UNIVERSITÀ DEGLI STUDI DI PADOVA
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Second Cycle Degree (MSc) in
Biotechnologies for Food Science

Molecular Assays for the Detection of Tick-Borne Protozoa in Cattle and Ticks in Benin

Supervisor
Prof. Rudi Cassini
Co-supervisors
Dr. Eric Roland Yessinou
Dr. Marika Grillini

Submitted by
Tingxi Zhang
Student n.2005987

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Abstract

Cattle are important domesticated animals, whose productions (i.e., milk and meat) are nutritious and commonly appreciated food products. The contribution of these products to the food security is particularly relevant in marginal and pastoral areas of African countries, such as in Benin, that constitutes the study area of the present thesis. Among the different health threats for cattle in these areas, they may play a role as hosts for several tick species. Ticks are obligate arthropods feeding on blood and they may act as vectors for bacterial, protozoan, and viral pathogens, transmitting diseases to domestic and wild animals, but also humans. Tick-borne diseases (e.g., babesiosis, anaplasmosis, Lyme borreliosis, rickettsiosis) may lead to severe and sometimes fatal negative impacts on cattle and humans, which in turn causes public health threat and economic losses. Piroplasms are the main concern of the study and they are induced by Babesiidae and Theileriidae families. Bovine babesiosis is a possible fatal disease caused by *Babesia bigemina* and *Babesia bovis*, transmitted by tick of the genus *Rhipicephalus*. Pathogens belonging to *Babesia* genus invade erythrocytes and may cause fever and anorexia in adult cattle. On the other side, *Theileria* spp. can infect both erythrocytes and white blood cells, leading to similar symptoms like fever, anorexia, and anaemia. Therefore, the investigation into the prevalence of tick-borne pathogens in Benin can contribute to a comprehensive understanding of their epidemiology and facilitate the development of more effective control strategies in the future. In this study, 2311 ticks and 81 blood samples were collected from cattle, in five areas from Benin, including Banikoara and Tanguiéta in Northern Benin and Kpobè, Ouidah, and Zakpota in Southern Benin. Ticks were collected during cattle blood sampling and morphologically identified. A representative number of ticks for each tick species from all geographical areas were analysed (n=246) to detect at least one positive sample. All samples (i.e., blood samples and ticks) were screened by real-time PCR targeting LSU gene of *Babesia* and *Theileria* genera. Subsequently, positive samples were analyzed by conventional PCR targeting the 18S gene in order to determine pathogens species.

Based on the molecular analysis, *Theileria velifera* (n=31, 44.9%), *Theileria mutans* (n=6, 8.6%), *Theileria* spp. (n=7, 10.1%), and *Babesia* spp. (n=2, 2.9%) were detected in blood samples.

Overall, *Rhipicephalus microplus* (n=987, 42.7%) and *A. variegatus* (n=1227, 53.1%) were the most collected species, followed by *Hyalomma rufipes* (n=45, 1.9%),

Hyalomma truncatum (n=37, 1.6%), and *Rhipicephalus sanguineus* (n=15, 0.6%). All five species were found in two or more of the considered sites, demonstrating a wide geographical distribution. Lastly, *T. velifera* was isolated in 1 *R. microplus* and *B. bigemina* in 1 *R. microplus* e 1 *H. rufipes*.

The findings of the present study provide new molecular data about the presence and distribution of piroplasms in Benin, which can contribute to improved tick control plans, although the sensitivity of this approach may be affected in case of co-infections.

Key words: tick-borne diseases, multiplex molecular assays, PCR, cattle, Benin

1. Introduction

1.1 Background

Benin is an agricultural country located in West Africa with most of the region suitable for cattle raising (De Clercq et al., 2012). In 2015, it was estimated that the cattle population in Benin exceeded two billion heads (Adinci et al., 2018). Indeed, livestock is crucial for the local economy, contributing through meat and milk production as well as plowing (Yessinou et al., 2018). Ticks and the tick-borne diseases (TBDs) pose a significant threat to cattle health and productivity Ticks affect livestock production through reduced body weight, growth rate, lower meat, milk yield and hide quality (Heylen et al., 2023). Ticks transmit many s pathogens such as protozoa, bacteria, and viruses to their vertebrate hosts, including humans (Yessinou et al., 2023). The most important TBDs in cattle include piroplasmoses (i.e., babesiosis and theileriosis), anaplasmosis and cowdriosis. Ticks of the genus *Rhipicephalus* play an important role in the transmission of *Babesia bigemina* and *Babesia bovis* causes Bovine babesiosisis (Heylen et al., 2023). Several species of *Theileria* were associated with the disease in cattle, and the more impactful one is the East Coast fever disease whom causative agent is *Theileria parva*. *Anaplasma marginale* infections are responsible for Bovine anaplasmosis, can cause severe anemia, until death. It were mainly detected in *R. microplus* which play important in transmission of this pathogen . Amblyomma ticks act as main vectors of Ehrlichia ruminantium responsible of Cowdriosis or heartwater disease (Kasaija et al., 2021). Many tick species have been reported across Benin, including *Amblyomma variegatum*, *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus microplus*, *Rhipicephalus sanguineus* and *Hyalomma spp.* (Adinci et al., 2018; Yessinou et al., 2018). De Clercq et al. (2012) corroborated the presence of four *Rhipicephalus* species; among them *Rhipicephalus microplus* predominantly infesting the Northern and central regions of the country. Other genera, such as *Amblyomma* and *Hyalomma*, have also been documented in multiple studies, indicating a wide geographical distribution of tick species in Benin (Yessinou et al., 2018).

1.2 The important vector-borne diseases in cattle

1.2.1 Babesiosis

Bovine babesiosis is caused by the protozoan parasites *Babesia bigemina* and *Babesia bovis*, which specifically invade the erythrocytes of their mammalian hosts (Bajer et al., 2022). The cycle starts with an infected tick that releases thousands of sporozoites during blood meal (Figure 1). These sporozoites penetrate the erythrocytes and develop into trophozoites and reproduce in them by binary fission generating merozoites, damaging the cell membrane and leading to anemia (Beugnet & Moreau, 2015; Almazane et al., 2022). Some merozoites continue to invade new erythrocytes and develop into trophozoites, although others are taken by adult ticks during blood feeding in which sexual reproduction of the parasite occurs. In the invertebrate host, gametes are released from erythrocytes to midgut lumen where after their fusion a zygote develops. The diploid infected phase invades the midgut epithelial cells and develops in motile kinetes that, after releasing, migrate through the hemolymph to different tissues, until ovaries of adult female ticks. Transovarian transmission occurs when kinetes infect embryos in the ovary, thus, infected eggs are laid by female ticks. Kinetes in newborn infected larvae migrate to salivary glands acini cells (i.e., cell type that produces saliva) and develop into sporoblasts then, after sporogony, into sporozoites (Almazane et al., 2022).

Babesia bovis is more virulent and primarily transmitted by the ticks *Rhipicephalus microplus* and *Rhipicephalus annulatus*. *Babesia bigemina*, relatively less virulent, is more widely distributed in Africa. It can be transmitted by *Rhipicephalus microplus*, *Rhipicephalus annulatus*, and *Rhipicephalus decoloratus* (Heylen et al., 2023). As reported above, transmission of *Babesia* spp. can occur transovarially, from one generation to the next via the eggs, or from eggs to larvae (Garcia et al., 2022) and this ensures the persistence of the pathogens within tick populations, enhancing their spread among cattle. The clinical symptoms of babesiosis in cattle include fever, hemolysis, anemia, which can lead to fatal outcomes in severe cases (Kasaija et al., 2021). Mildly infected cattle may recover on their own, but often suffer chronic debilitation, resulting in decreased productivity. Economically, babesiosis significantly impacts cattle production (Beugnet & Moreau, 2015). Infected animals experience reduced milk and meat yields, and the cost of veterinary care increases. These economic

burdens underscore the importance of effective control and prevention strategies for managing babesiosis in cattle populations.

Ouedraogo et al. (2021) identified *Babesia bigemina* and *Babesia bovis* in cattle blood samples in Northern Benin. The prevalence is significantly higher in Borgou and Donga, which may be related to the local prevalence of their primary vector *R. microplus*. Similarly, study confirmed the presence of *Babesia bigemina* and *Babesia bovis* in Southern Benin farms, with a positive rate of 13.89% and 5.56%, respectively (Safiou et al., 2016). *Babesia bigemina* and *Babesia bovis* have been molecularly identified in both animals and ticks in Benin, as well as in neighboring countries like Burkina Faso and Nigeria (Diarra et al., 2023). In Burkina Faso, both *Babesia bigemina* and *Babesia bovis* are present, but the prevalence of *Babesia* spp. in Northern Benin is notably higher than in Eastern Burkina Faso (Diarra et al., 2023; Ouedraogo et al., 2021). In contrast, Nigeria has a broader range of *Babesia* species, including *Babesia divergens*, indicating a *Babesia* variability in the region (Diarra et al., 2023). These findings highlight the regional differences in *Babesia* prevalence and species distribution, emphasizing the importance of localized studies for effective disease management and control.

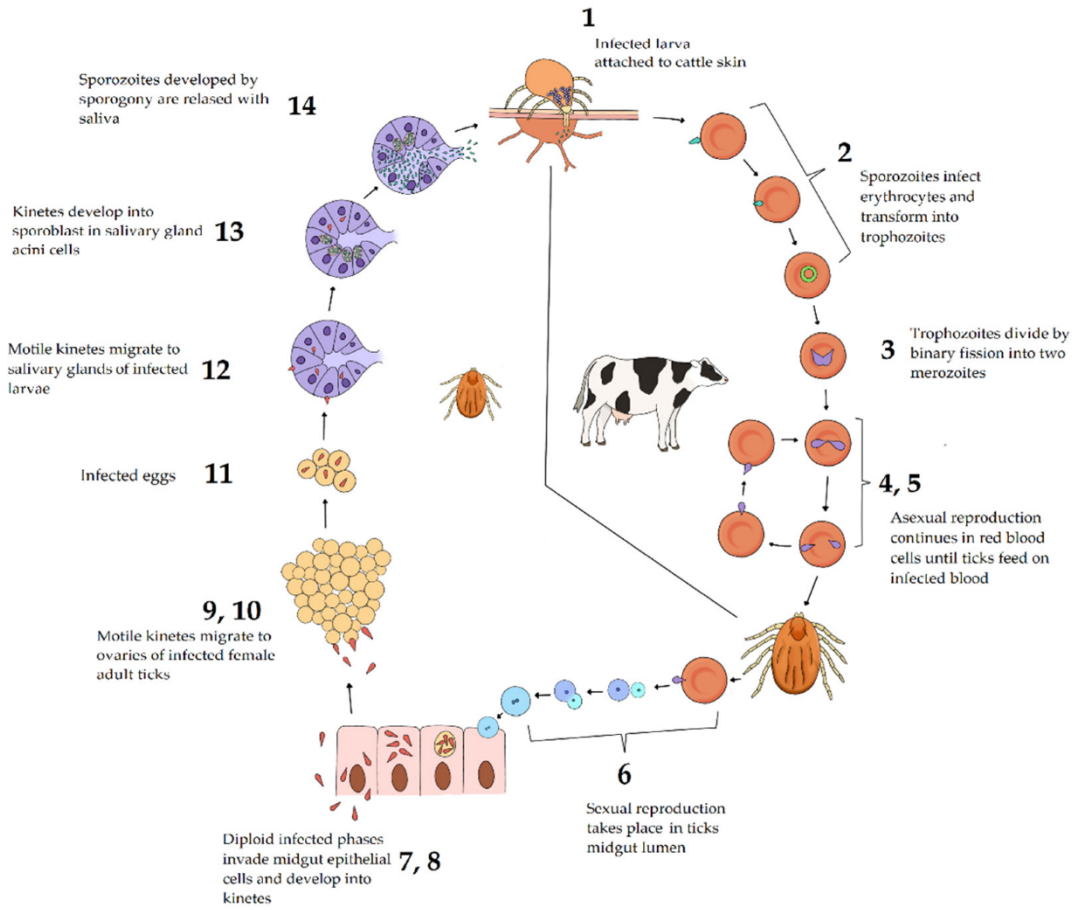

Figure 1. *Babesia bovis* life cycle (Almazán et al., 2022)

1.2.2 Theileriosis

Bovine theileriosis is caused by parasites belonging to Theileriidae family. Unlike *Babesia*, *Theileria* parasites can infect endothelial and white blood cells in addition to red blood cells (Heylen et al., 2023). The cycle starts with an infected tick that releases sporozoites during blood meal (Figure 2). These sporozoites penetrate a lymphocyte and develop into schizonts and reproduce in them by mitosis doubling the number of infected lymphocytes. Following merogony, multiple merozoites are generated from each schizont and released into the bloodstream until they reach erythrocytes and undergo asexual reproduction. Piroplasms are taken by nymph and/or adult ticks during blood feeding in which sexual reproduction of the parasite occurs. Transstadial transmission occurs when infected nymphs molt into adults. In the invertebrate host, gametes are released from erythrocytes to midgut lumen where after their fusion a zygote develops.

The diploid Infected phase Invades the midgut epithelial cells and develops In motile kinetes that, after releasing, migrate through the hemolymph to different tissues, until salivary glands. Kinetes in infected ticks migrate to salivary glands acini cells and develop into sporoblasts then, after sporogony, into sporozoites released with ticks saliva (Almazane et al., 2022)

As described, differently from Babesiidae, the transmission of Theileriidae can only occur by transstadial transmission, from larvae to nymphs and to adults (Beugnet & Moreau, 2015; Almazane et al., 2022). The most economically significant species is *Theileria parva*, which causes East Coast fever. Transmitted by the tick *Rhipicephalus appendiculatus*, this pathogen induces fever and lymphatic swelling (Kasaija et al., 2021). However, *T. parva* has not yet been detected in Benin, since it is more common in the Eastern African countries. Another major pathogen is *Theileria annulata*, transmitted by *Hyalomma* species ticks. This parasite also destroys red blood cells causing anemia and leading to similar symptoms and outcomes of babesiosis. In addition, other species can occur such as *Theileria mutans* and *Theileria velifera* which are transmitted by *Amblyomma* ticks. *Theileria mutans* is relatively mildly pathogenic, while *Theileria velifera* is generally nonpathogenic (Garcia et al., 2022).

A study conducted in four Northern regions of Benin—Alibori, Atacora, Borgou, and Donga—revealed the presence of six TBPs in cattle blood samples. The study

highlighted *Theileria mutans* as the most prevalent pathogen, with an overwhelming 97.4% of the sampled cattle testing positive. Following *T. mutans*, *Theileria velifera* was the next most prevalent pathogen detected. *Theileria annulata*, though less prevalent in the study, was also identified, further emphasizing the widespread impact of *Theileria* species in northern Benin (Ouedraogo et al., 2021). A cross-sectional study conducted in the Mono district of Benin also found that *Theileria mutans* was the most prevalent pathogen, detected in 22.22% of the samples. This result aligns with findings from other regions of Benin. Additionally, the study identified *Theileria ovis* in 5.56% of the samples, highlighting its presence, though at a much lower prevalence compared to *T. mutans* (Safiou et al., 2016). These findings underscore the significant presence of *Theileria* species in the region.

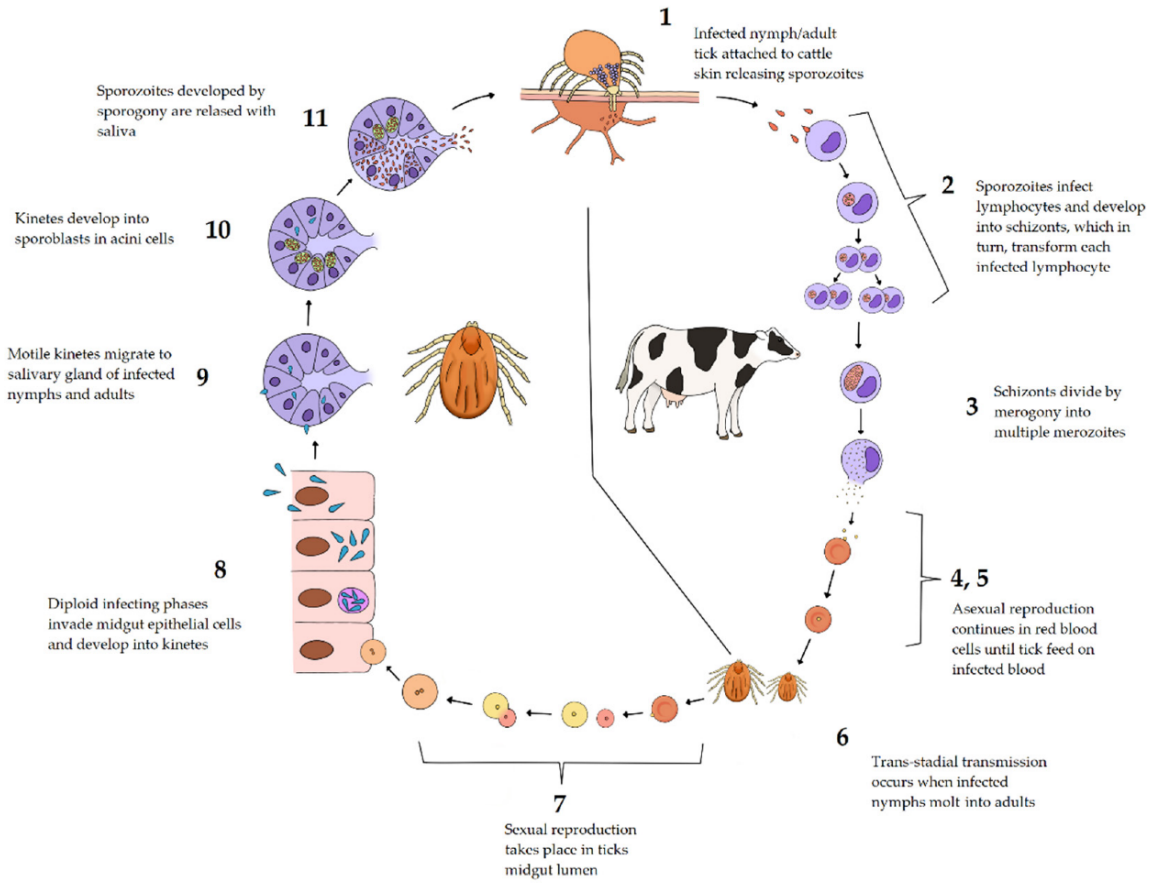

Figure 2. *Theileria* spp. life cycle (Almazán et al., 2022)

1.3 Diagnostic Methods for Tick-Borne Diseases

1.3.1 Conventional Diagnostic Methods

The first step to investigate tick-borne pathogens (TBPs) may be identify the presence of ticks on cattle. If present, ticks can be identified at the genus or species level by examining features such as suboral teeth, the shape of the glandular plates, and the scaly external spines on the basal segments of female ticks (Madder et al., 2012). Different ticks are able to transmit different pathogens. This information may help in understanding which pathogen cattle are exposed to. However, being parasitized by a tick does not necessarily indicate that a disease is present. Indeed, a more clinically useful and reliable diagnostic method is microscopy, used to observe the stained red blood cells on blood smears or of infected tissue. The red blood cells of cattle infected with babesiosis are usually morphologically altered and the parasitic inclusions can be observed in the stained red blood cells (Beugnet & Moreau, 2015). While this method is effective, it is less sensitive and requires precise sampling, e.g., the use of venous blood sampling may negatively affect the results.

Tick-borne diseases can also be detected serologically through methods such as enzyme-linked immunosorbent assay (ELISA), Indirect Fluorescent Antibody Test (IFAT), and western blot (Beugnet & Moreau, 2015). Compared to microscopic observation, serologic assays detecting antibodies, are more sensitive and useful for screening large populations. However, several factors can affect the accuracy of these results. For example, when using an ELISA to detect babesiosis, it may not distinguish between species and subspecies of the pathogen due to cross-reactivity, that can occur between antibodies of species phylogenetically closed (Garcia et al., 2022). This cross-reactivity can also produce false-positive results. In addition, different experimenters may choose various antigenic targets and thresholds for positivity, potentially resulting in biased results for the same pathogen (Beugnet & Moreau, 2015). Moreover, hosts may be infected with multiple TBPs simultaneously (called coinfections), necessitating multiple serological screens to identify all infections. This increases both the number and cost of experiments. Despite these challenges, serological tests remain a crucial tool in the detection and management of TBDs.

1.3.2 Molecular Diagnostic Methods

Molecular diagnostic methods, such as conventional (or end-point) PCR and real-time PCR, are more sensitive than traditional methods, enabling rapid identification at the subspecies or genotype level (Beugnet & Moreau, 2015). Various regions of the 18S rRNA gene have been used as targets for the molecular identification of piroplasms (Kumar et al., 2022). These sequences contain highly variable regions flanked by highly conserved ones, allowing for specific identification. However, the 18S rRNA gene sequences of distantly related evolutionary branches may differ significantly. To address this, molecular assays targeting the highly conserved regions of the mitochondrial genome (mtDNA) of *Babesia* spp. have also been developed. These assays provide higher sensitivity and a broader detection range (Qurollo et al., 2017). Other molecular target includes internal transcribed spacer (ITS), 16S rRNA, and heat shock proteins 70 (HSP70) have also been described (Kumar et al., 2022; Yabsley & Shock, 2013). Some of the primers and related target genes used in studies on *Babesia* and *Theileria* species are reported in Table 1.

Table 1. PCR assays developed for *Babesia* and *Theileria* detection

Pathogen	Target gene	Primer name	Primer sequence (5' – 3')	References
Babesia spp.	18S rRNA	BdiF	CAGCTTGACGGTAGGGTATTGG	(Øines et al., 2012)
		BdiR	TCGAACCCTAATTCCCCGTTA	
	18S rRNA	RLBH-F	GAGGTAGTGACAAGAAATAACAATA	(Schouls et al., 1999)
		RLBH-R	TCTTCGATCCCCTAACTTTC	
	ITS1	ITS1for	CGAGTGATCCGGTGAATTATTC	(Blaschitz et al., 2008)
		ITS1rev	CCTTCATCGTTGTGTGAGCC	
	HSP70	HSP70for	GCTATTGGTATTGACTTGGG	(Blaschitz et al., 2008)
		HSP70rev	CCTTCATCTTGATAAGGACC	
Theileria spp.	18S rRNA	*Therileria* forward	GGTAATTCCAGCTCCAATAG	(Sibeko et al., 2008)
		Therileria reverse	ACCAACAAAATAGAACCAAAGTC	
	18S rRNA	SetA	ACCTGGTTGATCCTGCCAGT	(Tian et al., 2014)
		setB	ACCTGGTTGATCCTGCCAGT	
	ITS1/2 of *Theileria* spp.	Forward	GAGAAGTCGTAACAAGGTTT	(Tian et al., 2014)
		reverse	GCTTCACTCGCCGTTACTAGG	

Both conventional PCR and Real-time PCR can only specifically detect a single pathogen. Multiplex PCR is being explored for the identification of coinfections and the simultaneous characterization of multiple pathogens. While this method is more complex due to the multiple amplification targets and primers involved, it increases the probability of non-specific amplification and primer interactions. Therefore, extensive testing is required to determine the optimal primer combinations (Garcia et al., 2022; Kumar et al., 2022). Molecular diagnostic methods represent a significant advancement in the detection and management of tick-borne diseases, offering higher accuracy and efficiency in identifying and studying these pathogens.

2. Objective of the study

Ensuring the health of cattle is essential for maintaining the quality of milk and meat products, as well as securing the income of farmers who raise cattle. Controlling tick-borne diseases requires accurate and timely monitoring of host and tick species distribution. Environmental and climate changes, along with social activities such as cross-border livestock trade, can influence tick density, range, and the spread of associated pathogens (Adinci et al., 2018; Yessinou et al., 2018). The most commonly used method for controlling ticks is synthetic acaricides. Yessinou et al. (2021) demonstrated that the resistance of *Rhipicephalus sanguineus sensu lato* to deltamethrin is linked to a genetic mutation in the sodium channel domain III, which is possibly related to the uncontrolled use of acaricides. Therefore, it is crucial to use acaricides appropriately, targeting cattle populations at high risk for life-threatening diseases, and to integrate this approach into a broader, more complex strategy. An in-depth understanding of the epidemiology of the most relevant cattle TBDs is the first step toward a cost-effective, integrated approach to tick and TBDs control. This study is part of an ongoing collaboration between the Department of Animal Medicine, Production and Health of the University of Padova (Italy) and the Communicable Disease Research Unit of the Polytechnic School of the University of Abomey-Calavi (Benin), aimed at investigating the epidemiological features of the tick-borne pathogens affecting bovines in Benin.

The specific objectives of this study are:

1. To investigate the occurrence and prevalence of *Babesia* and *Theileria* species in cattle from different regions of Benin.
2. To evaluate the effectiveness of molecular assays (PCR) in diagnosing these pathogens.
3. To contribute to the development of more effective control strategies for tick-borne diseases.

3. Materials and methods

3.1 Sample collection and DNA extraction

3.1.1 Study areas

Ticks and blood samples were collected from cattle, in five areas from Benin, including Banikoara and Tanguiéta in Northern Benin and Kpobè, Ouidah, and Zakpota in Southern Benin.

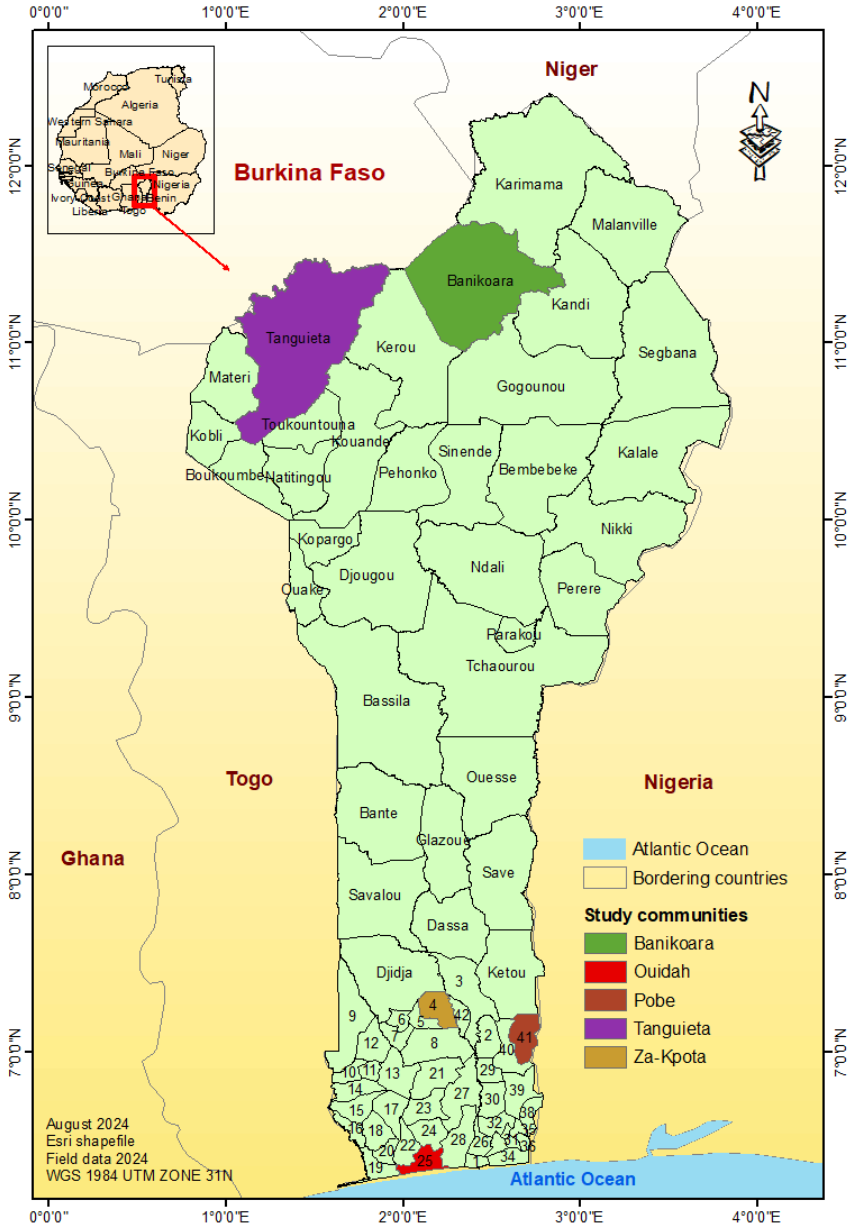

Figure 3. Map depicting the sample collecting sites in Benin: Banikoara, Tanguieta, Zakpota, Pobè, Ouidah.

District of Banikoara is located in the northwest of the Alibori Department (2º05’ and 2º46’; 11º02’ and 11º34’) and represents the agropastoral cotton production zone of Benin. It has a dry Sudanese climate, with annual rainfall ranging from 800 mm to 1100 mm, an average temperature of 24 °C to 31 °C, and a relative humidity of 18% to 99%. The area of the zone is 4397.20 km², of which approximately 49% is arable land and 50% is protected areas (Niger W National Park and the Atacora hunting zone). The Baribas and the Peulhs are the two communities that engage in cattle breeding activities in the commune of Banikoara (Sabai KATE et al 2017).

District of Tanguiéta is located in the northwest of Benin (1°50’58’’ and 1°55’19; 10°24’43’’ and 10°27’57’’). It has a Sudano-Sahelian climate with a rainy season from May to November and a dry season from November to May. The temperature varies between 18.9 °C and 37.6 °C. Annual rainfall ranges from 827 mm to 1898 mm. This climate is favorable to pastoral activity. The commune of Tanguiéta covers an area of 5,456 km² or 4.71% of the national territory (ABDOULAYE, 2016).

District of Za-Kpota is located in the northeast of the Abomey plateau (7°05' 24" and 7° 21' 57"; 2° 03'54" and 2°21' 57"). It enjoys a Benin-type subequatorial climate with two rainy seasons, a long one from mid-March to mid-July and a short one from September to November, and two dry seasons, the long one extending from December to March and the short one covering the second half of July and the month of August.The average annual rainfall of Zakpota is 980 mm, a source of climatic uncertainty for this area. Temperature varies between 28.90˚C to 34.18˚C, an average of 30.59˚C. The commune covers an area of 409 km2 (Ahlonsou et al 2024).

District of Pobè is located in the southeast of Benin (6°55' and 7°00'; 2°37' and 2°41'). The climate is subequatorial with two rainy seasons and two dry seasons that alternate. The temperature generally varies from 22 °C to 34 °C and precipitation is 1100 to 1200 mm per year. The commune covers approximately 400 km² (AKINDELE and TODOME, 2021).

District of Ouidah is located in the south of the Atlantique department (2° and 2°15; 6°15 and 6°30). It is characterized by a subequatorial climate comprising two rainy seasons (from April to July, then from September to November) and two dry seasons (from December to March then in August). The average rainfall is 1200 mm per year. The temperature fluctuates between 27 and 31°C while the relative humidity varies between 65% from January to March and 97% in June and July. Its area is 364 km2 (Ahokpossi et al 2020)

3.1.2 Study design and sampling methods

The study was conducted from June 2021 to March 2022 in Northern and Southern of Benin. The cattle farms were randomly selected from five districts including Banikoara, Tanguieta, Zakpota, Pobè and Ouidah (Fig.3). Cattle were restrained and ticks were collected on abdomen, external genitalia, tail, dewlap, ears, face and tests of animals. Blood samples were collected in EDTA-vacutainer tubes (2–5 ml). Ticks collected were preserved in 70% ethanol and transported to the Department of Animal Medicine, Production and Health, University of Padova, Italy. Tick species were identified under a microscope using morphological keys (Estrada-Peña et al., 2004; J.J. Walker et al., 2000; A.R.Walker et al., 2003), then preserved in 70% ethanol until the DNA isolation. The number of ticks to be individually analyzed was defined with the aim of identifying any pathogen circulating within the tick population at a certain prevalence (infection rate). Assuming an expected minimum prevalence of 4%, an infinite population and a 95% confidence level, the minimum overall number of ticks for each tick species was set at 74. A number of tick proportional to the overall ratio (ticks to be analysed/ticks collected) was randomly selected from each geographical area, including only adult ticks, both male and female, either not or slightly engorged. When the overall number of collected ticks was less than the minimum number, all ticks were individually analyzed.

Before DNA extraction, ticks were washed 3-4 times with PBS 1x. Then each tick was transferred into a new Eppendorf tube and crushed with pestles. DNA was extracted with NucleoSpin™ Tissue extraction kit (Macherey-Nagel, Düren, DE). The first step was the pre-lyse phase: in each tube, samples were mixed with 180 µl of Buffer T1 and 25 µl of Proteinase K, followed by an incubation at 56°C for 4 hours to digest chitin and proteins. Then, 200 µl of Buffer B3 was added and samples were incubated for 10 minutes at 70°C. After lysis, 210 µl of ethanol (97% concentration) was added in each tube and the mixture was spanned at 11,000xg for 30 seconds for adjusting DNA binding conditions. The resultant supernatant was transferred from the Eppendorf tube to the NucleoSpin™ Tissue column with a silica membrane, which was placed into a collection tube. All samples were centrifuged for 1 minute at 12,000xg, after which DNA was bind to the membrane. The silica membrane was washed in two steps, first with 500 µl of Buffer BW and then with 600 µl of Buffer B5. Tubes were centrifuged

at 12,000xg for 1 minute after both steps allowing to remove proteins and contaminants from DNA. Silica membrane was dried with another centrifugation for 1 min at 13,000xg. Finally, DNA was eluted by incubation with 100 µl of Buffer BE for 1 minute at room temperature. The NucleoSpin™ Tissue column was put into a new Eppendorf tube and centrifuged for 1 min at 11,000xg. Highly pure DNA was obtained and stored at -20°C.

3.2 Piroplasms detection (*Babesia* spp. and *Theileria* spp.)

3.2.1 Real-Time PCR

The real-time quantitative polymerase chain reactions (real-time PCR) mix was prepared with forward and reverse primers, DNase/RNase-free water and QuantiNova SYBR Green PCR Kit (QIAGEN Group, Hilden, DE). The primers used were based on *Babesia* and *Theileria* LSU-gene (primer pairs: 5'-ACCTGTCAARTTCCTTCACTAAMTT-3' and 5'-TCTTAACCCAACTCACGTACCA-3') described by Qurollo et al. (2017). The mixture was transferred to a 96-well plate, with 17µl of mix and 3 µl of DNA in each well. Two positive controls were used (i.e., *B. bovis* and *B. bigemina)* and one negative control with no DNA addition. After centrifuging the plate for 30 seconds to converge the liquid at the bottom, the plate was placed into the thermocycler Roche LightCycler® 96. Samples were amplified and analyzed under the cycle: pre-incubation at 95°C for 2 minutes, followed by 45 cycles of amplification at 95 °C for 5 seconds and 60 °C for 10 seconds, then melting at 95°C for 10 seconds, 65 °C for 1 minute and finally a temperature increase to 97 °C (1°C/sec).

Target pathogens (i.e., *Theileria* and *Babesia* genera) were identified by the temperature of melting (Tm). (Figure 3) comparing the Tm of positive controls that were preliminarily analysed and sequenced.

The samples with the closest Tm to positive controls and a proper cycle threshold (Ct) were selected for conventional PCR (cPCR) to confirm the specificity of melting curve temperature.

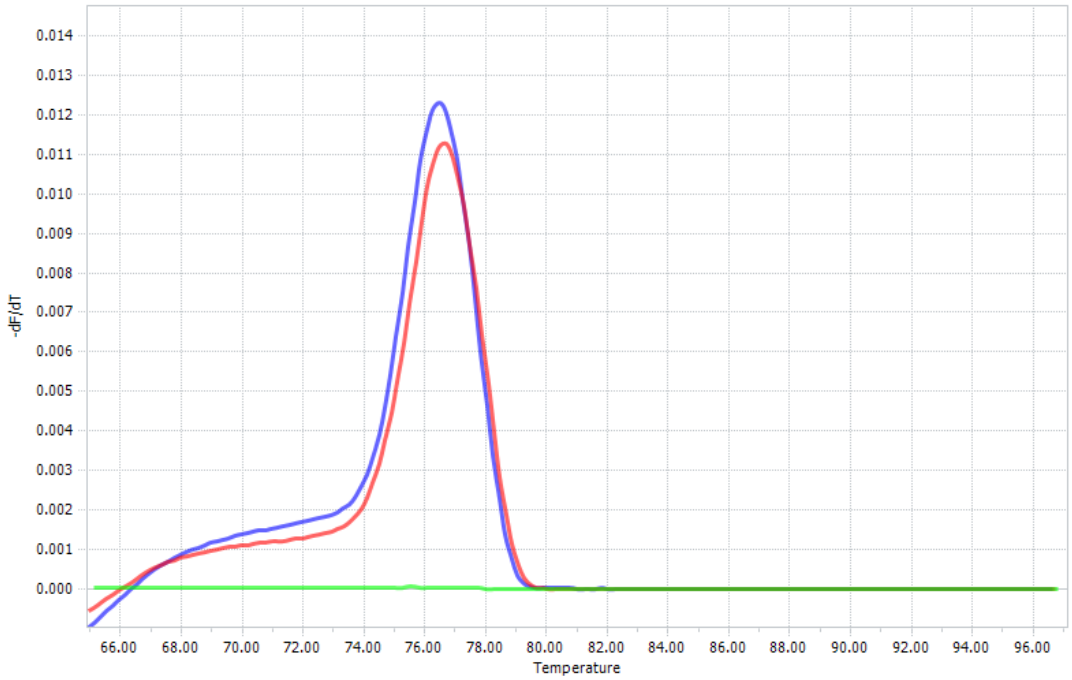

Figure 4. Specific temperature of melting (Tm) for *Babesia bovis* and *Babesia bigemina* (blue and red lines), and negative control (green line)

3.2.2 Conventional PCR assay

Since the parasitemia was low, following an initial test it was necessary to use a nested PCR which would therefore allow the PCR products to be reamplified. The reaction mix was prepared for both rounds with DNase/RNase-free water, Deoxyribonucleotide triphosphates (dNTPs), reagents (i.e., buffer, $MgCl_2$), forward and reverse primers targeting 18S gene (primer pairs 5'-AAGCCATGCATGTCTAAGTATAAGCTTTT-3' and 5'-GAATAATTCACCGGATCACTCG-3') of *Babesia* and *Theileria* species (Oosthuizen et al., 2008; Matila et al., 2008), and Taq DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA). Twenty-seven µl of mix and 3 µl of DNA was used in the first round and 29 µl of mix and 1 µl of PCR product in the second one. Positive (i.e., DNA of sequenced field sample) and negative (no DNA added) controls were analysed together with samples. The samples were amplified in both rounds with the cycle: initiation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 60° for 45 seconds, extension at 72°C for 1 minute, and a final extension cycle at 72°C for 5 minutes.

In the meanwhile, 2% agarose gel with Invitrogen SYBR® Safe DNA gel stain (Thermo Fisher Scientific Inc., Waltham, MA, USA) was prepared and used to visualize the DNA. Nine µl of each PCR product and 1.8 µl of Blue Gel Loading Buffer (Jena Bioscience GmBH, Jena, DE), allowing to tracked dye during electrophoresis, were loaded into gel wells, together with 4 µl of 1 kb DNA ladder (MassRuler™ Low Range DNA Ladder, Thermo Fisher Scientific Inc., Waltham, MA, USA). The gel was run in Tris/Borate/EDTA (TBE) buffer at 100V for 45 minutes. The gel was subjected to UV lighting in order to make visible the results. The samples that exhibited bands of the same molecular weight as the positive control were subsequently purified and sent for sequencing.

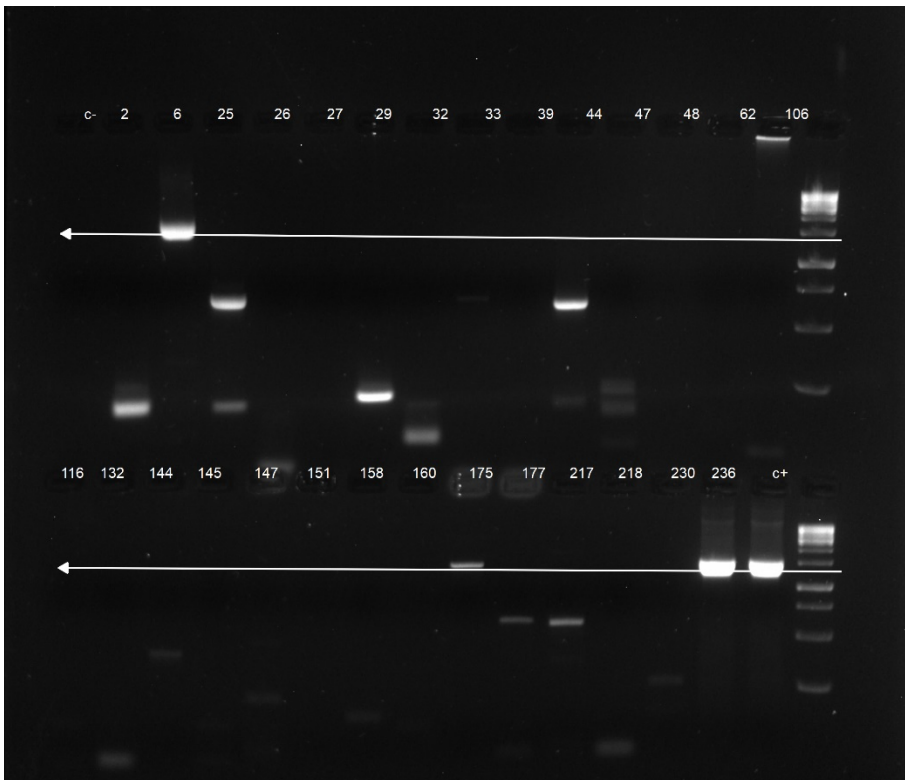

Figure 5. Electrophoretic gel depicting negative control, tested samples, and positive control (i.e, *Babesia bovis*). The arrowed lines stand for the molecular weight of the positive control.

3.3 Purifying and Sequencing

For the purification, 2 µl of ExoSAP (ExoSAP-IT PCR Product Cleanup Reagent, Thermo Fisher Scientific Inc., Waltham, MA, USA) were added to 5 µl of amplified

products. Following manufacturer instructions, an amplification cycle was run to activate the cleanup reagents. The cycles were set as follow: 35°C for 5 minutes and 80°C for 10 minutes. Then, 5 µl of purified mix was added to 5 µl of reverse primer (5µm concentration) and sent for sequencing. Purified products were sequenced following Sanger technology (Macrogen Spain, Madrid, ES) and the obtained nucleotide sequences were compared to those deposited in GenBank® using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast) (accessed date: 30 November 2023).

3.4 Data analysis

All collected data were entered into a Microsoft Excel (Microsoft Corporation, Albuquerque, USA) spreadsheet, edited and coded. Prevalence values and their 95% confidence intervals (95% CI) were calculated through EpiTools (www.epitools.ausvet.com.au) using the Wilson method for each identified pathogen.

4. Results

4.1 Tick species identification

A total of 2,311 ticks were collected and, among them, five tick species were identified: *Amblyomma variegatum* (n=1227, 53.1%), *Rhipicephalus microplus* (n=987, 42.7%), *Hyalomma rufipes* (n=45, 1.9%), *Hyalomma truncatum* (n=37, 1.6%), and *Rhipicephalus sanguineus* (n=15, 0.6%). Among these, *R. microplus* and *A. variegatum* were the most prevalent species across all sites, with *R. microplus* showing a particularly high abundance in Ouidah, in Southern Benin. The total number of ticks collected and analyzed as well as the relative percentage of the five tick species, data on each tick species at the five collection sites are presented in Table 2.

Table 2. Tick species identification results

	Sites						
Tick species	Banikoara N (n) [1]	Tanguieta N (n) [1]	Zakpota N (n) [1]	Kpobè N (n) [1]	Ouidah N (n) [1]	**Total N (n)** [1]	Relative percentage
Rhipicephalus microplus	216 (16)	193 (15)	143 (11)	83 (6)	352 (27)	**987 (75)**	42.7%
Amblyomma variegatum	353 (22)	137 (7)	225 (14)	316 (19)	196 (12)	**1227 (74)**	53.1%
*Hyalomma truncatum**	3	21	13	0	0	**37**	1.6%
*Hyalomma rufipes**	22	6	6	11	0	**45**	1.9%
*Rhipicephalus sanguineus**	0	1	14	0	0	**15**	0.6%

[1] N=Number collected; n=number analysed
* the number of analysed ticks is not reported since it corresponds to the collected ones

In Banikoara, a site in Northern Benin, *Amblyomma variegatum* and *Hyalomma rufipes* were equally dominant, while *Rhipicephalus microplus* was present in moderate numbers. Tanguieta, in the Northern as well, showed a high prevalence of *Hyalomma*

truncatum, alongside a significant presence of *Rhipicephalus microplus*. In contrast, the Southern regions, specifically Pobè and Ouidah, exhibited a higher concentration of *Rhipicephalus microplus* and *Amblyomma variegatum*, with *Hyalomma* species being absent or found in lower numbers.

4.2 TBPs identification in cattle blood samples

Totally, 81 cattle blood samples from five distinct regions in Benin were collected, but the laboratory analysis was successfully completed only for 69, mainly due to limited amount and poor quality of extracted material. The analysis revealed a significant presence of tick-borne pathogens, predominantly from the *Theileria* genus. The most prevalent pathogen identified was *Theileria velifera*, detected in 31 samples, representing 44.9% of the total (95% CI: 33.8-56.6). This species was particularly dominant in Tanguieta, where it was found in 10 out of 11 samples, and Banikoara, where 7 out of 10 samples testing positive. Additionally, *Theileria mutans* was identified in 6 samples (8.7%; 95% CI: 4.0-17.7), with its highest occurrence in Ouidah (4 out of 13 positive samples). Interestingly, a small proportion of samples tested positive for *Theileria* spp. (n=7; 10.1%; 95% CI: 5.0-19.5), indicating the presence of unidentified or less common *Theileria* species. *Babesia* spp., although less prevalent, was detected in 2 samples (2.9%; 95% CI: 0.8-10.0), specifically in Banikoara and Kpobè regions, where *Rhipicephalus* ticks, the primary vectors for *Babesia* pathogens, are known to be present. TBPs detected in blood samples from each site, as well as the detected numbers and positive ratios are reported in Table 3.

Table 3. TBP identification in cattle blood samples

Site	Total collected	Tested	*Theileria velifera* **N pos (%)**	*Theileria mutans* **N pos (%)**	*Theileria* spp. **N pos (%)**	*Babesia* spp. **N pos (%)**	**Total N pos (%)**
Banikoara	21	18	7 (38.9%)	0 (0%)	2 (11.1%)	1 (5.6%)	10 (55.6%)
Tanguieta	20	18	10 (55.6%)	0 (0%)	1 (5.6%)	0 (0%)	11 (61.1%)
Zakpota	5	4	0 (0%)	1 (25%)	1 (25%)	0 (0%)	2 (50%)
Kpobè	19	15	8 (53.3%)	1 (6.7%)	0 (0%)	1 (6.7%)	10 (66.7%)
Ouidah	16	14	6 (42.9%)	4 (28.6%)	3 (21.4%)	0 (0%)	13 (92.9%)
Overall	**81**	**69**	**31 (44.9%)**	**6 (8.6%)**	**7 (10.1%)**	**2 (2.9%)**	**46 (66.7%)**

4.3 TBPs identification in ticks

The molecular analysis of ticks collected from various regions in Benin revealed the presence of tick-borne pathogens, specifically *T. velifera* and *B. bigemina*. *T. velifera* was detected in a single specimen of *R. microplus*, representing an infection rate of 1.3% (95% CI: 0.2-7.2) in this tick species. Meanwhile, *B. bigemina* was identified in two ticks, *R. microplus* and *H. rufipes*, with an infection rate of 1.3% (95% CI: 0.2-7.2) and 2.2% (95% CI: 0.4-11.6), respectively.

5. Discussion and conclusions

The findings of our study highlighted the geographic variation in tick species distribution within Benin, which is crucial for understanding the epidemiology of tick-borne diseases in cattle. The predominance of *Rhipicephalus microplus* in Ouidah , the southernmost site investigated suggested a potential risk for the spread of diseases such as babesiosis, whose etiological agents are commonly transmitted by this tick species, in the area. Similarly, the significant presence of *Amblyomma variegatum* across all sites indicated the potential for widespread transmission of diseases like cowdriosis. The absence or low prevalence of *Rhipicephalus sanguineus* in most sites suggested limited involvement of this species in disease transmission in cattle in the surveyed areas. These data are pivotal for developing targeted control strategies to manage tick populations and mitigate the risk of tick-borne diseases in Benin.

Regionally, the distribution of pathogens varied, for instance Banikoara and Tanguieta showing higher prevalence of *Theileria velifera*, while Ouidah exhibited a more diverse presence of both *Theileria* and *Babesia* species. Zakpota had the lowest overall pathogen detection, with only two positive cases, suggesting a potentially lower tick burden or less exposure to infected vectors in this area. These findings highlighted the complex epidemiology of tick-borne diseases in Benin, emphasizing the need for region-specific control strategies and continued monitoring to mitigate the impact of these pathogens on cattle health and productivity.

Safiou et al., (2016) detected haemoparasites in cattles in Benin, showing a prevalence of 5.6% for *B. bovis*, 13.9% for *B. bigemina* and 22.2% for *Babesia* spp. Similarly, Heylen et al., (2023) observed *B. bigemina* and *B. bovis* in the cattle from Benin, with a positive rate of 20.7% and 9.1%, respectively. The low prevalence of *B. bigemina* detected in the blood samples of our investigation and the absence of *B. bovis*, compared to the expectations based on literature mentioned above, raises important questions about the factors that might have contributed to this discrepancy. One possible factor is co-infection, where cattle infected with multiple pathogens might show a competitive interaction between them, potentially affecting the detection rates of individual pathogens. Co-infections can complicate the clinical presentation and may lead to an underestimation of certain pathogens if they are outcompeted by more dominant ones (Van Wyk et al., 2014). This could result in lower detection rates of *Babesia* species, particularly when other haemoparasites are also present. Further research is needed to

explore the dynamics of co-infections and their impact on the prevalence of *Babesia* species in cattle populations. Another potential explanation could be related to the molecular diagnostic methods used in the study (Garcia et al., 2022). Molecular techniques, such as PCR, are highly sensitive and specific when properly optimized. However, several factors can affect their performance: 1) Sample quality and DNA extraction: The quality of the blood samples, including how they were collected, stored, and processed, can also impact the sensitivity of molecular assays (Raymaekers et al., 2009). Degraded DNA, or an insufficient amounts of pathogen DNA due to a low parasitemia, can result in undetectable levels of some pathogens, such as *Babesia* species. 2) Primers design and specificity: The accuracy of PCR-based detection depends heavily on the primers used to code for a specific region of a gene (Ma et al., 2021). In this study, we employed generic primers designed to detect *Babesia* species, which, while useful for broad screening purposes, may also introduce certain limitations. Generic primers are less specific to the genetic sequences of individual species, such as *Babesia bigemina*. This lack of specificity of the genetic sequences of *Babesia bigemina* can lead to reduced amplification efficiency and false negatives results. 3. PCR inhibitors: Blood contains various substances that can inhibit PCR reactions, such as hemoglobin or anticoagulants. If these inhibitors are not adequately removed during DNA extraction, they can reduce the effectiveness of the PCR assays (Raymaekers et al., 2009). 4. Regional genetic variability: There might be genetic differences in *B. bigemina* or *B. bovis* strains across different regions. If the local strains have variations in the gene regions targeted by the primers, this could reduce the assay's ability to detect the pathogen (Koonyosying et al., 2022). Considering these factors, it is possible that the low detection rate of *Babesia* species in the blood samples could be partially attributed to the limitations or issues associated with the molecular diagnostic method used. Further investigation, including the optimization of PCR protocols, re-evaluation of primers sets, and the use of complementary diagnostic methods, could clarify whether the observed low prevalence is a true reflection of the epidemiological situation or a result of methodological limitations.

The occasional finding of *T. velifera* and *B. bigemina* in ticks could suggest several possibilities. Firstly, it may indicate that these pathogens are not highly endemic in the study area, or that the transmission cycle is not fully understood in these areas. This could be due to various ecological factors, such as the density and distribution of the tick populations, climatic conditions that affect ticks survival and reproduction

(Koonyosying et al., 2022; Yessinou et al., 2023).

Additionally, the detection of *Babesia bigemina* in both *R. microplus* and *H. rufipes* is particularly noteworthy, as it suggests that multiple tick species may be involved in the transmission of this pathogen (Diarra et al., 2023), potentially increasing its spread among cattle populations. This finding underscores the complexity of controlling tick-borne diseases, as targeting a single tick species may not be sufficient to avoid the transmission of *Babesia bigemina*. The involvement of different tick species in pathogen transmission also raises concerns about the potential for wider dissemination of the disease, especially in regions where both tick species coexist.

The detection of *Theileria velifera* in *Rhipicephalus microplus* and *Babesia bigemina* in *Hyalomma rufipes* is an interesting finding. In the case of *R. microplus*, it is well-documented as a competent vector for *B. bigemina*, but its role in transmitting *T. velifera* is less clear. Similarly, while *H. rufipes* has been associated with a variety of pathogens, its competence as a vector for *B. bigemina* has not been conclusively established in the literature (Heylen et al., 2023; Kasaija et al., 2021). While the presence of these pathogens in these tick species has been confirmed by molecular analysis, this does not necessarily indicate that *R. microplus* is a competent vector for *T. velifera* or that *H. rufipes* is a competent vector for *B. bigemina*. Vector competence is determined by the ability of a tick species to acquire, maintain, and efficiently transmit a pathogen to a new host, a process often involving pathogen replication, typically sexual, within the vector (De La Fuente et al., 2017). In our study, however, we did not dissect the ticks, leaving the question of whether these pathogens undergo replication within these specific tick species unanswered. The mere presence of a pathogen's DNA in a tick does not confirm that the tick is a competent vector (De La Fuente et al., 2017); it only indicates that the pathogen was present in the tick at the time of testing. This presence could result from several factors, including incidental ingestion of the pathogen during a blood meal from an infected host, rather than an active infection within the tick. These findings highlight the importance of further studies to confirm vector competence. Such studies would need to demonstrate not just the presence of the pathogen in the tick but also its ability to multiply and be transmitted to a new host under natural conditions.

Tick species transmitting important pathogens, such as *R. microplus* and *A. variegatum*, were widely found in Benin (Heylen et al., 2023), whereas TBDs are reported in some areas of the country (Yessinou et al., 2023), but knowledge on their distribution is still

insufficient. This study investigated TBDs in cattle and associated ticks to update the epidemiological data in Benin.

References

Adinci, K. J., Akpo, Y., Adoligbe, C., Adehan, S. B., Yessinou, R. E., Sode, A. I., Mensah, G. A., Youssao, A. K. I., Sinsin, B., & Farougou, S. (2018). Preliminary study on the tick population of Benin wildlife at the moment of its invasion by the Rhipicephalus microplus tick (Canestrini, 1888). *Veterinary World*, *11*(6), 845–851. https://doi.org/10.14202/vetworld.2018.845-851

Bajer, A., Beck, A., Beck, R., Behnke, J. M., Dwużnik-Szarek, D., Eichenberger, R. M., Farkas, R., Fuehrer, H.-P., Heddergott, M., Jokelainen, P., Leschnik, M., Oborina, V., Paulauskas, A., Radzijevskaja, J., Ranka, R., Schnyder, M., Springer, A., Strube, C., Tolkacz, K., & Walochnik, J. (2022). Babesiosis in Southeastern, Central and Northeastern Europe: An Emerging and Re-Emerging Tick-Borne Disease of Humans and Animals. *Microorganisms*, *10*(5), 945. https://doi.org/10.3390/microorganisms10050945

Beugnet, F., & Moreau, Y. (2015). Babesiosis: -EN- -FR- La babésiose -ES- Babesiosis. *Revue Scientifique et Technique de l'OIE*, *34*(2), 627–639. https://doi.org/10.20506/rst.34.2.2385

Blaschitz, M., Narodoslavsky-Gföller, M., Kanzler, M., Stanek, G., & Walochnik, J. (2008). *Babesia* Species Occurring in Austrian *Ixodes ricinus* Ticks. *Applied and Environmental Microbiology*, *74*(15), 4841–4846. https://doi.org/10.1128/AEM.00035-08

De Clercq, E. M., Vanwambeke, S. O., Sungirai, M., Adehan, S., Lokossou, R., & Madder, M. (2012). Geographic distribution of the invasive cattle tick Rhipicephalus microplus, a country-wide survey in Benin. *Experimental and Applied Acarology*, *58*(4), 441–452. https://doi.org/10.1007/s10493-012-9587-0

De La Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A. G., Estrada-Peña, A., Johnson, N., Kocan, K. M., Mansfield, K. L., Nijhof, A. M., Papa, A., Rudenko, N., Villar, M., Alberdi, P., Torina, A., Ayllón, N., Vancova, M., Golovchenko, M., Grubhoffer, L., … Rego, R. O. M. (2017). Tick-Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for Tick-Borne Diseases. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00114

Diarra, A. Z., Kelly, P., Davoust, B., & Parola, P. (2023). Tick-Borne Diseases of Humans and Animals in West Africa. *Pathogens*, *12*(11), 1276. https://doi.org/10.3390/pathogens12111276

Garcia, K., Weakley, M., Do, T., & Mir, S. (2022). Current and Future Molecular Diagnostics of Tick-Borne Diseases in Cattle. *Veterinary Sciences*, *9*(5), 241. https://doi.org/10.3390/vetsci9050241

Heylen, D. J. A., Kumsa, B., Kimbita, E., Frank, M. N., Muhanguzi, D., Jongejan, F., Adehan, S. B., Toure, A., Aboagye-Antwi, F., Ogo, N. I., Juleff, N., Crafford, D., Fourie, J., Labuchange, M., & Madder, M. (2023). Tick-borne pathogens and body condition of cattle in smallholder rural livestock production systems in East and West Africa. *Parasites & Vectors*, *16*(1), 117. https://doi.org/10.1186/s13071-023-05709-0

Heylen, D. J. A., Kumsa, B., Kimbita, E., Frank, M. N., Muhanguzi, D., Jongejan, F., Adehan, S. B., Toure, A., Aboagye-Antwi, F., Ogo, N. I., Juleff, N., Fourie, J., Evans, A., Byaruhanga, J., & Madder, M. (2023). Tick communities of cattle in smallholder rural livestock production systems in sub-Saharan Africa. *Parasites & Vectors*, *16*(1), 206. https://doi.org/10.1186/s13071-023-05801-5

Kasaija, P. D., Estrada-Peña, A., Contreras, M., Kirunda, H., & De La Fuente, J. (2021). Cattle ticks and tick-borne diseases: A review of Uganda's situation. *Ticks and Tick-Borne Diseases*, *12*(5), 101756. https://doi.org/10.1016/j.ttbdis.2021.101756

Koonyosying, P., Rittipornlertrak, A., Chomjit, P., Sangkakam, K., Muenthaisong, A., Nambooppha, B., Srisawat, W., Apinda, N., Singhla, T., & Sthitmatee, N. (2022). Incidence of hemoparasitic infections in cattle from central and northern Thailand. *PeerJ*, *10*, e13835. https://doi.org/10.7717/peerj.13835

Kumar, B., Maharana, B. R., Thakre, B., Brahmbhatt, N. N., & Joseph, J. P. (2022). 18S rRNA Gene-Based Piroplasmid PCR: An Assay for Rapid and Precise Molecular Screening of Theileria and Babesia Species in Animals. *Acta Parasitologica*, *67*(4), 1697–1707. https://doi.org/10.1007/s11686-022-00625-2

Ma, H., Bell, K. N., & Loker, R. N. (2021). qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies. *Molecular Therapy - Methods & Clinical Development*, *20*, 152–168. https://doi.org/10.1016/j.omtm.2020.11.007

Madder, M., Adehan, S., De Deken, R., Adehan, R., & Lokossou, R. (2012). New foci of Rhipicephalus microplus in West Africa. *Experimental and Applied Acarology*, *56*(4), 385–390. https://doi.org/10.1007/s10493-012-9522-4

Øines, Ø., Radzijevskaja, J., Paulauskas, A., & Rosef, O. (2012). Prevalence and diversity of Babesia spp. In questing Ixodes ricinus ticks from Norway. *Parasites & Vectors*, *5*(1), 156. https://doi.org/10.1186/1756-3305-5-156

Ouedraogo, A. S., Zannou, O. M., Biguezoton, A. S., Kouassi, P. Y., Belem, A., Farougou, S., Oosthuizen, M., Saegerman, C., & Lempereur, L. (2021). Cattle ticks and associated tick-borne pathogens in Burkina Faso and Benin: Apparent northern spread of Rhipicephalus microplus in Benin and first evidence of Theileria velifera and Theileria annulata. *Ticks and Tick-Borne Diseases*, *12*(4), 101733. https://doi.org/10.1016/j.ttbdis.2021.101733

Qurollo, B. A., Archer, N. R., Schreeg, M. E., Marr, H. S., Birkenheuer, A. J., Haney, K. N., Thomas, B. S., & Breitschwerdt, E. B. (2017). Improved molecular detection of Babesia infections in animals using a novel quantitative real-time PCR diagnostic assay targeting mitochondrial DNA. *Parasites & Vectors*, *10*(1), 128. https://doi.org/10.1186/s13071-017-2064-1

Raymaekers, M., Smets, R., Maes, B., & Cartuyvels, R. (2009). Checklist for optimization and validation of real-time PCR assays. *Journal of Clinical Laboratory Analysis*, *23*(3), 145–151. https://doi.org/10.1002/jcla.20307

Safiou, B. A., Abel, B., Aquilas, D., Marc, N. A., Razacki, A., Hassane, A., Guy, A. M.,

Soua Iuml Bou, F., & Maxime, M. (2016). Blood survey of Babesia spp and Theileria spp in Monos cattle, Benin. *African Journal of Agricultural Research*, *11*(14), 1266–1272. https://doi.org/10.5897/AJAR2015.10055

Schouls, L. M., Van De Pol, I., Rijpkema, S. G. T., & Schot, C. S. (1999). Detection and Identification of *Ehrlichia* , *Borrelia burgdorferi* Sensu Lato, and *Bartonella* Species in Dutch *Ixodes ricinus* Ticks. *Journal of Clinical Microbiology*, *37*(7), 2215–2222. https://doi.org/10.1128/JCM.37.7.2215-2222.1999

Sibeko, K. P., Oosthuizen, M. C., Collins, N. E., Geysen, D., Rambritch, N. E., Latif, A. A., Groeneveld, H. T., Potgieter, F. T., & Coetzer, J. A. W. (2008). Development and evaluation of a real-time polymerase chain reaction test for the detection of Theileria parva infections in Cape buffalo (Syncerus caffer) and cattle. *Veterinary Parasitology*, *155*(1–2), 37–48. https://doi.org/10.1016/j.vetpar.2008.03.033

Tian, Z., Liu, G., Yin, H., Xie, J., Wang, S., Yuan, X., Wang, F., & Luo, J. (2014). First report on the occurrence of Theileria sp. OT3 in China. *Parasitology International*, *63*(2), 403–407. https://doi.org/10.1016/j.parint.2013.12.014

Van Wyk, I. C., Goddard, A., De C. Bronsvoort, B. M., Coetzer, J. A. W., Handel, I. G., Hanotte, O., Jennings, A., Lesosky, M., Kiara, H., Thumbi, S. M., Toye, P., Woolhouse, M. W., & Penzhorn, B. L. (2014). The impact of co-infections on the haematological profile of East African Short-horn Zebu calves. *Parasitology*, *141*(3), 374–388. https://doi.org/10.1017/S0031182013001625

Yabsley, M. J., & Shock, B. C. (2013). Natural history of Zoonotic Babesia: Role of wildlife reservoirs. *International Journal for Parasitology: Parasites and Wildlife*, *2*, 18–31. https://doi.org/10.1016/j.ijppaw.2012.11.003

Yessinou, R. E., Adoligbe, C., Akpo, Y., Adinci, J., Youssao Abdou Karim, I., & Farougou, S. (2018). Sensitivity of Different Cattle Breeds to the Infestation of Cattle Ticks *Amblyomma variegatum, Rhipicephalus microplus* , and *Hyalomma* spp. On the Natural Pastures of Opkara Farm, Benin. *Journal of Parasitology Research*, *2018*, 1–9. https://doi.org/10.1155/2018/2570940

Yessinou, R. E., Adoligbe, C., Atchade, F., Adinci, J., Brahi, H. D., Adehan, S., Mantip, S. E., & Farougou, S. (2021). Phenotypic and genotypic characterization of dog tick Rhipicephalus sanguineus sensu lato (Acari: Ixodidae) resistance to deltamethrin in Benin. *Veterinary Parasitology: Regional Studies and Reports*, *26*, 100638. https://doi.org/10.1016/j.vprsr.2021.100638

Yessinou, R. E., Cazan, C. D., Panait, L. C., Mollong, E., Biguezoton, A. S., Bonnet, S. I., Farougou, S., Groschup, M. H., & Mihalca, A. D. (2023). New geographical records for tick-borne pathogens in ticks collected from cattle in Benin and Togo. *Veterinary Medicine and Science*, *9*(1), 345–352. https://doi.org/10.1002/vms3.1022