Tick-borne pathogens in African cattle – novel molecular tools for diagnostics in epizootiology and the genetics of resistance

Dissertation

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To my beloved parents,
Abanda Ossee & Beck a Zock A. Michelle

And my siblings
Bilong Abanda
Zock Abanda
Betchem Abanda B.
Abanda Ossee R.J.
Abanda Beck E.G.

You are the hand holding me standing
when the ground under my feet is shaking

Thank you !

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Abbreviations

AMPs	Antimicrobial peptides
BV	Blood vessel
CCHFV	Crimean-Congo Hemorrhagic fever virus
CHO	Chinese hamster ovary (cell line)
DC	Dermal cells
DETC	Dendritic epidermal T-cell
Df	Dense form
EC	Endothelial cell
Fbt	Fibroblast
G	Gametocyte
Gut	Mid gut
GWAS	genome-wide association study
$h^2_{liab.}$	Heritability estimated on the liability scale
$h^2_{obs.}$	Heritability estimated on the observed scale
HO	Haller's organ
ILc	Innate lymphoid cells
K	Kinetes
L	Lymphocytes
LCD	Low cost, low density microarray
LCs	Langerhans cells
LV	Lymphatic vessel
M	Macrophage
MC	Mast cell
MHC	Major Histocompatibility Complex
Mo	Monocyte
Mz	Merozoite
NGS	Next-generation sequencing
ng	Nanogram
Nt	Neutrophil
PCR	Polymerase chain reaction
Pls	Piroplasms
R_0	Basic reproductive ratio

RBC	Red blood cells
REML	Restricted maximum likelihood method
Rf	Reticulate form
RLB	Reverse Line Blot
RO	Reproductive organ
SG	Salivary gland
SK	Strahlenkörper
SKz	Schizonts
SNP	Single Nucleotide Polymorphism
SZ	Sporozoite
T	Trophozoite
TBD	Tick-borne disease
TBEV	Tick-borne Encephalitis virus
TBP	Tick-borne pathogen
TBV	Tick-borne virus
V_A	Additive genetic variance
V_P	Phenotypic genetic variance
ZBst	Zygote of *Babesia* sensu stricto
ZTst	Zygote of *Theileria* sensu stricto

Summary

Tick-borne pathogens are among the most harmful micro-organisms responsible for losses in animal husbandry, with a significant threat to the human population. Their detection by blood smear or serology is more likely to allow the identification of individual species, whereas co-infections are more common. Limitations arise especially in laboratories with limited resources and without sustainable capacities. The present thesis presents the identification of seven organisms in the cattle population from Cameroon for the first time (*Borrelia theileri, Theileria mutans*, *T. velifera*, *Anaplasma platys*, *Anaplasma* sp. 'Hadesa') including the first published reports of *Rickettsia felis* and *Ehrlichia canis* in cattle worldwide. More than 80% of the infected studied population (1123/1260, 89.1%) were found being co-infected with at least two of the four studied groups of genera (903/1123, 80.4%), highlighting the caveats of the predominating single pathogen identification approach. Based on those observed limitations, a novel chip-based diagnostic array was developed through the platform of the commercial biochip manufacturer Chipron® in Berlin, Germany. The PCR-based tool allowed the simultaneous identification of co-infected samples of five genera, including novel species. Moreover, the array allowed the identification of significantly more pathogens in co-infection with increased specificity and sensitivity compared to traditional Sanger sequencing. This LCD-array can be easily implemented in small veterinary laboratories in endemic countries of Africa and elsewhere. The co-infection status and pathogen combinations was proven to differ between climatic zones and cattle populations, and being influenced by environmental factors (χ^2, regression). Different responses between individuals and breeds ($p < 0.05$) from the same environment motivated the test for heritability values. The observed low to moderate heritability based on genotyping dataset ($h^2_{obs.} = 0.1$ and $h^2_{liab.} = 0.6$) implied a genomic foundation of the trait of resistance to tick pathogens. More importantly, this result confirmed the possibility of improvement by breeding, which may be implemented as a control measure. The genome-wide analysis revealed the quantitative nature of the traits of resistance, exposing putative associated genomic regions with one of them not yet reported in the literature. Extended analyses and larger sample size will be advantageous for population differentiation and breed improvement through fine mapping of loci under natural selection and allele fixation related to resistance and susceptibility traits.

Zusammenfassung

Zecken-übertragene Pathogene gehören zu den schädlichsten Mikroorganismen, die für Verluste bei der Tierhaltung verantwortlich sind, mit einer erheblichen Bedrohung für die menschliche Bevölkerung. Ihr Nachweis mittels Blutausstrich oder Serologie erlaubt eher die Bestimmung einzelner Arten, während Ko-infektionen eher die Regel sind. Einschränkungen ergeben sich insbesondere in Laboren mit begrenzten Ressourcen und ohne nachhaltige Kapazität. Die vorliegende Arbeit beschäftigt sich mit der Identifizierung von 4 Gruppen von TBPs in Nordkamerun (Ehrlichien, Rickettsien, Spirochäten und Piroplasmen), wobei sieben Erstnachweise von Krankheitserregern in Rinderpopulationen (*Borrelia theileri, Theileria mutans*, *T. velifera*, *Anaplasma platys*, *Anaplasma* sp. 'Hadesa') in Kamerun sind, einschließlich den weltweiten Erstnachweisen von *Rickettsia felis* und *Ehrlichia canis* im Rind. Bei mehr als 80% (1123/1260, 89.1%) der untersuchten infizierten Rinder wurden mindestens zwei der vier untersuchten Genusgruppen (903/1123, 80.4%) von Krankheitserregern nachgewiesen, was die Einschränkung konventioneller Methoden unterstreicht. Über die Plattform eines kommerziellen Biochip-Anbieters (Chipron® in Berlin) wurde ein neuartiges Chip-basiertes Diagnostik-Array entwickelt. Dieses PCR-basierte Tool ermöglicht die gleichzeitige Identifizierung von fünf Erreger-Gattungen, einschließlich neuer Arten, im Blut mehrfach befallener Rinder. Deutlich mehr Krankheitserreger ließen sich so bei einer erhöhten Spezifität und Sensitivität nachweisen. Das LCD-Array kann problemlos in kleinen Veterinärlaboratorien in endemischen Ländern einschließlich Afrikas eingesetzt werden. Die häufigen Mischinfektionen mit je nach Region und Rinderrasse unterschiedlicher Zusammensetzung von Erregern werden zusätzlich von Umweltfaktoren beeinflusst (χ^2, regression, $p< 0,05$). Unterschiedliche Reaktionen zwischen Individuen und Rassen aus derselben Umgebung motivierten den Test auf Heritabilität. Niedrige bis moderate ($h^2_{obs.} = 0.1 \text{ and } h^2_{liab.} = 0.6$) Erbanlagen beobachtet, was eine genomische Grundlage für das Merkmal der Resistenz gegen Zecken-übertragene Pathogene darstellt. Dieses Ergebnis bestätigt die Möglichkeit einer Verbesserung der Resistenz durch Züchtung. Die genomweite Analyse erwies die quantitative Natur der Merkmale und verwies auf potentiell assoziierte genomische Regionen, von denen eine noch nicht in der Literatur beschrieben wurde. Erweiterte Analysen und eine größere Stichprobengröße wären nötig, um die Rinder Rassen besser zu charakterisieren (Feinkartierung von Lozi unter natürlicher Selektion und Allel-Fixierung).

Résumé

Les agents pathogènes transmis par les tiques comptent parmi les micro-organismes les plus nocifs responsables des pertes et de la détérioration des élevages, avec une menace importante pour la population humaine. Leur détection par frottis sanguin ou sérologie est plus susceptible de permettre l'identification d'espèces individuelle, alors que les co-infections sont plus fréquentes. Les contraintes sont tangibles dans les laboratoires aux ressources limitées et aux capacités non-durables. La présente thèse fait état de l'identification de sept agents pathogènes décris pour la première fois dans le cheptel bovin du Cameroun (*Borrelia theileri*, *Theileria mutans*, *T. velifera*, *Anaplasma platys*, *Anaplasma sp. 'Hadesa'*) y compris ceux identifiés pour la première fois dans l'hôte bovin (*Rickettsia felis* et *Ehrlichia canis*). Plus de 80% de (1123/1260, 89.1%) la population infectée étudiée était co-infectée par au moins deux groupes des genres (903/1123, 80.4%) des agents pathogènes étudiés, ce qui souligne les limites des méthodes d'identification d'un seul agent pathogène précédemment utilisées. Sur la base de ces contraintes, une nouvelle matrice de diagnostic a été mise au point, grâce à la plate-forme du fournisseur commercial de biochip Chipron® à Berlin, en Allemagne. L'outil (LCD-array) basé sur la PCR a permis l'identification simultanée d'échantillons co-infectés, y compris les nouvelles espèces. Il a également permis d'identifier un plus grand nombre de microorganismes en état de co-infection avec une spécificité et une sensibilité accrues. Cet outil peut facilement être utilisé dans des laboratoires vétérinaires à capacité réduite dans les pays endémiques d'Afrique et d'ailleurs. Il a été démontré que la co-infection ainsi que la combinaison de pathogènes responsables diffèrent selon les zones climatiques et les populations bovines, influencées par des facteurs environnementaux (χ2, régression). Des réponses différentes entre individus et espèces ($p< 0.05$) d'un même environnement ont motivé le test des valeurs d'héritabilité. Des héritabilités faibles à modérées ont été décelées ($h^2_{obs.} = 0.1 \text{ and } h^2_{liab.} = 0.6$), impliquant un fondement génétique du caractère de résistance. Ce résultat confirme la possibilité d'amélioration des facultés d'adaptation du bétail par gestion du système de production animale. Les analyses génétiques ont révélé les portions du génome responsables des phénotypes variés. Des analyses approfondies sur un plus grand échantillon seront nécessaires pour une différenciation des populations par représentation précise des loci sous sélection naturelle.

Disclosure of personal contribution

For all three manuscripts the same dataset was used. I (Babette Abanda) had a substantial role in the collection of samples in the field from all screened regions (except in the Far North) and in the microscopic analyses (including PCV measurements).

Own contribution to the published paper 1:

Abanda B., Paguem A., Abdoulmoumini M., Manchang T.K., Renz A. and Eisenbarth A. Molecular identification and prevalence of tick-borne pathogens in zebu and taurine cattle in North Cameroon. *Parasites and Vectors* (2019) 12:448. doi.org/10.1186/s13071-019-3699-x.

I (Babette Abanda: BA) performed all molecular analyses (PCR reactions, gel electrophoresis steps and sample preparation for sequencing). I made statistical analyses and drafted the manuscript before circulation to all co-authors. The BLAST searches were done with the substantial contribution of Dr. Albert Eisenbarth (AE) who produced the phylogenetic trees.

Author Contributions: BA designed the experiment and method, performed laboratory analyses and drafted the manuscript. BA and AE performed the statistical and phylogenetic analyses. BA, AP and Manchang Tanyi Kingsley (MTK) collected samples. BA, AP, AM, MTK, Alfons Renz (AR) and AE contributed to the interpretation of the results, wrote and corrected the manuscript. AR and AE supervised and managed the whole study. All authors read and approved the final manuscript.

Own contribution to the published paper 2:

Abanda B., Paguem A., Achukwi M.D., Renz A. and Eisenbarth A. Development of a low-density DNA microarray for detecting tick-borne bacterial and piroplasmid pathogens in African cattle. *Tropical Medicine and Infectious Diseases* (2019) 4:64. doi: 10.3390/tropicalmed4020064.

I (BA) performed all molecular analyses (PCR reactions, gel electrophoresis steps and sample preparation for sequencing). I have also tested, adjusted and validated the array protocol (detection limits, performance of synthetic inserts and PCR optimization). I made all statistical analyses and drafted the manuscript before

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Own contribution to the manuscript under finalization:

Abanda B., Schmid M., Paguem A., Iffland H., Achukwi M.D, Preuß S., Renz A. and Eisenbarth A. Genome-wide association studies on parasitic and microbial pathogen resistance of *Bos taurus* and *Bos indicus* cattle breeds in Cameroon.

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Abanda B., Paguem A., Achukwi M.D., Renz A. and Eisenbarth A. Development of a low-density DNA microarray for detecting tick-borne bacterial and piroplasmid pathogens in African cattle. *Tropical Medicine and Infectious Diseases* (2019) 4:64. doi: 10.3390/tropicalmed4020064.

c) Manuscript under finalization

Abanda B., Schmid M., Paguem A., Iffland H., Achukwi M.D, Preuß S., Renz A. and Eisenbarth A. Genome-wide association studies on parasitic and microbial pathogen resistance of *Bos taurus* and *Bos indicus* cattle breeds in Cameroon.

Not included in the thesis:

a) Submitted

Paguem A., **Abanda B**., Ndjonka D., Ngwasiri N.N., Eisenbarth A., Streit A., Renz A. and Achukwi M.D. Host specificity and Phylogeny of Trichostrongylidae of Domestic Ruminants in the Guinea savannah of the Adamawa Plateau in Cameroon. *Veterinary Parasitology: Regional Studies and Reports*.

Paguem A., **Abanda B**., Achukwi M.D., Baskaran P., Czemmel S., Renz A. and Eisenbarth A. Whole genome characterization of five autochthonous and introduced cattle breeds (*Bos taurus brachyceros* and *Bos indicus indicus*) from Cameroon regarding adaptive phenotypic traits and pathogen resistance. PLoS Genetics.

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Boursou D., Ndjonka D., Eisenbarth A., Manchang K., Paguem A., Ngwafu N.N., Dikti J.V., **Abanda B.**, Krumkamp R., Van Hoorn S., Renz A., Achukwi M.D., Liebau E. and Brattig N. *Onchocerca*-infected cattle produce strong antibody responses to excretory-secretory proteins released from adult male *Onchocerca ochengi* worms. *BMC Infectious Diseases* (2018) 18:200. doi: 10.1186/s12879-018-3109-6.

General Introduction

A. The biology of ticks

1. Taxonomy, morphology and life cycle

Ticks are worldwide distributed parasitic arthropods which obligately take blood meals from a wide range of vertebrate hosts. They belong to the class Arachnida, subclass Acari, order Parasitiformes and suborder Ixodida with its single superfamily Ixodoidea. This superfamily comprises three families, namely the Argasidae (soft ticks), Nuttalliellidae (Bedford, 1931; Guglielmone et al., 2010) and Ixodidae (hard ticks). The anatomy of all ticks (Fig. 1) consists of a main body (idiosoma), mouth parts (gnathosoma) and (for adults and nymphs) four pairs of segmented legs. Furthermore, ticks are furnished with paired articulated appendages on the gnathosoma, namely pedipalps and chelicerae, with the latter used to penetrate the host skin during the blood meal (Obenchain et al., 1982). To locate and identify their host, ticks are capacitated by specific chemosensory structures, on the foretarsus of their first pair of legs called Haller's organs (Fig. 2) (Carr et al., 2017; Nuttal et al., 1908; Woolley 1972).

Developmental stages, mating and lifespan vary widely according to the tick family. Female ticks need to take blood meals for survival and reproduction, although they can sustain long periods of food deprivation up to several months, especially members of the Argasidae family. Females are normally larger and thus ingest more blood than males necessary for the mass production of eggs. Depending on the family, the species and level of disturbance, the duration of a blood meal ranges from minutes (Argasidae) to days and weeks (Ixodidae). Afterwards, the tick detaches from its host and develops to the next stage or the adult female prepares for oviposition. Whereas soft ticks undergo many ovipositions, female hard ticks die soon after their first and unique oviposition leaving a single batch of 2,000 to 20,000 eggs (Wenk & Renz, 2003; Davey et al., 1980).

The life stages of a tick are divided into egg, larva (with three pairs of legs), nymphs (one to eight molts in soft ticks) and adult, requiring a fresh blood meal after each molting step (Lah et al., 2016). Some species leave the host before molting, and others only detach from their host to lay their eggs (Sonenshine & Roe, 2014). Therefore, species are categorized according to the number of hosts they parasitize: one-host (from larva to adult stage on the same host), two-host (larva and nymph stage on one

host and adult stage on another host) and three-host ticks (larva, nymph and adult each on a new host and/or host species).

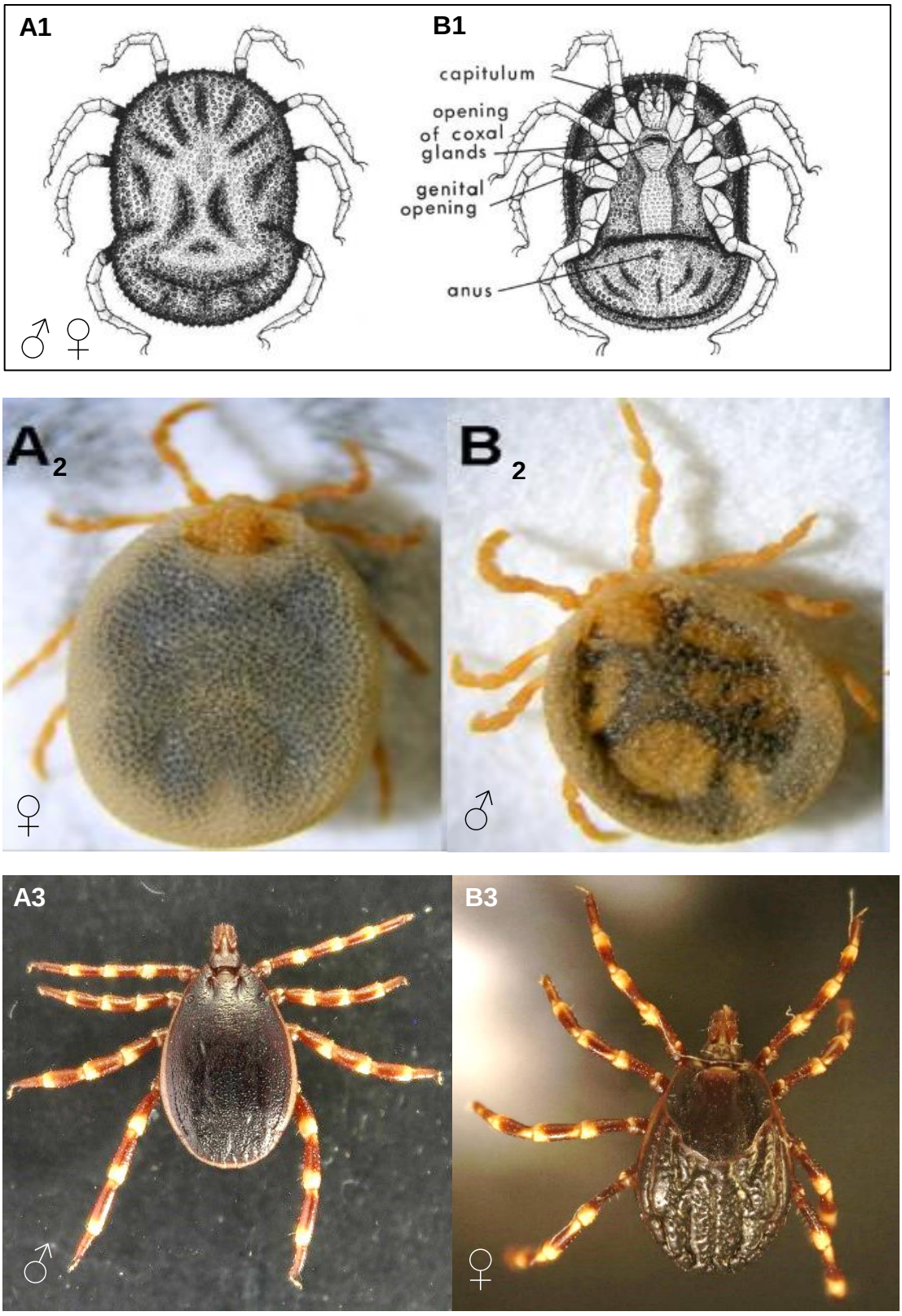


Figure 1. A1-B1: Tick superfamily Argasidae (soft ticks), drawing showing *Ornithodoros moubata*: no scutum, leading to the absence of clear sexual dimorphism in the adult stage.

Image: (Service, 1980), A2-B2: Nuttalliellidae with a single species included *Nuttalliella namaqua*: in males (♂), a pseudoscutum covers most of the dorsum, allows differentiation in the adult stage. Photo: (Latif et al., 2012), and A3-B3: Ixodidae (hard ticks), photo showing *Hyalomma rufipes*: both sexes have clearly defined scutum, completely covering the dorsum in males (♂) and incompletely in females (♀), leading to a clear sexual dimorphism in the adult stage. Photo: (B. Abanda).

2. Role as infectious disease vector

More than 896 tick species from about 20 genera are known worldwide (with widespread disagreement concerning the soft ticks genera), with the Ixodidae family being by far the most important, both in numbers and economically (Guglielmone et al., 2010; Nuttall, 2019). Because of their role as vectors for veterinary and medically relevant diseases, the most important genera are *Amblyomma*, *Dermacentor*, *Haemaphysalis, Hyalomma*, *Rhipicephalus* (*Boophilus*) and *Ixodes* (Mullen & Durden, 2002).

According to the synergic impact on the ecosystem and the nature of the association, microorganisms and their carrying host can be classified in three groups of symbiotic relationships: the mutualism/symbiosis with both involved parties benefiting, commensalism where only one is benefiting without or with a minimal detrimental effect on the other, and parasitism where only the microorganism is benefiting to the impairment of the host (Wenk & Renz, 2003). Similar relationships can be observed inside the host, between microbiota (Cowdry, 1925) interfering with a subsequent colonization including pathogens (Childs & Paddock 2002). Relationships between the existing, newly introduced organisms and even the organ involved plays a significant role in the successfully established microbiome (Noda et al., 1997; Sacchi et al., 2004).

Evolutionary processes have demonstrated the shift in organism's relationships from parasitism to mutualism, even though this development is not compulsory (de la Fuente et al., 2016; Wade, 2007; Sonenshine & Roe, 2014). An invertebrate species is termed vector for a given infectious disease when it is involved in the transmission to susceptible host. Flies, snails, mosquitoes, bugs, lice, mites and ticks are among the most important intermediate hosts and/or vectors in epidemiology. Evolutionary selection processes have created specific vector-host relationships based on the aptitude of a vector species to successfully transmit a particular pathogen to a susceptible host (vector capacity) or to other vectors. Moreover, the ability of the

infected host or vector to further develop and spread the pathogen is determined by its vector competence (Wilson et al., 2017).

Ticks are obligate ectoparasites taking blood meals from a wide range of host species including humans, depending on the development stage, host availability and environmental factors. Members of the tick endosymbiont have evolved to exploit this hematophagous nature to be transmitted to their host insuring propagation and survival (Šimo et al., 2017). Those endosymbionts can be pathogenic, commensal or mutualistic microorganisms to the host, interfering with each other with 'authentic' endosymbiotic relationship or not (Noda et al., 1997; Ahantarig et al., 2013). These relationships have been shown to be limited by the vector capacity and the effectiveness of the host immune response against the microbe (Kalil et al., 2017; Palomar et al., 2019). Pathogenic transmitted organisms are known as tick-borne pathogens (TBP) causing tick-borne diseases, and their importance on the biological, veterinarian and medical point of view determines the level of attention given to the interfaces vector – host – parasite.

3. Pathogen transmission among tick host

TBPs are known to affect the biology of the tick vector to facilitate their transmission. Pathogen transmission between the vector – host system can be complex. Cyclic transmission is the rule, although acyclic transmission can be observed (Elelu et al., 2016). Ticks are naturally infested by ingesting the parasite from the host during the blood meal. Once in the vector, these pathogen undertake strategies to be maintained alive, multiply, safeguarding their transmission as infective stage, thereby insuring further developmental stages.

Strategies allowing the pathogen circulation between tick hosts include stadial, transovarial, co-feeding and mechanical transmission.

3.1. Stadial transmission

This mode of transmission has three variations according to the state of the transmitted pathogen and the tick's developmental stage. The intrastadial transmission refers to the stage of the pathogen currently transmitted. A common example involves adult male ticks from the *Amblyomma* genus. After successful blood meal and mating, they eventually detach from their current host to infest a new one to mate with as many females as possible (Constable et al., 2017). If the parasitized host is patently infected

with the pathogen *Anaplasma marginale*, *A. centrale* or *Ehrlichia ruminantium*, the male ingests infective stages of the pathogen and becomes infective for any new host. For an intrastadial transmission to occur, the pathogens needs to be infective for the host in the life cycle stage it was acquired from (Ueti et al., 2008). This mode of transmission is most effective when a large population of mechanical vectors is present in the environment.

Pathogens may also with time evolve in their previously mechanical vector including developmental stages. It is the case for the non-pathogenic parasite *Trypanosoma theileri* transmitted by tabanids, yet identified in ticks (Latif et al., 2004) and recently in phlebotome flies (Calzolari et al., 2018) with both vectors presenting developmental stages of the parasite after dissection.

Transstadial transmission entails the sequential transmission between different development stages, such as larva to nymph and/or nymph to adult (Aguirre et al., 1994). Such transmission is common in the life cycle of the highly virulent piroplasms *Theileria parva* and *T. annulata*, because the pathogens become only infective after molting stages, when sexual gametes mate and develop to the infective sporozoite form. This mode of transmission is more common in *Rhipicephalus appendiculatus, R. zambeziensis* and in some mite species, remaining a pathogen feature*. Anaplasma centrale* can also undergo transstadial transmission while transmitted by *R.* (*Boophilus*) *microplus* and *R.* (*B.*) *simus* (Potgieter & van Rensburg, 1987).

3.2. Transgenerational transmission

Transgenerational transmission – also known as vertical transmission describes the passage of a parasitic microbe from an infected host or vector to its offspring (Burkett-Cadena, 2019). If the ovaries are infected it is a transovarial transmission which is one of the most sophisticated ways of pathogen maintenance in nature. Examples of transovarial transmission for TBDs include the protozoa *Babesia bovis* and *B. bigemina,* transmitted by *Rhipicephalus (Boophilus)* spp. The blood parasite is ingested from infected hosts both by adult or nymph/larval stages. In females of the premature stages the pathogen invades the ovarian germinal tissue and gets transmitted transstadially until the reproductive adult stage has been reached. When the female lays eggs after insemination, a proportion already carry the parasite ready to be transmitted onwards to susceptible blood hosts. Venereal transmission is another variant of the transgenerational transmission where the pathogen is passed from male

to female adults through the spermatophore. It has been shown for the transmission of spotted fever group Rickettsiae by infected *Ixodes ricinus* male ticks (Hayes et al., 1980).

3.3. Co-feeding transmission

Co-feeding transmission is defined as the transmission of a parasitic microbe from one competent vector to another in close proximity without necessarily creating a patent infection in the blood host. A number of short-living viruses which do not produce viremia in the vertebrate host use this strategy, considerably increasing the likelihood of vector transmission (Nonaka et al., 2010). Some groups of bacteria use this mode of transmission, such as *Borrellia afzellii* causing Lyme borreliosis in rodents (Belli et al., 2017), or *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever in Brazil. The advantage is that a large number of co-feeding tick vectors get infected while only a low number of systemic infections occur in the host population. The Tick-Borne Encephalitis virus has been a prime example of this phenomenon in rodents (Randolph, 2004).

Unlike mosquitoes which are arterial feeders, ticks belong to the group of pool feeders by cutting a small wound with their cheliceres. Apart from host blood and lymph the feeding pool contains other fluids, including saliva produced by the tick's glands and consequently the enclosed pathogen (Cutler et al., 2012). While feeding, ticks produce pheromones to attract conspecifics to share the feeding pool. This allows mating, pathogen transmission, and a stronger down-regulation of the host's sensorials (skin itching, pain) and immune response (Nuttall, 2019). The efficiency of co-feeding transmission has been shown to greatly depend on the involved strain (Eremeeva & Dasch, 2015; Wilson et al., 2017). High efficiency co-feeding transmission correlates with a higher R_0 value (Belli et al., 2017) suggesting the association of this phenotype to higher virulence or invasiveness (Norman et al., 2016; Voordouw, 2015; Walker, 2014).

3.4. Transmission by coxal fluid (Argasidae)

Like other blood feeding arthropods, ticks discard their bodies' aqueous excess to increase the nutritional value of blood meals (Šimo et al., 2017). In soft ticks, this is undertaken by the coxal glands (Fig. 1) while in hard ticks the excess fluid is alternatively with blood uptake injected in the feeding pool by salivary glands allowing

blood meal concentration (Sauer et al., 2000; Šimo et al., 2017). This mode of transmission has been observed in the pathogen – vector system *Borrelia duttonii* - *Ornithodoros moubata* responsible of the Tick-borne Relapsing fever, and African Swine fever virus transmitted by *Ornithodoros porcinus* (Schwan et al., 2009; Zheng et al., 2015).

4. Interaction interfaces

The same as in any organism, a parasitic microbe strives to maintain circulation in its habitat (susceptible vertebrate hosts). As a result, intricate interactions have evolved between the pathogens, its vectors and the vertebrate hosts to maximize their distribution (de la Fuente et al., 2017; Kazimírová et al., 2017; Wikel, 2018). Whereas the host acquired defensive strategies in response to pathogen invasion, the invader has developed mechanisms to circumvent these to increase fitness and survival (Keesing et al., 2006; Saito & Walker, 2016).

4.1. Tick – pathogen interface

The ability of an arthropod to transmit a pathogen to a susceptible host has been described as vectorial capacity and vector competence. The vectorial capacity is influenced by ecological and behavioral factors mainly affecting vector abundance, survival and competence, making the vector competence a component of the vectorial capacity (Beerntsen et al., 2000). In general, vectorial capacity describes the dynamics between the competent vectors of an infectious disease agents and its hosts (Dye, 1986; Rizzoli et al., 2019). In tick-borne infections, these relationships are considerably more complex implicating transmission dynamics and biological processes as compared to mosquito-borne ones (Hartemink et al., 2008; Rosà et al., 2003).

As other invertebrates, ticks activate their immune and cellular responses to challenge the invading pathogen through multiple pathways (Carr et al., 2017; Hajdušek et al., 2013). Antimicrobial peptides (AMPs) and defensins proteins (direct antimicrobial defense) are able to directly attack, kill or inhibit invaders from reproduction (Hajdušek et al., 2013). In almost all parasitic relationships between ticks and invading pathogens, the evasion plan of the pathogens overlap, with different speeds and cells involved (Belli et al., 2017; Gleim et al., 2016; Liu & Bonnet, 2014; Walker, 2014). The ingested pathogens enter the tick host through the blood meal, while some end up between epithelial cells, others become established within the midgut lumen. Ingested stages

(*i.e.* spirochetes of *Borrelia burgdorferi*) trapped outside of the peritrophic membrane are unlikely to survive (de la Fuente et al., 2017; Jalovecka et al., 2018; Blancou et al., 2004). Those established between epithelial cells use lipoproteins located on the host's cell surface to bind to a species-specific receptor (Pal et al., 2004). Albeit surviving spirochetes may start to undergo an initial phase of replication, these populations decline during the tick's post-feeding molt (Sonenshine & Macaluso, 2017).

The subsequent blood meal provides resources for further development, namely increasing the host tick's activity and triggering the pathogens' migration from the midgut to the hemolymph, and later to the salivary glands (Fig. 2). During the crossing of endothelial barriers, the vast majority is taken down by the immune system, *i.e.* by phagocytosis, before accessing the salivary glands where they have better control over the immune system (Coleman et al., 2006; Hajdušek et al., 2013; Sonenshine & Macaluso, 2017). Upon contact with the salivary glands, the survivors of the 'hemolymph crossing' quickly bind to immunosuppressive factors thus protecting them from potential host immune responses (Blisnick et al., 2017; Nuttall, 2019; Woldehiwet, 2010). Once they are established, their infectiveness is triggered by the vector's next blood meal.

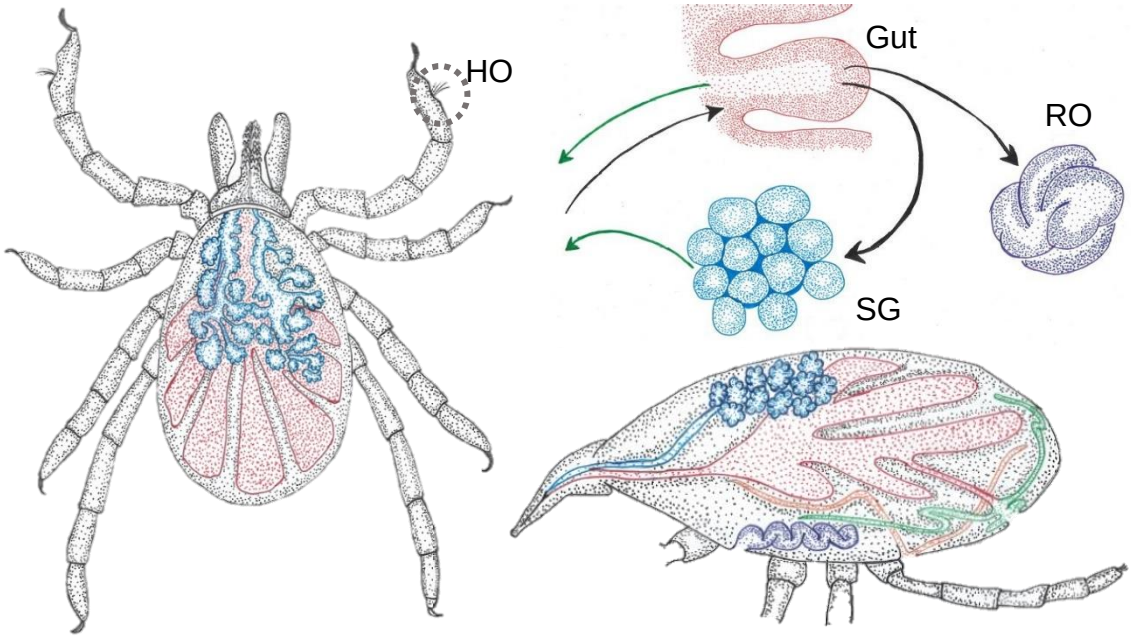


Figure 2. Schematic view presenting some relevant ticks inner organs associated to its physiological features and hosting the pathogen developmental stage until the transmission: midgut (red, Gut), salivary glands (blue, SG), reproductive organs (purple, RO). Haller's organ (black, HO), circle on the left. Image: Dorsal view: B. Abanda (reproduced based on

microscopic images); lateral view: modified from https://commons.wikimedia.org/wiki/File:Ixodid_tick_structure.jpg.

4.2. Host vertebrate – tick interface

The blood meal is recognized by the host as a detrimental process, triggering physiological and immunological reactions against potentially harmful invading pathogens (Fig. 3). As long lasting feeders (mostly hard ticks), ticks have developed myriad strategies to remain discreetly but solidly attached to their feeding hosts. The host's immune system naturally reacts by producing coagulant factors resulting in a hemostatic plug, vasoconstriction, inflammation and tissue remodeling aiming for wound healing and tick rejection (Šimo et al., 2017). Through evolutionary selection, ticks have developed effective ways to counterattack the host's immune system to ensure the completion of the blood meal. In the human host for example, ticks prevent the alert of sensorial organs (pain and itch) which may lead to manual removal. In cattle, the grooming behavior (rubbing, licking) may also expulse the tick from its host (Simonsen, 1979). Compounds in the salivary glands harbor all protective machinery against the host's line of protection. The mouth parts of some species are firmly attached to the host by a cement (Mans et al., 2014) produced by salivary glands (Kemp et al., 1982; Nuttall, 2019). Furthermore, they contain inhibitors targeting enzyme sites responsible of the activation of blood meal-mediated immune responses.

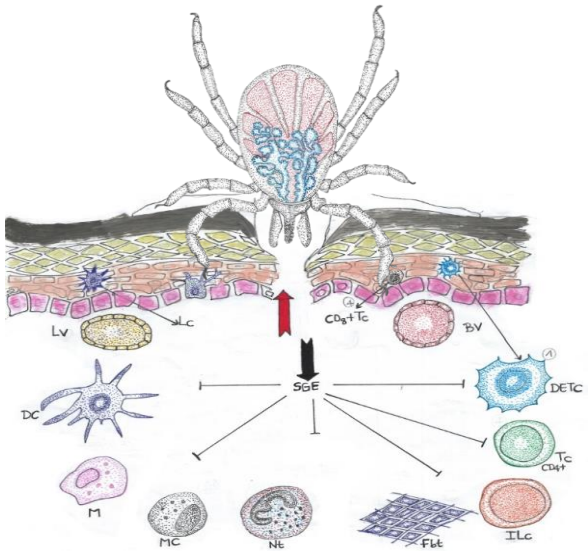


Figure 3. Schematic view, presenting the down regulation of the host protection by tick salivary glands secretions at the biting site favoring pathogen transmission. Langerhans cells (LCs), dendritic epidermal T-cells (DETC), CD8 T-cells, fibroblasts (Fbt), CD4 T-cells (CD4+ Tc), innate lymphoid cells (ILc), dermal cells (DC), macrophages (M) mast cells (MC), blood vessel

(BV), lymphatic vessel (LV) and neutrophils (Nt). Source: Modified from (Kazimírová et al., 2017).

During the vector-host contact the exchange of body fluids is most effective, thus enabling pathogen transfer from one host to the next. Tick salivary gland extracts have been reported to act as adjuvant, increasing the number of vectors on the feeding pool (see section 3.3. Co-feeding transmission).

4.3. Host vertebrate – pathogen interface

Pathogens transmitted by ticks are mostly obligatory intracellular parasites, entering and proliferating in cells and tissues, therefore relying completely on the host behavior to complete their life cycle. The intrusion of the foreign body (pathogen in the host) ignites a succession of immunological mechanisms including the massive clonal expansion of lineages of lymphocytes directed against the pathogen, and the recruitment and activation of different effectors systems: specific antibodies, macrophages, phagocytes and cytotoxic cells (de la Fuente, 2008; Hajdušek et al., 2013; Villar et al., 2016). During this process, diverse molecules (*e.g.* cytokines) are secreted to activate immune pathways all interacting with each other. Once the invasion is under control, a series of down-regulating actions are triggered with feedback mechanisms involving antibodies, regulatory cytokines and suppressor cells (Grenfell et al., 2004). Under such an intense line of protection, the pathogens had to evolve accordingly and develop multiple ways to allow their survival and propagation (Ayllón et al., 2015).

The assumption that parasites are inclined to rapid evolution is widely accepted, mainly based on their generally short generation time and large population (Battilani et al., 2017; Grenfell et al., 2004; Poulin & Randhawa, 2015). Based on the 'enhanced speed' of evolution, parasites have learned to quickly adapt in response to the immune response of the host, sometimes by overcoming or inducing mechanical barriers, or by manipulating the host to their own benefit. Pathogens are able to display phenotypic plasticity in response to new environments even in the same host (Bernard et al., 2018; Cangi et al., 2017). The decision to display a new life history (plasticity) or to maintain a fixed response to the environment's hostility greatly depends on the benefits and the costs necessary for the establishment of the new phenotype. For instance, an optimal allocation of resources in the pathogen development needs to be shared between growth and reproduction. When growth is retarded or receding, the pathogen invests

more energy in propagation to achieve a host change. In some evolved system, such as parasitoids, parasitic castrators, directly transmissible parasites and vector transmissible parasites, the resources are greatly provided by the carrying host more than often without its consent, sometimes at the cost of its survival (Grosman et al., 2008; Murgia et al., 2018; Poulin & Randhawa, 2015).

5. Selected tick-borne pathogens and their significance in the cattle host

Tick-borne pathogens (TBP) are a significant risk to human and veterinary public health (Lorusso et al., 2016; Parola & Raoult, 2001), including economic losses due to increased costs for treatment, prevention measures and control strategies (Allsopp, 2015; Ndip et al., 2005). The close relationship between humans and their domesticated livestock has greatly influenced the emergence of novel diseases and zoonoses in humans and animals alike. Moreover, the high level of habitat encroachment of human settlements, including urban environments facilitated closer contact with previously rather isolated wildlife and thereby an elevated risk of emerging infectious diseases (Baneth, 2014; Nyangiwe et al., 2019). Mosquitos and ticks are among the most important arthropods carrying and transmitting some of the most dangerous infectious agents globally (Davoust et al., 2010; Nyangiwe et al., 2019). Those infectious agents transmitted by ticks include viruses, bacteria and protozoans.

5.1. Tick-borne viruses

Both the retroviral genera Flavivirus and Orthonairovirus have species which are transmissible by ticks. A prominent member with zoonotic potential of the family Flaviviridae is the Tick-borne Encephalitis virus (de la Fuente, et al., 2017), whereas the Orthonairovirus Crimean-Congo hemorrhagic fever virus (CCHFV) is a TBP with an important distribution in Europe, Asia and Africa (Grard et al., 2011; Nyangiwe et al., 2019; Whitehouse, 2004). CCHFV is reported to be the most genetically diverse arbovirus making the molecular detection of the strains challenging (Kinsella et al., 2004). The main biological vector in endemic regions has been hard ticks of the *Hyalomma* genus, although also other tick vectors have been found carrying the virus (Bażanów et al., 2017; Hornok & Horváth, 2012). CCHFV has been identified in a wide range of vertebrates, with birds being generally resistant (Shepherd et al., 1987). Mammals such as small and large livestock species can be infected, however, no disease have been associated to the presence of the pathogen (Hornok & Horváth, 2012). Humans are by far the most vulnerable/susceptible host group. Infection can

occur by tick bites, mechanical vectors or contact with contaminated host tissue (Engin et al., 2019; Inci et al., 2016). In the most severe form the virus causes hemorrhagic fever syndrome with often fatal outcome (Karakus et al., 2019).

5.2. Tick-borne bacteria

5.2.1. *Anaplasma*

Anaplasmosis is an infectious disease affecting cattle, wildlife and human hosts. The pathogen is an intra-erythrocytic bacterium of the order Rickettsiales belonging to the genus *Anaplasma* (Fig. 4). The disease is of major constraint in cattle productivity and is endemic in tropical and subtropical areas all over the world (Jonsson et al., 2008). Young animals appeared to be more resistant to *Anaplasma* infection. In contrast, older animals experience a wide range of clinical signs, resolving between mild to severe outcome (Aubry & Geale, 2011).

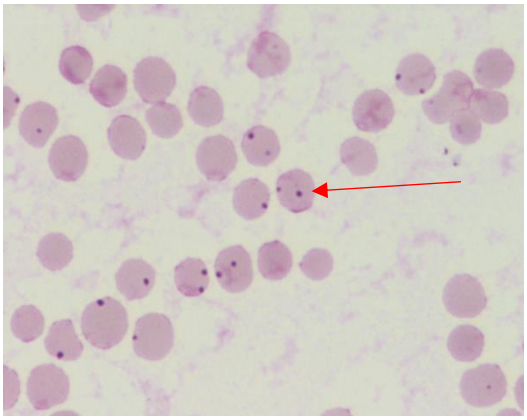


Figure 4. Giemsa-stained microscopic blood smear of cattle erythrocytes infected by *Anaplasma marginale* from Tunisia. The red arrow shows the typical phenotype. Photo: Mohamed Aziz Darghouth & Mohamed Gharbi, Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Tunisia.

After infection, cattle remain persistent carrier with low level of bacteria in the system, leading to strong immune responses if re-exposed (Aubry & Geale, 2011). *Anaplasma* can be identified by stained erythrocytes on blood smears, by serological test and molecular tools (Aubry & Geale, 2011). Anaplasmosis differs from most bovine TBPs by its ability to be transmitted cyclically and intra-stadially by mechanical vectors (*Stomoxys* stable flies, *Anopheles* mosquitoes and other dipterans) or blood-contaminated fomites (Aguirre et al., 1994). *Anaplasma marginale* is generally transmitted by *Rhipicephalus (Boophilus)* spp. and *Dermancentor* spp. ticks, depending on the environment (Aguirre et al., 1994).

5.2.2. *Borrelia*

The genus *Borrelia* covers a heterogeneous range of spirochetes bacteria with an increasing recognized biodiversity transmitted both by hard and soft ticks (Cutler et al., 2017). The probably most important member of the relapsing fever group belongs to the *Borrelia burgdorferi* complex and causes the zoonotic disease Lyme Borreliosis. It is transmitted by ticks from the *Ixodes* genus, and has been identified on all continents except Antarctica with an increasing incidence (Barbour, 2014). In Germany, Lyme borreliosis is the most frequently reported tick-borne disease, with annual costs of laboratory testing in the outpatient sector estimated to be 51 million Euros (Enkelmann et al., 2018). *Borrelia theileri* has been reported for years as a mild to non-pathogenic species in the cattle population worldwide. Its pathogenicity and characterization has been difficult to assess due to co-infections with other pathogens.

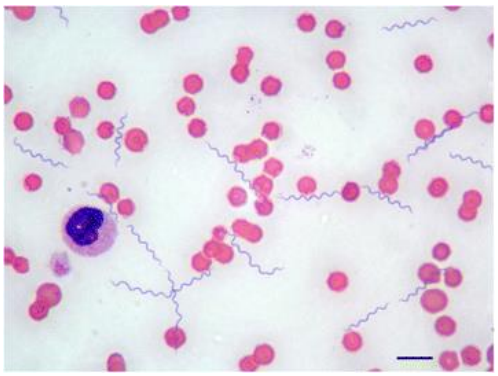


Figure 5. Higher magnification of spirochetemia with *Borrelia persica* in cat. Romanowsky stain. Scale-bar: 10 µM. Photo: (Baneth et al., 2016).

The transmission among vectors is transovarially and trans-stadially, with co-feeding described as one of the most important (Belli et al., 2017). In Africa, the identification of this bacteria greatly suffers from efficient diagnostics in areas where the pathogen have not yet been identified, mostly mixed up with other extracellular blood parasites such as trypanosomes. Misidentification by microscopic tools is not directly related to the shape of the parasite (Fig. 5) but mainly to the movements in a pool of erythrocytes without clear sight of the pathogen itself.

5.2.3. *Dermatophilus*

Dermatophilosis is an opportunistic bacterial disease established under low immune reactiveness in animals and humans. This severe skin condition is seen mostly on animals with high infestation levels of the tick *Amblyomma variegatum* (Merlin, Tsangueu & Ronsvoal, 1987; Stachurski et al., 1993a) and by contamination or bad hygiene to men (Ambrose, 1996; Ambrose et al., 1999). Disease progression of the ubiquitously occurring skin actinomycete bacteria *Dermatophilus congolense* (Fig. 6) can equally be favored by environmental conditions such as high humidity and poor health conditions (Ambrose, 1996).

Figure 6. Cow showing bovine dermatophilosis lesions all over the body possibly induced by the bacterium *Dermatophilus congolensis* following high *Amblyomma variegatum* tick infestation. Photo: Dr. Albert Eisenbarth, Programme Onchocercoses Ngaoundéré, Cameroon.

The massive suppression of the host's immune response by a high frequency of tick bites facilitates the uncontrolled propagation of *D. congolense* in the host which may recover or die according to the severity (Kemp et al., 1982). Calve infections by dermatophilosis involving *A. variegatum* have been shown to undergo more severe lesion and longer lasting recovery than environmentally infected individuals (Ambrose, 1996). The difference in immune response against the same bacteria displays the significant contribution of the intervention of salivary glands of *A. variegatum* in the virulence of the pathogen (Šimo et al., 2017). Dermatophilosis have been reported being responsible of losses in cattle livestock population in endemic African countries, with genes on the major histocompatibility complex (MHC) locus associated to susceptibility (acute clinical cases and epidemiological prevalence) (Maillard et al., 2003).

5.2.4. *Ehrlichia*

Ehrlichia spp. are obligate intracellular parasitic bacteria infecting mammals such as humans and ruminants through tick bites (Cangi et al., 2017). The symptoms include high fever and blood anemia, with a likely fatal outcome without treatment. Heartwater or cowdriosis is caused by the pathogen *Ehrlichia* (*Cowdria*) *ruminantium* which is transmitted by the ticks *Amblyomma variegatum* (Afrotropical region), *A. pomposum* (Angola) and *A. hebraeum* (South Africa, Botswana, Zimbabwe, Mozambique) (Bekker et al., 2002). Whereas *E. ruminantium* is highly virulent in cattle populations, *Ehrlichia canis* (Fig. 7) is mainly restricted to canine hosts (dogs), but has also been identified in humans (Dumler et al., 1991; Saito & Walker, 2016). A novel genotype closely related to *E. canis* was revealed in cattle from North America, however forming its own separate clade (Gajadhar et al., 2010).

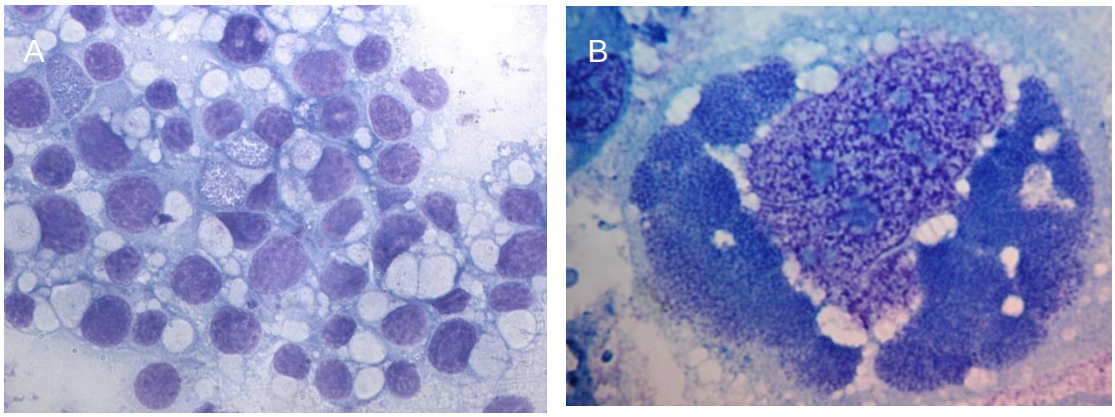


Figure 7. Stained microscopic smear of (A) IDE8 tick cell culture infected by *Ehrlichia canis*. (B) Dense form of *Ehrlichia ruminantium* in CHO cells. Photo: Dr. Erich Zweygarth, Freie Universität Berlin, Germany.

5.2.5. *Rickettsia*

Rickettsia spp. – also grouped as Rickettsiae – are small gram-negative obligate intracellular bacteria (Fig. 8) which are divided in two disease-inflicting groups: the Typhus group and Spotted fever group (Parola et al., 2013). Murine typhus causes endemic diseases in rats and can be transmitted to humans by rat fleas or other unidentified vectors (Sankasuwan et al., 1969). Most of the rickettsial Spotted fevers are transmitted by ticks. *Rickettsia*-harboring vectors are maintained in the population by horizontal and/or transovarial transmission, making the vector the main reservoir of the pathogen (Legendre & Macaluso, 2017). Ecological characteristics of the vector (passive and active) strongly influence the epidemiology of the disease and its

transmission (Behar et al., 2010). There are more than 30 recognized *Rickettsia* spp. plus more uncharacterized strains (Chisu et al., 2017). *Rickettsia africae* is the causative agent of African Tick Bite fever, and well known to infect humans with severe health implications (Ndip et al., 2004). Their principal vectors are *Amblyomma variegatum* and *A. hebraeum*, both autochthonous to large regions in Africa.

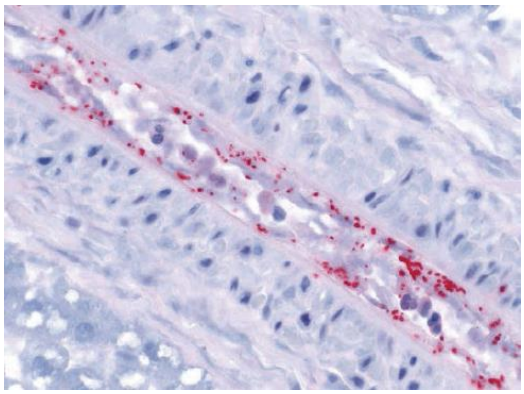


Figure 8. Immunohistochemical stain demonstrating *Rickettsia rickettsii* (red dots) in the infection of blood vessel endothelial cells. Photo: (Biggs et al., 2016).

5.3. Tick-borne protozoans

Unicellular apicomplexan protozoans of the family Piroplasmidae are obligately transmitted by ticks. For cattle livestock populations, the genera *Babesia* and *Theileria* have the highest significance.

5.3.1. *Babesia*

Bovine babesiosis is caused by the intra-erythrocytic piroplasms *Babesia* occurring in a wide range of climatic zones from temperate to tropical (Hauvin et al., 2009), in particular in traditional production systems (Bock et al., 2008; Mamoudou et al., 2017). Like *Plasmodium* in humans, the pathogen lives in the red blood cells of the carrying host (Fig. 9) and in the organs of the ticks where it can be transmitted through both the ovaries to the next generation and trans-stadially (Yu et al., 2016). The main species of cattle babesiosis in the tropics are *Babesia bigemina* and *B. bovis,* both mainly transmitted by the ticks *Rhipicephalus (Boophilus*) *microplus* and *R.* (*B.) annulatus* (Jonsson et al., 2008). Clinical signs of babesiosis include high fever and hemoglobinuria. Environmental factors such as humidity and rainfall are risk factors associated with the transmission of bovine babesiosis in areas where both the vector and pathogen are endemic. Climate have also been recorded to influence the lifespan of the ticks' larval stages, resulting in fluctuations in population size per generation and

year (Jalovecka et al., 2018). Some of the *Babesia* species have not been fully characterized, meaning their biological vectors and natural hosts are unknown. Previous assumptions that humans are accidental host to the pathogen have been proven wrong (Yabsley & Shock, 2013). The lack of molecular tools in endemic countries with sufficiently high specificity and sensitivity to the pathogen is one of the main reasons for diagnostic failure not always regarded as false negative (O'Connor et al., 2018).

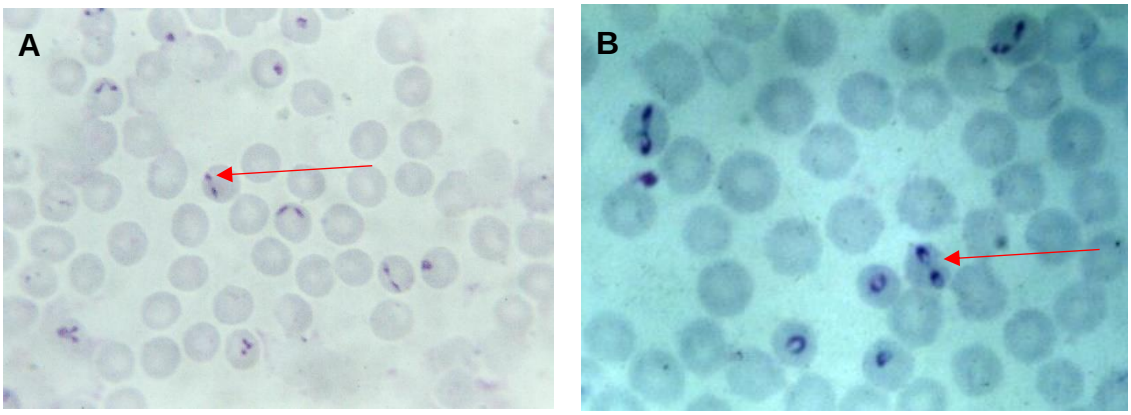


Figure 9. Stained microscopic blood smear of cattle erythrocytes infected with (A) *Babesia divergens* and (B) *Babesia bovis* from Tunisia. The red arrows show the typical phenotype, respectively. Photo: Mohamed Aziz Darghouth & Mohamed Gharbi, Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Tunisia.

5.3.2. *Theileria*

The most pathogenic theileriosis for cattle populations are tropical theileriosis and the East Coast fever, caused by *Theileria annulata* (Fig. 10) and *T. parva*, respectively (Muhanguzi et al., 2014). Those pathogens endemic respectively to North Africa and Southern Europe, through the Middle East and across Southern Asia for *T. annulata*, and South- East Africa for *T. parva* have been reported subject of substantial economic losses and significant anemia in infected indigenous animals (Yu et al., 2016). For imported breeds, immune-compromised and stressed animals, the disease is more severe, in many cases lethal (Gebrekidan et al., 2017). No transovarial transmission has been observed in the tick vector, but the pathogen can be transmitted transstadially. *Theileria velifera* and *Theileria mutans* are known as 'mild' or 'non-pathogenic' organisms, and are often found in co-occurrence with other TBPs (Neitz, 1957). Infection with *Theileria* spp. occur worldwide, and are determined by the distribution and seasonal activity of competent tick vectors. Animals who survive the

disease undergo a prolonged and incomplete convalescence, resulting in carrier stage and loss of productivity (Sahoo et al., 2017).

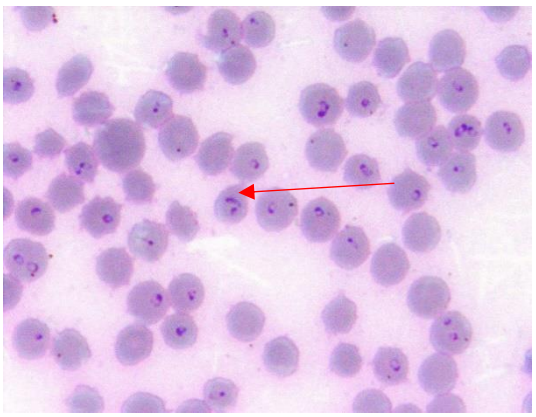


Figure 10. Stained microscopic blood smear of cattle erythrocytes infected with *Theileria annulata* from Tunisia. Infected erythrocytes contain several forms of piroplasms. Photo: Mohamed Gharbi, Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Tunisia.

5.4. Generic life cycle

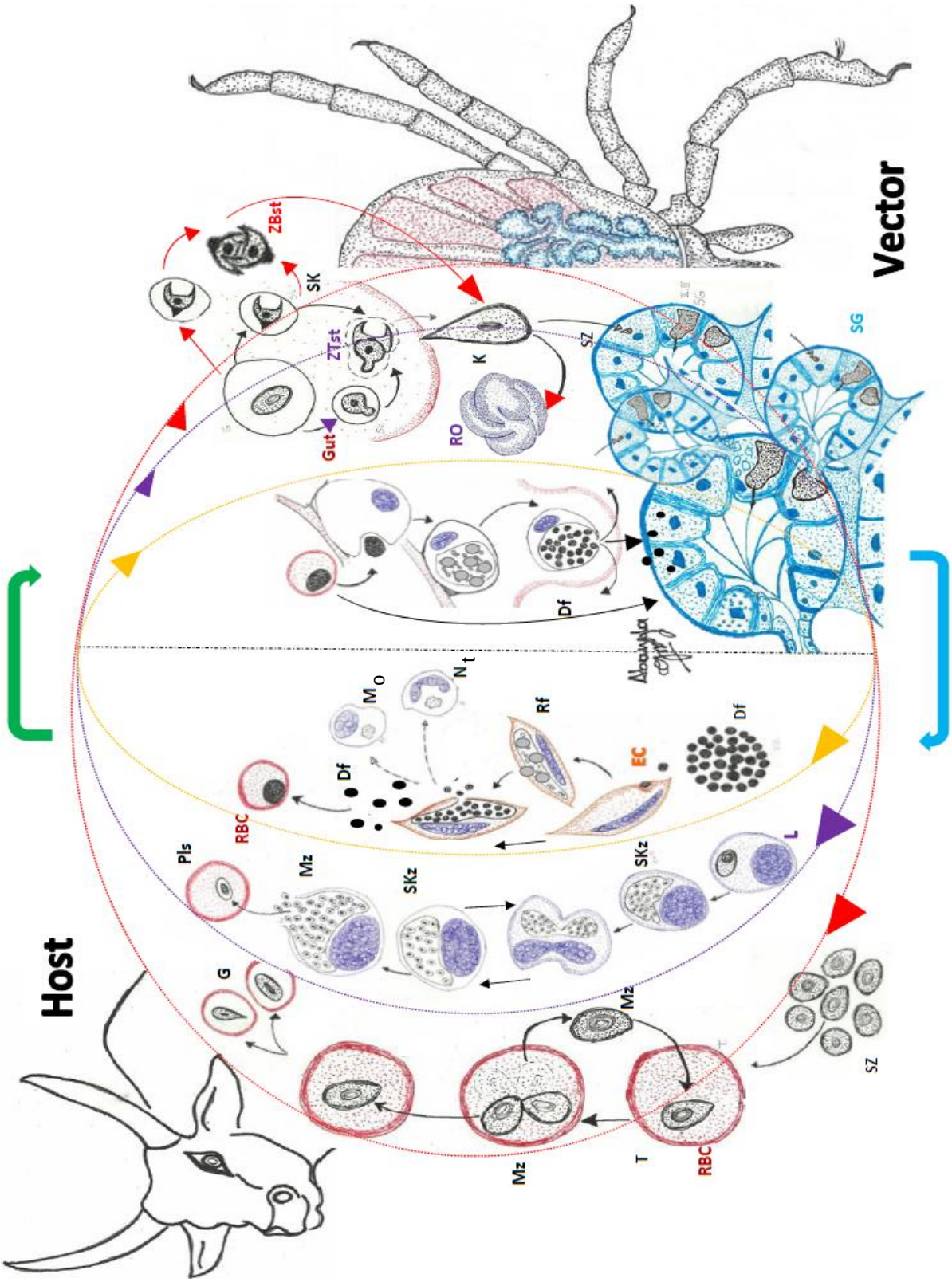


Figure 11. Schematic life cycles of *Babesia* (red arrow), *Theileria* (purple arrow) and Rickettsiales (Anaplasmataceae) (orange arrow). Captions and abbreviations are explained below.

Life cycle of piroplasms

Theileria sensu stricto

Sporozoites (SZ) enter the host lymphocytes (L) and develop into schizonts (SKz) by asexual reproduction then proliferate. Some of the schizonts undergo merogony, giving rise to merozoites (Mz), entering the erythrocytes (RBC) to form piroplasms (Pls) or gametocytes (G) which are infective to ticks. Gametocytes undergo sexual reproduction within the feeding larvae or nymph internal tissues, transstadially transmitted to the subsequent stage. The gametes of *Theileria* form a zygote (ZTst) from two morphologically distinct cell types (ray bodies) or Strahlenkörper, (Koch 1906) (SK), micro- and macro-gametes (no transovarial transmission). The infective stage 'Kinete' (K) migrates and invades the salivary glands and multiplies by asexual proliferation in a cavity (sporogony in a sporoblast). Its maturation starts after tick attachment to the host, resulting in sporozoites being released into tick saliva, transmitted to the mammalian hosts as sporozoites (SZ) during the blood meal (Smith and Kilborne, 1893; Mehlhorn et al., 1985; Starcovici 1893; Koch, 1898 and Theiler 1904, 1906; Jalovecka et al., 2018; Nene et al., 2016).

Babesia sensu stricto

Sporozoites (SZ) enter the host red blood cells (RBC) and develop into trophozoites (T). The asexual reproduction produces merozoites (Mz) which proliferate, penetrating new cells. Some of merozoites (Mz) cease dividing and form piroplasms (Pls) or gametocytes which are infective to ticks. Gametocytes undergo sexual reproduction within the feeding larvae, nymph or adult stage, and are transstadially and transovarially transmissible (except *B. microti*) (Uilenberg, 2006) to the subsequent stage or progeny, respectively. The gametes of *Babesia* parasites form a zygote (ZBst) from two morphologically identical cell types (SK). The final infective stage 'Kinete' (K) migrates and invades the salivary glands, and multiplies by asexual proliferation in a cavity (sporogony in a sporoblast). Its maturation starts after tick attachment to the host, resulting in sporozoites being released into tick saliva, transmitted to the mammalian hosts as sporozoites (SZ) during blood feeding (Jalovecka et al., 2018; Schnittger et al., 2012).

Life cycle of Anaplasmataceae (*Anaplasma* & *Ehrlichia*)

The dense form (Df) is released into the host bloodstream from infected ticks salivary glands and enters the endothelial cells (EC) of blood vessels. Dense forms are successively converted into reticulate form (Rf) and Df, under asexual reproduction inside a protective vacuole. Some of the converted infective Df released into the cytoplasm as extracellular forms infect circulating neutrophils (N) (*A. phagocytophilum)*, monocytes or RBCs. Colonized RBCs are engorged by the tick during the blood meal. Bacteria enclosed by RBC are released and infect tick tissues, including salivary glands (intrastadial transmission). Multiplication occurs alternating between

the Df and Rf inside the tick cells. The infective form, migrate to the salivary glands to be later transmitted during the blood meal (transstadial transmission) into the new host (Jalovecka et al., 2018; Mastronunzio et al., 2012; Prozesky & Du Plessis, 1987).

6. Epizootiology of tick-borne pathogens in Cameroon

Concerns about specific tick transmitting pathogens in Cameroon is a relatively new concept, even though cattle have been treated with acaricides ('tick baths') against the most devastating TBDs since many decades. Back then this was mainly directed to the good and healthy appearance of the animal skin for food and leather production. Indeed, cattle value considerably decreases based on apparent remnants of the tick infestation or associated skin conditions (Stachurski et al., 2000). In addition to the physical damage ticks created, cattle breeders are nowadays more aware of the detrimental impact of the pathogens they transmit. Tick-borne pathogens cause considerable economical losses in the livestock sector. In fact, they are considered one of the most detrimental causes impairing the agricultural sector in the livestock industry in the developing world, of which number have zoonotic potential (Esemu et al., 2018). Zoonoses are defined as often highly-pathogenic infections shared between other vertebrate animal species (livestock and wildlife) and humans (McDaniel et al., 2014). The presence of mechanical vectors, *e.g.* arthropods transmitting diseases by contamination with viable infectious agents, considerably increased the range of potential vectors to be targeted for disease eradication. Additionally, a number of emerging pathogens have been shown to originate from wildlife limiting their control (Foil & Gorham, 2000; Wells, 1972; Yabsley & Shock, 2013).

One of the most challenging factors in the relationship between the host and arthropod-transmitted pathogens is the detection of carrier animals without clinical symptoms. In the epizootiological context, carrier cattle are known to maintain the pathogen in the population, spreading by the surrounding vectors. Such animals serve as reservoir allowing the pathogen transition to other susceptible individuals (Ueti et al., 2008). The enzootic status is another limiting factor associated to losses of the livestock economic value (Mattioli et al., 2000; Jonsson et al., 2012). The pressure due to TBP on cattle livestock include animal weight loss, leading to lower yields, increased herd morbidity and mortality, and a reduced reproduction rate (including abortion), all of which ultimately lead to lower profitability of the domestic livestock sector. Those have a

noticeable impact on the farmer's life, health risk by zoonotic infections, and socio-economic development (Awa et al., 2015).

6.1. Tick-borne pathogens in the cattle population in Cameroon

The prevalence of different TBPs in cattle from Cameroon including information of the co-infection status is extremely scarce if present at all. Single studies on particular pathogen showed a high variation in the prevalence with fluctuation among and between seasons (Mamoudou et al., 2017; Ndi et al., 1998) and hosts including humans (Ndip et al., 2004). Pathogens transmitted by ticks have been reported to be endemic in the cattle population as a result of permanent exposure. A clear-cut between pathogenic and non-pathogenic species remains challenging, mainly due to the inability to effectively identify all microorganisms present in the host, to measure the level and impact of co-occurrence with other 'historical' TBPs, and the associated environmental factors leading to unpredictable responses among and between cattle breeds.

6.2. Diagnostic tools for identification of pathogens transmitted by ticks

In countries like Cameroon, the characterization of pathogens transmitted by ticks in the vertebrate host (cattle, sheep, goats, humans, etc.) and vector remains a strenuous attempt, mainly due to the lack of accuracy and sometimes reliability of most of the conventional diagnostic tools, but also the relatively high cost of the most reliable ones (Dwivedi et al., 2017; Speers, 2006). In parts of the world where resources are limited, the sustainability of livestock production (cattle and small ruminants) in a constant state of production is a myth (Eskezia & Desta, 2016). Among the most reported factors hindering successful TBP identification are the common state of co-infection, the pathogen density in the host (high or low systemic infection), and the animal state related to the pathogen's developmental stage (acute phase or carrier). Conventional diagnostic techniques include microscopy, polymerase chain reaction (PCR) and serology. Most routine diagnostic approaches for the identification of TBPs are still based on microscopic examination of blood smears and serological assays (Mamoudou et al., 2017; Ndi et al., 1998). The choice of those techniques rely on their moderate investments for equipment and infrastructure, and their robustness in practice. More sophisticated molecular tools based on PCR and high-throughput technologies such as next generation sequencing (NGS) are becoming widely spread,

at least in the developed and emerging countries. The latter being relatively economically viable when used for large sample sizes, and appropriate for the detection of multiple pathogens (Brinkmann et al., 2019). Nonetheless, a priori information on the screened pathogens is necessary, which is generally not applicable in situation of epidemiological studies or outbreaks (Cabezas-Cruz et al., 2018). Currently, the strength of the NGS technology in the area of TBP beside the detection of multiple pathogens is the possibility to study the synergy between pathogenic and non-pathogenic microorganisms associated with ticks, for correlation between ecosystem and interaction associations (Brinkmann et al., 2019; Nowrousian, 2010; Preidis & Hotez, 2015). As a result, NGS technology remains affordable only by a restricted range of laboratories with substantial resources and capacities in handling high cost and intensive labor and subsequent analyses (Nowrousian, 2010).

The microarray technology of PCR-amplified products by reverse line blot (RLB) combines high throughput, sensitivity, specificity and reproducibility (Beltramo et al., 2017). The currently used associated methodology involves a miniblotter (Nijhof et al., 2003). Its caveats as universal tool involve an extended protocol, lack of standard working solutions, and relatively demanding laboratory equipment. The first low-cost and low-density chip microarray kits (LCD-Array) produced were reported as a suitable solution allowing extensive usage. The reported drawback of this protocol however, is the challenge in simultaneous detection of *Babesia*/*Theileria* spp. and *Anaplasma*/*Ehrlichia* using a single microarray system (El-Ashker et al., 2015).

Based on those universal realities, identification of single species or ‘single pathogen’ epidemiology are still very popular (Nowrousian, 2010, Berry et al., 2019) leading to an incomplete picture of the epidemiology of TBDs and other diseases (Woolhouse et al., 2015).

6.3. Enzootic concept of the interfaces cattle – tick – pathogen

An enzootic status is a developmental stage of a disease in a population with epizootic or constant incidence displayed by a subset of the population (Jonsson et al., 2012). The enzootic status can fluctuate according to the interactions between the host (cattle) and his related parasite (ticks and associated pathogens, commensals or symbionts). The status can be of low instability (low tick population limiting the immunization of the majority of susceptible young animal and presence of clinical signs), high instability (low density of infected ticks leading to a low probability of an infected tick to be in

contact with a susceptible host increasing the morbidity and mortality in case of transmission) and stability (equilibrium between the susceptible host and the parasite, characterized by the absence of clinical disease, the high rate of infection and a low mortality and morbidity).

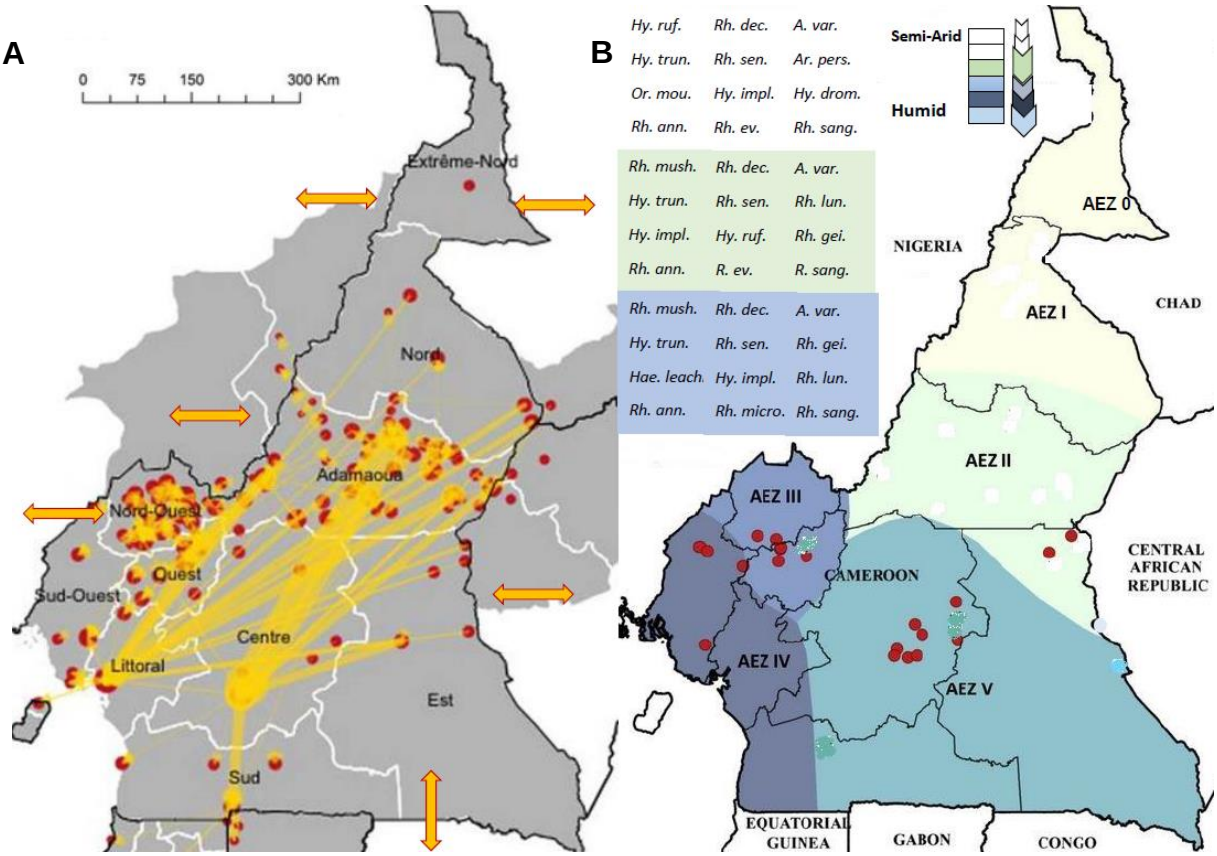
The enzootic status is determined and measured in the young population (2 to 9 months of age). The latter are exposed early in their lives to tick bites, among those, most are pathogens free (according to the birth season). Protected through the colostrum intake, they progressively acquire immunity, resulting in young population presenting high prevalence, with low or undetectable clinical signs. Critical cases can be due to impaired colostrum intake, enzootic instability at the time of birth (high percentage of biting ticks are infected, cancelling the expected immunization by progressive dosage), the introduction of a new strain more virulent or the co-infection with pathogens acquired before, during or after birth (Bram, 1983; Gharbi & Darghouth, 2015). Some of the prime factors influencing the enzootic stability are i) the high prevalence of the pathogen in the vector tick, ii) the presence of a susceptible vertebrate host constantly exposed to the pathogen, and iii) a high innate immunity (or resistance to the emergence of clinical disease, age related) at an early age in the vertebrate host for a resistant adult population (Jonsson et al., 2012; Uilenberg, 1995).

6.4. Pathogen prevalence and vector distribution

Cattle movements play a consistent role in the dynamics of vector-borne disease and their dispersion. These movements, also called transhumance, are not only meant to escape difficult environmental pressure but of great advantage for pathogen dispersion, including to the human host (Pamo, 2008; F'evre et al., 2006; Bronsvoort et al., 2004). Identified tick species from sample sites in Cameroon are reported in Figure 12B displaying the current knowledge on species distribution. Cattle movements reported in the Adamaoua regon in Cameroon are stated ranging between 53 and 170 km over 6 months to the grazing areas with potential resting periods (Motta et al., 2018). Livestock movements have been reported being even more globalized in the cattle trade network (Fig. 12A), favoring disease dispersion accross borders (Motta et al., 2017).

Cattle are infected by tick-borne microorganisms through bites of vectors or contaminations. Apart from human diseases, entomological data to correlate the vector distribution and the host history to the disease outbreaks have been scarce except the

report of new transmission cycles in foci of emergence and mathematical modeling for predictions (Pérez-Ramírez et al., 2017; Nguyen et al., 2019). The vectors' life span, the number of bites per individual in a lifetime, and the number of infective ones are important factors for the evaluation of vector-borne dynamics (Brand et al., 2016).



***Hy. ruf.**, Hyalomma rufipes; **Hy. trun.**, Hyalomma truncatum; **Or. mou.**, Ornitodoros moubata; **Rh. ann.**, Rhipicephalus annulatus; **Rh. dec.**, Rhipicephalus decoloratus, **Rh. sen.**, Rhipicephalus senegalensis; **Hy. impl.**, Hyalomma impeltatum, **Rh. ev.**, Rhipicephalus evertsi; **A. var.**, Amblyomma variegatum; **Ar. pers.**, Argas persicus; **Hy. drom.**, Hyamomma dromedarii, **Rh. sang.**, Rhipicephalus sanguineus; **Rh. mush.**, Rhipicephalus mushame; **Rh. lun.,** Rhipicephalus lunulatus; **Rh. gei.,** Rhipicephalus geigyi; **Hae. leachi.,** Haemaphysalis leachi; **Rh. micro.**, Rhipicephalus microplus*

Figure 12. (A) Cattle trading network in Cameroon and neighboring areas. The proportional volume of traded animals is indicated by the thickness of the arrow. Double sided arrows represent transboundary cattle movements. Map adjusted from Motta et al. (2017). (B) Reported tick distribution in Cameroon following the agro-ecological zones (AEZ). The red dots represent *Rhipicephalus (Boophilus) microplus* discovery in Cameroon. AEZ from 0 to V are grouped in three color codes representing semi-arid, semi-humid and humid climate. Cattle movement and tick distribution derived from Silatsa et al. (2019) and Walker et al. (2003).

Indeed, pathogens can be acquired from different reservoir ticks (three different development stages of two genders, multiple genera and species), wild mammal host (small or large), reptiles, birds (migratory and sedentary), amphibians and livestock (small and large ruminants) (de la Fuente et al., 2016) creating a 'melting pot'

challenging the predictions of infectious diseases co-occurences. Disease and vectors can therefore freely spread from counties with endemic state to the disease free/or not endemic ones as currently experienced with outbreaks and reports of comparable diseases reports (Rweyemamu et al., 2006).

6.5. Control of tick-borne pathogens

Tick-borne pathogens have been addressed through impressive attempts for control. Previously, interventions for eradication such as fencing, wildlife vaccination, refuge changes, mass use of acaricides and intentional bushfires have all shown their limitations in interrupting the tick-pathogen-host interface (Carreón et al., 2012; de la Fuente et al., 2017; Fischhoff et al., 2017). From then on, smaller steps have been undertaken focusing on each of the actors to weaken the interface system for ultimate control. In Cameroon, ticks are found in the vegetation and can be collected from their host. Their control was mainly based on acaricides and manual tick removal. Host dipping, pour-on or spraying are some of the currently applied techniques for vector control. Ticks are spreading due to climatic changes and increasing resistance to multi-acaricides are demanding new strategies. Pathogens transmitted by ticks need to be passed on from infected hosts to susceptible ones to complete their life cycle and proliferate. To date some of the drugs available have been proven limited in their effectiveness, because of compound residuals stemming from constant treatment and associated secondary effects facilitating antibiotic resistance. Furthermore, co-infection status and early diagnostics are limiting factors impairing effective treatment and pathogen control. Vaccines against TBP have been developed with some being successful and others withdrawn due to severe side effects (Barrett & Portsmouth, 2013). Current difficulties to fully rely on vaccine for TBP control concerned the necessity of one vaccine for each pathogen, the high to very high antigenic variability of the pathogen, and the possibility of pathogen evolution to become immune against the vaccine (de la Fuente et al., 2017).

6.6. Host heterogeneity and associated factors in host phenotype

In the bovine host, the breed, age and life history have an important impact on the response to the exposed pathogen. Indeed, genetic components (at individual and herd level) are responsible of the overall competence allowing the pathogen dispersion under diverse settings (Gervasi et al., 2015). Variable responses to both tick and pathogen pressures are observed within populations and environment highlighting

individual genetic characteristics. With the difference in cattle varying from more susceptible *Bos taurus taurus* to more resistant *B. t. indicus* breed, between bovine crosses as well as within a single cattle breed (George et al., 1985; Mwangi et al., 1998; Mattioli et al., 2000; Robbertse et al., 2017).

Attempts to distinguish between the host plasticity and the solely based genetic phenotype have been studied through epigenetics and heritability estimates. These permitted the differentiation between individuals according to their behavior and transmission capacity between and within environments. Different expression of particular immune responses at individual level is an evidence of their diversity. Ecological interactions modifying the dynamics of tick infestation (host attractive) and their transmitted pathogens can shape the fluctuation in disease risk associated to environment and genetic components.

As previously described, the determination of the pathogenicity of a microorganism can be difficult to assess in its carrying host. Its incidence in co-infection (or not) with other organisms vary greatly between populations, environment and host history (Laine & Mäkinen, 2018). Pathogen associations vary in frequency and abundance, making the immune system the only witness of all undergoing challenges through host-cell signaling pathways. Therefore, the promotion of a better adapted cattle breed with economically important heritable traits including pathogen resistance would allow to maintain a level of fitness for a sustainable livestock production under parasitic pressure. Studies have been undertaken using heritability measurement based on tick attractiveness by cross-sectional and longitudinal studies by tick count (Stachurski et al., 1993b; Stachurski et al., 2007). Molecular tools such as microsatellites (Singh et al., 2014), whole and partial genome sequencing amongst others were also used to underline the genetic component responsible of resistant traits in cattle populations (Zvinorova et al., 2016). Based on those findings, microarrays have been developed, and species and/or breeds labeled as more resistant, and others more susceptible (Berry et al., 2011; Gernand et al., 2012; Raszek et al., 2016); highlighting the role of the host genetics in response to pathogens.

6.7. Cameroon's genetic diversity in cattle as a source of host resistance

In Cameroon, the cattle population is dominated by more recently introduced zebu cattle and crosses with European taurine breeds. Today, the autochthonous taurine cattle population is estimated at less than 1% notifying their risk of extinction due to

widespread un-controlled admixture (Ibeagha-Awemu et al., 2004). The relatively recent introduction of European cattle to Africa, and the growing admixture between zebu (*Bos indicus*) x taurine (*Bos taurus*) increased the susceptibility of the population to local pathogens as signs of non-adaptability to the local environment (Achukwi & Musongong, 2009). Although there is a general standing agreement that zebu breeds are on average more resistant to tick infestation than European taurine breeds (Frisch & O'Neill, 1998; Wambura et al., 1998; Mwangi et al., 1998), there is growing evidence that African taurine breeds and crossbreeds thereof are less susceptible to the local tick populations than African zebu breeds (Rechav et al., 1991; Mattioli et al., 2000; Mattioli and Cassama, 1995; Achukwi et al.,2001). Furthermore, indigenous taurine breeds are being reported more resistant to trypanosomiasis, whereas gastrointestinal pathogens are equally reported detrimental for zebu and taurine breeds mostly in younger individuals (Ibeagha-Awemu et al., 2004).

To assess the genetic basis of this variation in disease resistance, variance components can be assessed in the studied populations. These have favored considerable discoveries in human and animal research on genetically based complex traits. Moreover, it allowed amongst others to draw more light on the inheritable character of the variation in phenotype expressed by single populations (Loh et al., 2015). Variance component is a complementary analysis to genome-wide association studies (GWAS) which utilize the generation of large datasets by NGS technology, such as high-density microarrays. The GWAS are known to associate loci from the genotype to the studied phenotype which are hence usable in animal breeding. Combination of both analyses allowed significant findings, with GWAS associating the genotype to its corresponding phenotype, and the variance component reporting the fraction of the phenotype explained by the associated genotype (Caballero et al., 2015). For variance component analyses, fixed and random effects are defined, with the fixed effect being the independent variables. The random effect is independent from the fixed effect and may arise from the choice of the genotyped single nucleotide polymorphisms (SNPs) or microarray dataset. Combination of both effects in a model is known as mixed model using linear equations where only significant fixed effects are introduced. This allows the estimates of variance components which may be assessed with the restricted maximum likelihood (REML) method as standard procedure. Different statistical software programs can be used for this estimate such as SAS, EMMA, R, JMP or GCTA with some being freely available.

Heritability has been defined as the proportion of phenotypic variance V_p explained by the additive genetic variance for a base population of unrelated individuals (Postma, 2006).

The phenotypic variance corresponds to

$$V_p = V_G + V_E + V_{GE}$$

with

V_G : Genetic variation that contributes to the total phenotypic variation

V_E : Environmental contribution to the total phenotypic variation

V_{GE} : Variation associated with the genetic and environmental factor interactions

The additive variation is the quantitative value of the effect of more than one gene on a trait. It belongs to the genetic variation which is expressed by the following equation

$$V_G = V_A + V_D + V_I$$

with

V_A : Additive genetic variance

V_D : Dominance genetic variance

V_I : Interaction genetic variance

Therefore, the heritability estimate in selective breeding and genetics can be reported as the fraction of the variability of the trait under genetic influence. Its estimate provides a better understanding of the causes underlying the differences between population individuals.

For an association between the phenotypic trait and the genotype, GWAS are performed and the obtained results are used for graphic representation of all significant SNPs according to chromosomes (Manhattan plots). The level of significance is determined by SNP p-values, based on the false-discovery rate threshold.

B. Objectives, methodologies, structure of doctoral research

The current thesis investigated the epizootiology of tick-transmitted microorganisms (bacteria and protozoa) present in cattle populations sampled from the Northern part of Cameroon, and the associated zoonotic risk for the human population in those areas. The thesis related the common knowledge on the tick biology and its role as vector, the characteristic host-pathogen relationship, the importance of identification tools, and the basis for variation in host resistance to infection. Under this light, it aimed to compare the identification of TBPs by conventional PCR with a novel developed chip-based diagnostic array. The thesis also studied the genetic foundation of the cattle resistance against tick-borne and other pathogens using NGS platforms.

To fulfil those objectives, three different analyses (grouped as chapters or peer-reviewed publications in scientific journals) are carried out as frame of the investigation.

The first chapter focused on the identification of TBPs from DNA isolates of 1260 cattle both of taurine and zebu breeds. This was done by molecular analysis using universal primers in conventional PCR coupled with Sanger sequencing and phylogenetics.

The second chapter documented the increased specificity and sensitivity for TBP detection by a newly developed and tested DNA array which was produced through the platform of a commercial biochip provider. The goal was to create a PCR-based tool which can be easily implemented in small veterinary laboratories in endemic countries in Africa and elsewhere.

The third chapter assessed the heritability estimates of the cattle population, measuring the level of genetic implication in the host resistance to TBPs. The methodology involved genotyping of approx. 50,000 SNPs by Illumina BovineSNP50 DNA bead chips with analyses based on association studies. The genotyping and phenotyping datasets produced heritability estimates and identified potential marker signatures responsible for the observed pathogen resistance.

C. Results and discussion

Chapter 1. Epidemiology of tick-borne pathogens infecting cattle in Northern Cameroon reveals emerging species of *Anaplasma*, *Rickettsia*, *Borrelia*, *Ehrlichia* and *Theileria*

Related publication

Abanda B., Paguem A., Abdoulmoumini M., Manchang T.K., Renz A. and Eisenbarth A. Molecular identification and prevalence of tick-borne pathogens in zebu and taurine cattle in North Cameroon. *Parasites and Vectors* (2019) 12:448. doi.org/10.1186/s13071-019-3699-x.

Extended summary

The present chapter deals with the epidemiology of TBP in the cattle host from Northern Cameroon assessed by PCR and subsequent DNA sequencing. This also entails the level of co-infection, and the host's reaction to the pathogens.

Epidemiology assesses the distribution of diseases, risk factors and methods for their control, usually based on pathogens identification, making the latter a crucial step in successful epidemiological studies (Thrusfield et al., 2007).

In the reported study the overall TBP prevalence was 89.1% (1123/1260) with every individual carrying at least one of the pathogen groups *Anaplasma*/*Ehrlichia*, *Borrelia*, *Babesia*/*Theileria* or *Rickettsia*. Pathogens such as *A. centrale*, *A. marginale*, *A. platys*, *R. africae* and *T. mutans* were found in all the study sites across North Cameroon. All except one of the studied cattle breeds (Bokolodji, n=6) were found to be infected with those TBPs. Their wide spread is explained by their effective establishment in the host and vector ticks (Muhanguzi et al., 2014), and probably their transmission by mechanical vectors (Foil & Gorham, 2000). Moreover, it shows the practical implications of national and transboundary cattle trade networks (Fig. 12A) being one of the major risk factors of pathogen dispersion (Motta et al., 2017).

Co-infections in the host influences the pathogenicity of the infection (Laine & Mäkinen, 2018; Mabbott, 2018) according to multiple factors associated to the host (age, treatment history, etc.), the genetic makeover, environmental factors (season, geographic regions), and the interacting pathogens (homologous, heterologous

infection) (Belongia, 2002). The associated pathogenicity was estimated by measurement of health parameters, with some co-infections resulting in subclinical status (absence of symptoms), triggered to anemia observed in association with other pathogens (*B. theileri* significantly associated to anemia). Apparently healthy animals can be carrier of TBP playing the role of reservoir hosts in the population (Davoust et al., 2010). Therefore, movements of such carrier animals to a region with an endemic competent vector population allows the spreading of the disease sustaining the pathogen transmission (Motta et al., 2017, 2018).

Heterogeneous reactivity and host response

In this dataset, the logistic regression reported a significant positive correlation between *Anaplasma* and *Theileria* species (Table 1). This co-infection was not found significantly associated to adverse health parameters, meaning it did not have a significant detrimental effect on blood anemia or weight loss. Therefore, the identified species and/or co-infections could be reported as 'non-pathogenic heterogeneous reactivity'. The comparison of the packed cell volume in the population according to co-infection level showed differences between individuals (high/low co-infestation), however, with no significant differences (Fig. 13). Albeit the infection status reported is limited to associated TBPs, the anemic status could also be caused by other detected or undetected co-infections in the study animals, such as trypanosomes (Paguem et al., 2019), gastrointestinal helminths, or other environmental factors. In the literature, inter-generic co-infection of *Theileria orientalis* and *Anaplasma marginale* have been reported in cattle from Algeria (Gale et al., 1997) as 'non-pathogenic' increasing the hosts' resistance to the generally pathogenic *Anaplasma* bacteria. *Anaplasma spp*. alone have been reported being of detrimental impact on livestock creating anemia, weight loss, morbidity, abortion and death with an increasing virulence when not in co-infection (Battilani et al., 2017; Gale et al., 1997).

Younger animals were reported less infected by TBPs, presumably due to an acquired immunological responses, and a possibly reduced exposition time (recent acquisition). Moreover, acquired immune protection is reported more effective in younger individuals in contrast to adults experiencing increased antigenic variability expressed by pathogens maintaining their persistent infection status (Mahan, 2003). This mechanism differ according to the pathogen and the host age during the first infestation. Accordingly, a different response is generally expressed during the

infection with *Theileria* spp. where younger individuals are more at risk to express the disease when infected by a more virulent strain of *Theileria* spp. In our dataset, only the less pathogenic *Theileria* spp. *T. mutans* and *T. velifera* were identified by Sanger sequencing. Less pathogenic *Theileria* spp. appeared to be more tolerated by the host making them more endemic (Gharbi et al., 2015). Furthermore, their establishment as primary infection can prevent the establishment of more pathogenic *Theileria* species, such as *Theileria annulata* and *T. parva*. Utilization of the attenuated Tunisian schizont-infected cell lines of *Theileria annulata* have been successfully used as a vaccine in calves with an optimal protection, however only observed against homologous challenges down to a single genotype of *Theileria annulata* (Darghouth et al., 1996). This natural vaccination also known as heterologous protection or premunition have been reported in Tunisian cattle (Gharbi et al., 2015), and has been possibly naturally reproduced in the studied Cameroonian cattle population as the co-infection with *T. mutans* and *T. velifera* was found in 60% with no pathogenic *Theileria* spp. identified.

Co-infections in general and those promoted by TBPs in particular are established in most of the cases through secondary and subsequent infections (Wikel, 2013). The immunology of the secondary infection extensively depends on the nature of the acquired pathogen and those already established in the host, the strains and the corresponding epitope targets for developing antigens (Brown, 2012).

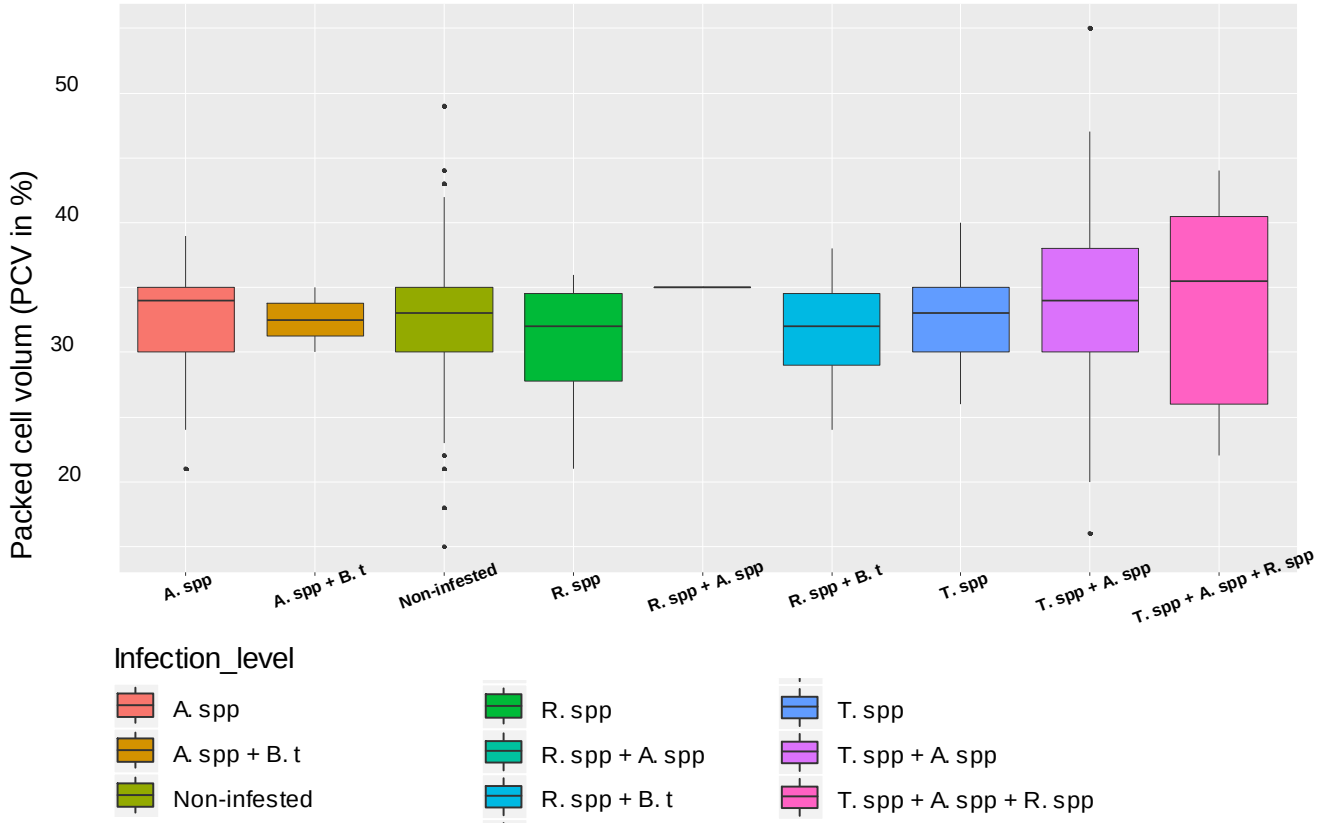


Figure 13. Packed cell volume (indicator of blood anemia) variability according to the level of co-infection in the studied population. A.spp., *Anaplasma* species; A. spp. + B. t, *Anaplasma* species and *Borrelia theileri* co-infection; R. spp., *Rickettsia* species; R. spp. + A. spp., *Rickettsia* species and *Anaplasma* species co-infection; R. spp. + B. t, *Rickettsia* species and *Borrelia theileri* co-infection; T. spp., *Theileria* species; T. spp. + A. spp., *Theileria* species and *Anaplasma* species co-infection; T. spp. + A. spp. + R. spp., *Theileria* species, *Anaplasma* species and *Rickettsia* species co-infection.

Host response and environmental changes

Landscape structure, ecological and bioclimatic zones are known to play a consistent role in the prevalence and distribution of TBPs as well as host fluctuation and wildlife management (Bertolini et al., 2018; Chan & Nagaraj, 2010; Rauw & Gomez-Raya, 2015). Environmental differences between regions of Cameroon have been reported (Ngwa et al., 2016; Penlap et al., 2004). For , the recent discovery of one of the most important cattle ticks *Rhipicephalus (Boophilus) microplus* according to climate and humidity level. This vector is believed having traveled from neighboring countries (Silatsa et al., 2019) and being progressively established in the country following suitable environmental patterns. Its recent identification showed a focus in the Southern equatorial part of the country, characterized by low temperatures and high rainfall. However, cattle movement networks displayed its possible and probably effective migration through the carrying cattle host (transboundary trading network). This observation may underline the minimal relative humidity in Northern Cameroon limiting the survival of larvae during the questing period (Leal et al., 2018). The relative humidity ranges from less than 50% in arid and semi-arid areas (Fig. 14B), with up to 30°C of diurnal range, to regularly above 80% in the South (https://www.globalsecurity.org/military/world/africa/cm-climate.htm).

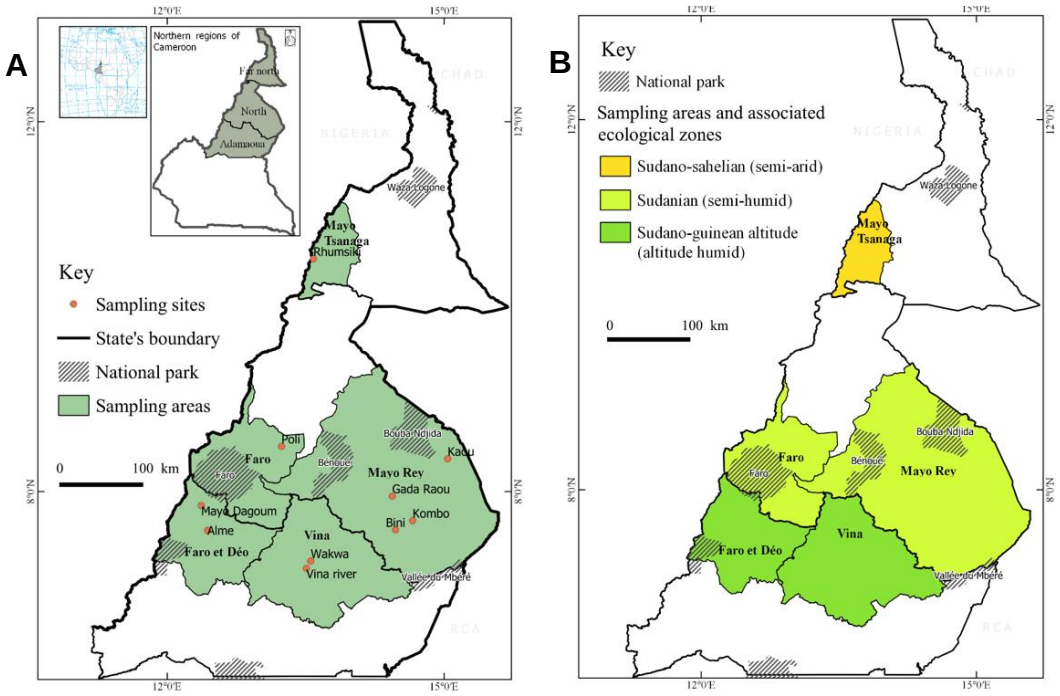
In the Northern part where the sampling for the study took place, the three involved regions are under semi-arid (Sudano-Sahelian in the Far-North region), semi-humid (Sudanian in the North region), and humid climate from high altitude (Sudano-Guinean in the Adamawa region) (Fig. 14). Those differences favor variable host pressure by endemic pathogens, including ticks, and hence leading to divergent prevalences. Therefore, besides the rightful incrimination of diagnostic tools, environmental factors are equally important factors to assess (Ebangi et al., 2002; Silatsa et al., 2019; Tawah

et al., 1999). The present study revealed a multitude of TBPs – some with a known zoonotic potential – of which *R. felis and E. canis* were previously unknown to occur in cattle in general, and in Cameroonian cattle in particular. However, a novel genotype closely related to *E. canis* was reported from North American cattle, forming its own separate clade (Gajadhar et al., 2010). The comparison with other reports of TBPs identification in Cameroon based mainly on microscopy showed a discrepancy not diagnosing *Theileria* species nor *A. platys*, *Anaplasma* sp. Hadesa, *E. canis* or *R. felis*. This makes the present report a valuable epidemiological intelligence for future investigations. The unexpectedly high prevalence of *Anaplasma* and *Theileria* spp. and previously unidentified species, as well as the level of co-infections calls for an in-depth identification of multi-pathogens transmitting ticks (*Boophilus microplus*) and screening with the latest diagnostic technologies available.

Table 1. Descriptive statistics screening for significance differences between the gender, age groups, cattle breeds, regions and study sites, together with the population prevalence of TBPs per sites included.

Variables	Categories	N. animal examined	Faro et Deo	Vina	Faro	Mayo Rey	Mayo-Tsanaga	Blood-samples pos. by PCR	($\chi 2$)	p-adjusted χ^2
Gender										
	female	970	104/108	276/324	107/123	225/265	149/150	861/970	0.44	0.5
	male	290	86/90	56/72	48/52	41/45	31/31	262/290		
Breed										
Bos indicus	Gudali	687	100/107	249/301	0	241/279	0	590/687	33.2	**< 0.01***
	Fulani	116	86/87	1/1	0	22/28	0	109/116		
	Bokolodji	6	3/3	2/2	0	1/1	0	6/6		
Bos taurus	Kapsiki	181	0	0	0	0	180/181	180/181		
	Namchi/Doayo	200	0	19/25	155/175	0	0	174/200		
	Charolais	31	0	26/30	0	1/1	0	27/31		
Hybrid	Cross-breeds	39	1/1	35/37	0	1/1	0	37/39		
Age group (years)										
	1-2.5	182	71/71	34/36	18/19	20/25	32/32	175/182	25.1	**< 0.01***
	>2.5-4.5	437	75/79	100/113	52/60	106/115	69/69	402/437		
	>4.5-8	539	38/42	158/200	63/68	138/163	65/66	462/539		
	>8	102	6/6	40/47	22/28	2/7	14/14	84/102		
Region										
	Adamawa	594	190/198	332/396	0	0	0	522/594	23.6	**< 0.01***
	North	485	0	0	155/175	266/310	0	421/485		
	Far North	181	0	0	0	0	180/181	180/181		

*significance: Association between the category and the pathogen acquisition.



Sources : Country boundaries : INC-Cameroon ; Sampling sites & Climatic zones : Babette Abanda & Anaba Banimb

Figure 14. Sampling areas in the northern part of Cameroon. (A) The Vina and Faro et Deo sites are located in the Adamawa region, the Faro and the Mayo-Rey in the North region, and the Mayo Tsanaga in the Far North region. (B) Ecological zones and climatic conditions of each of the sampling zone are shown. The colored zones represent the sampling areas, the zones with stripes the national parks, and the red dots the sampling sites.

Partial conclusion, perspectives and limitations

The identification of five yet undetected microorganisms in DNA extracted from blood samples of Cameroonian cattle is the best proof of the limitations of previously used identification tools over the applied PCR and sequencing protocol. The most prevailing pathogens were found in all breeds and regions, with no possibility of effective breed comparison (variable pathogen pressure and breed dispersion), because the cattle breeds are concentrated according to climatic zones. Ecological factors may have regulated the pathogen distribution with single appearances in all the studied zones, and legitimate similarities in pathogen distribution from the same study site. Although it is tempting to proclaim enzootic stability based on the observed prevalence (*e.g. A. platys* with 51.1%), and no apparent morbidity, it remains difficult to prove due to the high proportion of co-infections; with higher prevalence held by 'non-pathogenic' species (*e.g. T. mutans* with 92.2%). The distribution of the tick vector should be assessed to identify new transmission cycles for control. Further studies are required to measure the effective burden related to the presence of tick-borne

pathogens/microorganisms in the cattle population, along with measurements of herd management and health parameters.

Chapter 2. Development of an universally applicable microarray for the identification of co-infected tick-borne pathogens

Related publication

Abanda B., Paguem A., Achukwi M.D., Renz A. and Eisenbarth A. Development of a low-density DNA microarray for detecting tick-borne bacterial and piroplasmid pathogens in African cattle. *Tropical Medicine and Infectious Diseases* (2019) 4:64; doi:10.3390/tropicalmed4020064.

Extended summary

The present study allowed the comparison of two methods designed for effective identification of blood pathogens in DNA isolates. The first is by conventional PCR coupled with Sanger sequencing (Chapter 1), and the second uses an adjusted version of the previous PCR protocol with identical primers, coupled with the newly developed and tested microarray based on the widely used Reverse Line Blot (RLB) technique.

Tick borne pathogens are well-known for their co-occurrences in the infected host, with variations between an observed morbidity, triggered by specific co-infection patterns or herd management. Therefore, identifying most if not all pathogens in a host is an imposing necessity. Because of the usage of four pairs of generic primers, more than 6000 conventional PCR reactions were completed, including nested PCR for *Borrelia* associated pathogens. More than being a long procedure promoting contamination, positive samples (visible bands) of the expected size observed by agarose gel electrophoresis can encompass any of the pathogen enclosed in the associated genus group. Sanger sequencing can greatly enhance the diagnostic resolution for single infections. However, this approach has limitations in case of co-infections with pathogens of the same genus or related genera. Furthermore, a clear sequence does not exclude the presence of another related pathogen amplified by the same primer pair, possibly outperformed by the first (majority) template. In such cases, utilization of species-specific primer targets, next generation sequencing, real-time PCR or cloning are the given options to reveal any co-pathogens. Some of those tools are not universally accessible (Nowrousian, 2010, Berry et al., 2019). Moreover, using species-specific primers would lead to an exponential increase of the number of PCRs to be completed for each of the prevalent species; with a likelihood to miss yet unidentified ones.

Hence, the development of a universally accessible and robust tool, allowing the detection of bacterial pathogens and piroplasms in co-infected cattle DNA samples was endeavoured. A low-cost and low-density chip DNA microarray kit (LCD-Array) was designed and tested towards its specificity and sensitivity for five genera causing tick-borne diseases. Altogether, 12 species of the genera *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Theileria* were tested on a single LCD-Array. Genus-wide probes were designed and others retrieved from the literature as similar genotypes were found in our sequenced samples (Hailemariam et al., 2017).

LCD-Array validation: sensitivity and specificity

The validity consisted on a sensitivity test (Vanlalhmuaka et al., 2013), using twelve constructs on the plasmid vector pUC57 with inserts of the gene loci (16S rRNA or 18S rRNA) of all targeted species, including the most detrimental *Theileria* species and those previously identified by Sanger sequencing (Chapter 1) except for *Borrelia theileri*. Those constructs were synthesized by a commercial provider (BaseClear®) and used as positive controls to assess the sensitivity and specificity of the array by allowing concentration adjustment mimicking the number of copies in the solution (Yang & Rothman, 2004). The sensitivity test was meant to assess the detection limit of the PCR reaction, meaning the lowest concentration of the target which could still be amplified in the reaction and detected by agarose gel electrophoresis and the LCD-array. For this purpose, ten-fold serial dilutions in HPLC-grade water as solvent (Sigma-Aldrich) were prepared for each of the constructs and used as PCR templates, resulting in target concentrations ranging from 1 to 75 plasmid copies per reaction.

This result allowed the confirmation that a PCR product showing negative results on agarose gel does not necessary mean a sample free of pathogen DNA template (Table 2). Those are false negative undetectable by PCR only, as a negative gel electrophoresis product is ordinarily disqualified from sequencing.

Co-infection in the host is a limiting factor, playing a significant role in the successful PCR process and the obtained results. Hence, assessment for specificity was a fundamental step to eliminate false positives, rightfully expected from cross-hybridization in case of unspecific probes. For this purpose, 10 µL of the PCR amplification products of each recombinant positive control plasmid was submitted to the array, allowing the identification of the target probe. For each of the activated array fields, the corresponding probes were hybridized meaning each were specific, and the

associated ’catch all’ (representing a whole genus or family) probes showed also hybridization. For cross-hybridization tests, PCR products of different combinations, including different constructs from the three genera/groups, were mixed at equal volume. Once the probe specificity was confirmed, a maximum of three repetitions was considered. This was done mainly for single synthetic inserts and combinations between more than four species, mainly from different genera.

To test the practical performance of the LCD-Array, a subset of samples (n=31, retrieved from the dataset in Chapter 1.) was considered and evaluated under identical conditions used for the synthetic inserts. The obtained results were compared for analogy with the ones previously obtained by sequencing (Chapter 1). The LCD-array not only identified all pathogens previously detected by PCR and sequencing, but more pathogens could be revealed by the LCD-array showing its higher sensitivity and ability in simultaneous species identification.

Table 2. LCD-Array performance compared to Sanger sequencing results in percentages.

Primer group	Species	LCD-Array positive (in %)	Sanger sequencing positive (in %)
Anaplasma/Ehrlichia	*A. marginale*	61.3	12.9
	A. platys	41.9	29.0
	A. sp Hadesa	41.9	6.5
	A. centrale	41.9	12.9
	E. ruminantium	58.9	3.2
Rickettsia	*R. africae*	51.6	25.8
	R. felis	12.9	3.2
Babesia/Theileria	*T. mutans*	90.3	54.8
	T. velifera	77.4	3.2

Abreviations: A, Anaplasma; E, Ehrlichia;T, Theileria; R, Rickettsia; %, percent

Probes design

The probes were selected according to highest genus or species coverage in the GenBank database. The identified species by sequencing were aligned to the available GeneBank repository for similarities, allowing hybridization with the corresponding target if present after the PCR reaction. Parameters of probe selection and design were the exclusion of unintended hybridization with other genera or species, melting temperature optimum for the LCD-Array, and distance of the hybridization site to the biotinylated primer.

Limit of detection and copy number

To calculate the limit of detection, the page http://cels.uri.edu/gsc/cndna.html was used providing the following formulae and associated explanations. The successful copy number calculation would require the amount of the template in nanograms (ng) and its lenght in base pairs (bp).

$$Number\ of\ copies = \frac{amount * 6.022\text{x}\ 10^{23}}{(length * 1\text{x}10^{9}\ * 650)}$$

Based on the assumption that the average weight of 1 bp is 650 Daltons. That means one mole of a bp weighs 650 g, and that the molecular weight of any double stranded DNA template can be estimated by taking the product of its length (in bp) and 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro's number, 6.022x10^{23} molecules/mole, the number of molecules of the template per gram can be calculated:

$$\frac{\text{mol}}{\text{g}} * \text{molecules/mol} = molecules/g$$

Finally, the number of molecules or number of copies of template in the sample can be estimated when multiplying by 1x 10^{9} to convert to ng and then multiplying by the amount of template (in ng).

Adjusted PCR protocol and biotinylated primers

In the PCR reaction designed for LCD-Array hybridization, the primers were biotinylated (at the 5'end on the reverse strand). The primers ratio differ with the biotinylated primer introduced in higher concentration than its unlabeled opposite strand. That allowed the preferential amplification and therefore accumulation of the

DNA strand with the biotin label during the PCR process. The optimization of the PCR program included the increase in cycle numbers for the production of sufficient amounts of template.

Partial conclusion, perspectives and limitations

The produced array allowed the simultaneous detection of up to twelve species and four genera of TBPs, with two of the target species not yet described in Cameroon. All species previously identified by PCR were confirmed by the LCD-Array, with more co-infections identified by the latter. The main obstacle in the successful development of a microarray is the cross-hybridization, which once overcome allows the implementation of a higher number of probes. The present array did not cover the genus *Babesia*, not having been identified in the current dataset by sequencing. Previous reports based on microscopy of blood smears, however, suggested the presence of pathogens from this genus in the study area. Adaptation of the presently developed array may be necessary when used in a different geographic region with possibly different prevailing pathogens. Like Cameroon, many other developing countries have very limited access to sequencing facilities. Now, even a simple veterinary laboratory with PCR capabilities can establish this microarray for diagnostic screenings of TBPs.

Chapter 3. Molecular genetic variants associated with host resistance against tick-borne diseases

Related publication

Abanda B., Schmid M., Paguem A., Iffland H., Achukwi M.D, Preuß S., Renz A. and Eisenbarth A. Genome-wide association studies on parasitic and microbial pathogen resistance of *Bos taurus* and *Bos indicus* cattle breeds in Cameroon.

Note: The related publication estimates the fraction of the studied phenotypic traits (resistance against TBPs) attributable to genetic contribution with view to three groups of parasites (identified in the cattle population from Chapter 1). These include gastrointestinal nematodes, bovine onchocercosis and TBPs. The present third chapter of the thesis will mainly focus on the TBP group.

Extended summary

Epizootiology is the cornerstone of animal health by providing insights to prevent, treat and identify pathogen incidences in non-human hosts (Karstad, 1962, Kouba, 2003, Thrusfield, 2007). This term is generally associated with the assessment of control programs including the benefits of alternative (to drugs) control (Fuxa & Tanada, 1987; Artois et al., 2011; Gortazar et al., 2015; Pound et al., 2010). Besides intervention of the medical services, animal health can also be improved by breeding of better adapted individuals for higher resistance against diseases, without loosing other desirable productivity traits.

In the present study the variance components including phenotypic (V_P), additive (V_A) genetic variance and heritabilities (narrow sense) are calculated. Moreover, a GWAS was undertaken on the genotypic dataset generated from an Illumina 50k SNP BeadChip. Both analyses were conducted using the software GCTA (Yang et al., 2011). The results generated by association analyses served to locate all significant SNPs, revealing the quantitative nature of the traits of resistance presented by Manhattan plots.

The ultimate aim of those analyses was to assess the fraction of the genetic variability among studied individuals (57%; 720/1260 of the studied population in Chapter 1). In other words, the proportion of phenotypic variance explained by the differences in the genome, which can be used as genetic marker region in animal breeding (Getabalew et al., 2019). The dataset after quality control consisted of a total of 683 individuals

with complete genotyping data. The more the traits will be regulated by genetics, the better and straightforward could be the selection and breeding for a new genotype expressing the phenotypic trait. Inversely, a low genetic implication will denote important environmental contribution. Localized significant SNPs on the genome will confirm the presence of the trait in individuals.

Variance component estimates

Before being included in the evaluation model which was a linear equation (reported as equation 1 in the corresponding publication in the appendix), all available fixed effects were tested for significance ($p < 0.05$) using ANOVA. Those effects included the age, the sampling season and month, and the combined effect of breed and site as single fixed effect (breed_site). Heritabilities (h^2) were calculated using the phenotypes of the individuals on the liability scale (h^2_{liab}) (Falconer, 1965). Both estimates are disclosed in Table 3.

In order to estimate the level of association between the traits and the significant SNPs in GWAS, the model from the equation 1 was extended. It additionally includes the fixed effect of SNPs to be tested and the design matrix containing the number of 1-alleles. A leave-one-chromosome-out (loco) approach was applied to avoid a loss in mapping power by double-fitting the tested SNP. Corresponding *p*-values smaller than the threshold of $p = 5 * 10^{-5}$ were reported as significantly associated with the trait. This method has been recently developed (Lee et al., 2011) to estimate the genetic variation in quantitative traits when fitting all SNPs simultaneously (SNP heritability) by opposition to GWAS heritability (unable to recruit SNPs with small effect on present arrays) (Yang et al., 2010).

Table 3. Population specific parameters of the resistance for TBP. The estimated phenotypic (V_P) and additive genetic (V_A) variance, the heritability estimated for the observed ($h^2_{obs.}$) and liability scale ($h^2_{liab.}$) as well as their standard errors (in parentheses) are shown. The number of evaluated individuals (n) and the observed prevalence in the investigated population are given. Heritability estimates are considered low when the observed value is under or equal to 20% and moderate when this value is exceeded (Robinson et al., 1949).

Trait[1]	n	Prevalence	V_P (SE)	V_A (SE)	$h^2_{obs.}$ (SE)	$h^2_{liab.}$ (SE)
TBP	683	0.931	0.063 (0.003)	0.007 (0.006)	0.109 (0.103)	0.666 (0.631)

[1]tick-borne pathogens (TBP)

As expected for binary coded traits (observed scale), the heritability estimate was smaller (Dempster & Lerner, 1950). This result is explained based on the assumed combination of environmental and additive genetic components under liability scale. Thus, the result indicates a possibly high contribution of environmental factors in the presently observed phenotypic traits. This hypothesis has been reported before, and may be explained using epigenetics as presented by Barros and Offenbacher (2009) in the 'Epigenetics: Connecting environment and genotype to phenotype and disease'; where epigenetics is reported as the previously missing link among genetics, disease, and the environment.

GWAS and associated SNPs in the genome

The quantitative nature of the trait of resistance to TBPs was determined by association studies. In total, two SNPs were identified at the position 47,192,877 and 18,784,177 respectively on chromosome 20 and 24, to be significantly associated to the resistance against TBPs. The first chromosome (20) has been previously reported carrying markers associated with tick resistance in American Branford and Hereford cattle (Sollero et al., 2017), however, not located at the same position. The second chromosome (24) has not yet been associated with resistance to tick or their associated pathogens (Hu et al., 2019). Still, gene pleiotropy is reported in cattle and other organisms with view to resistance traits and biological pathways with variate genetic correlations (Mahmoud et al., 2018).

Partial conclusion, perspectives and limitations

Phenotypic traits in cattle populations, including resistance, tolerance and susceptibility, are difficult to measure with each of the traits likely being controlled by genetic factors. The estimated low heritability value (0.1) obtained, and the identification of two SNPs significantly associated to the resistance in TBPs, contributed to confirm the hypothesis that phenotypic variability in a population is controlled by genetics. Moreover, the estimated moderate heritability value (0.6) obtained on a liability scale allowed the confirmation these phenotypic differences are not only genetically-fixed, but equally influenced by environmental factors. The identification of the variance component, however, remains challenging considering the limited sample size. On the other hand, new standards have been requested for better adapted genotyping platforms, producing an appropriate panel of SNP datasets

enabling estimates with considerable reduced standard errors. Additional analyses will also be necessary for fine quantitative trait loci mapping aiming to detect loci under natural selection and allele fixation in specific populations. That may explain in better detail the presently concealed contribution of the environment to the expressed variance component (discrepancy between $h^2_{obs.}$ and $h^2_{liab.}$).

General conclusion

In North Cameroon, the epizootiology of tick-borne pathogens had been poorly documented. Their identification based for decades on conventional tools, including microscopy and serology, presents considerable limitations, mainly due to cross-reactions between antibodies (serology), misidentification (microscopy), or the focus on a single pathogen for identification (primer-specific PCR). The present identification of five yet unidentified pathogens from the cattle population in Cameroon inspired the development of a Low-cost and Low-density microarray (LCD-array). The ability to uncover the circulating pathogens in livestock is a starting point to the assessment of the level of exposure for the human population, as most of the emerging pathogens happen to have a zoonotic character. In the presently studied population of taurine and zebu cattle, the variance in response to the pathogens has been determined with a genetic and environmental contribution; in line with previous reports based on evolution and history (authochtonous and introduced breeds), or epigenetic factors. Estimated heritabilities produced results between low and moderate values highlighting the importance of environmental factors ($h^2_{obs.}$ and $h^2_{liab.}$) in the expressed phenotypic trait. This result can be considered valuable for achievable breed improvement based on their transmissible genetic material to the next generation.

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Appendix:

Parasites & Vectors

RESEARCH Open Access

Molecular identification and prevalence of tick-borne pathogens in zebu and taurine cattle in North Cameroon

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Abstract

Background: Public interest for tick-borne pathogens in cattle livestock is rising due to their veterinary and zoonotic importance. Consequently, correct identification of these potential pathogens is crucial to estimate the level of exposition, the risk and the detrimental impact on livestock and the human population.

Results: Conventional PCR with generic primers was used to identify groups of tick-borne pathogens in cattle breeds from northern Cameroon. The overall prevalence in 1260 blood samples was 89.1%, with 993 (78.8%) positive for *Theileria/Babesia* spp., 959 (76.1%) for *Anaplasma/Ehrlichia* spp., 225 (17.9%) for *Borrelia* spp., and 180 (14.3%) for *Rickettsia spp*. Sanger sequencing of a subset of positively-tested samples revealed the presence of *Theileria mutans* (92.2%, 130/141), *T. velifera* (16.3%, 23/141), *Anaplasma centrale* (10.9%, 15/137), *A. marginale* (30.7%, 42/137), *A. platys* (51.1%, 70/137), *Anaplasma* sp. 'Hadesa' (10.9%, 15/137), *Ehrlichia ruminantium* (0.7%, 1/137), *E. canis* (0.7%, 1/137), *Borrelia theileri* (91.3%, 42/46), *Rickettsia africae* (59.4%, 19/32) and *R. felis* (12.5%, 4/32). A high level of both intra- and inter-generic co-infections (76.0%) was observed. To the best of our knowledge, *B. theileri, T. mutans*, *T. velifera*, *A. platys*, *Anaplasma* sp. 'Hadesa', *R. felis* and *E. canis* are reported for the first time in cattle from Cameroon, and for *R. felis* it is the first discovery in the cattle host. *Babesia* spp. were not detected by sequencing. The highest number of still identifiable species co-infections was up to four pathogens per genus group. Multifactorial analyses revealed a significant association of infection with *Borrelia theileri* and anemia. Whereas animals of older age had a higher risk of infection, the Gudali cattle had a lower risk compared to the other local breeds.

Conclusion: Co-infections of tick-borne pathogens with an overall high prevalence were found in all five study sites, and were more likely to occur than single infections. Fulani, Namchi and Kapsiki were the most infected breed in general; however, with regions as significant risk factor. A better-adapted approach for tick-borne pathogen identification in co-infected samples is a requirement for epidemiological investigations and tailored control measures.

Keywords: Tick-borne pathogen, Cattle, Cameroon, *Anaplasma*, *Borrelia*, *Ehrlichia*, *Rickettsia*, *Theileria*

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Background

Tick-borne pathogens (TBPs) have severely impaired livestock productivity worldwide, with an increasing risk for the human population due to their potential zoonotic character [1]. In tropical Africa, ticks are vectors for a large variety of diseases, such as piroplasmoses caused by the protozoans *Babesia* and *Theileria*, bacterial infections with species of the genera *Anaplasma* (anaplasmosis), *Borrelia* (relapsing fever), *Ehrlichia* (heartwater), *Rickettsia* (spotted fever), and also many viral diseases, like Crimean-Congo hemorrhagic fever [2]. These infectious diseases cause considerable losses and diminish the economic value of livestock where the enzootic status remains unstable [2].

In Cameroon, which is one of the main regional providers of beef and other products derived from cattle, the population is dominated by zebu and crossbreeds (European taurine × zebu and African taurine × zebu), with the taurine cattle population at risk of extinction due to widespread and uncontrolled admixture [3]. The main local vectors for TBPs are hard ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* [4]. Pure *Bos taurus indicus* cattle have been reported less susceptible to TBPs than pure *Bos taurus taurus* cattle, based on attractiveness for the respective tick vectors and/or due to more effective immunological responses [5].

The prevalence of the various TBPs and their interdependences in Cameroon are not well investigated. Most of the studies used conventional microscopy of blood-smears, serology, or *post-mortem* analyses [6, 7] which all have considerable limitations. Identification of individual species of pathogens is almost impossible without the intervention of molecular tools, like PCR. Moreover, studies on the prevalence of the locally available TBPs in Cameroon and in particular on the level of co-infection is scarce. The present study aims to investigate the occurrence of TBPs in the cattle population, including "mild" and "non-pathogenic" conspecifics and their level of co-infection. Furthermore, the level of exposition and infection of different cattle breeds in Cameroon to TBPs, and the potential risk of exposure for the human population is highlighted.

Methods

Study sites and location

The sampling took place from April 2014 (end of the dry season) to June 2015 (middle of the rainy season). A total of 1260 cattle were examined in three different bioclimatic zones in the northern part of Cameroon. The corresponding sites (Fig. 1) were the Adamaoua highlands with 64,000 km^2 of surface, representing the subhumid Guinea savannah biotope, the North with 67,000 km^2, representing the semi-arid Sudan savannah, and the Far North with 34,000 km^2, representing the arid Sahel region. Sampling time was generally in the morning and mostly during the rainy season (April until October). Five sites were visited in the three regions: Vina (n = 396 cattle examined) and Faro et Deo (n =198) in the Adamaoua; Faro (n = 175) and Mayo-Rey (n = 310) in the North; and Mayo Tsanaga (n = 181) in the Far North.

Field work, sampling procedure and DNA isolation

For each herd visited, approximately 10% of the cattle were sampled. Parameters of age in years, sex, breed [Gudali; White and Red Fulani grouped as Fulani; Bokolodji (= Zebu *Bos taurus indicus*); Namchi/Doyao; Kapsiki (= autochtonous *Bos taurus taurus*); Charolais (= European *Bos taurus taurus* and cross-breed)], weight and body condition score (BCS) were taken from each animal. The BCS varied from 1 to 5 according to the fat and muscle appearance: 1–2, poor; 3–4, good; and 5, very good (convex look or blocky). The weight was standardized as recommended by Tebug et al. [8] using the formula LW = 4.81 HG–437.52 (where LW is live weight and HG is thoracic girth measurement in cm). The age was assessed by the dentition [9] and by the information of the herd keeper. Sampled animals were grouped as weaners (1–2.5 years-old), adults (2.5–4.5 years-old), old (4.5–8 years-old) and very old (> 8 years-old).

Approximately 5 ml of blood per animal was collected from the jugular vein in 9 ml ethylene diamine tetra acetic acid (EDTA) treated vacutainer tubes (Greiner Bio-One, Frickenhausen, Germany) and analyzed for packed cell volume (PCV) [10]. Briefly, approximatively 70 µl of collected whole blood was transferred into heparinized micro-hematocrit capillaries and centrifuged for 5 min at 12,000× *rpm* in a hematocrit centrifuge (Hawksley & Sons Limited, Lancing, UK). The solid cellular phase in relation to the liquid serum phase was measured using the Hawksley micro hematocrit reader (MRS Scientific, Wickford, UK). A PCV below the threshold level of 26% was considered anemic. The remaining whole blood was centrifuged at 3000× *rpm* for 15 min. Plasma was collected for immunological studies (not applicable here) and the remaining fraction (red blood cells and buffy coat) was used for DNA isolation.

Samples of 300 µl of the erythrocyte and cellular fraction were purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's instruction. For sample preservation, 50 µl of trehalose enriched 0.1× Tris EDTA (TE) solution (c = 0.2 M, Sigma-Aldrich, Taufkirchen, Munich, Germany) was added as DNA stabilizing preservative in the tube containing the extracted DNA [11], vortexed and spun down. All samples were stored at room temperature

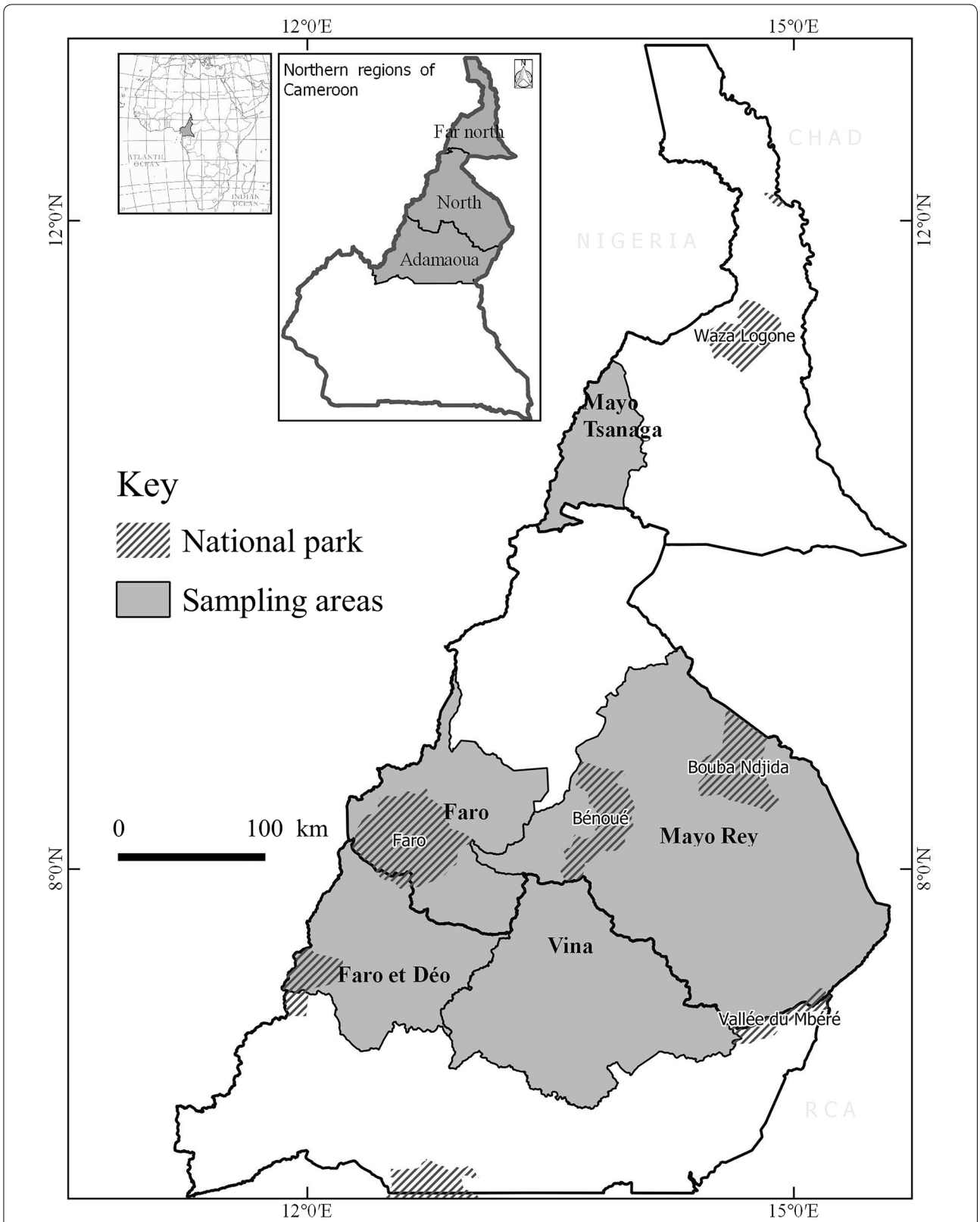

Fig. 1 Sampling areas in the northern part of Cameroon. The Vina and Faro et Deo sites are located in the Adamaoua region, the Faro and the Mayo-Rey in the North and the Mayo Tsanaga in the Far North region. The shaded zones represent the sampling areas and the zones with stripes the national parks

in a dry and light-protected environment after being left to dry at 37 °C. Rehydration was done in the laboratory in Tübingen using 75 µl 0.1× TE buffer at 35 °C for at least 10 min until the pellet was completely resolved, and immediately stored at − 20 °C.

Polymerase chain reaction for tick-borne pathogens

In 25 µl sample reaction tubes, 12.5 µl of the 2× Red-Master Mix (Genaxxon Bioscience, Ulm, Germany) were mixed with the corresponding primer pairs to the final concentration of 1 pmol/µl. One microliter of template DNA and molecular grade water (Sigma-Aldrich) were added to complete the volume at 25 µl. As a negative control, molecular-grade water (Sigma-Aldrich) was used, and positive controls were kindly shared by colleagues from the Freie Universität Berlin, Germany. For the detection of *Borrelia* spp., 1 µl of the first PCR reaction was used as a template for the second amplification in a nested PCR. The corresponding gene loci, primer pairs and annealing temperatures are shown in Table 1.

The PCR cycling conditions were: initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s and elongation at 72 °C for 30 s repeated 35 times, and final elongation at 72 °C for 10 min (MasterCycler EP S Thermal Cycler®, Eppendorf, Hamburg, Germany). All samples were visualized through electrophoresis on a 1.5% agarose gel stained with Midori Green (Nippon Genetics Europe, Düren, Germany). Selected positive reactions were prepared following manufacturer's recommendations (Macrogen, Amsterdam, Netherlands) and sent for sequencing. Obtained sequences were compared to the non-redundant database GenBank (NCBI) using BLASTN (http://blast.ncbi.nlm.nih.gov/) in the Geneious 9.1 software (Biomatters, Auckland, New Zealand).

Phylogenetic tree

Annotated sequences of the same genus and locus were extracted from the GenBank database, and aligned with the MUSCLE algorithm using standard parameters. Maximum Likelihood trees based on the Tamura-Nei model with 1000 bootstrap replications were generated using the software MEGA6 [15]. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. Furthermore, a discrete Gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed some sites to be evolutionary invariable. *Babesia bigemina* was selected as the outgroup for the *Theileria* tree, whereas *Wolbachia pipientis* was the outgroup for both *Anaplasma/Ehrlichia* and *Rickettsia* trees.

Statistical analysis

Descriptive statistics were performed to summarize TBP frequency, percentage, and proportion in study sites and co-infection levels according to region and breed. Multivariate logistic regression (MLG) analysis and descriptive statistics were performed using R v.3.4.2 (www.R-project.org) with the *ISLR* package for the MLG. The association between pathogen acquisition and independent variables were examined by computing the odds ratios (OR), 95% confidence intervals (CI) and *P*-value and using the logit equation in the logistic regression model. Each TBP species was used independently as outcome in separate equations. The other variables (PCV, BCS, age, sex, region and breed) were used as baseline predictors. All cattle breeds with less than 10 sampled individuals and all TBP species with less than 10 infected animals were

Table 1 Selected primer pairs and annealing temperature for the detection of mitochondrial target regions for the genera *Babesia/Theileria*, *Anaplasma/Ehrlichia*, *Rickettsia* and *Borrelia*

Genus	Primer	Target gene	Primer sequence (5′-3′)	Annealing T (°C)	Amplicon size (bp)	References
Babesia/Theileria	RLB-F2	*18S* rDNA	GACACAGGGAGGTAGTGACAAG	57	460–500	[12]
	RLB-R2		CTAAGAATTTCACCTCTGACAGT			
Anaplasma/Ehrlichia	AnaEhr16S_f	*16S* rDNA	AGAGTTTGATCMTGGYTCAGAA	55	460–520	This study
	Ana-Ehr16S_r		GAGTTTGCCGGGACTTYTTC			
Rickettsia	Rick-F1	*16S* rDNA	GAACGCTATCGGTATGCTTAACACA	64	350–400	[13]
	Rick-F2		CATCACTCACTCGGTATTGCTGGA			
Borrelia outer	16S1A	*16S* rDNA	CTAACGCTGGCAGTGCGTCTTAAG	63	1205	[14]
	16S1B		AGCGTCAGTCTTGACCCAGAAGTT			
Borrelia inner	16S2A	*16S* rDNA	AGTCAAACGGGATGTAGCAATAC	56	600–720	[14]
	16S2B		GTTATTCTTTCTGATATCAACAG			

Abbreviation: T, temperature

excluded from the logistic regression. A *P*-value below 0.05 was considered statistically significant.

Results

Cattle breeds examined and sampling sites

A total of 1306 cattle were examined in the three administrative regions of North Cameroon (Adamaoua, North, Far North) of which 1260 blood samples were used for molecular analyses. The different categories sex, age group, breed, region, BCS and PCV, together with the population prevalence of TBPs are summarized in Table 2. Data from seven different groups of cattle breed were gathered, including four zebu breeds Gudali (n = 687), White/Red Fulani grouped as Fulani (n = 116) and Bokolodji (n = 6), two indigenous taurine breeds Namchi/Doyao (n = 181) and Kapsiki (n = 200), cross-breeds (n = 37), and Charolais (n = 27). Most examined animals were female (76.9%). The age ranged from 1 to 16 years and the PCV from 11 to 55%.

Prevalence of TBPs by PCR

The blood samples of all 1260 animals were analyzed for TBP detection by conventional PCR with group-specific primer pairs for *Babesia/Theileria* spp., *Anaplasma/Ehrlichia* spp., *Borrelia* spp. and *Rickettsia* spp. The number of PCR-positive cases was 993 (78.8%) for *Babesia/Theileria* spp., 959 (76.1%) for *Anaplasma/Ehrlichia* spp., 225 (17.9%) for *Borrelia* spp., and 180 (14.3%) for *Rickettsia* spp. (Table 2). Nine hundred and three (80.4%, 903/1123) of all infected cattle were found to carry at least two of the screened pathogen groups, and the overall TBP prevalence was 89.1% (1123/1260) with every individual carrying at least one of the groups described above. The Adamaoua region had an overall prevalence of 87.9% (522/594) for all pathogens combined.

Logistic regression of pathogen acquisition with independent variables

Each of the identified pathogens (n = 7) was used as outcome in a logistic regression analysis. The results are reported in Table 3. Logistic regression analyzing the association of all TBPs as outcome to environmental and health factors highlighted the Kapsiki breed and older age as main risk factors (OR: 1.96, CI: 0.8–0.97, P = 0.01 and OR: 8.8, CI: 2.0–6.2, P = 0.002, respectively).

Table 2 Prevalence of TBPs per screened genera according to PCR results, sex, packed cell volume, body condition score, cattle breed, age and region

Variable	Category	Total	*Anaplasma/ Ehrlichia*	*Borrelia*	*Rickettsia*	*Babesia/ Theileria*
	PCR-positive		959/1260	225/1260	180/1260	993/1260
	Sequenced		187/959	46/225	63/180	167/993
	Identified		146/187	42/46	34/63	141/167
Sex	Female		736/959	166/225	139/180	760/993
	Male		223/959	59/225	41/180	233/993
PCV	≤ 25	114/1148	19/114	28/114	17/114	104/114
	≥ 26	1034/1148	107/1034	146/1034	123/1034	793/1034
BCS	1–2	82/1247	18/82	17/82	1/82	69/82
	3–4	1062/1247	111/1062	188/1062	135/1062	847/1062
	5	103/1247	7/103	17/103	15/103	72/103
Breed	Bokolodji	6/6	5/6	2/6	0/6	6/6
	Charolais	24/27	21/27	8/27	5/27	24/27
	Cross-breeds	35/37	29/37	9/37	2/37	35/37
	Fulani	107/109	97/109	22/109	10/109	107/109
	Gudali	480/590	480/590	88/590	103/590	472/590
	Kapsiki	171/180	171/180	54/180	32/180	169/180
	Namchi/Doayo	156/174	131/174	36/174	27/174	156/174
Age group (yrs)	1–2.5	157/175	152/175	48/175	31/175	157/175
	> 2.5–4.5	361/402	359/402	96/402	74/402	361/402
	> 4.5–8	398/462	376/462	68/462	58/462	398/462
	> 8	77/84	72/84	13/84	17/84	77/84
Region	Adamaoua		462/522	123/522	80/522	466/522
	Far North		171/180	54/180	32/180	169/180
	North		326/421	48/421	68/421	358/421

Table 3 Logistic regression model with all independent variables as exposure and their interaction with odds of being infected by the corresponding TBP species. *P*-values below 0.05 and level of significance are shown in bold

TBP	Region	Age	Sex	PCV	BCS	A.cn	A.H	A.mg	A.pl	B.th	R.af	T.mt	T.vl
A.cn OR	1	0.9	8.9	7.4	3.5		2.7	2.4	6.5	3.0	4.7	2.2	1.2
95% CI	−4.7–0.2	0.6–1.1	0.2–3.9	0.1–3.1	1.7–2.3		na	na	0.02–4.7	0.7–11	na	1.1–5.0	0.9–4.4
P	0.07	0.5	0.8	0.6	0.3		0.9	0.9	0.7	0.09	0.2	**0.002****	**0.002****
A.H OR	1.0	0.9	0.2	< 0.0001	4.3	2.7		2.3	1	1.3	6.7	8.5	5.6
95% CI	0.007–0.7	0.6–1.4	0.03–0.9	na	0.3–43.0	na		na	0.1–6.1	na	Na	1.8–3.7	6.7–5.5
P	**0.04***	0.8	0.05	0.99	0.2	0.9		0.9	0.9	0.9	0.9	**0.003****	**0.0001*****
A.mg OR	3.4	0.9	0.3	1.4	0.4	< 0.0001	< 0.001		0.3	2	0.8	14.8	4.2
95% CI	1.3–9.3	0.7–1.0	0.1–0.9	0.3–4.7	0.05–1.8	na	na		0.02–1.2	0.5–6.7	0.1–4.4	6.4–35.3	0.5–24.1
P	**0.009***	0.3	**0.03***	0.5	0.3	0.9	0.99		0.15	0.2	0.9	**< 0.0001*****	0.1
A.pl OR	1.9	0.8	2	0.9	0.3	1.1	1.2	0.2		1.2	0.7	22.4	2.6
95% CI	0.9–3.9	0.7–0.9	0.8–5.2	0.3–2.4	0.08–1.1	0.1–6.1	0.2–6.7	0.05–0.9		0.4–3.3	0.1–3.0	11.6–4.6	0.5–1.1
P	0.06	**0.02***	0.1	0.9	0.1	0.9	0.8	0.05		0.6	0.6	**< 0.0001*****	0.2
B.th OR	3.5	0.8	1.2	2.9	0.6	2.3	< 0.0001	1.8	1.35		2.1	0.5	1.1
95% CI	2.0–6.2	0.7–0.9	0.8–2.0	1.8–4.6	0.3–1.1	0.5–8.1	na	0.4–5.5	0.4–3.3		0.4–7.6	0.2–1.3	0.2–3.8
P	**< 0.0001*****	**0.003****	0.3	**< 0.0001*****	0.1	0.2	0.9	0.3	0.5		0.2	0.2	0.8
R.af OR	1.7	1	0.4	1	1	3.6	< 0.0001	1	1.1	1.9		8.4	2.06
95% CI	0.5–6.0	0.7–1.2	0.1–1.7	0.2–4.3	0.1–4.9	0.1–34.1	na	0.1–5.1	0.2-–.6	0.3–7.1		2.6–27.9	0.08–1.7
P	0.3	0.8	0.2	0.9	0.9	0.3	0.9	0.9	0.8	0.3		**0.0002*****	0.5
T.mt OR	0.8	1	1	0.4	1.5	12.8	9.3	16.4	21.2	0.6	7.9		6.4
95% CI	0.5–1.4	0.9–1.7	0.5–1.9	0.1–1.0	0.7–3.0	2.0–72.9	2.3–37.0	6.9–39.7	11.1–41.6	0.2–1.5	2.3–2.5		1.6–26.8
P	0.5	0.2	0.9	0.08	0.2	**0.004****	**0.001****	**< 0.0001*****	**< 0.0001*****	0.3	**0.0006****		**0.007****
T.vl OR	0.5	0.8	0.9	2.6	3.2	12.4	23.9	4	3.1	0.9	2	5.3	
95% CI	0.08–3.1	0.6–1.1	0.2–3.6	0.7–9.9	0.6–1.3	2.1–62.9	2.6–223	0.4–2.5	0.6–1.3	0.1–3.5	0.07–17.9	1.0–26.9	
P	0.4	0.3	0.8	0.1	0.1	**0.002****	**0.004****	0.1	0.1	0.9	0.5	**0.04***	

Abbreviations: A.cn, *Anaplasma centrale*; A.H, *Anaplasma* sp. 'Hadesa'; A.mg, *Anaplasma marginale*; A.pl, *Anaplasma platys*; B.th, *Borrelia theileri*; R.af, *Rickettsia africae*; T.mt, *Theileria mutans*; T.vl, *Theileria velifera*; na, not available; OR, odds ratio; CI, confidence interval

Pathogen identification and co-infections

For species identification, 296 of the 1123 PCR positive samples (26.4%) were selected for DNA sequencing, of which 240 (81.0%) could be successfully sequenced. Of these, 78.0% were generated for *Anaplasma*/*Ehrlichia* spp. (146/187), 84.4% for *Babesia*/*Theileria* spp. (141/167), 91.3% for *Borrelia* spp. (42/46), and 53.9% for *Rickettsia* spp. (34/63; Table 2). In total, 12 different species or genotypes were identified by matching with the GenBank database. Ranked after the most prevalent species, these were: *T. mutans*, *A. platys*, *A. marginale*, *B. theileri*, *A. centrale*, *Anaplasma* sp. 'Hadesa', *T. velifera*, *R. africae*, *R. felis*, *Theileria* sp. B15a, *E. ruminantium* and *E. canis*. The phylogenetic ML tree compares those genotypes with database entries from GenBank (Fig. 2a–c).

Co-infections with species of the same genus or group of genera were common. The highest percentage of animals with more than three of the five genera of parasites per individual was found in the Far North region (6.1%), followed by Adamaoua (2.8%) and North region (0.8%). The age was significantly associated to the pathogen acquisition ($P = 0.002$) with older animals being more infected. Kapsiki from the Mayo-Tsanaga division were more infected with TBPs (99.4% per region) than Namchi and zebu breeds from other regions ($P = 0.01$).

Single infections were detected in 264 (24.0%) of the 1123 infected cases. Intra-generic double infections that could still be delimitated to the respective species (Table 4), were most frequent for *T. mutans* + *T. velifera* (60.0%), followed by *A. platys* + *A. marginale* (17.3%), and *A. platys* + *Anaplasma* sp. 'Hadesa' (9.6%). In 45 cases (52%) of intra-generic co-infections, only one species could be identified. The most common inter-generic combinations were of *T. mutans* + *A. platys*, *T. mutans* + *Anaplasma* sp. 'Hadesa', *T. mutans* + *R. africae* and *T. mutans* + *A. marginale*. Gudali breed had less co-infections than Namchi and Kapsiki breeds.

Prevalence of *Anaplasma*/*Ehrlichia* species

PCR-positive samples from the *Anaplasma*/*Ehrlichia* group were found mostly in the Vina site on the Adamaoua Plateau (Table 4). Among the 146 positive sequences, 62.0% represented single infections and 38.0% represented co-infections. Single infections of *E. canis* and *E. ruminantium* were found in the sites Mayo Rey and Faro et Deo, respectively (Table 4). According to the proportions of the identified *Anaplasma*/*Ehrlichia* spp. in all study sites the total prevalence was 36.5% for *A. platys*, 21.9% for *A. marginale*, 7.8% for *A. centrale*, 7.8% for *Anaplasma* sp. 'Hadesa', 0.5% for *E. ruminantium*, and 0.5% for *E. canis*. Infection with *Anaplasma* spp. increases the likelihood of *Theileria* spp. infection and *vice versa* (Table 3). The age appeared being a risk factor for the acquisition of *A. platys*, with older animals being more infected (OR: 0.8, CI: 0.7–0.9, $P = 0.02$, Table 3).

Prevalence of *Borrelia* species

Borrelia pathogens were identified in all studied regions with the Adamaoua having significantly higher prevalence (OR: 3.5, CI: 2.0–6.2, $P < 0.0001$). The only identified species by sequencing was *B. theileri* with an overall prevalence of 17.9%. Gudali breeds were the least infected cattle with statistical support ($P = 0.02$). Younger animals were significantly less infected (OR: 0.8, CI: 0.7–0.9, $P = 0.003$). *Borrelia theileri* infection was significantly associated to anemia (OR: 2.9, CI: 1.8–4.6, $P < 0.0001$).

Prevalence of *Rickettsia* species

Rickettsia spp. were found in all the regions with no statistical difference. Cattle breed and age was not significantly associated to corresponding infected and non-infected groups. At least one individual from all examined breeds was positive for *Rickettsia* spp., except for Bokolodji ($n = 6$) which was excluded from the logistic regression analysis. The two species identified by sequencing were *R. africae* (prevalence 2.8%) and *R. felis* (prevalence 0.6%). For *R. africae*, the presence of *T. mutans* was a contributing risk factor (OR: 8.4, CI: 2.6–26.9, $P = 0.0002$).

Prevalence of *Theileria* species

Theileria mutans and *T. velifera* were detected in all screened regions. Furthermore, a closely related sequence of *T. mutans*, *Theileria* sp. B15a (GenBank: MN120896) has been detected (Fig. 2c). The overall prevalence of *Theileria* spp. was 57.3% for *T. mutans*, 2.7% for *T. velifera*, 0.5% for *Theileria* sp. B15a and 18.4% for *Theileria* spp. identified only to the genus level. *Theileria mutans* was highly associated with a number of TBP co-infections, including *A. centrale*, *A. marginale*, *A. platys*, *Anaplasma* sp. 'Hadesa', *R. africae* and *T. velifera*

(See figure on next page.)

Fig. 2 Molecular phylogenetic analysis of selected genera using rDNA markers by Maximum Likelihood method. Evolutionary analyses were conducted in MEGA6. Black stars indicate sequences generated in the present study. Annotations with asterisks indicate likely misidentifications. **a** *Anaplasma/Ehrlichia 16S* rDNA dataset (357 positions in final dataset) with *Wolbachia pipientis* as the outgroup. **b** *Rickettsia 16S* rDNA dataset (330 positions in final dataset) with *W. pipientis* as the outgroup. **c** *Theileria 18S* rDNA dataset (394 positions in final dataset) with *Babesia bigemina* as the outgroup

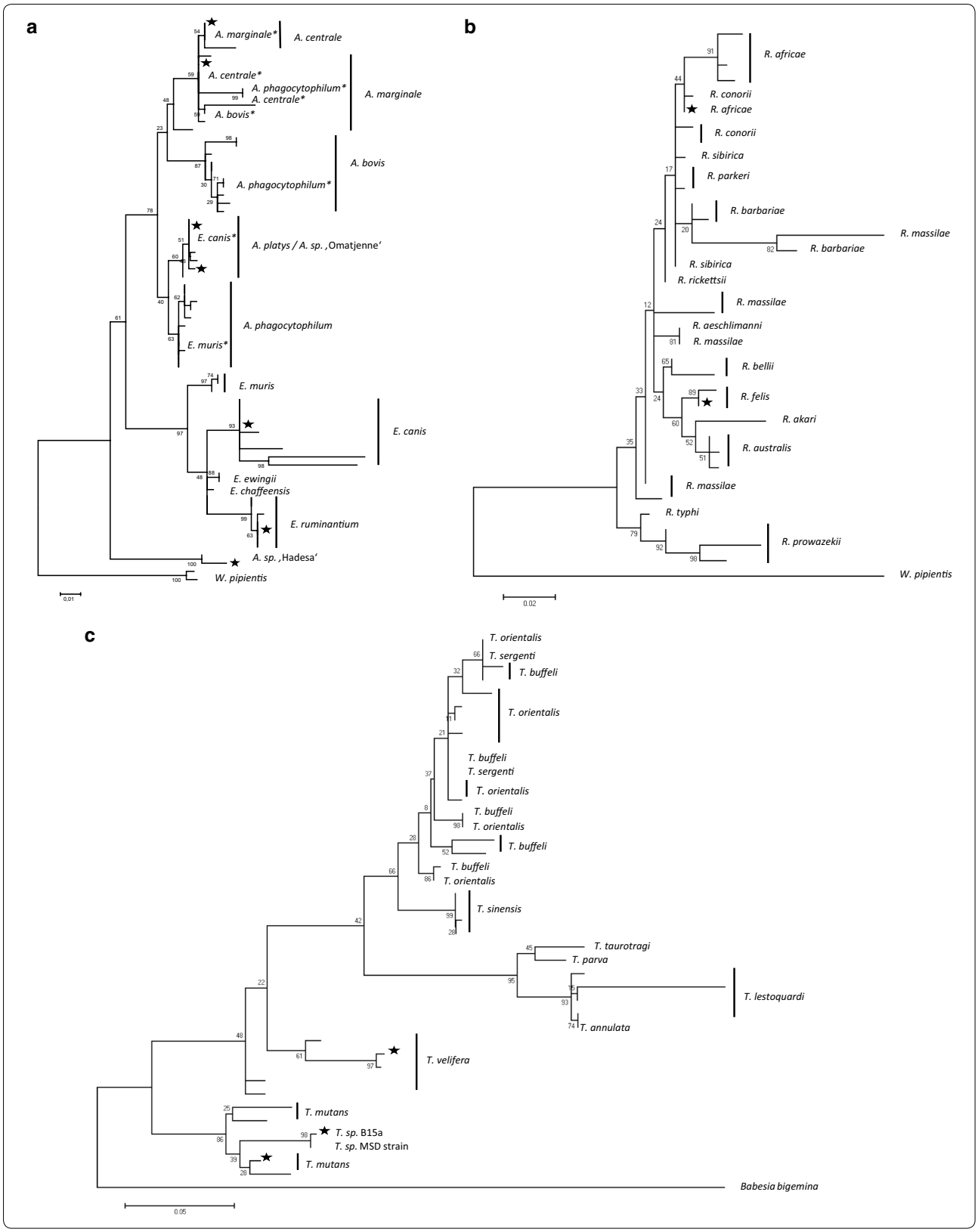
a
A. marginale*
A. centrale
A. centrale*
A. phagocytophilum*
A. centrale*
A. bovis*
A. marginale
A. phagocytophilum*
A. bovis
E. canis*
A. platys / A. sp. ‚Omatjenne'
E. muris*
A. phagocytophilum
E. muris
E. canis
E. ewingii
E. chaffeensis
E. ruminantium
A. sp. ‚Hadesa'
W. pipientis
0,01
b
R. africae
R. conorii
R. africae
R. conorii
R. sibirica
R. parkeri
R. barbariae
R. massilae
R. barbariae
R. sibirica
R. rickettsii
R. massilae
R. aeschlimanni
R. massilae
R. bellii
R. felis
R. akari
R. australis
R. massilae
R. typhi
R. prowazekii
W. pipientis
0.02
c
T. orientalis
T. sergenti
T. buffeli
T. orientalis
T. buffeli
T. sergenti
T. orientalis
T. buffeli
T. orientalis
T. buffeli
T. buffeli
T. orientalis
T. sinensis
T. taurotragi
T. parva
T. lestoquardi
T. annulata
T. velifera
T. mutans
T. sp. B15a
T. sp. MSD strain
T. mutans
Babesia bigemina
0.05

Table 4 Proportion of tick-borne pathogens in cattle blood from North Cameroon determined by DNA sequencing

Species	Positive ($n = 391$)	Proportion (%)[a]	Vina (%)[b]	Faro et Deo (%)[b]	Poli (%)[b]	Mayo-Rey (%)[b]	Mayo-Tsanaga (%)[b]
A. centrale	15	9.8	2 (13.3)	3 (20.0)	1 (6.7)	1 (6.7)	8 (53.3)
A. marginale	42	27.5	6 (14.2)	5 (11.9)	3 (7.1)	21 (50.0)	7 (16.7)
Anaplasma sp. 'Hadesa'	15	9.8	0	5 (33.3)	3 (20.0)	7 (46.7)	0
Anaplasma sp.	11	7.2	4 (36.4)	3 (27.3)	0	6 (54.5)	0
A. platys	70	45.8	20 (28.6)	3 (4.3)	6 (8.6)	33 (47.1)	8 (11.4)
E. canis	1	25.0	0	0	0	1 (100)	0
E. ruminantium	1	25.0	0	1 (100)	0	0	0
Ehrlichia sp.	2	50.0	0	0	0	2 (100)	0
R. africae	19	57.6	4 (21.1)	4 (21.1)	1 (5.3)	8 (42.1)	2 10.5)
R. felis	4	12.1	0	0	2 (50.0)	1 (25.0)	1 (25.0)
Rickettsia sp.	10	30.3	2 (20.0)	3 (30.0)	2 (20.0)	1 (10.0)	2 (25.0)
B. theileri	42	100	22 (52.4)	0	2 (4.8)	7 (16.7)	11 (26.2)
T. mutans	130	81.8	50 (38.5)	16 (12.3)	9 (6.9)	48 (36.9)	7 (5.4)
T. velifera	23	14.5	0	5 (21.7)	5 (21.7)	5 (21.7)	8 (38.1)
Theileria sp.	6	3.8	5 (83.3)	0	0	1 (16.7)	0

[a] Proportion of identified species in the respective group of pathogens

[b] Proportion of pathogen-positive samples per site

(Table 3). Furthermore, the taurine breeds, Namchi and Kapsiki were risk factors for *T. velifera* infection (OR: 9.0, CI: 1.4–64.4, $P = 0.02$) and (OR: 7.4, CI: 1.5–42.3, $P = 0.01$) respectively, as well as for co-infections with *A. centrale* and *Anaplasma* sp. 'Hadesa' (Table 3).

Phylogenetic analysis and genetic distances

Maximum Likelihood trees for the genera *Theileria*, *Rickettsia* and *Anaplasma/Ehrlichia* show the evolutionary relationships of the newly acquired sequences in comparison to published GenBank entries (Fig. 2a–c). Most matched very well with published sequences, but also a new genotype in the clade *A. platys*/*Anaplasma* sp. 'Omatjenne' (GenBank: MN120891), and another unrecorded genotype closely related to *Anaplasma* sp. 'Hadesa' (GenBank: MN124079), were found.

Discussion

Conventional PCR was used to assess the prevalence of circulating tick-borne parasites and bacteria in cattle from Cameroon's most important rearing sites in the northern regions. Four different primer pairs targeting ribosomal RNA loci allowed the identification of six genera of important species of TBPs. To the best of our knowledge, our study provides first molecular proof for the presence of *Borrelia theileri*, *Ehrlichia canis*, *Theileria mutans*, *Theileria velifera*, *Anaplasma* sp. 'Hadesa', *Anaplasma platys* and *Rickettsia felis* in cattle from Cameroon.

Generally, we found a high TBP prevalence, including a high level of co-infection with other TBP species. Many of the identified TBPs in those cattle are of major economic importance in Africa [16], while some are also causing zoonotic infections in humans. The investigated TBPs differed significantly depending on the cattle breed, age and geographical region, where indigenous taurine breeds, older age and the cattle-rich Adamaoua region were the highest risk factors, respectively. Although the detection and identification of co-infections by using generic primers without cloning can be at times challenging, a sample set of the presently identified species was confirmed by a reverse line blot DNA microarray, albeit with a lower detection rate than the microarray [17].

Anaplasma/Ehrlichia group

Anaplasma marginale and *A. centrale* are gram-negative bacteria of the order Rickettsiales, and known to cause bovine anaplasmosis in tropical and subtropical regions [6]. The prevalence in the present study (*A. marginale*: 21.9%, *A. centrale*: 7.8%) was significantly lower than reported in a recent study from North Cameroon with 62.2% and 53.3%, respectively [7], using Giemsa staining. Conversely, our results were higher than reported in the North-West region where the prevalence was 2.2% for *A. marginale* and 0% for *A. centrale*, respectively [6]. The limited mobility of cattle from the 'Centre de Recherche Zootechnique' ranch in the North-West region and possibly better husbandry management [6]

may explain the lower prevalence and TBP diversity in this area. Moreover, transhumance regularly undertaken by cattle holder in the Adamaoua region could explain the diversity of identified *Anaplasma* species, and the observed prevalence variability [18]. Different study results from the same sampling area in the Vina division are best explained by the alternative technical approaches used for identification. In comparison to molecular tools, microscopic analyses of blood smears are used for rapid diagnostic and informative purposes on the animals' health status. In fact, identification by microscopy is prone to errors in species identification, as pathogens may look very similar among and between genera leading to misidentification, or may be missed depending on the animals' patency or developmental status [19]. *Anaplasma marginale* and *A. centrale* are known to be mainly transmitted by ticks of the genus *Rhipicephalus*, in addition to other genera having also been reported as vectors [20]. In Cameroon, *R. appendiculatus* has been identified in the sampling regions as the second most common tick [21], correlating with the high prevalence of these pathogens in the corresponding sites. In our study, sex was significantly associated with the acquisition of *A. marginale*, although with a low odds ratio (OR: 0.3, CI: 0.1–0.9, $P = 0.03$, Table 3).

Anaplasma sp. 'Hadesa' identified in our sample set had been previously identified in blood samples from Ethiopian zebu cattle [22]. The phylogenetic tree grouped our sequence (GenBank: MN124079) to its clade in a relatively high evolutionary distance from other *Anaplasma* and *Ehrlichia* species (Fig. 2a). In our dataset *Anaplasma* sp. 'Hadesa' was inversely correlated with the Adamaoua region, significantly but with low support (OR: 1.0, CI: 0.007–0.7, $P = 0.04$).

Anaplasma platys is known as a canine pathogen, causing cyclic thrombocytopenia in dogs. However, it has also been identified in other mammals including cattle, humans and ticks worldwide [23]. In the present study, it was the most commonly detected *Anaplasma* species (prevalence of 36.5%). Two groups of genotypes were found, one of which had yet no listed entry in GenBank (GenBank: MN120882). The absence of detection of this pathogen in previous studies from Cameroon is very likely due to its misidentification for other TBPs [7]. Furthermore, the clade *A. platys* matched very well with *Anaplasma* sp. 'Omatjenne' (> 99% identity, GenBank: U54806, Fig. 2a), which was first isolated in sheep and *Hyalomma truncatum* ticks from South Africa [24] and later often diagnosed by its corresponding DNA probes used for reverse line blots assay [25]. In the study by Allsop et al. [24], the complete genome of *Anaplasma* sp. 'Omatjenne' (GenBank: U54806) shared 99.9% identity with *Anaplasma* (*Ehrlichia*) *platys* and closely resembled the genome of *E. canis*, most likely due to wrong species annotation [24]. *Rhipicephalus sanguineus* (*sensu lato*) is thought to be the most likely vector of the pathogen which is a tick species already identified in Cameroon [26]. *Anaplasma platys* was identified in 70 specimens of the sequenced subset resulting in a relatively high prevalence (36.5%) in comparison to the records in cattle from Algeria (4.8%) [27], Italy (3.5%) [28] and Tunisia (22.8%) [29]. As a rule, rather than exception, *A. platys* was found in co-infection with other TBPs of the genus *Theileria* with the infection rate increasing with age (Table 3).

Ehrlichia canis is a gram-negative bacterium causing canine monocytic ehrlichiosis in dogs and wild canids; these mammals can serve as a natural reservoir for human infections with *R. sanguineus* ticks as a natural vector in tropical and subtropical areas [30]. *Ehrlichia canis* has also been identified in other *Rhipicephalus* species [31]. Among others, the pathogen has been found in dogs from Cameroon [32], Nigeria, South Africa, Portugal, Venezuela [30]. To our knowledge, the present study provides the first evidence for the ocurrence of *E. canis* in cattle from Cameroon. Only one sample from our sequenced subset ($n = 187$) was identified to be *E. canis*. The infected host was a 2-year-old Gudali female cow from the North region in the Mayo Rey site. In fact, cattle paddocks include space for dogs, chicken and other domestic animals living in close proximity. As for most of the TBPs clinically healthy dogs in the subclinical stage can be carriers of *E. canis* for years [33], facilitating the infection of other susceptible hosts. According to the PCV and the BCS, the animal infected by *E. canis* was not suffering from illness albeit co-infected with *T. mutans*. In our study the *E. canis* strain shared 99.6% identity with the *E. canis* amplicon described in Italy and published under the GenBank accession numbers KY559099 and KY559100 [34] (Fig. 2a).

Ehrlichia (*Cowdria*) *ruminantium* is the etiological agent of heartwater, also called cowdriosis, in domestic ruminants. The evidence of *E. ruminantium* in Cameroon has been clearly demonstrated in cattle carcasses [6] and the tick vector *Amblyomma variegatum* [35]. Only one positive case of *E. ruminantium* could be identified from our samples subset, representing the second molecular evidence of this pathogen in cattle from Cameroon [36]. The prevalence in our data (0.5%), was significantly lower in comparison to the recently published data (6.6%) on cattle blood from the North and Southwest region of Cameroon [36]. The infected animal was a two years old Red Fulani breed from the Faro et Deo division on the Adamaoua plateau. The BCS was within the range characteristic for an asymptomatic animal, and the PCV level (23 %) indicated anemia. The pathogen was found in co-infections with *A. centrale*, *T. mutans*, *B. theileri* and

an unidentified *Rickettsia* sp. The identified strain (GenBank: MN120892) had > 99% sequence identity with the strain 'Welgevonden' as previously described from Cameroonian samples [36].

Babesia/Theileria group

Theileria mutans and *T. velifera* are known as mild to non-pathogenic species in cattle. *Amblyomma variegatum* ticks transmit *T. mutans*, with the vector being endemic in the northern part of Cameroon. Although age has been reported as a risk factor, our study did not show significant associations (OR: 0.1, CI: 0.9–1.7, $P = 0.2$). *Theileria mutans* is known as non-schizont-transforming of the *Theileria* spp. benign group [37]. However, studies have shown that the presence of the piroplasm at high density in red blood cells can cause disease associated to anemia [38]. The present study did not find any significant difference regarding the PCV level (OR: 0.4, CI: 0.1–1.0, $P = 0.08$). The genotype *Theileria* sp. B15a (GenBank: MN120896) detected, formerly isolated from African buffaloes in South Africa, grouped within the *T. mutans* clade (Fig. 2c) indicating it belongs to the same species.

No schizonts have been described for *T. velifera* [37], whose natural host is the African buffalo, found in high numbers in the Waza National park in the Far North region of Cameroon. This may be the reason for the higher *T. velifera* prevalence in the Kapsiki breed, which are the only cattle kept in this area. No highly pathogenic *Theileria* spp. such as *T. parva* and *T. annulata* was detected in the examined animals. This result indicates either its absence in Cameroon, or the presence below detection levels in cattle formerly or presently infected with *T. mutans* and/or *T. velifera*.

Borrelia group

Borrelia theileri is a member of the tick-borne relapsing fever group in contrast to the Lyme borreliosis group [39]. The present study reports for the first time the presence of *B. theileri* in blood samples from cattle in Cameroon. The spirochete bacterium is known to be transmitted to cattle by hard ticks of the genus *Rhipicephalus*, e.g. *R. microplus*, *R. annulatus* and *R. decoloratus* [40]. The pathogen has also been found in *R. geigyi*, however, its capacity as a vector is unknown [40]. Reported cases of tick-borne relapsing fever have been proven responsible for economic losses in livestock [41]. In cattle, *B. theileri* infections have been associated with fever and anemia [41]. In our study area, 17.9% of the studied cattle population was positive for *Borrelia* spp., with *B. theileri* being the only species identified by sequencing.

Furthermore, *B. theileri* was significantly associated with anemia (OR: 2.9, CI: 1.8–4.6, $P < 0.0001$), and present in co-infections with other TBPs in 62% of cases. The highest degree of co-infection comprised *T. velifera*, *T. mutans*, *R. felis*, *A. platys* and *A. centrale.* Similar TBP co-infections excluding *Rickettsia* spp. have been reported [42, 43]. Taurine cattle were significantly more infected than zebu cattle ($P < 0.01$) in line with previously published studies [44], and the difference was significant among age groups with old animals being more infected than their younger counterparts (Table 3). The genotype of *B. theileri* identified in our study (GenBank: MN120889) was 99.9% identical to the strain found in *Rhipicephalus geigyi* from Mali.

Spotted fever *Rickettsia* group

Rickettsia africae is known as the causative agent of African tick bite fever, and has been identified in Cameroon by PCR at a prevalence of 6% from human patients with acute febrile illness without malaria or typhoid fever [35], and at a prevalence of 51% in man from cattle-rearing areas [31]. In previous studies, the pathogen has been identified molecularly in 75% of *A. variegatum* ticks collected from cattle in southern Cameroon [35]. A recent study on ticks collected from cattle in the municipal slaughterhouse of Ngaoundéré in the Adamaoua region in northern Cameroon revealed the presence of *R. africae* among other *Rickettsia* species not identified in our survey [45]. However, the ML tree (Fig. 2b) illustrates the difficulty to clearly distinguish closely related *Rickettsia* spp. when using the *16S* rRNA marker [22]. The genotype of *R. africae* identified in our study (GenBank: MN124096) was 99.7% identical to the strain found in *Hyalomma dromedari* in Egypt and *A. variegatum* in Benin and Nigeria [46].

Rickettsia felis is known as an emerging insect-borne rickettsial pathogen and the causative agent of flea-borne spotted fever [47]. Four out of 34 sequenced *Rickettsia* spp. (11.8%) with a prevalence of 0.6% in the sequenced cattle population were detected. The infected animals were from the North region, more precisely from the Faro, Mayo Rey and Mayo-Tsanaga sites, and were in 75% of cases in autochthonous *B. taurus* breeds. The present study reports for the first time *R. felis* in cattle hosts, with previous identification from fecal samples in chimpanzees, gorillas and bonobo apes from Central Africa, including the southern part of Cameroon at a prevalence of 22% [48]. Furthermore, *R. felis* has been identified in *Anopheles gambiae* mosquitoes [49], and human cases were common in Kenya [50] and Senegal [51]. The strain reported in this study

(GenBank: MN124093) matches at 99.7% identity with the one described in a booklouse from England as rickettsial endosymbiont (GenBank: DQ652592) and in a cat flea from Mexico [52] indicating they are not predominantly transmitted by ticks, even though they have been found before in tick vectors.

Conclusions

In North Cameroon, we identified by sequencing of PCR-amplified rDNA from bovine blood at least 11 species of tick-borne pathogens, some of which are known to be pathogenic to livestock or humans alike. *Anaplasma platys*, *Borrelia theileri*, *Ehrlichia canis*, *Rickettsia felis*, *Theileria mutans* and *Theileria velifera* were identified for the first time in cattle from Cameroon. Furthermore, genuinely new genotype sequences related to *A. platys* and *Anaplasma* sp. 'Hadesa' were discovered. The high pathogen diversity and levels of co-infection in the livestock population is possibly a result from interaction between different host animals (transhumance or contacts between other domestic and wild animals) and their corresponding tick vectors. In addition to the identification of novel TBP species and genotypes, this study shows the necessity of a universally applicable method for TBP identification unbiased by co-infestations with other related pathogens, which appear in more than 75% of the infected cases.

Abbreviations

TBP: tick-borne pathogen; PCR: polymerase chain reaction; PCV: packed cell volume; LW: life weight; GH: thoracic girth; EDTA: ethylene diamine tetra acetic acid; TE: tris-EDTA; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; BCS: body condition score.

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Authors' contributions

BA designed the experiment and method, performed laboratory analyses and drafted the manuscript. BA and AE performed the statistical and phylogenetic analyses. BA, AP, MA and MTK collected samples. BA, AP, MA, MTK, AR and AE contributed to interpretation of the results, wrote and corrected the manuscript. AR and AE supervised and managed the whole study. All authors read and approved the final manuscript.

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Availability of data and materials

The sequences generated during the present study are available in the NCBI GenBank repository under the accession numbers MN120882, MN120888–MN120892, MN120895–MN120896, MN124079, MN124093–MN124096.

Ethics approval and consent to participate

The study has been carried out with the consent of the regional state representatives and traditional authorities from each of the sampling areas. Furthermore, oral consent was given by the cattle owners, herdsmen (who also helped in restraining the animals), and with the participation and approval of the National Institute of Agricultural Research for Development (IRAD) in Cameroon, which is the country's government institution for animal health and livestock husbandry improvement.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

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Development of a Low-Density DNA Microarray for Detecting Tick-Borne Bacterial and Piroplasmid Pathogens in African Cattle

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Abstract: In Africa, pathogens transmitted by ticks are of major concern in livestock production and human health. Despite noticeable improvements particularly of molecular screening methods, their widespread availability and the detection of multiple infections remain challenging. Hence, we developed a universally accessible and robust tool for the detection of bacterial pathogens and piroplasmid parasites of cattle. A low-cost and low-density chip DNA microarray kit (LCD-Array) was designed and tested towards its specificity and sensitivity for five genera causing tick-borne diseases. The blood samples used for this study were collected from cattle in Northern Cameroon. Altogether, 12 species of the genera *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Theileria*, and their corresponding genus-wide probes including *Babesia* were tested on a single LCD-Array. The detection limit of plasmid controls by PCR ranged from 1 to 75 copies per µL depending on the species. All sequenced species hybridized on the LCD-Array. As expected, PCR, agarose gel electrophoresis and Sanger sequencing found significantly less pathogens than the LCD-Array ($p < 0.001$). *Theileria* and *Rickettsia* had lower detection limits than *Anaplasma* and *Ehrlichia*. The parallel identification of some of the most detrimental tick-borne pathogens of livestock, and the possible implementation in small molecular-diagnostic laboratories with limited capacities makes the LCD-Array an appealing asset.

Keywords: tick-borne pathogen; low-cost and low-density-array; Reverse Line Blot; *Anaplasma*; *Ehrlichia*; *Rickettsia*; *Theileria*

1. Introduction

Tick-borne pathogens (TBP) are of high veterinary and medical importance worldwide. To evaluate the risk of exposure of TBPs in a livestock or human population, effective surveillance and monitoring practices are needed. For cattle and other livestock, the published literature highlights the importance of protozoa of the genera *Babesia* and *Theileria*, bacteria of the genera *Anaplasma*, *Ehrlichia* and *Rickettsia*, and arboviruses as etiologic agents of many diseases, of which a number of them have zoonotic potential [1]. Especially in developing countries, routine diagnostic approaches for the identification of TBPs are generally based on microscopic examination of blood smears [2,3] or serological assays [4,5].

While those techniques require only moderate investments for equipment and infrastructure, they have limitations regarding specificity and sensitivity (microscopy) [6–8], or tend to cross-react with closely related species (enzyme-linked immunosorbent assays) [9]. Furthermore, commercially available kits of the former are often not financially affordable for veterinary laboratories in low income endemic countries. Molecular tools based on PCR [10] and nowadays NGS are becoming more widespread, with NGS being economically viable when used for large sample sizes [11].

The DNA microarray technology of PCR-amplified products combines high throughput, sensitivity, specificity and reproducibility [12]. Its function is based on the reverse line blot (RLB), in which specific oligonucleotide spots (probes) are immobilized on a solid surface (Figure 1). When a target sample with complementary DNA sequence is added, it hybridizes with the probe where it is detected by a fluorescent, chemiluminescent or biotinylated label. The synchronous detection of a multitude of species in the same genetic material has contributed to its popularity in infectious disease diagnostics [10,13]. Low-density DNA microarrays such as the LCD-Array are designed to detect much lower numbers of pathogenic agents than high-density microarrays [14]. However, they are optimized for minimal input of equipment, workflow, costs and expenditure of time, and therefore suitable for small diagnostic laboratories in low and middle income developing countries [14,15].

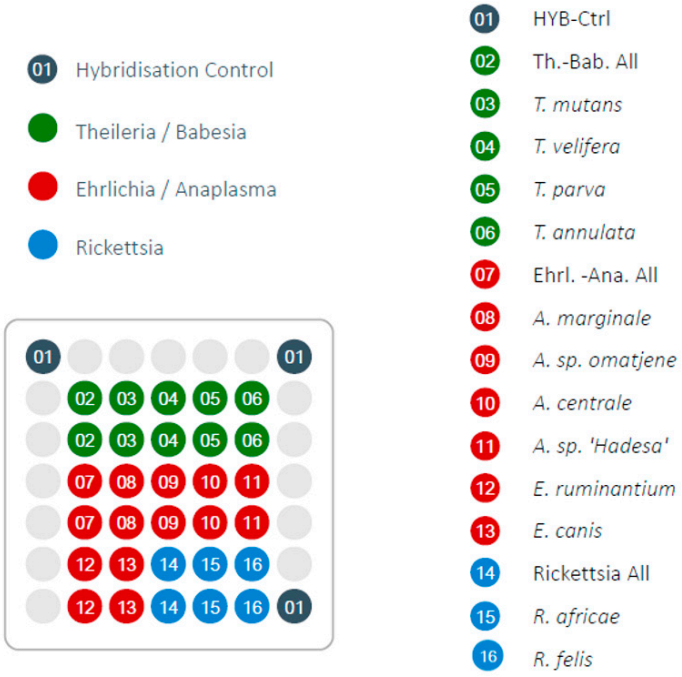

Figure 1. Design of LCD-Array for tick-borne pathogens indicating the screened species and genera. Light grey circles are blank positions.

In TBP epizootiology, the mostly used RLB application has been a mini-blotter coupled with a membrane where the probes of interest have been priorly linked to [10,13]. Although any desirable probes can be attached to the membrane prior to testing, the setup necessitates a high skill level in handling and optimization. Hence, for routine TBP identification a "ready to use" array or biochip for low to medium sample numbers with standardized protocol and reagents would be highly desirable.

In this paper we describe the development and testing of a novel LCD microarray for TBP, based on an already established biochip platform from a commercial provider (Chipron, Berlin, Germany). The same platform has been adapted for the detection of human mycobacteria [16], viruses [14,17], fungi [18] and in food safety [12]. In the field of TBP, this array has been tested once for the two piroplasmidae genera *Babesia* and *Theileria* [19]. In our study, the PCR and LCD-Array also detect ribosomal RNA fragments (18S) of the genera *Babesia* and *Theileria*, and additionally bacterial 16S fragments of the genera *Anaplasma*, *Ehrlichia* and *Rickettsia*. The array design, protocol specifications

and performance in comparison to PCR with Sanger sequencing are described and tested on a naturally exposed cattle population from North Cameroon.

2. Materials and Methods

2.1. Sample Origin, DNA Extraction, PCR and Sanger Sequencing

The tested blood samples ($n = 31$) were collected from cattle in Northern Cameroon. Blood samples (5 mL in EDTA tubes) were taken from the jugular vein of animals and tested by PCR and agarose gel electrophoresis. Briefly, blood samples were centrifuged at 3000 rpm using the Z380 laboratory centrifuge (Hermle Labortechnik, Wehingen, Germany) for 15 min and 300 µL of the erythrocyte and buffy coat was used for DNA extraction according to the manufacturer's instructions of the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Published primer pairs were used for the identification of the genera *Babesia/Theileria* [20] and *Rickettsia* [10]. Based on sequence alignments of the target species and ribosomal regions in GenBank, a new primer pair was designed for the detection of *Anaplasma/Ehrlichia*. The primer sequences and corresponding annealing temperatures are given in Table 1. To identify TBP-positive samples, a PCR reaction was done in 25 µL total volume combined as followed: 12.5 µL of the 2× RedMaster Mix (Genaxxon BioScience, Ulm, Germany) or 1 mM $MgCl_2$, 0.5 mM 5× buffer, 200 µM nucleotides mix and 1 U GoTaq DNA polymerase (Promega, Madison, WI, USA). To the master mix, 10 pmol of each primer was added per reaction. One microliter of template DNA was added to 24 µL of mastermix reagents, and HPLC-grade water (Sigma Aldrich, Taufkirchen, Germany) was used as PCR negative control. Temperature cycles were programmed on a MasterCycler EPS 96-well thermocycler (Eppendorf, Hamburg, Germany): initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, annealing temperatures (Table 1) for 30 s, 72 °C for 30 s, followed by a final elongation step of 72 °C for 10 min. Five microliter of the amplified products with 1 µL of loading buffer (Genaxxon BioScience, Ulm, Germany) were loaded on a 1.5% agarose gel with Tris Borate EDTA buffer (TBE) stained with Midori Green (Nippon Genetics Europe, Düren, Germany), run for about 40 min at 100 V, and photographed under UV light. The selected specimens with visible PCR product in the gel were prepared and submitted for DNA sequencing according to the provider's recommendation (Macrogen Europe, Amsterdam, Netherlands). The retrieved sequence data was edited manually, MUSCLE aligned and analyzed with Geneious v9.1 (Biomatters, Auckland, New Zealand) and the GenBank nucleotide database (National Center of Biotechnology Information, Bethesda, MD, USA).

Table 1. Primer pairs used for identification of tick-borne pathogens.

Genus	Gene Target	Primer Sequence	Annealing Temp.	Amplicon Size [bp]	Reference
Babesia/Theileria	18S rRNA	GAC ACA GGG AGG TAG TGA CAA G	57 °C	460–500	[20]
		b-CTA AGA ATT TCA CCT CTG ACA GT			
Anaplasma/Ehrlichia	16S rRNA	AGA GTT TGA TCM TGG YTC AGA A	55 °C	460–520	This study
		b-GAG TTT GCC GGG ACT TYT TC			
Rickettsia	16S rRNA	GAA CGC TAT CGG TAT GCT TAA CAC A	64 °C	350–400	[10]
		b-CAT CAC TCA CTC GGT ATT GCT GGA			

b- biotin label at 5′ end.

2.2. LCD-Array Specification and Validation

To allow the detection on the array, a similar PCR reaction was done with one of the paired primers being biotinylated at the 5'-end (Table 1) at a concentration 10-times higher than the corresponding non-biotinylated primer. Moreover, 10 more temperature cycles were added to increase template amplification for hybridization. For sensitivity tests, twelve constructs on the plasmid vector pUC57 (Baseclear, Leiden, Netherlands) with inserts of the following gene loci and species were used as positive controls: For 16S rRNA *Anaplasma centrale*, *A. marginale*, *A. platys* (*A. sp.* 'Ommatjenne'), *A. sp.* 'Hadesa', *E. canis*, *Ehrlichia ruminantium*, *Rickettsia africae* and *R. felis*. For 18S rRNA *Theileria annulata*, *T. mutans*, *T. parva* and *T. velifera* was used. The concentration of plasmid constructs was measured by the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and the number of copies calculated from the amount of DNA in ng and the length of the template in base pairs using the formulae described on the webpage http://cels.uri.edu/gsc/cndna.html (URI Genomics and Sequencing Center). Ten-fold serial dilutions in HPLC-grade water (Sigma Aldrich, Taufkirchen, Germany) as solvent were prepared and used as PCR templates, resulting in target concentrations ranging from 1 to 75 plasmid copies per reaction. Those dilutions of plasmids were amplified by PCR and loaded on gel electrophoresis, as well as tested on the LCD-Array using the first dilution with no detectable PCR product in the agarose gel, respectively for each of the species amplicons.

The LCD-Array consists of a transparent, pre-structured polymer support, with 50 by 50 mm dimensions. Each array had eight individually addressable sample wells where the probes are spotted on the surface as 19 to 28-meres of oligonucleotides using contact-free piezo dispensing technology [14]. The array presently used contained 33 probe spots of which three are proprietary kit controls ('hybridization controls'), and 30 genera- or species-specific probes in duplicates as controls in case of mechanical failure (Figure 1). Altogether, 12 TBP species and 3 genera or groups of genera ("catch all") were included. The probes were selected according to highest genus or species coverage in GenBank. Parameters of selection were the exclusion of unintended hybridization with other genera or species, melting temperature optimum for the LCD-Array, and distance of the hybridization site to the biotinylated primer.

2.3. LCD-Array Workflow

Single amplicons produced by each of the generic primer pairs or mixtures of the three species groups—each containing one biotinylated primer—were added at a final volume of 10 µL (for single product) and in equal proportions (3.3 µL for the mixture) to the LCD-Array according to the manufacturer's protocol (Chipron, Berlin, Germany). Briefly, 10 µL of the mixture was added to 24 µL Hybridization Mix (Chipron), and 28 µL thereof was applied per sample well. The chip was placed in the kit's humidity chamber and incubated in a 35 °C water bath for 30 min. Afterwards, washing steps were conducted with the supplied washing buffer for about 2 min successively in three small tanks filled with about 200 mL of 1× washing buffer. The slide was dried by spinning in the Chip-Spin centrifuge (Chipron, Berlin, Germany) for 15 s. Then, 28 µL of the previously combined horseradish peroxidase—streptavidin conjugate (Chipron) was added to the array for labeling, and incubated for 5 min. Subsequently, the array was washed and dried as previously indicated. Finally, 28 µL of the staining solution tetra methyl benzidine was added to each sample well. After 5 min incubation at room temperature, the staining process was stopped by washing once for 10 s and drying as described before. All tanks were filled with new washing buffer after each step. The LCD-Array was analyzed using the SlideScanner PF725u with the software package SlideReader V12 (Chipron, Berlin, Germany) for automated identification. By default, the cut-off value for positive detection was 2000 pixel values.

To test the specificity and the sensitivity of the assay, 10 µL of the PCR amplification products of each recombinant positive control plasmid was submitted to the array. The template concentrations were one order below the limit of detection by agarose gel electrophoresis as described above. For cross hybridization tests, PCR products of all three genera/groups of genera were mixed at equal volume.

Cattle field samples (n = 31) were PCR amplified and tested on the LCD-Array for analogy with previously obtained sequencing results.

The statistical analysis was done using R v.3.4.2 (www.R-project.org). Data produced from both tests (sequencing and LCD-Array chip) were considered as paired data. The paired t-test was used to assess the difference between both diagnostics. A statistical p-value below 0.05 was considered significant.

3. Results

3.1. LCD-Array Performance of Synthetic Inserts (Plasmids)

All twelve plasmid constructs hybridized only with their respective probes, including "catch all" on the LCD-Array (Figure 2). The tested concentration of plasmid template on the array was 10 to 1000 times lower than on agarose gel (Table 2). Onagarose gel electrophoresis the product was still visible at 10^{-8} dilution for Theileria and Rickettsia, and for dilutions between 10^{-5} and 10^{-7} for Anaplasma and Ehrlichia (Figure 3).

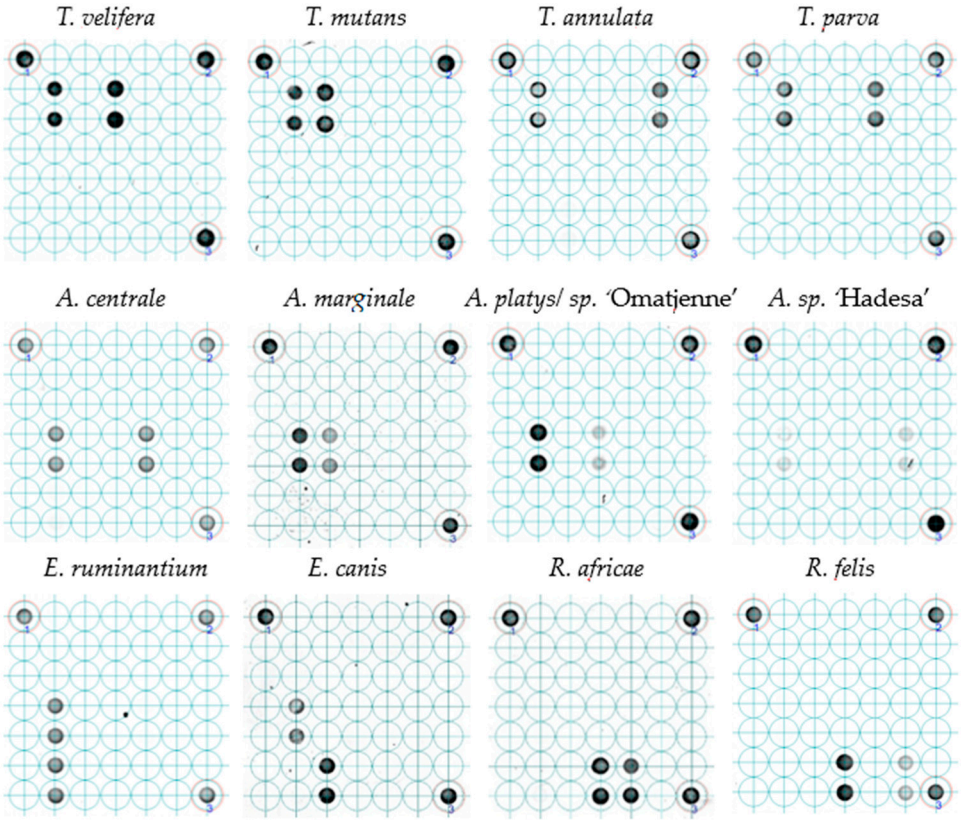

Figure 2. Probe hybridization of LCD-Array of tick-borne pathogens. The dark spots indicate hybridization of plasmids with species-specific inserts to the probe spotted on the array in duplicates. The faint spots indicate lower concentrations in the respective PCR products. The three spots in the corners are internal kit controls. For each of the tested positive controls (plasmids), the concentration came from the first dilution not producing a visible product in agarose gel.

Table 2. Limit of detection (LOD) of LCD-Array for tick-borne pathogens measured in the lowest detectable dilution of the PCR product.

Species	Copies/µL Pre-PCR *	LOD Post-PCR *	LOD LCD-Array
Anaplasma centrale	75	10^{-5}	10^{-8}
Anaplasma marginale	31	10^{-7}	10^{-8}
Anaplasma platys	28	10^{-7}	10^{-8}
Anaplasma sp. 'Hadesa'	34	10^{-7}	10^{-8}
Ehrlichia canis	60	10^{-6}	10^{-8}
Ehrlichia ruminantium	40	10^{-7}	10^{-8}
Rickettsia africae	3	10^{-8}	10^{-9}
Rickettsia felis	2	10^{-8}	10^{-9}
Theileria annulata	6	10^{-8}	10^{-9}
Theileria mutans	3	10^{-8}	10^{-9}
Theileria parva	7	10^{-8}	10^{-9}
Theileria velifera	1	10^{-8}	10^{-9}

* Detected on agarose gel electrophoresis.

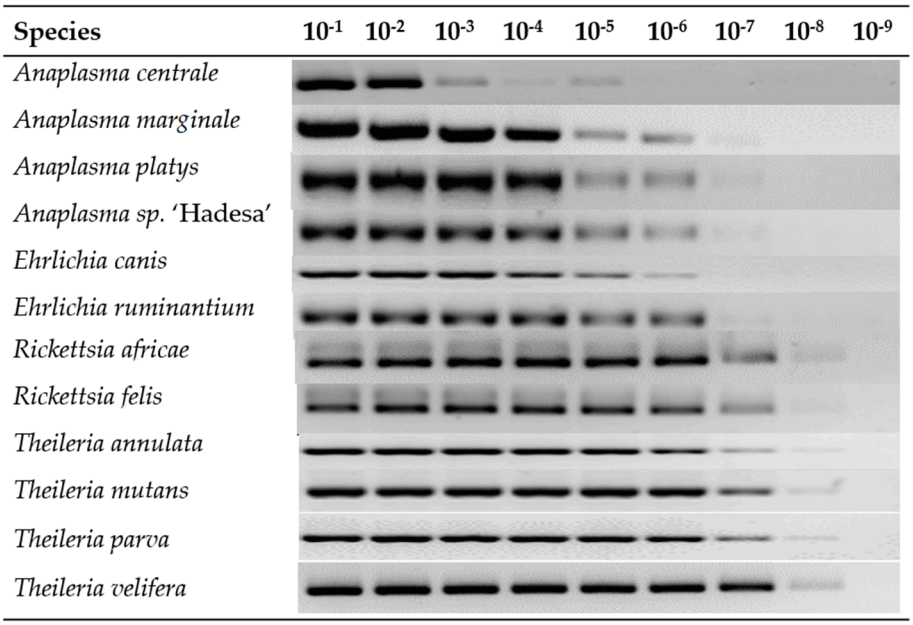

Figure 3. Serial dilution of plasmid amplicons in a 1.5% agarose gel electrophoresis. The last visible band determines the limit of detection which is the lowest dilution detectable on the agarose gel.

3.2. LCD-Array Performance of Cattle Blood Samples from North Cameroon

All pathogens identified by Sanger sequencing in the field-collected blood samples were also detected on the LCD-Array. Furthermore, the array revealed co-infections of more TBPs which were not detected by the sequencing (Figure 4). Statistical comparison showed significant lower detection rates by sequencing as compared to the LCD-Array.

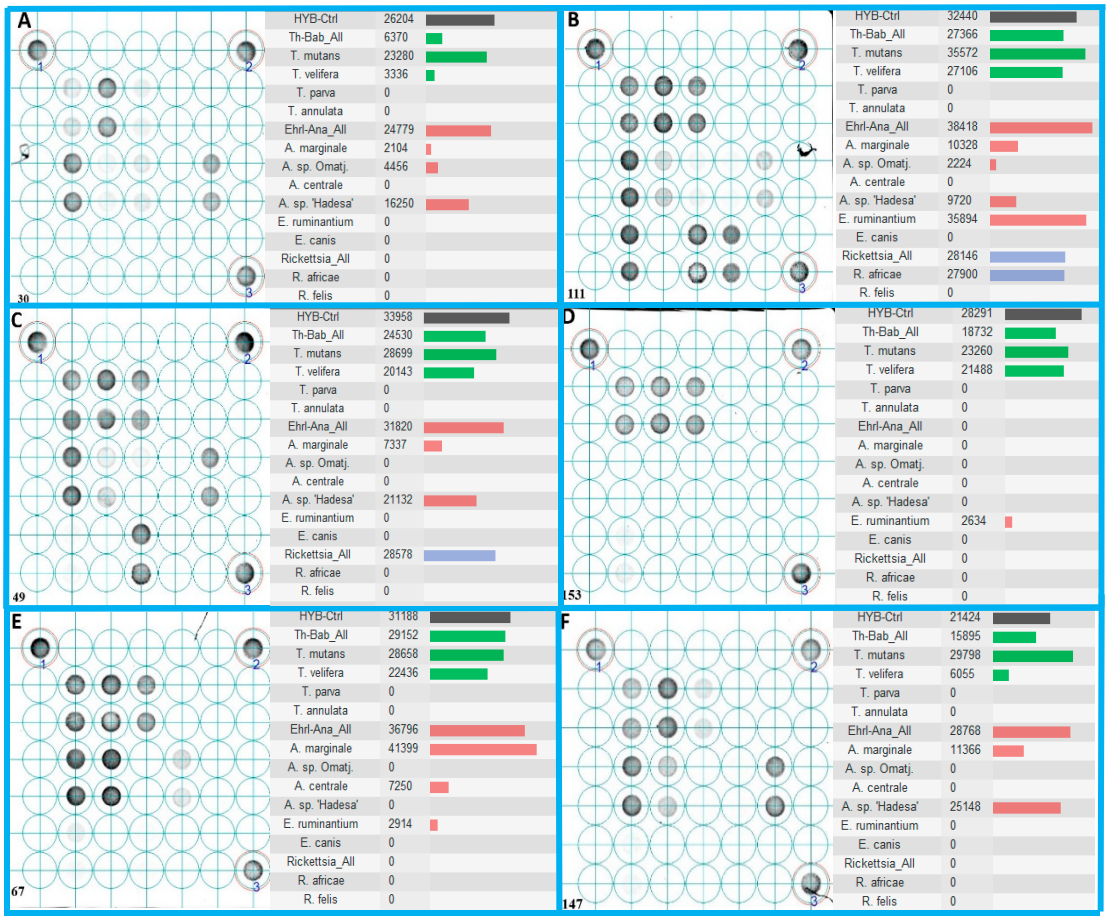

Figure 4. Probe hybridization of six field-collected blood samples (**A**–**F**) on LCD-Array detecting tick-borne pathogens, with 1–3 representing the proprietary kit controls. All shown specimens exhibit co-infections with a minimum of three tick-borne pathogens. The right half of each delimited box shows the hybridization intensity of the corresponding target probe duplicates (Kit control: Black color bar; *Babesia/Theileria*: green color bar; *Anaplasma/Ehrlichia*: red color bar; *Rickettsia*: blue color bar). Results below the cut off value of 2000 are considered negative.

3.2.1. *Anaplasma*

Of the 31 blood samples tested, *A. marginale* was detected in 61.3% (19/31), followed by *A. platys* 41.9% (13/31), *A. sp.* 'Hadesa' 41.9% (13/31), and *A. centrale* 41.9% (13/31). Sanger sequencing had consistently lower detection rates of 12.9%, 29.0%, 6.5% and 12.9% for the same species, respectively. In 26 of 29 positive cases (89.7%) both the species-specific and genus specific ("catch all") probes were hybridizing. The remaining 3 of 29 positive cases reacted only with the *Anaplasma/Ehrlichia* "catch all" probe. From the 31 screened samples, 12 from the *Anaplasma/Ehrlichia* could not be sequenced. Of those unsuccessfully sequenced samples the LCD-Array identified 8 species.

3.2.2. *Ehrlichia*

Ehrlichia species were detected in 17 (54.8%, 17/31) of the screened samples being significantly higher ($p < 0.001$) than the prevalence detected by Sanger sequencing (3.2%, 1/31). Among the unsuccessfully sequenced samples screened under the LCD-Array, *E. ruminantium* was found in co-infection with *A. centrale* and *A. marginale*. In another case *E. ruminantium* was found in co-infection with *A. marginale*. *E. canis* was found by sequencing and hybridized by its specific probe on the array in only one sample, however below the threshold of 2000 pixel values. From the 17 positive cases for *E. ruminantium*, 16 were also positive for the "catch all". From the 31 screened samples, 12 from

the *Anaplasma/Ehrlichia* primers could not be sequenced. The LCD-Array detected 8 of those samples being positive for *A. marginale* ($n = 3$), *E. ruminantium* ($n = 3$) and each co-infected specimens of *A. sp.* 'Hadesa', *A. marginale* and *A. platys*; *A. centrale*, *A. marginale* and *E. ruminantium*, and *A. marginale* and *E. ruminantium*.

3.2.3. *Rickettsia*

Rickettsia africae and *R. felis* were detected on the LCD-Array in 16/31 (51.6%) and 4/31 (12.9%) of cases, respectively, being higher than the detection rates by Sanger sequencing 8/31 (25.8%) and 1/31 (3.2%) of cases, respectively. Eighteen of 20 cases positive for *Rickettsia* species (90%) were also hybridizing with the *Rickettsia*-"catch all" probe. The other two out of 20 samples (10%) were only positive for *Rickettsia* "catch all". PCR amplicons identified by sequencing as bacteria related to *Klebsiella* or *Brevundimonas* did not hybridize with any probe on the LCD-Array. From the 21 PCR-positive samples with negative sequencing results 8 *R. africae* were detected by the microarray, 3 co-infected with *R. africae* and *R. felis*, and one with *R. felis*.

3.2.4. *Babesia*

None of the samples was positively tested and confirmed for *Babesia spp*. Hence, the present LCD-Array did not include probes specific to *Babesia*. However, the *Babesia/Theileria* "catch all" probe is complementary to the 18S loci of the bulk of *Babesia spp*.

3.2.5. *Theileria*

In accordance with the sequencing results, *Theileria mutans* and *T. velifera* were detected in high numbers (90.3%, 28/31, and 77.4%, 24/31, respectively). Detection by sequencing produced unknown *Theileria sp.* in 3 cases, *T. velifera* in one case, *T. mutans* in 17 cases, and *T. mutans* co-infected with *T. velifera* in 3 cases. In 85.7% (24/28) of the cases, *T. mutans* was found in co-infection with *T. velifera* which is significantly higher than recorded by Sanger sequencing of the PCR-product (13.6%; 3/22; $p < 0.001$). 26 of 28 positive animals (92.8%) were also signaling by the "catch all" probe. Both *T. annulata* and *T. parva* were not found neither by sequencing nor by LCD-Array. All PCR-positive samples with no outcome by sequencing ($n = 5$) were identified with the LCD-Array as *T. mutans* and co-infected with *T. velifera* ($n = 3$) and without ($n = 2$).

4. Discussion

The current LCD-Array based on the RLB method has been developed and used to test samples collected from cattle in the northern part of Cameroon. These samples have previously been screened for TBPs using conventional PCR and Sanger sequencing, and a subset of these samples is now being tested by the novel LCD-Array. Co-infection with up to six TBP per animal was common [20], yet difficult to detect by PCR and sequencing alone [13]. In such a scenario, utilization of generic primers poses the problem of correct allocation to the respective species or species complex. DNA sequencing without prior cloning of the less prevalent amplicons is often unsuccessful or distorts the whole readout making it at times incomprehensible [21]. Furthermore, the pathogen concentration in the host blood varies dramatically depending on the animal's state of infection, making the identification challenging when present in very low concentrations. For *Theileria spp.* it is known that carrier animals persist with a low number of infected erythrocytes [22]. Moreover, competition for multiple PCR templates are further limiting factors for the detection of pathogens in low concentrations. In this study, the sensitivity tested on the LCD-Array was between 10 and 1000 times higher than by PCR and Sanger sequencing (Table 2).

The hybridization in some cases of only the "catch all" probe (Figure 4C for *Rickettsia*) suggests the presence of bacteria or parasite species not addressed by the LCD-Array. If DNA sequencing of the PCR product cannot unveil the species responsible for the hybridization, alternative gene loci generally used for molecular taxonomy (e.g., *cox*-I, GAPDH, etc.) could pave the way. The highly pathogenic

piroplasmids *T. annulata* and *T. parva* were not confirmed in the blood samples, although three samples reacted with the corresponding hybridization spots below the cut-off value. Attempts to sequence those inconclusive specimens using primer pairs of species-specific target regions did not bring light to the effective presence of those pathogens. So far, outbreaks with high fatalities are only known in East Africa for *T. parva*, and North Africa for *T. annulata* [23]. By Sanger sequencing of the positively tested animals only *Theileria* species of low pathogenicity were discovered.

Specific probes for the genus *Babesia* were not included in the array because their presence could not be confirmed by PCR in our dataset. Previous infections of *Babesia* spp. may not be detectable by molecular tools as the pathogen can be completely cleared from the blood stream and even from organs [24]. The evidence of *Babesia* in a study from Northern Cameroon [2] could indicate current or very recent infection event in the sampled individuals, allowing its identification on Giemsa stained blood smears.

Reportedly more reliable than the real-time PCR for the detection of new pathogen strains [25], the LCD-Array for TBP can also detect unknown strains or species through conserved oligonucleotide "catch all" probes, representing a whole genus or family. Such amplicons hybridizing with "catch all" probes can be subjected to cloning and DNA sequencing to elucidate their origin. Most generic primer, however, are not able to amplify every variant and/or mutant of the species, genus or family of interest. This limits the detection of all available and yet undetected pathogens [26]. The current microarray was optimized for coverage of as many strains possible of its species or genus reported and deposited in the GenBank repository. Furthermore, the reliance of a species-specific and a genus group-specific probe minimizes the likelihood of false negatives at least on genus level. Since "catch all" probes are efficiently hybridizing with complementary amplicons, a depleting effect can occur if the DNA concentration of the respective pathogen is relatively low (Figure 4). Related to the tested concentration, the species-specific probes were able to hybridize in all cases, sometimes with a weaker intensity (Figure 2: *A. sp.* 'Hadesa'), however with a relatively high copy number. The reason of this discrepancy in comparison to other controls with the same copy number (Figure 2: *T. mutans*) which produce a stronger signal may be optimization issues for the amplification of the *Anaplasma/Ehrlichia* template.

In most of the cases the pathogen in the field-collected sample produced a hybridization signal above the cut-off value hence recognized by the software as a positive pathogen identification. Pathogens showing hybridization with a lower than the cut-off value were considered negative, even if in conformity with the previously obtained Sanger sequencing result. Such cases are better understood when used in a larger sample size. Therefore, recurrent appearance on the LCD-Array below the cut-off value of a doubtful pathogen and its distribution can be an indicator of its presence in the area.

In our sample subset, the inconclusive appearance of *E. canis* below cut-off may be due to the degradation of DNA in the original sample. The cattle samples were collected from April 2014 to June 2015, originally preserved in trehalose solution for transportation [27] and stored at −20 °C between analyses.

No cross reactivity among probes and plasmids were observed in the LCD-Array during testing. A number of the negative samples by gel electrophoresis and Sanger sequencing did not show probe hybridization. Some of the negative samples by PCR show hybridization on the array above the valid cut-off threshold. All field samples tested positive by PCR were confirmed by the LCD-Array being infected with TBPs.

One of the most critical aspects in epidemiological surveillance to avoid false positives and negatives relies on the workflow upstream the LCD-Array or sequencing. From the sampling to the DNA/RNA extraction, appropriate management of the samples is mandatory as inaccurate handling may lead to loss of DNA or contamination [28]. Amplification with Uracil instead of Thymine nucleotides and the addition of Uracil N-glycosylase is one approach to prevent carryover amplicon contamination [29]. Whereas the LCD-Array provided one false negative (*E. canis*), no false positives were confirmed. Optimization of calculation of the cut-off value could reduce the error rate further.

The addition of all three PCR products per sample at the same ratio helped the follow up of the sensitivity and possible cross contamination in case of high copy numbers. Tests using different ratios showed *Anaplasma* being the least sensitive followed by *Rickettsia* and *Theileria* having a higher sensitivity (Figure 2). Consequently, pathogens in low concentration may be overlooked, particularly of *Anaplasma*. This could be improved by protocol optimization or by starting the amplification using a higher template volume (2 or 5 µL) increasing the final concentration. Touch-down PCR program prior to hybridization have showed outstanding results in increasing sensitivity and yield which is of great value as long as the specificity is not hampered [30].

5. Conclusions

The presence of some of the most important non-viral TBPs for livestock on this LCD-Array, including those with zoonotic potential is a valuable asset. In the future, more groups of TBPs including arboviruses or helminths can be added. Although, the production of microarrays with species coverage of 100 and more is possible, the implementation of a running pipeline for diagnostic analyses is more challenging and herein not addressed. With the novel LCD-Array, a sequencing facility which is often lacking in developing countries is not compulsory. Additionally, post-PCR processing times are as short as 45 min, making immediate reporting and response after TBP outbreaks possible. Low- or non-pathogenic species must be incorporated for subsequent identification. Moreover, the better prospect to find endemic or newly introduced species can contribute to the understanding of possible heterologous reactivity responsible of the host health state.

Author Contributions: Conceptualization, A.E. and A.R.; methodology, B.A. and A.E.; validation B.A.; formal analysis, B.A.; investigation, B.A.; resources, B.A., A.P., M.D.A., A.R. and A.E.; data curation, A.E. and B.A.; writing—original draft preparation, B.A.; writing—review and editing, A.P., M.D.A., A.R. and A.E.; visualization, B.A.; supervision, A.R., M.D.A. and A.E.; project administration, A.R. and A.E.; funding acquisition, A.R. and A.E.

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Genetic analyses and genome-wide association studies on parasitic and microbial pathogen resistance of *Bos taurus* and *Bos indicus* cattle breeds in Cameroon

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Summary

Autochthonous taurine and later introduced zebu cattle from Cameroon differ considerably in their resistance to endemic pathogens with little to no reports of the underlying genetic makeover. Breed history and habitat variations are reported to contribute significantly to this diversity worldwide, presumably in Cameroon too, where locations diverge in climate, pasture and prevalence of pathogens. To investigate their genetic background for resistance, 719 cattle (472 *Bos indicus* and 247 *Bos taurus*) were collected from Northern Cameroon and phenotyped for pathogens transmissible by prevailing arthropod vectors, such as hard ticks (tick-borne diseases), and black flies (onchocercosis), and by oral-fecal ingestion (gastrointestinal nematodes) using a binary trait coding. Genotyping was done by Illumina BovineSNP50v3 BeadChip. Variance component estimation including heritability on the observed and liability scale as well as subsequent genome-wide association studies were conducted. Low to moderate heritabilities were observed, implying a genetic manifestation for pathogen resistance and therefore, possibility of improvement by breeding. The genome-wide analyses revealed the quantitative nature of the traits, exposing putative trait-associated genomic regions on five chromosomes, with both environment and genetics as associated factors. A total of 5 significant SNPs were detected on the chromosomes 12 for onchocercosis, 11 and 18 for gastrointestinal nematodes, and 20 and 24 for tick-borne diseases. For the latter no SNP association has been yet reported. Nonetheless, larger datasets are required to identify specific gene loci and to understand the responsible biological pathways.

Keywords: SNP chip, heritability, case control, parasitic diseases, cattle

Main text

In cattle breeds, the genetic makeup has been shown to create different phenotypes related to their ability to sustain environmental pressure, including pathogens. Cameroon is home to both *Bos taurus* and *Bos indicus* cattle breeds with reported differences in susceptibility according to the endemic parasites (Achukwi et al. 2001). The genetic differences play a significant role in their resistance towards parasites and their vectors (Mapholi et al. 2014). Resistance in human onchocerciasis has also been reported (Timmann et al. 2008), and in cattle, host resistance is known as putative immunity (Graham et al. 2006) but no gene association study was yet carried out. Although the bovine parasite has been reported non-pathogenic for its host (Wahl et al. 1994), it can be used as a model of the human parasite. Most traits associated to disease resistance have been found to have heritability potential, with a multitude identified in regions mapped by genome-wide association studies (GWAS) even if at times with low estimates (Porto Neto et al., 2011; Turner et al., 2010).

In tropical Africa, tick-borne pathogens (TBP), and gastrointestinal nematodes (GIN) are among the most detrimental infectious diseases impairing cattle husbandry, inducing increased morbidity and mortality (Pfeffer, Król & Obiegala 2018; Högberg et al. 2019). Those pathogens are dispersed according to climatic zones, habitat preference and vector abundance. The present study aims to investigate the genetic background and variance components underlining the traits of infection with vector-borne and oral fecal-transmitted pathogens in cattle breeds from Cameroon.

As large datasets are indispensable for reliable results in genomic studies (Schmid and Bennewitz 2017), a pooled multi-breed dataset was examined. It consisted of 719 individuals of the *Bos indicus* breeds Fulani (n = 100) and Gudali (n = 372) as well as the autochthonous *Bos taurus* breeds Kapsiki (n = 137) and Namchi (n = 110). DNA was extracted from blood, and phenotypic information about the infection status was recorded. For each individual,

related additional information and parasitological data of GIN, ONC and TBP have been published (Abanda et al., 2019). All phenotypes were binary coded as 1 (infected) and 0 (not infected).

Genotyping was conducted using the Illumina BovineSNP50v3 BeadChip (Illumina, San Diego, CA). After standard quality control, all annotated autosomal SNPs with a minor allele frequency (MAF) ≥ 0.05, no significant deviation from Hardy-Weinberg-equilibrium ($p < 0.001$), and segregating in all of the breeds, were favored for downstream analyses (Supplemental table 1). Individuals without reliable phenotypic records or more than 10 % missing genotypes were discarded. Recovered dataset contained the phenotypes of 608 to 683 animals depending on the trait, and their genotype status of 35,195 SNPs.

The statistical analysis included two major parts, the estimation of variance components, including the heritability, and the estimation of marker effects in order to infer marker-trait-association. Both were conducted using the software GCTA (Yang et al. 2011). Initially, all available fixed effects were tested for significance ($p < 0.05$) to determine the effects to be included in the evaluation model. Since not all breeds were present at all sites, the combined effect of breed and site (breed_site) was considered. For the variance component analyses, the following model was applied:

$$y = Xb + g + e \quad (1)$$

Where, vector y contains the phenotypes of the individuals. b denotes the fixed effect breed_site (additionally age for the trait ONC), and X is the corresponding design matrix. g is the random genetic animal effect, with $g \sim N(0, G\sigma_g^2)$ and G being the genomic relationship matrix. The vector e includes the residuals. Heritabilities were also calculated using the phenotypes of the individuals on the liability scale (λ) given an assumed prevalence of 0.8, 0.6 and 0,5 for the traits GIN, ONC and TBP respectively. In order to estimate the level of

association between the traits and the significant SNPs in GWAS, model 1 was extended towards

$$y = Xb + Wu + g + e \quad (2)$$

Where u denotes the fixed effect of the SNP to be tested and W the design matrix containing the number of 1-alleles. A leave-one-chromosome-out (loco) approach was applied to avoid a loss in mapping power by double-fitting the tested SNP. Those with *p*-values smaller than the threshold of $p = 5 * 10^{-5}$ were assumed to show significant trait association.

The fixed effect breed_site had a significant impact. This could be expected since site even differs in climate (humid, sub-humid and arid) (Bahbahani et al. 2017; 2018) and significant differences between breeds have been frequently reported (Mapholi et al. 2014). These significant factors were also confirmed applying the least significant difference (LSD) tests in the present dataset (details not shown elsewhere). Cattle distribution in Cameroon as in most parts of the world has been strongly influenced by history, climate, vector and parasite prevalence, feed and water scarcity (Chan & Nagaraj 2010; Ali et al. 2012; Li et al. 2013; Bahbahani et al. 2017).

The estimated variance components as well as the heritabilities based on the observed phenotypes and the liability scale for the investigated traits can be taken from Table 1. Phenotypic variances with low standard errors were estimated ranging from 0.063 to 0.181. As expected for binary coded traits, a high or low observed pathogen prevalence in the population came along with considerably smaller estimates. The greatest phenotypic variance of all studied traits was by far observed for ONC, for which the prevalence was rather close to an intermediate value, revealing the relatively low genetic intervention in the reduction of the prevalence (Dahlgwist et al. 2019). Analogous results could be observed for the estimated additive genetic variance resulting in low heritabilities on the observed scale for all traits but ONC ($h^2_{obs.} = 0.216$). The standard errors were generally large for the

estimates of the additive genetic variance and the heritabilities, mainly since the number of individuals was limited and the multi-breed data structure was complex. In agreement with Snowder et al. (2006), all heritabilities were substantially higher when the estimation was based on the liability, since these also capture parts of non-additive genetic variance particularly for a trait prevalence close to zero or unity (Dempster & Lerner 1950).

The quantitative nature of all studied traits can be observed in Figure 1 displaying GWAS results for each of the studied traits. Two SNPs on chromosome 11 and 18 exceeded the significance threshold indicating putative trait-associated genomic regions in the GIN trait and one on chromosome 12 for ONC. For TBP one significant SNP was seen on chromosome 20 and another one on chromosome 24, for which no association signal has been reported elsewhere (Hu, Park & Reecy 2019).

Generally, for all traits investigated, a relatively small amount of neighboring SNPs is in strong LD with the significant SNPs. This might be attributed to the multi-breed dataset, for which a large effective population size can be assumed and hence LD decays fast (Thévenon et al. 2007 ; Goddard and Hayes 2009). Furthermore, due to data filtering, the number of SNPs in chromosomal regions, where an increased number of SNPs do not segregate in all of the breeds, can be smaller.

The results imply that breeding for resistant animals might be possible for the pathogens investigated, however the results should be interpreted with caution due to the large standard errors of the estimates. The findings suggest that further research in this field using larger datasets will be worthwhile for the improvement of the livestock husbandry and to infer the genetic structures of African multi-breed populations. Special attention should be payed to LD consistency across populations and large-scale studies or meta-analyses might give a better insight into the architecture of the traits in future GWAS.

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Tables

Table 1 Population specific parameters of the investigated traits.

The estimated phenotypic (VP) and additive genetic (VA) variance, the heritability estimated for the observed ($h^2_{obs.}$) and liability scale ($h^2_{liab.}$) as well as their standard errors (in parentheses) are shown. The number of evaluated individuals (n) and the observed prevalence in the investigated population are given.

Trait[1]	n	Prevalence	V_P (SE)	V_A (SE)	$h^2_{obs.}$ (SE)	$h^2_{liab.}$ (SE)
GIN	675	0.890	0.087 (0.005)	0.006 (0.007)	0.079 (0.084)	0.265 (0.281)
ONC	608	0.694	0.181 (0.011)	0.039 (0.017)	0.216 (0.094)	0.393 (0.170)

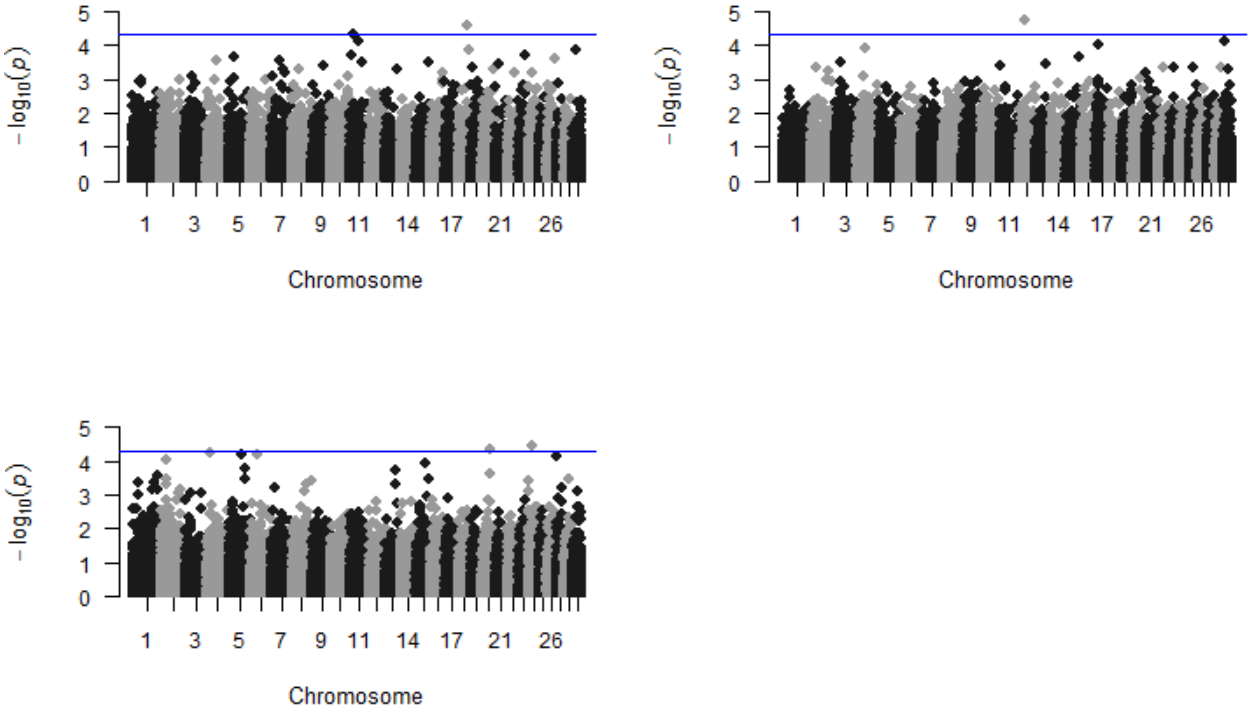
TBP	683	0.931	0.063 (0.003)	0.007 (0.006)	0.109 (0.103)	0.666 (0.631)

[1]gastrointestinal nematodes (GIN), onchocerciasis (ONC), tick-borne pathogens (TBP)

Legends to figures

Figure 1 Manhattan plots for the investigated traits.

The –log$_{10}$-*p*-values of the SNPs and their chromosomal positions are shown for the traits gastrointestinal nematodes (top, left), onchocerciasis (top, right) and tick-borne pathogens (bottom, left). The horizontal line corresponds to a nominal significance level of $p = 5 * 10^{-5}$.



Supporting Information

Parasitological data is available in the supplemental table 1.

Conflict of interest

None.

Supplementary table 1: Characteristics including parasite burden, husbandry and sampling sites of individuals used for analysis.

Site	Cattle breed	Number examined	Cattle species	GIN			ONC			TBP			Herd size/ movements	Treatm.	Season
				+	NA	-	+	NA	-	+	NA	-			
Kapsiki	Kapsiki	136	*B. taurus*	118	8	10	110	0	26	134	0	2	small/yes	No	Rainy
Poli	Namchi	106	*B. taurus*	80	0	26	52	1	53	95	0	11	small/no	No	Dry
Mayo Rey	Fulani	26	*B. indicus*	25	0	1	24	0	2	21	0	5	large/no	Yes	Rainy
	Gudali	189	*B. indicus*	188	0	1	149	2	38	169	0	20	large/no	Yes	Rainy
Vina du Sud	Gudali	123	*B. indicus*	117	0	6	75	7	41	116	0	7	large/no	Yes	Rainy
Faro et Deo	Fulani	68	*B. indicus*	47	2	19	1	66	1	66	1	1	large/no	No	Rainy
	Gudali	37	*B. indicus*	26	0	11	11	1	25	35	1	1	large/no	No	Dry
TOTAL		242	*B. taurus*	198	8	36	162	1	79	229	0	13			
		443	*B. indicus*	403	2	38	260	76	107	407	2	34			
		685		601	10	74	422	77	186	636	2	47			
ID tool				**McMaster egg c.**			**Palp / skin snips**			**PCR**					
Spp. incl.				*Toxocara* spp.			*O. ochengi* (mff)			*Theileria* spp.					
				Strongyle spp.			*O. gutturosa* (mff)			*Anaplasma* spp.					
				Strongyloides spp.			*O. dukei* (mff)			*Borrelia* spp.					
				Trichuris spp.			*O. armillata* (mff)			*Rickettsia* spp.					

GIN, Gastrointestinal nematodes; **ONC**, Onchocerca; **TBP**, tick-borne pathogens; **ID tool**, identification tool; **Spp. incl.,** pathogen species included; **mff**, microfilariae in the skin; **B.**, *Bos*; **O**., *Onchocerca*; **NA**, data not available

Herd size: small: less than 50 animals, large: more than 50 animals**;**

Herd movements: yes = migratory, transhumance, no = sedentary

Treatm.: No: veterinary surveillance absent, except very occasional treatments by the herdsman against ticks, TRP or GIN; Yes: by a qualified veterinarian; **Treatm**.: Treatment;

Identification (ID) tools: McMaster egg c.: counts by floatation technique in two chambers**; Palp / skin snips**: detection of *Onchocerca* nodules by palpation and/or *Onchocerca* microfilariae in three skin snips, taken from the animals inguinal region (Renz et al., 1995); **PCR:** for primers and conditions see Abanda et al. 2019; **Season:** Rainy or dry season during sampling