2$, 200 µl of each deoxynucleoside triphosphate, 1.25 U Taq (Qiagen, Switzerland), 50 pmol of each primer (Microsynth, Switzerland), and either 5 µl of purified tick DNA or one 1.2 mm disc for the blood samples. The reactions were performed in an Whatman Biometra Tgradient basic thermocycler 96 (Göttingen, Deutschland) thermal cycler. For each reaction, control samples were included, i.e. a positive sample, i.e. *B. bovis* or *A. marginale* (infected bovine blood obtained from Dr Louise Jackson, Department of Primary Industries and Fisheries, Tick Fever Center, Moorooka, Australia) and two negative samples, water, empty FTA cards or uninfected *I. ricinus* (laboratory strain of University of Neuchâtel). Each field sample was tested twice. Once with the primer pair (16S8FE, B-GA1B-new) to amplify sequences typical for pathogens belonging to the group of *Anaplasma* and *Ehrlichia* (AE) species and once with the primer pair (RLB-F2, RLB-R2) to amplify sequences typical for the pathogens belonging to the group of *Babesia* and *Theileria* (BT) species.
The two primer pairs were chosen to have similar melting temperatures and therefore a unique PCR reaction was performed. To increase specificity, a touchdown program modified from Bekker et al [15] was used (Table 2.1). Shortly: after 3 minutes of initial denaturation, cycles of 20s at 94°C denaturation, 30s at the annealing temperature (Ta) and an extension step of 30s at 72°C, whereby the annealing temperature was lowered from 67°C to 57°C by steps of 1°C, was performed. Sub-

sequently, forty cycles each of 20s at 94°C, 30s at 57°C and 30s at 72°C were run. The PCR was ended by a 10 minute elongation step.

The PCR products were visualized on an ethidium bromide stained 1.5% agarose gel.

Table 2.1: Touchdown PCR program for *Anaplasma/Ehrlichia* and *Babesia/Theileria*.

Number of cycle	Time length	Temperature	Initial denaturation
1 cycle	3 min	94°C	Initial denaturation
1 cycle	20 sec	94°C	Denaturation
	30 sec	67°C	Annealing
	30 sec	72°C	Extension
11 cycles	20 sec	94°C	Denaturation
	30 sec	67-57°C	Annealing
	30 sec	72°C	Extension
40 cycles	20 sec	94°C	Denaturation
	30 sec	57°C	Annealing
	30 sec	72°C	Extension
1 cycle	10 min	72°C	Final Extension

2.2.3 Analyses by Reverse Line Blot (RLB)

RLB is a modification of the reverse dot blot, and probes are applied to the membrane as lines instead of dots. In one assay, the reactivity of 45 PCR products with up to 45 different probes can be determined. The preparation and subsequent hybridization of the RLB membrane was done as described before [49] with the some modifications:

Probes:

For the RLB the following probes previously described were used (Table 2.2): *Anaplasma* and *Ehrlichia* (AE): Catch-all AE (GGGGGAAAGATTTATCGCTA); *Ehrlichia ruminantium* (AGTATCTGTTAGTGGCAG); *Anaplasma marginale* (GACCGTATACGCAGCTTG), *Anaplasma centrale* (TCGAACGGACCATACGC) and *Ehrlichia* sp (CGGATTTTTATCATAGCTTGC) previously described by Bekker at al [15]. *Anaplasma phagocytophilum* group (TTGCTATRAAGAATARTTAGTGG) previously described by Georges et al [44]; *Anaplasma bovis* (GTAGCTTGCTATGRGAACA) [56].

Babesia and Theileria (BT): Catch-all BT (TAATGGTTAATAGGARCRGTTG) previously described by Georges et al [44]. *Babesia bovis* (CAGGTTTCGCCTGT-

ATAATTGAG); *Babesia bigemina* (CGTTTTTTCCCTTTTGTTGG); *Babesia divergens* (GTTAATATTGACTAATGTCGAG); *Theileria annulata*; (ATTGCTTGTGTCCCTCTG); *Theileria parva* (TTCGGGGTCTCTGCATGT); *Theileria mutans* (CTTGCGTCTCCGAATGTT) and *Theileria velifera* (CCTATTCTCCTTTACGAGT) previously described by Gubbels et al [49].

Preparation of the membrane; Covalent coupling of oligonucleotide probes to the membrane:

All oligonucleotides, synthesized with a 5'-terminal aminogroup (Microsynth, Switzerland), were diluted to their optimized concentration in 150 µl 500 mM $NaHCO_3$, pH 8.4 before linking them to an activated negatively charged Biodyne C membrane following the protocol of Gubbels et al [49]. The concentration used for each individual probe is summarized in Table 2.2.

Shortly: The Biodyne C membrane (Pall Corporation, United States) was activated by 10 min incubation in 10 ml freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in demineralised water, in a rolling bottle at room temperature. The membrane was then rinsed with distilled water and placed on a support cushion in a clean miniblotter system (Miniblotter 45, US Patent No. 4834946, Immunetics, Cambridge). The slots of the miniblotter were then filled with 150 µl of the diluted oligonucleotide solutions leaving in each case an empty line between the probes. The remaining slots were filled with $NaHCO_3$. To help keeping the orientation of the membrane, the first and last slots were filled with diluted ink (1:100). After all samples were added they were allowed to incubate at least for 1 min at room temperature before removing them by aspiration.

To inactivate the membrane, the blot was incubated in 100 ml 100 mM NaOH for 10 min (maximum) in a rolling bottle. The membrane was then washed in a plastic container under gentle shaking in 100 ml 2x SSPE/0.1% SDS for 5 min at 60°C. The membrane was either directly used afterwards or washed in a plastic container under gentle shaking in 100 ml 20 mM EDTA pH 8 for 15 min at room temperature, sealed in a Saran-wrap to avoid dehydration and stored at 4°C until use.

Table 2.2: RLB species-specifi c probes. The probes are listed in the order in which they were applied onto the membrane. The odd numbered lines were kept empty to faciliated the interpretation of the results and to prevent cross-over hybridization. The concentration applied is noted. The following symbols are used to indicate degenerated positions: R = A/G, M = A/C, Y = A/G.

Line	Specifi c probe	Probe Sequence (5' - 3')	Ref.	Conc. pmol/ 150 µl
4	*A. phagocytophilum*	TTGCTATRAAGAATARTTAGTGG	[44]	500
6	*E. ruminantium*	AGTATCTGTTAGTGGCAG	[15]	500
8	*A. marginale*	GACCGTATACGCAGCTTG	[15]	500
10	*A. centrale*	TCGAACGGACCATACGC	[15]	500
12	*A. bovis*	GTAGCTTGCTATGRGAACA	[56]	500
14	*Ehrlichia*[a] sp.	CGGATTTTTATCATAGCTTGC	[15]	500
16	Catch-all AE	GGGGGAAAGATTTATCGCTA	[15]	100
18	Catch-all BT	TAATGGTTAATAGGARCRGTTG	[44]	50
20	*B. bovis*	CAGGTTTCGCCTGTATAATTGAG	[49]	500
22	*B. bigemina*	CGTTTTTTCCCTTTTGTTGG	[49]	500
24	*B. divergens*	GTTAATATTGACTAATGTCGAG	[49]	100
26	*T. annulata*	ATTGCTTGTGTCCCTCTG	[49]	100
28	*T. parva*	TTCGGGGTCTCTGCATGT	[49]	100
30	*T. mutans*	CTTGCGTCTCCGAATGTT	[49]	500
32	*T. velifera*	CCTATTCTCCTTTACGAGT	[49]	500

[a]strain Omatjenne

RLB:

Before use, the membrane was washed for 5 min at 42°C with 100 ml of 2xSSPE/0.1% SDS and placed in the miniblotter with the slots perpendicular to the previously applied oligonucleotides. A volume of 10 to 20 µl of PCR products was diluted to an end volume of 150 µl of 2xSSPE/0.1% SDS, heated for 10 min at 99°C and cooled on ice immediately. Denatured PCR samples were applied into the slots and incubated for 60 min at the hybridization temperature (42°C). Supernatants were aspirated and the blot was washed twice in 100 ml of 2xSSPE/0.5% SDS for 10 min at 42°C with gentle shaking. Subsequently the membrane was incubated in 10 ml of streptavidin-peroxidase conjugate (Roche, Switzerland) diluted 1:4000 in 2xSSPE/0.5%SDS for 30 min at 42°C. The membrane was washed twice in 100 ml 2xSSPE/0.5% SDS for 10 min at 52 °C. Afterwards the membrane was rinsed two times in 100 ml of 2xSSPE for 5 min each time at room temperature. The membrane was afterwards put on a transparency sheet and incubated for 1 min in 6 ml of

ECL detection fluid (Amersham Biosciences) before exposure to an ECL hyperfilm (Amersham Biosciences) for 15 - 30 min. The membrane was used up to ten times. Between experiments the membrane was stripped following the protocol: Wash twice in 1% SDS at 80°C for 30 min. Thereafter, the membrane was washed in 20 mM EDTA pH 8, for 15 min at room temperature, sealed in plastic or Saran-wrap to avoid dehydration of the membrane and stored at 4°C until use.

Measures to prevent contamination:

To allow an unidirectional workflow and to prevent contamination, different localities were used for the several steps of the manipulation procedure. Therefore, separate rooms were used for DNA extraction and storage (pre-PCR work), preparation of the PCR mastermix and handling of amplified products (post-PCR work).
Each room was equipped with dedicated pipette sets, reagents, lab coats and gloves. In the case of occurrence of cross-contaminations, work places and their equipments were cleaned thoroughly using bleach, UV-radiation and DNA zap (Ambion, USA). New PCR reagents were used to perform PCR again.

2.2.4 DNA sequencing

PCR amplified products were directly sent to Synergene Biotech GmbH, Switzerland for purification (Bind-wash-elute method, Qiagen) and sequence analyses. The obtained sequences were then analysed using various molecular tools: Finch TV Version 4.0, `www.geospiza.com`; ClustalW tool, `www.molbiol.net`, and compared to already known sequences using the NCBI Blast tool, `www.ncbi.nlm.nih.gov`.

2.2.5 Statistical analyses

All statistical analyses of the data were done using the S-Plus version 7.0 or SPSS version 11 statistical programs. The following test were used to analyse the data: Paired Student's test, Chi-square test, ANOVA analyses, regression analyses. All graphs were plotted using the same programs. The infestation degree (ID, ratio between the individual animal infestation and mean herd infestation) was calculated after Stachurski [109].

2.2.6 Bioinformatics

GenBank accession numbers of sequences used: The GenBank accession numbers of the 16S sequences used for deducing PCR oligonucleotides and species-specific RLB oligonucleotide probes for the *Anaplasma/Ehrlichia* group are as fol-

lows: X61659 for *E. ruminantium*; M60313 for *A. marginale*; AF414868 for *A. centrale*; M73220 for *A. phagocytophilum*; U03775 for *A. bovis* and U54806 for *Ehrlichia* sp [15].
The GenBank accession numbers of the 18S sequences used for deducing PCR oligonucleotides and species-specific RLB oligonucleotide probes for the *Babesia/-Theileria* group are as follows: L19077 for *B. bovis*; X59604 for *B. bigemina*; U16370 for *B. divergens*; AF078815 for *T. mutans*; AF098993 for *T. velifera*; M64243 for *T. annulata*; L02366 and AF013418 for *T. parva* [49].

Sequence alignments: Sequence alignments to verify the position of the probes and primers on the 16S rRNA or 16S rRNA subunit respectively, were made with the ClustalW tool (`www.molbiol.net`) using the following GenBank accession numbers:

For the 16S gene: X61659 for *E. ruminantium*; M60313 for *A. marginale*; AF414868 for *A. centrale*; U03775 for *A. bovis*; U54806 for *Ehrlichia* sp and M73220 for *A. phagocytophilum*.

For the 18S gene: AF013418 for *T. parva*; M64243 for *T. annulata*; AF078815 for *T. mutans*; AF098993 for *T. velifera*; X59604 for *B. bigemina*; L19077 for *B. bovis* and U16370 for *B. divergens*.

Chapter 3

Verification and adaption of the Polymerase chain reaction (PCR) and Reverse Line Blot (RLB) assay

3.1 Optimization of PCR and RLB

Based on the publications of Gubbels et al [49] and Bekker et al [15], the suggested PCR programs and RLB method were optimized and adapted to the Laboratory of molecular Parasitology, Institute of Biology, Neuchâtel, Switzerland. Different samples of tick-borne pathogens relevant to cattle throughout the world were used as positive controls to set up both assays, PCR and RLB. The following samples were used: *B. divergens* (infected bovine blood) obtained from Prof. Dr med. vet. Kurt Pfister, Ludwig-Maximilians-Universität, Tierärztliche Fakultät, Institut für Vergleichende Tropenmedizin und Parasitologie, Munich, Germany; *T. annulata* (cell culture, infected macrophages) and *T. parva* (cell culture, infected lymphocytes) obtained from Prof. Dirk Dobbelaere, University of Bern, Institute of Animal Pathology, Molecular Pathology, Bern, Switzerland; *A. phagocytophilum* (DNA) obtained from B. Rutti, University of Neuchâtel, Institute of Biology, Laboratory of immunodiagnostics, Neuchâtel, Switzerland; *A. marginale*, *A. centrale*, *B. bovis*, *B. bigemina* (infected bovine blood) obtained from Dr Louise Jackson, Department of Primary Industries and Fisheries, Tick Fever Center, Moorooka, Australia and *E. ruminantium* (DNA extracted from infected blood and ticks) obtained from Dr Dominique Martinez, CIRAD-EMVT, Guadeloupe, France.

3.1.1 PCR

The PCR program was set up separately for the two individual pathogen groups, i.e. the group containing all *Anaplasma* and *Ehrlichia* pathogens (AE) and the group containing all *Babesia* and *Theileria* (BT) pathogens following the recommendation for setting up PCR by Qiagen, Switzerland.
The primers previously described by Schouls et al [104], i.e. the forward primer 16S8FE (5'-GGAATTCAGAGTTGGATCMTGGYTCAG-3') and by Bekker et al [15], i.e. the reverse primer B-GA1B-new (5'biotin-CGGGATCCCGAGTTTGCC-GGGACTTYTTCT-3'), were used to amplify a fragment of the 16S rRNA gene of *Anaplasma* and *Ehrlichia* pathogens.
On the other hand, the primer pair, i.e. the forward primer RLB-F2 (5'-GACACAG-GGAGGTAGTGACAAG-3') and the reverse primer RLB-R2 (CTAAGAATTTCA-CCTCTGACAGT -3'), previously described by Georges et al [44] were used to amplify a fragment of the 18S rRNA gene of *Theileria* and *Babesia* organisms.

The position of the respective primer pairs on the 16S rRNA or 18S rRNA gene sequences are shown in Table 3.1 and 3.2.

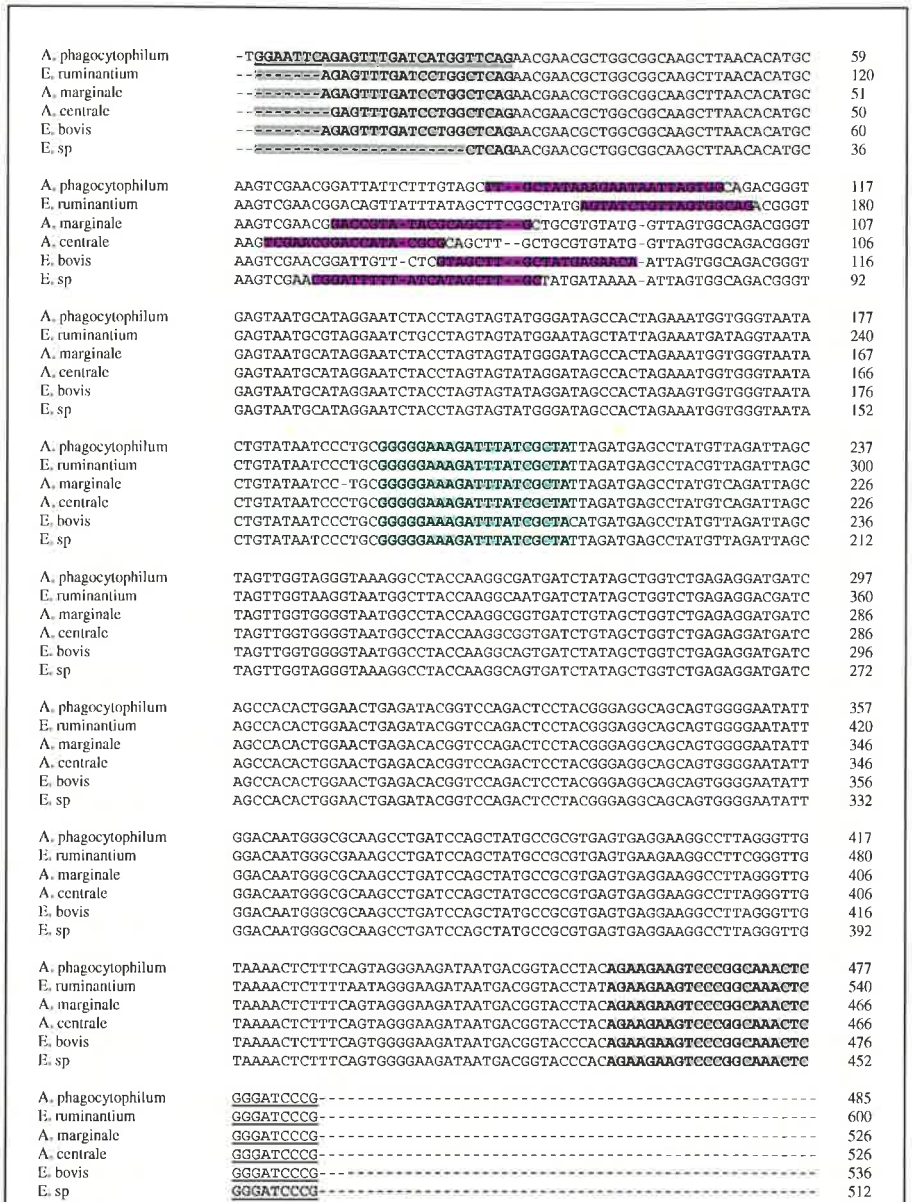

Table 3.1: The location of the primer pair (gray), catch-all probe AE (cyan) and the species-specifi c probes (magenta) are shown on the 16S rRNA sequences. The GenBank accession numbers of the 16S sequences used are as follows: X61659 for *E. ruminantium*; M60313 for *A. marginale*; AF414868 for *A. centrale*; U03775 for *A. bovis*; U54806 for *Ehrlichia sp* and M73220 for *A. phagocytophilum*. The underlined sequence at the end represents the sequence of a restriction enzyme.

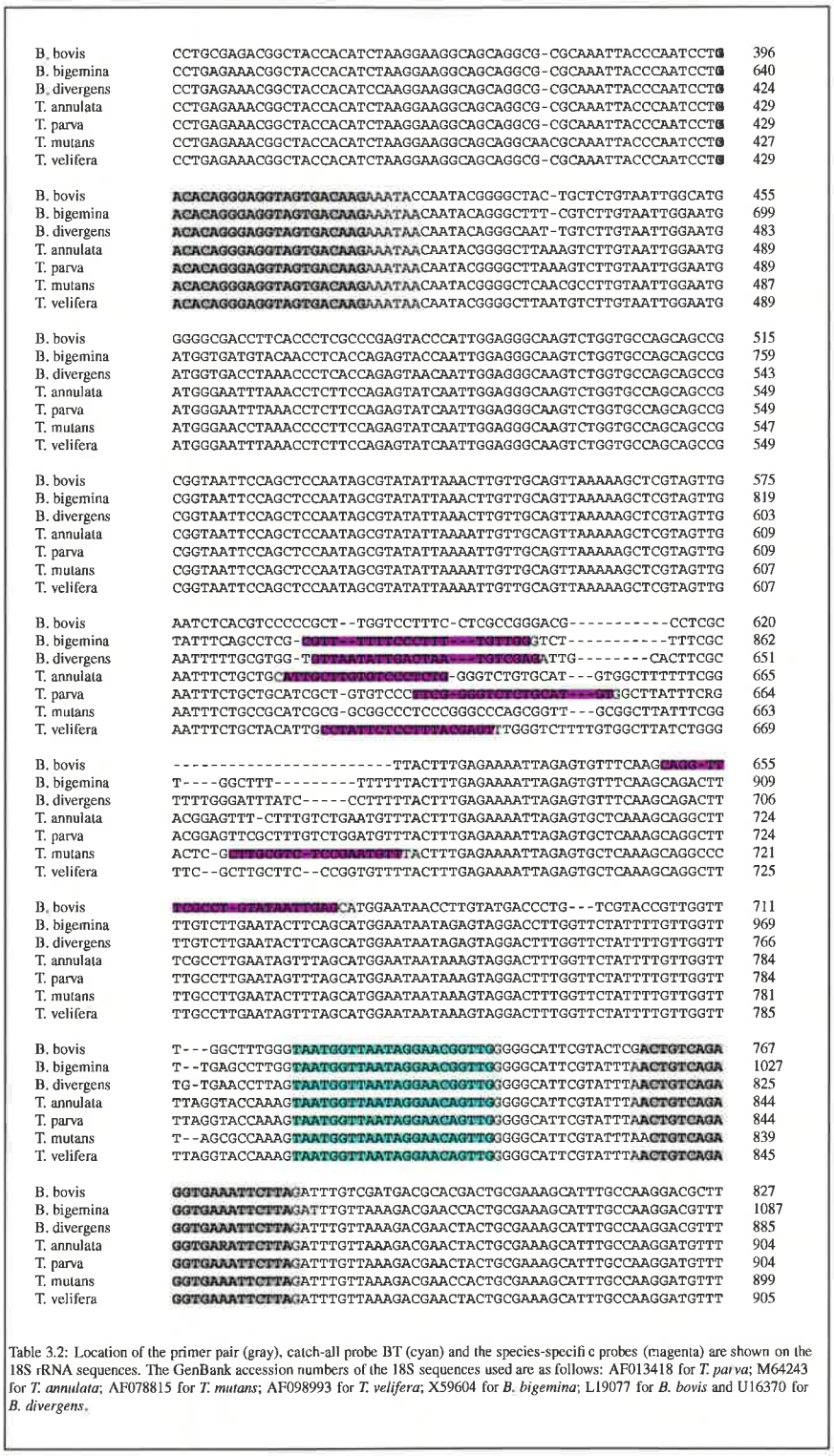

Table 3.2: Location of the primer pair (gray), catch-all probe BT (cyan) and the species-specifi c probes (magenta) are shown on the 18S rRNA sequences. The GenBank accession numbers of the 18S sequences used are as follows: AF013418 for *T. parva*; M64243 for *T. annulata*; AF078815 for *T. mutans*; AF098993 for *T. velifera*; X59604 for *B. bigemina*; L19077 for *B. bovis* and U16370 for *B. divergens*.

The following conditions were used in a 50 µl reaction volume to set up the PCR (Table 3.3).

Table 3.3: PCR components used for the master mix

Component	Volume for 1 reaction (µl)	Final concentration
10x PCR buffer	5	1x
20 mM $MgCl_2$	variable	
dNTP mix (10 mM each)	1	200 µM of each
Forward primer (10 µM)	1	200 pmol
Reverse primer (10 µM)	1	200 pmol
Taq DNA polymerase	0.25	1.25 U
Distilled H_2O	variable	
Template DNA	variable	

Additional magnesium was noticed to have a beneficial effect on the PCR reaction. Therefore, an additional 2 µl of 25 mM $MgCl_2$ was added to the PCR mix, which resulted in a final concentration of 2.5 mM $MgCl_2$. Good hybridization signals were obtained for all positive samples by adding 1 µl of DNA starting amount or 1 punched circle of the FTA cards.

Based on the melting temperature of the four primers, optimal annealing temperature was tested using a temperature gradient varying from 50°C to 60°C. This gradient was applied to most of the positive samples, i.e. *A. marginale*, *A. centrale* and *A. phagocytophilum* in the group AE, and *T. annulata*, *T. parva*, *B. divergens* and *B. bigemina* in the group BT (Table 3.4).

Table 3.4: PCR temperature gradient from 50° to 60°C for *A. marginale* (Am), *A. centrale* (Ac), *A. phagocytophilum* (Ap), *T. annulata* (Ta), *T. parva* (Tp), *B. bigemina* (Bbig) and *B. divergens* (Bdiv). X is indicating the successful amplification by PCR.

Pos.	50	50.9	52	53.2	54.4	55.6	56.8	58	59.1	60
Am	x	x	x	x	x	x	x	x	x	x
Ac	x	x	x	x	x	x	x	x		
Ap	x	x	x	x	x	x	x	x	x	x
Ta	x	x	x	x	x	x	x	x	x	x
Tp	x	x	x	x	x	x	x	x	x	x
Bbig	x	x	x	x	x	x	x	x	x	x
Bdiv	x	x	x	x	x	x	x	x		

The primer pairs for both groups, i.e. AE and BT, were working nearly over the full range of temperature from 50°C to 60°C. However, above 58°C signals could not be obtained for all positives. There were also minor intensity changes which varied with the temperature and positive utilised.
Based on these verifications, the final hybridization temperature was set to 57°C for

both primer pairs as suggested by other authors [44] [56]. Although, the annealing temperature for the group BT was also satisfying at 50°C [49], a common hybridization temperature of 57°C was preferable to facilitate the screening of large scales of samples and also to allow comparing the obtained results with data of other studies. After the evaluation of « short » and « longer » times for denaturation, annealing and elongation (30s, 30s and 1 min against 20s, 30s and 30s) as well as testing various cycling numbers, i.e. 20, 30, 40 or 50 cycles, the PCR program was optimised and used for all subsequent studies (Table 3.5).
A touchdown program with varying annealing temperatures was used for the amplification of DNA to increase the specificity of the PCR. Details of the PCR program are explained in Table 3.5.

Table 3.5: Touchdown PCR program for *Anaplasma/Ehrlichia* and *Babesia/Theileria* species.

Number of cycles	Time length	Temperature	Step
1 cycle	3 min	94°C	Initial denaturation
1 cycle	20 sec	94°C	Denaturation
	30 sec	67°C	Annealing
	30 sec	72°C	Extension
11 cycles	20 sec	94°C	Denaturation
	30 sec	67-57°C	Annealing
	30 sec	72°C	Extension
40 cycles	20 sec	94°C	Denaturation
	30 sec	57°C	Annealing
	30 sec	72°C	Extension
1 cycle	10 min	72°C	Final Extension

These PCR conditions gave good signals for all positive samples on a 1.5 % agarose gel stained with ethidium bromide (Fig. 3.1) as well as in the subsequent RLB assay (Fig. 3.2).

3.1.2 RLB

In the RLB assay species-specific probes were included exclusively for tick-borne pathogens which are relevant for causing diseases in cattle such as cowdriosis, anaplasmosis, babesiosis, and theileriosis. The concentrations of the probes applied onto the Biodyne C membrane were tested empirically in the range of 12.5 to 500 pmol and chosen in a way to obtain more or less equally intense hybridization signals for all positive control samples. The probes and their respective concentrations in which they were applied onto the Biodyne C membrane are summarized in Table

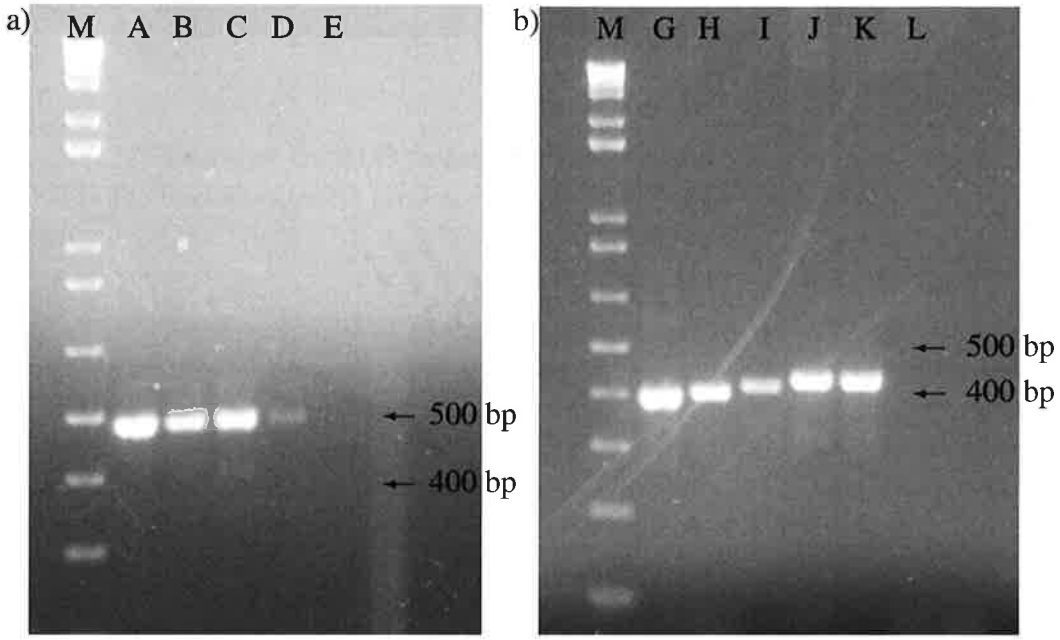

Figure 3.1: PCR using touchdown program (Table 3.5) showing typical signals for the diverse tick-borne pathognes. Figure a) shows typical results for diverse pathogens of the group AE, whereas Figure b) shows typical results for diverse pathogens of the group BT. a) M: 1 Kb Plus DNA Ladder (Invitrogen); A, *A. marginale*; B, *A. centrale*; C, *A. phagocytophilum*; D, *E. ruminantium*; E, Negative control. b) M: 1 Kb Plus DNA Ladder (Invitrogen); G, *B. bovis*; H, *B. bigemina*; I, *B. divergens*; J, *T. annulata*; K, *T. parva*; L, Negative control.

2.2 (see chapter 2). The position of all species-specific probes are indicated on their respective gene sequence (16S rRNA or 18S rRNA) in Tables 3.1 and 3.2.
For the preparation of the Biodyne C membrane and the subsequent RLB hybridization, the protocol from Gubbels et al [49] was followed. However, the posthybridization temperature was set to 52°C. For the exact protocol see chapter material and methods. A typical RLB film showing good hybridization signals for all applied positive samples is shown in Figure 3.2.

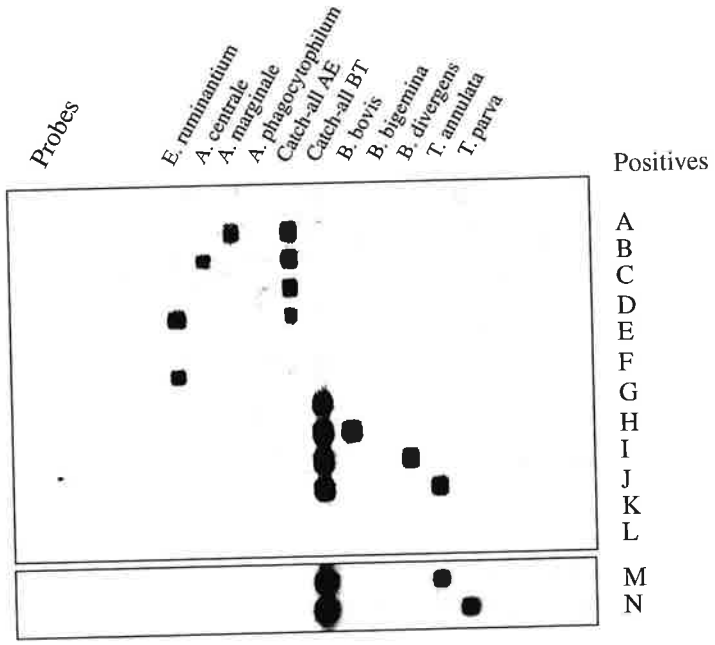

Figure 3.2: Results of the RLB membrane after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical lines: Different positive controls for tick-borne pathogens. A: *A. marginale*; B: *A. centrale*; C: *A. phagocytophilum*; D: *E. ruminantium*; E: Negative control; F: *E. ruminantium*; G: *B. bigemina*; H: *B. bovis*; I: *B. divergens*; J: *T. annulata*; K: *T. parva*; L: Negative control; M: *T. annulata*; N: *T. parva*. Note : *T. parva* sample K was lost during manipulation.

All positive samples gave good hybridization signals. The exception was the *B. bigemina* sample (Australia) which did not hybridize with the species-specific probe. It was also very difficult to obtain a good signal for the *A. phagocytophilum* positive. In contrast to the positive for *B. bigemina* which gave good signal, but did never hybridize with the species-specific probe, very weak hybridization with the species-specific probe was obtained with the positive for *A. phagocytophilum*

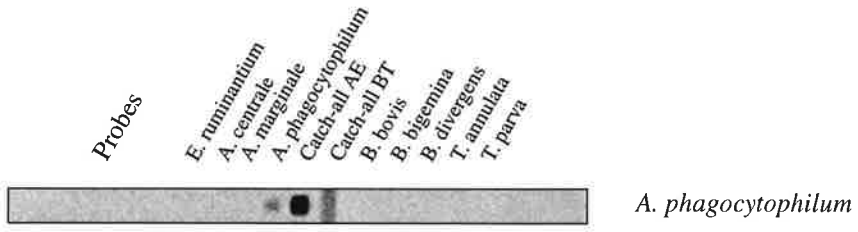

Figure 3.3: Results of the RLB membrane after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical line: Positive control for *A. phagocytophilum*.

(Fig. 3.3). Sequence analyses of the positive control for *B. bigemina* showed a difference in the probe region to the sequence (X59604) utilised to design the probe in 2 nucleotide positions (Fig. 3.4). This might explain, why the sample did not hybridize with the species-specific probe. The sequence of the positive used for *A. phagocytophilum* however was identical to the sequences (M73220) used to design the probe (Fig. 3.5).

Figure 3.4: Alignment of the sequence of *B. bigemina* used to design the species-specific probe (X59604) and the sequence of positive for *B. bigemina* (F07) used in the RLB assay. The region of the probe for *B. bigemina* is underlined in yellow, the differences in the nucleotides between the sequences X59604 and F07 are marked in blue.

The RLB assay was also tested with a mix of positive plasmid controls (obtained with the TBD-RLB kit, see chapter 5) to assure that all probes gave good hybridization signals. The mix contained the following positives: Control plasmids for the group *Anaplasma* and *Ehrlichia* with positives such as *A. phagocytophilum*, *E. ruminantium*, *Ehrlichia* sp, *A. marginale*, *A. centrale*, *A. bovis* and control plasmids for diverse *Babesia* and *Theileria* species such as *B. bovis*, *B. bigemina*, *B. divergens*, *T. annulata*, *T. parva*, *T. mutans* and *T. velifera* (Figure 3.6).

```
M73220  CTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATA   60
F03     ---------------------------TAACACATGCAAGTCGAACGGATTATTCTTTATA   34

M73220  GCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGT   120
F03     GCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGT    94

M73220  AGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGAT   180
F03     AGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGAT   154

M73220  TTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAA   240
F03     TTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAA   214

M73220  GGCGATGATCTATAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGATACGGTC   300
F03     GGCGATGATCTATAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGATACGGTC   274

M73220  CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG   360
F03     CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG   334

M73220  CTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAACTCTTTCAGTAGGGAAGATAA   420
F03     CTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAACTCTTTCAGTAGGGAAGATAA   394

M73220  TGACGGTACCTACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGA   480
F03     TGACGGTACCTACAGAAAAAGTCCCGGCAAACTCGGGATCCCGA----------------   438
```

Figure 3.5: Alignment of the sequence of *A. phagocytophilum* used to design the species-specific probe (M73220) and the sequence of the positive for *A. phagocytophilum* (F03) used in the RLB assay. The region of the probe for *A. phagocytophilum* is underlined in yellow. There are no differences between the sequences.

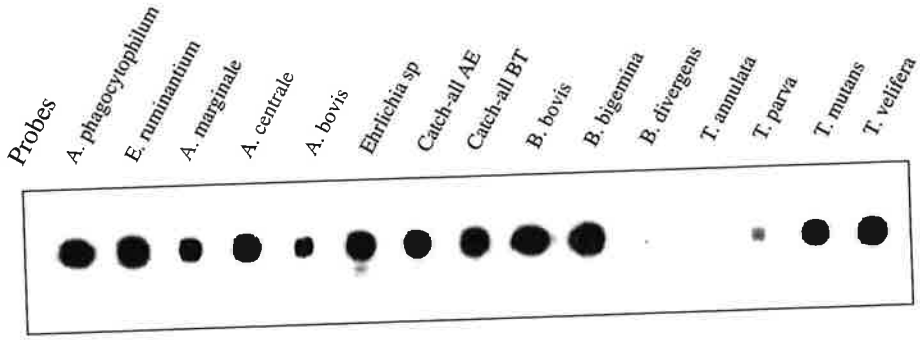

Figure 3.6: Results of the RLB membrane after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical line: Different positive control plasmids for tick-borne pathogens.

The positive plasmid control samples gave good hybridization signals. Only the control plasmids for *B. divergens*, *T. annulata* and *T. parva* did not (*B. divergens*, *T. annulata*) or very weakly (*T. parva*) hybridize with the species-specific probe. All control samples were working fine with the TBD-RLB kit membrane (see chapter 5). However, the TBD-kit used different probe sequences for this three species, namely « ACT RAT GTC GAG ATT GCA C » for *B. divergens*, «CCT CTG GGG TCT GTG CA » for *T. annulata* and « GGA CGG AGT TCG CTT TG » for *T. parva*.

3.2 Preliminary analyses of blood and tick samples collected of cattle by RLB.

A preliminary study with field samples collected on cattle in the Center and East of the Ivory Coast as well as Bangladesh was made to finalise the PCR-RLB methods before applying them to the epidemiological field survey.

3.2.1 Blood samples from the Ivory Coast and Bangladesh

To test the RLB method on field samples, blood was collected and stored on FTA cards in 4 different districts of the East Ivory Coast, Abengourou, Agnibilekro, Bondoukou and Tanda, respectively, in 2005. Blood was collected from cattle belonging to different herds by Dr L. Achi (LANADA). The animals were of the local cattle breeds and held under traditional farming. Totally 16 blood samples (Table 3.8) were subsequently tested several times with the Reverse line blot (RLB).

Table 3.8: Details of the animals selected for the pilote study, including district, location, age, sex and presence of ticks and clinical signs.

Sample	District	Place	Age	Sex	Ticks present/Clinical signs
1	Abengourou	Yakasse	2 years	F	Yes
2	Abengourou	Appoisso	3 years	M	Yes
3	Abengourou	Abengourou	1 year	M	
4	Abengourou	Niablé	1 year	F	
5	Agnibilekro	Yobouakro	2 years	M	Yes
6	Agnibilekro	Damé	4 years	F	Yes
7	Agnibilekro	Damé	4 years	F	Yes
8	Agnibilekro	Kongodia	1 years	F	Yes
9	Bondoukou	Songorie	2 years	F	Yes
10	Bondoukou	També	5 years	F	Yes
11	Bondoukou	Méré	5 years	F	Yes
12	Bondoukou		1 year	M	Yes; Clinical signs, (signe de la bouteille)
13	Tanda	Tanda	2 years	F	Yes
14	Tanda	Dokanou			
15	Tanda	Dokanou			
16	Tanda	N'gorato			Yes ; Animal skinny

Blood samples were extracted from FTA cards and then directly used for PCR. Two circles were punched for each blood sample, one to be used to perform PCR amplification using the primer pair targeting the 16S rRNA gene for all *Ehrlichia* and *Anaplasma* organisms, and one to perform PCR amplification using the primer pair targeting the 18S rRNA gene for all *Babesia* and *Theileria* organisms. PCR products were then made visible by agarose gel electrophoresis.

PCR

Of the total of 16 blood samples, only one sample (no.14) tested positive for *Ehrlichia* and *Anaplasma*, all other 15 were negative (Fig. 3.7). In the second group, *Babesia* and *Theileria*, 12 of the 16 blood samples applied were positive. Only no.7, no.11, no.14 and no.16 showed no bands on the gel (Fig. 3.8). No unspecific bands were observed.

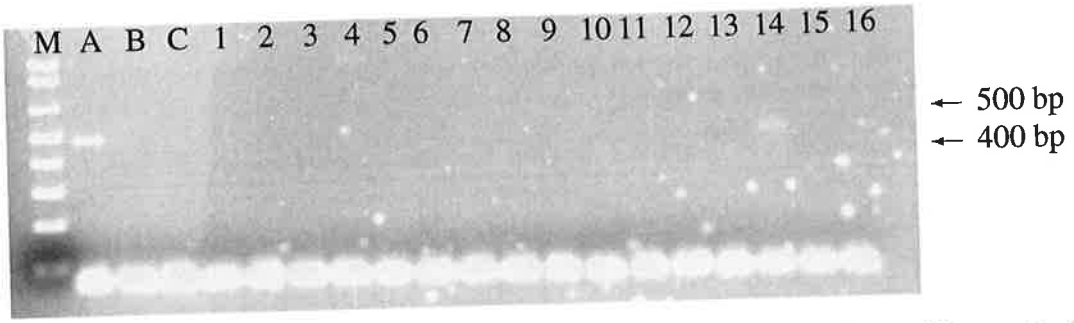

Figure 3.7: PCR for the group AE. M, 1 Kb Plus DNA Ladder; A, Positive control for *A. marginale*; B, negative control (Negative FTA card circle); C, negative control (water); line 1 - 16. Blood samples number 1 to 16.

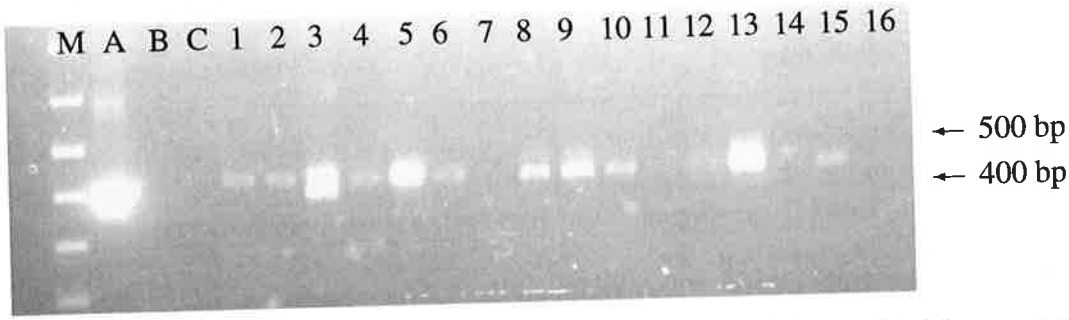

Figure 3.8: PCR for the group BT. M, 1 Kb Plus DNA Ladder; A, Positive control for *B. bovis*; B, negative control (Negative FTA card circle); C, negative control (water); line 1 - 16. Blood samples number 1 to 16.

RLB

The PCR products of the 16 blood samples were applied on a reverse line blot assay. Only sample no.14 reacted with the catch-all probe for *Anaplasma* and *Ehrlichia* (AE). In the other group, 15 samples reacted with the catch-all probe for *Babesia* and *Theileria* (BT). Out of these, 3 samples were positive for *B. bigemina* (no.3, no.6 and no.12). 14 samples tested positive for *T. mutans* and 15 tested positive for *T. velifera*. Only one sample was negative (no. 16) (Fig. 3.9).

The animal from which the blood sample no. 12 was taken presented clinical signs of a possible infection with a tick-borne pathogen. Indeed, by RLB it was possible to detect the presence of *B. bigemina* parasites in this blood sample. This allowed to confirmed the presence of the tick-borne infection.

Overall, no crossreactions were observed and no background lines did occur. The RLB assay was giving good hybridization signals. The assay could be repeated several times (data not shown).

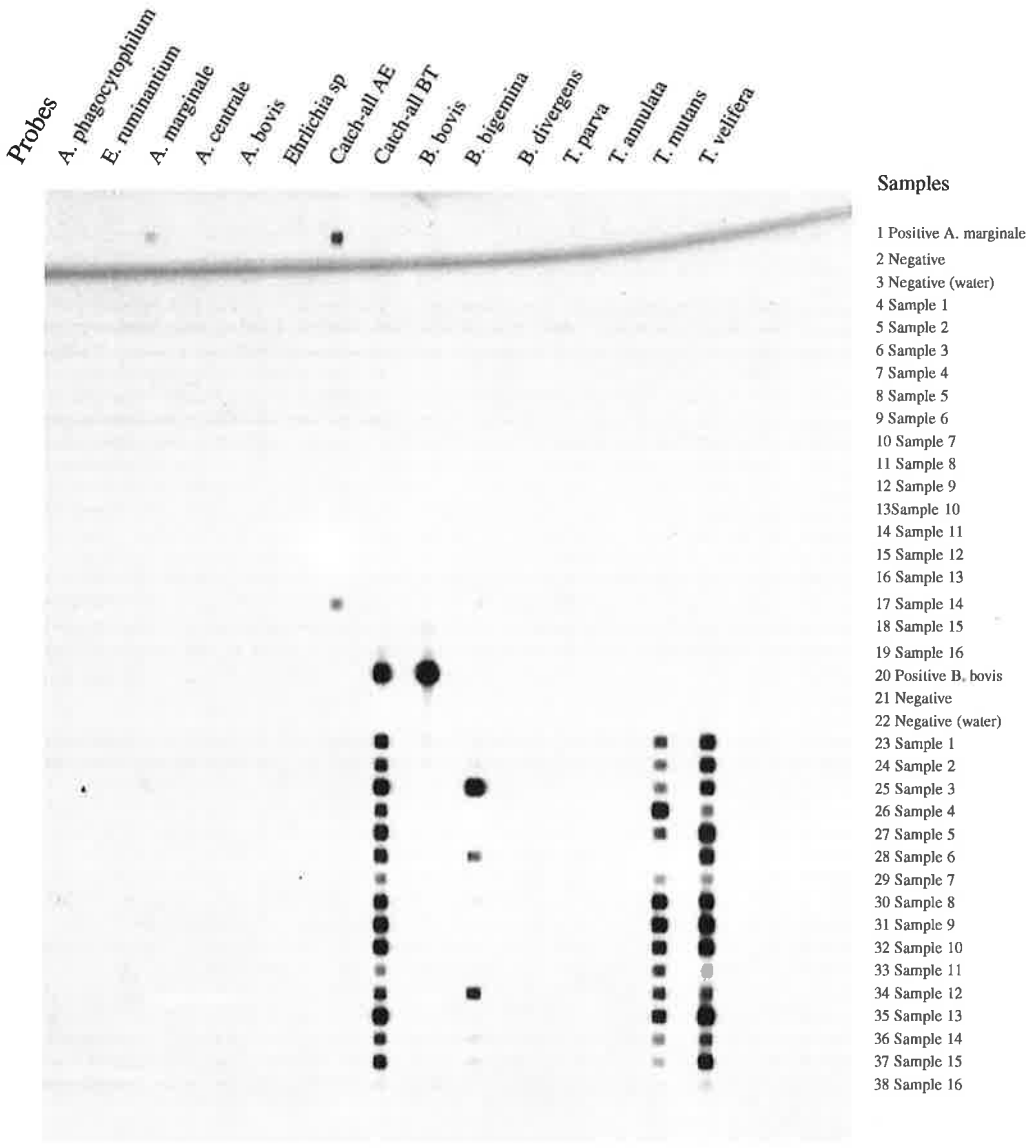

Figure 3.9: Results of the RLB membrane of bovine samples of the Ivory Coast after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical line: line 1, positive control for *A. marginale*; line 2, 3, negative controls; line 4 - 19: Blood samples no.1 - 16, tested for the group AE; line 20, positive control for *B. bovis*; line 21,22, negative controls; line 23 - 38: Blood samples no.1 - 16, tested for the group BT.

In a further assay, the RLB membrane was also used to test bovine blood samples originating from Bangladesh, Asia (Dr Nadira Akhtar) (Fig. 3.10). The samples were known to contain *Theileria* sp and *Anaplasma* sp pathogens (blood smear examination by microscopy). A total of 16 blood samples was tested by RLB for the presence of tick-borne pathogens. Two samples did hybridize with the catch-all probe for the group AE. One sample was positive for *A. marginale*, the second did not react with any of the species-specific probes. All 16 samples reacted positive with the catch-all probe for BT. Nine samples reacted with the probe for *T. annulata*. The other blood samples possibly did contain other pathogen species occurring in the Asian region for which no specific-probes had been included.

The RLB assay confirmed the findings of the blood smear analyses and the samples gave good hybridisation signals with the specific probe. (All material and results for the RLB with the Asian blood samples were obtained from Dr Nadira Akhtar, University Rajashi, Bangladesh, data not shown).

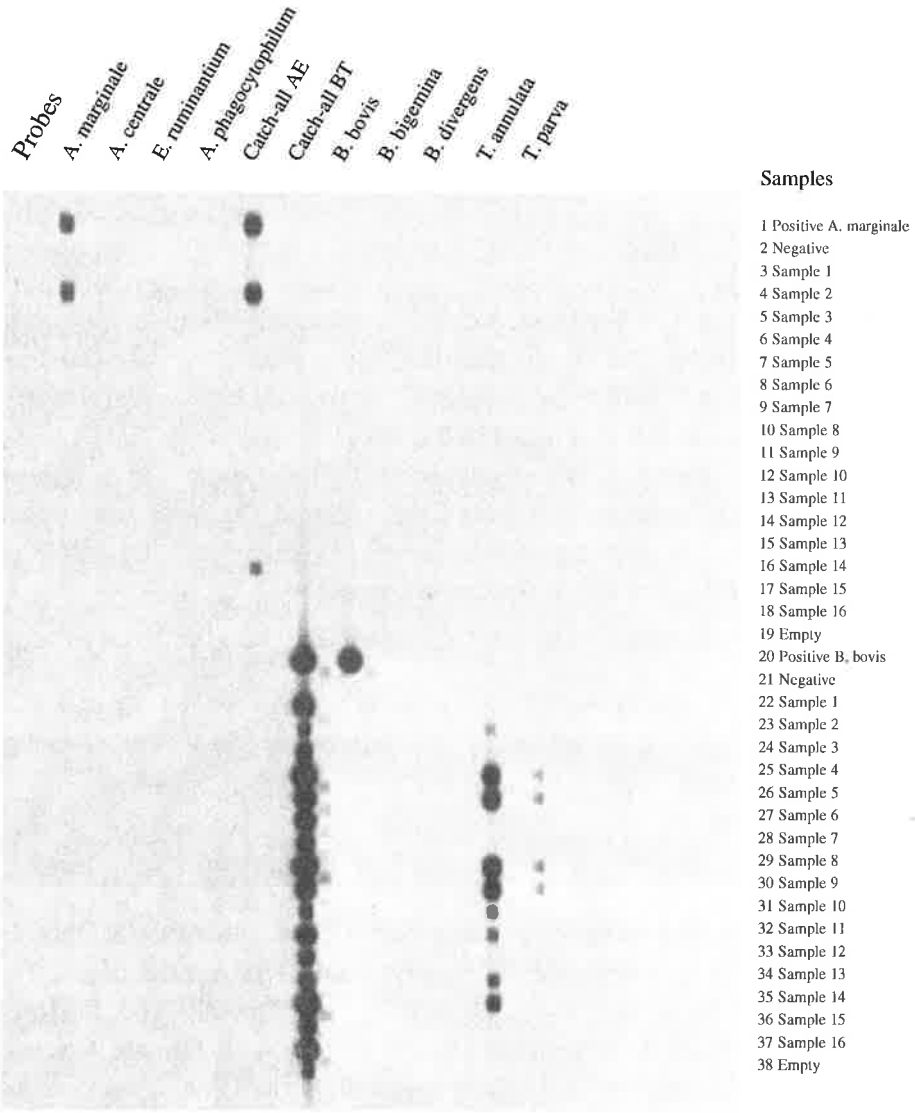

Figure 3.10: Results of the RLB membrane of bovine samples of Bangladesh after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical lines: line 1, positive control for *A. marginale*; line 2, negative control; line 3 - 18: Blood samples no.1 - 16, tested for the group AE; line 19, empty; line 20, positive control for *B. bovis*; line 21, negative control; line 22 - 37: Blood samples no.1 - 16, tested for the group BT; line 38, empty. Note: the diverse points next to the catch-all line for BT and in the region of the probe for *T. parva* probes are artefacts due to pushing the film during the development process.

3.2.2 Tick samples of the Ivory Coast

Ticks are known to have many components that can inhibit PCR reactions including blood, body fat and cuticular components. To exclude any inhibition or unspecific PCR amplifications, the RLB assay was tested with DNA extracted from ticks which have been collected from cattle in the Centre of the Ivory Coast (Bouaflé) in 2005 by Dr L. Achi. A traditional phenol-chloroform extraction (modified after Wen et al [125]) was chosen. The DNA was eluted in a TE buffer.

PCR

DNA of 11 individual *A. variegatum* ticks, 7 males and 4 females, was amplified by PCR (Bouaflé, Ivory Coast). In addition, three wild *I. ricinus* (NE) and two laboratory reared ticks known to be pathogen free (one *A. hebraeum* (Novartis, CH) and one *I. ricinus* (NE)) were included in the assay.
None of the samples gave a positive signal in the PCR for the group *Anaplasma* and *Ehrlichia* (Fig. 3.11). In the second group, *Babesia* and *Theileria*, only one sample (male *A. variegatum* tick) tested positive (sample 4) (Fig. 3.12). One PCR product was found in sample 11, but not of the correct size.

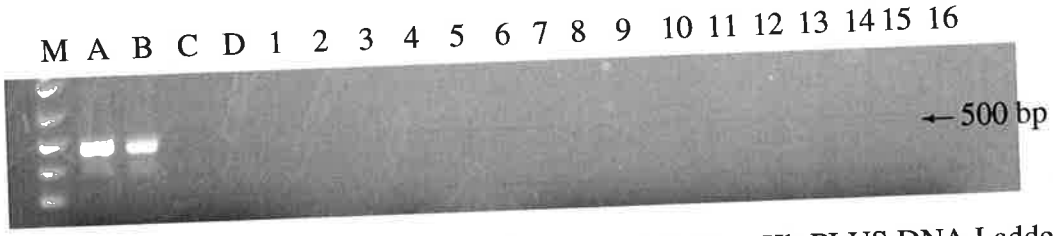

Figure 3.11: PCR for tick samples of the group AE. M, 1 Kb PLUS DNA Ladder; A, Positive control for *A. marginale*; B, Positive control for *A. centrale*; C, Negative control (water); D, Negative control (*I. ricinus* NE); line 1 - 16. Different tick samples. line 1 - 7, Male *A. variegatum* (flat) IC; line 8 - 11, Female *A. variegatum* IC (line 8, 9 engorged and line 10, 11 flat individuals); line 12, *A. hebraeum* Novartis CH; line 13, 14, engorged and flat female *I. ricinus*; line 15, male *I. ricinus*; line 16, negative control (water).

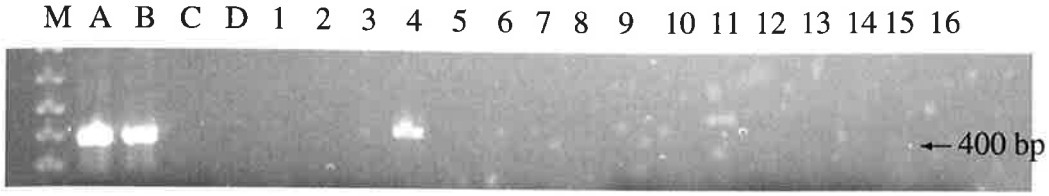

Figure 3.12: PCR for tick samples of the group BT. M, 1Kb Plus DNA Ladder; A, Positive control for *B. bovis*; B, Positive control for *B. bigemina*; C, Negative control (water); D: Negative control (*I. ricinus* NE); line 1 - 16. Different tick samples. Line 1 - 7, Male *A. variegatum* (flat) IC; line 8 - 11, Female *A. variegatum* IC (line 8, 9 engorged and line 10, 11 flat individuals); line 12, *A. hebraeum* Novartis CH; line 13, 14, engorged and flat female *I. ricinus*; line 15, male *I. ricinus*; line 16, negative control (water).

RLB

No sample reacted positive for the group AE, and only one sample (sample 4 (line 28), Fig. 3.12) was positive in the group BT. However, the sample only hybridized with the catch-all probe for BT (Fig. 3.13). It is suspected that the tick might have been infected with *T. mutans* or *T. velifera* organisms, since the specific probes for these species were only included later on. The sample no. 11 did not react at all in the RLB analyses.

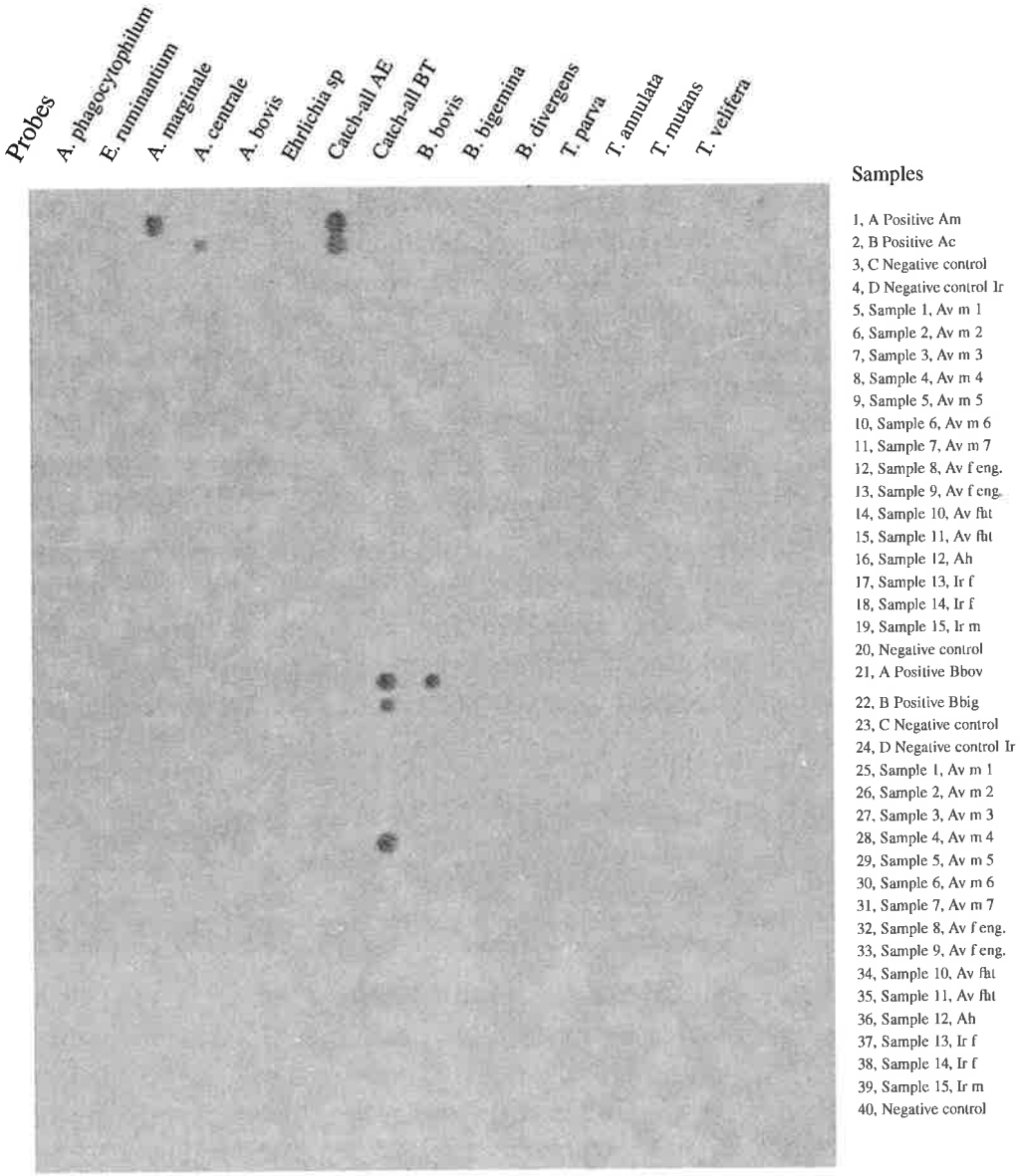

Figure 3.13: Results of the RLB membrane for tick samples of the Ivory Coast after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical line: line 1, positive control for *A. marginale* (Am); line 2, positive control for *A. centrale* (Ac) 3, 4, 20 negative controls; line 5 - 19: Various Tick samples tested for the group AE; line 21, positive control for *B. bovis* (Bbov); line 22, positive control for *B. bigemina* (Bbig), 23, 24, 40, negative controls; line 25 - 39: Various tick samples tested for the group BT. Ir: *I. ricinus*; Av: *A. variegatum*; Ah: *A. hebraeum*; m: male; f: female; eng: engorged.

3.2.3 Conclusion

The results confirm the optimal PCR conditions as well as the subsequent RLB assay for the application of both blood and tick samples. There were no indications of inhibition problems neither for blood nor tick (flat or engorged) samples. Positive samples on agarose gel and the hybridization gave good signals. The tested RLB assay allows therefore to carry out epidemiological studies on tick borne diseases in endemic areas.

Chapter 4

Optimisation of DNA extraction for different tick species.

4.1 Introduction

Tick species carry many components, such as hem, lipids and polysaccharide, which might contaminate the DNA and thereby inhibit the subsequent PCR reaction. To assure a good detection sensitivity for pathogens in ticks, the extraction method has to yield a good purification as well as sufficient DNA.

DNA extraction methods vary with the tick species used [106]. Three of the most commonly used methods were selected and compared on four different tick species varying in size and body composition (i.e. the amount of fat, chitin, etc.): 1) DNA extraction with the Qiagen Tissue Kit (Protocol for DNA extraction, QIAGEN, Switzerland), 2) DNA extraction by ammoniumhydroxide [104] and 3) Classical DNA extraction by phenol-chloroform [11].

4.2 Material and methods

4.2.1 Laboratory reared ticks

The following species were obtained from Novartis AG, St. Aubin, CH. *A. hebraeum* (adult male and female), *Ornithodoros moubata* (adult male), *R. sanguineus* (adult male and female), *I. ricinus* (adult male and female), and *R. (B.) annulatus* (larvae). *Ixodes ricinus* (adult male and female, pathogen-free), Parasitology, University of Neuchâtel. Purified DNA of *T. parva* (Qiagen kit) obtained from Dirk Doebbelaere, University of Berne was used as a positive control. 1 µl was used with DNA equivalent to be detected by PCR.

4.2.2 DNA extraction methods:

All ticks were washed in 70 % ethanol, rinsed twice with distilled water and let to dry on individual paper disc. DNA was extracted following three different protocols:
Method 1: QIAamp DNA mini kit (Qiagen, Switzerland). For DNA extraction the Qiagen tissue protocol was followed.
Method 2: Extraction by ammoniumhydroxide (0.7 M). A modified method from Schouls et al [104] was used. Shortly: Ticks were boiled for 15 min in 100 µl of 0.7 M ammoniumhydroxide to free the DNA. After cooling, the vial with the lysate was left open for 15 min at 100°C to evaporate the ammonia. The lysate was directly used for PCR or was stored at -20°C until use.
Method 3: Digestion with proteinase K, followed by a phenol-chloroform extraction [125] [11] (See chapter two for more details).

4.3 Comparison of DNA extraction by kit (Qiagen), by ammoniumhydroxide and by phenol-chloroform

Both, fed and unfed individual *I. ricinus* ticks were extracted following the three extraction protocols.
The purified tick DNA was spiked with DNA of a positive control, *T. parva*. Furthermore, a sample containing a crushed tick was also spiked with DNA of *T. parva*. A positive control (DNA of *T. parva*) as well as a negative control were included into the subsequent PCR assay.

All three methods gave a positive signal for *T. parva* for fed and unfed tick samples (Fig. 4.1). In contrast, the sample with the crushed and spiked tick did not yield a positive PCR signal (line 9). The tick contains PCR inhibiting substances, which have to be removed before the PCR reaction. All signals had the same intensity as the positive control (line 1) with the exception of line 2, where a slightly weaker signal was obtained.

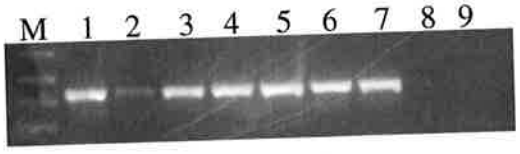

Figure 4.1: PCR to test three DNA extraction methods for their capability to remove inhibitory substances of *I. ricinus*. M: Marker 1 Kb Plus DNA Ladder (Invitrogen); lane 1: Positive control for *T. parva*, lane 2, 3: method 2 (ammoniumhydroxide), 2 unfed, 3 fed tick; lane 4, 5: method 1 (kit), 4, unfed, 5 fed tick; lane 6, 7: method 3 (phenol-chloroform), 6, unfed, 7 fed tick; lane 8: negative control; lane 9: crushed tick.

4.3.1 Influence of the extraction method on the RLB assay

PCR products obtained by using DNA extraction methods 1, 2 and 3 for different tick species, were applied on the Biodyne membrane for RLB analyses. DNA was obtained from four different tick species, i.e. *A. hebraeum*, *O. moubata*, *R. sanguineus* and *I. ricinus*. In one assay the whole tick was used to extract DNA and in the second assay individual ticks were first pulverized prior to the DNA extraction process (Fig. 4.2, 4.3).

Frequently, when the whole ticks were used for the DNA extraction, black lines appeared on the membrane (Fig. 4.2). This phenomenon did not occur, when the ticks were pulverized prior to the DNA extraction (Fig. 4.3). However, in the case of *O. moubata* for the extraction with ammoniumhydroxide neither the pulverization step was able to avoid black lines on the membrane.

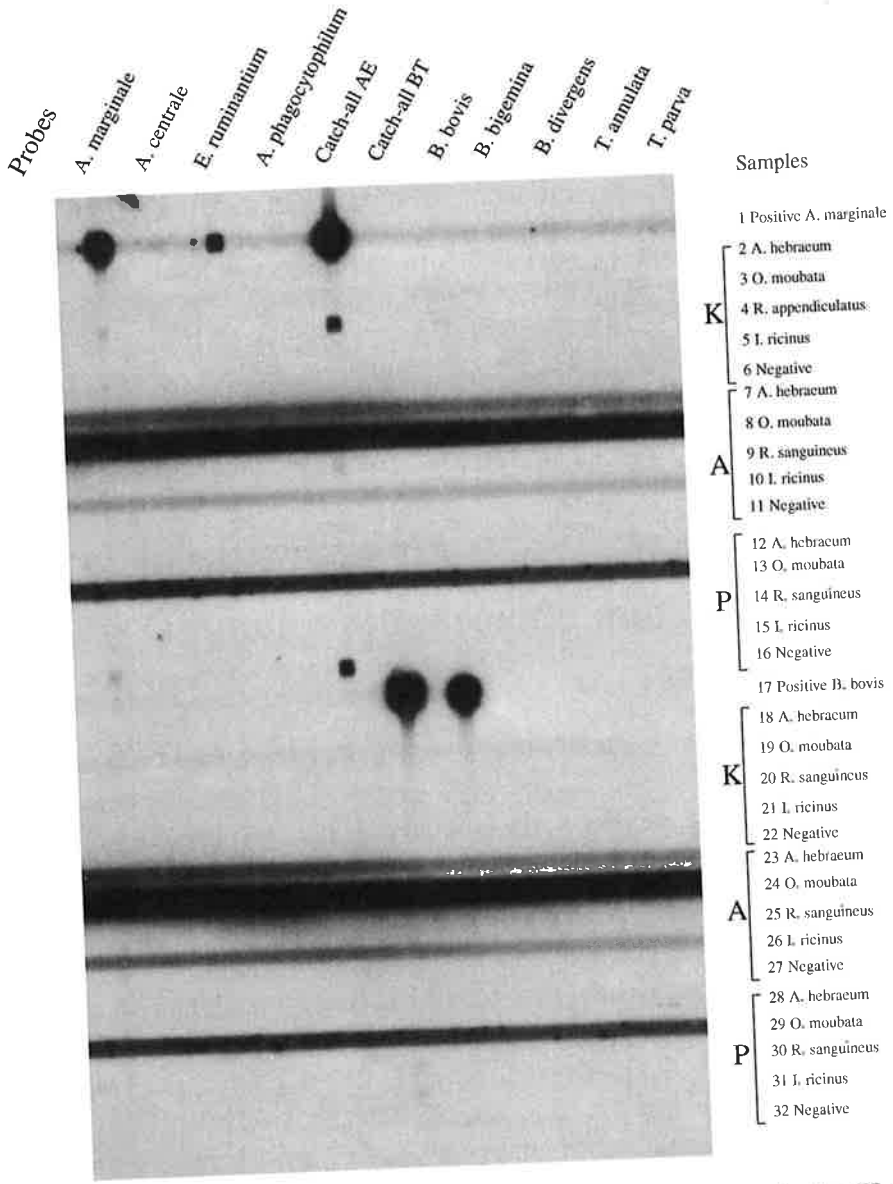

Figure 4.2: Results of the RLB membrane after exposure to the X-ray film. DNA was extracted from whole ticks. Horizontal lines: Species-specific probes. Vertical lines: Different tick samples : *A. hebraeum*, *O. moubata*, *R. appendiculatus*, *I. ricinus*. Line 1, Positive control for *A. marginale*; line 2-6, method 1 (K); line 7-11, method 2 (A); line 12-16, method 3 (P); line 17, positive control for *B. bovis*; line 18-22, method 1 (K); line 23-27, method 2 (A); line 28-32, method 3 (P). K: Qiagen kit, method 1; A: ammoniumhydroxide, method 2; P: phenol-chloroform, method 3. Note: Sample no. 16 (negative) is reacting positive due to a contamination by *A. marginale*

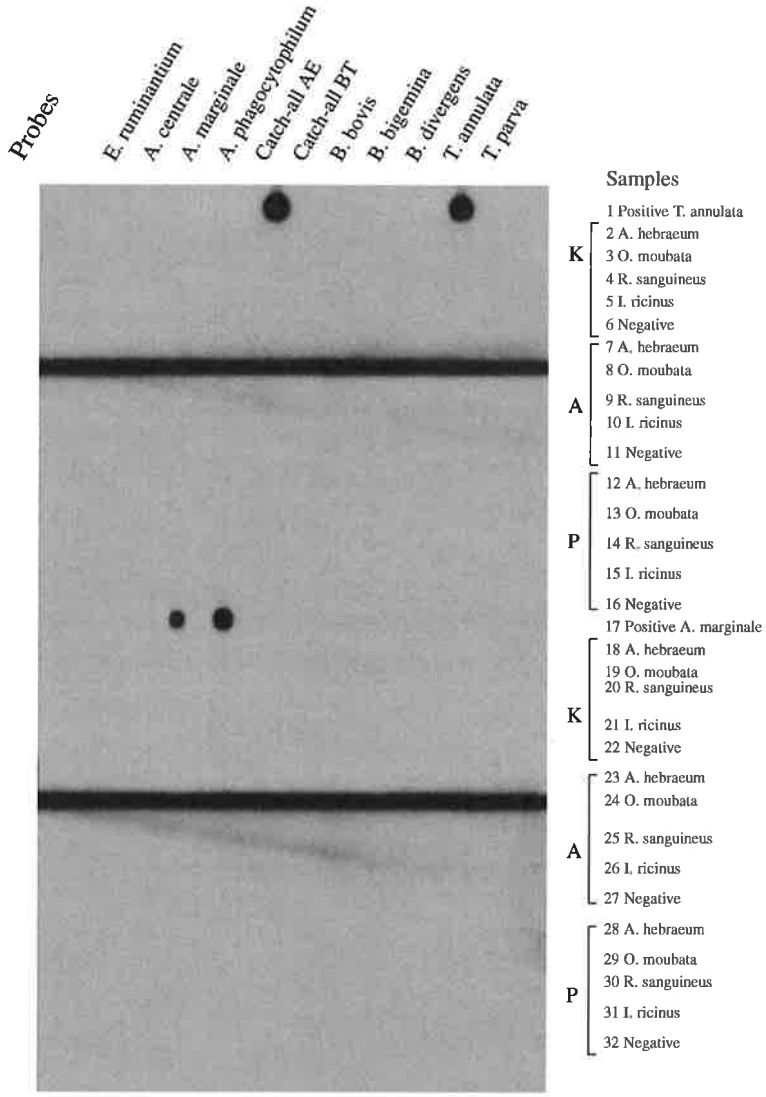

Figure 4.3: Results of the RLB membrane after exposure to the X-ray film. Ticks were pulverized prior to the DNA extraction. Horizontal lines: Species-specific probes. Vertical line: Different tick samples : *A. hebraeum*, *O. moubata*, *R. appendiculatus*, *I. ricinus*. Line 1, Positive control for *A. marginale*; line 2-6, method 1 (K); line 7-11, method 2 (A); line 12-16, method 3 (P); line 17, positive control for *B. bovis*; line 18-22, method 1 (K); line 23-27, method 2 (A); line 28-32, method 3 (P). K: Qiagen kit, method 1; A: ammoniumhydroxide, method 2; P: phenol-chloroform, method 3.

4.4 Optimised DNA extraction using digestion with proteinase K followed by phenol-chloroform extraction

The purity and yield of the DNA extraction were verified using *A. hebraeum*, *A. variegatum*, *O. moubata*, *R. sanguineus*, *B. annulatus* and *I. ricinus* (Table 4.1).

Table 4.1: Ratio 260 nm /280 nm for DNA extracted from different tick species

Species[a]	Ratio	Yield µg/ml
Amblyomma hebraeum	1.7	130.20
Ornithodoros moubata	1.3	91.05
Rhipicephalus sanguineus	2.0[b]	2.90
Ixodes ricinus NE	1.6	10.85
A. variegatum female	1.7	88.33
A. variegatum male	1.6	84.30
R. (B.) annulatus female	1.6	112.90
R. (B.) annulatus male	1.6	27.89

[a]One tick was used with the exception of *A. variegatum* where only half a tick was used

[b]Absorption at 260nm was 0.058. Therefore the measurements are not reliable

DNA of *A. centrale* was mixed with DNA of a *A. variegatum* to test whether inhibitory factors remained in the solution after this extraction procedure. Both, the positive control for *A. centrale* as well as the mixture of tick DNA with the positive control gave a clear and strong signal on an ethidium bromide stained agarose gel. There was no difference in the band intensity between the two samples (Figure 4.4). This is an indication that possible inhibitory factors could be eliminated during the extraction process by phenol-chloroform.

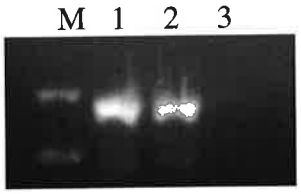

Figure 4.4: PCR reaction to test for inhibitory factors left in the extracted tick DNA (*A. variegatum*) using phenol-chloroform. M : Marker ; Lane 1 : Positive control for *A. centrale*, Lane 2 : Mixture of DNA of *A. variegatum* and *A. centrale*, lane 3 : Negative control.

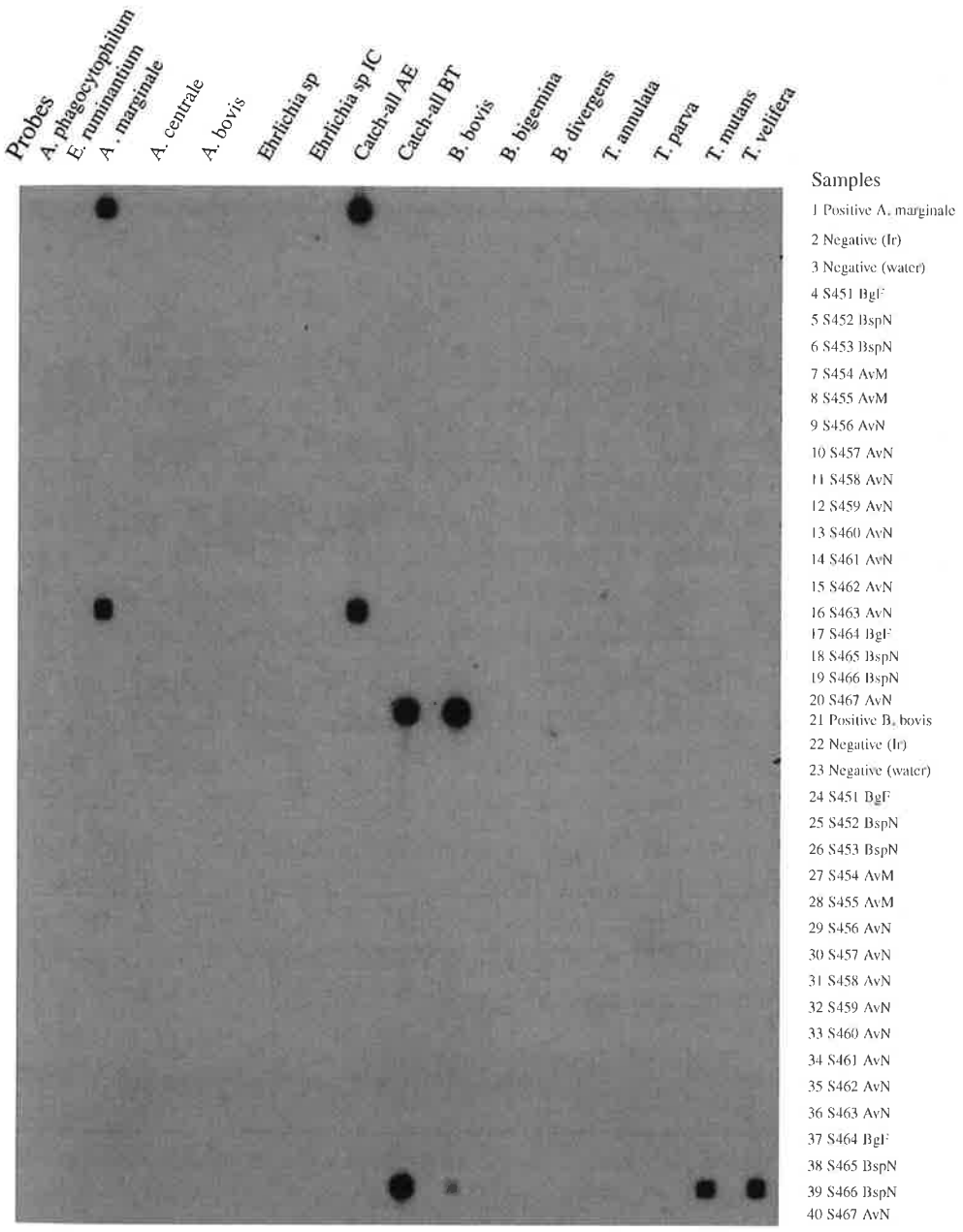

Figure 4.5: Results of the RLB membrane after exposure to the X-ray film. Vertical line: Different tick samples: BgF: female *R. (B.) geigyi*; BspN: *Rhipicephalus (B.)* sp nymph; AvM: *A. variegatum*, male; AvN, *A. variegatum*, nymph; Ir: *I. ricinus*. Line 1, Positive control for *A. marginale*; line 2, 3, negative control samples; line 4 - 20, tick samples for the group AE; line 21, positive control for *B. bovis*; line 22,23, negative control samples; line 24 - 40, tick samples for the group BT. Horizontal lines: Species-specific probes.

Unspecific black lines were no longer detected on the RLB membrane. The DNA yield and purification degree were sufficient to detect pathogens in the analysed tick samples. In the tick sample (No 464) *A. marginale* pathogens could be detected. In the samples (no 467), both *T. mutans* and *T. velifera* were detected. It also reacted very weakly with *B. bovis* (Figure 4.5).

4.5 Conclusion

All of the three methods tested here were giving satisfying results for the detection of pathogens in ticks for small size tick species. Although, pathogens could be detected by RLB in samples where tick DNA has been liberated by ammoniumhydroxide (data not shown, see also [119]), the method is nevertheless prone for inhibitory effects by blood (fed ticks) or lipid. With the big African ticks of the type *Amblyomma*, inhibition problems have been encountered. Typically the solution got coloured greenish or brownish and the PCR products were giving black lines on the RLB membrane, rendering difficult, if not impossible, to interpret the results.

The kit of Qiagen is suitable for all types of ticks in all stage of feeding. Only for ticks of the species *O. moubata* it was impossible to avoid black lines of the RLB membrane.

The digestion by proteinase K followed by phenol-chloroform extraction took away successfully PCR inhibiting components and allowed the detection of pathogen by RLB. Considering that the main species found on cattle in the Ivory Cost were *Amblyomma* sp and *Rhipicephalus (B.)* sp ticks and that they might not always be truly unfed, this method was chosen for all following DNA extractions. It was chosen for its good performance and the low cost.

Chapter 5

Comparative validation of the RLB-PCR method and the ICTTD TBD-RLB kit

5.1 Introduction

The reverse line blot test to detect various tick-borne pathogens [49] [15] adapted to our needs (chapter 3) was validated by using the kit of the Integrated Consortium on Ticks and Tick-borne Diseases (ICTTD) RLB Kit (TBD-RLB kit) provided by Isogen Life Sciences, Netherlands. Blood and tick samples collected from cattle of the Ivory Coast in the framework of the collaborative project carried out with Dr Louise Achi, LANADA, Bingerville, were used. The specificity and the sensitivity of the two methods were evaluated.

5.2 Material and methods

Blood samples were collected from cattle in various regions in the East (Abengourou, Agnibilekro, Bondoukou, Tanda) and South (Sikensi, Dabou) of the Ivory Coast in 2005. The samples were stored on FTA cards (Whatman International LTD) until further analysis. DNA was directly extracted as described in the protocol provided by Whatman. For details see Chapter « Material and methods ». Positive (*A. marginale*, *B. bovis*) and negative (empty FTA cards and water) control samples were included in all assays.
The RLB analysis was carried out using the protocol obtained with the Isogen TBD-kit for the PCR and RLB reactions (Isogen Life Science, The Netherlands).
Before testing our own blood samples, the start-up protocol with cloned plasmid controls (*Ehrlichia/Anaplasma* sp, *Theileria* sp and *Babesia* sp) as suggested in the

kit manual was carried out. Additional tests were performed using identical samples for both methods.

5.3 Results

5.3.1 Start-up protocol

A RLB-PCR analysis was carried out using the control plasmids. The results were as expected (Fig. 5.1). Mix 1 contained controls for *Ehrlichia/Anaplasma* sp, *Theileria* sp and *Babesia* sp, mix 2 contained the control for *Ehrlichia/Anaplasma* sp, mix 3 contained the control for *Babesia* sp and the mix 4 contained *Theileria* sp. All control samples were positive. The horizontal lines in probe number 3 (*A. marginale*), 15 (*B. divergens*) and 32 (*T. parva*) correspond to unspecific background signals.

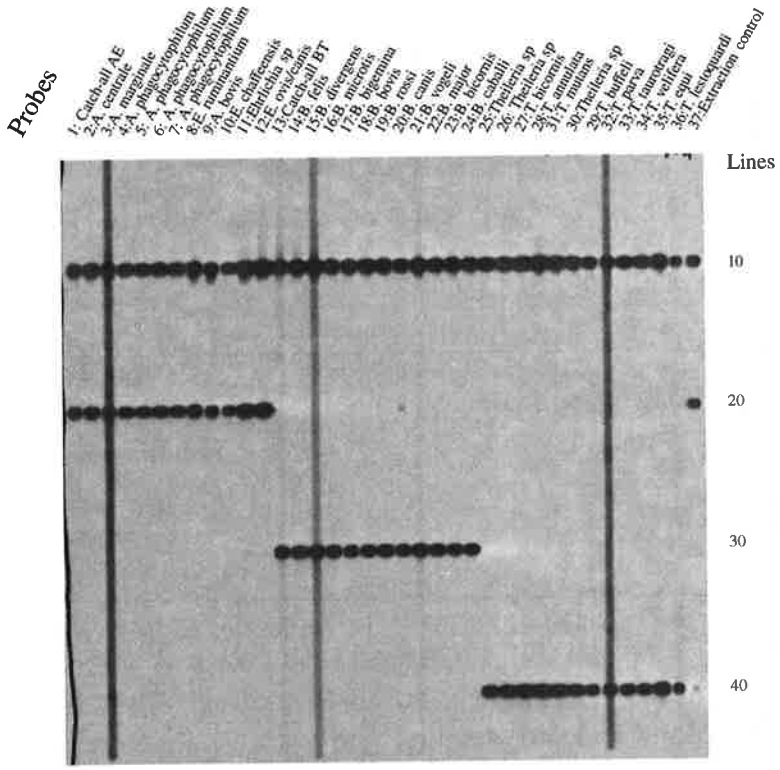

Figure 5.1: Results of the TBD-RLB membrane after 5 min exposure time to the X-ray film. Vertical lines: Probes no. 1 to 37. Horizontal lines: line 10, mix 1 (*Ehrlichia/Anaplasma* sp, *Theileria* sp and *Babesia* sp), line 20, mix 2 (*Ehrlichia/Anaplasma* sp), line 30, mix 3 (*Babesia* sp) and line 40, mix 4 (*Theileria* sp).

5.3.2 Blood sample analysis

Sixteen blood samples collected from cattle of different farms located in the East district of the Ivory Coast were used with the TBD-RLB membrane (Fig. 5.2).
Various probes, including probe numbers 3, 13, 15, 21, 32, and 36 showed black lines on the x-ray film developed for 15 minutes. The results could therefore not be interpreted completely.
Ten blood samples reacted with the probe for catch-all *Anaplasma/Ehrlichia*. The probe for *A. marginale* (line number 3) was a black line. One blood sample reacted with *Ehrlichia* sp, 14 blood samples were positive with catch-all *Theileria/Babesia* of which 6 reacted with *B. bigemina*, one with *B. bovis*, 2 with *B. rossi*, 13 with *Theileria* sp and all 14 with *T. mutans* and *T. velifera*. Our own method detected only 7 samples with the catch all *Anaplasma/Ehrlichia* and 14 with the catch-all *Theileria/Babesia* (Fig. 5.3). Six samples were positive for *B. bigemina* and 1 for *B. bovis*, whereas probes for *T. mutans* and *T. velifera* were only included at a later time. However, samples detected positive for *B. bigemina* and *B. bovis* did not correspond with the two methods. The same 5 samples were positive for *B. bigemina* with both methods, whereas the methods differed in one positive *B. bigemina*. The same was true for *B. bovis* where the two methods did not detect the same sample as positive (Table 5.1). It was not clear, if this was due to a mistake in the membrane charging process, due to a possible contamination or a problem of the membrane itself. No unspecific background lines were observed with the membrane of Neuchâtel (Figure 5.3). On the basis of the black background lines, the customer service was contacted to report this problem and a new membrane was obtained in January 2006 (no.2005-020).

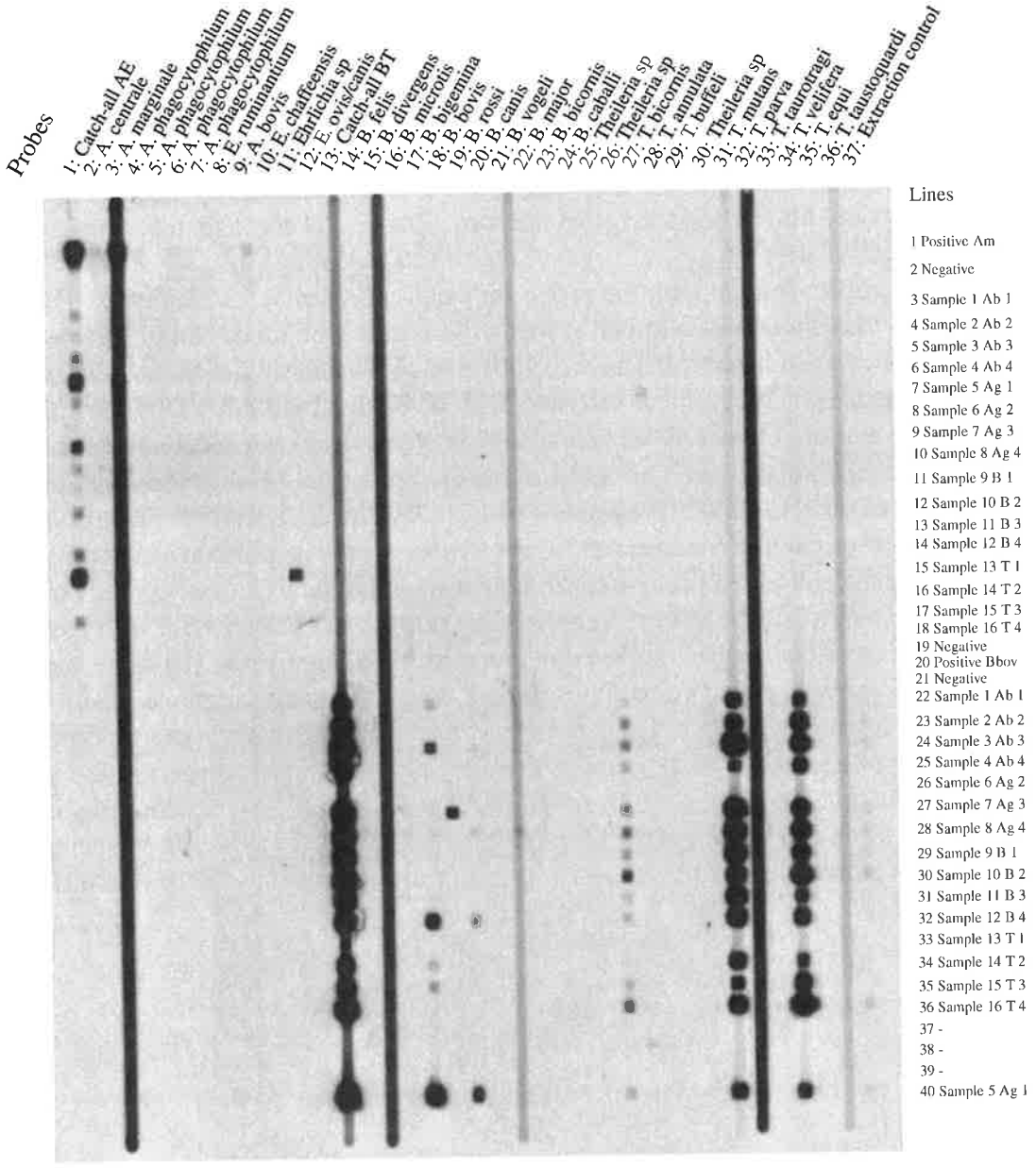

Figure 5.2: Results of the TBD-RLB membrane after 15 min exposure time. Vertical lines: Probes no. 1 to 37. Horizontal lines: Lines 1 - 19, PCR products for the group *Anaplasma/Ehrlichia*; lines 20-40, PCR products for the group *Babesia/Theileria*. Line 1, positive control for *A. marginale*; line 20, positive control for *B. bovis* (lost during the manipulation); lines 2, 21, negative control (FTA card); lines 19, 37, negative control (water); lines 3-18 and 22-36 and 40, blood samples from bovines; line 38, 39, empty. Blood samples were collected from the following districts: Ag, Agnibilekro; Ab, Abengourou; B, Bondoukou; T, Tanda.

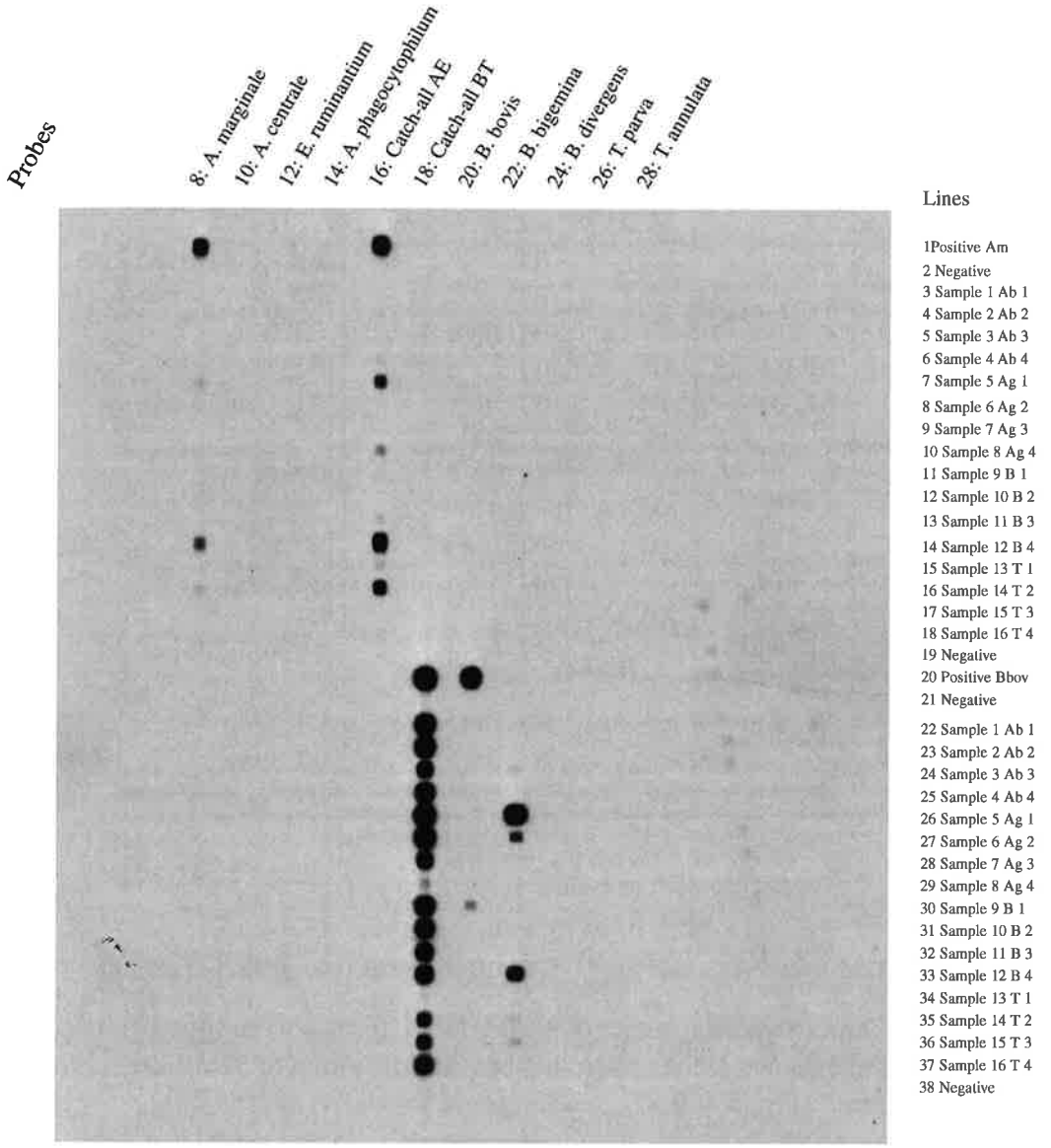

Figure 5.3: Results of the RLB membrane NE after 15 min exposure time. Vertical lines: Specific probes. Horizontal lines: Lines 1-19, PCR products for the group *Anaplasma/Ehrlichia*; lines 20-38, PCR products for the group *Babesia/Theileria*. Line 1, positive control for *A. marginale*; line 20, positive control for *B. bovis*; lines 2, 21, negative control (FTA card); lines 19, 38, negative control (water); lines 3-18 and 22-37, bovine blood samples. Blood samples were collected from the following districts: Ag, Agnibilekro; Ab, Abengourou; B, Bondoukou; T, Tanda.

Table 5.1: Comparison of the results of RLB with the TBD kit membrane (TBD) and the membrane of the Laboratory of Neuchâtel (NE). AE : catch-all AE, Am: *A. marginale*, BT: catch-all BT, Bbov: *B. bovis*, Bbig: *B. bigemina*, Bross: *B. rossi*, Tm: *T. mutans*, Tv: *T. velifera*.

	Group AE		Group BT	
Sample	NE [a]	TBD [b]	NE	TBD
1 Ab 1	-	-	BT	BT, Bbig (weak)
2 Ab 2	-	AE	BT	BT
3 Ab 3	-	-	BT, Bbig (weak)	BT, Bbig
4 Ab 4	AE (weak)	AE	BT	BT
5 Ag 1	AE, Am	AE	BT, Bbig	BT, Bbig, Bross
6 Ag 2	-	AE	BT, Bbig	-
7 Ag 3	-	-	BT	BT, Bbov
8 Ag 4	AE	AE	-	BT
9 B 1	-	AE	BT, Bbov	BT
10 B 2	-	-	BT	BT
11 B 3	AE	AE	BT	BT
12 B 4	AE, Am	-	BT, Bbig	BT, Bbig, Bross
13 T 1	AE	AE	-	-
14 T 2	AE, Am	AE, Esp	BT, Bbig	BT, Bbig
15 T 3	-	-	BT, Bbig	BT, Bbig
16 T 4	-	AE	BT	BT

[a]Probes for Tm, Tv, Esp were not included yet
[b]Positives for Am could not be evaluated

5.3.3 Sample analysis using the membrane no. 2005-020

The new membrane was tested twice with the DNA from bovine blood (Samples collected from Sikensi and Dabou in November 2005). A total of 31 different blood samples were tested (Fig. 5.4, 5.5).
Of the 31 blood samples tested, 3 samples reacted with the probe for catch-all *Anaplasma/Ehrlichia* and 1 blood sample reacted with *A. marginale*. Sixteen blood samples reacted with the probe for catch-all *Theileria/Babesia*, 1 blood sample reacted with *B. bigemina*, 12 blood samples reacted with *T. mutans* and 8 blood samples reacted with *T. velifera* (Tables 5.2, 5.3).
All positive control, i.e. *A. marginale* and *B. bovis*, reacted well with their respective probes.

Table 5.2: Comparison of the results of RLB with the TBD kit membrane (TBD) and the membrane of the Laboratory of Neuchâtel (NE). AE: catch-all AE, Am: *A. marginale*, BT: catch-all BT, Bbov: *B. bovis*, Big: *B. bigemina*, Tm: *T. mutans*, Tv: *T. velifera*. Miss.: sample is missing

	Group AE		Group BT	
Sample	NE	TBD	NE	TBD
N1	AE	AE	-	-
N2	-	-	BT, Tm, Tv	BT, Tm, Tv
N3	AE	AE (weak)	BT, Bbig, Tm, Tv	BT, Bbig, Tm, Tv
N4	-	-	-	-
N5	-	-	-	-
N6	-	-	-	-
N7	-	-	BT, Tm	BT, Tm
N8	-	-	-	-
N9	-	-	-	-
N10	-	-	-	-
N11	-	-	-	-
N12	-	-	BT, Tm, Tv	BT, Tm, Tv
N13	-	-	BT, Tv	BT, Tv
N14	-	-	BT	BT
N15	miss.	miss.	miss.	miss.
N16	-	-	BT, Tm	miss.
N17	-	-	BT, Tm	BT, Tm
N18	-	-	BT, Tm	BT, Tm
N19	-	-	-	-
N20	-	-	BT, Tv (weak)	BT, Tv
N21	-	-	-	-
N22	-	-	miss.	miss.
N23	-	-	-	-
N24	-	-	BT, Tm, Tv	BT, Tm, Tv
N25	-	-	-	-
N26	-	-	BT, Tv (weak)	BT
N27	-	-	-	-
N28	-	-	BT, Tm, Tv	BT, Tm, Tv
N29	-	-	BT, Tm	BT, Tm
N30	-	-	BT, Tm, Tv	BT, Tm, Tv
N31	AE, Am	AE, Am (weak)	BT, Tm	BT, Tm
N32	AE	-	BT, Tm, Tv	BT, Tm, Tv
N33	-	-	-	-

The same 31 samples were applied to the second membrane (Neuchâtel) (Fig. 5.6, 5.7). Of the 31 blood samples tested, 4 samples reacted with the probe for catch-all *Anaplasma/Ehrlichia* and 1 blood sample reacted with *A. marginale*. Seventeen blood samples reacted with the probe for catch-all *Theileria/Babesia*, 1 blood sample reacted with *B. bigemina*, 13 blood samples reacted with *T. mutans* and 9 blood samples reacted with *T. velifera* (Table 5.3).
All positive controls, i.e. *A. marginale* and *B. bovis*, reacted well with their respective probes.

Table 5.3: Summary of the positive blood samples

Probes that reacted	Total number of blood samples	Number of positives in the RLB TBD-Kit	Number of positives in the RLB Neuchâtel[a]
Catch-all AE	31	3	4
A. marginale	31	1	1
Catch-all BT	31	16	16
B. bigemina	31	1	1
T. mutans	31	12	12
T. velifera	31	9	10

[a]Sample no 16 was not counted; lacking in TBD-kit

The two test showed identical results, i.e. the same samples were positive for the same pathogens. The RLB with the membrane of Neuchâtel could detect an additional positive for the catch-all AE as well as an additional positive for *T. velifera*. Even though the number of unspecific background lines was reduced with the new TBD membrane, probe numbers 13, 21 and 36 continued to give unspecific background lines, a phenomenon which could not be observed with the membrane of Neuchâtel.
As far as evaluated the TBD-kit test is working fine and giving specific results. However, even with the second membrane background lines were still obtained for some of the probes. Since both methods gave identical results, data evaluated with the membrane of NE can be compared to studies carried out with the TBD-kit RLB.

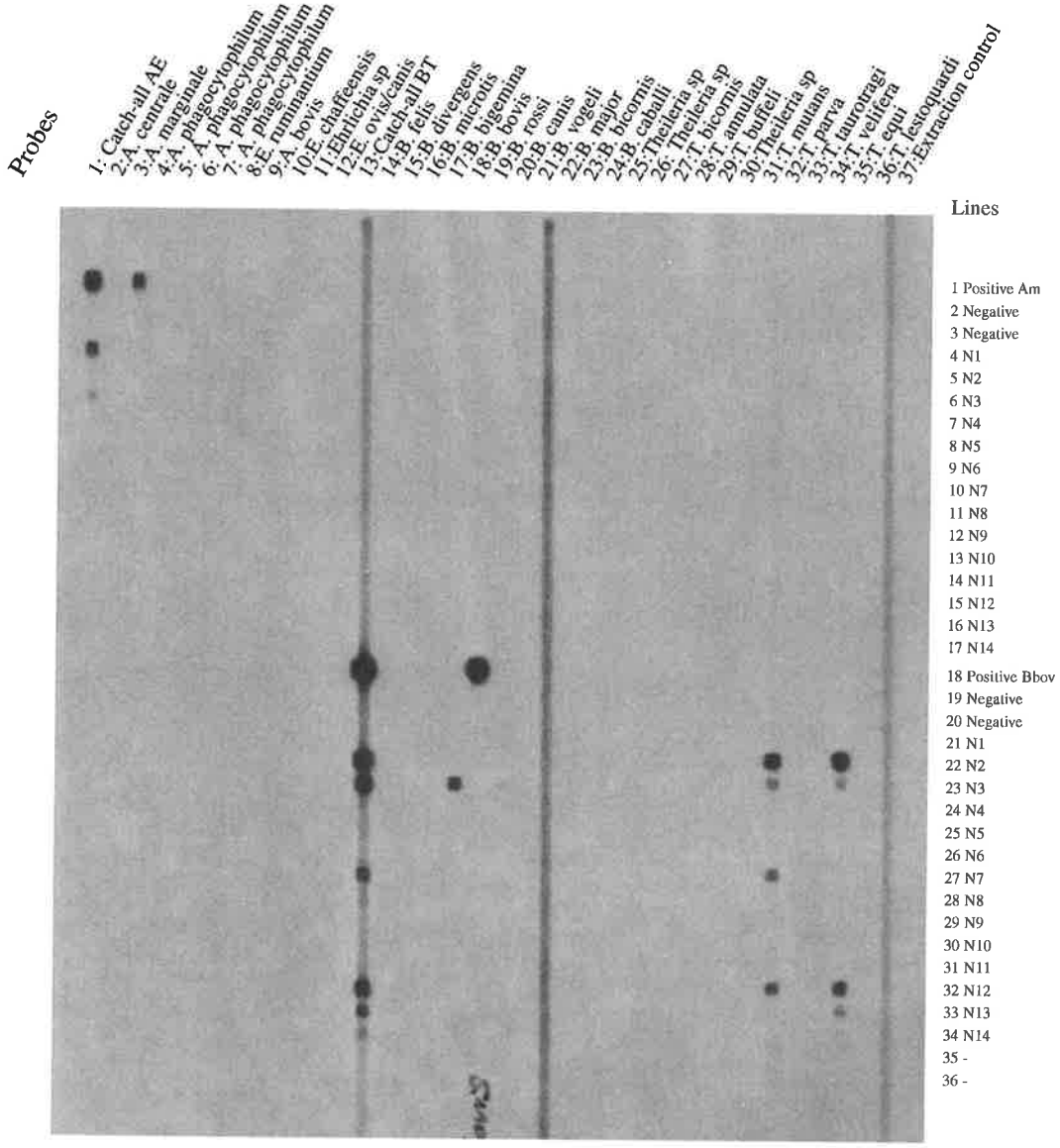

Figure 5.4: Results of the TBD-RLB membrane after 15 min exposure time. Vertical lines: Probes no. 1 to 37. Horizontal lines: Lines 1-17 PCR products for the group *Anaplasma/Ehrlichia*; lines 18-34 PCR products for the group *Babesia/Theileria*. Line 1, positive control for *A. marginale*; line 18, positive control for *B. bovis*; lines 2, 19, negative control (FTA card); lines 3, 20, negative control (water), lines 4-17 and lines 21-34 blood samples 1 to 14 (Sikensi); Sample no. 15 is missing; lines 35-36, empty slots.

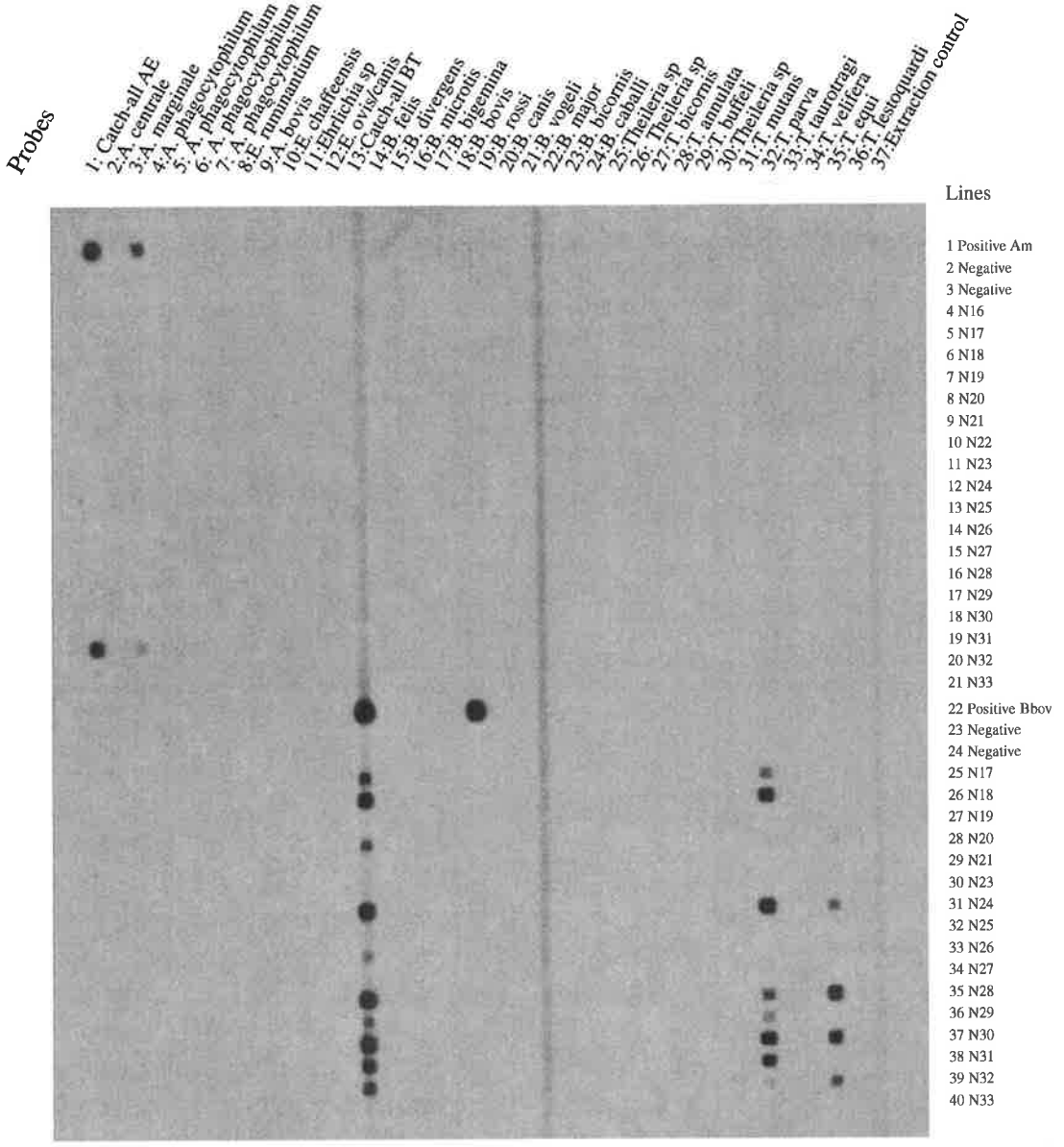

Figure 5.5: Results of the TBD-RLB membrane after 15 min exposure time. Vertical lines: Probes no. 1 to 37. Horizontal lines: Lines 1-21, PCR products for the group *Anaplasma/Ehrlichia*; lines 22-40, PCR products for the group *Babesia/Theileria*. Line 1, positive control for *A. marginale*; line 22, positive control for *B. bovis*; lines 2, 23, negative control (FTA card); lines 3, 24, negative control (water); lines 4-21 and 25-40 blood samples 16 to 33 (Dabou); Samples no 16 and 22 are missing for the group BT.

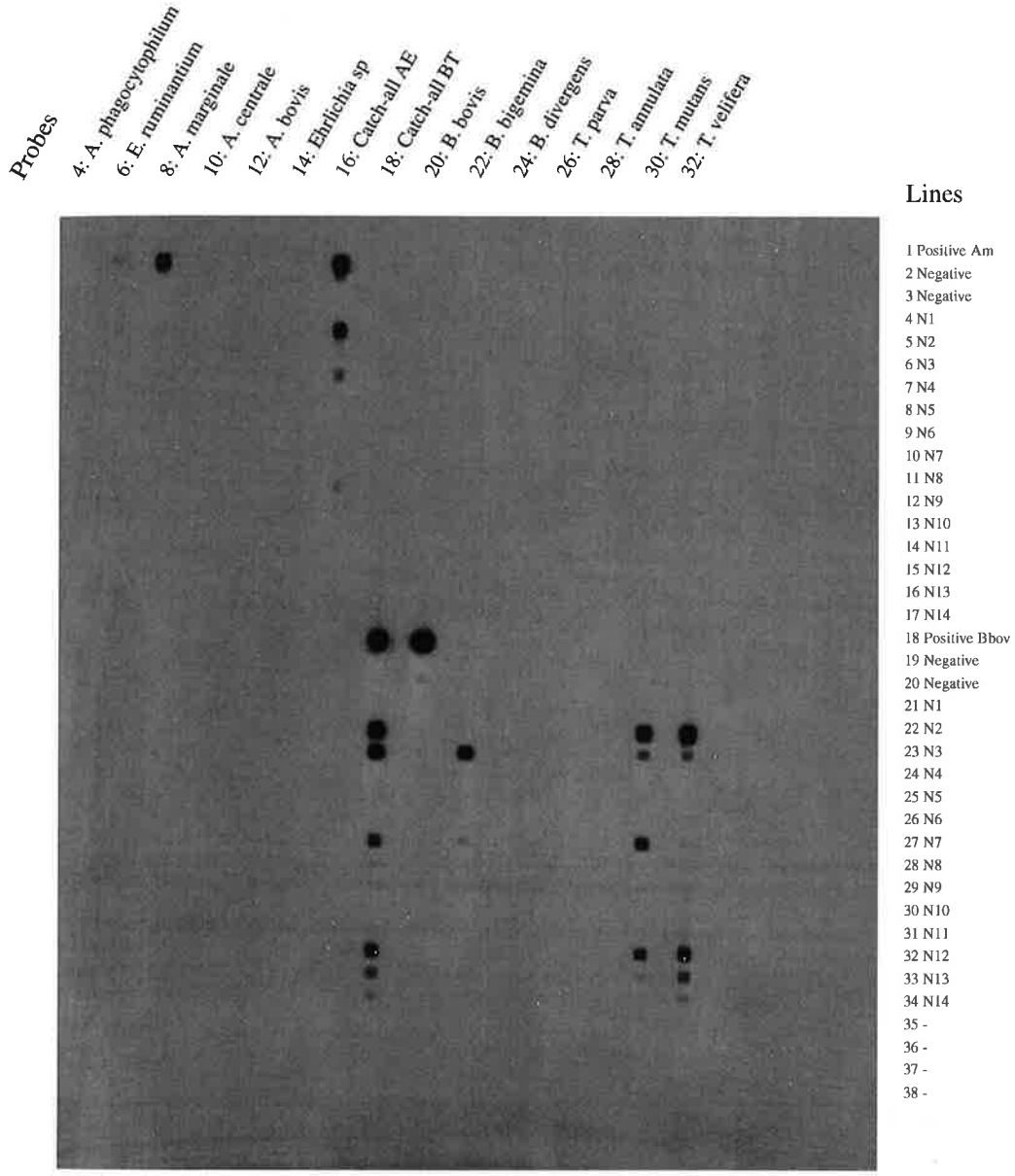

Figure 5.6: Results of the RLB membrane NE after 15 min exposure time. Vertical lines: Probes. Horizontal lines: Lines 1-17, PCR products for the group *Anaplasma/Ehrlichia*; lines 19-34, PCR products for the group *Babesia/Theileria*. Line 1, positive control for *A. marginale*; line 22, positive control for *B. bovis*; lines 2, 23, negative control (FTA card); lines 3, 24, negative control (water); lines 4-17 and 21-34, bovine blood samples 1 to 14 (Sikensi); Number 15 is missing.

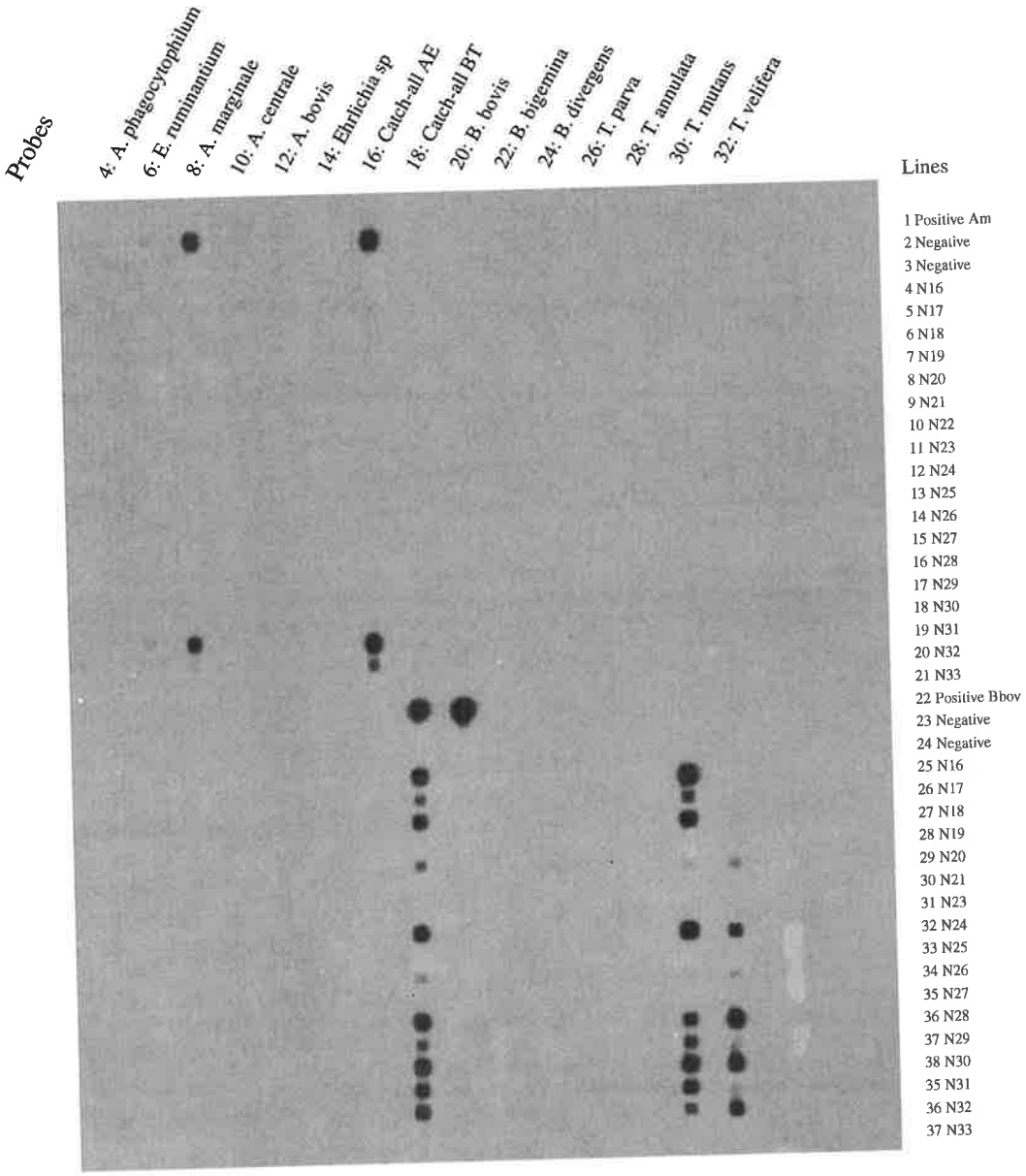

Figure 5.7: Results of the RLB membrane NE after 15 min exposure time. Vertical lines: Probes. Horizontal lines: Lines 1-21, PCR products for the group *Anaplasma/Ehrlichia*; lines 22-40, PCR products for the group *Babesia/Theileria*. Line 1, positive control for *A. marginale*; line 22, positive control for *B. bovis*; lines 2, 23, negative control (FTA card); lines 3, 24, negative control (water); lines 4-21 and 25-40 bovine blood samples 16 to 33 (Dabou); Samples no 22 is missing.

Chapter 6

Study of the abundance, variety and seasonal variation of tick species collected from cattle in the Southern zone of the Ivory Coast.

6.1 Tick genus and species found on cattle in the southern region of the Ivory coast

Five tick species were identified to be present on cattle in the Southern area of the Ivory Coast. These are *A. variegatum*, *R. (B.) decoloratus*, *R. (B.) geigyi*, *R. (B.) annulatus* and *R. lunulatus*. Only at one occasion a female *H. truncatum* tick was found on one of the investigated cattle. Since this was a single occurrence, this tick species is not regarded to be endemic in the area.

Altogether a total of 31'886 individual ticks were collected over the study period (November 2005 - October 2006) (Table 6.1). The tick species are cited in the order of their frequency (paired students' test, for details see annex A.1.1). *A. variegatum* was, with an overall prevalence of 70.9%, by far the most abundant tick species found in this region, followed by *R. (B.) decoloratus* (12.8%). The other two *Rhipicephalus (B.)*, i.e. *R. (B.) geigyi* and *R. (B.) annulatus* species, were present in equal numbers, though are slightly less numerous than *R. (B.) decoloratus* (7.4 and 6.5 %, respectively). *R. lunulatus* figured at the very end with a frequency of less than 1 % (Fig. 6.1).

All four developmental stages, i.e. male, female, nymph and larva, could be found for most of the tick species presented. The exception was *R. lunulatus*, where only adult ticks were found on cattle, the host for the immature stages being rodents

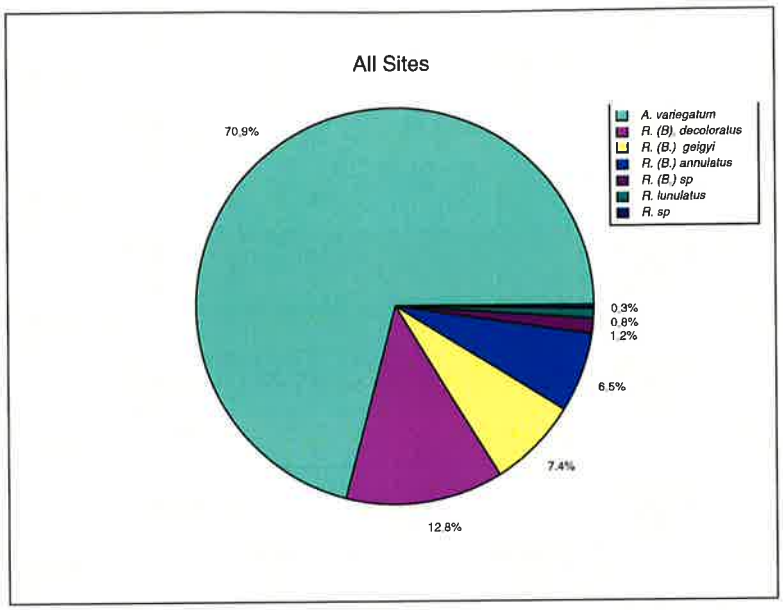

Figure 6.1: Diagram showing the abundance (%) of the different tick species in the Southern zone of the Ivory Coast.

and hares. In Table 6.2 total numbers for the individual stages of all tick species collected during the sampling period are listed in detail (see also figure 6.2).

Table 6.1: Diversity, total counts and abundance (%) of the diverse tick species collected on cattle in the Ivory Coast.

Tick species	Total number of individuals[a]	Abundance %
Amblyomma variegatum	22607	70.90
Rhipicephalus (B.) decoloratus	4096	12.85
Rhipicephalus (B.) geigyi	2364	7.41
Rhipicephalus (B.) annulatus	2068	6.48
Rhipicephalus (B.) sp	397	1.25
Rhipicephalus lunulatus	249	0.78
Rhipicephalus (B.) sp	104	0.33
Hyalomma truncatum	1	0
Total number	31886	100

[a]counting adults, nymphs and larvae

Table 6.2: Diversity and total numbers of the different developmental stages of tick species collected on cattle in the Ivory Coast.

Tick species	Male	Female	Nymph	Larva
Amblyomma variegatum	3416	1515	13058	4618
Rhipicephalus (B.) decoloratus	423	1659	1841	173
Rhipicephalus (B.) geigyi	471	1216	662	15
Rhipicephalus (B.) annulatus	402	821	829	16
Rhipicephalus (B.) sp		193	180	24
Rhipicephalus lunulatus	152	97	0	0
Rhipicephalus (B.) sp	2	102	0	0
Hyalomma truncatum	0	1	0	0

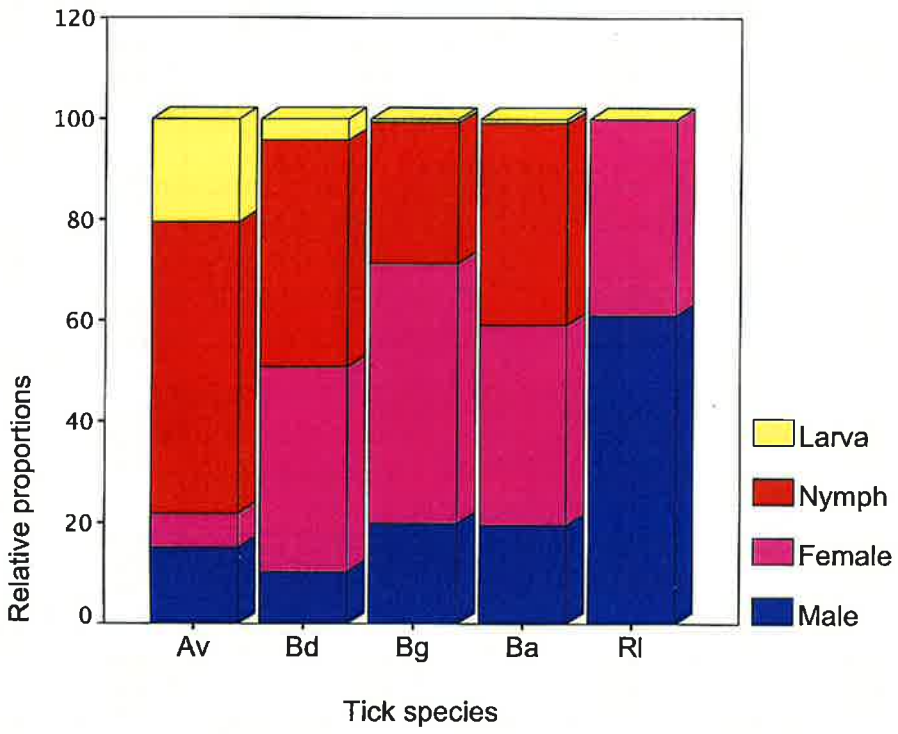

Figure 6.2: Relative proportion of the different developmental stages of the diverse tick species found on cattle. Av: *A. variegatum*; Bd: *R. (B.) decoloratus*; Bg: *R. (B.) geigyi*; Ba: *R. (B.) annulatus*; Rl: *R. (B.) lunulatus*

6.2 Distribution of the different tick species and their abundance in the four different study sites.

ANOVA analyses were carried out in order to present variations among the different study sites in the distribution and frequency of the tick species (Annex A.1.2). The distribution of the divers tick species and their respective presence significantly varied among the different farms (Table 6.3).

Table 6.3: Total counts of the different tick species for the four study sites. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Tick species	Me	Ca	Br	Az
Amblyomma variegatum	8033	7818	4846	1910
Rhipicephalus (B.) decoloratus	60	43	55	3938
Rhipicephalus (B.) geigyi	582	0	1696	86
Rhipicephalus (B.) annulatus	2056	0	7	5
Rhipicephalus lunulatus	150	62	37	0
Rhipicephalus (B.) sp	310	12	64	11
Rhipicephalus sp	32	60	12	0
Total count	11223	7995	6717	5950

Even though *A. variegatum* was found on all four study sites, a difference in the tick burden (F-value of 70.34) was noticed. Whereas *A. variegatum* was the most frequent tick species in the three sites Sikensi, Dabou and Brofodoumé with a frequency of 71.6% to 97.8%, it was clearly outnumbered by *R. (B.) decoloratus* in the farm of Azaguié. Here, *A. variegatum* was only present with a frequency of 32.1% (Fig. 6.3 - 6.6).

Likewise, the occurrence and abundance of the three different *Rhipicephalus (B.)* species varied greatly between the different farms (Annex A.1.2). The farms seem to have their specific *Rhipicephalus (B.)* species.
Nevertheless *R. (B.) decoloratus* can be found on all four farms and it is the most abundant *Rhipicephalus (B.)* species in Azaguié (66.2%) and only occurs with a frequency of less than 1% in the other three farms (F-value of 72.21). *R. (B.) geigyi* is the most abundant species in Brofodoumé (25.2%) and absent in Dabou, whereas the frequency is of 5.2% and 1.4% respectively in Sikensi and Azaguié. Last but not least, *R. (B.) annulatus*, which is absent in Dabou and hardly present in the two other sites (Brofodoumé and Azaguié), is by far the most abundant *Rhipicephalus (B.)* species (18.3%) in Sikensi (Fig. 6.3 - 6.6).
Overall, it is interesting to note that Dabou hosted almost exclusively *A. variegatum* (97%) ticks and had a very small number of *Rhipicephalus (B.)* ticks.

R. lunulatus ticks were not found in the farm of Azaguié, but were present in all

other farms, most frequently in Sikensi (F-value of 18.32) (Fig. 6.3 - 6.6).

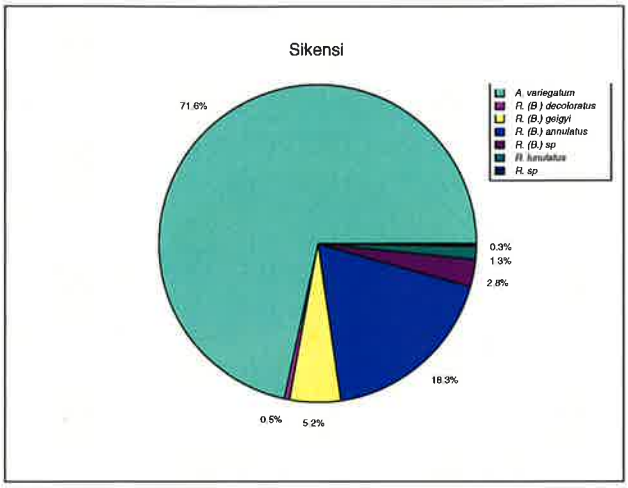

Figure 6.3: Distribution and frequency (%)of the diverse tick species present in Sikensi.

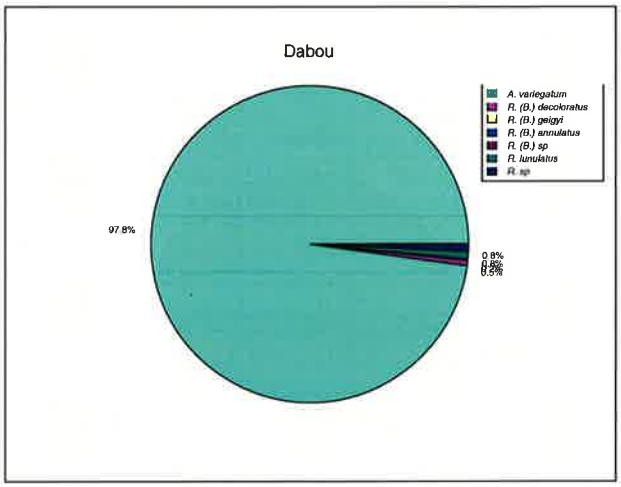

Figure 6.4: Distribution and frequency (%) of the diverse tick species present in Dabou.

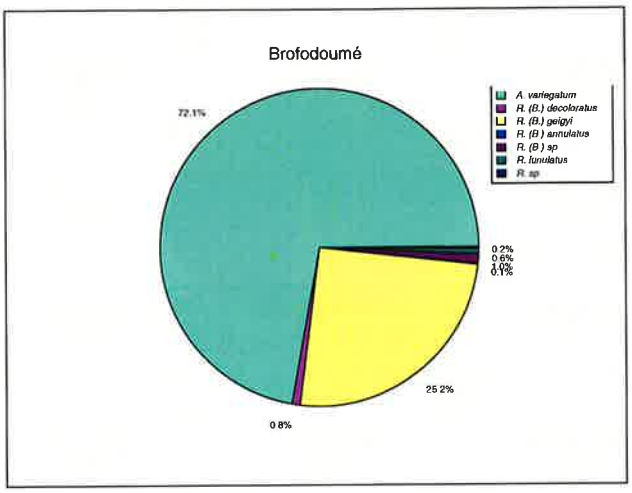

Figure 6.5: Distribution and frequency (%) of the diverse tick species present in Brofodoumé.

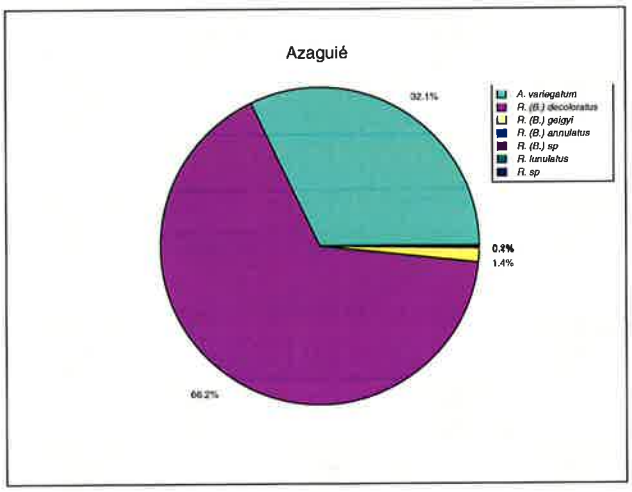

Figure 6.6: Distribution and frequency (%) of the diverse tick species present in Azaguié.

6.2.1 Tick infestation on individual cattle and herds of the different study sites

Infestation by *A. variegatum*

The mean tick infestation per herd was calculated by dividing the total count of *A. variegatum* individuals found on the half body on all cattle of a site by the total sample size (number of cattle analysed) for a given sampling time [109]. The average mean tick infestation was highest on cattle of the farm in Dabou (23.28) and Sikensi (21.65). Cattle in the farm of Brofodoumé had about half the mean infestation (12.61) whereas Azaguié had a very low mean infestation (5.26). The ranges of the mean tick infestation by *A. variegatum* per farm are summarized in Table 6.4. The highest tick number sampled in one go (per herd) at one time of the bimonthly samplings, was during February for Dabou (1319 individual ticks) and in January for Sikensi (1102 individual ticks) whereas the maximum number sampled for Brofodoumé was 430 and for Azaguié 210 ticks (Annexes A.1.3).

Table 6.4: Mean tick infestation per herd per sampling for *A. variegatum* on cattle in the different farms for the study period (Nov 05 - Oct 06); Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

	Me	Ca	Br	Az
Average	21.65	23.28	12.61	5.26
Max.	78.71	87.93	28.67	15.00
Min.	3.36	1.29	1.50	0.07

The infestation degree (ID) for a given cow was calculated by dividing the total half body count of *A. variegatum* of an individual animal by the mean infestation by *A. variegatum* of the farm at a given time [109]. This allowed to compare the infestation between individual cows (Annex A.1.3).
The average infestation degree (ID) for *A. variegatum* over the whole study period was 0.997, 1.012, 0.996 and 1.001 for Sikensi, Dabou, Brofodoumé and Azaguié, respectively.
The ID varied from 0 to 5.2 for Sikensi, from 0 to 4.6 for Dabou, from 0 to 5.5 for Brofodoumé and from 0 to 14.3 for Azaguié (Fig. 6.7).
The maximum of *Amblyomma* ticks counted on the half body of an individual cow at a specific sampling was of 223 in Sikensi, 251 in Dabou, 101 in Brofodoumé and 46 in Azaguié. In all four farms there were cattle which did not carry any ticks at some time of collecting, but none of the cattle stayed without ticks throughout the study period. In Azaguié six of the fifteen animals followed were calves younger

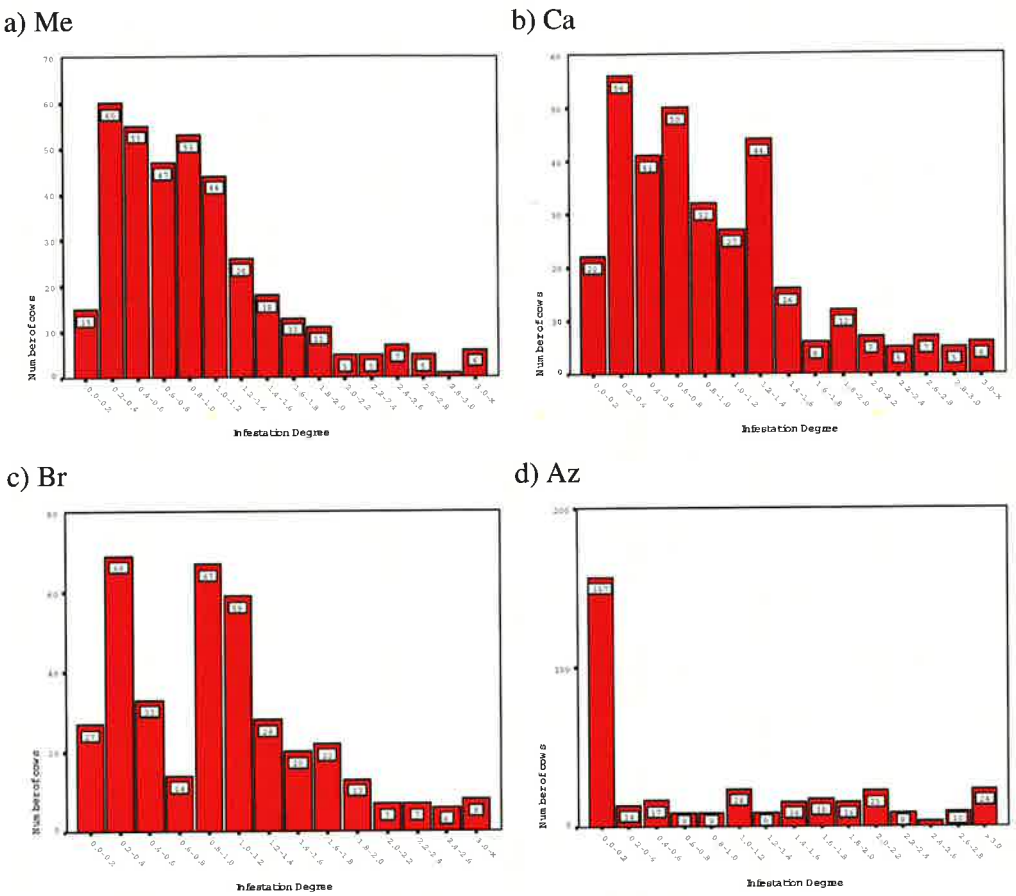

Figure 6.7: Infestation degrees for the four farms for *A. variegatum*. a) Sikensi (Me) b) Dabou (Ca) c) Brofodoumé (Br) d) Azaguié (Az) (for details see annex A.1.3).

than one year which did not or rarely go to the pasture and which were not or only lightly infested.

Infestation by *Rhipicephalus (B.)* sp

The mean tick infestation per herd was calculated by dividing the total count of *Rhipicephalus (B.)* sp individuals found on the half body on all cattle of a site by the total sample size (number of cattle analysed) for a given sampling time [109]. In the case of *Rhipicephalus (B.)* sp Azaguié was the farm with the highest mean tick infestation (11.47) followed by Sikensi (8.55) and Brofodoumé (4.74). As already mentioned earlier in this chapter, hardly any *Rhipicephalus (B.)* individuals were found in Dabou (0.17). The ranges of the mean tick infestation by *Rhipicephalus*

(B.) sp per farm are summarized (Table 6.5). The highest number of tick individuals sampled (per herd) at one time of the bimonthly samplings were collected during February in Azaguié, namely 595, during December in Sikensi (484) and in February (387) in Brofodoumé, whereas the highest number of *Rhipicephalus (B.)* ticks was 19 in Dabou (Annex A.1.3).

Table 6.5: Mean tick infestation per herd per sampling of *Rhipicephalus (B.)* sp on cattle in the different farms for the study period (Nov 05 - Oct 06); Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié (for details see annex A.1.3).

	Me	Ca	Br	Az
Average	8.55	0.17	4.74	11.47
Max.	48.40	1.90	25.80	39.67
Min.	0	0	0	0.53

The infestation degree (ID) for a given cow was calculated by dividing the total half body count for *Rhipicephalus (B.)* sp of an individual animal by the mean infestation by *Rhipicephalus (B.)* sp of the farm at a given time [109]. The average infestation degree (ID) for *Rhipicephalus (B.)* sp over the whole study period was 1.220 for Sikensi, 0.400 for Dabou, 0.958 for Brofodoumé and 0.937 for Azaguié (Annex A.1.3).
The ID varied from 0 to 71.4 for Sikensi, from 0 to 40.0 for Dabou, from 0 to 13.2 for Brofodoumé and from 0 to 14.3 for Azaguié (Fig. 6.8).

For *Rhipicephalus (B.)* the maximum tick count on the half body of a single cow at a specific sampling was of 135 for Sikensi, 12 for Dabou, 87 for Brofodoumé and 164 for Azaguié. There were cattle which did not carry any *Rhipicephalus (B.)* ticks at some time of collecting, but none stayed free of ticks throughout the study period. In Azaguié six of the fifteen animals followed were calves younger than one year which did not or rarely go to the pasture and which were not or only lightly infested.

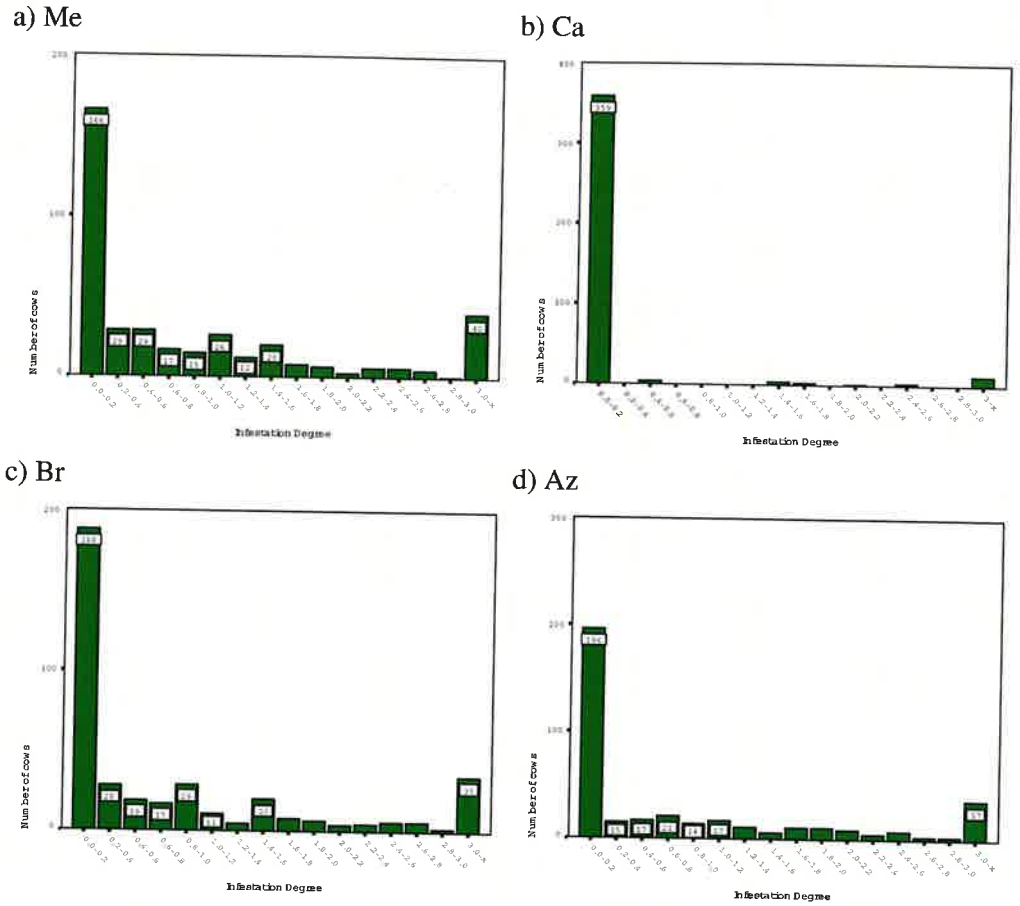

Figure 6.8: Infestation degrees for the different farms for *Rhipicephalus (B.)* sp. a) Sikensi (Me) b) Dabou (Ca) c) Brofodoumé (Br) d) Azaguié (Az)

6.3 Predilection sites of *A. variegatum*, *Rhipicephalus (B.)* sp and *R. lunulatus* on the body of the cattle.

A. variegatum ticks were mainly found in the following regions of the cattle: groin and udder, axillae, dewlap and abdomen. Less frequently, they could also be found on the hooves. Larvae were often observed on the head.

Rhipicephalus (B.) sp species were observed most frequently on dewlap, abdomen, head and axillae as well as to a lesser extend on groin and udder, axillae and hooves.

R. lunulatus was observed on head and between the hooves.

(Personal observation during sampling process; observation of Dr Louise Achi (data not shown).)

6.4 Seasonality of the diverse tick species.

ANOVA analyses were performed to test for a possible influence of the different seasons (months) as well as the local conditions of the different farms on the annual distribution and density of the tick species studied (Annex A.1.4).

The annual distribution of *A. variegatum* was similar for all four study sites (Fig. 6.9). *A. variegatum* ticks were present throughout the year with two intensity peaks during the study period. For all farms an increase in numbers could be observed before the beginning of the main rainy season, in February, and after the main rain season was finished, in August/September. In the case of Sikensi and Dabou, the number was much higher in February than August, whereas for Brofodoumé and Azaguié the two peaks are more or less similar for both months (Fig. 6.9).

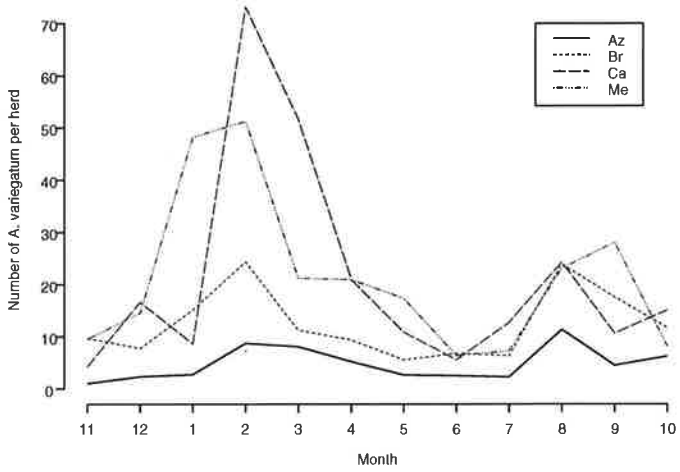

Figure 6.9: Annual distribution of *A. variegatum* in all study sites. Numbers represent the mean count of *A. variegatum* per herd per month. Me: Sikensi; Ca: Dabou; Br: Brofodoumé; Az: Azaguié.

All *Rhipicephalus (B.)* species were also present the whole year round. However, as already mentioned in the section before, not all *Rhipicephalus (B.)* species could be found in all study sites and the annual distribution is therefore discussed for the respective farm for each specific species.

The distribution of the *R. (B.) decoloratus* species followed the same distribution trend as *A. variegatum*. A high number occurred in the month of February and the

numbers raised again, though to a lesser extend, in August for the site Azaguié. In the other three farms the numbers of *R. (B.) decoloratus* were too low to observe any seasonal trend (Fig. 6.10).

In contrast, *R. (B.)geigyi* and *R. (B.) annulatus* showed more than two peaks in their annual distribution. For *R. (B.) geigyi* the main peak was observed in February, whereas smaller ones occurred in December and August in Brofodoumé. In Sikensi, an increase was seen around January and a second rise in May. After June *R. (B.) geigyi* was only found sporadically in Sikensi anymore. For Azaguié 86 ticks were collected over the time period of November to February. Afterwards, these ticks were not longer present on the animals (Fig. 6.11). None were found in Dabou.

R. (B.) annulatus was only present on one farm, Sikensi, where it was present the whole year round. Its highest peak occurred earlier in the year compared to the species discussed before, namely in January, and was followed by two smaller peaks in April and August (Fig. 6.12).

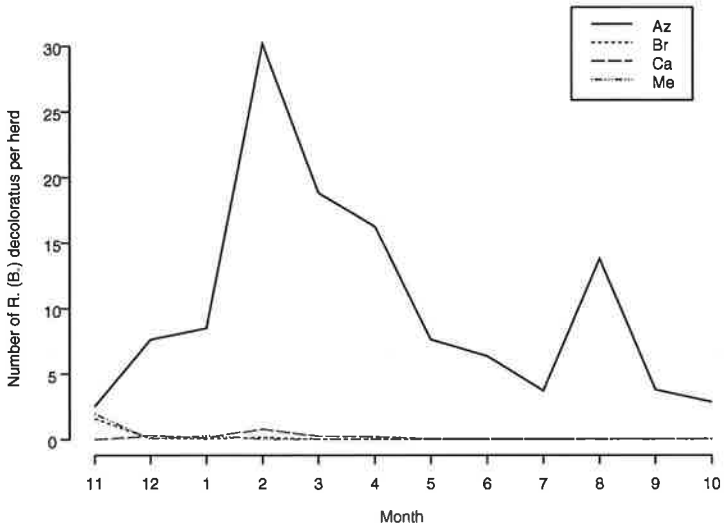

Figure 6.10: Annual distribution of *R. (B.) decoloratus* in all study sites. Numbers represent the mean count of *R. (B.) decoloratus* per herd per month. Me: Sikensi; Ca: Dabou; Br: Brofodoumé; Az: Azaguié.

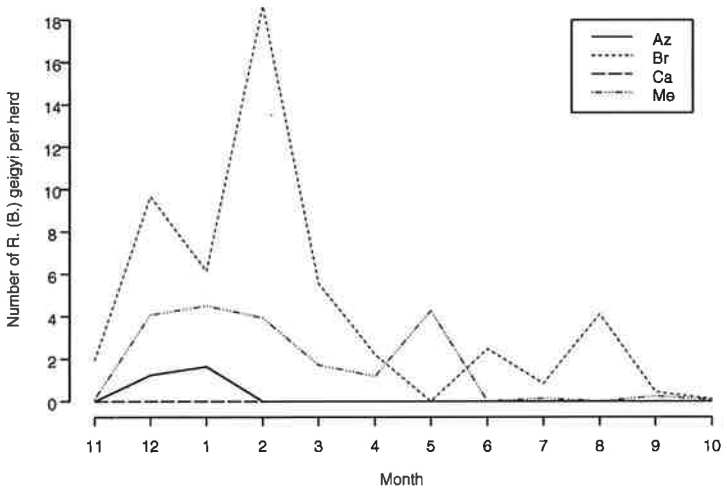

Figure 6.11: Annual distribution of *R. (B.) geigyi* in all study sites. Numbers represent the mean count of *R. (B.) geigyi* per herd per month. Me: Sikensi; Ca: Dabou; Br: Brofodoumé; Az: Azaguié.

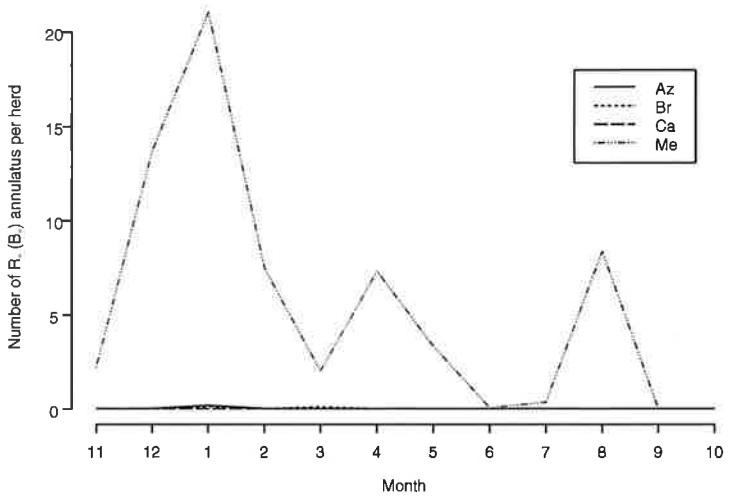

Figure 6.12: Annual distribution of *R. (B.) annulatus* in all study sites. Numbers represent the mean count of *R. (B.) annulatus* per herd per month. Me: Sikensi; Ca: Dabou; Br: Brofodoumé; Az: Azaguié.

In contrast to the *Amblyomma* and *Rhipicephalus (B.)* species being present throughout the year, *R. lunulatus* was not found over the whole study period. The first individuals were only found at the beginning of March. Only shortly after their first appearance, they already had their highest peak (April), whereas a rather small second increase was seen in August before *Rhipicephalus* ticks disappeared again from the diverse study sites. The species was not present on cattle of all sites during December, January and February of the study period (Fig. 6.13). However, in Dabou tick numbers were found to increase at the end of our study period, in October.

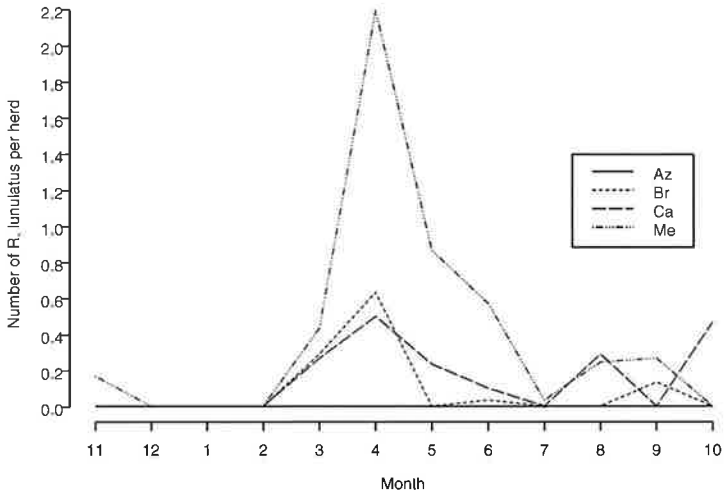

Figure 6.13: Annual distribution of *R. lunulatus* in all study sites. Numbers represent the mean count of *R. lunulatus* per herd per month. Me: Sikensi; Ca: Dabou; Br: Brofodoumé; Az: Azaguié.

Depending on climatic conditions and the biology of the ticks, adult and immature stages are often seen to follow an inverse trend in their annual distribution. This is why the seasonal distribution was analysed separately for the different stages, i.e. for male, female, nymph and larval stages, and for the different tick species found. In our study area, for *A. variegatum* species no trend for a successive occurrence of the diverse developmental stages could be observed. All stages were present simultaneously the whole year round. Adult male and female tick were observed year round in a more or less constant number and they did not show any real intensity peak at any time during our study period. The two increases in density are therefore mainly due to an increase in the number of nymphs and larvae during these months (Fig. 6.14). Only for Sikensi the increase of larval ticks occurred slightly earlier than the increase in nymphal ticks (Figure 6.15).

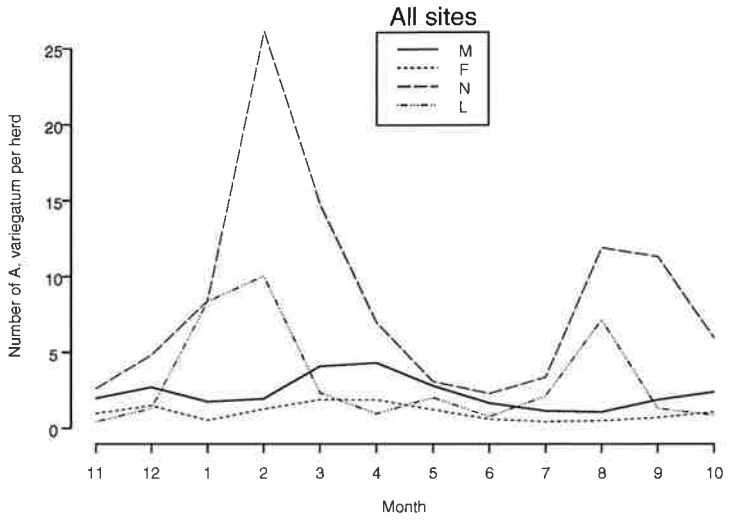

Figure 6.14: Annual distribution of different stages of *A. variegatum*. Numbers represent the mean count of *A. variegatum* per herd per month. M: male; F: female; N: nymph; L: larva.

R. (B.) decoloratus is the only *Rhipicephalus (B.)* species for which sufficient numbers for each developmental stage were found. The number of larva found (around 15 each) for *R. (B.) geigyi* and *R. (B.) annulatus* was too low to be further investigated.

It is in the nature of the life cycle of *R. (B.) decoloratus* that the different developmental stages did coexist. However, the larval stage had a single rise in their density during February and was more or less absent during the rest of the year. The other three stages were found throughout the year having their intensity peak also at the same time as the larval stage, though the nymphal intensity peak is extended towards March. The nymphal stage showed a second small increase in August as do the adult stages. Both males and females had an additional third increase in their density during April (Fig. 6.16). See annex A.1.4 for individual farms.

A similar distribution was observed for the other two *Rhipicephalus (B.)* species (Fig. 6.17, Fig. 6.18). See annex A.1.4 for individual farms.

In the case of *R. lunulatus*, no immature stage was found. Therefore, it is unknown at which time point they can be found on their rodent hosts and if larva and nymph will be present simultaneously or in succession to the adult stages (Fig. 6.19).

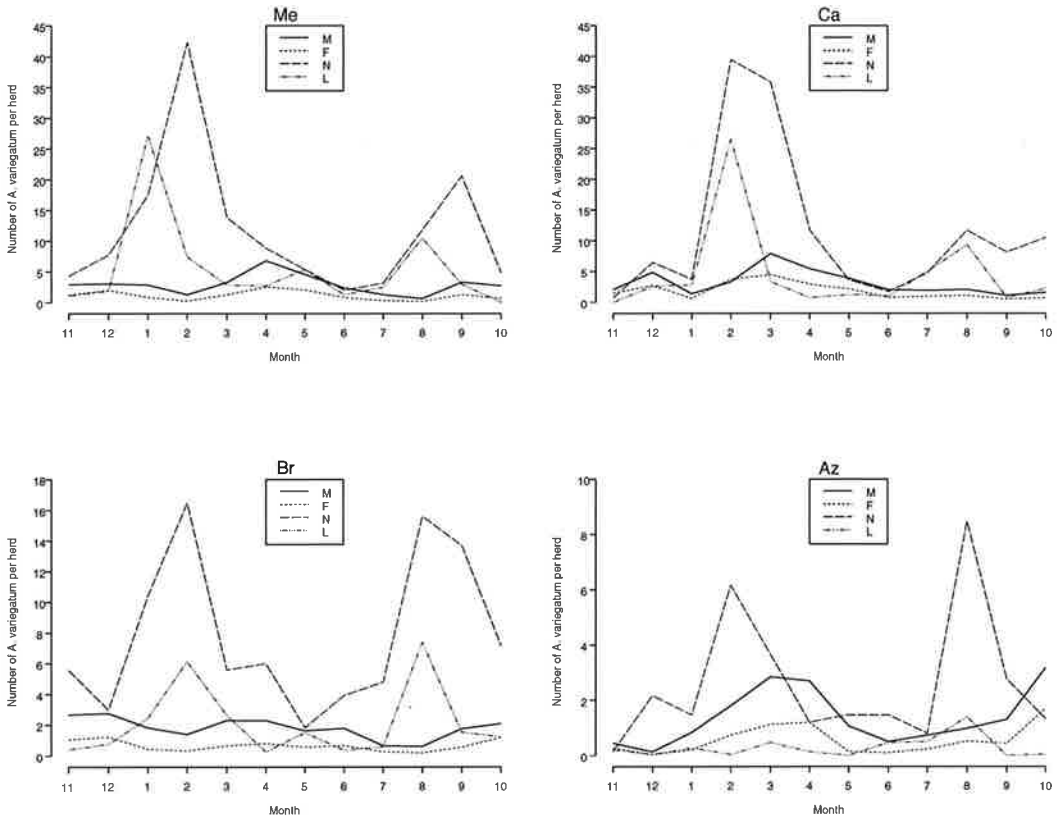

Figure 6.15: Annual distribution of the different developmental stages of *A. variegatum* (M: male; F: female; N: nymph; L: larvae) for the four sites Sikensi (Me), Dabou (Ca), Brofodoumé (Br) and Azaguié (Az). Numbers represent the mean count of *A. variegatum* per herd per month.

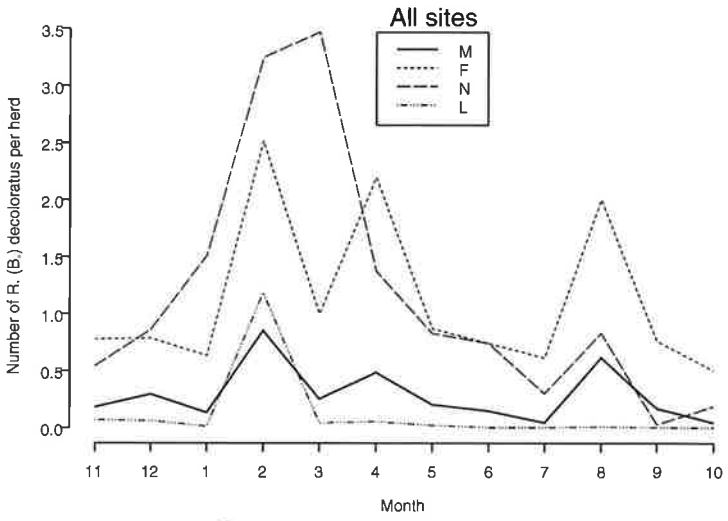

Figure 6.16: Annual distribution of different stages of *R. (B.) decoloratus*. Numbers represent the mean count of *R. (B.) decoloratus* per herd per month. M: male; F: female; N: nymph; L: larva.

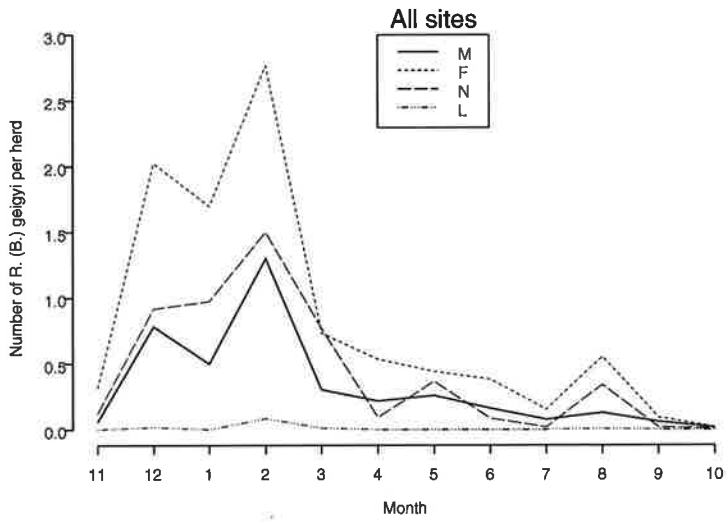

Figure 6.17: Annual distribution of different stages of *R. (B.) geigyi*. Numbers represent the mean count of *R. (B.) geigyi* per herd per month. M: male; F: female; N: nymph; L: larva.

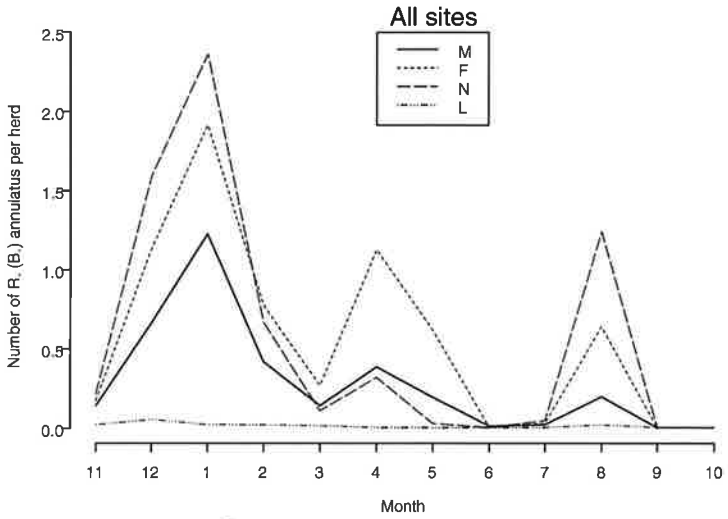

Figure 6.18: Annual distribution of different stages of *R. (B.) annulatus*. Numbers represent the mean count of *R. (B.) annulatus* per herd per month. M: male; F: female; N: nymph; L: larva.

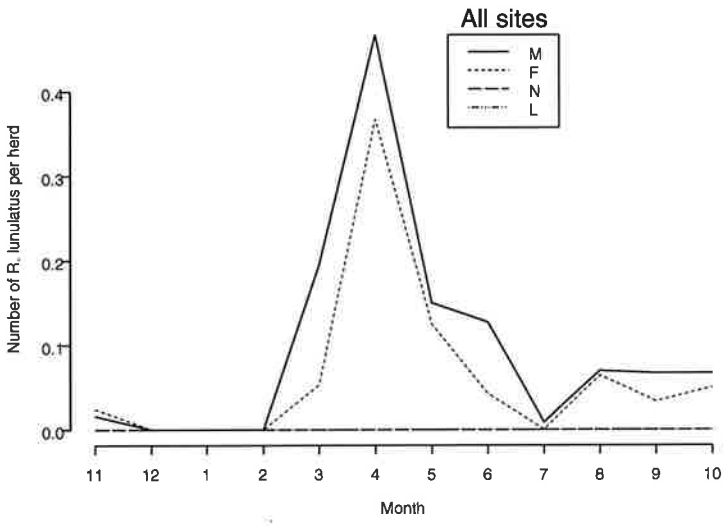

Figure 6.19: Annual distribution of different stages of *R. lunulatus*. Numbers represent the mean count of *R. lunulatus* per herd per month. M: male; F: female; N: nymph; L: larva.

6.4.1 Acaricide treatment schedule

All cattle of the herds included in the study were treated with diverse acaricides. Normally, the herds were treated monthly. During some selected time points of the study period additional treatments were undertaken to decrease the tick burden. However, whereas the farms in Dabou and Azaguié were following a strict treatment schedule, the farm Brofodoumé treated their cattle irregularly depending upon the availability of acaricide products and also the farm in Sikensi was treating their animals on an irregular basis. Since the treatment schedules were not standardized for the four farms, differences existed not only in the frequency of product application, but also in the acaricide products used. However, at the study start all four farms were treating their animals with alphacypermethrin (Dominex). Following products were used by the farmers:

- Farm Mesics in Sikensi:
  Alphacypermethrin (Dominex), amitraz (Antitic), delthamethrin (DeltaVet).
- Farm Calys in Dabou:
  Alphacypermethrin (Dominex), amitraz (Antitic, Tick-Net 100).
- Farm in Brofodoumé:
  Alphacypermethrin (Dominex), amitraz (Antitic, Abotic).
- Farm in Azaguié:
  Alphacypermethrin (Dominex), amitraz (Antitic), deltamethrin (DeltaTick).

Generally spoken the current treatment does not seem to influence the presence of the two tick species studied, i.e. *A. variegatum* and *Rhipicephalus (B.)* sp. However, in Dabou no specimens of *Rhipicephalus (B.)* sp can be found and Azaguié shows a significant lower number of *A. variegatum* whereas the number of *Rhipicephalus (B.)* sp is higher than in the other three farms.

When asked the farm owners did not think their acaricide treatment to be efficient. Shortly after the treatment the cattle were found to be mostly free of ticks, but apparently there is no longterm effect of the acaricide.

Chapter 7

Longitudinal study of the presence of tick-borne parasites in the blood of cattle raised in the southern region of the Ivory Coast

7.1 Prevalence of tick-borne parasites in bovine samples using a reverse line blot (RLB) assay

7.1.1 Prevalence of the parasites present in the Southern region of the Ivory Coast

Blood samples were collected monthly from cattle in the Southern area of the Ivory Coast from November 2005 to October 2006. For this purpose, four herds located on different farms were selected prior to the study start. Each of the four herds followed was represented by 15 individual cows and the blood was analysed simultaneously for the presence of diverse tick-borne blood parasites belonging either to the group *Anaplasma* and *Ehrlichia* (AE) or *Babesia* and *Theileria* (BT) with a reverse line blot method.

In total 707 blood samples were analysed, whereof 147 samples were negative and 560 samples positive by RLB. Out of the 560 positives, 501 samples were positive for either AE or BT. Out of these only nine were positive for AE in comparison to 492 positives for BT. Only 59 samples were positive for both catch-all.

The following parasites could be detected in the blood samples by RLB in the order of their frequency (number of positives per total number of samples analysed):
T. mutans (74.12 %), *T. velifera* (66.90 %), *B. bigemina* (9.76 %), *B. bovis* (8.06

%), *A. marginale* (2.26 %), *Ehrlichia* sp (1.69 %) (Table 7.1; Annex A.2.2). None of the samples reacted positive neither for *E. ruminantium*, *A. centrale* nor *E. bovis*. Though at least *E. ruminantium* is known to be present in the Ivory Coast.

Table 7.1: Distribution and prevalence (%) of tick-borne parasites in the blood of cattle in the Southern area of the Ivory Coast.

Parasite	Positive[a] Samples	Negative[b] Samples	Total Number[c] of Samples	Prevalence %
Catch-all AE	68	639	707	9.62
A. phagocytophilum	0	707	707	0
E. ruminantium	0	707	707	0
A. marginale	16	691	707	2.26
A. centrale	0	707	707	0
A. bovis	0	707	707	0
Ehrlichia sp	12	695	707	1.69
Catch-all BT	551	156	707	77.93
B. bovis	57	650	707	8.06
B. bigemina	69	638	707	9.76
B. divergens	0	706	707	0
T. annulata	0	707	707	0
T. parva	0	707	707	0
T. mutans	524	183	707	74.12
T. velifera	473	233	707	66.90

[a]Number of blood samples testing positive by RLB analyses
[b]Number of blood samples testing negative by RLB analyses
[c]Total number of blood samples analysed by RLB

7.1.2 Single & mixed infection

Overall 560/707 blood samples were infected with at least one parasite, i.e. 79.2 % of the blood samples analysed carried a parasite. The majority (97%) of this samples were infected mainly with *T. mutans* and/or *T. velifera*, whereas all other pathogens were present only in smaller numbers. Single and mixed infections are listed separately for *T. mutans* and *T. velifera*, which are considered to be not or mildly pathogenic to cattle, and for the remaining parasites, which are pathogenic to cattle (Tables 7.2, 7.3).
Although, the different pathogens could be found at the same time in the blood samples analysed, more often we were dealing with single infections. Only 18/131 infected samples carried mixed infections (Table 7.2).
In contrast, *T. mutans* and *T. velifera* were mostly found together in the same animal (Table 7.3).

Table 7.2: Single and mixed infections without *T. mutans* and *T. velifera* in the blood of cattle.

Single infections [a]	*(# of cases)*	*Mixed infections*	*(# of cases)*
A. marginale	8	*A. marginale & B. bovis*	3
E. sp	9	*A. marginale & B. bigemina*	4
B. bovis	45	*A. marginale & E. sp*	1
B. bigemina	69	*B. bovis & B. bigemina*	9
		A. marginale & Esp & Bbov	1
Total single infections	**131**	**Total mixed infections**	**18**

[a]Infections with *T. mutans* and *T. velifera* were not considered given their high prevalence

Table 7.3: Single and mixed infections for the *Theileria* species in the blood of cattle.

Infections with T. mutans and T. velifera	*(# of cases)*
T. mutans	70
T. velifera	20
T. mutans & T. velifera	454
Neither *T. mutans* nor *T. velifera*	163
Total samples infected with *T. mutans* and/or *T. velifera*	**544**

Coinfections of the various parasites could be observed in all possible combinations, when all parasites were considered together (see annex A.2.2 for details). It is not possible to describe in full length the number and combinations of all in this context. However, the figures in Table 7.4 may give an idea of how often and how many different parasites could be found to be present simultaneously in the blood samples analysed.

Table 7.4: Frequency of single and mixed infections in blood sample of cattle.

Number of different parasites	Number of cases [a]
5	3
4	24
3	109
2	334
1	79
0	146

[a]The 12 samples which reacted not with any species-specific probe were not considered

7.1.3 Clinical symptoms and other (not tick-borne) diseases

The following clinical symptoms were observed during the study period: Fever, anorexia, death, death with nervous signs, apathy, skinny animals, hypothermia, cough, dermatophilosis, subcutaneous knots, further trypanosomosis and infestation with strongles and coccidies (observations of clinical symptoms and blood smears by Dr L. Achi).

During the study period, five mortalities were reported. One animal died by accident, one died after showing clinical symptoms and three animals died after showing nervous signs. Unfortunately, those did not participate in the study. For the cattle showing clinical symptoms, one infection by *B. bigemina* and *T. mutans* could be detected by RLB.
In addition, three additional animals were reported to show clinical symptoms of tick-borne diseases. In all three cases diagnosis by RLB showed the presence of *B. bovis* and therefore confirmed the clinical symptoms (Table 7.5).

Twelve cows were infected by Dermatophilosis or showed subcutaneous knots (field observation). Only three of these had an infection by either *B. bovis* and/or *B. bigemina* (Table 7.5). Another 16 animals were diagnosed with Trypanosomosis (blood smears). Out of these, five animals were coinfected by tick-borne parasites. All findings are summarized in Table 7.6.

In general, animals of the farm Sikensi were reported to be in bad health and animals were skinny. In April, all cattle of the farm Dabou had to be treated against trypanosomosis, since a majority of the herd was infected.

Table 7.5: Reported cattle with noteworthy clinical signs and other diseases. The following abbreviations are used for the parasites: *B.bovis*, Bbov; *B. bigemina*, Bbig; *T. mutans*, Tm; *T. velifera*, Tv.

No	Farm	Month	Clinical symptoms	Diagnosis (RLB)	Hematocrite
71	Ca	07	Death by accident		
12	Me	06	Death	Bbig, Tm	44
62	Az	07	Anorexia, Dermatoph.	Bbov, Tv,	36
6	Me	08	Fever	Bbov, Bbig, Tm, Tv	37
8	Me	01	Apathy, hypothermia	Bbov, Tm, Tv	40
35	Br	05	Dermatophilosis	Tm, Tv	36
32	Br	06	Dermatophilosis	Tm, Tv	42
24	Ca	08	Dermatophilosis	Tm, Tv	45
30	Ca	08	Dermatophilosis	Tm, Tv,	40
72	Ca	08	Dermatophilosis	Tm, Tv	35
5	Me	07	Dermatophilosis	Bbig, Tm, Tv	36
24	Ca	07	Dermatophilosis	no parasites	30
74	Ca	07	Dermatophilosis	no parasites	44
44	Br	06	Subcutaneous knots	no parasites	34
69	Me	06	Subcutaneous knots	Bbov, Bbig, Tm, Tv	39
46	Az	05	Subcutaneous knots	Tm, Tv	44

Table 7.6: Reported cattle with Trypanosomosis. The following abbreviations are used for the parasites: *A. marginale*, Am; *B.bovis*, Bbov; *B. bigemina*, Bbig; *T. mutans*, Tm; *T. velifera*, Tv.

No	Farm	Month	Blood smears	Diagnosis (RLB)	Hematocrite
6	Me	9	*T. brucei*	Bbig, Tm, Tv	24
11	Me	10	*T. brucei*	Bbig, Tm, Tv	34
4	Me	9	*T. brucei*	Am, , Tv	
13	Me	9	*T. brucei*	Am, Bbig, Tm, Tv	32
4	Me	10	*T. brucei*	Am, Bbov, Tm, Tv	27
7	Me	10	*T. brucei*	Tm, Tv	38
2	Me	11	*T. brucei*	Tm, Tv	49
6	Me	11	*T. brucei*, *T. congolense*	no parasites	42
5	Me	8	*Trypanosoma* sp	Tm, Tv	37
71	Ca	5	*Trypanosoma* sp	Tm,	38
37	Br	7	*Trypanosoma* sp	Tm, Tv	30
39	Br	7	*Trypanosoma* sp	Tm, Tv	34
64	Br	7	*Trypanosoma* sp	Tm, Tv	37
98	Br	7	*Trypanosoma* sp	Tm, Tv	35
52	Az	7	*Trypanosoma* sp	Tm, Tv	33
60	Az	7	*Trypanosoma* sp	Tm, Tv	29

Every time blood samples were taken, the hematocrite levels were analysed for individual animals. The hematocrite levels varied from 13 to 58 (mean=36.35, SD=7.12) for Sikensi, from 10 to 63 (mean=39.18, SD=7.36) for Dabou, from 26 to 55 (mean=37.46, SD=5.3) for Brofodoumé and from 25 to 62 (mean=36.58, SD=6.67) for Azaguié, respectively. In 13 (Me), 21 (Ca), 15 (Br) and 14 (Az) cases the hematocrite levels were above 46 and in 7 (Me) and 4 (Ca) cases the hematocrite was below 24. All other measurements were situated somewhere in the normal range between 24 and 46.

7.1.4 Prevalence of the diverse parasites in the different study sites

The Pearsons's Chi-square test was used to test, if the various parasites were equally present in all four study sites or if local differences occurred among Sikensi, Dabou, Brofodoumé and Azaguié (Annex A.2.3).
Regarding the samples reacting positive with the catch-all probe for AE there was a strong presence of parasites in the sites of Sikensi (12.92 %) and Brofodoumé (17.78 %), and a significantly lower presence in Azaguié (5.2 %) and Dabou (2.26 %)(Chi = 30.85, p value of 0).

The positives reacting with the catch-all probe for BT were more numerous and were also more equally distributed among the different farms. Although they had nevertheless a higher presence in some farms (Chi = 15.62, p value of 0.0014). For details see Table 7.7.

Table 7.7: Distribution and prevalence (%) of the different bovine parasites in the four study sites Sikensi, Dabou, Brofodoumé and Azaguié

	Sikensi	Dabou	Brofodoumé	Azaguié
Catch-all AE	12.92	2.26	17.78	5.2
A. phagocytophilum	0	0	0	0
E. ruminantium	0	0	0	0
A. marginale	2.81	0	6.11	0
A. centrale	0	0	0	0
A. bovis	0	0	0	0
Ehrlichia sp	0.56	0	2.22	4.05
Catch-all BT	80.34	79.66	83.89	67.05
B. bovis	16.85	1.1	8.33	5.78
B. bigemina	18.54	0	20.0	0
T. mutans	78.10	76.8	80.56	60.12
T. velifera	69.66	67.23	78.33	51.45

Table 7.8: Distribution and total number of positives found in cattle in the four study sites Sikensi, Dabou, Brofodoumé and Azaguié

	Sikensi n=178	Dabou n=177	Brofodoumé n=180	Azaguié n=173
Catch-all AE	23	4	32	9
A. phagocytophilum	0	0	0	0
E. ruminantium	0	0	0	0
A. marginale	5	0	11	0
A. centrale	0	0	0	0
A. bovis	0	0	0	0
Ehrlichia sp	1	0	4	7
Catch-all BT	143	141	151	116
B. bovis	30	2	15	10
B. bigemina	33	0	36	0
T. mutans	139	136	145	104
T. velifera	124	119	141	89

Within the group AE, both *A. marginale* and *Ehrlichia sp* were not present equally in all four study sites (*A. marginale* Chi = 20.37, p value of 0.0001 ; *Ehrlichia* sp Chi = 10.53, p value of 0.014). *A. marginale* could only be detected in Sikensi (2.81 %) and Brofodoumé (6.11 %), whereas on the other hand, *Ehrlichia* sp was detected in all farms except Dabou, with 7 cases in Azaguié, 4 in Brofodoumé and 1 in Sikensi. Great variations existed also in the distribution of the various parasites among the four study sites inside the protozoan group BT. Although *B. bovis* was detectable in all sites, it nevertheless had a very low prevalence in Dabou (1.1 %) and a high presence in Sikensi (16.85 %), compared to Brofodoumé (8.33 %) and Azaguié (5.78 %) (Chi = 31.22, p value of 0). In contrast, *B. bigemina* could not be detected in all sites. It was absent in Azaguié and Dabou (Chi = 74.75, p value of 0), but was equally present in the two other sites Sikensi (18.54 %) and Brofodoumé (20.0 %).

T. mutans and *T. velifera* were present in all four farms in very high numbers (*T. mutans*: Chi = 22.76, p value of 0; *T. velifera*: Chi = 27.80, p value of 0). However, in Azaguié the prevalence for both parasites were slightly lower compared to the other three sites.

7.1.5 Seasonal distribution of the tick-borne parasites in cattle

The Pearson's Chi-square test was used to detect a possible difference in the abundance of the diverse parasites during the study year. Unfortunately, the overall prevalence of the individual parasites was too low to detect variations in their seasonal distribution for the different study sites (Annex A.2.3).
To get nevertheless an idea of the seasonal variations, the distribution of the positives detected by the two catch-all probes AE and BT were plotted for the study year (November 2005 to October 2006) for each study site (Figures 7.1 - 7.4).

The graphs show the seasonal distribution for positives with the catch-all probes for AE and BT for the different farms. The distribution of AE shows higher numbers in December and July in Sikensi. In Dabou cases were higher in January and then disappeared for the rest of the time. In Brofodoumé cases were higher in February and August and in Azaguié a peak can be seen in July.
The positives for the catch-all BT were generally spoken more equally distributed over the year. In Sikensi numbers were lower in November and February. In Dabou numbers were also low in November and February. In Brofodoumé numbers were low in December. In contrast, numbers were lower in Azaguié at the beginning of the study and rised afterwards.
The parasite distribution following each month of the year is indicated for every single study site, Sikensi, Dabou, Brofodoumé and Azaguié, respectively in Table 7.9 - 7.12. However, the number of positives is to low to indicate clear peaks in the distribution over the year.

Analyses of individual parasites for differences in their annual distribution could not be done correctly due to the low prevalences (Annex A.1.3). When the study area was analysed as a whole, the samples testing positive for the catch-all probe of diverse *Anaplasma/Ehrlichia* species, showed a variation in the distribution over the study period. Numbers were higher during January and July (Table 7.13). The positives reacting with the catch-all probe for BT showed a significant difference in their distribution over the study period (Chi = 48.56, p value of 0.0) (Table 7.13).
The distribution of *A. marginale* showed no difference between the months. Similarly, *Ehrlichia sp* was equally dispersed over the whole study period. However in both cases higher numbers are found in the period from June to October.
On the other hand, seasonal variations could be observed for *Babesia bovis*, whereas *B. bigemina* showed a equal distribution over all 12 months. *T. mutans* (Chi = 52.47, p value of 0) and *T. velifera* (Chi = 38.45, p value of 0) showed statistically significant variations in their distribution over the study period.

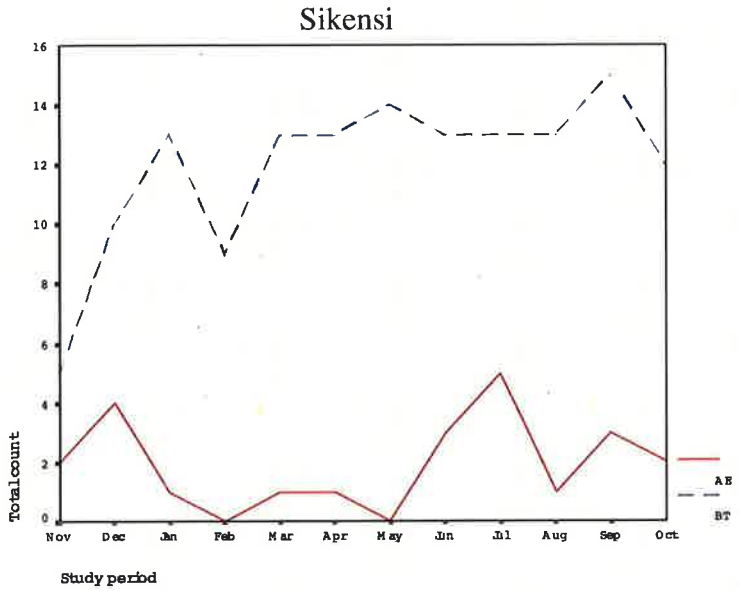

Figure 7.1: Graph showing the seasonal distribution of samples reacting positive with catch-all probe for AE and BT for the site Sikensi

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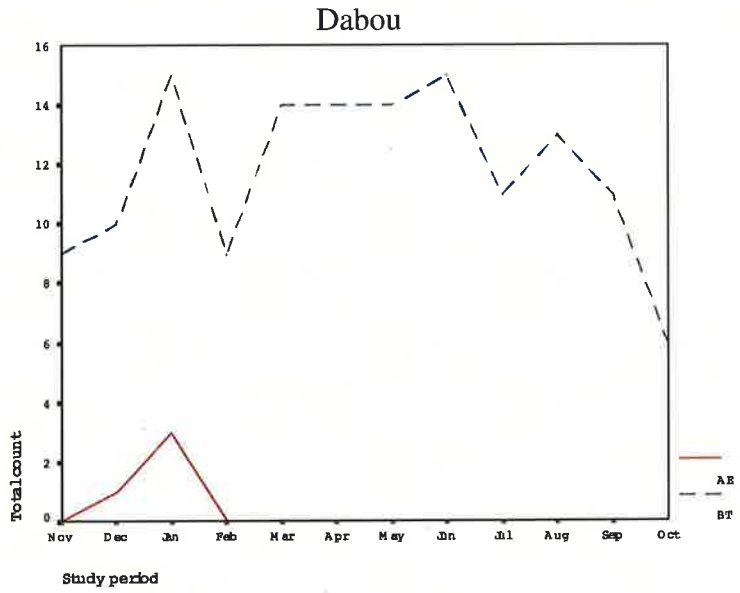

Figure 7.2: Graph showing the seasonal distribution of samples reacting positive with catch-all probe for AE and BT for the site Dabou

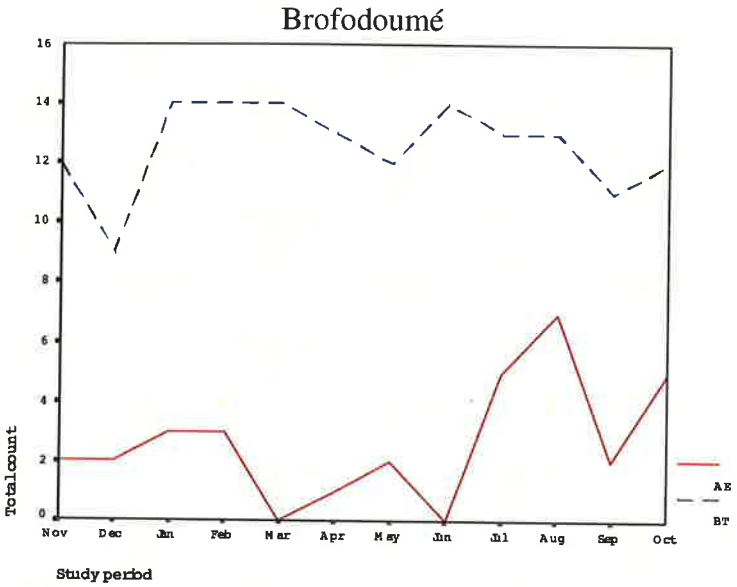

Figure 7.3: Graph showing the seasonal distribution of samples reacting positive with catch-all probe for AE and BT for the site Brofodoumé

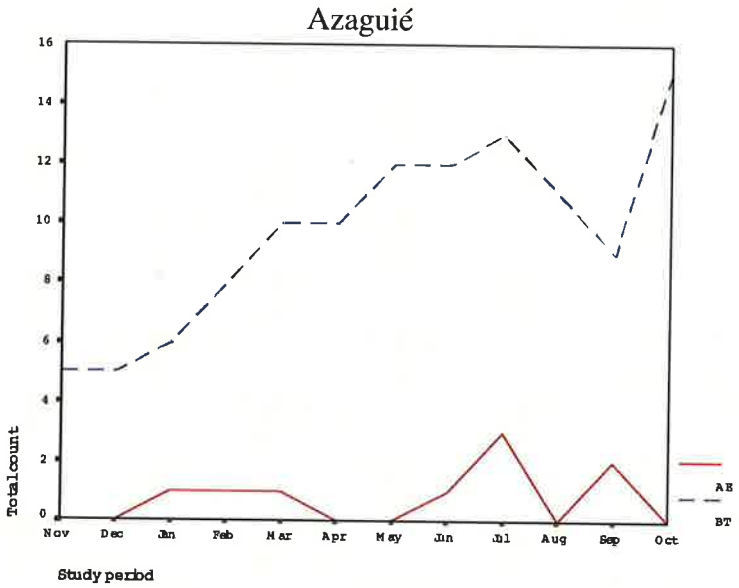

Figure 7.4: Graph showing the seasonal distribution of samples reacting positive with catch-all probe for AE and BT for the site Azaguié

Table 7.9: Distribution of the different parasites over the study period in Sikensi.

	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Catch-all AE	2	4	1	0	1	1	0	3	5	1	3	2
A. marginale	0	0	0	0	0	0	0	1	1	0	2	1
Ehrlichia sp	0	0	0	0	0	0	0	0	1	0	0	0
Catch-all BT	5	10	13	9	13	13	14	13	13	13	15	12
B. bovis	0	2	3	0	2	2	2	3	2	3	5	6
B. bigemina	1	2	2	0	3	3	3	5	5	3	3	3
T. mutans	4	10	12	9	12	13	14	13	13	14	13	12
T. velifera	4	9	12	6	10	10	11	13	12	13	13	11

Table 7.10: Distribution of the different parasites over the study period in Dabou.

	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Catch-all AE	0	1	3	0	0	0	0	0	0	0	0	0
A. marginale	0	0	0	0	0	0	0	0	0	0	0	0
Ehrlichia sp	0	0	0	0	0	0	0	0	0	0	0	0
Catch-all BT	9	10	15	9	14	14	14	15	11	13	11	6
B. bovis	0	1	0	0	0	0	0	0	1	0	0	0
B. bigemina	0	0	0	0	0	0	0	0	0	0	0	0
T. mutans	8	10	15	9	13	14	14	15	10	13	9	6
T. velifera	5	9	12	7	12	10	12	14	11	11	10	6

Table 7.11: Distribution of the different parasites over the study period in Brofodoumé.

	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Catch-all AE	2	2	3	3	0	1	2	0	5	7	2	5
A. marginale	1	1	2	1	0	1	0	0	1	2	0	2
Ehrlichia sp	0	0	1	0	0	0	0	0	0	2	0	1
Catch-all BT	12	9	14	14	14	13	12	14	13	13	11	12
B. bovis	0	1	0	0	0	0	1	2	1	4	6	0
B. bigemina	4	1	2	3	8	1	4	4	3	2	3	1
T. mutans	11	7	14	14	14	13	10	14	13	13	10	12
T. velifera	11	9	13	13	13	12	10	14	12	12	10	12

Table 7.12: Distribution of the different parasites over the study period in Azaguié.

	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Catch-all AE	0	0	1	1	1	0	0	1	3	0	2	0
A. marginale	0	0	0	0	0	0	0	0	0	0	0	0
Ehrlichia sp	0	0	1	0	0	0	0	1	3	0	2	0
Catch-all BT	5	5	6	8	10	10	12	12	13	11	9	15
B. bovis	0	0	0	0	0	0	0	1	2	0	0	7
B. bigemina	0	0	0	0	0	0	0	0	0	0	0	0
T. mutans	3	5	5	7	9	10	11	11	11	10	8	14
T. velifera	4	3	5	7	10	9	10	9	10	7	6	10

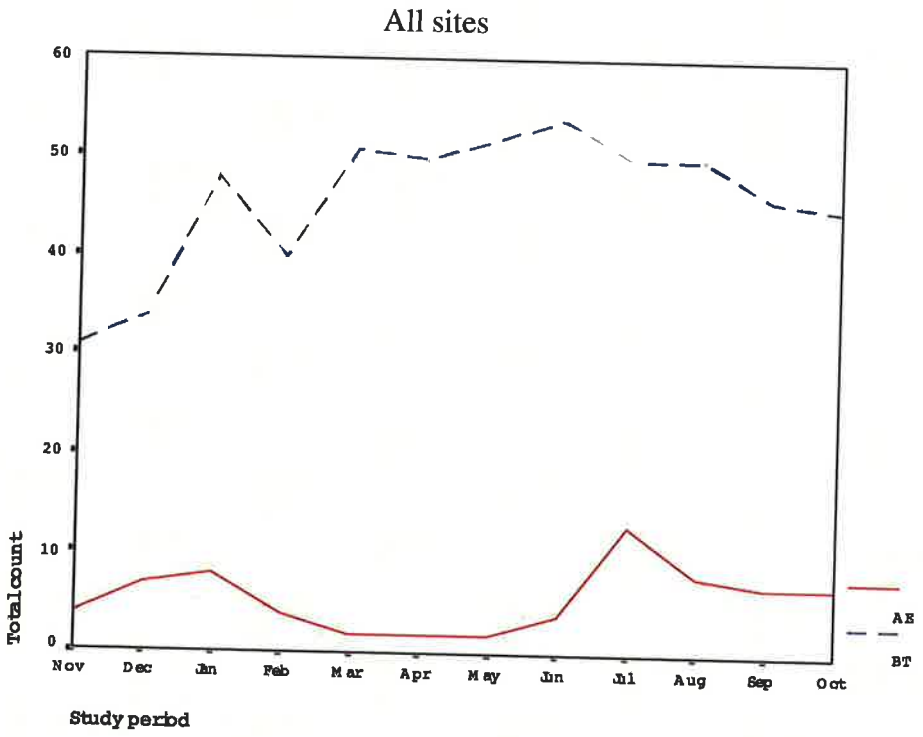

Figure 7.5: Graph showing the seasonal distribution of bovine samples reacting positive with catch-all probe for AE and BT for the study area

Table 7.13: Distribution of the parasites over the study period. Numbers are representing number of positive samples found.

	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Catch-all AE	4	7	8	4	2	2	2	4	13	8	7	7
E. ruminantium	0	0	0	0	0	0	0	0	0	0	0	0
A. marginale	1	1	2	1	0	1	0	1	2	2	2	3
A. centrale	0	0	0	0	0	0	0	0	0	0	0	0
Ehrlichia sp	0	0	2	0	0	0	0	1	4	2	2	1
Catch-all BT	31	34	48	40	51	50	52	54	50	50	46	45
B. bovis	0	4	3	0	2	2	3	6	6	7	11	13
B. bigemina	5	3	4	3	11	4	7	9	8	5	6	4
T. mutans	26	32	46	39	48	50	49	53	47	49	41	44
T. velifera	24	30	42	33	45	41	43	50	45	43	39	39

7.1.6 Course of the detection of pathogens in the blood of cattle

In general a selected number of 15 animals were followed for each study site for the duration of one year. However, in practise, some of the cattle had to be replaced during the study time due to their aggressiveness, because they were sold or died.
This is the attempt to examine, how the infections were progressing during the study year for individual animals. It was focused on the major disease causing pathogens, *B. bovis* and *B. bigemina* in the group BT and *A. marginale* in the group AE. However, since it was difficult to detect infection with *A. marginale* and a high number of samples reacted only with the catch-all probe AE, the infection course was studied with the samples hybridizing with the catch-all probe for AE.
Positive and negative blood results are shown graphically for 15 cows each for the sites Sikensi and Brofodoumé. The two farms have been selected for the presence of a greater variety of parasites in these sites compared to Dabou and Azaguié.

Infection course for cattle in the four study sites

Diagrams showing the infection course of *B. bovis*, *B. bigemina* and *A. marginale* (represented by AE) in 15 animals of the two sites Sikensi and Brofodoumé.
It is impossible to say, whether in the case of several detections of a given pathogen, this is the same infection circulating in the blood of the cow or if the cow actually got reinfected. However, often a cow did test recurrently positive for a given pathogen.
Graphs 7.6 and 7.7 illustrate infection with BT and graphs 7.8 and 7.9 aim to illustrate infection with AE.

Sikensi

For the study site Sikensi, a total of 20 different animals were followed in the study year, with 15 cows being sampled at each visit.
In the case of infection with one of the protozoan organisms *B. bovis* and *B. bigemina*, only a single cattle did not get infected with any of the two protozoa. The other

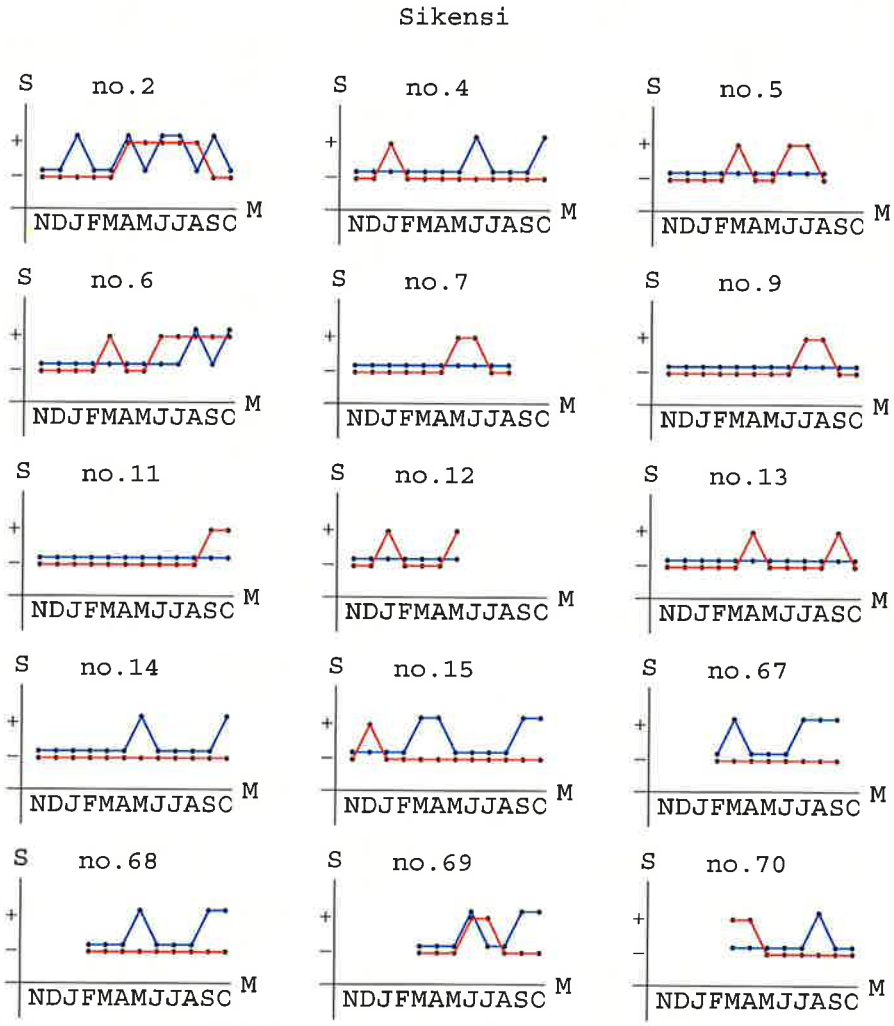

Figure 7.6: Infection course for *B. bovis* (red lines) and *B. bigemina* (blue lines) is shown for individual cows of the farm Sikensi. Numbers represent the individual cows. A positive blood test is indicated by + and a negative blood test by −.

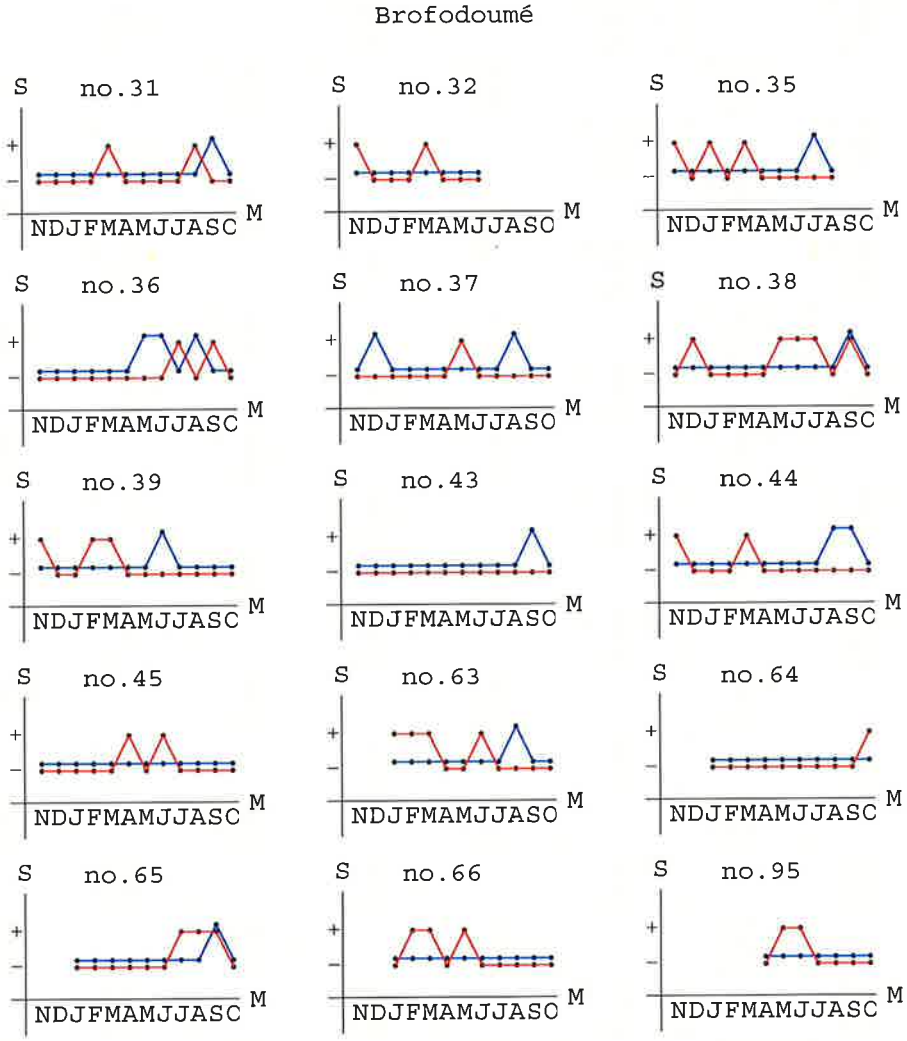

Figure 7.7: Infection course for *B. bovis* (red lines) and *B. bigemina* (blue lines) is shown for individual cows of the farm Brofodoumé. Numbers represent the individual cows. A positive blood test is indicated by + and a negative blood test by –.

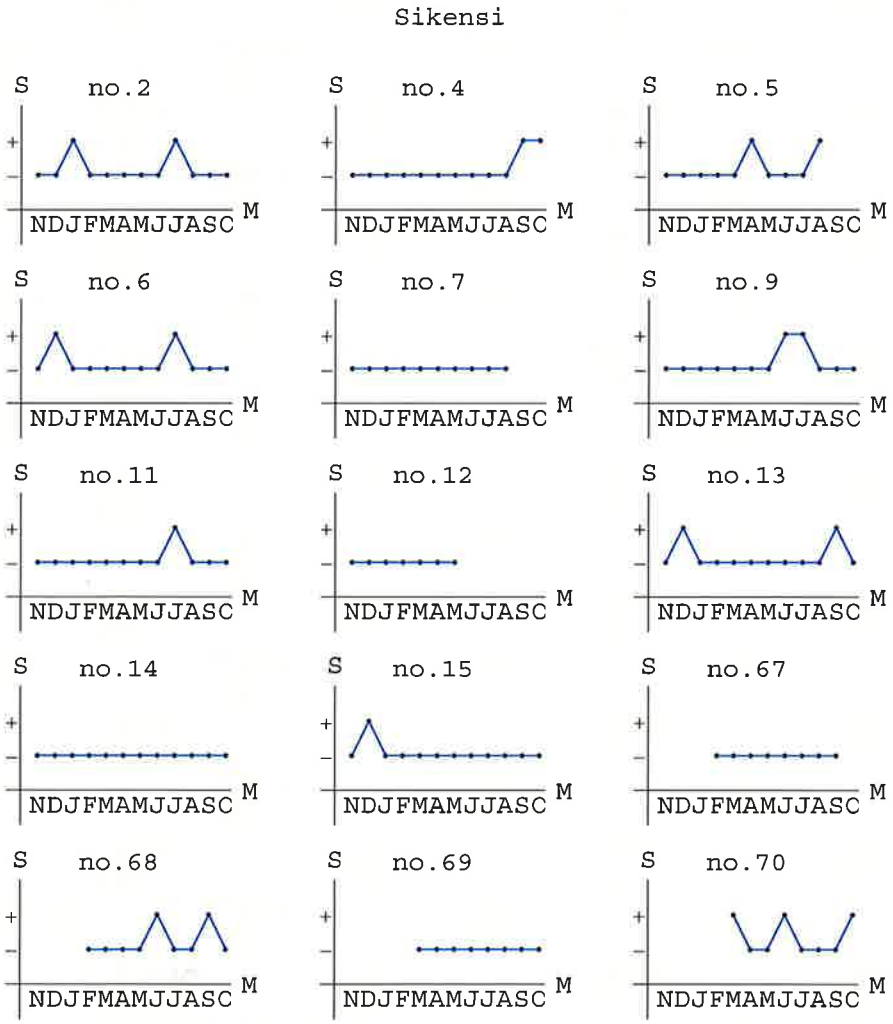

Figure 7.8: Infection course for the group *Anaplasma* and *Ehrlichia* (blue lines) is shown for individual cows of the farm Sikensi. Numbers represent the individual cows. A positive blood test is indicated by + and a negative blood test by −.

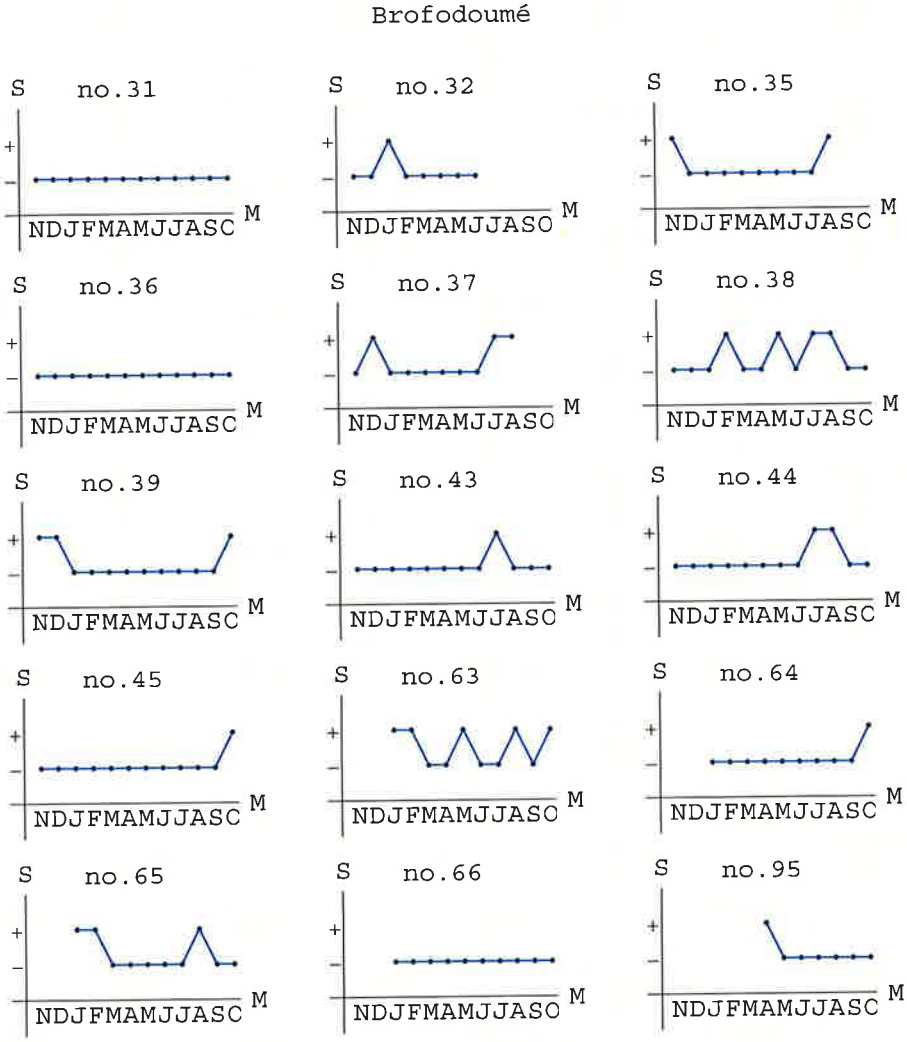

Figure 7.9: Infection course for the group *Anaplasma* and *Ehrlichia* (blue lines) is shown for individual cows of the farm Sikensi. Numbers represent the individual cows. A positive blood test is indicated by + and a negative blood test by −.

all carried at least one of the protozoa at some time of the study year.
Seven cattle got infected with both, *B. bovis* and *B. bigemina*, though not necessarily at the same time point. Five cattle were only infected with *B. bovis* but not with *B. bigemina* and 7 got infected with *B. bigemina* but not with *B. bovis*.
A. marginale was only detected in 4 different cattle, the other 16 cattle tested negative. However, in the case of AE, 12 tested positive and only 8 stayed negative throughout the study period.
T. mutans and *T. velifera* were detectable in all cattle participating in the study.

Dabou

In Dabou hardly any pathogens were found. All of the four positive samples for AE occurred in different cattle. The same was true for the two cases of *B. bovis* which were found in different cattle.

Brofodoumé

Seventeen different cattle were followed for this study site. Out of these, 9 cattle tested positive for both *B. bovis* and *B. bigemina*, 6 were only positive for *B. bigemina* and 1 only for *B. bovis*. None of the cattle never tested positive over the study year.
In the second group (AE), 7 different cattle were found positive for *A. marginale*. Additionally 3 cattle tested positive for AE only. Here, only 4 cattle stayed negative for the group AE during the whole study period.

Azaguié

Out of 23 animals studied, 7 cattle were found positive for *B. bovis*, whereas all others never tested positive for *B. bovis* for the whole study time.
Whether *A. marginale* nor *B. bigemina* could be detected in this farm.

7.2 Comparison of the detection capacity of the three methods: Microscopy, PCR and RLB in bovine field blood samples

7.2.1 Examination by thin and thick blood smears

All blood samples collected (a total of 707 samples) were analysed by microscopy for the presence of blood parasites. Both thin and thick blood smears were prepared for the analyses. Out of the 707 thin blood smears analysed, only 15 blood smears

tested positive for the presence of *B. bovis* and 1 for *Babesia* sp. No positive sample was found for *Anaplasma* sp, *Ehrlichia* sp nor *Theileria* sp species.
In addition to the tick-borne parasites, different *Trypanosoma* species were detected in the blood samples, i.e. 2 cases of *T. brucei* and 2 cases of *T. congolense*. In the remaining 691 samples, no parasites could be detected.
In the thick blood smears, however, no parasites of the species *Babesia* sp, *Anaplasma* sp, *Ehrlichia* sp or *Theileria* sp were detected. Only in the case of the *Trypanosoma*, a slightly higher number of positives was detected in the thick blood smears. Additionally to the 2 positive samples for *T. brucei* and 2 positive samples for *T. congolense* detected in thin smears, a further 6 positive samples for *Trypanosoma* sp were found on thick smears.

7.2.2 Detection of the various parasites by PCR prior to the RLB

No species-specific PCR's were carried out in this study. The primer pairs amplified directly more (if present) than one parasite belonging to a specific group of tick-borne protozoa or bacteria. All parasites of interest could be classified into one of two groups. One group was named AE and contained the diverse *Anaplasma* and *Ehrlichia* species and the second group was named BT and contained the diverse *Babesia* and *Theileria* species. In consequence, all blood samples had to be tested twice, once using the primer pair amplifying all parasites belonging to the group AE, and once using the primer pair amplifying all members belonging to the group BT.
The prevalence of infection was of 3.82% (27/706) in the group AE and of 54.61% (385/705) in the group BT (Table 7.14).

Table 7.14: Results of the PCR analyses of the bovine blood samples for the two groups AE and BT

Group	Positive	Negative	%	Total
AE	27	679	3.82	706
BT	385	320	54. 61	705
Total	412	999		1411

In total, 19 samples tested positive in both groups, AE and BT.

7.2.3 Comparison of the three methods

With PCR only 27/707 samples tested positive whereas by RLB 68/707 samples tested positive for the group AE. For the group BT, 385/707 samples were detected positive by PCR only, whereas 551/707 samples were detected positive by RLB. Therefore, in the group AE 41 additional samples were detected positive whereas in the group BT 166 additional samples were tested positive. In all cases (except one) the samples testing positive by PCR tested also positive by RLB. Overall, the RLB had the better detection rate over the PCR assay as well as over the screening of the blood smears by microscopy where only few samples were detected positive.

Chapter 8

Parasites detected by RLB-PCR in *A. variegatum*, *R. (B.) annulatus* and *R. (B.) geigyi* ticks collected from cattle in the South of the Ivory Coast

8.1 Prevalence of the different parasites detected in the most abundant tick species

A restricted number of ticks (423 in total) collected from cattle in the Southern region of the Ivory Coast, were analysed for the presence of parasites by RLB. Only the most abundant tick species, i.e. *A. variegatum*, *R. (B.) annulatus* and *R. (B.) geigyi*, were analysed for two selected farms (Sikensi and Brofodoumé) where the greatest variety of parasites was found in the blood sample analyses of the cattle (Table 7.7). For those tick species, adult and nymphal stages collected during the peak of January/February 2006 were analysed by RLB. The nymphal stages for *R. (B.) annulatus* and *R. (B.) geigyi* were analysed together as *Rhipicephalus (B.)* sp.

In adult *A. variegatum* ticks (14 females and 46 males) only one male was infected with *T. mutans*. In contrast 13.2 % (17/129) of the nymphs were positive for one *E. ruminantium*, 9 *T. mutans* and 11 *T. velifera* parasites. Besides 3 male *A. variegatum* and 4 nymphs tested positive for *B. bovis*, a pathogen which is not transmitted by *A. variegatum* (Table 8.1).
Four nymphs carried following coinfection: all four were double infected with *T. mutans* and *T. velifera* and one additionally carried *B. bovis*.

Females and males of *R. (B.) annulatus* carried practically no parasites of the group AE, except one male tick which was positive for the catch-all AE. However, 21%

(9/42) of the females carried parasites of the BT grouped compared with 14.8% (4/27) of male *R. (B.) annulatus*. Three females were positive for *B. bovis*, 6 for *T. mutans* and 3 for *T. velifera* whereas 3 males were positive for *B. bovis* and 1 for *T. mutans* (Table 8.2).
Three females carried following coinfection: all three were double infected with *T. mutans* and *T. velifera*.

Females and males of *R. (B.) geigyi* carried parasites of the group AE, namely 14.3 % (19/70) of the females and 10 % (5/50) of the males. Six females were positive for *A. marginale* and 2 for *Ehrlichia* sp. On the other hand, 4 males were positive for *A. marginale* and 3 for *Ehrlichia* sp. Both females and males were also positive for the group BT. 18.6% (13/70) of females and 22 % (11/50) of the males were positive for the catch-all BT. Eight females were positive for *B. bovis*, 1 for *B. bigemina* and 4 for *T. mutans*. Three males were positive for *B. bovis*, 2 for *B. bigemina*, 5 for *T. mutans* and 5 for *T. velifera* (Table 8.2).
Five males carried the following coinfection: 2 were double infected with *T. mutans* and *T. velifera*, 1 was quadruple infected with *A. marginale*, *B. bovis*, *T. mutans* and *T. velifera* and 1 was multiply infected with *Ehrlichia* sp, *A. marginale*, *B. bovis*, *T. mutans* and *T. velifera*.

One of 45 nymphs of *Rhipicephalus (B.)* sp was positive with the catch-all AE and 5 of 45 nymphs tested positive with the catch-all BT. Two of a total of seven infections could not be determined at the species level. Three nymphs were positive for *B. bovis* and two for *T. velifera* (Table 8.2).

Table 8.1: Distribution of *Anaplasma, Ehrlichia, Babesia* and *Theileria sp* species in *A. variegatum* ticks collected from cattle in the Ivory Coast by RLB. The infection rate is indicated in parenthesis (%).

	Female (n=14) (0%)	Male (n=46) (6.5%)	Adults (n=60) (5%)	Nymphs (n=129) (15.5%)	Total (n=189) (12.2%)	Infection rate (%)
Catch-all AE	0	0	0	1	1	0.5
A. phagocytophilum	0	0	0	0	0	0
E. ruminantium	0	0	0	1	1	0.5
A. marginale	0	0	0	0	0	0
Ehrlichia sp	0	0	0	0	0	0
Catch-all BT	0	3	3	19	22	9.5
B. bovis	0	2	2	4	6	3.2
B. bigemina	0	0	0	0	0	0
T. mutans	0	1	1	9	10	5.3
T. velifera	0	0	0	11	11	5.8

Eighty of the 423 ticks (19%) analysed were detected positive for parasites belonging either to the group *Anaplasma/Ehrlichia* (AE) and/or to the group *Babesia/Theileria* (BT). Namely, 12.2% (23/189) of the *A. variegatum* ticks and 24.4% (57/234) of the *Rhipicephalus (B.)* sp ticks were found to host parasites.

The overall frequency of parasites detected in ticks are summarized in Table 8.3 counting total numbers of positives regardless of the tick species analysed.

Table 8.2: Distribution of *Anaplasma, Ehrlichia, Babesia* and *Theileria sp* species in adult *R. (B.) annulatus*, *R. (B.) geigyi* and nymphal *Rhipicephalus (B.)* sp and ticks collected from cattle in the Ivory Coast by RLB. The infection rate is indicated in parenthesis (%).

	R. (B.) annulatus			*R. (B.) geigyi*			*R. (B.)* sp	*R. (B.)* sp	
	Female (n=42) (21%)	Male (n=27) (18.5%)	Adult (n=69) (20.3%)	Female (n=70) (33%)	Male (n=50) (28%)	Adult (n=120) (31%)	Nymphs (n=45) (13.3%)	Total (n=234) (25)	Infection
Catch-all AE	**0**	**1**	**1**	**10**	**5**	**15**	**1**	**17**	**7.3**
E. ruminantium	0	0	0	0	0	0	0	0	0
A. marginale	0	0	0	6	4	10	0	10	4.3
E. sp	0	0	0	2	3	5	0	5	2.1
Catch-all BT	**9**	**4**	**13**	**13**	**11**	**24**	**5**	**42**	**17.9**
B. bovis	3	3	6	8	3	11	3	20	8.5
B. bigemina	0	0	0	1	2	3	0	3	1.3
T. mutans	6	1	7	4	5	9	0	16	6.8
T. velifera	3	0	3	0	5	5	2	10	4.3

Table 8.3: Frequency of the parasites detected in different tick species. Figures represent total numbers found and frequency (%) in the 423 ticks analysed regardless of the tick species.

Parasite	Total number	Frequency (%)
Catch-all AE	18	4.25
E. ruminantium	1	0.24
A. marginale	10	2.36
Ehrlichia sp.	5	1.18
Catch-all BT	64	15.1
B. bovis	26	6.1
B. bigemina	3	0.7
T. mutans	26	6.1
T. velifera	21	5

In the majority of cases, infection with a single parasite was observed. However, double infections and less frequent coinfections by several parasites were obeserved (Fig.8.4).

Table 8.4: Frequency of single and mixed infections in ticks collected from cattle.

Number of different parasites	Number of cases [a]
5	2
4	0
3	3
2	8
1	61
0	343

[a]Samples reacting not with any species-specific probes are not included

The level of infection with the diverse parasites was compared among the species *A. variegatum*, *R. (B.) annulatus*, *R. (B.) geigyi* and *Rhipicephalus (B.)* sp by logistical regression and Pearson's chi-square analyses. Infection was measured by the number of positives with the catch-all AE and/or BT, whereas double infections were counted as one infection (Annex A.3.1).

There was a significant difference in the total number of infections between the different *Amblyomma sp.* and *Rhipicephalus (B.)* sp species (Chi = 17.72, p = 0.0005)(Annex A.3.1). The infection of *A. variegatum* was significantly different from *R. (B.) geigyi* (Chi = 15.12, p = 0.0001), i.e. *R. (B.) geigyi* was more infected than *A. variegatum*. No significant difference could be seen between *A. variegatum* and *R. (B.) annulatus* (Chi = 2.09, p = 0.15). Their infection levels were statistically

similar. There was also a difference in the total number of infection between adult *R. (B.) annulatus* and *R. (B.) geigyi* species (Chi = 13.10, p = 0.0003). The infection of the nymphs was not different from adult *R. (B.) annulatus* (Chi = 0.49, p = 0.48) and adult *R. (B.) geigyi* (Chi = 4.33, p = 0.04) species.
The number of positives for the respective individual parasites are too low for statistical analyses. Nevertheless *A. marginale*, *Ehrlichia* sp and *B. bigemina* were only detected in *R. (B.) geigyi*. *E. ruminantium* only in *A. variegatum*. *B. bovis*, *T. mutans* and *T. velifera* could be detected in all three species.

8.2 Correlation of tick infection and cattle infection

To verify, whether the infection of the tick population on a given cattle correlated with the infection of a given animal, ticks collected from specific cattle at given times, were analysed by RLB and the results were compared with the blood analyses of the respective animal.

The cattle belonging either to the farm Sikensi or Brofodoumé were grouped as positive or negative on the basis of their blood analyses (either January or February 2006) and classified into three different groups: Blood test positive for one or several pathogens (Group 1), blood test negative for all pathogens at least at one specific time point (Group 2), blood test negative over a time period of at least 4 months (at the beginning of the year 2006) (Group 3). Emphasis was put on infections with *A. marginale*, *Ehrlichia* sp, *B. bovis* and *B. bigemina*. The infections with *T. mutans* and *T. velifera* were not considered, since their overall prevalence was very high and they are of minor importance for causing diseases in animals.

All ticks found on these animals at the time of the blood test were analysed by RLB. Due to the high number of ticks found on some animals, the maximum amount of tick individuals analysed for a species and specific stage, was ten. Only non-fed or partially fed ticks were selected for the analyses. This was to minimise to detect parasites which were ingested by the blood meals and to actually detect the parasites present in the tick before the blood meal.
The prevalence of parasites in the tick analysed by RLB are shown for each individual cow on Figures 8.1, 8.2 and 8.3.
The infection of the ticks analysed was compared between the three groups by logistical regression analyses. Infection was measured by the number of positives with the catch-all AE and/or BT, whereas double infections were counted as one infection.
No significant differences in the infection rate (infection with AE and/or BT) of ticks belonging to the different groups (group 1, 2 or 3) could be found (p-values > 0.5 for all three groups) (Annex A.3.1).

Figure 8.1: **Group 1:** Blood test positive for one or several parasites especially for *A. marginale*, *B. bovis* and *B. bigemina*. The following cattle were selected during the period of high number of tick infestation, i.e. December to February 2005/2006: Cattle no. 2, no. 8 , no. 12, no. 37 and no. 39. The respective disease history for each individual cattle can be consulted in chapter 7, Fig. 7.6-7.9 with the exception of cattle no. 8. The following abbreviations are used: Female *A. variegatum*: AvF ; Male *A. variegatum*: AvM; Nymphal *A. variegatum*: AvN; Female *R. (B.) annulatus*: BaF; Male *R. (B.) annulatus*: BaM; Female *R. (B.) geigyi*: BgF; Male *R. (B.) geigyi*: BgM; Nymphal *Rhipicephalus (B.)* sp: BspN. For each cattle the total number (adults, nymphs and larvae) of ticks collected on the half body of the cattle is indicated. Total number of ticks analysed: 194.

Cattle no. 2, Sikensi, January; RLB: Positive for catch-all AE, catch-all BT, *B. bovis*, further *T. mutans* and *T. velifera*. Total number of ticks present: *A. variegatum*: 63; *Rhipicephalus (B.)* sp : 76

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	1	3	10	10	11	9	2	3	49
Catch-all AE	0	0	0	0	1	0	0	0	1
E. ruminantium	0	0	0	0	0	0	0	0	0
A. marginale	0	0	0	0	0	0	0	0	0
Ehrlichia sp	0	0	0	0	0	0	0	0	0
Catch-all BT	0	0	2	1	2	4	2	0	11
B. bovis	0	0	0	0	1	4	1	0	6
B. bigemina	0	0	0	0	0	0	0	0	0
T. mutans	0	0	2	1	1	0	1	0	5
T. velifera	0	0	0	1	0	0	0	0	1

Cattle no. 8, Sikensi, January; RLB: Positive for catch-all BT, *B. bovis*, further *T. mutans* and *T. velifera*. Total number of ticks present: *A. variegatum*: 223; *Rhipicephalus (B.)* sp: 51

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	3	6	10	6	3	10	9	10	57
Catch-all AE	0	0	0	0	0	0	0	0	0
E. ruminantium	0	0	0	0	0	0	0	0	0
A. marginale	0	0	0	0	0	0	0	0	0
Ehrlichia sp	0	0	0	0	0	0	0	0	0
Catch-all BT	0	0	1	1	1	1	0	2	6
B. bovis	0	0	0	0	1	0	0	2	3
B. bigemina	0	0	0	0	0	0	0	0	0
T. mutans	0	0	0	1	0	1	0	0	2
T. velifera	0	0	1	0	0	0	0	1	2

Cattle no. 12, Sikensi, January; RLB: Positive for catch-all BT, *B. bigemina*, further *T. mutans*. Total number of ticks present: *A. variegatum*: 64; *Rhipicephalus (B.)* sp: 10

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	1	2	9	4	4	2	-	-	22
Catch-all AE	0	0	0	0	0	0	-	-	0
E. ruminantium	0	0	1	0	0	0	-	-	1
A. marginale	0	0	0	0	0	0	-	-	0
Ehrlichia sp	0	0	0	0	0	0	-	-	0
Catch-all BT	0	0	1	1	0	1	-	-	3
B. bovis	0	0	0	0	0	0	-	-	0
B. bigemina	0	0	0	0	0	0	-	-	0
T. mutans	0	0	0	0	0	1	-	-	1
T. velifera	0	0	1	0	0	0	-	-	1

Cattle no. 37, Brofodoumé, December; RLB: Positive for catch-all AE, *A. marginale*, catch-all BT, *B. bovis*, further *T. mutans* and *T. velifera*. Total number of ticks present: *A. variegatum*: 12; *Rhipicephalus (B.)* sp: 53

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	2	6	-	-	-	10	10	-	28
Catch-all AE	0	0	-	-	-	0	2	-	2
E. ruminantium	0	0	-	-	-	0	0	-	0
A. marginale	0	0	-	-	-	0	1	-	1
Ehrlichia sp	0	0	-	-	-	0	1	-	1
Catch-all BT	0	0	-	-	-	3	1	-	4
B. bovis	0	0	-	-	-	1	0	-	1
B. bigemina	0	0	-	-	-	0	0	-	0
T. mutans	0	0	-	-	-	2	0	-	2
T. velifera	0	0	-	-	-	0	1	-	1

Cattle no. 39, Brofodoumé, February; RLB: Positive for catch-all BT, *B. bigemina*, further *T. mutans* and *T. velifera*. Total number of ticks present: *A. variegatum*: 34; *Rhipicephalus (B.)* sp: 69

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	-	10	-	1	10	7	10	38
Catch-all AE	-	-	1	-	0	4	2	1	8
E. ruminantium	-	-	0	-	0	0	0	0	0
A. marginale	-	-	0	-	0	3	2	0	5
Ehrlichia sp	-	-	0	-	0	0	2	0	2
Catch-all BT	-	-	2	-	1	0	4	0	7
B. bovis	-	-	1	-	1	0	1	0	3
B. bigemina	-	-	0	-	0	0	1	0	1
T. mutans	-	-	1	-	0	0	3	0	4
T. velifera	-	-	1	-	0	0	3	0	4

Figure 8.2: **Group 2:** Blood test negative for any important pathogen, i.e. *A. marginale*, *B. bovis* and *B. bigemina* at a given time point. All cattle carried the less pathogenic *T. mutans* and *T. velifera*. The following cattle were selected: Cattle no. 2, no. 8, no. 12, no. 32 and no. 37. The respective disease history for each individual cattle can be consulted in chapter 6, Fig. 6.6-6.9 with the exception of cattle no. 8. The following abbreviations are used: Female *A. variegatum*: AvF; Male *A. variegatum*: AvM; Nymphal *A. variegatum*: AvN; Female *R. (B.) annulatus*: BaF; Male *R. (B.) annulatus*: BaM; Female *R. (B.) geigyi*: BgF; Male *R. (B.) geigyi*: BgM; Nymphal *Rhipicephalus (B.)* sp: BspN. For each cattle the total number (adult, nymphs and larvae) of ticks collected on the half body of the cattle is indicated. Total number of ticks analysed: 86.

Cattle no. 2, Sikensi, December ; RLB: Negative (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 18; *Rhipicephalus (B.)* sp: 1

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	2	5	6	-	-	-	-	1	14
Catch-all AE	0	0	0	-	-	-	-	0	0
E. ruminantium	0	0	0	-	-	-	-	0	0
A. marginale	0	0	0	-	-	-	-	0	0
Ehrlichia sp	0	0	0	-	-	-	-	0	0
Catch-all BT	0	1	2	-	-	-	-	0	3
B. bovis	0	1	2	-	-	-	-	0	3
B. bigemina	0	0	0	-	-	-	-	0	0
T. mutans	0	0	1	-	-	-	-	0	1
T. velifera	0	0	0	-	-	-	-	0	0

Cattle no. 8, Sikensi, December ; RLB: Negative (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 42; *Rhipicephalus (B.)* sp: 22

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	1	10	7	4	2	3	6	2	35
Catch-all AE	0	0	0	0	0	0	0	0	0
E. ruminantium	0	0	0	0	0	0	0	0	0
A. marginale	0	0	0	0	0	0	0	0	0
Ehrlichia sp	0	0	0	0	0	0	0	0	0
Catch-all BT	0	0	0	0	0	1	1	1	5
B. bovis	0	1	0	0	0	0	0	0	1
B. bigemina	0	0	0	0	0	1	0	0	1
T. mutans	0	1	0	0	0	0	1	0	2
T. velifera	0	0	0	0	0	0	0	1	1

Cattle no. 12, Sikensi, December; RLB: Negative (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 14; *Rhipicephalus (B.)* sp: 0

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	1	1	5	-	-	-	-	5	12
Catch-all AE	0	0	0	-	-	-	-	0	0
E. ruminantium	0	0	0	-	-	-	-	0	0
A. marginale	0	0	0	-	-	-	-	0	0
Ehrlichia sp	0	0	0	-	-	-	-	0	0
Catch-all BT	0	0	0	-	-	-	-	-	0
B. bovis	0	0	0	-	-	-	-	0	0
B. bigemina	0	0	0	-	-	-	-	0	0
T. mutans	0	0	0	-	-	-	-	0	0
T. velifera	0	0	0	-	-	-	-	0	0

Cattle no. 32, Brofodoumé, December; RLB: Negative (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum* : 6; *Rhipicephalus (B.)* sp: 22

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	1	3	2	-	-	9	2	-	17
Catch-all AE	0	0	0	-	-	2	0	-	2
E. ruminantium	0	0	0	-	-	0	0	-	0
A. marginale	0	0	0	-	-	0	0	-	0
Ehrlichia sp	0	0	0	-	-	1	0	-	1
Catch-all BT	0	0	0	-	-	0	1	-	1
B. bovis	0	0	0	-	-	0	0	-	0
B. bigemina	0	0	0	-	-	0	1	-	1
T. mutans	0	0	0	-	-	0	0	-	0
T. velifera	0	0	0	-	-	0	0	-	0

Cattle no. 37, Brofodoumé, November; RLB: Negative (except for *T. mutans*, *T. velifera*). Total number of ticks present : *A. variegatum*: 5; *Rhipicephalus (B.)* sp: 3

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	4	2	-	-	2	-	-	8
Catch-all AE	-	0	0	-	-	1	-	-	1
E. ruminantium	-	0	0	-	-	0	-	-	0
A. marginale	-	0	0	-	-	1	-	-	1
Ehrlichia sp	-	0	0	-	-	0	-	-	0
Catch-all BT	-	0	0	-	-	1	-	-	1
B. bovis	-	0	0	-	-	1	-	-	1
B. bigemina	-	0	0	-	-	0	-	-	0
T. mutans	-	0	0	-	-	0	-	-	0
T. velifera	-	0	0	-	-	0	-	-	0

Figure 8.3: **Group 3:** Blood test negative for any important pathogen, i.e. *A. marginale*, *B. bovis* and *B. bigemina* over a time period of at least 4 month (beginning of the year 2006). Cattle carried nevertheless the less pathogenic *T. mutans* and *T. velifera*. The following cattle were selected: Cattle no.9, no. 11, no. 13, no. 14, no. 31, no. 36,no. 42, no. 65. The respective disease history for each individual cattle can be consulted in chapter 6, Fig. 6.6-6.9 with the exception of cattle no. 42. The following abbreviations are used: Female *A. variegatum*: AvF ; Male *A. variegatum*: AvM; Nymphal *A. variegatum*: AvN; Female *R. (B.) annulatus*: BaF; Male *R. (B.) annulatus*: BaM; Female *R. (B.) geigyi*: BgF; Male *R. (B.) geigyi*: BgM; Nymphal *Rhipicephalus (B.)* sp: BspN. For each cattle the total number (adults, nymphs and larvae) of ticks collected on the half body of the cattle is indicated. Total number of ticks analysed: 155.

Cattle no. 9, Sikensi, February; RLB: Negative for at least 4 months. Total number of ticks present: *A. variegatum*: 38; *Rhipicephalus (B.)* sp: 10

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	1	10	5	3	-	-	2	21
Catch-all AE	-	0	0	0	0	-	-	0	0
E. ruminantium	-	0	0	0	0	-	-	0	0
A. marginale	-	0	0	0	0	-	-	0	0
Ehrlichia sp	-	0	0	0	0	-	-	0	0
Catch-all BT	-	0	2	5	0	-	-	1	8
B. bovis	-	0	0	1	0	-	-	0	1
B. bigemina	-	0	0	0	0	-	-	0	0
T. mutans	-	0	2	4	0	-	-	0	6
T. velifera	-	0	1	2	0	-	-	0	3

Cattle no. 11, Sikensi, January; RLB: Negative for at least 4 months (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 38; *Rhipicephalus (B.)* sp: 6

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	-	10	3	-	-	-	-	13
Catch-all AE	-	-	0	0	-	-	-	-	0
E. ruminantium	-	-	0	0	-	-	-	-	0
A. marginale	-	-	0	0	-	-	-	-	0
Ehrlichia sp	-	-	0	0	-	-	-	-	0
Catch-all BT	-	-	0	0	-	-	-	-	0
B. bovis	-	-	0	0	-	-	-	-	0
B. bigemina	-	-	0	0	-	-	-	-	0
T. mutans	-	-	0	0	-	-	-	-	0
T. velifera	-	-	0	0	-	-	-	-	0

Cattle no. 13, January; RLB: Negative for at least 4 months (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 46; *Rhipicephalus (B.)* sp: 6

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	-	9	3	2	-	-	1	20
Catch-all AE	-	-	0	0	0	0	-	0	0
E. ruminantium	-	-	0	0	0	-	-	0	0
A. marginale	-	-	0	0	0	-	-	0	0
Ehrlichia sp	-	-	0	0	0	-	-	0	0
Catch-all BT	-	-	7	0	1	-	-	0	7
B. bovis	-	-	1	0	0	-	-	0	0
B. bigemina	-	-	0	0	0	-	-	0	0
T. mutans	-	-	3	0	0	-	-	0	3
T. velifera	-	-	6	0	0	-	-	0	6

Cattle no. 14, Sikensi, January; RLB: Negative for at least 4 months (except for *T. velifera*). Total number of ticks present: *A. variegatum*: 36; *Rhipicephalus (B.)* sp: 12

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	-	10	7	2	-	-	1	19
Catch-all AE	-	-	0	0	0	-	-	0	0
E. ruminantium	-	-	0	0	0	-	-	0	0
A. marginale	-	-	0	0	0	-	-	0	0
Ehrlichia sp	-	-	0	0	0	-	-	0	0
Catch-all BT	-	-	0	1	0	-	-	1	2
B. bovis	-	-	0	2	0	-	-	1	3
B. bigemina	-	-	0	0	0	-	-	0	1
T. mutans	-	-	0	0	0	-	-	0	0
T. velifera	-	-	0	0	0	-	-	0	0

Cattle no. 31, Brofodoumé, December; RLB: Negative for at least 4 months. Total number of ticks present: *A. variegatum*: 5; *Rhipicephalus (B.)* sp: 65

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	1	3	-	-	10	10	10	34
Catch-all AE	-	0	0	-	-	2	1	0	3
E. ruminantium	-	0	0	-	-	0	0	0	0
A. marginale	-	0	0	-	-	1	1	0	2
Ehrlichia sp	-	0	0	-	-	1	0	0	1
Catch-all BT	-	0	0	-	-	0	1	0	1
B. bovis	-	0	0	-	-	0	0	0	0
B. bigemina	-	0	0	-	-	0	0	0	0
T. mutans	-	0	0	-	-	0	0	0	0
T. velifera	-	0	0	-	-	0	1	0	1

Cattle no. 36, Brofodoumé, February; RLB: Negative for at least 4 months. Total number of ticks present: *A. variegatum*: 26; *Rhipicephalus (B.)* sp: 10

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	-	8	-	-	2	4	2	16
Catch-all AE	-	-	0	-	-	0	0	0	0
E. ruminantium	-	-	0	-	-	0	0	0	0
A. marginale	-	-	0	-	-	0	0	0	0
Ehrlichia sp	-	-	0	-	-	0	0	0	0
Catch-all BT	-	-	1	-	-	0	1	0	2
B. bovis	-	-	0	-	-	0	1	0	1
B. bigemina	-	-	0	-	-	0	0	0	0
T. mutans	-	-	0	-	-	0	0	0	0
T. velifera	-	-	1	-	-	0	0	0	1

Cattle no. 42, Brofodoumé, January; RLB: Negative for at least 4 months (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 54; *Rhipicephalus (B.)* sp: 3

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	2	3	10	-	-	2	-	-	17
Catch-all AE	0	0	0	-	-	0	-	-	0
E. ruminantium	0	0	0	-	-	0	-	-	0
A. marginale	0	0	0	-	-	0	-	-	0
Ehrlichia sp	0	0	0	-	-	0	-	-	0
Catch-all BT	0	0	0	-	-	2	-	-	2
B. bovis	0	0	0	-	-	2	-	-	2
B. bigemina	0	0	0	-	-	0	-	-	0
T. mutans	0	0	0	-	-	0	-	-	0
T. velifera	0	0	0	-	-	0	-	-	0

Cattle no. 65, Brofodoumé, February; RLB: Negative for at least 4 months (except for *T. mutans*, *T. velifera*). Total number of ticks present: *A. variegatum*: 44; *Rhipicephalus (B.)* sp: 3

	AvF	AvM	AvN	BaF	BaM	BgF	BgM	BspN	Total
Number analysed	-	2	8	-	-	1	-	2	15
Catch-all AE	-	0	0	-	-	1	-	0	1
E. ruminantium	-	0	0	-	-	0	-	0	0
A. marginale	-	0	0	-	-	1	-	0	0
Ehrlichia sp	-	0	0	-	-	0	-	0	0
Catch-all BT	-	0	0	-	-	0	-	0	0
B. bovis	-	0	0	-	-	0	-	0	0
B. bigemina	-	0	0	-	-	0	-	0	0
T. mutans	-	0	0	-	-	0	-	0	0
T. velifera	-	0	0	-	-	0	-	0	0

8.2.1 Correlation of the parasite infection between bovine and tick samples

Correlation between tick and cattle infection

In a comparative analyses, the presence of parasites in ticks were compared to the presence of parasites in the blood of the animal from which the respective ticks were collected. The process is illustrated in figure 8.4.

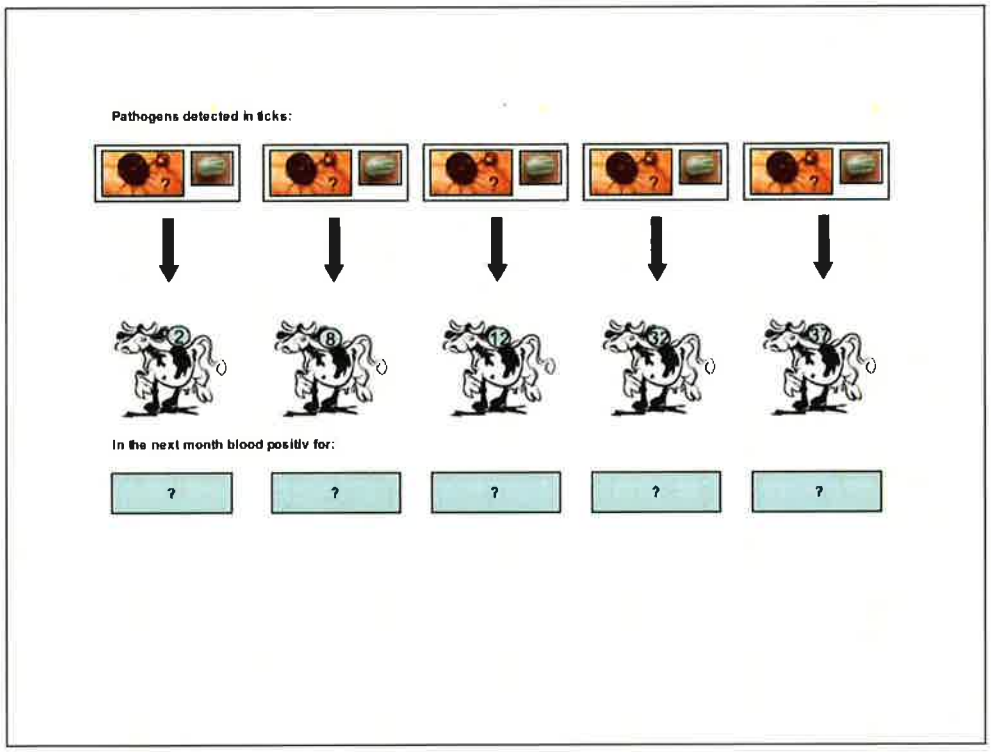

Figure 8.4: Comparison of the parasites detected in tick and blood samples analysed which were collected at the same time from a given cattle. Ear tags symbolise a given cattle, results of the tick analyses and blood test (light blue) are indicated in boxes. The arrow indicates that first the tick sample was analysed and then the blood sample.

Five cattle (no. 2, 8, 12, 32, 37) were selected on the basis of their infections with *B. bovis*, *B. bigemina*, *A. marginale*. Ticks collected one month before these parasites were detected in the blood analyses, were analysed by RLB for the presence of parasites. Hence at the time when the tick sample was collected, the cattle had no apparent infection (with the exception of *T. mutans* and *T. velifera*).

Cattle no 2: of a total of 14 ticks analysed by RLB, none was positive for the group AE and 3 were positive for the group BT. Three were positive for *B. bovis* and one for *T. mutans*. *B. bovis* was detected in one male *A. variegatum* and two nymphal *A. variegatum*. *T. mutans* was present in one nymphal *A. variegatum*. Both, *B. bovis* and *T. mutans* were present in the blood during the next month (Table 8.23).

Cattle no 8: of a total of 35 ticks analysed by RLB, none was positive for the group AE and 5 for the group BT. One was positive for *B. bovis*, one for *B. bigemina*, two for *T. mutans* and one for *T. velifera*. *B. bovis* was present in one male *A. variegatum*. *B. bigemina* was present in one female *R. (B.) geigyi*. *T. mutans* was present in one male *A. variegatum* and in one male *R. (B.) geigyi*. *T. velifera* was present in one nymphal *Rhipicephalus (B.)* sp. In the next month the following parasites were present in the blood analysed by RLB: *B. bovis*, *T. mutans* and *T. velifera*. *B. bigemina* was not present. *B. bigemina* could not be detected in the blood until February, when the cow was expelled from the study (Table 8.23).

Cattle no 12: of a total of 12 ticks analysed by RLB, none was positive for the group AE nor group BT. No parasite was detected in the ticks analysed. In the next month, the following parasites were present in the blood: *B. bigemina* and *T. mutans* (Table 8.23).

Cattle no 32: of a total of 17 ticks analysed by RLB, two were positive for the group AE and one for the group BT. One was positive for *Ehrlichia* sp and one for *B. bigemina*. *Ehrlichia* sp was present in one female *R. (B.) geigyi*. *B. bigemina* was present in one male *R. (B.) geigyi*. The blood analyses of the next month showed the presence of the following parasites: *A. marginale*, *Ehrlichia* sp, *T. mutans* and *T. velifera*. *B. bigemina* was detected only three months later (Table 8.23).

Cattle no 37: of a total of 8 ticks analysed by RLB, one was positive for the group AE and one for the group BT. One was positive for *A. marginale* and one for *B. bovis*. *A. marginale* was present in one female *R. (B.) geigyi*. *B. bovis* was present in one female *R. (B.) geigyi*. In the next month the following parasites were present in the blood: *A. marginale*, *B. bovis*, *T. mutans* and *T. velifera* (Table 8.23)

Table 8.23: Comparison of the parasites detected in blood and tick samples. Ticks were collected at the time, when no parasites were detected in the blood by RLB. Blood test were positive by RLB one month after the time of tick collection. The following abbreviations are use: AE, catch-all AE; Am, *A. marginale*; Esp, *Ehrlichia* sp; Bbov, *B. bovis*; Bbig, *B. bigemina*; Tm, *T. mutans*; Tv, *T. velifera*. *Detected in *A. variegatum*

Animal	Parasite present in ticks	Tick sampling	Parasite present in cow	Blood sampling
No. 2	Bbov*, Tm	Dec.	Bbov, Tm	Jan.
No. 8	Bbov*, Bbig, Tm, Tv	Dec.	Bbvo, Tm, Tv	Jan.
No. 12	Negative	Dec.	Bbig, Tm	Jan.
No. 32	AE, Esp, Bbig	Dec.	Am, Esp, Tm, Tv (Bbig)[a]	Jan.
No. 37	Am, Bbov	Nov.	Am, Bbov, Tm, Tv	Dec.

[a]three months later

Ticks carried diverse parasites even though the cattle from which they were collected had no apparent infection at that time point. In four cases (no 2, no 8, no 32, no 37) several of the parasites present in the ticks were also present in the blood samples one month later. Although *B. bigemina* was only detected three months later in the blood of the cattle no 32. No 8 left the study early and therefore it is not known, if it got infected with *B. bigemina* later on. Cattle no. 12 was infected with *B. bigemina* the next month, even though all ticks were negative. The vector species, i.e. *Rhipicephalus (B.)* sp was absent at the time of collection.

Correlation between cattle and tick infection

In a second assay, ticks, which have been collected at the same time as the blood test of a given animal was positive by RLB, were analysed by RLB.
Five cattle (no.2, 8, 12, 37, 39) were selected on the basis of their infections with *B. bovis*, *B. bigemina*, *A. marginale*.

Cattle no 2 was positive for group AE, group BT, *B. bovis*, *T. mutans* and *T. velifera*. Of a total of 49 ticks analysed by RLB, one was positive for the group AE and 11 for the group BT. None was positive for a specific parasite in group AE, 6 were positive for *B. bovis*, 5 for *T. mutans* and one for *T. velifera*. All parasites detected in the cow were hence also detected among the analysed ticks. One male *R. (B.) annulatus* was positive for the group AE. *B. bovis* was found in one male *R. (B.) annulatus*, in four female *R. (B.) geigyi* and in one male *R. (B.) geigyi*. *T. mutans* was found in two *A. variegatum* nymphs, in one female *R. (B.) annulatus*, in one male *R. (B.) annulatus* and in one male *B. geigyi*. *T. velifera* was present in one female *R. (B.) annulatus* (Table 8.24).

Cattle no 8 was positive for the group BT, *B. bovis*, *T. mutans* and *T. velifera*. Of a total of 57 ticks analysed by RLB, none was positive for the group AE and 6

for the group BT. Three were positive for *B. bovis*, 2 for *T. mutans* and 2 for *T. velifera*. All parasites present in the cow were hence also detected among the ticks analysed. *B. bovis* was found in one male *R. (B.) annulatus* and in two nymphal *Rhipicephalus (B.)* sp. *T. mutans* was found in one female *R. (B.) annulatus* and in one female *R. (B.) geigyi*. *T. velifera* was found in one *A. variegatum* nymph and in one *Rhipicephalus (B.)* sp nymph (Table 8.24).

Cattle no 12 was positive for group BT, *B. bigemina* and *T. mutans*. Of a total of 22 ticks analysed by RLB, none was positive for the group AE and 3 for the group BT. One was positive for *E. ruminantium*, one for *T. mutans* and one for *T. velifera*. *B. bigemina* was not detected in the ticks, whereas *T. mutans* and *T. velifera* were present. In addition *E. ruminantium* was present in the tick. *E. ruminantium* was found in one nymph *A. variegatum*. *T. mutans* was found in one female *R. (B.) geigyi* and *T. velifera* was found in one nymph *A. variegatum* (Table 8.24).

Cattle no 37 was positive for group AE, *A. marginale*, group BT, *B. bovis*,*T. mutans* and *T. velifera*. Of a total of 28 ticks analysed by RLB, 2 were positive for group AE and 4 for the group BT. One was positive for *A. marginale*, one for *Ehrlichia* sp, one for *B. bovis*, two for *T. mutans* and one for *T. velifera*. All parasites detected in the cow were hence also present in the ticks. In addition *Ehrlichia* sp was found which was not detected in the cow. *A. marginale* was found in one male *R. (B.) geigyi*. *Ehrlichia* sp was found in one male *R. (B.) geigyi*. *B. bovis* was found in one female *R. (B.) geigyi*. *T. mutans* was found in two female *R. (B.) geigyi*. *T. velifera* was found in one male *R. (B.) geigyi* (Table 8.24)

Cattle no 39 was positive for group BT, *B. bigemina*, *T. mutans* and *T. velifera*. Of the 38 ticks analysed by RLB, 8 were positive for group AE and 7 for group BT. Five were positive for *A. marginale*, two for *Ehrlichia* sp, 3 for *B. bovis*, one for *B. bigemina*, four for *T. mutans* and 4 for *T. velifera*. All parasites detected in the cow were hence also present in the ticks. Additionally, *A. marginale*, *Ehrlichia* sp, and *B. bovis* were present in the ticks, which were not detected in the cow. *A. marginale* was found in three female *R. (B.) geigyi* and in two male *R. (B.) geigyi*. *Ehrlichia* sp was present in two male *R. (B.) geigyi*. *B. bovis* was present in one nymphal *A. variegatum* and in one male *R. (B.) annulatus* and in one male *R. (B.) geigyi*. *B. bigemina* was present in one male *R. (B.) geigyi*. *T. mutans* was present in one nymphal *A. variegatum* and in three male *R. (B.) geigyi*. *T. velifera* was present in one nymph *A. variegatum* and in three male *R. (B.) geigyi* (Table 8.24).

Table 8.24: Comparison of the parasites detected in blood and tick samples which were collected at the same time from the animal. The following abbreviations are use: AE, catch-all AE; Am, *A. marginale*; Esp, *Ehrlichia* sp; Bbov, *B. bovis*; Bbig, *B. bigemina*; Tm, *T. mutans*; Tv, *T. velifera*.

Animal	Parasite present in cow	Parasite present in ticks
No. 2	AE, Bbov, Tm, Tv	AE, Bbov, Tm, Tv
No. 8	Bbov, Tm, Tv	Bbov, Tm, Tv
No. 12	Bbig, Tm	Erum, Tm, Tv
No. 37	Am, Bbov, Tm, Tv	AE, Esp, Bbov, Tm
No. 39	Bbig, Tm, Tv	AE, Esp, Bbov[a], Bbig, Tm, Tv

[a]detected in *A. variegatum*

By analysing a tick population of a cattle at the same time as the blood of the animal, a good correlation between the parasites detected in the blood by RLB and the parasites detected in the tick populations could be shown. The same species of parasite could be found in both samples in all 5 cases. Only in one case, *B. bigemina* was detected in the blood, but not among the tick species analysed. Ticks carried further parasites (cattle no. 12, 37 and 39), i.e. *E. ruminantium* (no 12), *Ehrlichia* sp (no 37, no 39), *A. marginale* and *B. bovis* (no 39). These parasites could not be detected in the blood of the respective animals during the next three months.

Chapter 9

Identification of *Anaplasma* & *Ehrlichia* species found in tick and blood samples of the Ivory Coast by 16S rRNA sequence analyses.

During the screening of the blood samples over 50% (43/68 samples) of the samples hybridizing with the catch-all probe for the group *Anaplasma* and *Ehrlichia* (AE), did not react with any of the specific probes. The same phenomenon could be observed with DNA samples of ticks (5/18). Twelve samples showing good hybridization signals with the catch-all probe and no reaction with any of the specific probes were selected at random and subsequently sequenced using 16S rRNA gene sequences to identify the organisms present (Table 9.1).

Table 9.1: Details of the samples which have been analysed for their 16S rRNA sequences.

Number	Farm	Month	Sample no.	Sample type
1	Me	Nov.	1	Blood
2	Me	June	68	Blood
3	Me	March	70	Blood
4	Me	July	99	Blood
5	Br	Sep.	37	Blood
6	Br	Feb.	38	Blood
7	Br	July	44	Blood
8	Br	Feb.	63	Blood
9	Br	April	95	Blood
10	Me	Feb.	226	Tick *R. (B.) annulatus*)
11	Br	Dec.	13	Tick (*R. (B.) geigyi*)
12	Br	Dec.	162	Tick (*R. (B.) geigyi*)

9.1 Identification of 16S rRNA gene sequences

All DNA sequences were blasted against all up to date known *Ehrlichia* and *Anaplasma* organisms using the NCBI Blast tool (`www.ncbi.nlm.nih.gov`). The 12 DNA sequences could afterwards be classified into four different clusters.

The analysis of the blood samples no. 2, 3, 4, 5 and 6 identified sequences identical with the sequence of *A. marginale* (DQ000616.1). The variable region was identical except for samples no. 2 and 3 which differed in one nucleotide position (Fig. 9.1). In sample no. 4 the region of the probe was missing and therefore no conclusion could be made.

The DNA sequences of blood samples no. 1 and 8 showed a 97% similarity with an *Anaplasma sp* (AY837740.1) sequence which has been identified in the midgut of field-caught *Anopheles gambiae* sensu lato and *A. funestus* mosquitoes (Fig. 9.2) [66].

The two tick samples no. 11 and no. 12 showed a 98% and 99% similarity with the *Ehrlichia sp* (AF414399.1) sequence which has been discovered in *R. (B.) microplus* ticks from Tibet (Fig. 9.3) [125].

The samples no. 7 (blood) and 10 (tick) had 92% and 96% similarity with *E. phagocytophilum* (AF336220.2) which was discovered in the blood of a Norwegian sheep [112]. The two however also showed variations in their respective sequences (Fig. 9.4).

The sequences were classified into four clusters and named after the most closely related organisms, i.e. Group A: *A. marginale*, Group B: *Anaplasma* sp, Group C: *Ehrlichia* sp and Group D: *A. phagocytophilum*-like (Table 9.2, Annex A.4.1).

Table 9.2: List of samples sequenced and their closest match using the NCBI Blast tool

Group	Sample	Closest sequence identifi ed by BLASTn	Similarity (%)
A	5	*A. marginale*	99 % similarity
A	6	*A. marginale*	99 % similarity
A	2	*A. marginale*	98 % similarity
A	3	*A. marginale*	98 % similarity
A	4	*A. marginale*	98 % similarity
B	1	*Anaplasma sp*	97 % similarity
B	8	*Anaplasma sp*	97 % similarity
C	11	*Ehrlichia sp*	98 % similarity
C	12	*Ehrlichia sp*	99 % similarity
D	7	*A. phagocytophilum*-like	92 % similarity
D	10	*A. phagocytophilum*- like	96 % similarity
-	9	Not to be identifi ed	

9.1.1 Alignment of 16S rRNA sequences

Subsequently all newly identified sequences of the same cluster were aligned together with the respective most closely matching 16S rRNA sequence as well as the corresponding 16S rRNA sequence which has been used to design the probes for the RLB assay using the ClustalW alignment tool.

```
M60313  AGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAGTA  112
5       AGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAGTA  119
6       AGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAGTA  115
2       AGTCGAACGGACCATATACGCAGCTTGCTGCGTATATGGTTAGTGGCAGACGGGTGAGTA  120
3       AGTCGAACGGACCATATACGCAGCTTGCTGCGTATATGGTTAGTGGCAGACGGGTGAGTA  120
4       -------------------------TTGCTGCGTGTACGGTTAGTGGCAGACGGGTGAGTA   36

M60313  ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTA  172
5       ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTA  179
6       ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTA  175
2       ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTA  180
3       ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTA  180
4       ATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTA   96

M60313  TAATCC-TGCGGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGTT  231
5       TAATCCCTGCGGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTT  239
6       TAATCCCTGCGGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGTT  235
2       TAATCCCTGCGGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGTT  240
3       TAATCCCTGCGGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGTT  240
4       TAATCCCTGCGGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGTT  156

M60313  GGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCA  291
5       GGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCA  299
6       GGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCA  295
2       GGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCA  300
3       GGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCA  300
4       GGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCA  216
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Figure 9.1: Alignment of the sequences belonging to the cluster A. M60313 is the sequence of *A. marginale* which was used to design the probe. Samples: No. 2, 3, 4, 5 and 6. The region of the probe is underlined in yellow, the difference in the nucleotides between M60313 and the sample sequences are marked in green and the region of the catch-all probe is underlined in gray.

The alignment of the sequences identified as *A. marginale* with the reference sequence of *A. marginale* (M60313) confirmed the blast result (Fig. 9.1). Samples number 2 and 3 differed in one nucleotide position in the probe region (yellow) when compared to the reference sequence. In contrast, samples 5 and 6 were identical to the reference sequence. For sample 4, the probe region was missing.

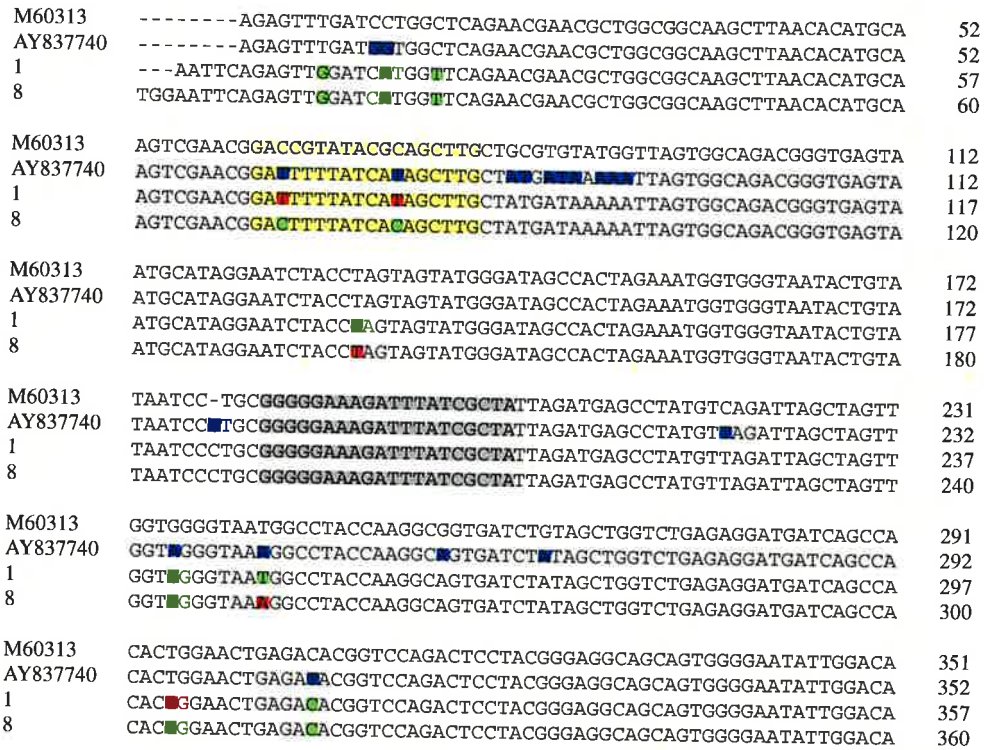

Figure 9.2: Alignment of the sequences of the cluster B. M60313 is the sequence of *A. marginale* which was used to design the probe and AY837740 the sequence of *Anaplasma* sp to which the sequences showed the highest homology. Samples no. 1 and 8. The region of the probe for *A. marginale* is underlined in yellow, the differences in the nucleotides between M60313 and AY837740 are marked in blue, differences between the samples and AY837740 are marked in green and differences between the samples 1 and 8 are marked in red. The region of the catch-all probe is underlined in gray.

In group B, the sequences were aligned with both the reference sequence for *A. marginale* (M60313) as well as the closest match (*Anaplasma* sp; AY837740) of the blasting result (Fig. 9.2). The sequence no. 1 differed in 7 positions and the sequence no. 8 in 8 positions compared to the *Anaplasma sp* sequence no. AY837740. Moreover, the two newly identified sequences differed among themselves in 5 positions. In the probe region sequence no. 1 was identical to *Anaplasma* sp and differed from *A. marginale* in two positions whereas the sequence no. 8 was identical to *A. marginale*. In attend for a good classification, these organisms were named *Anaplasma sp* Ivory Coast (IC) I and II.

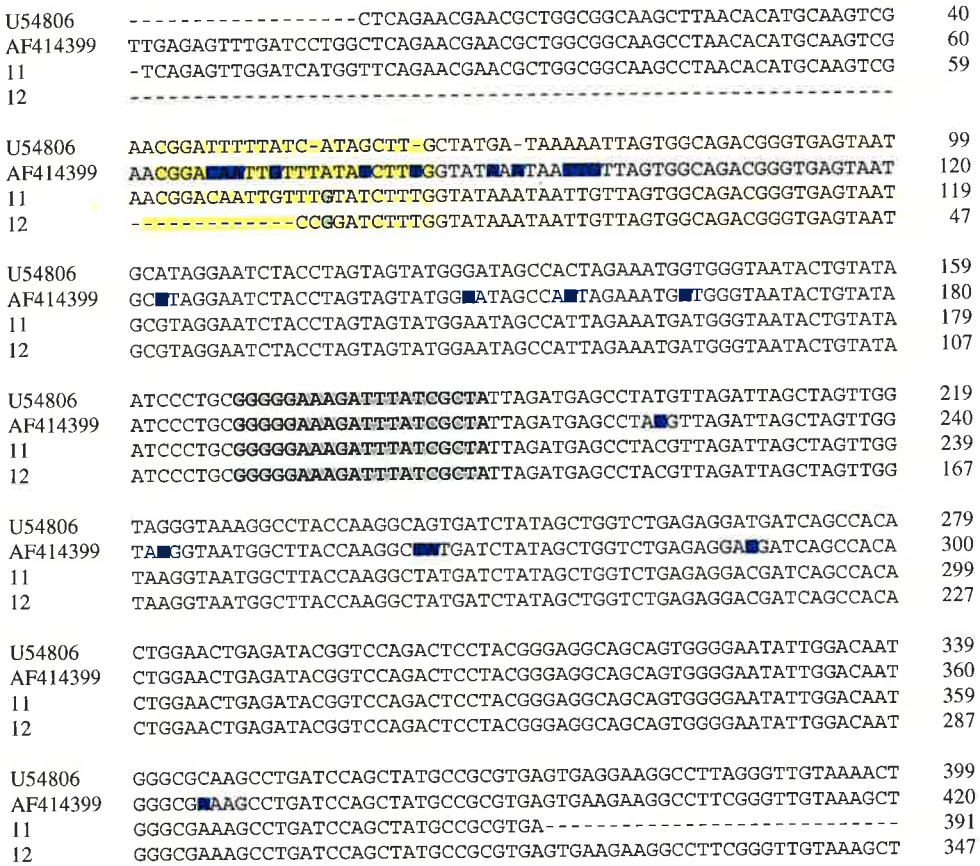

Figure 9.3: Alignment of the sequences of the cluster C. U54806 is the sequence of *Ehrlichia* sp which was used to design the probe and AF414399 the sequence of *Ehrlichia* sp to which the sequences had the highest homology. Samples no. 11 and 12. The region of the probe for *Ehrlichia sp* is underlined in yellow, the differences in the nucleotides between U54806 and AF414399 are marked in blue, the differences in nucleotides between the samples 11 and 12 and AF414399 are marked in green. The region of the catch-all probe is underlined in grey.

The alignment of samples 11 and 12 with the published sequences of *Ehrlichia* sp AF414399, showed that these sequences could clearly be identified as *Ehrlichia* sp identical to the already found *Ehrlichia* sp in Tibetan ticks (Fig. 9.3). These findings are therefore the first report of these *Ehrlichia* sp in *R. (B.) geigyi* ticks of the Ivory Coast.

The low similarities (92% and 96%) of the sequences of the samples no. 7 and no. 10 to the sequence of the *A. phagocytophilum* organism AF336220 showing differences in several nucleotide positions (24 differences for sample no. 7, and 8 differences for sample no. 10) indicate that these sequences are most possibly two new strains of *A. phagocytophilum* organisms (Fig. 9.4). In the probe region, sample no 7 showed 5 differences in nucleotide positions whereas sample no. 10 showed 3 differences in nucleotides. Further analyses are needed to clarify the situation. In the meantime, the two organisms were named *A. phagocytophilum*-like IC.

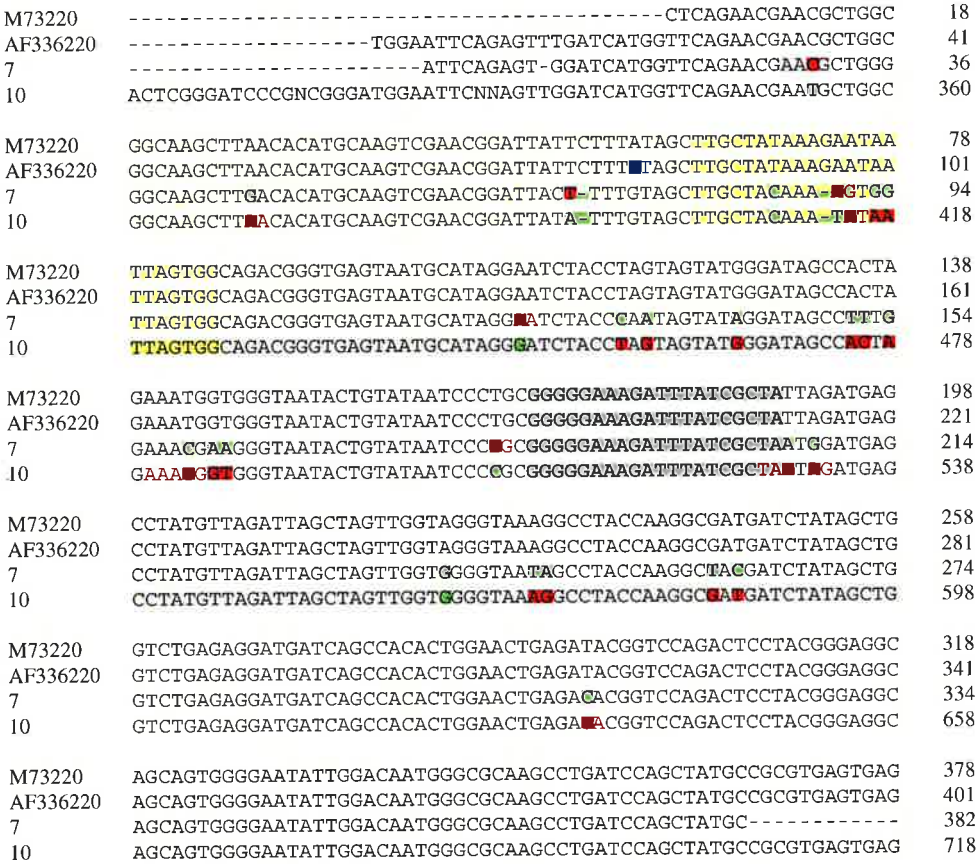

Figure 9.4: Alignment of the sequences for the cluster D. M73220 is the sequence of *A. phagocytophilum* which was used to design the probe and AF336220 the sequence of *A. phagocytophilum* to which the sequences were most closely homolog. Blood sample no. 7, tick sample no. 10. The region of the probe for *A. phagocytophilum* is underlined in yellow, the differences in the nucleotides between M73220 and AF336220 are marked in blue, differences between samples and AF336220 are marked in green and differences between the samples 7 and 10 are marked in red. The region of the catch-all probe is underlined in grey.

9.2 Design of two novel probes for the detection of a new *Ehrlichia* sp and a new *A. phagocytophilum*-like species.

Two novel probes were designed to be added to the RLB assay in the aim of retesting the samples which did not react before with any of the specific-probes for the group AE.

Design of new probes:

The following area in the highly variable region of the 16S rRNA gene was chosen in order to design an additional probe to detect the new *Ehrlichia* species found (Table 9.3). The sequence was chosen in a way that the hybridization temperature matched the average hybridization temperature of the other probes already used in the RLB assay.

Table 9.3: Sequences used for *Ehrlichia* sp: U54806 and AF414399. Sample 13 (Positive for *E. sp*). The already existing probe of the RLB assay is underlined in yellow, the new designed probe is indicated in cyan and the nucleotide differences are indicated in magenta.

U54806	AACGGATTTTTATC-ATAGCTT-GCTATGA-TAAAAATTAGTGGCAGACGGGTGAGTAAT	99
AF414399	AACGGACAATTGTTTATATCTTTGGTATAAATAATTGTTAGTGGCAGACGGGTGAGTAAT	120
Sample 11	AACGGACAATTGTTTGTATCTTTGGTATAAATAATTGTTAGTGGCAGACGGGTGAGTAAT	119

The novel probe was named *Ehrlichia* sp Ivory Coast (IC) and had the following sequence: CGG ACA ATT GTT TGT ATC TTT G with a hybridization temperature of 57.8 °C which is similar to the hybridization temperature of the already existing probe for *Ehrlichia* sp which is 57.5 °C.
The second probe, which was named *A. phagocytophilum*-like Ivory Coast, was also designed in the hypervariable region of the 16S rRNA gene. The novel probe has the following sequence TTG CTA CAA AAG TGG TTA G and a hybridization temperature of 54.25 °C, which is similar to the hybridization temperature (54.6°C) of the already used *A. phagocytophilum* probe (Table 9.4).

Table 9.4: Sequences used for *A. phagocytophilum* M73220 and AF336220. Sample no. 44 (Positive). The already existing probe of the RLB assay is underlined in yellow, the new probe is underlined in cyan and the nucleotide differences to the old probe are underlined in magenta.

M73220	CGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAA	100
AF336220	CGGATTATTCTTTGTAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAA	123
44	CGGATTACT-TTTGTAGCTTGCTACAAA-ACTGGTTAGTGGCAGACGGGTGAGTAA	116

Both probes were incorporated into the RLB assay and applied onto a Biodyne C membrane in a concentration of 500 pmol. The probe was then firstly tested with the known (sequenced) positives and afterwards applied to rescreen the samples in the

group AE which did only react with the catch-all probe for AE. The probe for the new *Ehrlichia* sp proved to be effective in detecting these parasites in tick (5 times in *R. (B.) geigyi*, data not shown) and blood samples (Fig. 9.5, 9.6), whereas the probe for the new *A. phagocytophilum* needs to be redesigned. It gave no hybridization signal with the PCR product of the positive sample (9.5) and only very weak signals with 2 of the field samples (Fig 9.6).

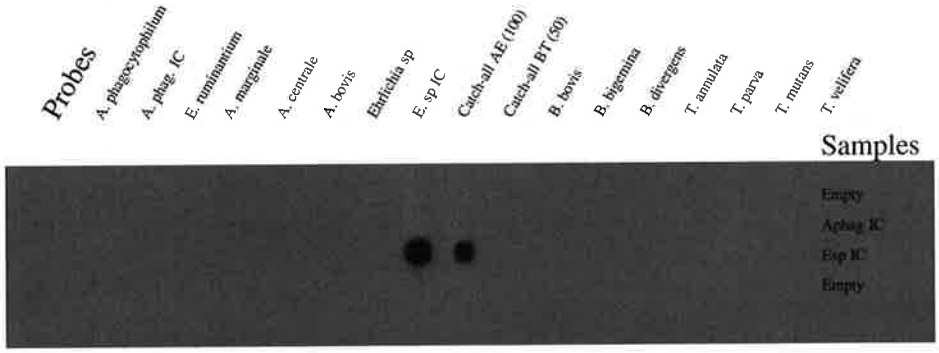

Figure 9.5: Results of the RLB membrane: Horizontal lines : Species-specific probes. Vertical lines : PCR products of the positive samples. Aphag, *A. phagocytophilum*; Esp, *Ehrlichia* sp.

In total 38 samples were rescreened using the RLB assay including the novel probes. None of these samples reacted with any of the species-specific probes of the group AE at the first time. However, the intensities of the hybridization signals with the catch-all probe for AE varied from hardly visible to very strong (details are explained in Table 9.5).

Table 9.5: Details of the 38 samples which have been retested using new probes. AE, catch-all AE; Am, *A. marginale*; Aphag, *A. phagocytophilum*; Esp, *Ehrlichia* sp.; None: no species-specific hybridization

Sample	No	Farm	Month	1st time intensity[a] of catch-all AE	2nd time Intensity of catch-all AE	2nd time positive for
1	5	Me	04	**	**	none
2	37	Br	09	****	*	
3	2	Me	07	**	***	none
4	9	Me	07	***	****	none
5	5	Me	08	**	*	
6	70	Me	06	*****	*****	Am
7	68	Me	09	****	*****	none
8	70	Me	10	**	*	
9	15	Me	12	**	**	none
10	29	Ca	01	****	****	none
11	28	Ca	01	****	*	
12	3	Me	01	**	**	none
13	2	Me	01	*****	****	Aph IC
14	6	Me	12	**	*****	Aph IC, Esp IC
15	13	Me	12	**	*	
16	47	Az	2	*****	*****	Esp
17	3	Me	11	*	*	none
18	27	Ca	12	****	**	none
19	37	Br	09	****	*	
20	26	Ca	01	*****	***	none
21	37	Br	07	***	******	Am
22	98	Br	07	***	******	Am
23	38	Br	07	***	**	none
24	37	Br	08	****	****	none
25	38	Br	08	****	***	none
26	44	Br	08	****	****	Am
27	65	Br	08	****	****	Am
28	98	Br	10	**	*	none
29	39	Br	10	****	*****	Am, Esp
30	45	Br	10	**	*****	Am
31	63	Br	02		*****	Am
32	39	Br	12	**	***	none
33	39	Br	11	******	******	Am
34	63	Br	05	******	******	Esp
35	38	Br	05	******	******	Am
36	65	Br	01	*****	****	none
37	48	Az	03	*	******	Esp
38	44	Br	07	******	*****	none

[a]Intensities of the hybridization signal with the catch-all probe for AE (* no signal ** hardly visible *** very weak/weak **** medium ***** strong /very strong)

During the rescreening of the blood samples only one of the blood samples reacted positive with the new probe for *Ehrlichia* sp Ivory Coast (sample 14) and two other samples are suspected to be positive for the new *A. phagocytophilum*-like Ivory Coast (samples 13, 14), though they gave very weak hybridisation signal (Fig. 9.6). In this second assay, 30 samples reacting again positive with the catch-all probe for AE and 7 tested negative. One sample which was negative in the first round remained negative. In contrast to the first time, now 10 samples could be identified as *A. marginale* (mostly for the farm Brofodoumé) and 4 as *Ehrlichia* sp (Brofodoumé, Azaguié).
Nevertheless, we are still left with twenty samples where the parasites present in the blood samples could not be identified.

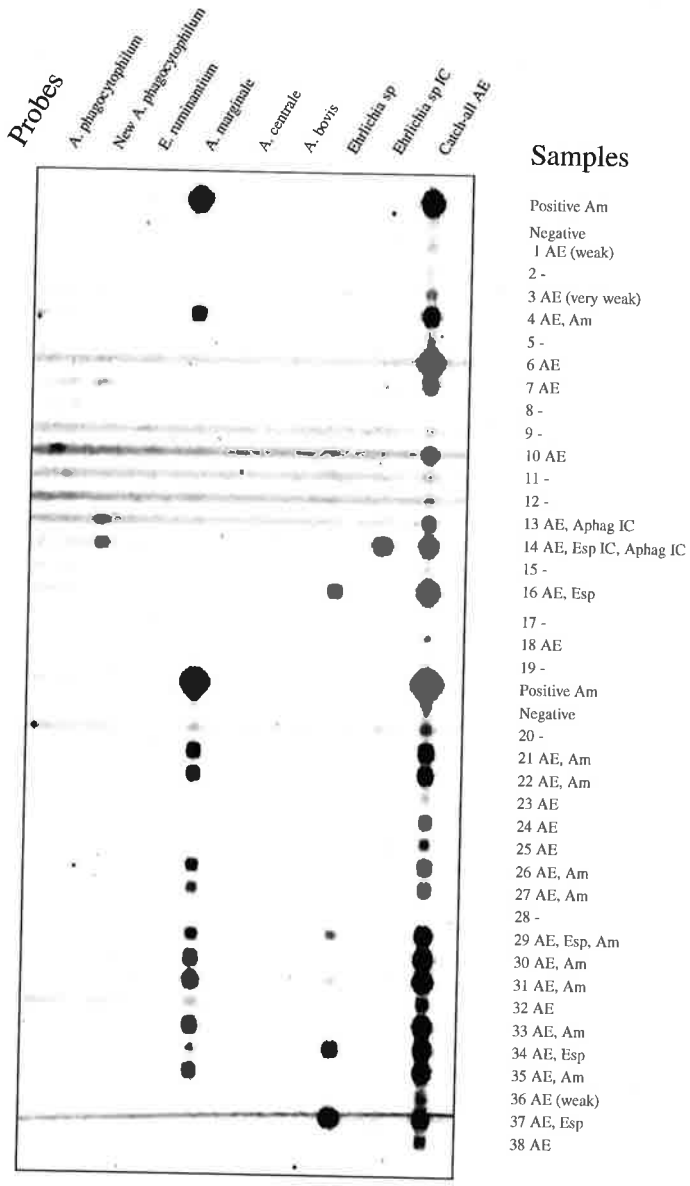

Figure 9.6: Results of the RLB membrane after exposure to the X-ray film. Horizontal lines: Species-specific probes. Vertical line: Samples no.1 -38. AE, catch-all AE; Am, *A. marginale*; Aphag, *A. phagocytophilum*; Esp, *Ehrlichia* sp.

Chapter 10

Discussion

Based on the reverse line blot assays developed by Gubbels et al [49] and Bekker et al [15], a reverse line blot assay which simultaneously detects the principal tick transmitted protozoan and rickettsial cattle parasites was established in the Laboratory of molecular Parasitology, Neuchâtel, Switzerland and evaluated for its use in an epidemiological study on ticks and tick-borne parasites in cattle in the Ivory Coast. The aim was to have a sensitive and specific diagnostic tool for screening large scale of samples, since the current diagnostic methods used in the Ivory Coast lack both sensitivity and specificity and certain species may be entirely excluded from routine surveys.
The species-specific PCR's, which exist for the majority of economically important parasites, are only designed to detect one specific pathogen. However, tick-borne parasites are known to coexist in animals [130] and it would be too time consuming to test animals for each parasite individually.
On the other hand, serodiagnosis does not detect the parasite itself and therefore the animal may already have cleared the parasite but still remains seropositive. Parasitaemia usually drops to a very low level once the acute infection has passed and the animal becomes a carrier. This latent infections are a perpetual source for infections for the feeding tick species and a danger for transferring parasites from an endemic region into a previously uninfested area by means of introducing carrier animals. The routine use of blood smear analyses can only detect parasites in acute disease, but not in latent infected cattle [12].

10.1 Study design

Information on ticks and tick-borne parasites is still scarce for the different regions in the Ivory Coast. Although studies on the prevalence of ticks as well as the microorganisms they host, have been done in various regions from South to North, these

studies now already date from 20 to 40 years ago [81] [2] [47]. Studies investigating the presence of haematoparasites in cattle have been restricted to the North [80] (Achi, unpublished data) and the Center of the Ivory Coast [60]. However, the study in the district of Toumodi is the only recently published (2002) study on ticks and tick-borne diseases in the Ivory Coast [60]. Further, studies investigating the detection of tick-borne pathogens in ticks were mainly focused on rickettsiales of the type *R. conorii* and *R. africae* [29]. To date, no study has addressed the topic in the Southern coastal region of the Ivory Coast where the vegetation and climate are quite different from conditions of the humid savanna (Central) and the more arid savanna in the North. A difference in the presence of tick species, their seasonal pattern and the prevalences of tick-borne parasites can therefore be expected. The South of the Ivory Coast was chosen for the present study due to the lack of knowledge on ticks and tick-borne parasites in this area, the specific climatic conditions as well as the increasing interest in the area for livestock production.

For convenience, study sites were chosen to be only a day trip away from Abidjan. Herd owners were contacted by a local Veterinarian (Dr L. Achi) and selected upon their interest and willingness to participate in a one-year study. Finally, herds were chosen in order to represent different cattle breeds, namely Zebu, N'Dama, crosses of N'Dama and Zebu as well as crossbreed cattle of N'Dama with Abondance, Holstein or Montbéliard breeds. During the study, no interference was made in the individual tick control scheme of the farms, in order to represent local daily conditions. However, herdsmen were advised not to treat immediately before the day fixed for tick collection. It was not possible to include non-treated cattle, due to the high production losses this would have been representing for local cattle owners. An earlier study has shown that collection of ticks should be done on a bimonthly basis, in order to get an accurate idea of the seasonal distribution pattern (Achi, personal communication). The number of cattle included per herd (15) was a trade-off between minimal estimated numbers needed for statistical analyses and the number of animals that could be sampled in an adequate time.
For the analyses of the prevalence of tick-borne diseases in ticks and blood samples, a PCR-RLB assay was chosen a) since molecular methods have proved to be very efficient and sensitive in previous studies and b) since the assay permitted the screening of multiple samples for the presence of several tick-borne parasites.

10.2 DNA origin and extraction

The ideal situation for evaluating any DNA extraction, PCR or RLB assay for different tick-borne parasites, would have been to have cell cultures of diverse parasites and/or artificially infected animals and ticks on the hand. Obviously, the detection sensitivity of a method can only be evaluated correctly, if the parasitaemia level is

known for a specific parasite. However, it would still have been a problem to isolate the intracellular parasites from the contaminated host DNA. In the Laboratory of molecular parasitology in Neuchâtel, neither cell cultures nor artificially infected animals or ticks are kept for tick-borne diseases relevant to cattle. All DNA for the different tick-borne parasites as well as all tick species (except *I. ricinus*) used in the experiments were therefore generously obtained from other laboratories. It was not possible to determine parasitaemia levels for any of the parasites obtained and therefore neither the sensitivity of the PCR nor RLB could be determined in this study.

It is important to mention that any PCR amplification is only as good as the DNA extraction process. Tick tissues and blood carry PCR inhibitory substances, such as hem, lipids and polysaccharide, which contaminate the DNA and thereby inhibit the subsequent PCR reaction. DNA extraction of ticks is particularly difficult due to their hard exoskeleton. To date there are many different methods to « free » the DNA from the ticks with the hard chitinous exoskeleton. A review on different DNA extraction methods for ticks as well as molecular detection of parasite DNA in ticks was given by Sparagano et al. [106].
A DNA extraction method needs to yield a good purification as well as a sufficient DNA amount. DNA extraction from blood using the Whatman extraction kit did not pose any problems. It is, however, more difficult to extract DNA from the tick samples [53]. Generally, it was found beneficial to homogenise the tick samples mechanically prior to the extraction step with better results in the subsequent PCR and RLB assay. A study carried out by Halos et al (2004) also showed that enzymatic protein degradation using proteinase K before DNA extraction was not sufficient for maximal isolation of DNA [52]. An initial step of fine crushing by either mortar crush or bead beating was therefore added [52]. The crushing is probably needed to destruct mechanically the polysaccharide chains of the tick exoskeleton [52]. These findings therefore confirm the experience made with DNA extraction of ticks in this study.
Most DNA extraction methods described by Sparagano et al (1999) gave good results for any small tick species such as *Ixodes*, *Rhipicephalus* etc., *Amblyomma* are ticks of a bigger size and it was difficult to take away all inhibitory substances. This is why besides of the crushing in liquid nitrogen, the use of digestion by proteinase K and sodium dodecyl sulfate extraction together with phenol extraction and DNA concentration by ethanol precipitation was necessary. With this DNA extraction method subsequent PCR and RLB gave good results. Protein digestion and DNA extraction techniques using chloroform/phenol was also highly efficient compared to other methods [52]. However, the method is labour intensive and time consuming. Though for cohort studies quicker methods, such as extraction by bead beater [52], Chelex-Based Resins (Biorad, Switzerland) or ammoniumhydroxide [92] [97]

might be desirable, they seem to date to be particularly well adapted for samples of small tick sizes such as nymph and larva [52] (Biorad, Switzerland, pers. communication).

10.3 RLB as a powerful tool for epidemiological studies

In the previous years, molecular biology has been the subject of intense studies that have provided many useful tools for the study of tick-borne parasites. In fact, PCR made it possible to amplify small amounts of the target parasite DNA even within abundant host DNA. 16S rRNA and 18S rRNA sequences have proved to be good target sequences for group specific PCR amplification and subsequent species-specific probe detection [25]. The PCR-RLB assay makes use of species-specific variations in these rRNA genes as well as the abundance of ribosomes and therefore RNA in cells to detect the specific parasites [25].
While testing the first membrane of the ICTTD TBD-RLB (TBD) kit (Isogen, Netherlands), we noticed how important it is to evaluate correct concentrations for the species-specific probes before applying them on the membrane. On the TBD kit membrane, black lines, masking possible positive hybridization signals, occurred which made it impossible to interpret results. The TBD kit helped to verify the method established in Neuchâtel. With the exception of two samples one hybridizing with the catch-all AE only and one hybridizing with the probe for *T. velifera*, which were found positive in addition by our method, both tests gave similar results for all samples tested. However, nevertheless, the membrane of the TBD kit showed black lines at the place of specific probes, a phenomenon that was never observed with the membrane prepared in Neuchâtel. Results obtained by both RLB assays, suggest that results obtained in different studies can be compared. However, different RLB assays apply different probe concentrations onto their membranes.
Both, PCR and RLB were found to be extremely prone to contamination during manipulation processes. Extreme care needs to be taken to avoid contamination and separate rooms and equipment need to be available. The requirements restrict the use of PCR and RLB to highly equipped laboratories of high standards and is therefore not suitable as quick field diagnosis. Decontamination of the working surfaces should be made on a regular basis using commercial products to inactivate DNA, such as for instance DNAzap (Ambion), together with bleach and UV radiation. A further improvement to avoid contamination would be to incorporate an Uracil DNA glycosylase as suggested by Bekker et al [15] into the PCR assay. Uracil DNA glycosylase eliminates in combination with dUTP contaminations caused by previous PCR amplifications. Natural DNA does not contain Uracil and therefore the original

probe stays completely intact.

During the evaluation, PCR amplification and subsequent RLB hybridization was specific for selected positive DNA samples (*A. marginale*, *A. centrale*, *E. ruminantium*, *B. bovis*, *B. divergens*, *T. annulata*, *T. parva*), positive plasmid controls (*A. phagocytophilum*, *E. ruminantium*, *A. marginale*, *A. centrale*, *A. bovis*, *Ehrlichia* sp, *B. bovis*, *B. bigemina*, *T. mutans*, *T. velifera*) as well as for field samples. As already previously shown [49] [15] all probes were hybridizing well with the respective positive samples and no cross-reactions occurred. Two group specific catch-all probes, one for the group *Anaplasma/Ehrlichia* (AE) and one for the group *Babesia/Theileria* (BT), assured the detection of parasites which did not react with any of the species-specific probes, i.e. when possible nucleotide differences between strains would occur or novel species would be present. However, it was difficult to obtain an intense hybridization signal with the species specific probe for *A. phagocytophilum*. Furthermore, did the *B. bigemina* DNA sequence, obtained from infected cattle from Australia, differed in 2 nucleotide positions to the included probe and failed to hybridize. This shows the importance of point mutations or geographical differences in strains which might provoke a failure in the species-specific recognition by a probe.

A critical point of the RLB is the sensitivity, in particular the capacity to detect parasites at low concentrations such as subclinical cases. Due to a lack of pure DNA, that was not contaminated by host DNA, it was not possible to evaluate the sensitivity of the RLB assay. However, the sensitivity of the RLB has already been analysed before for some pathogens [87] [15] [49]. Detection limit for *T. parva* was 4 x 10^{-5}% parasitaemia [87], for *B. bovis* 10^{-6}% and for *B. bigemina* 10^{-8}% parasitaemia [49] and for *T. annulata* 10^{-6}% [44]. Sensitivity of probes seemed to differ and sensitivity analyses should be performed for all individual probes. In essence, it is not known, if in negative samples the respective pathogen was really absent or if it was present in a quantity that lied below the threshold of the PCR-RLB assay. In contrast, positive hybridization signals could be clearly interpreted.
Different authors mentioned a possible lack of sensibility of the RLB probe for the detection of *E. ruminantium* in blood of cattle [15] [36]. In a comparative assay between the pCS20 PCR, the PCR which shows the highest sensitivity to detect *E. ruminantium* to date, and the RLB, the latter showed a significant lower sensitivity. During the field study in the South of the Ivory Coast no positive blood sample was detected for *E. ruminantium* and DNA from only one nymphal *A. variegatum* tick hybridized with this probe in this study. Similarly, no tick was detected positive for *E. ruminantium* by RLB in a study in Guinea [117]. Both the Ivory Coast and Guinea are known to be heartwater endemic countries.
To date 8 different 16S genotypes of *E. ruminantium* are known [25]. The probe used in the present RLB assay was designed to detect them all. However, there

is always a possibility that a different genotype of *E. ruminantium* exists in West Africa which might not be recognised by the current probe. Allsopp et al showed the presence of multiple 16S genotypes in field blood samples [6]. Furthermore, a clear separation of genotypes of southern and eastern as well as western Africa could be observed [6].
The parasitaemia level of our positive control for *E. ruminantium* was not known and detection capacity of the RLB could not be evaluated. There is a strong evidence that the RLB in its current form is not well adapted to detect *E. ruminantium* in cattle. Generally, it is difficult to detect these pathogens in low level parasitaemia. Only few pathogens are circulating in the peripheral blood and pathogens are more attracted to internal visceral structures and capillaries of the brain [26] [20]. The lack of positive samples in this study could also simply reflect these facts.

The threshold for detecting *A. marginale* by RLB is also not known. Although, positive samples seemed always to react well with the catch-all probe for AE, several samples were observed with no species-specific hybridization. In a few cases, the lack of hybridization with the species-specific probe for *A. marginale* could be explained by point mutations found in the region of the probe on the basis of sequence data. However, some samples, which had not reacted with the species-specific probe during the screening process, were retested by RLB in order to confirm the results. A few samples of the ones reacting only with the catch-all probe before could now be confirmed to be either *A. marginale* or *Ehrlichia* sp. This phenomenon is most possibly due to parasitaemia levels in the samples which were close to the detection limit of RLB. It is important to determine the threshold for the *Anaplasma/Ehrlichia* group to clarify the situation. If necessary, PCR sensitivity could be possibly optimised by a nested PCR or the specificity of the probe for *A. marginale* could be improved by redesigning it at a different location.
The importance of *A. phagocytophilum* or *A. phagocytophilum*-like species might have been underestimated. Since *A. phagocytophilum* was not known to play any important role in causing disease in African cattle, only one probe was included for this pathogen, although the occurrence of different variants was well known [15]. Current findings suggest that more emphasis should be put on the detection of these pathogens and their role in cattle disease in the Ivory Coast.

In general, the sensitivity of the RLB seemed to be at least similar to the detection by PCR for specific parasites, if not higher [87]. In any way, the sensitivity of molecular methods is way above the detection by microscopy with the added advantage of a high specificity.
RLB has proven to be an important tool to obtain baseline data on the presence of tick-borne parasites in ticks and cattle in the Ivory Coast. Further, it permitted to study coinfections and led the way in discovering possible new strains or species of parasites in cattle of the Ivory Coast (see below).

10.4 Prevalence and seasonality of tick species on domestic cattle in the Ivory Coast

The general spectrum of ticks found in the South of the Ivory Coast matches well with previous findings [2] [81] as well as with findings from other parts of the Ivory Coast [60], [21] (Achi, unpublished data) and is in accordance with tick species generally reported from other West African countries [24] [122] [63] [50] [51] [117] [77] [14] [115]. *R. (B.) decoloratus*, a frequent species in West Africa [81], but previously reported as absent from the Ivory Coast by Aeschlimann (1967), was now found to be present in the Ivory Coast. Early records by Morel (1958) already mentioned *R. (B.) decoloratus* and the species was also present in the North of the Ivory Coast (Achi, unpublished data), whereas it was not found in areas in the Centre of the country [60] [21] nor in the Eastern area [21]. *R. (B.) decoloratus* can be found in the neighbouring Ghana in a similar climatic zone than this study [122], so its presence is not really surprising. However, it is unknown if the species was newly introduced into the country or if it was already endemic.
Although, a single *H. truncatum* was found, the species as well as other more xerophilous species found north on from the present study area [60] [2] (Achi, unpublished data) and in more arid countries of West Africa, were absent or only sporadically present in the humid South of the Ivory Coast.

A. variegatum, which is the most common tick species throughout West African countries, is well present in the whole Ivory Coast [2] [60] and the most frequent species in the South of the Ivory Coast as well as in other regions [2] [21]. The species is known to adapt to a wide variety of climates from rain forest to steppe [123], which does explain its omnipresence. Although, *A. variegatum* was the most frequent species in the majority of the study sites, it was nevertheless clearly outnumbered by *R. (B.) decoloratus* in Azaguié.
Both *R. (B.) annulatus* and *R. (B.) geigyi* are typical species of West Africa and can not be found outside the humid West African regions [123] [35]. In contrast, *R. (B.) decoloratus* has a much wider range of distribution and extends into much of Africa and was associated with many vegetation categories [35]. *R. (B.) annulatus* and *R. (B.) geigyi* are mainly associated with woodlands and forest with secondary grasslands and have preferences towards high temperatures [35].
The three *Rhipicephalus (B.)* species did not occur in the same frequencies on all farms and the differences were remarkable. In Sikensi, all three species, i.e. *R. (B.) annulatus*, *R. (B.) geigyi* and *R. (B.) decoloratus* were present in this decreasing order. In contrast, in Dabou hardly any *Rhipicephalus (B.)* ticks were collected and all individuals were *R. (B.) decoloratus*. In Brofodoumé, the order was *R. (B.) geigyi*, *R. (B.) decoloratus* (3%) and *R. (B.) annulatus* (0.4%). In Azaguié, however *R. (B.) decoloratus* represented 97% , *R. (B.) geigyi* 2% and *R. (B.) annulatus*

0.1%. It is unknown, if competitive factors between the species play a role in the differences of numbers present of the three species on each farm. Specific ecological niches on individual farms may also play a role as do other factors such as tick control, herd management, local vegetation or cattle breed. Yet further investigations are needed to clarify the situation. However, cattle in the farm of Dabou were more isolated from the environment and therefore acaricide treatment, which was applied rigidly, might have been more successful. This would explain, why hardly any *Rhipicephalus (B.)* ticks were found on cattle of this farm.
Recent research (2007) identified a fourth *Rhipicephalus (B.)* species in the South of the Ivory Coast, *R. (B.) microplus* [70]. Originally a typical species of Southeastern Africa, *R. (B.) microplus* is believed to be on the spread into different African countries [35]. However, no specimen was found on cattle during the present study (2005/2006) and the species is generally thought to be absent from West Africa [123]. This is in contrast with the study by Madder et al (2007) who collected *R. (B.) microplus* as only member of this genus and did not find any of the other *Rhipicephalus (B.)* in Azaguié (on a different farm than in the present study) [70]. *R. (B.) decoloratus*, which was the most abundant tick species identified for Azaguié in this study, however on a different farm, is distinguished from *R. (B.) microplus* by the following characteristics : it is the only species of the *R. (B.)* with 3+3 columns of teeth of the hypostome [123]. Further it can be distinguished from *R. (B.) microplus* by a setae bearing protuberance on the internal margin of the first articles of the palps [123] and the adanal plates of the two species differ in their shape and size [123]. All of these features could be clearly identified by microscopy in this study and the species was therefore classified as *R. (B.) decoloratus*. Further investigations are needed to clarify the presence of *R. (B.) microplus* in the Ivory Coast. However, one can imagine that the tick species was introduced by the importation of animals coming from a *R. (B.) microplus* endemic country. *R. (B.) microplus* (3 females) has already been reported beforehand on Zebu in the region of Korhogo (North) in 1993, most likely on importated livestock [29]. The settlement of a tick species in an area after it has been introduced by livestock coming from endemic countries, is indeed the way how *R. (B.) microplus* arrived in Southern Africa from Asia and has continued its spread [35]. Furthermore, *R. (B.) microplus* has displaced successfully *B. decoloratus* in other countries such as Tanzania [69] and South Africa [116].

The cumulative mean tick infestation for both *A. variegatum* and *Rhipicephalus (B.)* sp was highest in Sikensi, followed by Dabou. The two other herds, i.e. the one in Brofodoumé and the one in Azaguié, had lower tick burden. During the sampling, some cattle were found to be free of any ticks at specific times, but none of the animals did stay free of ticks throughout the study period.
Some cattle breeds, such as Zebu and trypanotolerant breeds, are thought to acquire resistance to ticks such as *Rhipicephalus (B.)* sp, whereas exotic breeds are highly

susceptible to tick infestation [23]. The natural resistance against ticks is expressed in a reduction of the number of females reaching engorgement, in a reduced weight of engorged female, in a reduction in tick fecundity and the number of larvae that hatches from the eggs [23]. Generally, N'Dama are considered to be less susceptible than Zebu to local species of ticks [75]. Significantly lower numbers of *A. variegatum* were found on N'Dama cattle than on Gobra Zebu in The Gambia [74], [75]. The cumulative tick count on Zebu cattle raised in Sikensi was much higher than on the cattle in Dabou and Brofodoumé, which supports the hypothesis that Zebu breeds are less adapted to tick infestation than the N'Dama breed or N'Dama/Zebu crosses raised in Dabou and Brofodoumé, respectively. However, no direct comparison between the three herds can be made, since they were raised in different zones and great variations existed in the acaricide treatment.
The low infestation degree in Azaguié, a farm which raised crossbreed cattle is in contrast to the findings in the literature, where crossbreeds are considered to be highly susceptible to tick infestation [23], [74]. However, six of the cattle were young calves which did not go to the pastures and carried hardly no ticks, which influences the total tick count. Despite the regular acaricide treatment, nevertheless a great number of *Rhipicephalus (B.)* ticks were found on these animals. It is most likely due to the regular acaricide treatment that infestation by *A. variegatum* of the crossbreed cattle was low.

Climatic factors such as humidity and temperature are known to influence and control the presence of tick species and the number of generations occurring per year [23]. In the South of the Ivory Coast, where no marked differences between dry and wet seasons exist and where climatic conditions are more equal than in the Northern savannah, the prevailing conditions favour the presence of ticks all year round. In general, the absence of an unfavourable season in equatorial Africa allows evolution throughout the year and generations are normally overlapping [23]. Indeed, *A. variegatum* was present throughout the year and all stages were found simultaneously on the cattle. In the neighbouring Ghana, in the plain of Accra, which lies in the same climatic zone as this study, *A. variegatum* was also present throughout the year [63]. Therefore, a year round challenge of *A. variegatum* is experienced by the livestock production in these zonal latitudes. Though the absence of a very marked seasonality as it was found in the North (Achi, unpublished data), seasonal variations in infestations could nevertheless be observed. Nymphs and larvae had a tendency to be more present during the dry season. Adult *A. variegatum* were present throughout the year in constant numbers, showing a slight peak in March/April depending on the location. Increase in numbers was therefore mainly due to an increase in numbers of larvae and nymphs. Numbers of immatures were higher before the beginning of the main rain season in February and again after the rainy season was finished in August. Sikensi was the only farm, where first the num-

ber of larvae increased (January and August) and was then followed by an increase in numbers of nymphs (February and September). Moving northwards in the Ivory Coast, already in the district of Toumodi, inverse trends for immature and adult ticks were reported [60]. Adults were most frequent during the rainy season (April-June), whereas nymphs were present mainly during the dry season. In October a second small peak of adults was observed at the end of the small rainy season, which implicates nevertheless the presence of two generations per year [60]. In the very North, only one generation was present, the appearance of larvae, nymphs and adults was successive and very marked, and generations did not overlap anymore (Achi, unpublished data). The evolution was marked by the unfavourable conditions during the dry season. This is in accordance with what was described in drier countries, such as Mali [115] and Cameroon [84]. In the North, *A. variegatum* therefore follows the typical tick-population dynamic of West Africa, i.e. adults synchronise with the rainy season and immatures are more frequent during the dry season [77]. The reason is, for example in *A. variegatum*, that females need a certain humidity rate to activate host seeking behaviour. This prevents engorgement and egg laying by females during a period when rains are still sparse, which might put in danger the survival of desiccation-sensitive eggs [108].
In West Africa, *Rhipicephalus (B.)* species are generally present throughout the year [117] [81] [77]. Also in the Ivory Coast, *Rhipicephalus (B.)* species did not seem to be affected by the seasonal conditions. All three *Rhipicephalus (B.)* species were present throughout the year and the short cycles allowed several generations per year. All *Rhipicephalus (B.)* showed several infestation peaks (in general three) during the year. The main peaks occurred in the same periods as for *A. variegatum*. Likewise, *Rhipicephalus (B.)* species were not affected by seasonal changes in the northern savanna.
R. lunulatus was the only species with a marked seasonal activity. Adults were only present during the months of rain and disappeared afterwards. Nothing is known about the seasonal pattern of activity of the immatures, since they do not feed on cattle.

Acaricide control is a widespread practice throughout Africa and is also the number one strategy to control the tick burden in the Ivory Coast. The treatment is not standardized and therefore the component and number of products used and the frequency of application varied among the study sites. A big problem of the acaricide control is the misuse of products, since the income is often very low and the acaricides are considered to be too expensive [108]. This often led to inadequate dilutions and large between-treatment intervals. Beside a possible resistance in ticks against the acaricides, this might be another reason, why farm owners reported the acaricides to be inefficient. Indeed, the current treatment scheme did not seem to influence greatly the presence of the two main tick species, i.e. *A. variegatum* and

Rhipicephalus (B.) sp. However, it can be assumed that tick burden would nevertheless have been higher without any treatment. At the current knowledge, it is difficult to know, if the two facts, that hardly no specimens of *Rhipicephalus (B.) sp* could be found in Dabou and that significant lower numbers of *A. variegatum* were present in Azaguié, can be attributed to the acaricide treatment or if ecological or cattle breed factors played a major role.
Extensive use of acaricides always carries the risk that local tick species develop resistance against the acaricide in use. Evidence exists (Achi, person. communication) that certain tick strains might have become resistant to specific acaricides in the Ivory Coast. The main formula applied in the study sites are acaricides belonging to the classes pyrethroides or amidines. The pyrethroides are highly effective and known to have prolonged residual activity (7-10 days) [79]. However, resistance is widespread especially in 1-host ticks. Within the amidines, Amitraz is also very effective against ticks and known for its prolonged residual activity (7-10 days) [79]. Unfortunately, cross-resistance to DDT precluded or abbreviated the use of pyrethroids in countries such as Australia and South-Africa [45]. There were also reports of Amitraz resistance in *R. (B.)* populations of South Africa [45]. Resistance has progressively eliminated or limited the use of arsenic, organophosphates, carbamated and pyrethroids in different countries and the spectrum of chemical groups to which ticks have evolved resistance continues to broaden [45]. It therefore seems necessary to evaluate the resistance to the diverse acaricides in the Ivory Coast.

10.5 Prevalence of tick-borne parasites in cattle

Data available for the prevalence of tick-borne parasites such as *Anaplasma*, *Ehrlichia*, *Babesia* or *Theileria* in the Ivory Coast are scarce. In particular, the epidemiology of tick-borne parasites has never been addressed in the South of the Ivory Coast. In addition, this is the first time ever, that molecular methods have been used to study the prevalence of tick-borne parasites in cattle in this country. PCR followed by RLB hybridization, designed to detect parasites of the species *Anaplasma/Ehrlichia* and *Babesia/Theileria*, showed the presence of *A. marginale*, *Ehrlichia sp*, *B. bovis*, *B. bigemina*, *T. mutans* and *T. velifera* in the blood of cattle raised in the South of the Ivory Coast. *Ehrlichia* sp and *T. velifera* detected in this study are reported for the first time in the Ivory Coast.
The range of tick-borne haemoparasites found in domestic cattle in the Ivory Coast complements the earlier findings by blood smear analyses [60] [80] (Achi, unpublished data) and is in agreement with the spectrum of tick-borne parasite species generally reported for West African countries [17] [51] [50] [117] [77] [88] [84] [3].
In terms of frequency, *T. mutans* and *T. velifera*, outnumbered by far the more

pathogenic species such as *B. bigemina*, *B. bovis*, *A. marginale* and *Ehrlichia sp* in this decreasing order. In a blood smear survey in the neighbouring Ghana, *T. mutans* and *T. velifera* were also the most common parasites (97% and 87%, respectively) [17]. *B. bigemina* was the next frequent species, as in the Ivory Coast, but the overall prevalence was much higher (61%). *Anaplasma* sp had also a much higher incidence in Ghana (60%) than in the Ivory Coast.

To our knowledge, no epidemiological surveys using RLB to study the presence of tick-borne parasites in cattle have been done in any other West African country. The closest comparisons to this study are therefore the application of RLB in the Sudan [101] and in Uganda [87]. Oura et al have found similar high level of *T. mutans* and *T. velifera* in indigenous cattle (African Shorthorn) by RLB (95 and 91% respectively) [87]. Equally, *T. mutans* had a high prevalence (73%) in Sudan with *T. velifera* being less frequent (45.3%) [101]. Besides, 18% of cattle carried *A. marginale*, 23% *E. bovis*, 1 cattle was positive for *B. bigemina* in Uganda, whereas no positive was found for *B. bovis*, *A. centrale* and *E. ruminantium* [87]. In Sudan the prevalence for *B. bovis* and *B. bigemina* were of 1.7% and 0.3%, respectively [101]. No indications were given about parasites of the group AE.

The high level of prevalence for *T. mutans* and *T. velifera* could reflect a high and continuous challenge by these two parasites. Probably cattle do not develop resistance to *T. mutans* and *T. velifera*, since they normally induce no clinical disease. Whereas reports exist for pathogenic strains of *T. mutans*, *T. velifera* is always involved in subclinical disease. Sugimoto and Fujisaki (2002) suggest that in endemic area, all calves acquire *T. mutans* early in life and remain life-long carriers [113]. This fact is supported by the high prevalence of the parasite in cattle and the results of a study in Ghana, where at the end of the study 100% of the surviving animals were infected with both species [17]. The lower rates of detection for *A. marginale*, *B. bovis* and *B. bigemina* would suggest either lower challenge by these parasites or reflect the capability of the animals to develop resistance.

An advantage of the RLB assay is the detection of coinfections. Indeed, coinfections were frequent, specially for *T. mutans* and *T. velifera*. Although, pathogenic forms, i.e. disease causing parasites, were found together in the same animals, the majority of animals did not carry more than one pathogenic form at the time. Multiple infections were also reported in other studies [101]. Further, multiple parasitaemic episodes for the same pathogen, i.e. *B. bovis*, *B. bigemina* or the catch-all AE could be observed in animals when infections were followed over the time span of 12 months. Animals would show an infection with the same pathogen once, twice or several times during the study period. It is not clear, whether these episodes were due to recrudescence or reinfection. In the case of *B. bovis* infection was often seen to persist for several months.

The frequency as well as the combination of individual parasites varied greatly from farm to farm. The difference was specially marked for infections by *A. marginale*,

Ehrlichia sp, *B. bovis* and *B. bigemina*. Generally spoken, the study sites Sikensi and Brofodoumé had a higher prevalence and greater spectrum of parasites present than Azaguié and Dabou. However, it is difficult to evaluate the cause for this. The differences in the prevalence of the diverse parasites might be due to differences in the management of a farm, to tick control practices, cattle breeds as well as climatic factors such as local humidity and temperature which influence the presence or absence of vector species.

Sensibility for tick-borne diseases is long known to vary among different breeds and genotypes within a breed [23]. Local breeds are more robust, better adapted to climatic and nutritional conditions and have coevolved with vectors and vector-borne parasites, whereas exotic breeds are selected for their productivity and are less adapted to tropical climates and the poor nutrition often found in tropical regions [23]. Even though that, Zebu are less sensible to the effects of tick-borne parasites than exotic breeds in many parts of Africa as well as Australia [23], evidence shows, that N'Dama might be the best adapted cattle breed in West Africa [75] [74]. N'Dama breeds are well adapted to their environment and also resist the attachment of long-hypostome ticks better than Zebu breeds [74]. N'Dama might even have a certain tolerance to babesiosis and other tick-borne diseases. Not only were they less susceptible to local tick species than Zebu, but also serological prevalence for *A. marginale* was lower in N'Dama [74]. For *B. bigemina* no difference could be found [74]. More deleterious effects on animal production and on health by ticks and tick-borne diseases were shown for Zebu than N'Dama [74].

Oura et al noticed differences in the prevalence of *T. mutans* and *T. velifera* in indigenous and crossbred cattle in Uganda [87]. The prevalence for both species were also lower in Azaguié, which raises crossbred cattle designed to improve the milk and meat production, compared to the other farms. This is most possibly due to the fact that some of the cattle were calves younger than one year which did not go to the pasture. On the other hand, the infestation by *A. variegatum*, the vector species for *T. mutans* and *T. velifera*, was also low compared to the other farms.

The nearly complete absence of *A. marginale*, *Ehrlichia* sp, *B. bovis* and *B. bigemina* from Dabou can possible be attributed to the negligible presence of vector species. This might be the consequence of a very strict acaricide treatment of N'Dama, which are already known to have a certain resistance to both tick infestation by *Rhipicephalus (B.)* ticks and the pathogen they transmit. However, attention should be paid in maintaining an endemic stability.

The overall tick infestation was very high in the Zebu cattle raised in Sikensi and both, *B. bovis* and *B. bigemina*, as well as *A. marginale* and *Ehrlichia* sp were present in important numbers. Zebu breeds are generally believed to be less resistant to tick-borne diseases [74]. In addition treatment was undertaken rather irregularly in this farm which did favour both the higher numbers of infections and higher infestation of ticks.

The crosses of N'Dama and Zebu held in Brofodoumé, were infected with *B. bovis*, *B. bigemina*, *A. marginale* and *Ehrlichia* sp. Treatment was irregularly practised, but cattle were well adapted to the tick-borne parasites and no mortalities were observed.

The different cattle breeds raised in the farms of the present study, are very likely to contribute to the fact that tick infestation and prevalence of the different parasites did vary between the study sites. However, cattle were kept in different zones and acaricide treatments were handled differently in the four farms which does not allow to compared tick infestation and the prevalence of the various pathogens between the cattle breeds.

The fact that no *E. ruminantium* could be detected in the blood of cattle is in contrast to the findings in the district of Toumodi where blood analyses of N'Dama cattle showed a seroprevalence of 30.9% for *E. ruminantium*. *E. ruminantium* was also present in brain smears in the North (Achi, unpublished data). Although heartwater is known to exist in the Ivory Coast, *E. ruminantium* could be detected only once in a nymphal *A. variegatum* in the present study. However, animals died showing nervous signs during the study year.

Nevertheless, the high seroprevalence of *E. ruminantium* in Toumodi could also be due to a known crossreactivity of the ELISA with other *Ehrlichia* sp species, which have been shown to be present in this study. It is unknown, if the absence of *E. ruminantium* in the blood samples analysed, reflects the general difficulty to detect *E. ruminantium* in living cattle or questions the capacity of the RLB method in detecting this pathogen. However, the prevalence of *E. ruminantium* might have also been reduced by the acaricide treatment [76] in this study compared to the untreated N'Dama cattle studied in the Center [60].

The capacity to detect *E. ruminantium* in *A. variegatum* of a nested pCS20 PCR, a nested map1 PCR and a nested RLB assay have been compared in a study in The Gambia [36]. The RLB detected a significantly lower proportion of *E. ruminantium* positive samples and showed a lower sensitivity, whereas the nested pCS20 PCR showed the highest performance[36]. The same results were obtained for carrier animals [36]. *E. ruminantium* was successfully detected in experimentally infected sheep during the clinical phase of infection, but could not be detected in the carrier stage [15]. The results suggest the need for further optimisation of the RLB for application in epidemiological investigations of heartwater. To further evaluate the RLB and the heartwater situation in the Ivory Coast, it might be good to apply the pCS20 PCR in parallel, since it is known to give reliable results. The lack of *E. ruminantium* positive samples might have many reasons and one should not conclude from it that the disease is absent from the South of the Ivory Coast. The heartwater situation in neighbouring Ghana is similar to that in the Ivory Coast. Although cattle were found to be positive for heartwater by ELISA, only in a few cases the presence of *E. ruminantium* could be confirmed by brain smear analyses

[16].

In the North of the country, where the contrast between the rainy and dry seasons are marked and therefore for instance adult and nymphal *A. variegatum* ticks showed inversed seasonal patterns, infection by *Theileria* sp seemed to vary with the fluctuation in tick infestation (Achi, unpublished data). Only little is known for other countries of West Africa. The seasonal variation of ticks in the North West Province of Cameroon did not seem to correspond with any variation of *Babesia* in cattle [84]. In contrast, in Senegal haematoparasites of the type *Theileria* were more abundant during the rainy season and detection of positive blood smears decreased during the dry season [50]. This was especially marked for *T. mutans*. No effect was seen in the prevalence of Anaplasmosis. Further, in The Gambia, *A. marginale* and *B. bigemina* showed a seasonal peak at the end of the rainy season [24]. Pronounced effect of seasons can be seen in infection rate of *T. parva* which could be attributed to the seasonal activity of the vector *R. appendiculatus* in Sudan [101]. The same situation is present in Zambia, where *T. parva* occurs mainly during the rainy period [101].
In the South of the Ivory Coast, seasons are less marked and favourable conditions are prevailing for most of the year. The main tick species (*A. variegatum* and *Rhipicephalus (B.)* sp) can be found whole year round, showing several peaks during the study year. Most likely, the number of infections will possibly raise together or shortly after heavy tick infestation, but since no marked seasonality could be shown in the prevalence of tick species, a marked seasonality is most likely also absent in the prevalence of haematoparasites in the South of the Ivory Coast. Unfortunately, the overall prevalence of individual parasites was too low to give statistically significant seasonal data. However, the overall prevalence of the most frequent species *T. mutans* and *T. velifera* did fluctuate during the study year. Indications exist, that pathogens such as *A. marginale*, *B. bovis* and probably also *B. bigemina* might fluctuate in numbers with the seasons. An important difference could be seen in the number of positive samples detected either by microscopy or RLB. Most of the blood smears were negative, whereas the RLB allowed to detect and simultaneously determine many different species. In fact, surprisingly few positives were detected by blood smear analyses, namely 15 blood smears were found positive for *B. bovis*. Ruling out a lack of technical skills as well as bad quality of blood smears, it is most possible that the absence of observed parasites in the blood smears does indicate the prevalence of low parasitaemia levels in the South of the Ivory Coast. This is in accordance to the few acute clinical cases observed during the study. In the acute phase, parasites can normally easily be diagnosed in routine blood smear, whereas subclinical infections with very low levels of parasites do persist in the peripheral blood, which can not be detected microscopically [5]. Animals with subclinical infections are a perpetual source of infection for ticks [5]. Molecular methods with

their high sensitivity help to detect these low levels of parasitaemias.

10.6 Prevalence of tick-borne parasites in their vector species

No real lead exists for the prevalence of tick-borne parasites relevant to cattle in vector ticks for the Ivory Coast. This is the first time that *A. variegatum*, *R. (B.) geigyi* and *R. (B.) annulatus* ticks collected from cattle were analysed by RLB for the presence of tick-borne parasites in the Ivory Coast. Not only *A. variegatum* but also the different *Rhipicephalus (B.)* species are well known cattle ticks and are known to transmit various pathogens [23] [123].
In this study, the presence of *T. mutans*, *T. velifera* and in a single occasion of *E. ruminantium* could be shown in *A. variegatum*. These findings are in accordance to the widespread abundance of *T. mutans* and *T. velifera* in cattle which suggested their presence in the vector species *A. variegatum*. It matches also well the absence of *E. ruminantium* in cattle. Furthermore, the presence of these parasites is in accordance with the fact that *A. variegatum* is a known vector for *E. ruminantium*, *T. mutans* and *T. velifera* [23] [123]. Although, it was not possible to show *E. ruminantium* in the blood of the studied cattle, it would nevertheless have been expected to detect it in the vector species.
In addition, 6 cases of *B. bovis* were detected in adult and nymphal *A. variegatum*. This was rather unexpected, since *A. variegatum* is not a vector species for *B. bovis* and the pathogen is normally transmitted by species of the genus *Rhipicephalus (B.)* sp, the most famous being *R. (B.) microplus* and *R. (B.) annulatus* [23] [123]. It is unknown, if *B. bovis* was detected in the ingested blood meal, acquired through co-feeding [96] or if this represents a contamination by the positive for *B. bovis* during the manipulation. However, all controls, for detecting a possible contamination, were negative during the analyses of these samples. Great care was taken in preventing contaminations, but it can nevertheless not be completely ruled out. The detection of parasites in *A. variegatum* in the Ivory Coast did so far concentrate on rickettsiales of the type *R. conorii* and *R. africae* [46] [29] and did not permit any conclusions for the parasites studied in the present survey. However, other organisms, namely *T. theileri*, which are not thought to be transmitted by *A. variegatum* were reported to be present in the tick beforehand [46].
In West Africa, the presence of parasites in *A. variegatum* by RLB was assessed before in Guinea [118] and The Gambia [36]. However, the latter concentrated mainly on heartwater, whereas the study in Guinea is the only example where the RLB assay did also include probes for the group BT. In The Gambia, 6.2% (9/145) of the adult ticks analysed were infected by *E. ruminantium* [36]. Also one case

of coinfection with *A. marginale*, a parasite not transmitted by *A. variegatum*, was detected [36]. In the study, no PCR amplification for the group BT was performed. On the other hand, in Guinea, neither *E. ruminantium* nor other parasites belonging to *Ehrlichia* and *Anaplasma* species could be detected [117] even though the sample size of the ticks analysed was greater than in The Gambia. *T. velifera* was detected in 1% of the ticks. In addition, in 2% *B. caballi* was present, a parasite which is transmitted by *Hyalomma* and not known to be transmitted by *A. variegatum* ticks [117].
These findings together with our own results, give strong evidence that RLB allow detection of small numbers of parasites ingested with the blood meal in ticks that are not recognized as their vectors. Even ticks that were apparently unfed at the time of collection, might nevertheless already have ingested a small amount of blood which makes detection possible. The knowledge of the presence of parasites in ticks and the role of species to act as vectors, was until now mainly obtained by PCR analyses. PCR is designed for targeting a specific species and studies concentrated on parasites having major impacts on cattle health, such as *A. marginale*, *E. ruminantium*, *B. bovis*, *B. bigemina*, *T. parva* and *T. annulata*. It is only now, by simultaneous detection of parasites by RLB, that more coinfections are discovered.

The spectrum of parasites present was greater in *R. (B.) geigyi* than in *R. (B.) annulatus*. Nymphal *Rhipicephalus (B.)* sp showed the same spectrum of parasites as *R. (B.) geigyi*. Only *B. bovis* was detected in *R. (B.) annulatus*, whereas *A. marginale*, *Ehrlichia* sp, *B. bovis* and *B. bigemina* were present in *R. (B.) geigyi*. The absence of *B. bigemina* and *A. marginale* in *R. (B.) annulatus* ticks, which have been collected from cattle of Sikensi, a farm where the prevalence for both *B. bigemina* and *A. marginale* were shown in cattle, was unexpected. All the more, since between the two *Rhipicephalus (B.)* species studied, *R. (B.) annulatus* is a well known vector species for *B. bovis*, *B. bigemina* as well as *A. marginale* [123]. The vector role for *R. (B.) geigyi* is only poorly known [123]. Most likely, the very short timeframe for which *R. (B.) annulatus* was studied for the presence of tick-borne parasites, did falsify the spectrum of parasites detectable in this species. The ticks analysed were sampled restrictively during the peak of abundance of this tick species, in January and February. During these months, no cases of *A. marginale* nor *Ehrlichia* sp were found in cattle in Sikensi and only two cases of *B. bigemina* occurred. Indeed, one *R. (B.) geigyi* infected with *B. bigemina* was found in Sikensi. It is necessary to enlarge the sample of ticks analysed and to expand it to cover different months of the year to get a clear idea of the parasites present in *R. (B.) annulatus* species.
On the other hand, findings suggest *R. (B.) geigyi* playing an important role in parasite transmission in the Ivory Coast. However, it needs to be confirmed that the species actually transmits the parasites. This is the first time, *Ehrlichia* sp was reported in *R. (B.) geigyi*, whereas the presence of *B. bigemina* as well as *T. theileri* in

the hemolymph of *R. (B.) geigyi* was already reported beforehand [47].
In both, *R. (B.) annulatus* and *R. (B.) geigyi*, the protozoa *T. mutans* and *T. velifera* were detected by RLB. Further investigations are needed to see, if the parasites were simply acquired with the ingested blood or were actually present in the tick beforehand. To our knowledge, so far no report exists of this species being present in *Rhipicephalus (B.)* ticks which are not known to transmit either *T. mutans* nor *T. velifera*. However, these *Theileria* species were so far of minor interest, since they are not believed to be involved in serious disease outbreaks. It is only now with the use of RLB that coinfections were discovered. Until now, no *Rhipicephalus (B.)* species was so far studied by RLB for the presence of protozoan parasites in West Africa or Africa. The only RLB study of a *Rhipicephalus (B.)* analysed *R. (B.) microplus* in Tibet, but was restricted to the detection of *Anaplasma* and *Ehrlichia* [125]. It needs to be awaited for other studies by RLB to be able to compare the present findings.
Hardly no *B. bigemina* parasites were found in ticks. This might be due to the restricted time frame and the low number of ticks analysed. In addition, in unfed ticks the number of sporozoites present in the salivary glands is low and only increases during feeding [86]. This might lead to an underestimation of prevalence of tick-borne parasites in ticks in cases where parasite numbers are below the detection limits of the RLB.
Good correlations were obtained of the presence of parasites in ticks collected from cattle at the time when infections could be detected by RLB in the blood. A great majority of parasites present in the animal could also be detected in the ticks feeding on the cattle. There is evidence for the transport of parasites during the feeding process as known from vector-host models. It was more difficult to show the evidence for the inverse process, i.e. a correlation between an infected tick feeding on an animal which was apparently negative at that time and the cattle becoming positive for this parasite afterwards. However, it was possible for a few cases. Infections with *Babesia* sp are the only one known to be transmitted transovarially, whereas all other parasites are transmitted transstadially. In the cases of *Babesia* feeding females do get infected or reinfected and pass the parasites on to their progeny [23]. Even though *Rhipicephalus (B.)* ticks normally pass their entire development cycle on the same animal, there is nevertheless a certain percentage of ticks which fall from an animal accidentally and can then transfer infections from one animal to another [23]. This could explain, why an animal still can become infected even, if the tick population on it was apparently negative. Further, it is also wise to keep in mind, that in case of negative samples, it can never be completely ruled out, that the parasite was simply present in a number which was to low for detection by RLB. Further, in the case of *A. marginale* and *Ehrlichia* sp and possibly also other parasites, it can not be ruled out that other species, such as tabanide flies might be involved in transmitting parasites [130].

10.7 Identification of new *Anaplasma* and *Ehrlichia* species

For a proportion of samples, a catch-all only signal without any species-specific hybridization was obtained, indicating the presence of potentially novel species or a variant of an existing species in these samples. Indeed, 16S rRNA sequence analyses discovered three new groups of « sequences » for the Ivory Coast, namely two *Anaplasma sp* sequences showing 97% similarity with an *Anaplasma sp* sequence which has been identified in the midgut of a field-caught *Anopheles* [66]; two *Ehrlichia sp* sequences showing 98% and 99% similarity with the *Ehrlichia sp* (strain Tibet) sequence which has been discovered in *R. (B.) microplus* ticks from Tibet [125] and two *A. phagocytophilum*-like sequences showing 92% and 96% similarity with *A. phagocytophilum* discovered in the blood of a Norwegian sheep [112]. On the other hand, 16S rRNA sequence analyses revealed also sequences identical with the sequence of *A. marginale*, with two sequences showing a point mutation in the region of the probe sequence. While the point mutations could explain why samples did not hybridize with the species-specific probe, the identical samples indicate that the parasitaemia level in the blood was probably close to the threshold of the detection capacity of the probe for *A. marginale*.

In general, it is very difficult to classify species of the Anaplasmataceae and the determination of novel strains, variants or species cannot be made on the analyses of a single gene sequence. Further data have to be accumulated in order to propose a better classification system for these groups. The identified sequences were therefore classified as belonging to *Anaplasma sp* IC; *A. phagocytophilum*-like IC and *Ehrlichia* sp IC.

Two species of the type *Anaplasma* sp were discovered in cattle in the Ivory Coast. It is not known, if the same sequences were also present in the ticks. The sequence was similar to one discovered in the gut of field-caught *Anopheles* mosquito in Kenya [66]. However, in the study it could not be evaluated, if the bacterium can actually be transferred by the mosquito or if it was ingested with the blood meal. Further analyses are needed to see, if these *Anaplasma* sp are transmitted by mosquitoes and/or ticks and whether the organism has any harmful effect on cattle.

In the present study, two types of *Ehrlichia* sp were detected in cattle, one being similar to the *Ehrlichia* sp of the strain Omatjenne from Mozambique [15] and one the newly discovered *Ehrlichia* sp IC, which is similar to the one discovered in Tibetan *R. (B.) microplus* [125]. *Ehrlichia* sp IC was first discovered in *R. (B.) geigyi*, but could subsequently also be detected in one animal. *Ehrlichia* species are known as important pathogens of medical as well as veterinary importance [125]. Phylogenetic tree analyses performed by Wen et al (2002) showed that the novel ehrlichial

agent, they discovered, belonged to the *E. canis* group (most closely related to *E. chaffeensis*) and is not closely related to the *A. phagocytophilum* group [125]. Their role in cattle disease in the Ivory Coast needs to be further evaluated. So far an *Ehrlichia* sp (Germishuys) was described as causing heartwater in sheep by Allsopp et al [9] implicating that *Ehrlichia* sp might play a role in heartwater, a theory which should be investigated further. In any case, the *Ehrlichia* sp are a possible source for cross-reactions in ELISA tests [9].

Two sequences of *A. phagocytophilum*-like pathogens were discovered in one *R. (B.) annulatus* as well as in one animal and suspected in two other cows. In essence, the sequences did already vary among themselves and were also rather distantly homologous to the *A. phagocytophilum* to which they were most closely related by Blast analyses [112]. Possibly the difference can be attributed to strain differences between European and African *A. phagocytophilum*, but might as well represent a species on its own. Further analyses are needed to determine the position of these novel organisms in the classification of the Anaplasmataceae.
A. phagocytophilum is known to cause tick-borne fever (TBF) in sheep and is a well-known disease in domestic ruminant in Europe [112]. In sheep, TBF is characterized by high fever, reduced milk yield abortion and reduced fertility. To date, many variants are known for *A. phagocytophilum* [112] [9]. The pathogen was mainly considered as European and was not thought to be involved in the African tick-borne disease complex so far.
In another study, a new *Anaplasma* species closely related to *A. phagocytophilum* was reported to occur in Africa [55]. Organisms closely related to this new *Anaplasma* were also detected in the blood of a goat in Mozambique that died with clinical signs suggesting heartwater due to *E. ruminantium* [55] and also in sheep in South Africa which had signs of heartwater. In the goat, *E. ruminantium* could not be detected, whereas the sheep was coinfected by *E. ruminantium* [9].

Although, the role of the newly discovered species in the heartwater diseases complex must be investigated further, there is a strong evidence that possibly not only *E. ruminantium* is causing heartwater disease in domestic animals. This would explain, why instead of the presence of clinical symptoms, it was not possible to detect pathogens of the type *E. ruminantium*. Additional samples should be used to confirm species-specific determination. There were still samples left, which did not react with any of the species-specific probes by RLB. Investigation of these samples is expected to either confirm the presence of the novel agents or to broaden the range of organisms present in the Ivory Coast even more. Conclusive, the presence of these pathogens suggests their involvement in causing diseases in cattle and suggest that tick-borne diseases in the Ivory Coast might be more complex than assumed so far. However, it needs to be evaluated, if they remain asymptomatic in cattle or if they might contribute to the disease complex of tick-borne diseases in the Ivory Coast.

The reverse line blot in combination with DNA sequence analyses has shown to be a powerful tool in clarifying and determining novel organisms. Two newly designed probes for *Ehrlichia* IC and *A. phagocytophilum*-like IC were included into the RLB. However, the latter needs to be further improved in order to assure its capability to hybridize with the corresponding samples. Possibly, more than one probe needs to be designed in order to cover the spectrum of variances in these sequences. It is suggested also to incorporate a probe for *Anaplasma* IC.

10.8 Conclusion and outlook

The here established RLB-PCR assay has proved a valuable tool in gaining baseline data on the prevalence of tick-borne parasites in the Ivory Coast and has permitted to discover novel parasite sequences which were not reported to be present in the Ivory Coast before. In addition, a great diversity of parasites could be detected in *R. (B.) geigyi* ticks, a species of which the vector role is only poorly understood. The prevalence of parasites present in the cattle was moderate, animals did rarely show clinical disease and the tick infestation of the diverse tick species was reasonable.

A follow-up study should address the following objectives:

- To study a larger sample of ticks for the presence of tick-borne parasites including samplings at different times throughout the season in order to get an accurate picture of the prevalence of parasites in the vector ticks, including *R. (B.) decoloratus*, by RLB-PCR.
- To study the vector-host relationship for the parasites detected in ticks and cattle in this study. Elaborate for instance, if *T. mutans* and *T. velifera* can be transmitted by *Rhipicephalus (B.)* species or if they are leftovers in the blood meal.
- Address the topic of the presence of *R. (B.) microplus* in the Ivory Coast.
- Set up a study to monitor closely possible occurrence of acaricide resistance in the local tick species in the four sites studied.
- Study in more detail the causes for the differences of local tick species and the prevalence of tick-borne parasites in order to explain it.
- Address the question of heartwater in more details in the Ivory Coast, since this study was not able to give any real answers. It is suggested to apply pCS20 PCR and ELISA in addition to the RLB-PCR in order to better evaluate the findings by RLB-PCR. Investigate the possible role of the here discovered *Ehrlichia* sp and *A. phagocytophilum* organisms in causing heartwater disease.

- Exploit the spectrum of additional tick-borne pathogens.
- Establish detection threshold for individual species-specific probes of the RLB-PCR. Incorporate Uracil DNA glycosylase into the PCR assay to avoid contamination by previous PCR amplification.

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Appendix A

Statistical Analyses

Abbreviations used for appendix:

The following abbreviations are utilised to describe the parasites:

For the tick species:

Av = *A. variegatum*;
Bd = *R. (B.) decoloratus*;
Bg = *R. (B.) geigyi*;
Ba = *R. (B.) annulatus*;
Rl = *R. lunulatus*;

For the parasites:

Aph, *A. phagocytophilum*;
Eru, *E. ruminantium*;
Ama, *A. marginale*;
Ace, *A. centrale*;
Abo. *A. bovis*;
Ehr.sp or Esp *Ehrlichia* sp;
AE, Catch-AE;
BT, Catch-BT;
Bbo or Bbov, *B. bovis*;
Bbi or Bbig, *B. bigemina*;
Bdi, *B. divergens*;
Tan, *T. annulata*;
Tpa, *T. parva*;
Tmu, *T. mutans*;
Tve, *T. velifera*;
Tryp, *Trypanosoma* sp;
Tbru, *T. brucei*;
Tcon, *T. congolense*;
Frottis, thin blood smear;
G.paisse, thick blood smear;

For the farms:
Me, Sikensi;
Ca, Dabou;
Br, Brofodoumé;
Az, Azaguié;

A.1 Statistical analyses of Chapter 6

A.1.1 Tick genus and species found on cattle in the southern region of the Ivory Coast

A paired t-test was used to evaluate the frequency of the different tick species found in the Southern region of the Ivory Coast. The following abbreviations are used to indicate the species: Av = *A. variegatum* ; Bd = *R. (B.) decoloratus* ; Bg = *R. (B.) geigyi* ; Ba = *R. (B.) annulatus* ; Rl = *R. lunulatus*. All statistical analyses were performed using the S-Plus Version 7.0 statistic program.

Paired t-Test
data: Av and Bd
t = 15.1412, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
10.66827 13.84705
sample estimates:
mean of x - y
12.25766

Paired t-Test
data: Av and Rl
t = 20.8334, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
13.38103 16.16540
sample estimates:
mean of x - y
14.77321

Paired t-Test
data: Av and Bg
t = 19.0805, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
11.98910 14.73931
sample estimates:
mean of x - y
13.36421

Paired t-Test
data: Bd and Bg
t = 2.6138, df = 717, p-value = 0.0091
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
0.2753975 1.9376944
sample estimates:
mean of x - y
1.106546

Paired t-Test
data: Av and Ba
t = 19.8913, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
12.21441 14.88958
sample estimates:
mean of x - y
13.552

Paired t-Test
data: Bd and Ba
t = 3.0117, df = 717, p-value = 0.0027
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
0.4505748 2.1380975
sample estimates:
mean of x - y
1.294336

Paired t-Test
data: Bd and Rl
t = 6.959, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
1.805867 3.225238
sample estimates:
mean of x - y
2.515552

Paired t-Test
data: Bg and Rl
t = 6.8312, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
1.004063 1.813950
sample estimates:
mean of x - y
1.409006

Paired t-Test
data: Bg and Ba
t = 0.6863, df = 717, p-value = 0.4927
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
-0.3494027 0.7249830
sample estimates:
mean of x - y
0.1877902

Paired t-Test
data: Ba and Rl
t = 5.7784, df = 717, p-value = 0
alternative hypothesis: mean of differences is not equal to 0
95 percent confi dence interval:
0.806293 1.636140
sample estimates:
mean of x - y
1.221216

A.1.2 Distribution of the different tick species and their abundance in the four different study sites.

ANOVA analyses were performed to test the influence of the factor month and farm. The following abbreviations are used : mois = month, ferm = farm. The different months are represented by numbers : -1 = Nov 05, 0 = Dec 05, 1 = Jan 06, 2 = Feb 06, 3 = March 06, 4 = April 06, 5 = May 06, 6 = Jun 06, 7 = Jul 06, 8 = Aug 06, 9 = Sep 06, 10 = Oct 06. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié. Analyses were done using S-Plus Version 7.0

[1] "Amblyomma variegatum"

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Ferm	3	34757.1	11585.69	70.34684	0
as.factor(Mois)	11	60963.3	5542.12	33.65103	0
Ferm:as.factor(Mois)	33	54074.6	1638.62	9.94953	0
Residuals	670	110344.8	164.69		

Az	Br	Ca	Me
4.775641	12.43148	21.37853	21.40596

-1	0	1	2	3	4	5
5.967742	10.31667	19.01724	39.36667	23.03056	14.10833	9.1
6	7	8	9	10		
5.305085	7.042373	20.61111	15.19167	10.29167		

	-1	0	1	2	3	4
Az	1.058824	2.366667	2.766667	8.733333	8.088889	5.233333
Br	9.666667	7.766667	15.100000	24.333333	11.222222	9.366667
Ca	4.266667	16.600000	8.615385	73.166667	51.633333	20.933333
Me	9.533333	14.533333	48.200000	51.233333	21.177778	20.900000
	5	6	7	8	9	10
Az	2.666667	2.533333	2.266667	11.35556	4.50000	6.233333
Br	5.533333	6.766667	6.333333	23.82222	17.56667	11.700000
Ca	10.86666	7 5.600000	12.714286	24.20000	10.63333	15.033333
Me	17.333333	6.392857	7.233333	23.06667	28.06667	8.200000

[1] "Rhipicephalus (B.) decoloratus"

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Ferm	3	13419.53	4473.178	72.21450	0.000000e+000
as.factor(Mois)	11	2848.73	258.975	4.18087	5.311591e-006
Ferm:as.factor(Mois)	33	8616.94	261.119	4.21548	0.000000e+000
Residuals	670	41501.77	61.943		

Az	Br	Ca	Me
10.09524	0.1527778	0.1308851	0.1871508

-1	0	1	2	3	4	5
1.556452	1.991667	2.275862	7.8	4.758333	4.108333	1.908333
6	7	8	9	10		
1.618644	0.9491525	3.45	0.95	0.725		

	-1	0	1	2	3	4
Az	2.529412	7.63333333	8.50000000	30.20000000	18.8222222	16.2666667
Br	1.600000	0.06666667	0.00000000	0.16666667	0.0000000	0.0000000
Ca	0.000000	0.26666667	0.07692308	0.80000000	0.2111111	0.1666667
Me	1.966667	0.00000000	0.23333333	0.03333333	0.0000000	0.0000000
	5	6	7	8	9	10
Az	7.633333	6.366667	3.733333	13.8	3.8	2.86666667
Br	0.000000	0.000000	0.000000	0.0	0.0	0.00000000
Ca	0.000000	0.000000	0.000000	0.0	0.0	0.03333333
Me	0.000000	0.000000	0.000000	0.0	0.0	0.00000000

[1] "Rhipicephalus (B.) geigyi"

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Ferm	3	2144.51	714.8381	33.75325	0.000000e+000
as.factor(Mois)	11	1973.95	179.4501	8.47328	4.396483e-014
Ferm:as.factor(Mois)	33	3439.00	104.2122	4.92069	1.110200e-016
Residuals	670	14189.50	21.1784		

Az	Br	Ca	Me
0.2362637	4.340741	0	1.690875

-1	0	1	2	3	4	5
0.4919355	3.741667	3.172414	5.65	1.816667	0.8416667	1.066667
6	7	8	9	10		
0.6271186	0.2457627	1.025	0.1666667	0.04166667		

	-1	0	1	2	3	4
Az	0.000000	1.233333	1.633333	0.000000	0.000000	0.000000
Br	1.933333	9.666667	6.133333	18.666667	5.555556	2.200000
Ca	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
Me	0.100000	4.066667	4.500000	3.933333	1.711111	1.166667
	5	6	7	8	9	10
Az	0.000000	0.000000	0.0000000	0.0	0.0000000	0.00000000
Br	0.000000	2.466667	0.8333333	4.1	0.4333333	0.10000000
Ca	0.000000	0.000000	0.0000000	0.0	0.0000000	0.00000000
Me	4.266667	0.000000	0.1333333	0.0	0.2333333	0.06666667

[1] "Rhipicephalus (B.) annulatus"

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Ferm	3	4069.50	1356.501	72.10567	0.000000e+000
as.factor(Mois)	11	1808.97	164.452	8.74155	1.365574e-014
Ferm:as.factor(Mois)	33	5201.20	157.612	8.37798	0.000000e+000
Residuals	670	12604.50	18.813		

Az	Br	Ca	Me
0.01373626	0.01574074	0	5.513035

-1	0	1	2	3	4	5
0.5322581	3.416667	5.517241	1.875	0.5222222	1.825	0.8333333
6	7	8	9	10		
0.008474576	0.08474576	2.088889	0	0		

	-1	0	1	2	3	4	5
Az	0.0	0.00000	0.1666667	0.0	0.00000000	0.0	0.000000
Br	0.0	0.00000	0.1000000	0.0	0.08888889	0.0	0.000000
Ca	0.0	0.00000	0.0000000	0.0	0.00000000	0.0	0.000000
Me	2.2	13.66667	21.0666667	7.5	2.00000000	7.3	3.333333
	6	7	8	9	10		
Az	0.00000000	0.0000000	0.000000	0	0		
Br	0.00000000	0.0000000	0.000000	0	0		
Ca	0.00000000	0.0000000	0.000000	0	0		
ME	0.03571429	0.3333333	8.355556	0	0		

[1] "Rhipicephalus lunulatus"

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Ferm	3	15.6271	5.209044	18.32098	1.960099e-011
as.factor(Mois)	11	35.5626	3.232962	11.37081	0.000000e+000
Ferm:as.factor(Mois)	33	41.7889	1.266330	4.45387	1.921000e-014
Residuals	670	190.4952	0.284321		

Az	Br	Ca	Me
0	0.09074074	0.1572505	0.3975791

-1	0	1	2	3	4	5
0.04032258	0	0	0	0.2472222	0.8333333	0.275
6	7	8	9	10		
0.1694915	0.008474576	0.1333333	0.1	0.1166667		

	-1	0	1	2	3	4
Az	0.0000000	0	0	0	0.0000000	0.0000000
Br	0.0000000	0	0	0	0.2888889	0.6333333
Ca	0.0000000	0	0	0	0.2666667	0.5000000
Me	0.1666667	0	0	0	0.4333333	2.2000000
	5	6	7	8	9	10
Az	0.0000000	0.00000000	0.00000000	0.0000000	0.0000000	0.0000000
Br	0.0000000	0.03333333	0.00000000	0.0000000	0.1333333	0.0000000
Ca	0.2333333	0.10000000	0.00000000	0.2888889	0.0000000	0.4666667
Me	0.8666667	0.57142857	0.03333333	0.2444444	0.2666667	0.0000000

A.1.3 Tick infestation on individual cattle and herds of different study sites

The mean tick infestation per herd was calculated by dividing the total count of *A. variegatum* individuals found on the half body on all cattle of a site by the total samples size (number of cattle analysed) [109]. Statistical analyses have been done using SPSS Version 11.

Descriptive Statistics (SPSS)
The mean tick infestation per herd was calculated by dividing the total count of *A. variegatum* individuals found on the half body on all cattle of a site by the total sample size (number of cattle analysed) for a given sampling time [109].

Table A.21: Mean tick infestation of *A. variegatum* for all cattle of a given herd. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Farm	N	Min	Max	Mean	SD
Me	26	3.36	78.71	21.62	17.70
Ca	24	1.29	87.93	23.29	20.99
Br	26	1.5	28.67	12.61	8.34
Az	26	0.7	15.00	5.26	3.98

Table A.22: Minimal, maximal and mean total count per month for a given herd for *A. variegatum*. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Farm	N	Min	Max	Mean	SD
Me	26	47	1102	308.96	256.4
Ca	24	18	1319	325.75	304.7
Br	26	15	430	186.38	124.5
Az	26	1	210	73.46	57.7

Table A.23: Mean tick infestation of *A. variegatum* on cattle in the different study sites. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Sampling	Month	Me	Ca	Br	Az
1	Nov	19.90	-	4.60	2.10
2	Nov	4.64	4.27	14.73	.33
3	Dec	11.00	18.13	5.07	4.67
4	Dec	14.53	13.91	10.47	.07
5	Jan	19.86	8.62	10.33	1.47
6	Jan	78.71	-	19.87	6.88
7	Feb	56.53	47.90	27.53	3.93
8	Feb	45.93	87.93	21.13	12.33
9	Mar	32.92	57.80	3.47	9.87
10	Mar	14.33	53.23	13.13	3.00
11	Mar	20.07	44.93	17.07	10.62
12	Apr	28.20	28.40	7.20	5.20
13	Apr	13.60	13.47	11.53	7.18
14	Mai	13.40	5.93	3.07	1.93
15	Mai	21.27	15.07	8.00	3.40
16	Jun	3.36	9.80	4.20	2.33
17	Jun	9.43	1.29	9.33	2.73
18	Jul	11.00	12.57	1.50	1.0
19	Jul	3.47	11.21	9.53	2.87
20	Aug	38.20	14.40	27.40	7.93
21	Aug	21.33	27.93	24.67	10.33
22	Aug	9.67	31.20	19.42	15.00
23	Sep	35.33	5.47	28.67	5.00
24	Sep	19.69	15.40	6.47	4.00
25	Oct	10.67	14.20	15.87	9.60
26	Oct	5.73	15.87	3.69	2.87
Total N		26	24	26	26

Table A.24: Total count of *A. variegatum* at a given sampling time

Sampling	Month	Total count Me	No sampled Me	Total count Ca	No sampled Ca	Total count Br	No sampled Br	Total count Az	No sampled Az
1	Nov	199	10	-	-	69	15	21	10
2	Nov	65	14	64	15	221	15	5	15
3	Dec	110	10	272	15	76	15	70	15
4	Dec	218	15	153	11	157	15	1	15
5	Jan	278	14	112	13	155	15	22	15
6	Jan	1102	14	-	-	298	15	55	8
7	Feb	848	15	479	10	413	15	55	14
8	Feb	689	15	1319	15	317	15	185	15
9	Mar	395	12	867	15	52	15	148	15
10	Mar	215	15	692	13	197	15	42	14
11	Mar	301	15	674	15	256	15	138	13
12	Apr	423	15	426	15	108	15	78	15
13	Apr	204	15	202	15	173	15	79	11
14	May	201	15	83	14	46	15	29	15
15	May	319	15	226	15	120	15	51	15
16	Jun	47	14	147	15	63	15	35	15
17	Jun	132	14	18	14	140	15	41	15
18	Jul	165	15	88	7	15	10	6	6
19	Jul	52	15	157	14	143	15	43	15
20	Aug	573	15	216	15	411	15	119	15
21	Aug	320	15	391	14	370	15	155	15
22	Aug	145	15	468	15	233	12	210	14
23	Sep	530	15	82	15	430	15	75	15
24	Sep	256	13	231	15	97	15	60	15
25	Oct	160	15	213	15	238	15	144	15
26	Oct	86	15	238	15	48	13	43	15
Total		8033		7818		4846		1910	

The infestation degree (ID) for a given cow was calculated by dividing the total half body count of *A. variegatum* of an individual animal by the mean infestation by *A. variegatum* of the farm at a given time [109].

Table A.25: Infestation degree for *A. variegatum*. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Farm	Min	Max	Mean	SD
Me	0	5.2	0.997	0.715
Ca	0	4.6	1.012	0.739
Br	0	5.5	0.996	0.781
Az	0	14.3	1.001	1.356

Descriptive Statistics (SPSS)

Table A.26: Mean tick infestation of *Rhipicephalus (B.)* sp for all cattle of a given herd. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Farm	N	Min	Max	Mean	SD
Me	26	0	48.40	8.55	11.15
Ca	24	0	1.9	0.17	0.41
Br	26	0	25.80	4.74	5.89
Az	26	0.53	39.67	11.47	11.50

Table A.27: Minimal, maximal and mean total count per month for a given herd for *Rhipicephalus (B.)* sp. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

Farm	N	Min	Max	Mean	SD
Me	26	0	484	115.69	132.7
Ca	24	0	19	2.20	4.5
Br	26	0	387	70.08	88.1
Az	26	8	595	155.38	164.0

Table A.28: Mean tick infestation of *Rhipicephalus (B.)* sp on cattle in the different study sites

Sampling	Month	Me	Ca	Br	Az
1	Nov	7.00		5.13	1.87
2	Nov	2.00	0	1.93	2.70
3	Dec	0.93	0.09	15.67	10.53
4	Dec	48.40	0.67	4.0	7.20
5	Jan	32.21	0.3	8.60	27.75
6	Jan	20.50		3.87	5.33
7	Feb	13.93	0	11.93	39.67
8	Feb	11.60	1.90	25.80	20.79
9	Mar	6.13	0.6	1.80	10.69
10	Mar	4.73	0.8	5.40	10.29
11	Mar	4.00	0	10.33	34.27
12	Apr	4.73	0.4	4.67	11.55
13	Apr	14.47	0	1.07	24.07
14	Mai	9.27	0	0.53	1.27
15	Mai	11.20	0.14	0	14.00
16	Jun	0.07	0	4.40	2.13
17	Jun	2.0	0	0.53	10.60
18	Jul	1.27	0	1.33	1.27
19	Jul	0.40	0	0.4	10.33
20	Aug	0.93	0.13	7.75	33.64
21	Aug	13.80	0	3.93	1.40
22	Aug	10.33	0	1.47	2.93
23	Sep	0.38	0	1.33	0.53
24	Sep	1.3	0	0.73	7.47
25	Oct	0.73	0.07	0.46	1.47
26	Oct	0	0	0.13	4.47
Total N		26	24	26	26

Table A.29: Total count of *Rhipicephalus (B.)* sp at a given sampling time

Sampling	Month	Total count Me	No sampled Me	Total count Ca	No sampled Ca	Total count Br	No sampled Br	Total count Az	No sampled Az
1	Nov	98	14	-	-	29	15	28	15
2	Nov	20	10	0	15	77	15	27	10
3	Dec	14	15	1	11	235	15	158	15
4	Dec	484	10	10	15	60	15	108	15
5	Jan	451	14	4	13	129	15	222	8
6	Jan	287	14	-	-	58	15	80	15
7	Feb	209	15	0	15	179	15	595	15
8	Feb	174	15	19	10	387	15	291	14
9	Mar	92	15	9	15	27	15	139	13
10	Mar	71	15	1	13	81	15	144	14
11	Mar	48	12	0	15	155	15	514	15
12	Apr	71	15	6	15	70	15	127	11
13	Apr	217	15	0	15	16	15	361	15
14	May	139	15	0	15	8	15	19	15
15	May	168	15	2	14	0	15	210	15
16	Jun	1	14	0	14	66	15	32	15
17	Jun	28	14	0	15	8	15	159	15
18	Jul	19	15	0	14	20	15	19	15
19	Jul	6	15	0	7	4	10	62	6
20	Aug	14	15	2	15	93	12	471	14
21	Aug	207	15	0	14	59	15	21	15
22	Aug	155	15	0	15	22	15	44	15
23	Sep	5	13	0	15	20	15	8	15
24	Sep	19	15	0	15	11	15	112	15
25	Oct	11	15	1	15	6	13	22	15
26	Oct	0	15	0	15	2	15	67	15
Total		3008		55		1822		4040	

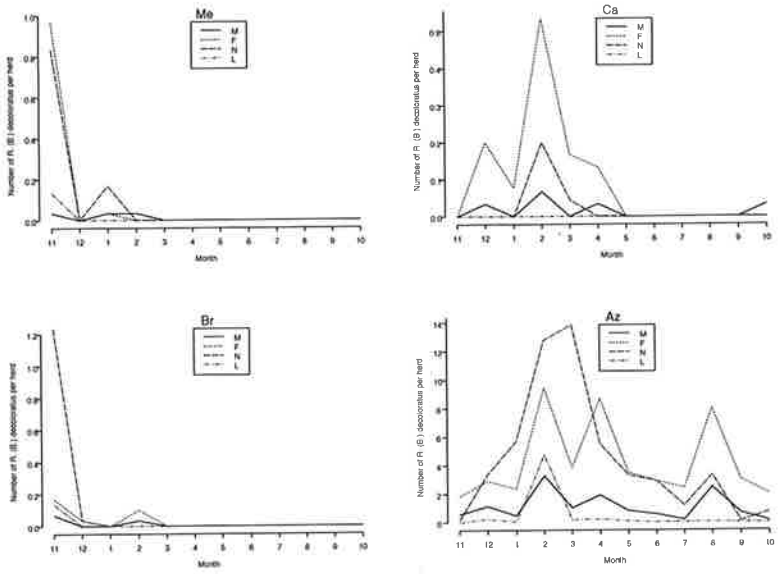
The infestation degree (ID) for a given cow was calculated by dividing the total half body count of *Rhipicephalus (B.)* sp of an individual animal by the mean infestation by *Rhipicephalus (B.)* sp of the farm at a given time [109].

Table A.30: Infestation degree for *Rhipicephalus (B.)* sp. Me: Sikensi, Ca: Dabou, Br: Brofodoumé, Az: Azaguié.

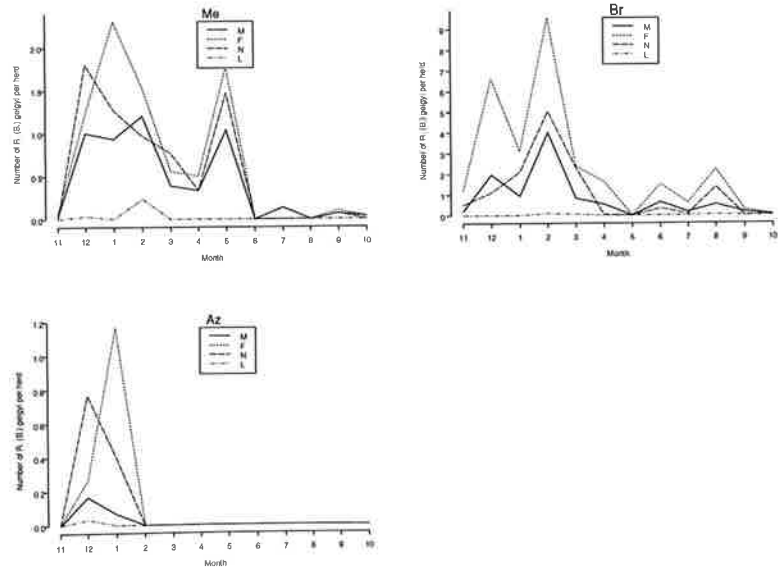
Farm	Min	Max	Mean	SD
Me	0	71.4	1.220	4.018
Ca	0	40.0	0.400	2.474
Br	0	13.2	0.958	1.793
Az	0	14.3	0.937	1.592

A.1.4 Seasonality of the diverse tick species

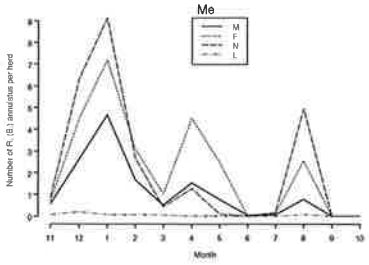
Seasonal distribution of the different stages for *R. (B.) decoloratus* in the four study sites Sikensi (Me), Dabou (Ca), Brofodoumé (Br) and Azaguié (Az).



Seasonal distribution of the different stages for *R. (B.) geigyi* in the three study sites Sikensi (Me), Brofodoumé (Br) and Azaguié (Az).



Seasonal distribution of the different stages for *R. (B.) annulatus* in the study site Sikensi (Me).



A.2 Statistical analyses of Chapter 7

A.2.1 Prevalence of the parasites present in the Southern region of the Ivory coast

The following abbreviations are utilised to describe the parasites: Aph, *A. phagocytophilum* ; Eru, *E. ruminantium* ; Ama, *A. marginale* ; Ace, *A. centrale* ; Abo. *A. bovis* ; Ehr.sp, *Ehrlichia* sp ; AE, Catch-AE; BT, Catch-BT; Bbo, *B. bovis* Bbi, *B. bigemina*; Bdi, *B. divergens*; Tan, *T. annulata*; Tpa, *T. parva*; Tmu, *T. mutans*; Tve, *T. velifera*; Tryp, *Trypanosoma* sp; Tbru, *T. brucei*; Tcon, *T. congolense*; Frottis, thin blood smear; G.paisse, thick blood smear; Samples testing negative are indicated by zero and samples testing positive are indicated by one.

[1] "********** Frottis"
0, Bab, Bbov, Tbru, Tcon
508, 1, 15, 2, 2

[1] "********** G..paisse"
0, Tbru, Tcon, Tryp
519, 2, 1, 6

[1] "********** PCRAE"
0, 1
679, 27

[1] "********** PCRBT"
0, 1
320, 385

[1] "********** Aph"
0
707

[1] "********** Eru"
0
707

[1] "********** Ama"
0, 1
691, 16

[1] "********** Ace"
0
707

[1] "********** Abo"
0
707

[1] "********** Ehr.sp"
0, 1
695, 12

[1] "********** Catch.AE"
0, 1
639, 68

[1] "********** Catch.BT"

```
0, 1
156, 551

[1] "********** Bbo"
0, 1
650, 57

[1] "********** Bbi"
0, 1
638, 69

[1] "********** Bdi"
0
706

[1] "********** Tan"
0
707

[1] "********** Tpa"
0
707

[1] "********** Tmu"
0, 1
183, 524

[1] "********** Tve"
0, 1
233, 474

[1] "********** Table(fPCRAE, fCatch.AE)"
     0   1
 0 637  42
 1   1  26

[1] "********** Table(fPCRBT, fCatch.BT)"
     0   1
 0 155 165
 1   1 384
```

A.2.2 Mixed infections

Single infections:

Parasite	Number
T. mutans	58
T. velifera	17
B. bovis	2
B. bigemina	2

Double infections:

Parasite	Number
Tm & Tv	321
Bbov & Tm	5
Bbig & Tm	4
Bbov & Tv	1
Am & Tv	1
Esp & Tv	1
AE & Bbig	1

Triple infections:

Parasite	Number
Bbig & Tm & Tv	40
Bbov & Tm & Tv	33
Esp & Tm & Tv	8
Am & Tm & Tv	7
Am & Bbig & Tm	1
AE & Tm & Tv	18
AE & Bbig & Tm	2

Quadruple infections:

Parasite	Number
Bbov & Bbig & Tm & Tv	7
Am & Bbig & Tm & Tv	3
Am & Bbov & Tm & Tv	2
Am & Esp & Tm & Tv	1
Esp & Bbig & Tm & Tv	1
AE & Bbov & Tm & Tv	4
AE & Bbig & Tm & Tv	6

Quintuple infections:

Parasite	Number
Am & Esp & Bbov & Tm & Tv	1
AE & Bbov & Bbig & Tm & Tv	2

No species-specific hybridization:

Parasite	Number
Only AE	9
Only BT	2
AE & BT	1

No parasite detected:

Parasite	Number
Without	146

A.2.3 Prevalence of the diverse parasites in the different study sites

Pearson's chi-square test was used to test, if a given parasite's distribution does vary between the different farms. See beginning of the chapter for abbreviations.

[1] "********** Ferm Ama"

	Az	Br	Ca	Me
0	172	169	177	173
1	0	11	0	5

Pearson's chi-square test without Yates' continuity correction

data: tab

X-square = 20.3709, df = 3, p-value = 0.0001

[1] "********** Ferm Ehr.sp"

	Az	Br	Ca	Me
0	165	176	177	177
1	7	4	0	1

Pearson's chi-square test without Yates' continuity correction

data: tab

X-square = 10.5312, df = 3, p-value = 0.0146

[1] "********** Ferm Catch.AE"

	Az	Br	Ca	Me
0	163	148	173	155
1	9	32	4	23

Pearson's chi-square test without Yates' continuity correction

data: tab

X-square = 30.8502, df = 3, p-value = 0

[1] "********** Ferm Catch.BT"

	Az	Br	Ca	Me
0	56	29	36	35
1	116	151	141	143

Pearson's chi-square test without Yates' continuity correction

data: tab

X-square = 15.6273, df = 3, p-value = 0.0014

[1] "********** Ferm Bbo"

	Az	Br	Ca	Me
0	162	165	175	148
1	10	15	2	30

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 31.2281, df = 3, p-value = 0

[1] "********** Ferm Bbi"

	Az	Br	Ca	Me
0	172	144	177	145
1	0	36	0	33

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 74.757, df = 3, p-value = 0

[1] "********** Ferm Tmu"

	Az	Br	Ca	Me
0	68	35	41	39
1	104	145	136	139

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 22.746, df = 3, p-value = 0

[1] "********** Ferm Tve"

	Az	Br	Ca	Me
0	82	39	58	54
1	90	141	119	124

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 27.8019, df = 3, p-value = 0

The Pearson's chi-square test was also applied to test, if the month had an influence of the distribution of the divers parasites. Note: The approximations is good when each expected cell counts exceeds about 5. Due to low numbers the test results give indications, but are not statistically valuable.

[1] "********** Mois Ama"

	1	2	3	4	5	6	7	8	9	10	11	12
0	56	58	60	59	60	58	57	57	57	57	53	59
1	2	1	0	1	0	1	2	2	2	3	1	1

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 6.6013, df = 11, p-value = 0.8304

[1] "********** Mois Ehr.sp"

	1	2	3	4	5	6	7	8	9	10	11	12
0	56	59	60	60	60	58	55	57	57	59	54	60
1	2	0	0	0	0	1	4	2	2	1	0	0

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 18.3208, df = 11, p-value = 0.0744

[1] "********** Mois Catch.AE"

	1	2	3	4	5	6	7	8	9	10	11	12
0	50	55	58	58	58	55	46	51	52	53	50	53
1	8	4	2	2	2	4	13	8	7	7	4	7

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 23.1772, df = 11, p-value = 0.0167

[1] "********** Mois Catch.BT"

	1	2	3	4	5	6	7	8	9	10	11	12
0	10	19	9	10	8	5	9	9	13	15	23	26
1	48	40	51	50	52	54	50	50	46	45	31	34

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 48.5653, df = 11, p-value = 0

[1] "********** Mois Bbo"

	1	2	3	4	5	6	7	8	9	10	11	12
0	55	59	58	58	57	53	53	52	48	47	54	56
1	3	0	2	2	3	6	6	7	11	13	0	4

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 40.8522, df = 11, p-value = 0

[1] "********** Mois Bbi"

	1	2	3	4	5	6	7	8	9	10	11	12
0	54	56	49	56	53	50	51	54	53	56	49	57
1	4	3	11	4	7	9	8	5	6	4	5	3

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 13.2334, df = 11, p-value = 0.2783

[1] "********** Mois Tmu"

	1	2	3	4	5	6	7	8	9	10	11	12
0	12	20	12	10	11	6	12	10	18	16	28	28
1	46	39	48	50	49	53	47	49	41	44	26	32

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 52.4762, df = 11, p-value = 0

[1] "********** Mois Tve"

	1	2	3	4	5	6	7	8	9	10	11	12
0	16	26	15	19	17	9	14	16	20	21	30	30
1	42	33	45	41	43	50	45	43	39	39	24	30

Pearson's chi-square test without Yates' continuity correction
data: tab
X-square = 38.4572, df = 11, p-value = 0.0001

A.3 Statistical analyses of Chapter 8

A.3.1 Prevalence of the different parasites detected in the most abundant tick species

Logistical regression analyses were performed using S-PLUS V6.0

[1] "———"
[1] "p-value"
(Intercept) as.factor(f$Groupe)1 as.factor(f$Groupe)2 f$Species1 f$Species2 f$Species3
0, 0.977438, 0.5102473, 0.1321023, 0.001750519, 0.2901311
[1] "———"
$"(Intercept)":
(Intercept)
-1.54428

$"as.factor(f$Groupe)":
1 2 3
0.07915322 0.07094561 -0.1500988
$"f$Species": Av Ba Bg Bsp
-0.4521954, 0.1185577, 0.6996908, -0.3660532

Call:
crosstabs(formula = f$maxCatch + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
f$maxCatch,f$Species

	Av	Ba	Bg	Bsp	RowTotl
0	166	55	83	39	343
	0.88	0.8	0.69	0.87	0.81
1	23	14	37	6	80
	0.12	0.2	0.31	0.13	0.19
ColTotl	189	69	120	45	423
	0.45	0.16	0.28	0.11	

Test for independence of all factors

Chi$\hat{2}$ = 17.72226 d.f.= 3 (p=0.0005018389)
Yates' correction not used

Pearson's chi-square test without Yates' continuity correction
data: f$maxCatch and f$Species
X-square = 17.7223, df = 3, p-value = 0.0005

Ba & Bg
Pearson's chi-square test with Yates' continuity correction
data: matrix(c(55, 14, 83, 73), ncol = 2)
X-square = 13.0759, df = 1, p-value = 0.0003

Av & Bsp
Pearson's chi-square test with Yates' continuity correction
data: matrix(c(166, 23, 39, 6), ncol = 2)
X-square = 0.0015, df = 1, p-value = 0.9691

Av & Bg
Pearson's chi-square test with Yates' continuity correction
data: matrix(c(166, 23, 83, 37), ncol = 2)
X-square = 15.1693, df = 1, p-value = 0.0001

Av & Ba
Pearson's chi-square test with Yates' continuity correction
data: matrix(c(166, 23, 55, 14), ncol = 2)
X-square = 2.0926, df = 1, p-value = 0.148

Bsp & Bg
Pearson's chi-square test with Yates' continuity correction
data: matrix(c(39, 6, 83, 37), ncol = 2)
X-square = 4.3329, df = 1, p-value = 0.0374

Bsp & Ba
Pearson's chi-square test with Yates' continuity correction
data: matrix(c(39, 6, 55, 14), ncol = 2)
X-square = 0.4937, df = 1, p-value = 0.4823

Call:
crosstabs(formula = f$Bbo + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
fBbo, fSpecies

	Av	Ba	Bg	Bsp	RowTotl
0	183 0.97	63 0.91	109 0.91	42 0.93	397 0.94
1	6 0.032	6 0.087	11 0.092	3 0.067	26 0.061
ColTotl	189 0.45	69 0.16	120 0.28	45 0.11	423

Test for independence of all factors
Chi$\hat{2}$ = 5.589406 d.f.= 3 (p=0.1333879)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value

Call:
crosstabs(formula = f$Ama + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
fAma ,fSpecies

	Av	Ba	Bg	Bsp	RowTotl
0	189 1	69 1	110 0.92	45 1	413 0.98
1	0 0	0 0	10 0.083	0 0	10 0.024
ColTotl	189 0.45	69 0.16	120 0.28	45 0.11	423

Test for independence of all factors
Chi$\hat{2}$ = 25.86138 d.f.= 3 (p=0.0000101965)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value

Call:
crosstabs(formula = f$Tmu + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
fTmu , fSpecies

	Av	Ba	Bg	Bsp	RowTotl
0	179 0.95	62 0.9	111 0.92	45 1	397 0.94
1	10 0.053	7 0.1	9 0.075	0 0	26 0.061
ColTotl	189 0.45	69 0.16	120 0.28	45 0.11	423

Test for independence of all factors

Chi$\hat{2}$ = 5.480142 d.f.= 3 (p=0.1398312)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value

Call:
crosstabs(formula = f$Tve + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
fTve , fSpecies

	Av	Ba	Bg	Bsp	RowTotl
0	178 0.94	66 0.96	115 0.96	43 0.96	402 0.95
1	11 0.058	3 0.043	5 0.042	2 0.044	21 0.05
ColTotl	189 0.45	69 0.16	120 0.28	45 0.11	423

Test for independence of all factors

Chi$\hat{2}$ = 0.5365635 d.f.= 3 (p=0.9107882)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value

Call:
crosstabs(formula = f$Bbi + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
fBbi , fSpecies

	Av	Ba	Bg	Bsp	RowTotl
0	189 1	69 1	117 0.98	45 1	420 0.99
1	0 0	0 0	3 0.025	0 0	3 0.0071
ColTotl	189 0.45	69 0.16	120 0.28	45 0.11	423

Test for independence of all factors

Chi$\hat{2}$ = 7.629107 d.f.= 3 (p=0.0543323)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value

Call:
crosstabs(formula = f$Eru + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
fEru , fSpecies

	Av	Ba	Bg	Bsp	RowTotl
0	188	69	120	45	422
	0.99	1	1	1	1
1	1	0	0	0	1
	0.0053	0	0	0	0.0024
ColTotl	189	69	120	45	423
	0.45	0.16	0.28	0.11	

Test for independence of all factors

Chi$\hat{2}$ = 1.241029 d.f.= 3 (p=0.7431816)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value

Call:
crosstabs(formula = f$Nesp + f$Species, margin = list("Col%" = 2))
423 cases in table
N
Col%
f$Nesp , f$Species

	Av	Ba	Bg	Bsp	RowTotl
0	189	69	115	45	418
	1	1	0.96	1	0.99
1	0	0	5	0	5
	0	0	0.042	0	0.012
ColTotl	189	69	120	45	423
	0.45	0.16	0.28	0.11	

Test for independence of all factors

Chi$\hat{2}$ = 12.77602 d.f.= 3 (p=0.005146891)
Yates' correction not used
Some expected values are less than 5, don't trust stated p-value
Fisher's exact test
data: matrix(c(108, 10, 43, 0), ncol = 2)
p-value = 0.0635
alternative hypothesis: two.sided

Fisher's exact test
data: matrix(c(108, 10, 39, 4), ncol = 2)
p-value = 1
alternative hypothesis: two.sided

A.3.2 Mixed infections in tick species

Single infections:

Parasite	Number	Tick species
B. bovis	22	10x Bg, 6x Ba, 3x Bsp, 3x Av
T. mutans	14	6x Bg, 4x Ba, 4x Av
T. velifera	11	7x Av, 2x Bg, 2x Bsp
Am	8	8x Bg
Esp	3	Bg
Bbig	2	2x Bg
Erum	1	Av

Double infections:

Parasite	Number	
Tm & Tv	6	3x Ba, 3x Av
Bbov & Tm	2	1x Ba, 1x Bsp

Triple infections:

Parasite	Number	
Bbov & Tm & Tv	1	Av
Esp & Tm & Tv	1	Bg
Am & Tm & Tv	1	Bg

Quintuple infections:

Parasite	Number	
Esp & Am & Bbov & Tm & Tv	1	Bg
Am & Bbov & Bbig & Tm & Tv	1	Bg

Parasite	Number	
AE only	5	2x Bg, 1x Av, 1x Ba, 1x Bsp
BT only	5	2x Av, 1x Ba, 1x Bg, 1x Bsp

No parasite detected:

Parasite	Number
Without	343

(Note: Two times positives for *B. bovis* did not hybridize with the catch-all BT. They are leftout in calculations for infections which are based on the hybridization with catch-all AE and/or BT.)

A.4 Statistical analyses of the Chapter 9

A.4.1 Identification of 16S rRNA sequences

Sample	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
No 37	DQ000616.1	Anaplasma marginale strain IG42 16S ribosomal RNA gene, partial sequence	680	680	97%	0.0	98%
No 38	DQ000616.1	Anaplasma marginale strain IG42 16S ribosomal RNA gene, partial sequence	745	745	100%	0.0	99%
No 68	DQ000616.1	Anaplasma marginale strain IG42 16S ribosomal RNA gene, partial sequence	671	671	98%	0.0	98%
No 70	DQ000616.1	Anaplasma marginale strain IG42 16S ribosomal RNA gene, partial sequence	667	667	98%	0.0	98%
No 99	DQ000616.1	Anaplasma marginale strain IG42 16S ribosomal RNA gene, partial sequence	712	712	97%	0.0	99%
No 1	AY837740.1	Uncultured Anaplasma sp. clone G2.12.35 16S ribosomal RNA gene, partial sequence	686	686	98%	0.0	97%
No 63	AY837740.1	Uncultured Anaplasma sp. clone G2.12.35 16S ribosomal RNA gene, partial sequence	628	628	96%	3e-177	97%
No13	AF414399.1	Ehrlichia sp. Tibet 16S ribosomal RNA gene, partial sequence	697	697	99%	0.0	98%
No 162	DQ324547.1	Ehrlichia sp. Fujian 16S ribosomal RNA gene, partial sequence	725	725	96%	0.0	99%
	AF414399.1	Ehrlichia sp. Tibet 16S ribosomal RNA gene, partial sequence	725	725	96%	0.0	99%
No 44	AF336220.2	Ehrlichia phagocytophilum 16S ribosomal RNA gene, partial sequence	553	553	100%	2e-154	92%
No 226	AF336220.2	Ehrlichia phagocytophilum 16S ribosomal RNA gene, partial sequence	715	1132	89%	0.0	97%

Appendix B

Life cycle of *Ixodidae*

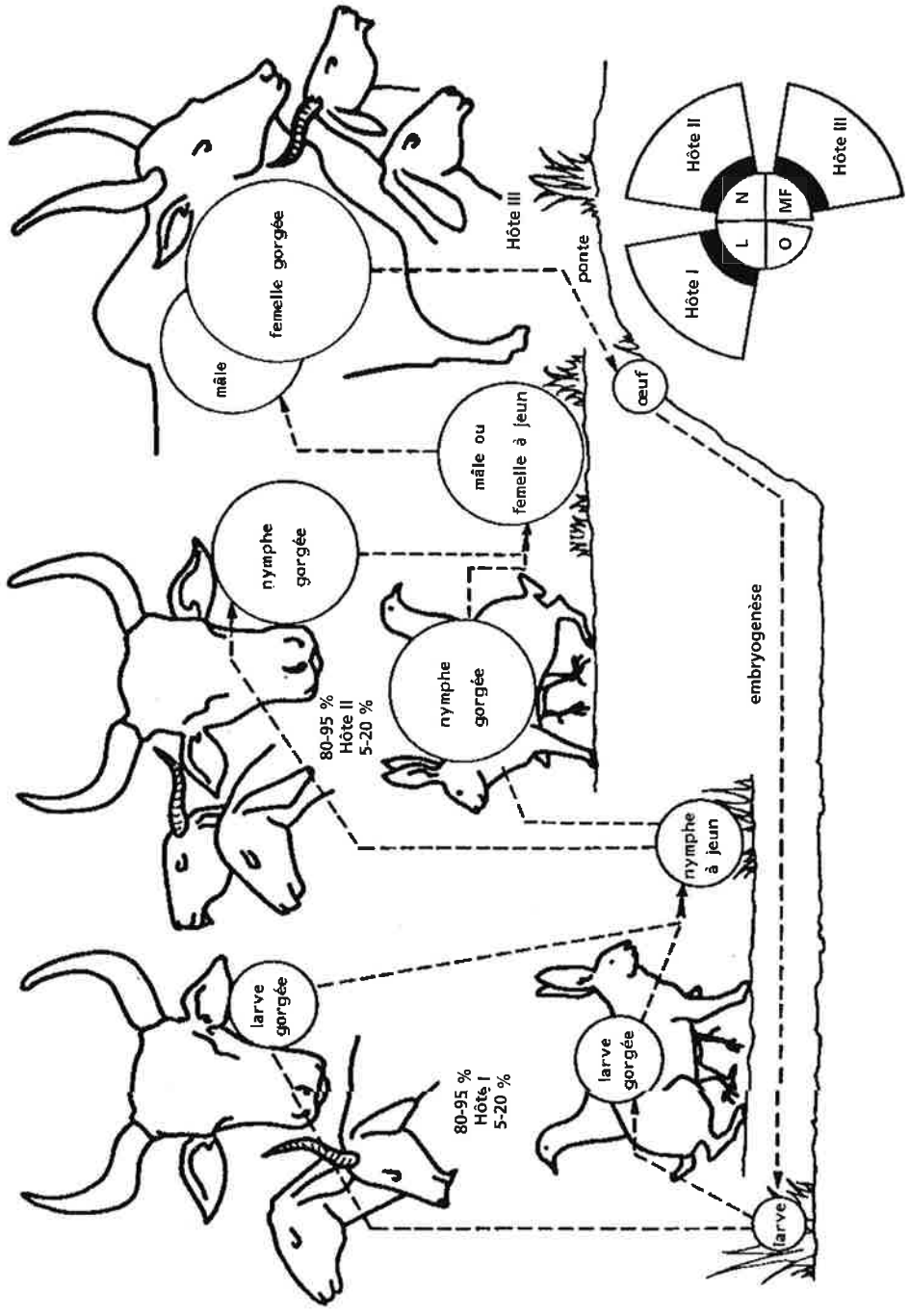


Figure B.1: Triphasic cycle of *A. variegatum* on cattle [23]

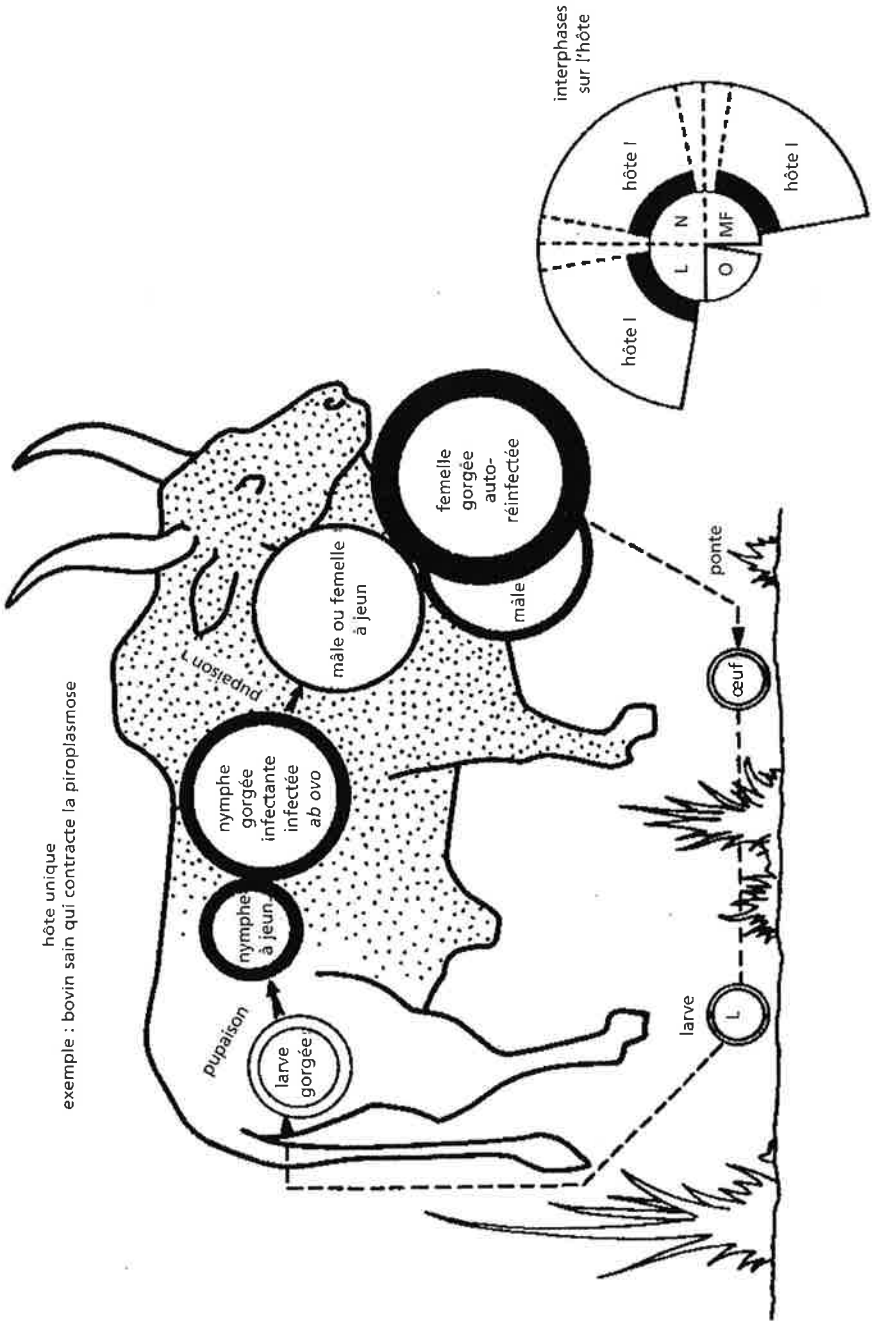


Figure B.2: Monophasic cycle of *Rhipicephalus (Boophilus)* on cattle [23]

Appendix C

Life cycle of diverse parasites

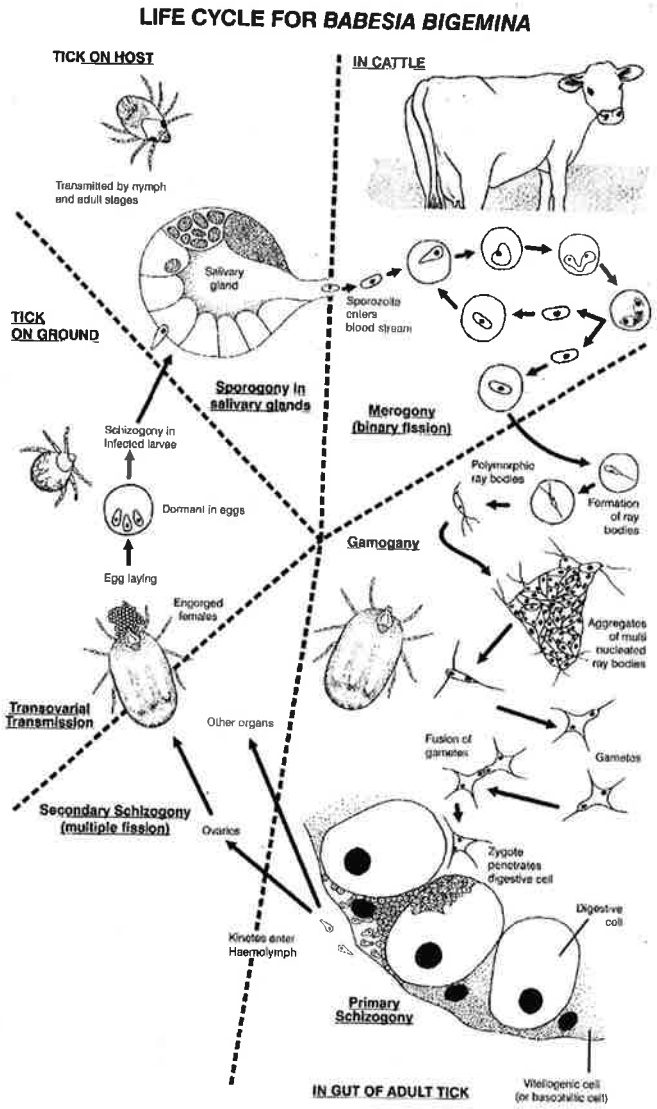


Figure C.1: Life cycle of *B. bigemina* after Bock et al (Parasitology (2004) 129, S247-S269)

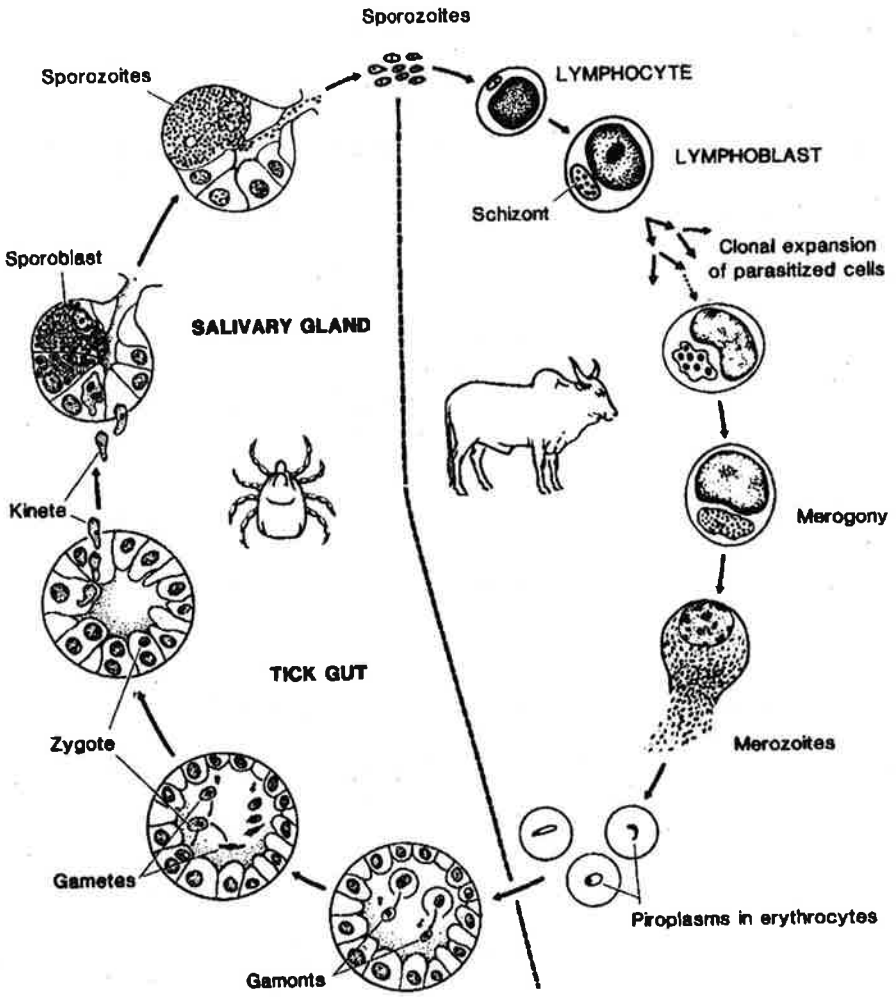


Figure C.2: Life cycle of *T. parva* after Bishop et al (Parasitology (20049 129 S271-S283)

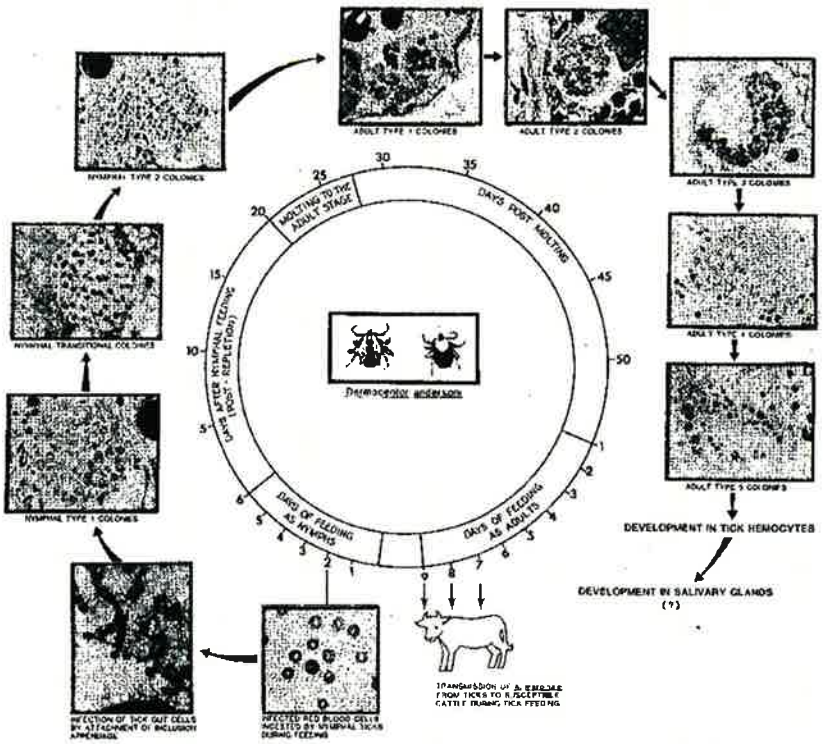


Figure C.3: Life cycle of *A. marginale* after Kocan, KM.

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