Comparative Analysis of Tick-Borne Relapsing Fever Spirochaetes from Ethiopia and Nigeria

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DEDICATION

Dedicated to God Almighty, and to my Family, Friends, Supervisors, and everyone that supported me throughout this journey. I appreciate every one of you.

DECLARATION

This work has not been submitted elsewhere for any other degree nor is it being currently submitted for any other degree.

Signed… 

Name……Adefolake A. Bankole

Date…6 January 2023

Statement

This thesis is as a result of my investigations, except where otherwise stated. All supporting citations are appropriately listed in the bibliography.

Signed…… 

Name……Adefolake A. Bankole

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ABSTRACT

In recent decades, the frequency of tick-borne relapsing fever (TBRF) borreliae and distribution of its tick vectors has been increasing in Africa. Soft ticks of the *Ornithodoros* genus are the predominant vector for these spirochaetes and are endemic in many regions in Africa including East and West Africa. While TBRF of veterinary importance are exclusively transmitted by hard ticks. Despite this, TBRF is still a neglected disease in many regions such as Nigeria and Ethiopia where the disease epidemiology is still largely unknown. In Nigeria, *O. savignyi* ticks infected with a human TBRF specie *Candidatus* B. kalaharica has been reported. While in Ethiopia, the agent of relapsing fever is the louse-borne relapsing fever (LBRF) that is endemic in some parts of the country, with no evidence of TBRF available.

To demonstrate the presence of TBRF of clinical and veterinary importance in Nigeria, 550 livestock, 152 patients presenting with recurrent fever, 550 livestock sera, 71 dog sera, and 251 soft ticks of the genus's *Ornithodoros* and *Argas* were sampled. Conversely, in Ethiopia, to demonstrate the possible coexisting of TBRF with LBRF, 312 *Ornithodoros* ticks were sampled from soil around cattle shelter. Samples were screened for *Borrelia* infection on RT-PCR, with reactive samples subjected to further confirmation on conventional PCR using the 16S rRNA flagellin B genes, and the 16S-23S intragenic spacer region of *Borrelia* genome. While ticks were identified to the molecular level using tick 16S rRNA and tick mtrrs genes. *Borrelia* infection rate in livestock from Nigeria after initial screening was 3.8% (21/550), and 14% (3/21) after final confirmation. While the prevalence of infection in ticks from Ethiopia was 3.5% (11/312), and 36% (4/11) upon final confirmation. No infection was reported in the patients recruited.

Sequencing analysis revealed the borreliae from Nigerian livestock as *B. theileri* 'a bovine borreliosis" specie. Whereas the borreliae from the Ethiopian ticks was identified *C*. B.

kalaharica, a human species. Phylogenetic analysis revealed the identity of the *Ornithodoros* ticks as *O. savignyi* with >98% similarity for the Nigerian ticks, while the Ethiopian species had a 94% similarity. Conspicuously, the Ethiopian ticks formed a sister clade from the Nigerian ticks and other *O. savignyi* species available in the GenBank. This raises the question of whether the Ethiopian ticks represent a distinct species. The *Argas* ticks were identified as *A. persicus* using the 16S rRNA gene sequences.

This is the first documented evidence of a *C*. B. kalaharica and its *O. savignyi* vector in Ethiopia. This is significant for both diagnostic and public health, as the possible coexisting of both RFs may likely increase the burden of the diseases. The gold standard for RF diagnosis in the country is microscopy, which is unable to differentiate between LBRF and TBRF. Concurrently, this is also the first report of TBRF in livestock from Nigeria.

This study has clearly revealed the possible coexisting of LBRF and TBRF in Ethiopia. With this evidence, additional research into the vector distribution and disease epidemiology is vital to better understand its true burden in the country. This applies for Nigeria as there is a need for additional research to determine the true clinical and veterinary significance of the disease, especially in high-risk areas where these ticks are known to be endemic.

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CHAPTER 1: LITERATURE REVIEW

1.1 General Introduction

Ticks in general are obligate parasites that are important vectors for zoonotic pathogens such as bacteria, viruses, and protozoans that causes mild to life threatening diseases in humans and animals globally. They are only second to mosquitoes as vectors of human pathogenic agents (Brites-Neto et al., 2015), and are considered the most economic important ectoparasite of animals in sub-Sahara Africa (Lorusso et al., 2013). These ticks are either of the Ixodidae group that contains the hard-body ticks of the genus's such as *Amblyomma*, *Rhipicephalus, Hyalomma* or the Argasidae group that consists of soft ticks from the genus's *Ornithodoros* and *Argas*. Asides transmitting pathogens and causing painful swelling in humans after bites, these ticks also damage hides and skins of livestock, introduction of toxins (Marufu et al., 2008), and paralyses or in extreme cases cause death in poultry due to anaemia (Lodos et al., 2000).

Tick-borne relapsing fever (TBRF) is mostly a zoonosis caused by the bacteria of the genus *Borrelia* that are majorly vectored by soft *Ornithodoros* ticks. The one exception is *Borrelia miyamotoi* that is transmitted by hard ticks of the genus *Ixodes* (Fukunaga et al., 1995; Kahlig et al., 2021). All relapsing fever spirochaetes are vector transmitted without causing any significant harm to these vectors or even providing them with benefits, with soft ticks being the predominant vectors of relapsing fever (Cutler, 2010). Human RF is either the endemic tick-borne (TBRF), or the epidemic louse borne (LBRF) *B. recurrentis* (Vial et al., 2006). Most of these spirochaetes are transmitted within the tick transstadial, however, a few species such as B. *anserina* can undergo transovarial transmission than ensures their longevity (Lisboa et al., 2009).

TBRF species are divided into two groups based on phylogenetic information: old world group (*B. recurrentis*, *B. duttonii*, *B. crocidurae*, *B. hispanica*), and the new world group (*B.*

hermsii, *B. coriaceae*, *B. parkeri*, *B. turicatae*, *B. miyamotoi*, B. anserina, *B. lonestari*). In addition, *B. theileri* and *C*. B. kalaharica both of which were generated from this study, fall under the new world group (Fig. 1.1).

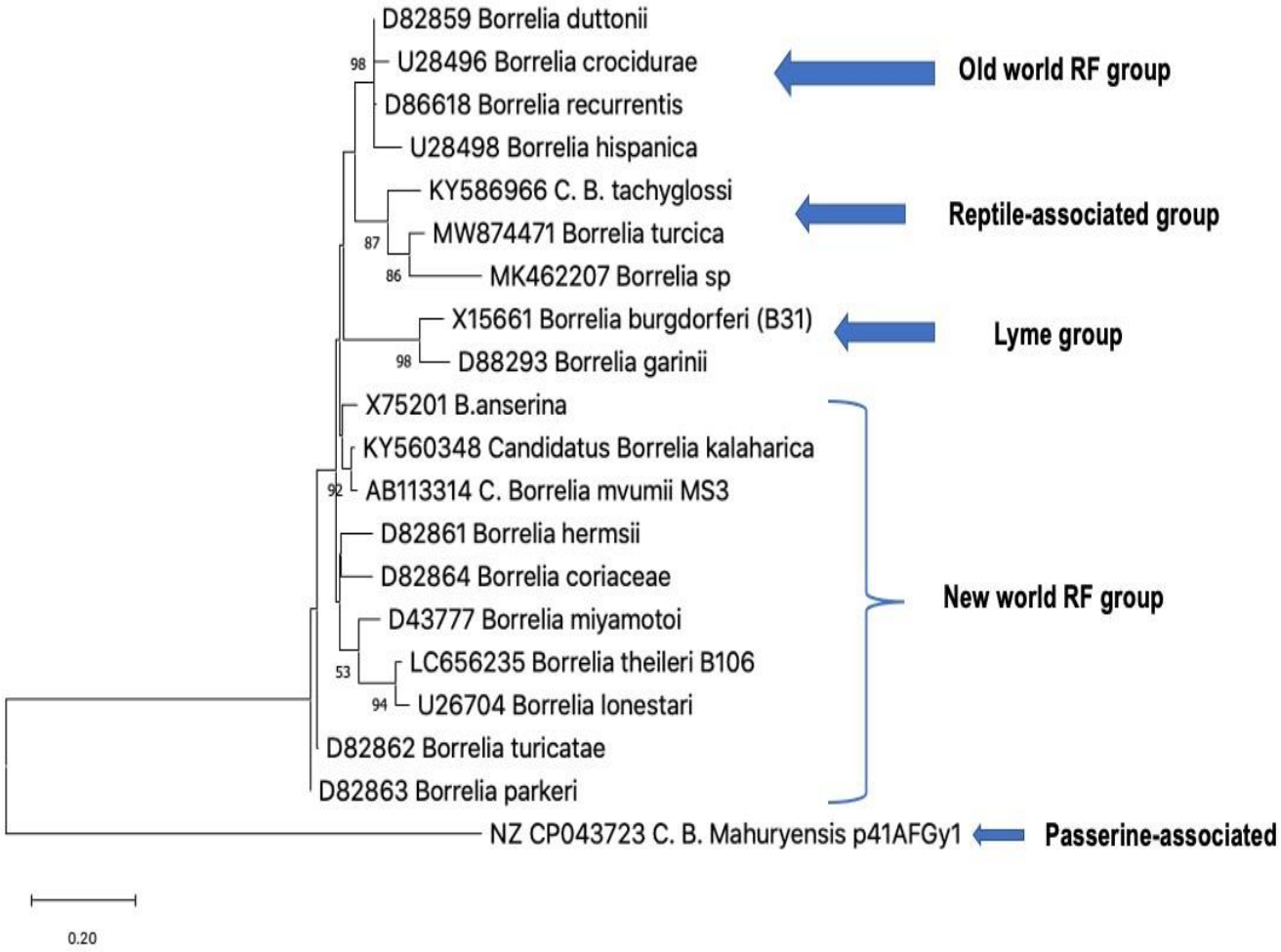


Figure 1.1: Phylogenetic classification of *Borrelia* species of the flagellin partial sequences and constructed using the maximum likelihood method and Tamura-3 Model on MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes (Tamura et al., 2021).

Over the years, the literature on the population and distribution of soft ticks, and TBRF in Africa has been increasing of which majority of the predominant spirochaetes documented in the continent are from the old-world group (Parola et al., 2011)

In West Africa, the predominant spirochaete circulating is *B. crocidurae* vectored by *Ornithodoros sonrai*, however, the exact distribution and incidence of TBRF remains unclear even with the presence of the tick vectors in the region (Trape et al., 2013). The disease is the second highest cause of febrile illnesses only after malaria in Senegal (Hadji et al., 2021; Parola et al., 2011).

In Nigeria, there have been few historical documentations of borreliae infection in poultry such as chicken and Muscovy ducks (Barnes, 1975; Van-Geldorp, 1975), experimental infection in cattle (Trees, 1978), and in ruminants from this study that will be further discussed in (chapter 2). More recently, the confirmation of the presence of spirochaetes pathogenic to human *Candidatus* Borrelia kalaharica was reported in its *Ornithodoros savignyi* tick vector (Cutler et al., 2018), albeit no evidence currently exists for its clinical relevance in the country.

In East Africa, the endemic TBRF spirochaete is *B. duttonii* that is exclusively vectored by *O. moubata* (Bryceson. et al., 1969; Scott et al., 2005). The incidence of TBRF in some regions in Africa such as areas within Tanzania can reach 384/1000 among children <1 year, and perinatal death of up to 436/1000 births over a 5-year period (Jongen. et al., 1997). In Ethiopia, and in neighbouring Sudan and Somaliland, LBRF still ravages many parts, especially in the highlands, and in younger men in the rural areas (Cutler, 2010; Warrell, 2019). Since 2015, there has been increasing awareness of LBRF in Europe (~100 cases) among migrants from the above countries (Goldenberger et al., 2015; Isenring et al., 2018). Historical evidence from the region showed the mortality rate was between 10-70% in untreated and ~5% in treated patients (Bryceson. et al., 1969; Raoult & Roux, 1999) .

Small mammals, insectivores, livestock, domestic pets, and humans can serve as reservoirs or hosts for TBRF spirochaetes without causing harm or benefitting them (Diatta et al., 2015; Trape et al., 2013), even whilst maintaining the spirochaetes and remaining infectious for up to 30days (Burgdorfer & Mavros, 1970).

Whereas humans are believed to be the only known reservoir for the LBRF spirochaete (Kahlig et al., 2021; Talagrand-Reboul et al., 2018). However, some studies have been able to successfully develop a LBRF model that mimic infection in immunocompromised mice (Larsson et al., 2009).

Human exposure to soft tick bites is usually nocturnal. Rodent burrows within households are a risk factor and can be common in traditional mud-built dwellings. Furthermore, proximity of livestock can contribute towards increased risks for tick exposure (Parola & Raoult, 2001). In many cases the ticks stay hidden in the burrows or crevices of their animal host and feed indiscriminately on them, albeit can also travel to the outside of mud huts to feed on children sleeping outside. This is opposite for hard ticks that show questing behaviour and can be found on leaves or blades of grass waiting to attach to suitable preys such as livestock and domestic animals (Bouattour et al., 2010).

Ornithodoros ticks are predominantly rapid feeders within 15 to 90 minutes depending on the life stages, except for a few species like *O. coriaceus*, *O. kelleyi* that may require extended feeding period to complete their blood which could last for a few days (Cutler, 2010). The ability of soft ticks to survive for up to 7 years without another meal and remaining infectious for up to 5 years after initial acquisition of the spirochaetes (Francis, 1942) makes them a better suitable reservoir for *Borrelia* compared to mammals. Upon attachment, some of these ticks depending on their life stage can transmit the spirochaete in as little as 1 minute after attachment due to the persistent infection of their salivary glands for ~144 days after initial spirochaetes acquisition (Schwan & Hinnebusch, 1998). For instance, *O. moubata* nymphs transmit the pathogens via the salivary gland, while the adults primarily transmit infection through the coxial fluid (Burgdorfer & Mavros, 1970). In addition, the spirochaetes like *B. duttonii* can be found in the tick midgut, coxial fluid, central ganglion, malphigian tubules, ovaries, and coxial organs of species such as *O. moubata* (Schwan & Piesman, 2002).

TBRF is transmitted in humans by infected soft tick via their saliva, coxial fluid, or by consuming infected animal products(Schwan & Piesman, 2002; Telmadarraiy et al., 2015). Host range for these ticks depends upon the species, for example, species such as the bat tick *O. turcica* are specific with their preferred host for feeding, while *O. turcicata* are more generalised depending on the range of available host. Furthermore, the natural habitat for these ticks also varies as some require specific environmental conditions. For instance, *O. moubata* are predominantly found around human dwellings in East Africa (Cutler et al., 2010), *O. savignyi* are mostly distributed in the arid/semi-arid regions (Bakkes et al., 2018), *O. turcica* are endemic in soils under human dwellings, while *O. hermsii* are mostly found during the summer in rusty cabins and human shelters around high-altitude regions in the USA (Dworkin. et al., 2002).

Clinically, relapsing fever (RF) is characterized with several episodes of recurrent fever usually between 2 to 7 days, spaced with an asymptomatic period of up to 10 days (Dworkin et al., 2008; Lopez et al., 2016). This is often accompanied by a wide range of symptoms such as headache, nausea, vomiting, etc., and in severe cases of neurological complications, Jarisch-Herxheimer reaction (JHR), and sudden abortion in women can occur (Goutier et al., 2013). Historically, due to the overlapping clinical manifestations between TBRF and LBRF and microscopic method of diagnosis, it was impossible to differentiate between the two RF spirochaetes. Diagnosis was based on microscopic blood smear, geographical and epidemiological factors (endemics, outbreaks) for LBRF, and exposure to tick bites for TBRF (Cutler, 2010). In addition to this, TBRF was mostly obliterated by LBRF that was more notable and epidemiologically pertinent in many regions across the world (Cutler et al., 2009; Jakab et al., 2022; Nordmann et al., 2018; Warrell, 2019). However, as the decades go by and hygiene improved, LBRF significantly reduced and is now restricted to few regions within countries in Africa particularly Ethiopia (Cutler et al., 2010). Concurrently, TBRF continued to receive more attention due to improved diagnostic capabilities via the birth of molecular techniques such as PCRs and genome sequencing

that can differentiate between the two RFs and identify them to the species level (Scott et al., 2005).

In animals, the predominant vector of TBRF of veterinary and economic importance worldwide is *B. theileri* that is primarily vectored by some hard ticks from the *Rhipicephalus* (formerly *Boophilus*) genus (Trees, 1978), and *B. anserina* that is primarily transmitted by the soft tick *Argas persicus* (Sakharoff., 1891).

Borrelia. theileri, the specie of bovine relapsing fever has been reported in domestic and wild animals in many parts of Africa (Abanda et al., 2019; Abdullah. et al., 2021; Qiu et al., 2021). , South America (Morel et al., 2019; Paula et al., 2022), Asia (Kumagai et al., 2018), Australia (Callow, 1967), and Europe (Uilenberg, 1995; Uilenberg et al., 1988). Often presenting as a milder disease compared to other piroplasm's, symptoms include elevated temperature up to 39°C, minor depression, anaemia, anorexia for 1–2days, haemoglobinuria, and diarrhoea (Callow, 1967; Sharma et al., 2000). This study also describes the presence of *B. theileri* infection in small ruminants from Nigeria (chapter 2).

Argas persicus the vector of *B. anserina* is considered the most important ectoparasite of poultry (Pantaleoni et al., 2010). These ticks direct and indirectly cause havoc in birds through excessive blood mean that reduces egg production, as well as via the transmission of disease such as the *Borrelia* avian spirochaetosis that is a highly fatal particularly for chickens and domestic turkeys (Lisboa et al., 2009). Avian spirochaetosis has been reported in North America (Cooper & Bickford, 1993), Asia (Aslam et al., 2015), Africa (Barnes, 1975; Cutler et al., 2010; Leeflang & Ilemobade, 1977). However, majority of the cases today are now concentrated in the tropical and warm temperate regions such as Africa where tick infestation is high because of the traditional style of breeding which involves free range breeding (Pantaleoni et al., 2010). Free range bred birds are usually at higher risk of tick infestation as they are allowed to roam freely compared to the restricted or semi-restricted ones (Usman et al., 2012). The disease symptoms can be mild but, in many cases, can

present with severe symptoms such as paralysis of legs and wings, diarrhoea, anorexia, hyperthermia, and in some cases sudden death (Chauhan & Roy, 1996).

Ticks of veterinary burden frequently parasitize farm animals, thereby causing serious financial havoc especially in resource poor settings in Africa such as Nigeria and Ethiopia, where livestock and poultry industry makes up a huge part of the economy (Reye et al., 2012; Vesco et al., 2011). Farm animals are bred not only for their primary products such as meat and milk, but also for their by-products and hides such as cheese and cotton respectively (Rodino et al., 2020).

In Nigeria, there is little knowledge on TBRF in both clinical and veterinary settings. Given the recent description of C. B. kalaharica and its vector *O. savignyi*, TBRF in Nigeria clearly has a knowledge gap. Secondly, based on the clarity surrounding TBRF in Ethiopia, despite the country remaining endemic for LBRF, the co-existence of TBRF as not been categorically investigated in the region. The aim of this study was to determine the diversity of borreliae and its associated vectors in Ethiopia, and to compare this with the borreliae from Nigeria.

1.2 LITERATURE REVIEW

1.2.1 Definition of Zoonosis

Zoonotic pathogens such as those from the genus *Borrelia* are culpable in many emerging and re-emerging infections worldwide continues to threaten global health (Taylor et al., 2001). Between the 1940's and mid 2000's, zoonotic diseases accounted for (60.3%) of all diseases, of which (71.8%) originated from wildlife and (22.8%) from arthropod vectors (Jones et al., 2008). Zoonotic infections or diseases can be transmitted only from animals to humans (Karesh et al., 2012). Zoonosis are believed to be driven by the continuous increase of agriculture, particularly in poorer settings where animal shelters are in proximity to humans which puts them at higher risk of exposure to biting arthropods that transmits harbouring pathogens (Wolfe et al., 2007). TBRF has been described in every continent except for Antarctica (Jacob et al., 2022; Lopez et al., 2016) however, when looking at its pathogenicity in humans, there is no data for Australia as the only evidence is on bovine borreliosis in livestock (Callow, 1967).

Just like other pathogenic agents, borreliae transmission is vector dependent, some of which are exclusive to their vectors, i.e., *B. duttonii* and *O. moubata* (Cutler et al., 2010); *B. crocidurae* and *O. sonrai* (Trape et al., 2013), or can be transmitted by more than one tick such as *B. theileri* in *Rhipicephalus* and *Amblyomma* ticks (Cutler et al., 2012; Kumsa et al., 2015).

1.2.2 Historical Context of Tick-borne Relapsing Fever (TBRF)

The clinical description of relapsing fever was first introduced in 1843 by David Craigie during a disease outbreak in Edinburgh, while Otto Obermeier discovered *B. recurrentis* the louse-borne relapsing fever (LBRF) spirochaete during an outbreak between 1867-1868 in Berlin, albeit the evidence wasn't published until 1873 (Bryceson. et al., 1969). Obermeier's evidence was further confirmed by Gregor Munch in 1874, who were the first to suggest that LBRF was transmitted by fleas, lice, and bugs (Burgdorfer, 2001). The role of body lice '*Pediculus humanus*' as the vector of the disease was confirmed by Sergent and Foley in 1910 (Sergent & Foley, 1910). The LBRF spirochaete was initially named *Spirocheta recurrentis*, then *Spirocheta Obermeier*, before being grouped into the *Borrelia* genus that was developed in 1907 by Swellengrebl and reconfirmed by Johnstone in the early 1940s (Johnstone, 1942).

Tick-borne relapsing fever was first identified in 1857 independently by both Dutton and Todd in Kenya, and Ross and Milne in Uganda, with these becoming known as the 'human tick disease' transmitted by *Ornithodoros moubata* ticks (Livingstone, 1861). It was Robert Koch's discovery of *B. duttonii* in infected monkeys as well as providing evidence of transovarial transmission in the *O. moubata* ticks (Koch, 1905) that led to a global scale investigation of soft ticks as a potential vector of TBRF.

In 1971, the Barbour-Stonner-Kelly (BSK-H) medium was developed and the first borreliae '*B. hermsii*' cultivation was conducted (Kelly, 1970). An optimised version of the artificial growth medium allowed for the continued serial passage of the spirochaetes (Barbour, 1984). (Burgdorfer et al., 1982) discovered the agent of Lyme disease *B. burgodorferi* in 77% (61/126) *Ixodes scapularis* (formerly *I. dammini*) ticks from an area in the US endemic for the disease. The spirochaetes were predominantly found in the midgut of the ticks, and none were observed in the salivary glands as is the case for soft ticks.

1.2.3 Classification of Relapsing fever Spirochaetes

Initially, the *Borrelia* genus was divided into two groups based on their known associated tick vectors. The spirochaetes were classified into the *Borrelia* RF group comprising of the Argasid ticks, and the *Borreliella* Lyme disease group consisting of Ixodid ticks (Adeolu & Gupta, 2014). However, due to the discovery of new borreliae in nontraditional tick vectors, this stirred up controversies about the division and warranted additional revision of the genus. One of such discoveries is the RF specie *B. miyomotoi* that was reported in Ixodes persulcatus ticks in Japan (Fukunaga et al., 1995). This borreliae is widely distributed and pathogenic to humans and has been reported in North America
(Kingry et al., 2018; Krause et al., 2013, 2014), Europe (Fonville et al., 2014; Jahfari et al., 2014; Platonov et al., 2011).

Other RF species such as the bovine strain *B. theileri* and its genetically related specie *B. lonestari* are both primarily transmitted by hard ticks of the *Rhipicephalus* and *Amblyomma* genus's (Gü-Ner et al., 2003; Kumsa et al., 2015; Trees, 1978).

Apart from the relapsing fever and the Lyme disease groups, there is also the reptile-associated *B. turcica*, *B. tachyglossi* group phylogenetically different from these two groups as they form a separate clade on the phylogenetic tree as evident in (Fig. 1.1). Species within this group was first reported in *Hyalomma aegyptium* ticks parasitising tortoise in Turkey (Gü-Ner et al., 2003; Güner et al., 2004) and has subsequently been reported elsewhere (Panetta et al., 2017; Takano et al., 2010). In addition, a novel *Borrelia* species reported in *Bothriocroton concolor* ticks (Fig. 1.2.3) (Loh et al., 2016) aligned separately from other groups using the 16S sequences, while *C*. B. mahuryensis reported in neotropical passerine birds (Binetruy et al., 2020) also aligned separately using the flagellin sequences aligned separately on the phylogenetic tree as shown in (Fig. 1.1). This suggests a possibly forth clade within the Borreliaceae family, and hence shows how diverse and complicated the *Borrelia* genus is.

To resolve some of the discrepancies within the genus, (Margos et al., 2020) proposed a revised classification into: (i) Ixodid-associated *Borrelia* (ii) Matestraite-associated *Borrelia* (hard-tick RF), and (iii) Relapsing fever group. However, this is not feasible as it does not take into consideration the reptile-associated group nor the Australian species.

Presently, there are 24 validated species within the RF group, and an additional 5 species including *C*. B. kalaharica under the Candidatus status (Fig. 1.2.1) which will be discussed further (chapter 3). Prokaryotes species are assigned the Candidatus status when there is more than one sequence available for them. However, they lack other characteristics such as pathogenicity, morphology, natural habitat, reproductive feature needed for their full description based on the *Bacteriological code* (1990 version). In essence, the Candidatus status is used to describe borreliae partly described but not yet cultured as is the case of *C*. B. kalaharica, or have a low passage capacity (Labeda, 1997; Murray & Stackebrandt, 1995).

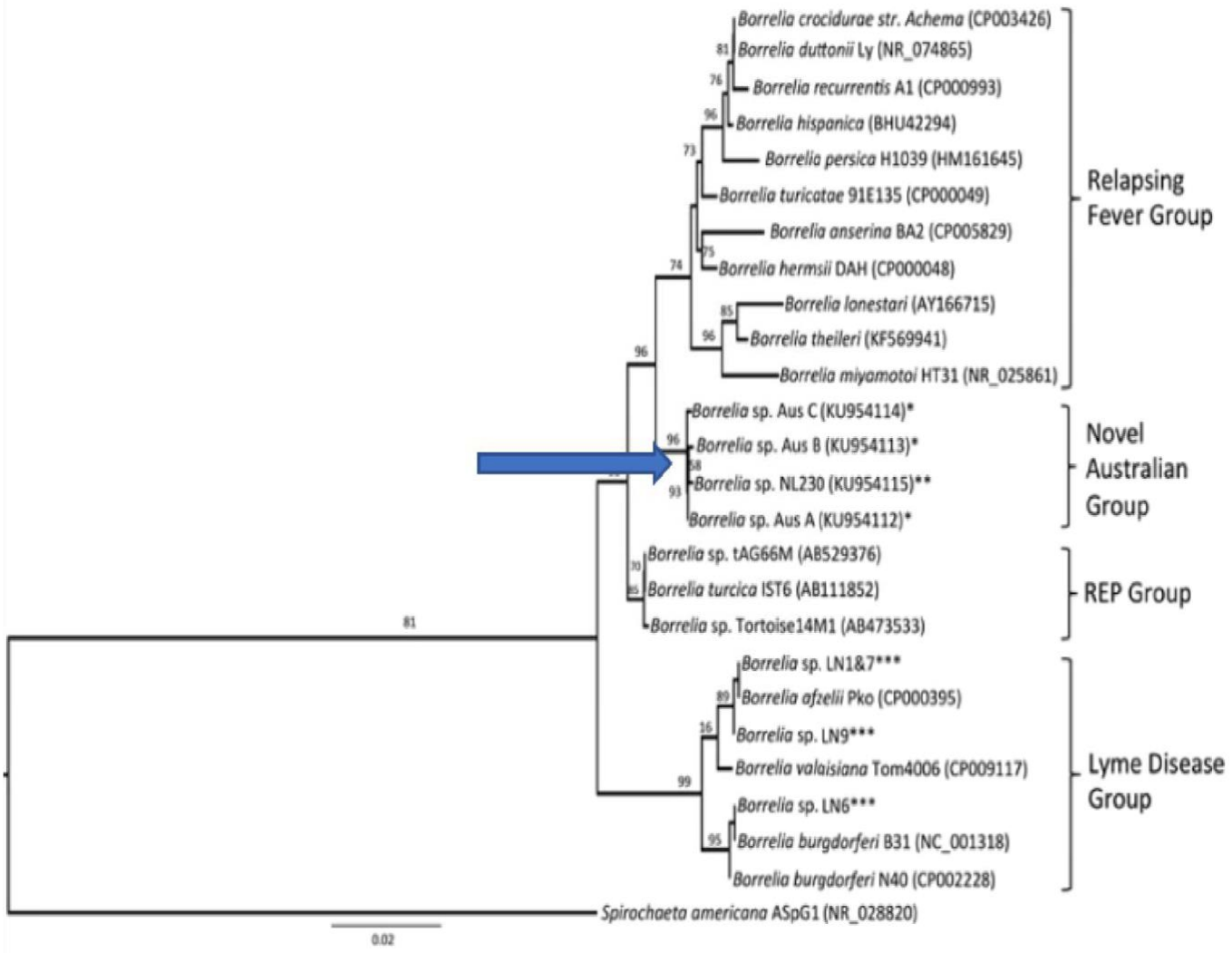


Figure 1.2.3: Phylogenetic classification of *Borrelia* species using the 16S rRNA partial sequences amplifying a 1067bp. The novel *Borrelia* species from Australia depicted by the arrow (Adapted from Loh et al., 2016).

1.2.4 Epidemiology of tick-borne relapsing fever, a Global view

Presently, TBRF has been documented in almost all the continents of the worldwide.

1.2.4(a) TBRF in North America

In North America, TBRF is endemic in the southern British Colombia, mountainous region in Mexico, and the western hemisphere where soft ticks predominantly O*. hermsii* and its borreliae *B. hermsii* are widely distributed (Goubau, 1984; Lopez et al., 2016). These ticks are the most researched and small rodent such as chipmunks, squirrels, deer mice serve as suitable hosts for the ticks (Burgdorfer & Mavros, 1970; Johnson et al., 2016; Nieto & Teglas, 2014). In the southern hemisphere, *O. turicata* and its borrelia *B. turicatae* are the predominant specie, however, the tick distribution pattern is not very clear (Lopez et al., 2016).

In the US, the first case of TBRF was in 1905 in traveller who visited Texas (Meador, 1915). However, the disease wasn't considered endemic there until the discovery of *O. hermsii* in 1939 (Edell. et al., 1979).

Historically, several cases were reported from states such as Montana, Texas, Arizona, Kansas, Utah, Oregon etc. during the 1920s till the early 1940s (Palmer and Crawford, 1933; Davis, 1936b, 1936a, 1940). The disease typically occurs in forested and mountainous regions in Arizona, Colorado, Montana, Nevada; in caves around central Texas, and in the north-western region of Washington and Idaho where the highest number of cases was recorded in 1990s (Dworkin. et al., 1998). It is estimated that 13 counties such as Rocky Mountain, San Bernardino Sierra Nevada from the endemic regions, make up around 50% of all TBRF cases (Dworkin. et al., 1998).

Another predominant route for infections in humans occurs around cabins and mostly in tourists who visit these endemic regions. One of such outbreaks was in 48% (11/23) tourists from the same family who had visited a cabin in Rocky Mountain Colorado. Similar symptoms were reported in 17% (5/30) other patients who vacationed in the same cabin and *B. hermsii* was cultured from the blood one of the patients and in chipmunks around the

cabin (Trevejo et al., 1998). Between 2013–2018 six cases were reported in tourists that visited cabins around the White Mountain area of Arizona, with *B. hermsii* reported as the causative agent (Mafi et al., 2019). An outbreak was also described among a high school football team that went on an educational camping trip in Arizona, with a total of 10 cases recorded (Jones. et al., 2016). Between 1990 and 2011, there were 504 cases of TBRF recorded in 12 states in the US with *B. hermsii* accounting for majority of them (Forrester et al., 2015; Wagemakers et al., 2015).

There have also been several isolated cases of *B. hermsii* RF in many parts of the country particularly in the endemic regions (Chowdri et al., 2013; Christensen et al., 2015). Infection with *B. hermsii* have been described in a pregnant woman (Lam et al., 2020), as well as in a newborn and its mother (Leggiadro, 2012).

Infection with other RF species such as *B. miyamotoi*, *B. turicatae* are also well documented in the US. For example, several studies have reported the presence of *B. miyamotoi* infection in 3.9% (25/635) asymptomatic (Krause et al., 2014), 2% (18/875) symptomatic people (Krause et al., 2013). From 1991 to 2019, there have been 101 cases of *B. miyamotoi* associated RF (Hoornstra et al., 2022). Infection with *B. turicatae* has been described in several cases (Bechtel et al., 2021; Bissett et al., 2018; Kingry et al., 2018).

Presently, the principal TBRF spirochaetes circulating the US are *B. hermsii, B. turicatae* vectored by *O. hermsii* and *O. turicata* respectively (Dworkin et al., 2008), and *B. miyamotoi* that is transmitted by several *Ixodes* ticks such as *I. scalpularis*, *I. ricinus*, *I. persulcatus* (Krause et al., 2014). *Borrelia parkeri* have been described in its *O. parkeri* vector, however, its pathogenicity in humans is still unknown with only one case reported so far (Dworkin et al., 2008).

TBRF is also common in animals from the US, with *B. parkeri* and *B. turicatae* been the predominant causative agent. Several infections of *B. parkeri* have been described in

domestic animals such as dogs and cats (Whitney et al., 2007; Kelly et al., 2014; Piccione et al., 2016; Esteve-Gasent et al., 2017). Infections of *B. turicatae* have also been reported in 1.9% (12/553) (Modarelli et al., 2019) and 0.68% (8/1171) (Esteve-Gasent et al., 2017b) dogs. Antibodies of *B. hermsii* was discovered in 36% (73/202) chipmunks and 21% (18/87) ground squirrels (Fritz et al., 2013). 71% (42/59) of wild turkey (*Meleagris gallopavo*) were infested with *A. americanum*, of which 59% (35/59) were infected with *B. miyamotoi* (Scott. et al., 2010). In the wild, several animals have shown competence as reservoir for *Borrelia* such as *Carios kelleyi* (Gill et al., 2008; Loftis et al., 2005), however, a few deaths have been documented for a few. The DNA of a *B. hermsii* was isolated from the carcasses of a two northern spotted owl (Fischer et al., 2009; Thomas et al., 2002).

In Canada, British Columbia is a known region for *B. hermsii* (Banerjee et al., 1998), with the first case reported in the early 1930s (Palmer & Crawford, 1933), and subsequently in 1984 (Spiller, 1986). More recently, *B. miyamotoi* infection was reported in ~5% of the 1541 *Ixodes* ticks collected around companion animals from Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia (Jacob et al., 2022).

In Mexico, the first evidence of TBRF *B. turicatae* was described in *O. turicatae* ticks by (Brumpt & Brumpt, 1939), while (Pilz & Mooser, 1936) documented the first evidence in humans. Since then, there have been 398 cases of RF documented in humans between 1939 to 2020, of which 1.3% (5/398) were TBRF infections, albeit the infecting species were not specified (Colunga-Salas et al., 2020). Notwithstanding, the primary species circulating in the region are *B. turicatae*, *B. duguesii*, *B. mazzottii, B. brasilliensis* (Brumpt and Brumpt, 1939; Martins et al., 2011a; Colunga-Salas et al., 2020) all of which are pathogenic to humans and animals. During the same period, a total of 949 infections were reported in animals of which 58% (551/949) were caused by *B. turicatae*, *B. duguesii,* and *B. mazzottii.*

1.2.4(b) TBRF in South America

In Central America, little evidence is available on the distribution and incidence of TBRF. One of the earliest descriptions of the disease in the region was recorded in 1907 from patients who were canal workers from Panama (Darling, 1909). It wasn't until 1921 that the disease was confirmed as tick-borne, after several cases were reported in hunters from the Arraijan district of the country who had bite marks on their bodies (Bates et al., 1921). *O. talaja* the vector of *B. mazzottii* were discovered around the shelters of the farmers, and healthy volunteers were either inoculated with an infected rodent blood or exposed to tick bites at the endemic sites to confirm the transmission by these vectors (Bates et al., 1921). The tick ecology was defined in 1933 after infections were detected in mammals such as horse, calves, armadillo, opossum, with the highest infection rates found in armadillos (9/61) and opossum (10/61). To verify whether the human spirochaetes were pathogenic for animals, two armadillos were injected with an infected human blood of which one of them became infected within 24hrs and succumbed to the disease on day. The second armadillo maintained recurrent episodes of infection for a month before the spirochaetes became undetectable in the blood. These historical studies from Panama by (Dunn & Clark, 1933) birthed much of the framework used for understanding TBRF ecology today. Presently, the significance of the disease remains unclear with only a case reported (Heerdink et al., 2006). Likewise in countries like Brazil and Bolivia, historical evidence shows the disease remains endemic with the report of *O. brasilliensis* in the early 1930s as the vector for infection in symptomatic patients after finding them around burrows of rodents close to human shelters. The ticks were also able to infect guinea pigs that also became symptomatic. The resulting spirochaete was name *B. brasillensis* by (Davis, 1952).

There was no further report of the ticks until recently in Brazil where patients presented with intense systemic reactions to tick bites, as well as the death of a pet that was exposed (Martins et al., 2011b). The implicated tick was *O. brasilliensis*, and they are largely distributed in Brazil in soils under human dwellings, for example, these ticks were found in

~17% of the 30 farms sampled (Reck, Marks, Guimarães, et al., 2013). Asides the ability to transmit *B. brasillensis*, the ticks are known to be aggressive towards humans and animals as they can cause necrosis at the site of bite (Reck et al., 2011, 2014) and delay the healing of wound (Reck, Marks, Termignoni, et al., 2013).

More recently, the novel *C*. B. mahuryensis that is phylogenetically different from the RF and Lyme disease group was reported in 16.4% (20/290) *Amblyomma* longirostre and *A. geayi* ticks from Brazil collected between 2012 to 2018. At least one tick from each of these years was infected with this spirochaete (Binetruy et al., 2020). This new specie has close identity to a *Borrelia* specie previously reported in *A. longirostre* ticks from Brazil (Pacheco et al., 2019) and a *Borrelia* specie in *A. maculatum* from the US (J. K. Lee et al., 2014). Using the flagellin partial sequence, the *C*. B. mahuryensis is more closely related to the reptile group compared to the RF and Lyme group, albeit different.

1.2.4(c) Tick borne relapsing fever in Europe

In Europe, the most common cause of tick-borne disease is Lyme borreliosis (Glass et al., 2023), however, TBRF is also of clinical concern with the predominant species being *B. hispanica*, *B. persica*, *B. miyamotoi*, *B. caucasia*, and *B. crocidurae* (Anda et al., 1996; Platonov et al., 2011; Rebaudet & Parola, 2006) that causes sporadic infections after accidental exposures to ticks (Wyplosz et al., 2005). While the most widely distributed ticks are *O. sonrai, O. asperus*, *O. erraticus*, *O. persica*, *O. tholozani,* (Assous et al., 2006; Kleinerman et al., 2021; Rebaudet & Parola, 2006) and all *Ixodes* species that vectors *B. miyamotoi* (Rollend et al., 2013; Wagemakers et al., 2017). The areas with the highest risk of exposure for *Ornithodoros* ticks are those around the Iberian Peninsula especially the Mediterranean areas such as Spain, Portugal, Cyprus, where *B. hispanica* is present (Rebaudet & Parola, 2006).

Historically, there were sporadic reports of TBRF in Spain caused by *B. hispanica* (Aznar, 1926), but in 1994, a *Borrelia spp*. genetically homologous to *B. duttonii*, *B. crocidurae*, and

B. hispanica was detected in three patients and *O. erraticus* ticks collected from a pig farm (Anda et al., 1996). In addition to soft tick RF species, *B. miyamotoi* infected ticks have been reported from many European countries. For example, infection in people from the Netherlands is estimated at 10% (12/120) which would extrapolate to some 30,000 people yearly are exposed to ticks infected *B. miyamotoi* (Jahfari et al., 2014), and in Russia 17% (51/302) where the first ever human infection was reported (Platonov et al., 2011). This raises the question regarding the extent of exposure and what conditions are required for an infection to occur even given the wide spread of tick vectors. In addition to this, *B. miyamotoi* coexists with the agents of Lyme disease *B. burgdorferi* sensu lato that are endemic in Europe (Baranton et al., 1992; Canica et al., 1993), with a seroprevalence rate of 34% (104/304) in some places (Platonov et al., 2011).

RF have also been documented in animals with *O. tholozani* have been implicated in the transmission of *B. persica* in pets such as cats and dogs from Israel (Schwarzer et al., 2015; Baneth et al., 2016). *Borrelia miyamotoi* infection have been reported in 5.5% (4/72) wild rodents from France (Cosson et al., 2014), 3% (3/108) from Switzerland (Burri et al., 2014), and (2/251) from Slovenia (Cerar et al., 2015). Infection with *B. miyamotoi* in the *Ixodes* tick vectors ranges from 10% (36/360) in Germany (Glass et al., 2023), to 2.7% (15/561) and 0.4% (8/20161) in *I. persulcatus* and *I. ricinus* from Estonia (Geller et al., 2012), to 3% (8/267) in *I. ricinus* from France, etc (Cosson et al., 2014).

1.2.4(d) TBRF in the Middle East and Central Asia

In the Middle East and Central Asia, the predominant TBRF circulating is *B. persica* (Aznar, 1926). In addition, other species such as *B. microti*, *B. latyschewii*, and *B. baltazardii* have also reported (Baltazard et al., 1955; Trevisan et al., 2021). Generally, in Eurasia, *B. persica* is the primary agent of TBRF that is vectored by *O. tholozani* ticks, of which 30 to 60% of the ticks were reported to harbour *Borrelia* (Assous & Wilamowski, 2009). *Borrelia miyamotoi*

is the primary agent of TBRF in Japan (Gugliotta et al., 2013; Jahfari et al., 2014; Platonov et al., 2011). The most widely distributed ticks in the region *O. persica*, *O. erraticus O. tartakovsky,* and *O. tholozani* (Baltazard et al., 1955; Moradi-Asl & Jafari, 2020; Trevisan et al., 2021).

1.2.5(e) Tick borne relapsing fever in Africa

In Africa, literatures documenting the incidence, frequency and distribution of RF tick vectors is sporadic, often relating to certain study areas. However, some studies have described the geographic distribution *of Borrelia* tick vectors and their associated host animals, and their relevance to clinical and veterinary medicine (Diatta et al., 2015a; Elbir et al., 2015; Godeluck et al., 1996; Vial et al., 2006). The primary TBRF spirochaetes of clinical concern circulating the region are *B. duttoni* in the East*, B. crocidurae* in the West, and *B. hispanica* in the North of Africa, that are vectored by *O. moubata*, *O. sonrai*, and *O. erraticus* respectively (Trape et al., 2013). The disease is largely pronounced in regions that surrounds the Sahel and Saharan regions such as Senegal where the temperature is high, the climate is drier with less than 750 mm of annual rainfall as a result on the ongoing droughts (Trape et al., 1996). This dry climate created a suitable environment for *O. sonrai* ticks to be more frequent and widely distributed, and an estimated 87% of animal burrows in the villages are infested with these ticks (Fig 1.2.5e[1]) (Trape et al., 2013).

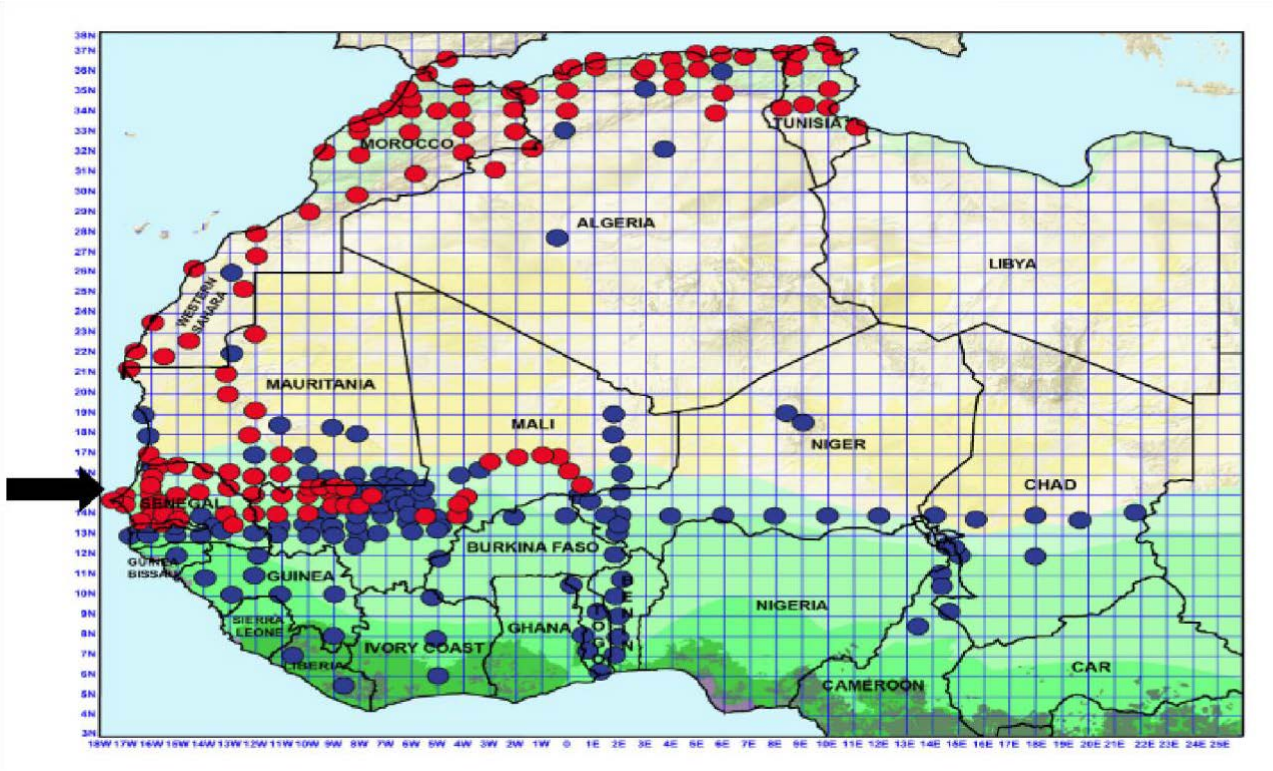


Figure 1.2.5(e[1]): Infestation of *Ornithodoros* ticks in small animal burrows in North-West Africa. Red circles indicate presence of ticks, blue circle indicates absence of ticks. Senegal is signified by the black arrow. (Adapted from Trape et al., 2013).

In East Africa, *B. duttoni* is the endemic specie (Cutler et al., 2009; Walton, 1962). This specie is known to be associated with a higher mortality rate when untreated (up to 8%) only second to the LBRF *B. recurrentis* (30-70%) if left untreated (Gouban, 1984; Southern & Sanford, 1969) due to their high density in the blood (Cutler et al., 2009). The infection rate in its *O. moubata* tick vector can be as high as 60% (8/13) (Fukunaga et al., 2001). *B. duttonii* is endemic in some countries in the region such as Tanzania, with certain areas such as the Mvumii area in the Dodoma district showing particularly high infections levels. Here an annual incidence of TBRF in children <1 year can be up to 384/1000, 163/1000 in children under 5 years, and one of the top ten causes of death in children <5 years old (McConnell, 2003). Another study conducted in Mvumii and Ikombolinga also reported similar infection level of 3% (29/960) in children under 5 years (Talbert et al., 1998), perinatal mortality of 436/1000 births, and 475/1000 total pregnancy loss in other areas of the country (Jongen.

et al., 1997). In addition, *C*. Borrelia. mvumii was described in 17.6% (9/51) infected *O. porcinus* ticks collected around human dwellings in the country. This new specie aligned closely to the new world RF fever group on the phylogenetic tree as previously shown (Fig 1.1).

Recently, *C*. B. kalaharica have been reported in patients that visited the Kalahari Desert region areas of Southern Africa (Fingerle et al., 2016; Stete et al., 2018). The vector of this borreliae '*O. savignyi'* was initially hypothesised to have been introduced into Africa through camel migration from the middle east (Hoogstraal, 1956b), but (Theiler, 1962) argued otherwise and considered it emergence from either the Sahelian regions in North Africa, or the Kalahari region of southern Africa. The discovery of *C*. B. kalaharica aligns with Theiler's hypothesis, however, recent literature reveals *O. savignyi* is endemic in Egypt, Saudi Arabia, and Yemen (Bakkes et al., 2018) which supports Hoogstraal's claim. A more recent study reported the presence of a borreliae in a *Hyalomma rufipes* tick collected from a giraffe in Kenya that had a 100% identity to the Lyme disease *B. garinii* isolated from *Ixodes ricinus* ticks in Finland (Olivieri et al., 2021).

In Ethiopia, the significance of TBRF is unclear, and even if it was present, it would be obscure by the diagnostically indistinguishable endemic LBRF that is one of the commonest causes of death, and seventh cause of hospital admission (Cutler et al., 2009). A retrospective study conducted in central Ethiopia reported the case fatality of 13% (8/63) in confirmed LB cases within the space of six month (Nordmann et al., 2018). Historically, there were suspected outbreaks of TBRF in Somaliland between 1913 and 1917, due to its cyclic features and the report of bites. The biting arthropods were suggested to be ticks imported into the country by Ethiopian refugees and their livestock fleeing famine at the time (Mohamed, 1999). However, due to soft ticks being rapid feeders, it is not feasible for them to have attach to their prey for long periods. Whereas it was more likely that the endemic clothing lice was the transmitting vector, and as the report did not confirm the transmitting

vector nor did they rule out the possibility of LBRF (Clark, 1937), the infections cannot be attributed to ticks.

In Zambia, this first case of TBRF is a novel human pathogenic specie *Candidatus*. Borrelia. faini isolated from the blood of a symptomatic patient who reported tick bite after visiting a cave in the region. This specie had above e 98% similarity to B*. parkeri* and *B. turicatae* using the 16S and flagellin sequences respectively. For vector identification, 50 *Ornithodoros* ticks were collected from the cave of interest. Of these, (20/50) were infected with the same *Candidatus*. Borrelia. faini detected in the patient with a 100%, while the tick identity was confirmed as *O. faini* morphologically and molecularly. To identify the potential animal reservoir for the species, a total of 277 bats around the caves where recovered, of which 177 were *R. aegyptiacus*, 47 *Hipposideros sp*., 12 *Miniopterus sp*., and 1 *Rhinolophus sp*. Of these, a total of 64 bat consisting of 59 *R. aegyptiacus,* 3 *Hipposideros sp*., and 2 *Miniopterus sp.* were infected with the same borreliae in the human and ticks (Qiu et al., 2019).

The discovery of the novel C. B. kalaharica occurred in similar circumstances after foreign tourist who visited areas in the Kalahari Desert of southern Africa presented with TBRF symptoms (Fingerle et al., 2016; Stete et al., 2018). These discoveries warrant for further studies on TBRF of clinical concerns in the southern African.

In West Africa, *B. crocidurae* (Godeluck et al., 1996; Schwan & Hinnebusch, 1998) is the predominant specie circulating, with majority of the cases reported from Senegal (Mediannikov et al., 2014) (Fig. 1.2.5[2]) where most studies have been conducted. Its *O. sonrai* tick vector is the only specie documented in the region. This borreliae specie is known to have a high transmission rate within its tick vector (Baltazard et al., 1955; Vial et al., 2006), albeit with a milder clinical effect due to low spirochaetemia (<20 spirochaete per 200 microscopic field) (Vial et al., 2006). Ideally, the minimum level of microscopic detection for

TBRF is 10^4 spirochaetes (Dworkin. et al., 2002) but many RF species like *B. crocidurae*, *B. hispanica* have less than 200spirochaetes/ml which makes them more difficult to detect as reported by (Nordstrand et al., 2007). In contrast, the level of spirochaetemia during infection with *B. duttonii* is often 10X higher than the above species, and even significantly higher for LBRF, which makes them much easier to detect using microscopic tools (Cutler et al., 2009). Furthermore, the high fatality rate of LBRF and *B. duttonii* RF is in part due to severe symptoms exhibited during this high spirochaetemia in acute infections. The study of (Anda et al., 1996) confirmed this after 10^5 of RF spirochaetes were inoculated into mice which became detectable microscopically after day 2 of inoculation and up to day 20 (Fig. 1.2.5e[2]). Since the drought began in the 1970s, the frequency and distribution of soft ticks in Senegal has been increasing steadily yearly from 4 per 100 persons-yearly in 1990, to 25 per 100 person-years in 1997, and an overall 11 per 100 persons-yearly in which the highest incidence of 1.4 per 100 persons-month occurred during the dry months (Vial et al., 2006). The high infestation of *O. sonrai* ticks in animal burrows opened around human dwellings in Senegal (Trape et al., 2013), one can directly link it to the high infection rate, with at least one infected tick reported in 75% (15/20) of similar animal burrows (Vial et al., 2006).

There have been isolated reports of infection elsewhere in West Africa. One of such is in the southern part of Togo where 9.8% (5/51) children and 16.3% (16/98) adult presenting with RF symptoms alongside 14.3% (2/14) asymptomatic were seropositive by ELISA using (glpQ) gene that is specific for RF (Trape et al., 1991). In addition, 10% (9/90) of children from the northern part of the country also tested positive for the infection, with 88.9% (8/9) confirmed of PCR using the 16S and flagellin partial sequences. While 11 of the 23 ELISA positives from the southern patients were also confirmed on PCR. Sequence analysis of the southern cases revealed the borreliae as *B. duttoni* and *B. crocidurae*, while *B. duttoni* was reported for the northern patients (Nordstrand et al., 2007). The presence of the East African *B. duttonii* in a West African state shows that the geographical distribution of these

spirochaetes may be wider than initially suggested. In addition, (Trape et al., 1991) report of the increasing distribution of *O. savignyi* and its borreliae in Senegal is largely attributed to the droughts, this could be the same for Togo. For instance, the South of Togo in recent years have also experienced episodes of droughts with a mean temperature of 26.8–28.9°C during the dry months in places like Atakpame and Dapaong respectively (Nordstrand et al., 2007).

In Mali and Mauritania, 12.4% (34/273) and 9.7% (7/72) of ticks respectively collected around animal burrows were infected with *B. crocidurae*, albeit the clinical importance in the regions is still unknown.

In North Africa, the predominant borreliae circulating is *B. hispanica* (Sarih et al., 2009), but recent reports have also described the presence of other species like *B. crocidurae* and *B. merionesi* (Fig. 1.2.5e[3]) (Diatta et al., 2012; Trape et al., 2013). Human exposure to these ticks is the same as other species (Cutler et al., 2009), albeit the pathogenicity of *B. merionesi* is not yet known as few experiments have been unable to establish infection in humans (Blanc & Maurice, 1948). Between the year 2000 to 2005, TBRF was recorded in 0.11% (102/89,995) of patients presenting with fever in Morocco, with those aged 15 years accounting for 60% of the cases (Diatta et al., 2012). Similar report was documented in any region in Morocco where the infection rate with *B. hispanica* was 20.5% (26/127) in patients with unknown febrile symptoms (Sarih et al., 2009).

A novel specie *Candidatus* Borrelia Algerica was described in 1 of 257 febrile patient blood from Oman, Algeria. This specie had a 99% homology to the old-world spirochaetes using the intergenic spacer sequences and glpQ gene (Fotso et al., 2015).

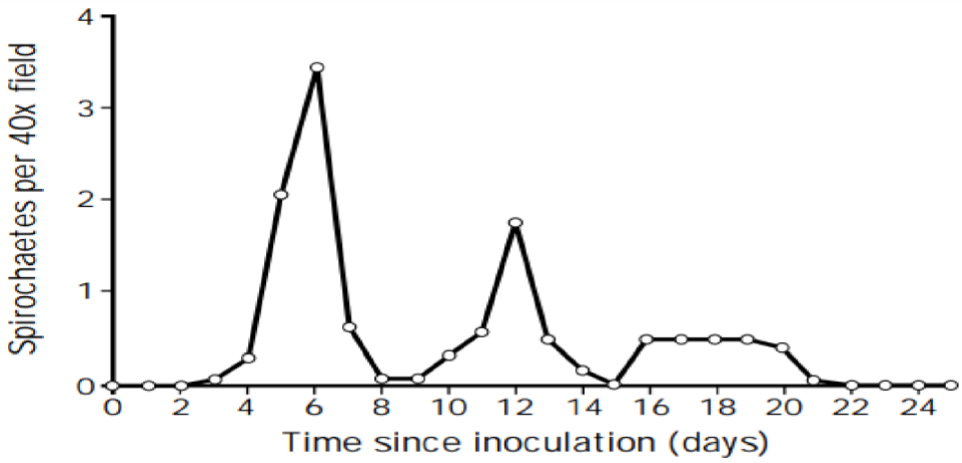


Figure 1.2.5(e[2]): Density of a TBRF spirochaetes in mice blood smear after artificial infection. (Adapted from Anda *et al.,* 1996).

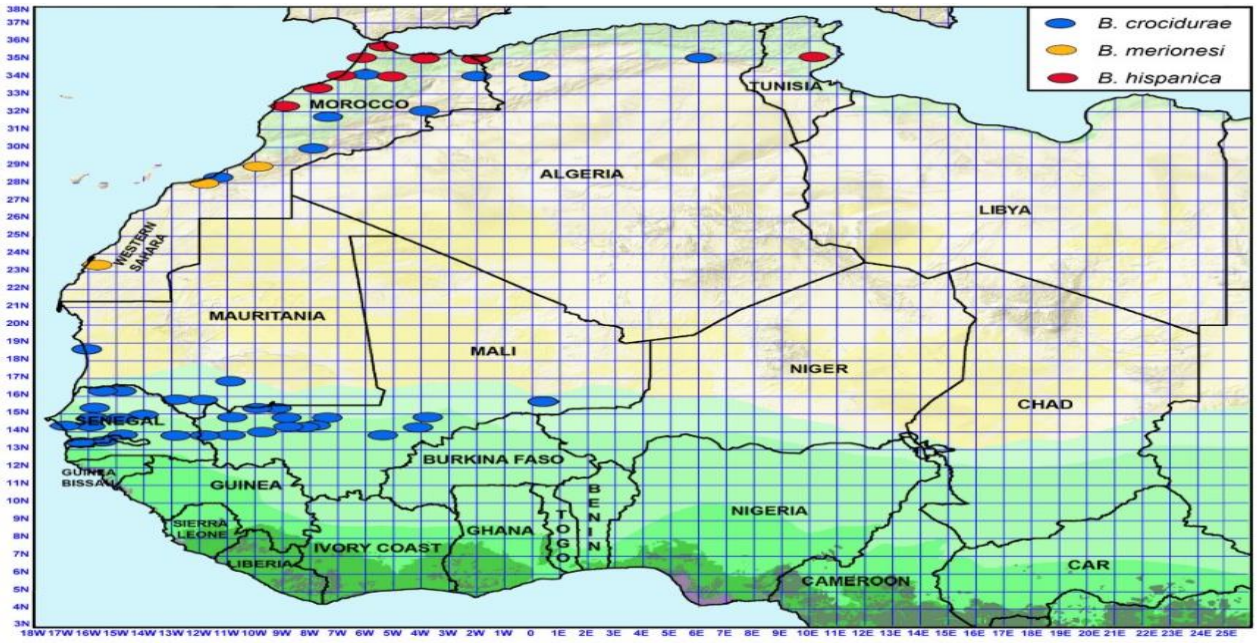


Figure 1.2.5(e[3]): Borrelia species in ticks and or small animals in North-Western Africa. (Adapted from Trape et al., 2013).

1.2.5 (f) Vectors of TBRF in Africa

In many parts of Africa, the predominant RF vectors are usually from the Argasidae family (*Ornithodoros*, *Argas)*, except for hard-tick RF vectors. Currently, around 128 and 62 species of the *Ornithodoros* and *Argas* (fowl tampan) species respectively have been documented,

many of which are vectors of clinical and veterinary importance (Estrada-Pena et al., 2010; Estrada-Peña, Mihalca and Petney, 2018) such as the agents of TBRF.

In East and Southern Africa, the most widely distributed Ornithodoros ticks are those within the *O. moubata* complex (vectors: *O. moubata* sensu lato, *O. porcinus, O. phacochoerus n. sp., O. waterbergensis, O. apertus, O. compactus*) (Leeson, 1952), with *O. moubata* s.l serving as both the vector and reservoir for *B. duttonii* (Walton, 1962; Morel, 1968). The *O. savignyi* complex comprising of (*O. savignyi*, *O. kalahariensis*, *O. pavimentosus*, *O. noorsveldensis*) are widely endemic in the Southern Africa region (Bakkes et al., 2018). Vectors for the hard-tick RF group such as species from the *Rhipicephalus, Hyalomma,* and *Amblyomma genera* are widely distributed in the continent (Kamani et al., 2011, 2013, 2015; Kumsa et al., 2015, 2019).

In West Africa, *O. sonrai* is the most abundant vector reported in available literatures. However, *O. savignyi* is also endemic in Nigeria and widely spread in the arid and semi-arid regions (Bunza et al., 2008; Cutler et al., 2018). *Ornithodoros moubata* have also been reported in poultry; 4.9% (3/61) chickens, 14.3% (4/28) in guinea fowls, using the entomological keys (Bunza et al., 2008).

In North Africa, there is abundant diversity of *Ornithodoros* ticks with the endemic species being the *O. erraticus* complex (*O. erraticus*, *O. sonrai*) the vectors of *B. hispanica, B. crocidurae* respectively (Fig. 1.2.5f). These ticks are known to colonise burrows of their small mammal host with a high infestation rate of 39.5% (239/605) (Diatta et al., 2012). In Morocco, *O. erraticus* is distributed around the wet areas in the north, while *O. sonrai* is found in the arid areas in the south (Diatta et al., 2012) with the first report documented in the 1920s (Hornus, 1928; Nicolle & Anderson, 1928). Other RF agents like *O. normandi,* also widely distributed in the coastal areas of Algeria and Tunisia, while O*. marocanus* is endemic in the northwestern regions of Morocco (Trape et al., 2013). Furthermore, newly described

species *O. kairouanensis* 20% (1/5), *O. costalis* 4.6% (3/65), *O. occidentalis* 5.5% (3/55), *O. rupenstris,* and *O. merionesi* 18.8% (3/16) are also reported to harbour *Borrelia* spirochaetes (Trape et al., 2013). *O. savignyi* is also widely distributed in Sudan and Egypt (Bakkes et al., 2018). The distribution of these ticks is dependent on temperature, climate condition, elevation, rainfall, and longitude. For instance, *O. sonrai* thrives best in areas of drier climates and high temperature with a low precipitation level (between 50-100mm). Whilst the distribution of *O. erraticus*, *O. normandi* are negatively impacted by high rainfalls and elevation. For *O. marocanus*, *O. costalis*, *O. occidentalis*, *O. merionesi*, they thrive best in environments of low altitudes, as elevation negatively impacts their dispersal (Trape et al., 2013).

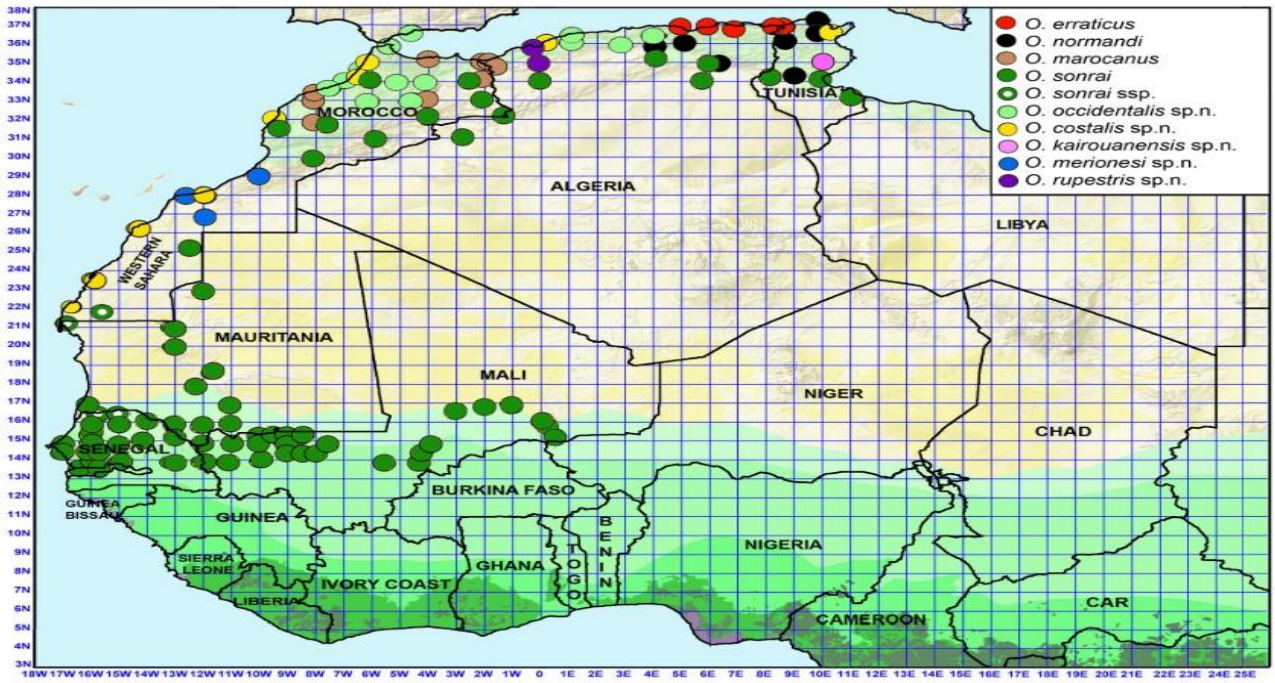


Figure 1.2.5 (f): Distribution of *Ornithodoros* species in North-Western regions in Africa. (Adapted from Trape et al., 2013).

In addition to *Ornithodoros* species, the fowl tick '*Argas persicus*' are widely distributed in the Northern hemisphere; Tunisia, Algeria, Egypt, Libya (Boucheikhchoukh et al., 2018; Kaboudi et al., 2019; Lafri et al., 2018; Ouchene et al., 2019); Western Nigeria, BurkinaFaso,

Senegal; Southern; South Africa, Zimbabwe, and Eastern Ethiopia, Tanzania (Ledwaba et al., 2022; Walker et al., 2003) regions. They are considered the most important ectoparasites of poultry in many regions including in Africa.

For the hard-tick RF transmitting ticks, species from the genus *Rhipicephalus* such as *R. annulatis*, R. microplus, *R. evertsi, R. decoloratus R. geigyi* are the primary vectors of bovine RF *B. theileri* (Cordeiro et al., 2018; McCoy et al., 2014; Qiu et al., 2021; Trees, 1978). Several ticks from this genus are also vectors for many *Rickettsia* species that are agents of spotted fever groups (SFG) in humans and animals worldwide (García-García et al., 2010; Oteo & Portillo, 2012; Rolain et al., 1998). This tick was initially discovered in South Africa a century ago as the vector of *B. theileri* in cattle (Theiler. A, 1904).

In addition, species from the *Amblyomma* genus have shown competence as potential hosts for RF species of veterinary concern. A study in Ethiopia reported RF borreliae in 7.3% (8/109) *Amblyomma cohaerens* ticks collected from cattle. This new *Borrelia* had 85-86% similarity to the *B. theileri*/*B. lonestari* group (Mediannikov et al., 2013). A similar *Borrelia* specie was reported in 6.7% (20/298) *Amblyomma* ticks (Kumsa et al., 2015). Although, literatures have revealed the incompetency of *Amblyomma* ticks as vectors for Lyme disease (Masters, 2006; Wormser et al., 2005), they have shown competency as potential vectors for RF.

With the abundance of tick vectors, many borreliae spirochaetes have animals that can serve as reservoir or host for them to preserve the continuous transmission cycle in their natural habitat. However, soft ticks can be considered the most suitable reservoir for
TBRF spirochaetes because of their longevity and the ability to remain infectious for several years after initial infection (Francis, 1938). Some mammals especially small rodents and insectivores serve as host or reservoirs for *Borrelia* due to the huge tick infestation of their burrows by *Ornithodoros* tick vectors (Trape et al., 2013). Many rodents belonging to the genus *Rarrus spp.*, *Cricetomys spp.*, *Gerbillus* spp., *Mastomys* spp.; insectivores from the

family Soricidae, Erinaceidae are well documented hosts for *Borrelia* (Diatta et al., 2015b; Godeluck et al., 1994; Trape et al., 2013). Prevalence of *Borrelia* infection in the above rodents was 4.1% (57/1386), (Trape et al., 2013).

In addition to rodents, other mammals both domestic and in the wild such as porcupines, warthogs, antbears, camels, tortoise, horses, livestock, dogs, humans (Bakkes et al., 2018; Lopez et al., 2016; Walton, 1962) have shown competence as probable host for the African spirochaetes due to their susceptibility for infection. Some bats Bats such as *Megaderma cor* from Kenya (Heisch, 1952) can serve as reservoirs too.

1.2.5 (g) TBRF of Veterinary and Economic importance in Africa

TBRF in animals have been documented in several regions, albeit many infections may still be underreported in veterinary medicine (Piccione et al., 2016). The burrows, cages, and shelters of animals are often infested with RF soft tick vectors (Trape et al., 2013), while hard tick vectors tend to quest for their host in open vegetation (Elelu et al., 2022). In farm animals, the predominant RF species of economic and veterinary importance are *B. theileri*, *B. anserina*, the aetiological agents of bovine and avian spirochaetosis respectively (Sakharoff., 1891; Sharma et al., 2000).

Avian spirochaetosis has been reported in 1.8% (7/390) poultry birds from Nigeria (Sa'idu et al., 1995). In Tanzania 11% (11/100) of chickens and 8.9% (16/180) pigs from were reported to be infected with *B. duttonii,* however, the authors did not conduct any transmission studies to confirm that these spirochaetes were viable and capable of being transmitted onwards (McCall et al., 2007). A similar study by (Larsson et al., 2006) also illustrated *B. duttonii* infection in rodents after artificially infection, and the volume of spirochaetemia detected in the blood was like that of humans. Notwithstanding the onward transmission of the spirochaete by the rodent was not determined.

In Ethiopia, TBRF *B. anseria* in found in 7.5% (3/40) *Argas persicus* ticks collected around poultry, while *B. theileri* was in 12.5% (2/16 pools) *Amblyomma* and *Rhipicephalus* species collected from cattle (Cutler et al., 2012). Bovine relapsing fever infections have been documented in livestock: 3.3% (42/1260) cattle from Cameroon (Abanda et al., 2019), 4.1% (20/488) cattle from Zambia (Qiu et al., 2021), 0.9% (5/557) cattle and sheep from Egypt (Abdullah. et al., 2021).

1.2.6 Afrotropical *Ornithodoros* Species

Ornithodoros ticks in Africa are usually xerophilous as they are most likely distributed around arid and semi-arid regions where the annual precipitation can be as low as ~50mm for some species such as *O. sonrai* (Hoogstraal, Salah and Kaiser., 1954), and not >750mm for extreme cases (Trape et al., 1996).

Presently, Afro-tropical *Ornithodoros* species are subdivided into two; *O. savignyi* complex, and *O. moubata* complex comprising a total of ten species (Bakkes et al., 2018). Initially comprising of two species *O. moubata* and *O. savignyi* distinguished by the presence or absence of eyes (A. Murray, 1877), before (Walton, 1962) described an additional three species (*O. compactus*, *O. apertus*, *O. porcinus*). Subsequently, Walton described two subspecies of *O. porcinus* (*O. porcinus domesticus*, *O. porcinus porcinus*) based on ecological and geographical factors. Current literature confirms the geographical restrictive characteristics of *O. porcinus* as a specie but not subspecies (Bastos et al., 2009; Boshoff et al., 2014). Phylogenetic information from these two studies revealed a separation of species based solely on the geographical location. These ticks include four additional species were redescribed by (Bakkes et al., 2018) using the morphology and tarsus 1 lobes traits of the female and nymphs.

O. moubata Complex

This group contains O*. moubata*, *O. porcinus*, *O. apertus*, *O. compactus*, *O. Phacochoerus, and O. waterbergensis* that are differentiated by the lack of eyes

I. *O. moubata*: Widely distributed around regions in the south and some eastern parts of Africa where the annual rainfall is >400mm with an elevation of 1200m above sea level. Their primary animal hosts are warthog, pangolin, ruminants, humans, etc. it is the primary vector of TBRF in the region.

II. *O. porcinus*: Fairly distributed around the eastern part of Africa where the annual rainfall is >600m. Their associated hosts include warthog, pigs, elephant, ruminants, humans, etc. known vector of African swine fever (ASF).

III. *O. Phacochoerus*: Fairly distributed the southern part of Africa where the annual rainfall is >400mm with an elevation level <800m. Often found around warthogs but can adapt to human and livestock shelters. Potential vector of African swine fever.

IV. *O. waterbergensis*: Geographical restricted to regions in southern Africa where the annual rainfall is >400mm with an elevation level >700m. Often found around warthogs but can adapt to human and livestock shelters. Potential vector of African swine fever.

V. *O. compactus* and *O. apertus*: These is currently to evidence of disease associated with these two species. *O. compactus are* fairly distributed around southern Africa where the annual rainfall is <200mm, and their preferred host is tortoise. whereas *O. apertus* is a rare species that is restricted to regions East Africa where the annual rainfall

is >900mm and an elevation of 1800m above sea level. Their only known host is porcupine.

The O. savignyi complex

This group contains of *O. savignyi*, *O. pavimentosus n. sp.*, *O. noorsveldensis n. sp.*, and *O. kalahariensis n. sp*. that are differentiated by the presence of eyes.

I. *O. savignyi*: Widely distributed around North Africa (Egypt, Sudan, Somali), and the Arabian Peninsula where the annual rainfall is <500mm. This species is free living found burrowed into sandy soils. Primary hosts include Camel, ruminants, pigs, dog, humans etc, are warthog, pangolin, ruminants, humans, etc. it is the primary vector of TBRF in the region. Recently described vectors of TBRF and a potential vector of sand tampan toxicosis.

II. *O. pavimentosus n. sp.*: Fairly distributed around southern Africa where the annual rainfall is <500mm. This species is free living found burrowed into gravelly soils. Primary hosts include Cattle, humans, can also be found around pig, dog, fowl, etc. A potential vector of sand tampan toxicosis.

III. *O. noorsveldensis n. sp*.: Restricted to a single location in Southern Africa (Eastern Cape) where the annual rainfall is ~400mm. This species is free living found burrowed into clay soils. Primary host include wildlife such as Kudu, potential hosts are ruminants, pig, dog, fowl, human, etc.

IV. *O. kalahariensis n. sp.*: Moderately distributed in countries around the Kalahari region where the annual rainfall is <500mm. This species is found free living and burrowed into sandy soils. This is the closest related specie to *O. savignyi* based on morphological traits.

Indeed, it is evident that ecology and climatic factors play a huge role in the maintenance of *Ornithodoros* ticks in Africa. For the *O. moubata* complex, high level of rainfall (except for *O. compactus*) and altitude are the major drivers for their distribution and frequency. While the abundance of the *O. savignyi* group largely dependent on lower altitude and rich soil topology. In addition, the size of the tarsus 1 lobes of *O. savignyi* group are directly correlated

to their soil topology. O. *pavimentosus n. sp.* has the biggest lobe required to dig through the coarse soil, while *O. noorsveldensis n. sp.* has the smallest lobes due to the smooth texture of clay soil.

1.2.6(b) North-Western African *Ornithodoros* ticks

Presently, there are nine *Ornithodoros* (including 5 newly described) species distributed across the north-western parts of Africa (Fig. 1.2.5f). These ticks are either spread cross the most arid regions in north Africa or restricted to a specific geographical location. They include the previously described *O. sonrai*, *O. normadi*, *O. marocanus*, *O. erraticus*, and the newly described O*. kairouanensis*, *O. occidentalis*, *O. merionesi*, *O. rupestris*, and *O. costalis* (Trape et al., 2013)

I. *O. sonrai* and *O normadi*: These species are found in dry arid climates where the temperature is high, and the precipitation level is low (between 50-100mm). These two factors play a huge role in their distribution.
II. *O. erraticus*: This species is widely spread in warmer climates with low rainfall. elevation
III. *O. marocanus*, *O. costalis*, *O. occidentalis*, *O. merionesi*: These species thrive best in environments of low altitudes, as elevation negatively impacts their dispersal.

1.2.7 Borrelia maintenance in tick vector

Once spirochaetes have been acquired after an infectious blood meal, they colonise the tick midgut and multiply (Fig. 1.2.8) (Lopez et al., 2016), albeit it is unclear what volume is required before they become infected. A study in their animal model suggested that ticks require ~30 spirochaetes before becoming infected, as 50% of the ticks in their study became infected after continuous feeding on infected mice for 13days (Lopez et al., 2011).

A study conducted on *O. moubata* ticks suggested that *Borrelia* or borrelia-component induced the upregulation of genes for all defensin isoforms. Furthermore, increased antimicrobial activities against gram positive bacteria and not for gram negative was observed in the tick after immunization (Nakajima et al., 2003). From day 10 of acquiring the pathogen, some RF spirochaetes exit the midgut and congregate in the salivary and coxial glands. Once a blood meal occurs, the ticks can infect the host in as low as a few seconds after attachment (Boyle et al., 2014). The salivary gland plays a vital role in ensuring the maintenance and continuation of *Borrelia* life cycle in their animal host.

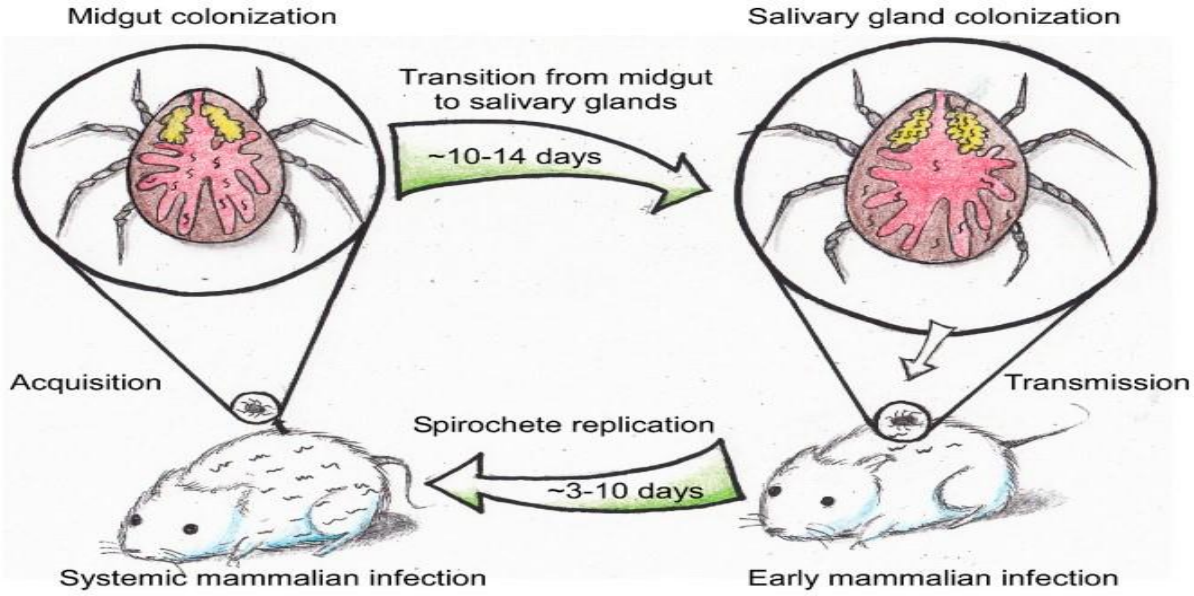


Figure 1.2.7: Tick acquisition and transmission of RF spirochaetes (Adapted from Lopez et al, 2016).

1.2.8: Borrelia maintenance in host animal

The propensity of TBRF spirochaetes to maintain their infectiousness in an associated animal host that supports their acquisition and transmission by a tick vector is important for their consistent survival in nature. The rapid feeding characteristics of their soft tick vectors together with the ability to transfer the spirochaetes in as little as 15secs as described for *B.*

turicatae (Boyle et al., 2014) after attachment increases the likelihood of transmission and preserves their maintenance within their natural habitat. Even whilst the host innate immune (macrophages, dendritic cells) cells are established to counter RF spirochaetes, these spirochaetes can multiply within the first few days of infection before the host immune cells are fully deployed (Fig. 1.2.8). The ability of the spirochaete to evolve from initial onset infection to a systemic one is because of their ability to switch off their surface proteins and upregulate their variable major proteins (Vmps) which allows them to evade the host system. The cyclic episode typical of the RF is a result of the several antigenic variations that occurs during host invasion. Once the spirochaetes achieve high density, the host immune cells clear them out except for the ones coated with the antigenic proteins. The spirochaetes can then reproduce new populations in high numbers coated with the new Vmp that ensures a relapse occurs (Raffel et al., 2014). The time between initial clearance (low spirochaetemia) and the production of new spirochaetes is the afebrile period.

This mechanism allows the spirochaetes replicate in high volume in the host bloodstream, and the recurrent nature of the infection is propelled by these antigenic variations that ensures the spirochaetes can persist within their vertebrate host (Barbour et al., 2006). Relapsing fever spirochaetes have more than 14 Vtps that allows ensures their persistence when a host, whereas the Lyme disease group has only one surface protein (OspC) (Hinnebusch et al., 1998).

1.2.9: Clinical presentation, diagnosis, and treatment of TBRF

Clinical presentations

In humans, the onset of the infection begins with recurrent episodes of fever of ~40°C within the first 18days of infection after This is usually accompanied by several symptoms such as headache, generalised body, vomiting, but can be complicated by abortion in women, and

neurological involvement (section 1.1). In few cases, bilateral infiltrates leading to respiratory failure (Davis. et al., 1992), involvement of the central nervous system such as facial palsy, myocarditis, vertigo, and hepatosplenomegaly has been described (Dworkin et al., 2008).

Diagnosis

Presently, no commercial kit is available for the diagnosis of RF *Borrelia*. The two primary method of diagnosis are microscopy and molecular tools. Serology diagnosis for retrospective diagnosis can be achieved either by immunofluorescence or enzyme-linked assays. This can be used for seroprevalence studies against specific antigens exclusive to RF spirochaetes such as the glycerophosphodiester phosphodiesterase (GlpQ) protein or *Borrelia* immunogenic protein A (BipA) (TG. et al., 1996). Microscopy is the predominant diagnostic tool in resource limited settings as is the case for most countries in Africa (Cutler et al., 2012). Relapsing fever spirochaetes are detected during acute infection Giemsastained blood smear (Lopez et al., 2016). The number of the spirochaetes in the blood is highest during acute infection but reduces below the level of detection during the afebrile periods (McCoy et al., 2010). This in part contributes to the current underdiagnosis of the disease in Africa as some RF such as *B. crocidurae* are associated with lower blood density, thus making microscopy more difficult (Cutler, 2010). Secondly, TBRF and LBRF spirochaetes are not distinguishable using microscopic tools, which can lead to misdiagnosis based on historical evidence (Cutler et al., 2012).

Molecular tools such as PCRs and genome sequencing are more reliable and sensitive for borreliae detection and can be used for species delineation (Gama et al., 2007), albeit more costly. The issue of underdiagnosis and/or misdiagnosis will continue to persist until a more economical and commercial test is available.

Treatment of RF

Prior to the discovery of penicillin, arsenicals and emetine bismuth iodide were the drugs used for the treatment of RF. Subsequently, antibiotics (tetracycline, penicillin, macrolides) were the preferred treatment. Presently, a more recent antibiotic B-lactams has been added to the pool of available treatment (Hasin et al., 2006).

1.3 Aim and Objectives of the Study

Aim: To investigate and compare tick-borne relapsing fever spirochaetes of importance from Ethiopia and Nigeria.

Objectives:

I. To investigate and compare the *Borrelia* diversity in Nigeria and Ethiopia.

II. To investigate and compare the soft tick diversity in Nigeria and Ethiopia.

III. To screen febrile patients in Nigeria for the evidence of tick-borne relapsing fever.

IV. Investigate livestock from Nigeria for possible exposure to borreliosis that might pose economic burden or serve as a zoonotic risk.

1.4 Description of Study Sites

1.4.1 Nigeria

Nigeria is situated in the west coast of Africa between latitude 4°16'-13°53' north and 2°40'14°41' east, with an estimated landmass of 923,768km^2 that stretches from the Gulf of Guinea in the south, to the fringes of the Sahara Desert in the north (NPC, 2014). The country is bound to the south by the Atlantic Ocean, east by Cameroon, northeast by Chad, north by Niger, and west by Benin (Asadu, 2015). Administratively, the country is divided into six geopolitical zones (North-West, North-East, North- central, South-West, South-East, and South-South) that consists of 37 states and 774 local government areas (LGAs) (Ofem, 2012) (Fig. 1.4.1a). The population is estimated to be over 162,000,000 (Etebong, 2018) people, of which 48% live in urban areas (Ofem, 2012) while majority live in the rural regions. It is the fourth largest exporter of crude oil, despite this, majority of its population live in poverty with an estimated 71% living under a dollar per day (WHO, 2007). More than 70% of the population are engaged in agricultural practices as their primary source of livelihood (Asadu, 2015). The country has an estimated 45.3 million livestock of which 23 million are goat, 12.3 million cattle, 9 million sheep, and 1 million pigs, with over 90% of them in the savannah region (Fasanmi & Onyima, 1992).

The topography is made up of lowlands and highlands. The lowlands stretch from the Sokoto and Borno plains, to the coastal regions of the west and the Cross-River basin in the east. While the highlands extend from 600 to 1300meters in the north-central such as Plateau and Adamawa states, to the east highlands of Oban hills and Obudu Plateau in the southeast region (NPC, 2014). Nigeria has a tropical climate made up of wet and dry seasons. Its climate is dependent upon the rain-bearing southwest winds, and the cold, dry, dusty winds of the northeast commonly referred to as Harmattan. The dry season occurs from October to March, while the wet season is from April to September. The average temperature ranges between 25°C or less for the highland regions, and up to 40°C in the northern regions, particularly areas that border the Sahara Desert such as Borno. The annual rainfall ranges

from <600mm in many parts of the North, to 2650mm in the south and east regions (NPC, 2014).

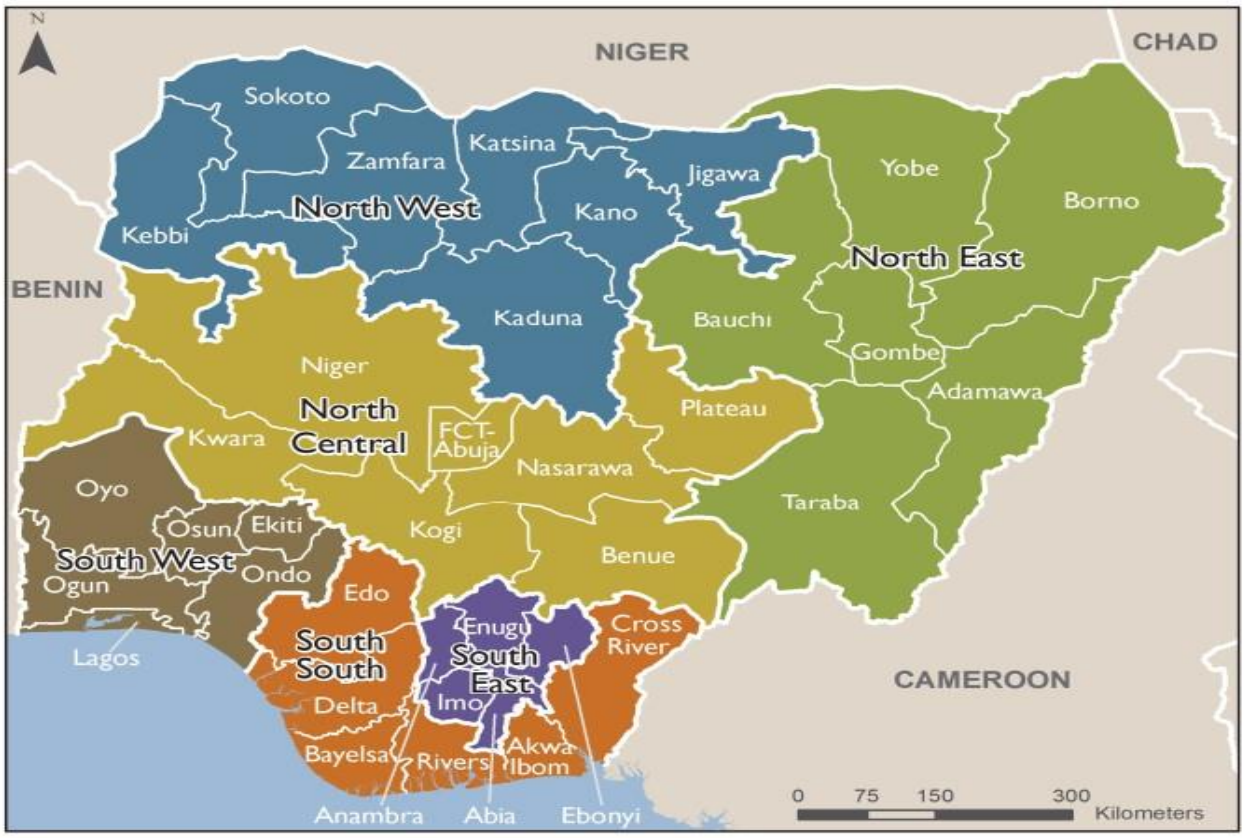


Figure 1.3.1(a): Map of Nigeria showing the six geopolitical zones and the neighbouring countries (Adapted from Nigeria Population Commission, 2014).

1.4.1(a) Plateau state

Plateau state is situated in the North Central region of Nigeria (locally known as the middlebelt) at latitude 80°24N and longitude 80°32 and 100°38E (Folorunso & Makwin, 2022). The state lies between the predominantly Muslim north and the majority Christian south, with the Berom's and Hausa/Fulani's being the predominant tribes. Their economy is largely centred around agriculture and pastoralism (Krause., 2011). The state is made up of 17 (LGAs) such as the study sites Jos (state capital), Kanke, Shendam, Bokkos, and Barkin-Ladi, etc.

With an estimated landmass of 26,899km^2 and over 3 million residents, majority of whom are into agricultural practices (Folorunso & Makwin, 2022). Its altitude ranges from 1200m to an optimum of 1828m above sea level in the Hilly regions around Jos. The mean annual rainfall range between 1347mm in the south, to 1460mm around the plateau areas, with the highest rainfall recorded between July and August (Kosshak et al., 2020). Although, the rest of the country experiences a much temperate climate, Plateau has an average temperature between 18 and 22°C, with the lowest temperature occurring between December and February during the harmattan period (Kosshak et al., 2020). The low temperature has led to a reduction in the incidence of some endemic temperate diseases such as malaria particularly during low these three months (https://www.plateaustate.gov.ng/plateau/at-aglance). In fact, a study reported a cyclical trend of malaria in the state with the lowest incidence occurring during the coldest months compared to other parts of the country (Nanvyat et al., 2018). Similar trends were also reported in Ethiopia where a significant correlation was observed between malaria with temperature, relative humidity, and rainfall (Midekisa et al., 2015),

1.4.1(b) Borno state

Borno state is in the North-East region of Nigeria between longitude 110°3 and 140°4E and latitude 10°0 and 14°0N (Idrisa et al., 2012). The state is the largest in the country with an estimated landmass of 69,436km^2, and an estimated 5,860,200 population size, majority of whose livelihood depends on agriculture and pastoral farming (Fausat & Naphtali, 2014). The state is made up of 27 LGAs such as our study sites Maiduguri (state capital), and Jere, etc., with an estimated population of 521,492 and 211,204 respectively (Ahmed & Abah, 2014; Idris et al., 2009). The state occupies the largest part of the Chad Basin, and shares border with Chad, Niger, and Cameroon (https://bornostate.gov.ng/about-borno/). The state is divided by three ecological zones; the northern guinea savannah (NGS) in the southeast, to the

Sahel in the north, and the Sudan savannah zone in the south where majority of the state lies in (Amaza. et al., 2006). The farming in the state is largely dependent on the level and duration of the wet season that continues to decline each year due to drought, with an estimated precipitation level of 600mm in the north and 1200mm in the south (Amaza. et al., 2009). The climate is distinguished by high temperatures and low humidity for most part of the year, with an average daily temperature of 34.7°C. The hottest temperature of 40.5°C occurs in April, while the lowest temperature between 13°C and 12°C occurs during December and January respectively (Mohammed & Alibaba, 2018).

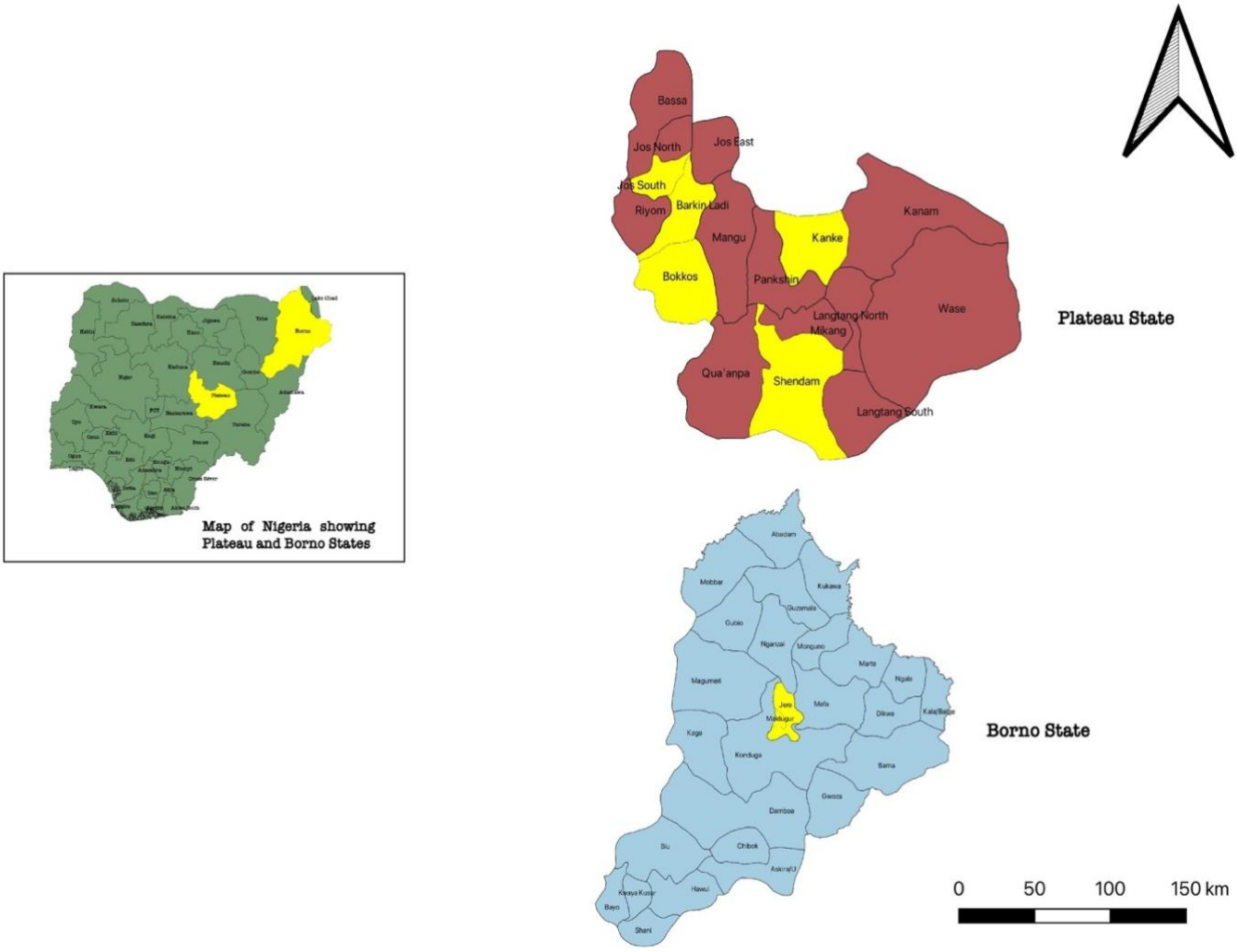


Figure 1.4.1(b): Map of Nigeria showing Plateau and Borno states, and the study locations. Map was produced using QGIS version 3.0.

1.4.2 Ethiopia

Ethiopia is situated in East Africa between latitude 3° and 15°N and longitude 33° and longitude 48°E with an estimated landmass of 1.13 million km^2. The country is characterised by a broad range of elevation from ~125m below sea level in the Afar region in the east to 4533m in the mountain highland regions of the north due to its complex topography and positioning (Ababa, 2005; Berhanu et al., 2014). The region is characterised by diverse climate and vegetation such as the humid and warm lowland rainforest in the southwest, to Afroalpine forest in the southeast and north, and the semi-arid woodlands in the east (Asefa et al., 2020). The climate is tropical with a rainfall of high spatial and temporal variability (Alemayehu et al., 2020). The annual rainfall ranges from ~141 to 500mm in the arid, semiarid areas, and lowlands to ~1200mm to 2275mm in the highlands (Berhanu et al., 2013; Hijmans et al., 2005), with the most rainfall occurring between June to August (up to 350mm monthly), while lesser rainfall is from October to December (~100mm/month) (Berhanu et al., 2014). The climate of the country is diverse based on the multi temperature and altitude of its five climatic zones. The annual temperature in the cold to moist zone (Wurich) is less than 11.5°C, cool to humid zone (Dega) is 11.5–17.5°C, cool to sub-humid. (Weynadega) is 17.5–20°C, warm semi-arid zone (Kola) is 20–27.5°C, and the hot-arid zone (Berha) is above 27.5°C (NRMRD, 1998).

Around 30% of the population reside in the rural areas of the arid and semi-arid regions where agriculture and livestock farming are the major source of livelihood (Beyene & Meissner, 2010; Fita et al., 2012). In fact, Ethiopia has the biggest livestock population in Africa which includes 60 million cattle, 42 million sheep and goats, 57 million poultry, 1 million camels (CSA, 2017) and 1.4 million horses (Pegram et al., 1981). Livestock trading makes up ~15% of the total yearly export earnings and 36% of the total agriculture GDP of the country (Leta & Mesele, 2014).

1.4.2(a) Eastern Afar State

The eastern Afar state is a pastoral region is in the north-east of Ethiopia between latitude 8°49’ to 14°30’N and longitude 39°34’ to 42°28’E, and it is characterised by its arid and semiarid climatic weather as previously stated (section 1.4.2). The state is bordered by Djibouti in the east, and Eritrea in the north-east, with an average rainfall of 150mm and 500mm below sea level in the arid and semi-arid regions respectively (Mamo et al., 2013). The population size is pegged around 1.5 million majority of whom are pastoralists by profession, with an estimated 1.9 million Afar bred cattle of which 90% is under the control of the pastoralists (ANRS, 2011). The pastoralists own 42% of the country’s livestock in the arid and semi-arid regions. The vast pastureland and rivers in these areas promotes the breeding of livestock such as cattle, goat, sheep, camel, and coexisting with wildlife such as zebra, warthog, etc. Areas such as Amibara (our study site), Dubti, etc. are major regions for the country’s cotton production, which after every harvest also becomes grazing and feeding fields for these animals (Mamo et al., 2013). Ecological niche like this plays a vital role in the maintenance of *Borrelia* in nature due to the abundance of tick vectors, because of the large presence of their animal host in the environment.

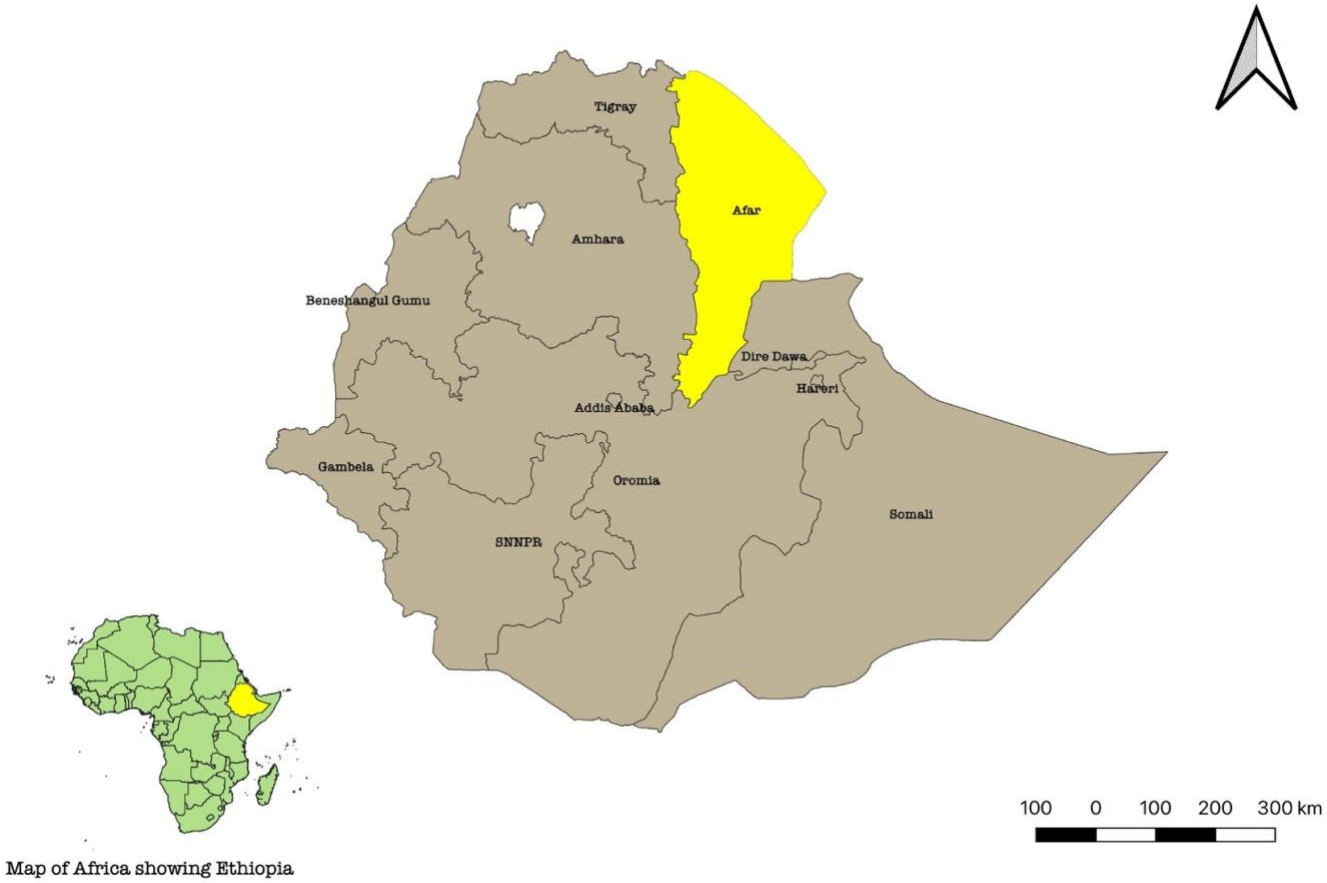


Figure 1.4.2: Map of Africa showing Ethiopia, and map of Ethiopia showing the study location. Map was produced using QGIS version 3.0.

CHAPTER 2: NIGERIAN TICK-BORNE RELAPSING FEVER STUDIES

2.1: Introduction

In Nigeria, there is considerable presence of tick fauna particularly ixodid ticks of the genera *Amblyomma*, *Hyalomma*, and *Rhipicephalus* (nee *Boophilus*) that are the most widely spread and destructive species of farm animals (table 1) (Dipeolu, 1975; Lorusso et al., 2013). However, studies on soft ticks are still porous. Around 40-50% of the livestock in the country are believed to be infested by ticks (Fasanmi & Onyima, 1992) with the highest infestation found in male animals 36.9% (735/2175) vs 33.8% (360/975) (Opara & Ezeh, 2011), 64% (102/159) vs 61% (28/46) (H. I. Musa et al., 2014). This disparity between male and female livestock could be attributed to the breeding pattern in the tropical regions where male animals are mostly used for farm activities and they must forage for long distances, hence, the likelihood for tick attachment. Whereas female livestock are mostly kept for breeding purposes as well as their by-products such as milk production (H. I. Musa et al., 2014). However, some studies in Asia contradicts this as female livestock were observed to have a higher infestation rate compared to the male 78% vs 65% (Sarkar et al., 2010), 69.74% (83/132) vs 41% (49/132) of which the free range bred accounted for 94% (125/132) of the total infested animals (Rony et al., 2010). These ticks are culpable in the spread of livestock diseases such as rickettsiosis, babesiosis, ehrlichiosis (cowdriosis), anaplasmosis, etc., that are the most important livestock diseases (Dipeolu, 1975; Leeflang & Ilemobade, 1977; Pullan, 1980). In addition, these ticks are also competent vectors for *B. theileri* the agent of bovine relapsing fever in livestock. Risks for these diseases varies among animals with exotic livestock known to be highly susceptible, while the impact on indigenous animals is still ambiguous (Ajayi et al., 1982). A study reported *Anaplasma* infection in ticks from the above genera collected from cattle as 11% (15/136), while *Rickettsia* infection was 12.5% (17/136) (Reye et al., 2012b). Another study carried out in Plateau state reported a 100% infestation rate on all 228 cattle (60% adult) sampled during the latter part of the rainy season

and a total of 5011 Ixodid ticks from the *Amblyomma* (6.3%), *Hyalomma* (7.6%), and *Rhipicephalus* (82%) genera were recovered (Lorusso et al., 2013). This present study also reports the first documented evidence of a bovine relapsing fever agent *Borrelia theileri* in goats (section 2.3.4) that is primarily transmitted by some *Rhipicephalus* species (Trees, 1978).

Most *Rhipicephalus* species are widely distributed in the northern part of Nigeria including Plateau state due to the favourable environmental conditions such as the topography (highlands/semi-highlands) and annual rainfall of above 800mm where these ticks strive best (Pegram et al., 1981). While (Okwuonu et al., 2017) reported a 60.9% (930/1528) infestation rate of *Hyalomma spp*., 32% (488/1528) *Rhipicephalus spp*., and 6.4% (97/1528) *Amblyomma spp*. in cattle. Approximately 90% of the country's livestock are bred under traditional normardic method by the Fulani pastoralists in the northern part of the country (Awogbade, 1979; Fasanmi & Onyima, 1992). This management style means livestock are exclusively grazed in open pastures and forests, which increases the likelihood of tick infestation on the animals particularly those from the previously mentioned genera (Fasanmi & Onyima, 1992). The study of (Pukuma et al., 2011) reported higher rate of tick infestation among Adamawa (193/434) and Sokoto (166/386) gudali cattle that were bred free range compared to the Bunaji cattle (113/298) that were restricted. Tick infestations on livestock is at the highest one month (May and June) after the heavy rains (April to October) and lowest during the dry months of November to February (Bayer & Maina, 1984; Maina, 1986; Pukuma et al., 2011) (Fig. 2.1). This could be because of robust vegetation cover provided during the wet season that creates a conducive environment for the ticks. They are known to drop off host and moult whilst awaiting another suitable host on the vegetation blade to intestate. In addition, the incidence of the previously mentioned livestock diseases follows the same pattern as the tick infestation, with the highest infection rate recorded during these peak wet months (Fasanmi & Onyima, 1992). The pastoralists are aware of the hazards posed by ticks to their herds particularly the adult animals, which they manage by hand

removing manually trice a week during the rainy season and twice during the dry season (Maina, 1986; Pullan, 1980). This method of tick management is still predominant amongst pastoralist compared to the use of acaricides or dip tanks (Bayer & Maina, 1984; Lorusso et al., 2013).

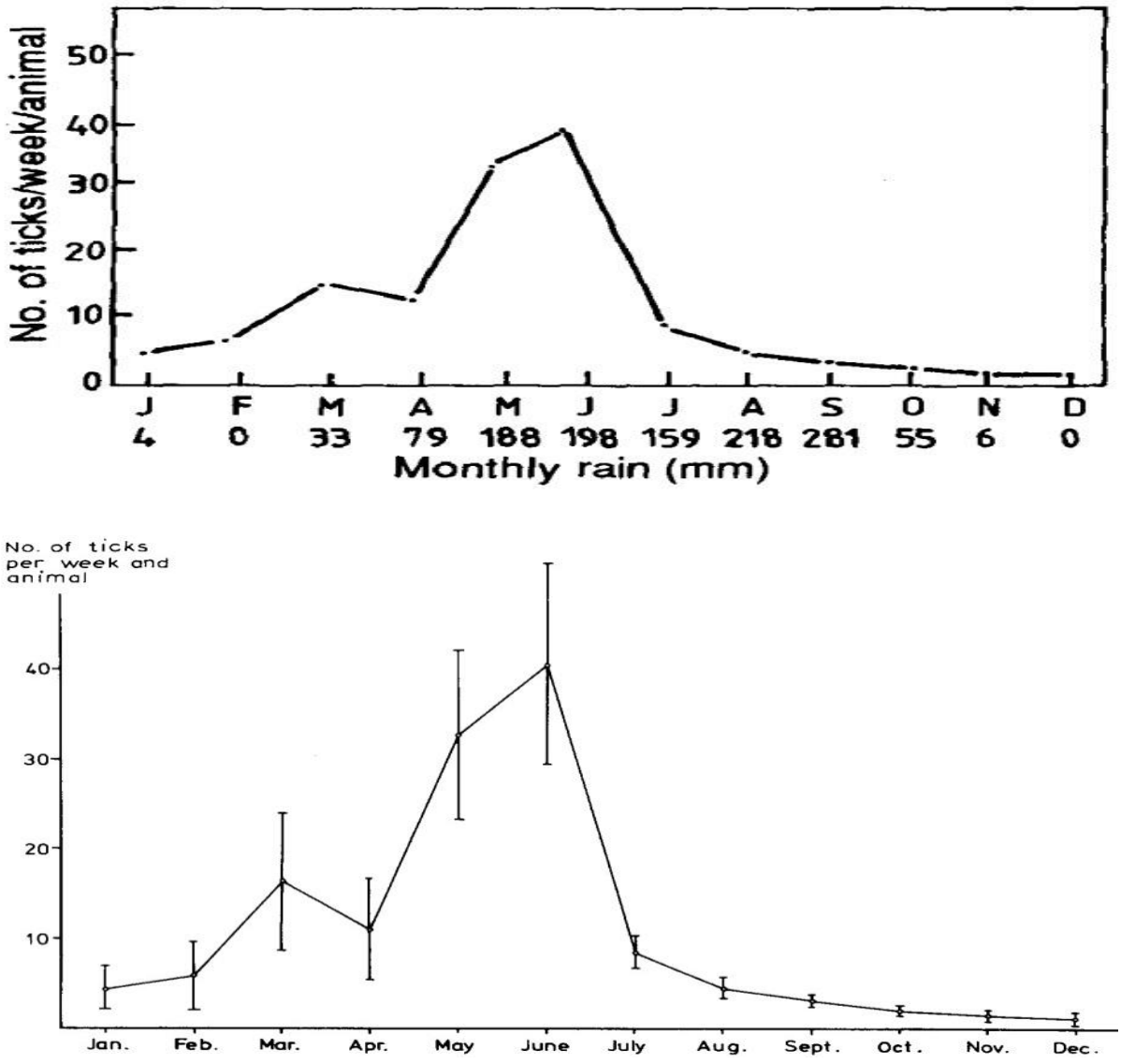


Figure 2.1: Average tick infestation on cattle in the sub-humid region of Nigeria. (Adapted from Bayer and Maina, 1984).

Table 2.1.1: Tick fauna in livestock

Hard tick species	Infestation rate in livestock	Tick specie vs total no of ticks harvested	Specie identification method	References
R. decoloratus *R. annalatus* *R. geigyi* *R. guihoni* *H. truncatum* *A. variegatum* *Rhipicephalus. spp*	Cattle 228/228 (100%)	1890/5011 819/5011 306/5011 434/5011 681/5011 361/5011 494/5011	Morphology	(Lorusso et al., 2013)
A. variegatum *R. sanguineus* *A. variegatum* *Hyalomma spp*.	Cattle 130/205 (63%)	N/D	Morphology	(H. I. Musa et al., 2014)
A. variegatum *Hyalomma spp*. *R. microplus* *D. variabilis*	Cattle (1095/3150) (35%)	480/3150 270/3150 240/3150 105/3150	Morphology	(Opara & Ezeh, 2011)
Amblyomma spp. *Rhipicephalus spp.* *Hyalomma spp.* *Boophilus spp.*	Cattle (30/43) Sheep (12/45) Goat (24/45) Pigs (26/44)	257/626 17/626 125/626 70/626	Morphology	(Omudu & Amuta, 2007)
A. variegatum *H. truncatum* *R. decoloratus* *R. sanguineus*	Cattle (299/500) (60%)	798/2119 395/2119 453/2119 473/2119	Morphology	(Bura-Thlama et al., 2017)

Amblyomma spp *Rhipicephalus spp* *H. leachi* *B. decoloratus* *Hyalomma spp*	Cattle (1800/2000)	719/5391 1442/5391 437/5391 1174/5391 1583/5391	Morphology	(James-Rugu & Jidayi, 2004) (James-Rugu & Jidayi, 2004)
Amblyomma spp *B. decoloratus*	Sheep (25/500)	473/947 474/947	Morphology	
A. variegatum *B. microplus* *Hyalomma spp* *D. variabilis*	Cattle (1095/3150) (35%)	480/1095 240/1095 270/1095 105/1095	Morphology	(Opara & Ezeh, 2011)
Rhipicephalus spp *Boophilus spp* *A. variegatum* *H. leachi*	Cattle (305/317) (96%)	153/347 126/347 50/347 18/347	Morphology	(Ameen et al., 2014)
Rhipicephalus spp *Boophilus spp* *A. variegatum* *H. leachi*	Goat (170/210) (80%)	83/204 31/204 25/204 6/204	Morphology	
Rhipicephalus spp *Boophilus spp* *A. variegatum* *H. leachi*	Sheep (70/104) (67%)	39/85 31/85 10/85 5/85	Morphology	
A. variegatum	Cattle	135	Morphology	(Kamani et al., 2011)

Note: ND (not disclosed)

Ticks are also common ectoparasites of domestic animals such as dogs and only second to mosquitoes (Hoogstraal, 1956a), where they transmit haemoparasitic infections like *Babesia canis*, *Haemobatonella, Ehrlichia*, Anaplasmosis, borreliosis, that are of significant health concerns particularly the ones that pose a zoonotic risk (Okubanjo et al., 2013; Patz et al., 2000; Robertson et al., 2000). In Nigeria, ixodid tick infestation in dogs is endemic, with the infestation pattern like that of livestock and other farm animals, with the highest infestation observed during the rainy season (Arong et al., 2011). Ticks of the genera *Rhipicephalus* (formerly *Boophilus*)*, Hyalomma*, and *Amblyomma* are the most prevalent ectoparasites of dogs in Nigeria (table 1), with *R. sanuineus* (brown dog tick) being the most prevalent due to its host preference (Oguntomole et al., 2018). However, there is no evidence of *Ixodes spp*. of medical or veterinary importance documented (Kamani et al., 2013).

Table 2.1.2: Tick infestation in Dogs from Nigeria

Hard tick species	Infestation rate in dogs	Tick specie/total no of ticks collected	Identification tool	References
R. sanguineus *Haemaphysalis. leachi* *Boophilus. decoloratus*	155/300 (51.7%)	465/1146 297/1146 384/1146	Morphology	(Shitta et al., 2018)
Boophilus. sp. *R. sanguineus* *Hyalomma sp.* *A. variagetum*	352/400 (88%)	3712/4216 456/4216 36/4216 12/6216	Morphology	(Konto et al., 2014)
H. leachi *R. sanguineus*	ND	77/258 181/258	Morphology	(Kamani et al., 2013)

R. sanguineus *Rhipicephalus sp*.	66/100 (66%) 9/100 (9%)	107/146 9/146	Morphology	(Adamu et al., 2014)
H. leachi	27/100 (27%)	27/146		
Amblyomma sp. *Rhipicephalus sp.* *Boophilus sp.*	25/44 (56.8%)	38/626 69/626 50/626	Morphology	(Omudu & Amuta, 2007)
R. sanguineus	160/200 (80%)	980	Morphology	(Abah & Audu, 2014)
R. sanguineus H. leachi Other hard tick species	199/202 (98.5%)	847/1358 451/1358 60/1358	Morphology	(Agbolade et al., 2008)
Rhipicephalus sp. *H. leachi* *A. variagetum* *B. decoloratus*	208/230 (90.4%)	673/3100 215/3100 728/3100 1265/3100	Morphology	(James-Rugu & Jidayi, 2004)

Note: ND (not disclosed)

Tick infestation have been described in camels; 30% (51/170) of camels infested with 197 *Hyalomma sp.* (Kamani et al., 2015), 71% (1138/1600) infested with 3620 ticks of which 876 were *B. decoloratus* and 2744 were *Hyalomma sp* (James-Rugu & Jidayi, 2004).

Nigeria is a country were cohabiting with farm animals is common practice particularly in the rural regions, and with the high level of ixodid tick distribution in animals, the likelihood of humans being at higher risk of tick-borne diseases cannot be overlooked.

In comparison to the studies conducted on ixodid ticks, there is scarcity of literatures on soft ticks and their importance in clinical and veterinary medicine in Nigeria. Few studies have described the presence of soft ticks such *Argas persicus*, *O.savignyi*, *O. moubata* in domestic birds and semi-wild domestic using entomological tools (table 3). More recently,

(Cutler et al., 2018) reported the presence of 47% (23/49) *O. savignyi* ticks around animal and human dwellings using molecular tools. This current study presents the first molecular identified *A. persicus* ticks collected around poultry farms.

Table 2.1.3: Description of soft ticks in Nigeria

Soft tick species	Infestation rate in animals/total no of animals	Tick specie/total no of ticks collected	Identification tool	References
Argas persicus	Chicken (3.6%) Guinea fowl (3%)	9/253 7/234	Morphology	(Okaeme, 1988)
A. persicus *A. walkerae* *O. moubata* *O. savignyi*	Chicken (90/150) (60%)	38/61 20/61 3/61 0	Morphology	(Bunza et al., 2008)
A. persicus *A. walkerae* *O. savignyi* *O. moubata*	Guinea fowl (45/150) (30%)	16/28 6/28 2/28 4/28		
A. persicus	Chicken (66/200) (33%)	ND	Morphology	(Usman et al., 2012)
O. savignyi	N/A	23/49 (47%)	Molecular	(Cutler et al., 2018)
A. persicus	*Meleagris gallapavo* (domestic turkey) (8/300) (2.67%)	ND	Morphology	(Lawal et al., 2019)
O. savignyi	N/A	267	Molecular	This study
A. persicus	N/A	138	Molecular	This study

A. persicus	*Meleagris gallapavo* (domestic turkey) (132/265) (50%)	ND	Morphology	(Fabiyi et al., 2017)

Note: ND (not disclosed), NA (not applicable)

Argas species are one of the important ectoparasites of poultry in Nigeria that can hamper productivity (meat, protein production) due to morbidity, and in severe cases leads to fatality because of anaemia (Bunza et al., 2008). Poultry is known to be the most important source of protein in Nigeria (Usman et al., 2012). and possibly elsewhere. Poultry farming in the country is either done by raising indigenous breeds (free range birds) or semi-intensive breeds (partially restricted birds) that constitutes a major aspect of farming in developing nations including Nigeria (Copland & Alders, 2005). Tick infestation pattern in poultry is also like that of cattle, with the free range bred birds having the highest infestation rate (66/100) compared to the commercial bred restricted birds (0/100) (Usman et al., 2012).

In addition, these ticks are culpable in the transmission of *Borrelia anserina* an agent of avian spirochaetosis in poultry (Leeflang & Ilemobade, 1977). Historically, *Borrelia anserina* infection was reported in domestic chickens, and fatality due to spirochaetosis was documented in Muscovy ducks in a north-western village where *A. persicus* was present (Barnes, 1975).

On the other hand, the role of *Ornithodoros spp*. as vectors of clinical and veterinary importance particularly for TBRF is still sketchy, with the only confirmed report being *C. Borrelia kalaharica* an agent of relapsing fever in humans described in its *O. savignyi* tick vector (Cutler et al., 2018). There was an unconfirmed report of infection with a 99% *B. burgodoferi* sensu lato specie in 0.4% (3/700) questing *R. evertsi* ticks using the 16S and flagellin genes, however, specie identification was impossible due of poor sequences (Reye et al., 2012b). Unrelatedly, two studies on *Ornithodoros* ticks described the presence of *O.*

moubata antibodies in pigs infected with African swine fever (AFSV) that is known to be endemic in the country (Ayas et al., 2016; Luka et al., 2017).

Clearly, there is a need to upscale the research on soft ticks and associated pathogens. More specifically, the role of these ticks in the transmission of TBRF needs to be investigated to increase awareness on their disease, as well as and putting in place adequate intervention and control measures against tick bites in humans and animals alike.

The objectives of the study on animals were to determine:

- The identification and diversity of soft ticks' fauna
- The identification and diversity of *Borrelia*

2.2 MATERIALS AND METHODS

2.2.1 Tick sampling

To determine the role of Argasidae ticks as possible agents of TBRF transmission, soft ticks previously collected around cattle and poultry farms in Plateau state were obtained from the laboratory of the Nigerian Veterinary Research Institute (NVRI), Vom. In addition, soft ticks previously collected around camel shelters from Borno state were also obtained from the same institution. A total of 318 *Ornithodoros spp*. and *Argas spp*. (adults and nymphs) were obtained from both states (table 2.2.1) and transported to the UK under the DEFRA licence (ITIMP19.0310). The minimum sample size was determined based on the prevalence of 6.1% reported in pooled ticks (Cutler et al., 2018) using the formular $n = Z^2 \frac{4pq}{d2}$ where q is (1-p) (Cochran, 1977).

- Z or Z score is based on the confidence level at 95% or 1.96
- p is the assumed population proportion of the variable of interest (level of infection)
- d is the margin of error set at ±5% or 0.05
- 100% attrition rate was allowed to obtain the minimum sample size required because the reference study was conducted on unspecified number of pooled ticks.

z= 1.96, p= 6.1% or 0.061, q=1 - 0.061 (0.939), $d=0.05^2$

$$= \frac{(1.96)^2 \text{ X } (0.061) \text{ X } (0.939)}{(0.05)^2}$$

$$= \frac{0.2200}{0.0025}$$

= 88 + 88(100% of 88)

= 176 (minimum sample size)

Ticks from Plateau state were sampled from Doemak, Qua'an Pan local government area LGA at latitude 8.6377N and longitude 9.1013E, and Vom, Jos South LGA at latitude 9.7376N and longitude 8.8087E. While ticks from Borno state were sampled from Maiduguri at latitude 11.8311N and longitude 13.1510E (chapter 1, section 1.4.1b).

Table 2.2.1(a): Soft ticks sampled from Nigeria

Sampling location	Tick specie	Total number of ticks	Developmental stage		
			Adult	Nymph	Larvae
Doemak, Qua'an pan, Plateau	*Ornithodoros* specie	25	23	2	0
Vom, Jos South, Plateau	*Ornithodoros* specie	45	44	1	0
Vom, Jos South, Plateau	*Argas* specie	211	124	87	0
Maiduguri, Borno	*Ornithodoros* specie	37	25	12	0

Upon receipt, each tick was visually inspected to ensure intactness, rinsed with phosphate buffered saline (PBS) and placed into individual labelled tubes according to their sizes. Ticks were assigned into four developmental stages based on the length of the body as described previously (Kisinza, 2006).

- Stage 1 (Larvae): <3mm in length
- Stage 2 (Nymph 1-2): ~3.1–6mm
- Stage 3 (Nymph 3-4): ~6.1–9mm
- Stage 4 (Adult): >9mm (table 2.2.1b)

Table 2.2.1(b): Classification of ticks into developmental stages

Tick life stages	Tick specie	Body length	No. of ticks (N=215)
Stage 1	*Ornithodoros sp.*	2	
	Argas sp.	14	16
Stage 2	*Ornithodoros sp.*	3	
	Argas sp	18	21
Stage 3	*Ornithodoros sp*	6	
	Argas sp.	55	61
Stage 4/Adult	*Ornithodoros sp.*	66	
	Argas sp.	124	190

2.2.2 DNA extraction of ticks

Each tick was rinsed in phosphate buffered saline (PBS), air dried, and halved into equal halves using individual disposable scalpels. Each halved tick was placed into designated plain tubes containing homogenising beads (Matrix H) (MP biomedicals) and freshly made PBS (200µl for stages 3–4, 100µl for all other ticks). The other halved ticks were stored in 70% ethanol at -20°C for re-extraction purposes if required. Tubes containing ticks were then crushed at a speed of 6.5min/sec for 5 cycles at 1min/cycle depending on the tick size, using the FastPrep-24® 5G (MP biomedicals) tissue homogenizer. Ticks were visibly checked after each cycle to ensure crushing was successful, otherwise, another round of homogenisation was done. Once completed, homogenates were pulsed briefly centrifuged for 1min at 6000g to collect samples at the bottom of the tubes. Homogenates were then resuspended in proteinase K and lysis buffer at a volume of 1:10 (10µl in 90µl for smaller and 20µl in 180µl for bigger ticks). Resuspended ticks were incubated overnight at 56°C in the water bath as previously described (Cutler et al., 2018; Elelu et al., 2022). DNA extraction was carried out using the DNeasy blood and tissue kit according to manufacturer's guidelines (Qiagen, Hilden, Germany®) (complete DNA extraction protocol in appendix 3). Extracted DNA were quantified based on the A260/280 and A260/230 ratio using the

Nanodrop the ND-1000 spectrophotometer (Thermo Scientific®) before freezing at -20°C pending analysis.

2.2.3 Animal sampling

To determine if the soft ticks are also of veterinary importance, cattle, goat, sheep, and dogs were sampled from Plateau and Borno States during April and May 2019. Animal sampling was carried out during this period after the dry season had ended, and the beginning of the rainy season. Ticks including the agents of TBRF have difficulty surviving during the dry season months of November to March, when the vegetation is hot and dry, and in some cases burnt (Ogo et al., 2012). In addition, tick infestation in animals is at the highest during the wet period as stated previously, which can increase the likelihood of infection with borreliae. A total of 622 animal sera were obtained from both states (table 2.2.3). Two hundred and five cattle were sampled from Bokkos (9°19'024N-8°55'197E; 9°19'078N-8°54.891E), Barkin-Ladi (9°31'334N-8°49'014E; 9°26'292N-8°56'289E) and Jos-South (10°8'679N-8°59.766E) LGAs, and 5ml blood were obtained from each cattle via jugular vein puncture using individual syringes.

Concurrently, 200 goats were also sampled from Jos-South (9°32'00N-8°54'00E), Shendam (8.8955°N, 9.4537°E), and Kanke (9.3702°N, 9.5896°E) in Plateau state. While in Borno state, 145 cattle blood were obtained from Jere LGA at latitude 11.8469N and longitude 13.15712E (figure, 2.2.3). Seventy-two dog sera previously collected from local dogs from Barkin-Ladi, Plateau state were obtained from the laboratory of the NVRI, albeit one leakage occurred during transportation reducing the total to 71. The minimum sample size of 118.4 was determined based on the prevalence of 4.1% in cattle from Zambia (Qiu et al., 2021) using the formular and parameters from section 2.2.1.

z= 1.96, p= 4.1% or 0.041, q=1 - 0.041 (0.939), $d=0.05^2$

$$= \frac{(1.96)^2 \text{ X } (0.041) \text{ X } (0.939)}{(0.05)^2}$$

$$= \frac{0.15110}{0.0025}$$

= 59.2 + 59.2(100% of 60.4)

= 118.4 (minimum sample size)

Table 2.2.3: Distribution of animal sampled from Plateau and Borno states between April and May 2019.

State	**Plateau**					**Borno**	
Sampling location	**Shendam**	**Kanke**	**Jos-South**	**Bokkos**	**BarkinLadi**	**Jere**	**Total**
Cattle	NC	NC	70	62	73	145	350
Goat	60	74	66	NC	NC	NC	200
Dog	NC	NC	NC	NC	71	NC	71
Total	85	65	120	62	144	145	621

Note: **NC** (not collected).

Figure 2.2.3: Livestock farms in Plateau and Borno states where animals were sampled between April and May 2019.

Animal blood were temporarily stored in boxes containing wet ice during sampling before transferring to the Parasitology department of the NVRI, while animal blood from Borno were stored at the State Specialist Hospital, Maiduguri prior to transport to NVRI. Bloods were left to clot in the syringes before transferring each serum into sterile 2ml tubes and stored at -20°C. Prior to transport to the UK, all serum samples were heat inactivated at 56°C for 30mins and placed in the approved UN bio-bottle pathopack to prevent leakage. Upon arrival in the UK, DNA were extracted from all animal sera by using 100µl of each animal sample. Each 100µl animal sera were digested in 20µl proteinase K, and the final volume adjusted to 220µl with PBS before incubating for 1hour at 56°C in the water bath to allow the breakdown of all cellular material. All other DNA extraction steps are described in (appendix 3).

2.2.4 Tick DNA Amplification for Species Identification

A 450bp tick amplicon was amplified using tick 16S rRNA primers previously described (Black & Piesman, 1994) (table 2.2.4). A final PCR volume of 25µl of each sample containing reaction buffer (1X), dNTPs (0.2mM each), $MgCl_2$ (2.5mM), 2µl of tick DNA template, 2.5 µl each primer at (5uM), and 0.15U taq DNA polymerase were analysed. Conventional PCRs were run on the Bio-Rad T100^{TM} thermal cycler using the following cycling conditions; denaturing at 94°C for 5mins, followed by 35 cycles of (94°C for 45sec, annealing of 50°C for 45secs, 72°C for 45secs), and a final extension of 72°C for 10mins. DNA of an *Ixodes ricinus* tick was used as the positive template, while nuclease free water was used as the negative template control. Amplified PCR products were viewed on 1.5% agarose gel electrophoresis stained

with SYBR safe (Invitrogen®), while a 100bp DNA ladder was used to determine the sizes of the amplicons (either Invitrogen®; or New England BioLabs®) at 100volts for 80mins. All

positive tick amplicons were subjected to PCR clean-up using the QIAquick® PCR Purification kit (Qiagen) that can isolate up to 10µg of DNA and between 100bp–10kb in sizes according to the manufacturer's guideline stated in (appendix 4). Purified PCR products were quantified on Nanodrop to determine the final DNA purity level. Samples were run on 1.5% agarose gel as previously stated, at a ratio of 1:5 with DNA loading dye (1X) (New England BioLabs™) (5µl of PCR product in 1µl of dye) to reconfirm the size of the amplicon.

PCR was performed using the Bio-Rad T100® thermal cycler, and amplicons were viewed using the Bio-Rad ChemiDocÔ MP imaging system. Amplified products at a volume of 10µl were each transferred into 0.5ml Eppendorf's, and tick 16S primers (forward and reverse) were each diluted to a final concentration of 3.2uM before submitting for Sanger sequencing to the DBS Genomics unit at Durham University.

Table 2.2.4: Primers and Probes used in this study.

Gene Target	Nucleotide sequences	Band Size	Reference
Flagellin B (flaB)	**First round:** F: TAATACGTCAGCCATAAATGC R: GCTCTTTGATCAGTTATCATTC **Nested:** F: GCTGAAGAGCTTGGAATGCAACC R: TGATCAGTTATCATTCTAATAGCA	**655bp** **350bp**	(Assous et al., 2006) (Takano et al., 2010)
16S rRNA	**First round:** F: GCGAACGGGTGAGTAACG R: CCTCCCTTACGGGTTAGAA **Nested:** F: GAGGCGAAGGCGAACTTCTG R: CTAGCGATTCCAACTTCATGAAG	**600-650bp**	(Zhai et al., 2017)
Tick 16S rRNA	F: CTGCTCAATGATTTTTTAAATTGC R: CCGGTCTGAACTCAGATCATGTA	**450bp**	(Black & Piesman, 1994;

			Dupraz et al., 2016)
RT-PCR 16S rRNA	F: AGCTTTAAAGCTTCGCTTGTAG R: GCCTCCCGTAGGAGTCTG P [**HEX**] CCGGCCTGAGAGGGTGACGG-BHQ1	**475bp**	(Diatta et al., 2016)

NB: BHQ 1 (black hole quencher) are traditional linear probes made of 20-30 bases in length, used to quench yellow and green dyes on HEX, FAM channels during the PCR amplification process.

2.2.5 Borrelial Screening of all Samples

All ticks and animal samples were screened for the presence of *Borrelia* by using an updated version of *Borrelia*-specific real-time PCR (qPCR) primers (Merck) designed to amplify a 148-bp fragment of the 16S rRNA of *Borrelia* (table 2.2.4) as previously described (Kingry et al., 2018). A final PCR reaction volume of 25µl containing reaction buffer (1X), dNTPs (0.2mM each), MgCl2 (2.5mM), 2µl DNA template, each primer at (0.6µM), probe (0.2µM), and 0.15U taq polymerase. Cycling conditions were as follow: 95°C for 5mins, 40cycles of 95°C for 15secs, and 60°C for 30secs as described (Kingry et al., 2018). DNA of *B. burgdoferi senso stricto* B31 strain was used as positive control and nuclease free water (Invitrogen) as negative control. Amplification was conducted using the Agilent AriaMx 1.2 Real-Time thermocycler. Samples with a cycle threshold (CT) value of £36 were considered as positive. All *Borrelia*-positive samples from the initial qPCR screening were retested and subjected to further confirmation on nested PCR using the flagellin B (flaB) and the 16S rRNA genes. Cycling conditions were as follow: 16S rRNA nested **first round:** 94°C for 4 mins, 35 cycles of 94°C for 30 secs, 50°C for 30 secs, 72°C for 45secs and a final extension of 72°C for 10mins; and for the **second round**: 94°C for 4 mins, followed by 30 cycles of 94°C for 30 secs, 60.2°C for 30 secs, 72°C for 35 secs, and a final extension of 72°C for 10 mins (Zhai et al., 2017); flaB nested **first round**; 30cycles of 94°C for 40secs, 63°C for 60secs, 72°C for 60secs; **second round**: 30cycles of 94°C for 40 secs, 50°C for 40secs, 72°C for 40secs (Takano et al., 2010).

Amplified products were viewed on 1.5% agarose gel and borrelial amplicons successfully generated were purified using the Qiaquick PCR purification kit (Qiagen), and DNA concentration measured using the nanodrop as previously stated. Amplification was conducted using the Bio-Rad T1000 thermal cycler, and amplicons were viewed using the Bio-Rad ChemiDoc^{TM} MP imaging system. Amplified products at a volume of 10µl were each transferred into 0.5ml Eppendorf's, and both *Borrelia* primers (flagellin and 16S nested forward and reverse primers) were each diluted to a final concentration of 3.2uM before submitting for Sanger sequencing to the DBS Genomics unit at Durham University.

2.2.6 Phylogenetic Analysis

The Borrelial infection in ticks and animals was determined using descriptive statistics, and statistical significance based on tick developmental stage was set at $p < 0.05$. All resulting sequences derived from this study were compared to those in the GenBank® using the basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST). Multiple sequences alignments were carried out using the MUSCLE program, and phylogenetic construction using the Neighbor-joining and Maximum Likelihood methods at a confidence test of 1000 bootstrap on MEGA 11 (Tamura et al., 2021).

2.3 RESULTS

2.3.1 Tick Identification

Of the 318 ticks investigated from Plateau and Borno states, 20 and 42 of the *Ornithodoros* and *Argas* ticks respectively yielded amplifiable products at 450bp on conventional PCR using tick 16S rRNA primers (table 2.3.1, Fig. 2.3.1a, 2.3.1b). Of these, a random convenience subset of ten *Ornithodoros* ticks from both states and 10 *Argas* ticks from Plateau were submitted for Sanger sequencing. The sequenced ticks contain ticks of all four developmental stages. BLAST analysis of the *Ornithodoros* 16S sequences revealed the tick identities as >97% similarity to *O. savignyi* KJ133578 (Mans & Neitz, 2004), and MG256662 (Cutler et al., 2018), from Sudan and Nigeria respectively (Fig, 2.3.1c). While the *Argas* 16S sequences revealed a 100% identity to *Argas persicus* GU451248 from Italy (Pantaleoni et al., 2010) and L34321 from the USA (Fig. 2.3.1d) (Black & Piesman, 1994).

Table 2.3.1: Tick Identification using the 16S rRNA gene

Sampling site	**Species**	**Amplified tick DNA**	
		Yes	**No**
Plateau	*Ornithodoros sp.*	13	57
Plateau	*Argas sp.*	31	180
Borno	*Ornithodoros sp.*	7	30

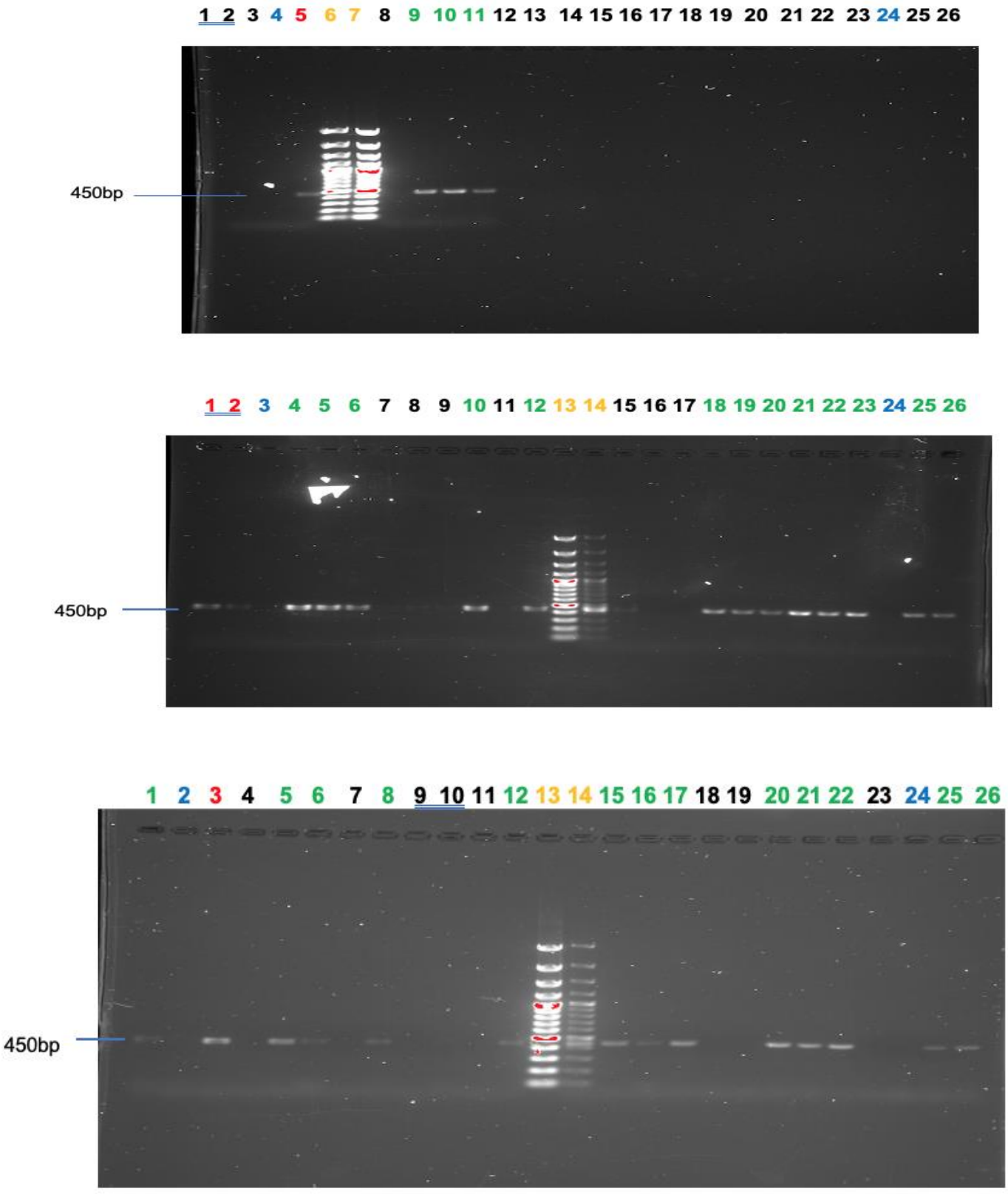


Figure 2.3.1(a): *Ornithodoros savignyi* tick DNA amplicons using 16S rRNA gene at 450bp viewed on 1.5% agarose gel. Positive control template in lanes with numbers highlighted in red, negative template controls in blue, DNA 100bp ladder in orange, amplified tick DNA lanes in l green, and ticks unsuccessfully amplified in black.

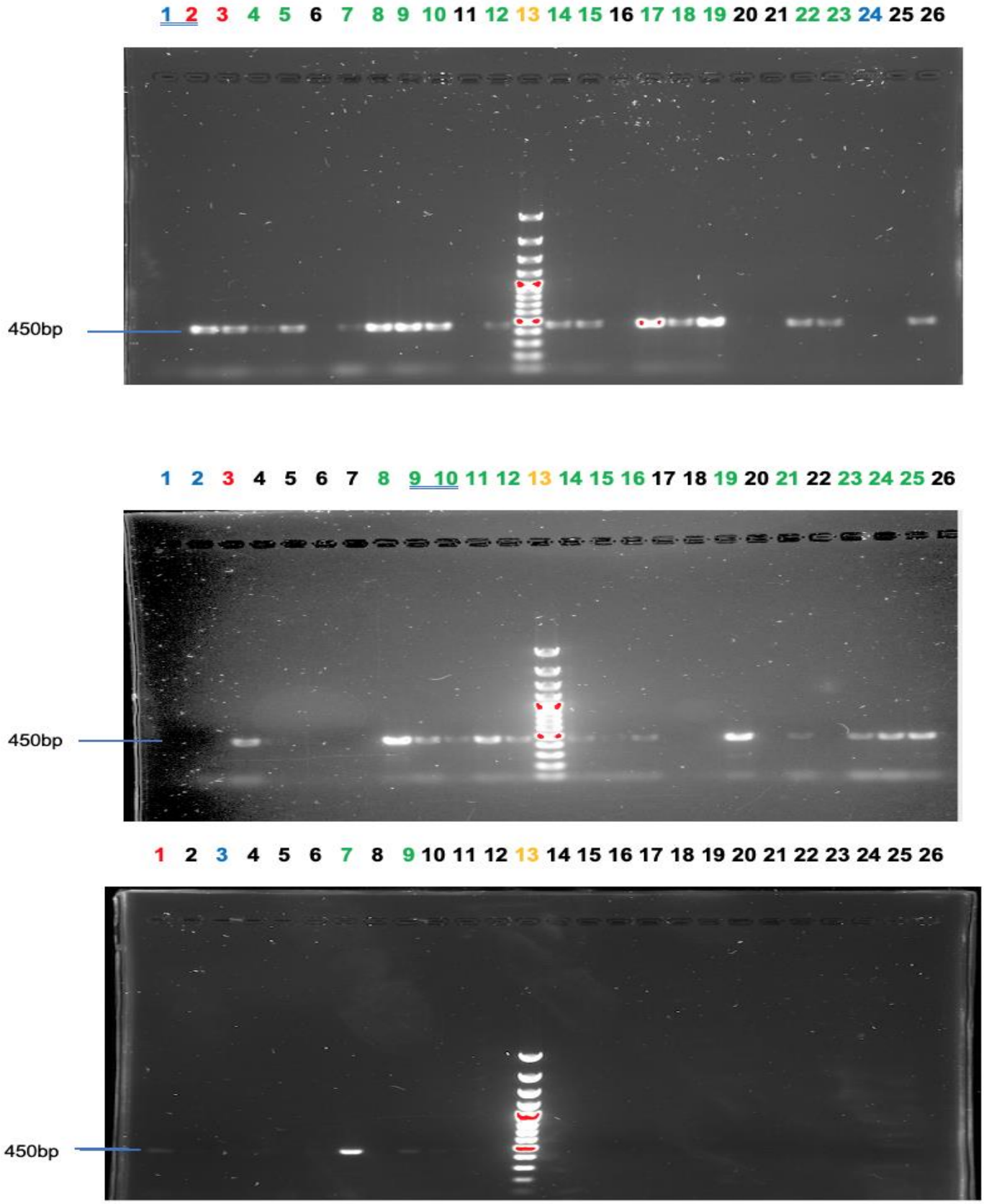


Figure 2.3.1(b): *Argas persicus* tick amplicons on 16S rRNA gene at 450bp viewed on 1.5% agarose gel. Highlighted number lanes are same as described in figure 2.3.1(a).

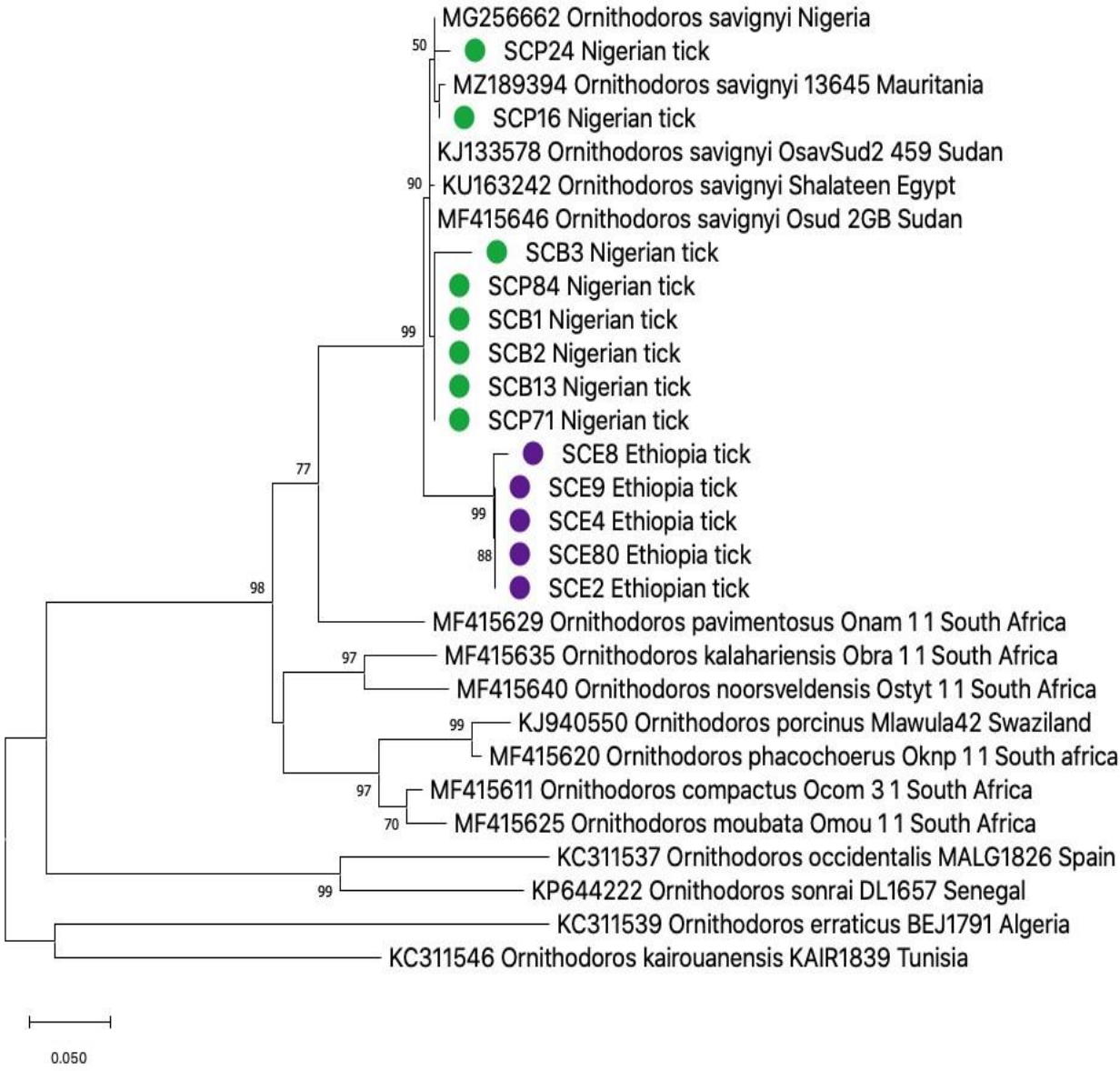


Figure 2.3.1(c): Phylogenetic analysis of Neighbor-joining tree of tick 16S rRNA sequences (450bp), comparing the Nigerian *Ornithodoros* ticks with other *Ornithodoros* species. The tree with the highest log likelihood (-1978.44) is shown. Evolutionary distances were calculated using the maximum likelihood method and Hasegawa-Kishino-Yano model in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The green circles represent the Nigerian while the purple circles represent the Ethiopian ticks from this study.

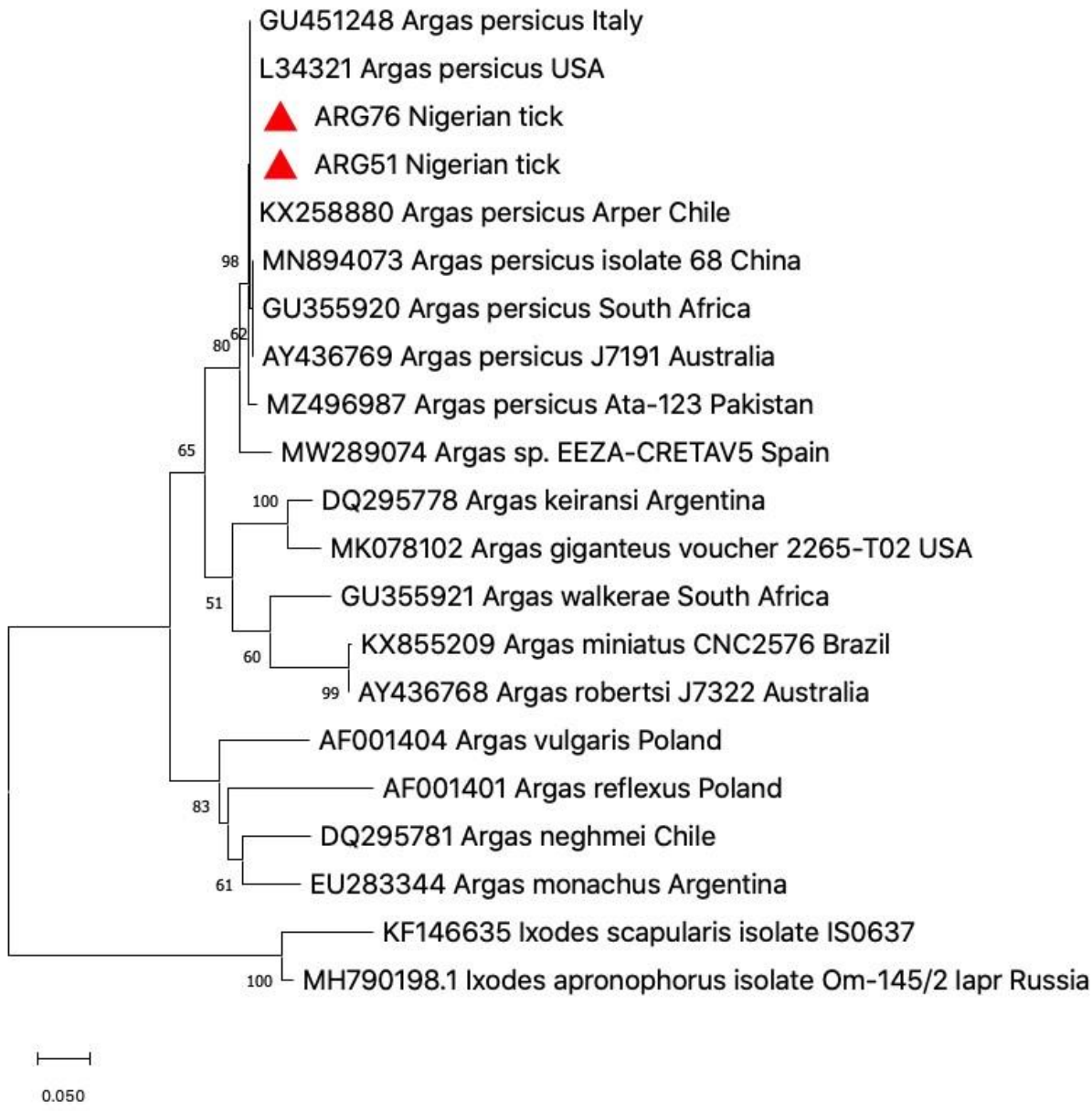


Figure 2.3.1(d): Phylogenetic analysis of Neighbor-joining tree of tick 16S rRNA sequences (450bp), comparing the Nigerian *Argas* tick with other *Argas* species. The tree with the highest log likelihood (-1667.99) is shown. Evolutionary distances were calculated using the maximum likelihood method and Hasegawa-Kishino-Yano model in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The red triangles represent the Nigerian and Ethiopian tick sequences respectively from this study.

2.3.2 Sequence heterogeneities in ticks

Using the 16S rRNA tick-specific gene target, few heterogeneities were observed among the *O. savignyi* tick sequences from Plateau (SCP71, SCP16, SCP24, SCP84) and Borno (SCB13, SCB2, SCB1, SCB3) state highlighted with black arrows (Fig. 2.3.2a). Most of the nucleotide polymorphisms were observed in sample SCB3 and SCP24 which is evident on the phylogenetic tree. Whereas no polymorphism was observed in the *A. persicus* sequences. Representative sequences for both tick species were deposited into the GenBank under accession numbers (*Ornithodoros* OP688109, OP688110, OP688112, OP688115; *Argas* OQ133461, OQ133462). In addition, the tick sequences between the Nigerian (SCP16, SCP24, SCBI, SCB3) and Ethiopian ticks (chapter 3, section 3.3.1) (SC2, SC4, SC8, SC9, SC46, SC80) revealed several polymorphisms at positions 86bp, 105bp, 148bp, 151bp, 180bp, 182bp, 210bp, 213-214bp, 223bp, 225-226bp, 234bp, 272bp, 276bp, 287bp, and 300bp (Fig. 2.3.2b). These sequence divergences caused a divide between the two countries as they formed separate clades on the phylogenetic tree. (Fig. 2.3.1c).

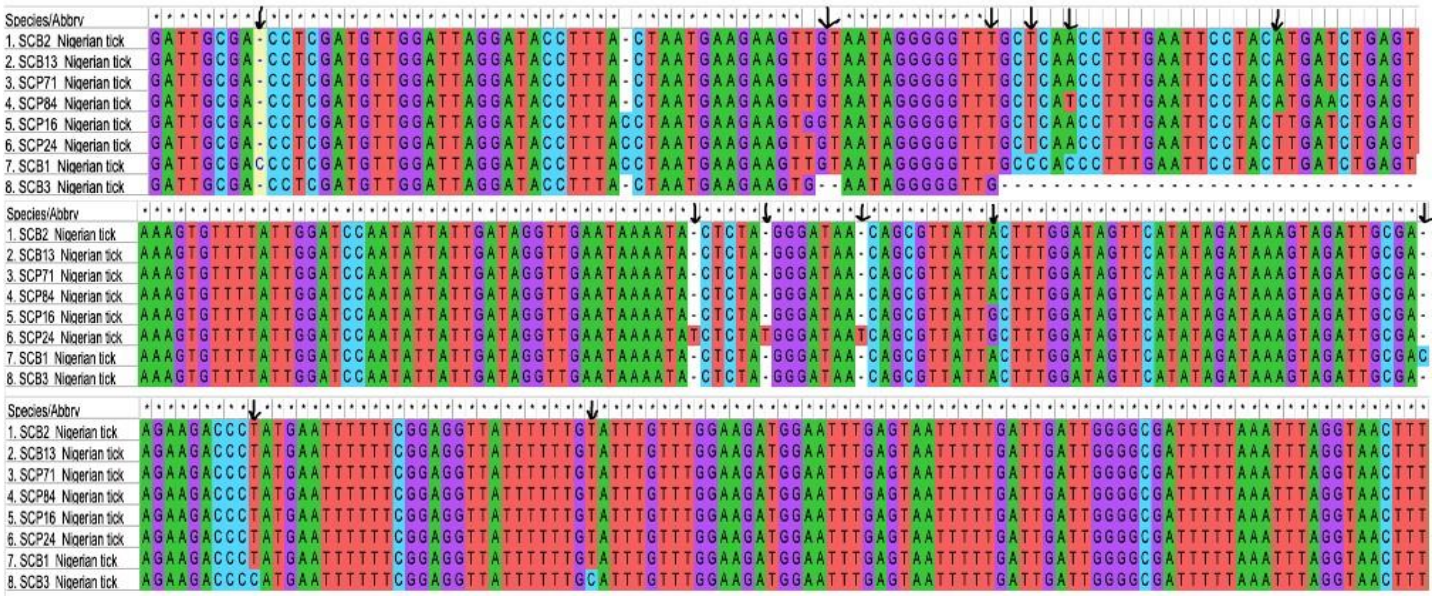


Figure 2.3.2(a): Differences in 16S rRNA (450bp) sequences between the ticks from Plateau and Borno states, with the polymorphisms depicted with black arrows.

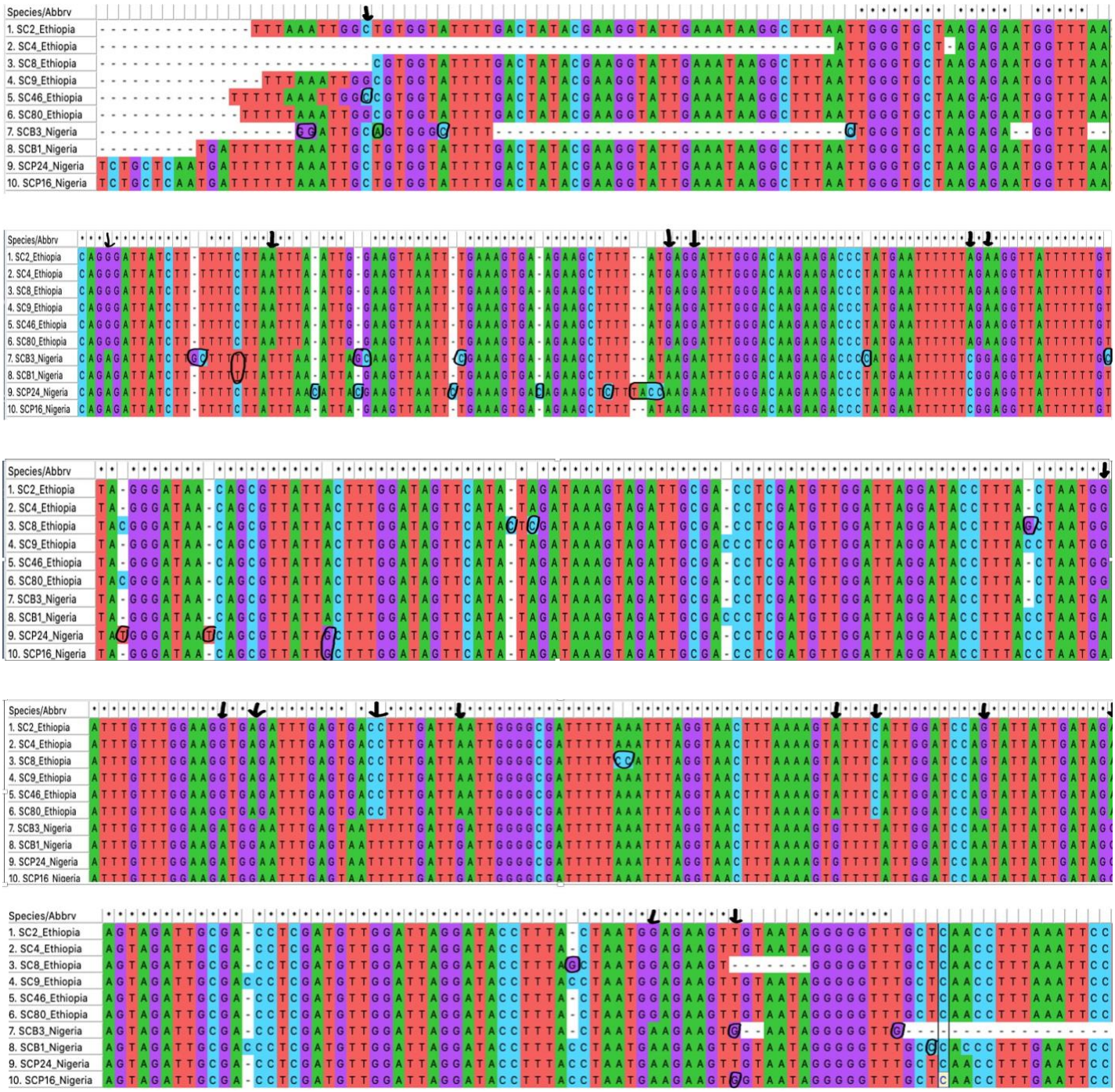


Figure 2.3.2(b): Differences in 16S rRNA (450bp) sequences between the Ethiopian ticks (numbers 1-6) and Nigerian ticks (numbers 7-10), with the black arrows depicting areas of polymorphisms.

2.3.3 Polymerase Chain Reactions for Borrelia infection

Of the 621 animals screened *Borrelia* infection were as follows; 18 (5%) of cattle and 3 (1.5%) of the goats were positive after initial screening on 16S *Borrelia*-specific real-time PCR. All cattle amplification occurred after cycle 36 which is the cut-off, while the goat amplification cycles were less than cycle 36 (Fig. 2.3.3a; Table 2.3.3).

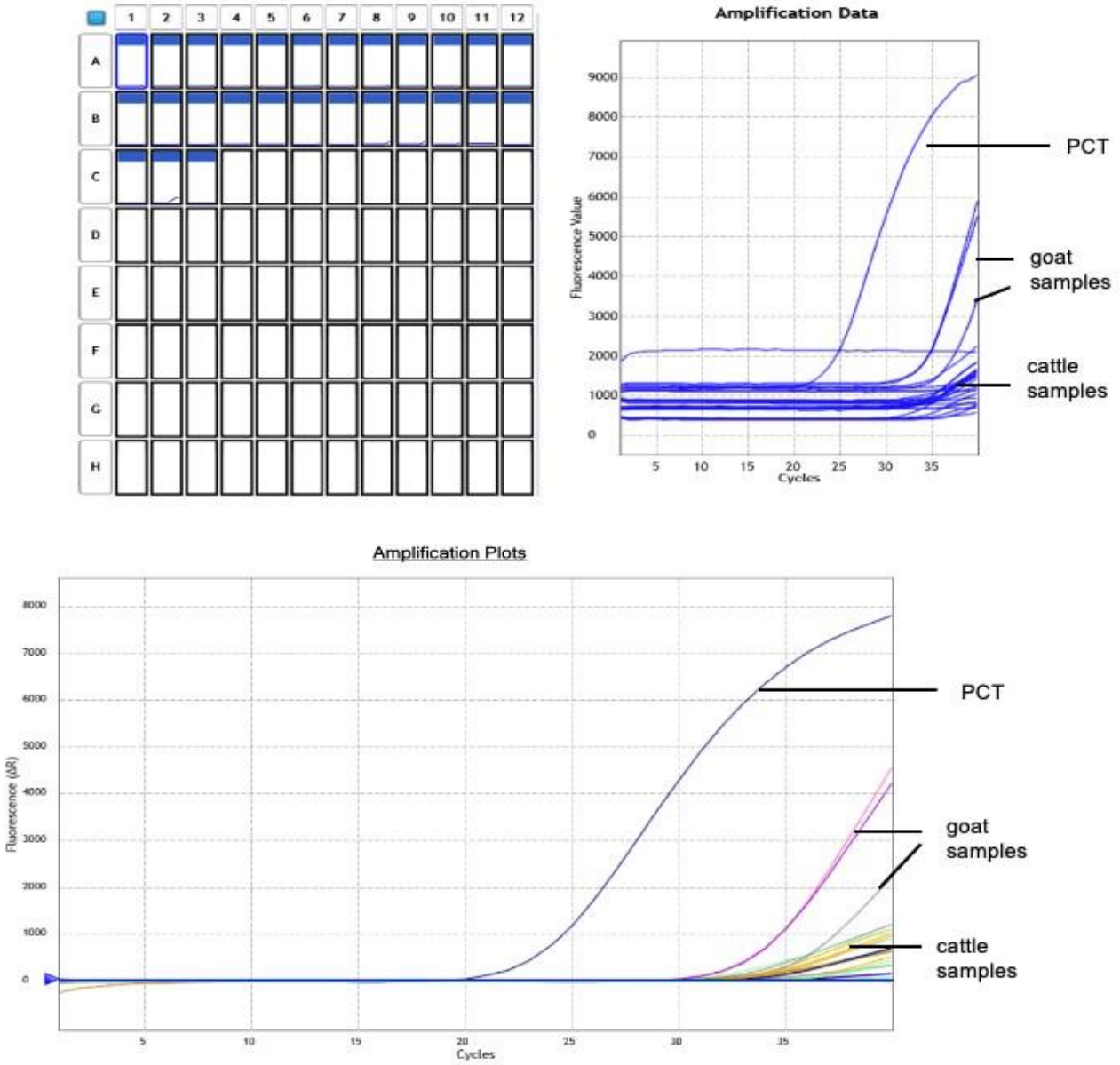


Figure 2.3.3(a): Screen-positive borrelial infection in animals on real-time 16S *Borrelia* PCR.

All 21 (3.4%) initial positive animal samples were subjected to additional confirmation on conventional PCR using the flagellin B and 16S genes as previously stated (section 2.2.5). After further confirmation, the three goat samples (CS19, CS51, KAD8) yielded amplifiable products of 323bp on the flagellin, while only CS19 and CS51 yielded amplicons on 16S gene targets (Table 2.3.3, Fig. 2.3.3b, 2.3.3c). None of the ticks from both states were positive for borreliae infection.

Table 2.3.3: Borrelial PCR result of all animal samples

Animal	**RT-PCR**	**Flagellin B**	**16S rRNA**
Cattle	5% (18/350)	0/18	0/18
Goat	1.5% (3/200)	3/3	3/3
Dog	0/71	N/A	N/A

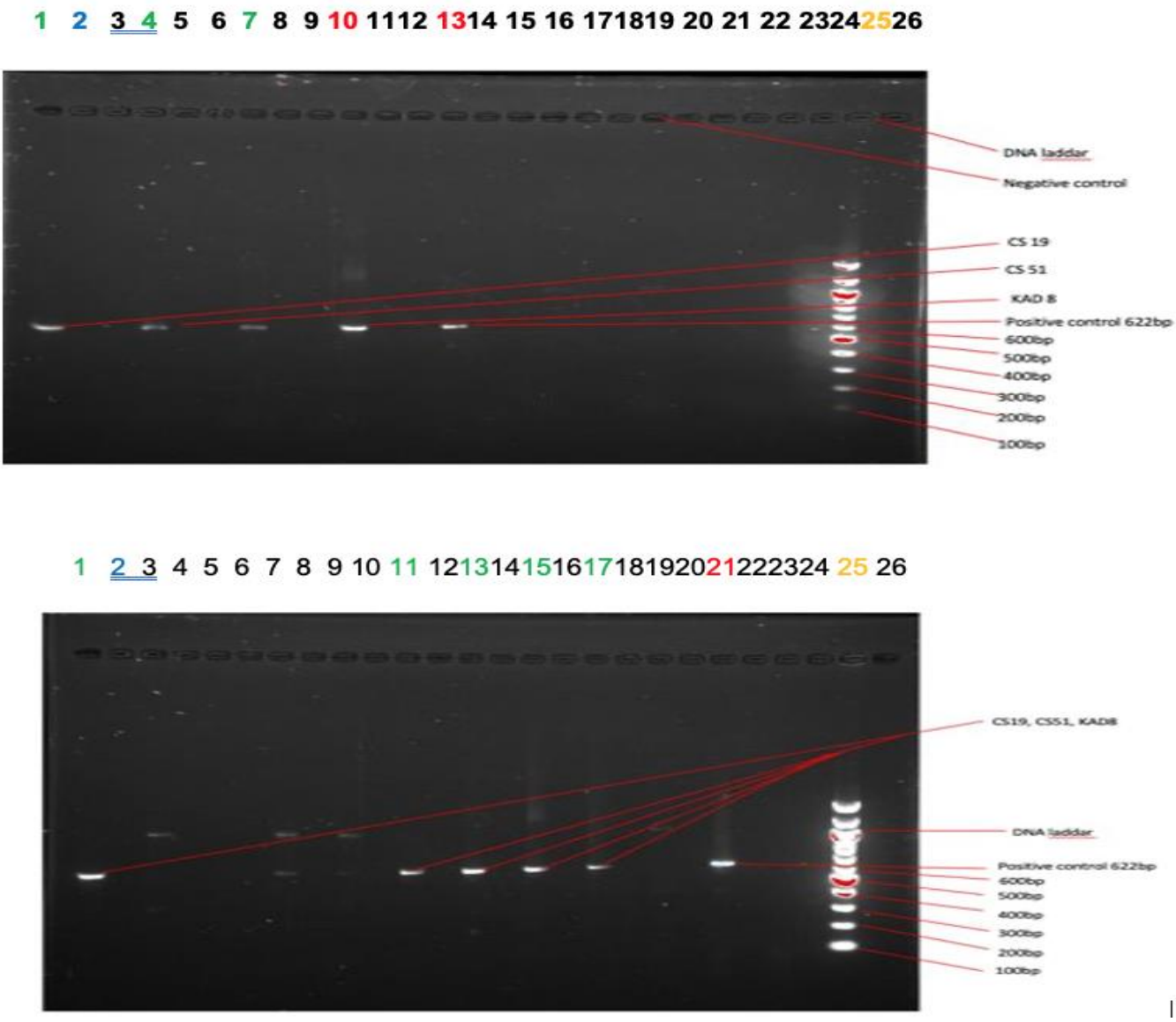
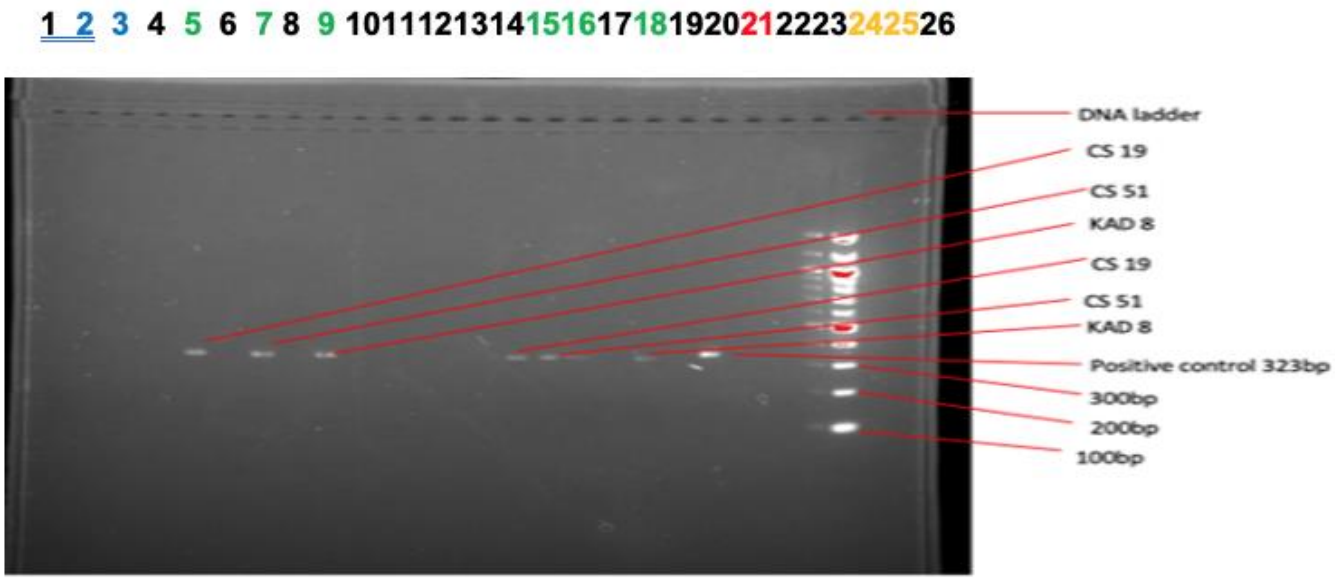


Figure 2.3.3(b): *Borrelia* confirmation in goat on conventional PCR amplifying a 622bp of the 16S gene viewed on 1.5% agarose gel. Highlighted number lanes are same as described in figure 2.3.1(a).



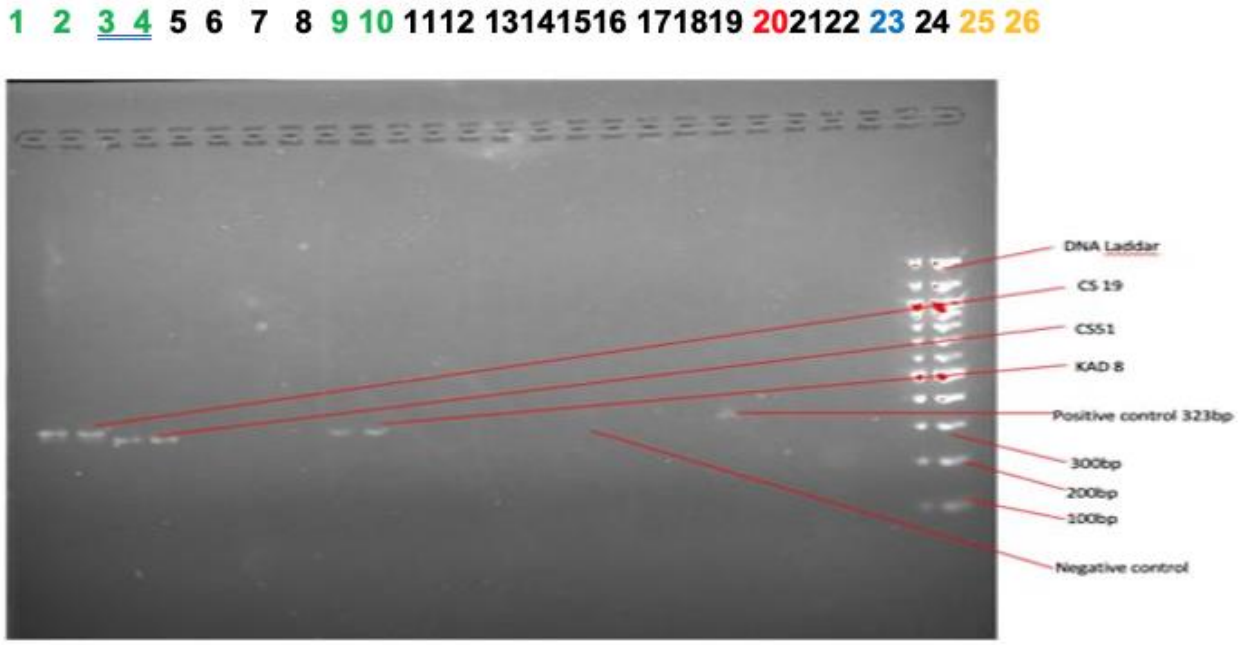


Figure 2.3.3(c): *Borrelia* confirmation in goat on conventional PCR amplifying a 350bp on the flagellin B gene viewed on 1.5% agarose gel. Highlighted number lanes are same as described in figure 2.3.1(a)

2.3.4 Sequence Analysis for Borrelia Identification

BLAST analysis of the goat *Borrelia flaB* sequences at 350bp (CS19, CS51, KAD8) revealed a >97% similarity to *Borrelia theileri* ON191583 reported in Cattle from Brazil (Paula et al., 2022). While CS51, CS19 the 16S sequences revealed >99% similarity with the *B. theileri* LC656246 reported in cattle from Zambia (Qiu et al., 2021). Phylogenetic analysis of the *B. theileri* flagellin sequences aligned closely to the Brazilian, Argentine, and Malian strains (Fig, 2.3.4a), while the 16S sequences aligned closely with the Columbian (Ramírez-Hernández et al., 2022) and Thailand (Fig. 2.3.4b) (Takhampunya et al., 2021) species. Representative sequences of the *Borrelia* 16S sequences were deposited into the GenBank under the accession numbers (OP703387 and OP703388), while a representative of the flagellin was deposited under the accession number OP839115.

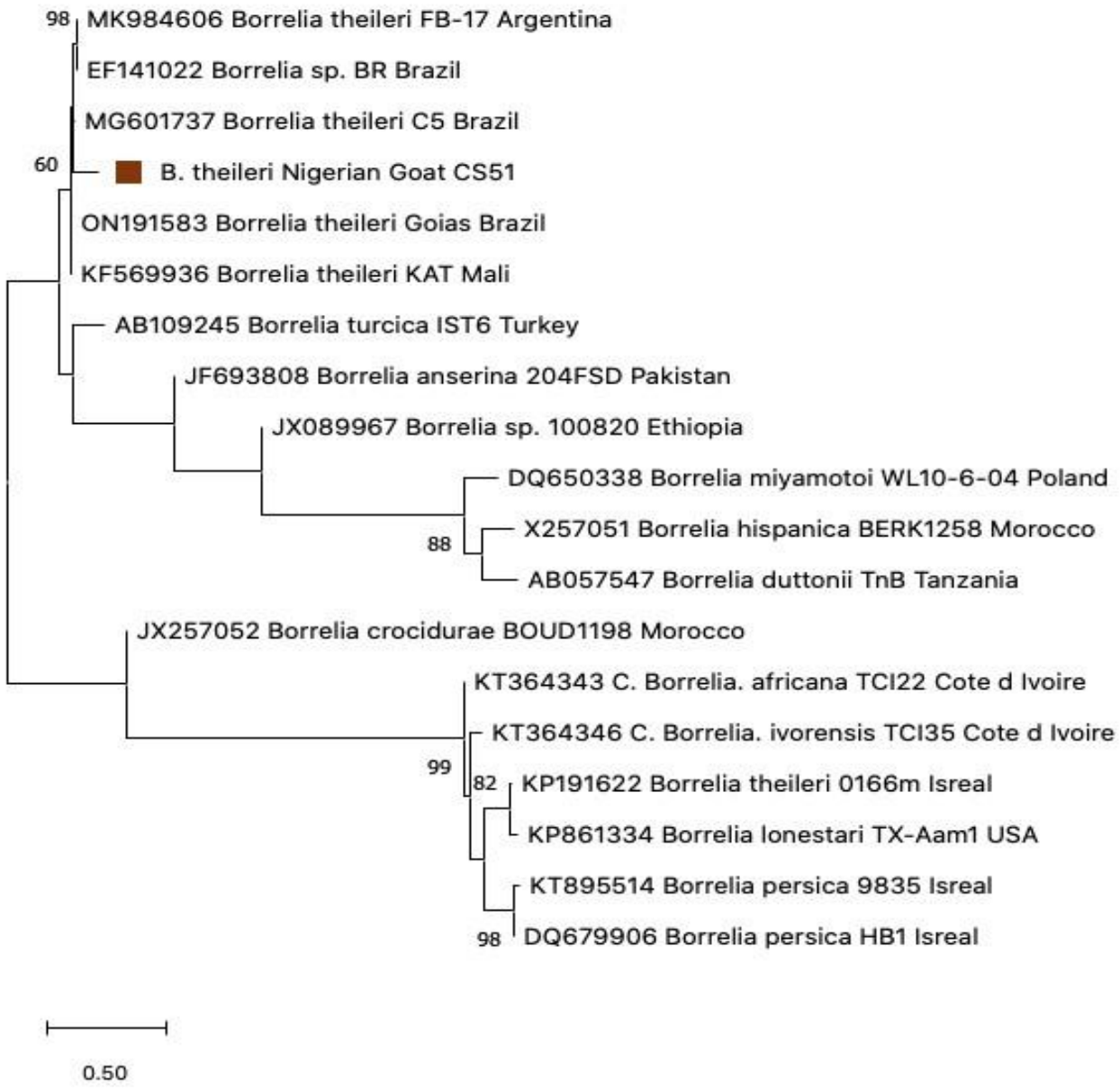


Figure 2.3.4(a): Phylogenetic analysis of Neighbor-joining *Borrelia* flagellin goat DNA sequences (350bp) from Nigeria. The tree with the highest log likelihood (-2182.93) is shown. Evolutionary distances were calculated using the maximum likelihood method and Tamura 3 models in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The square signifies the goat *Borrelia* sequence from this study.

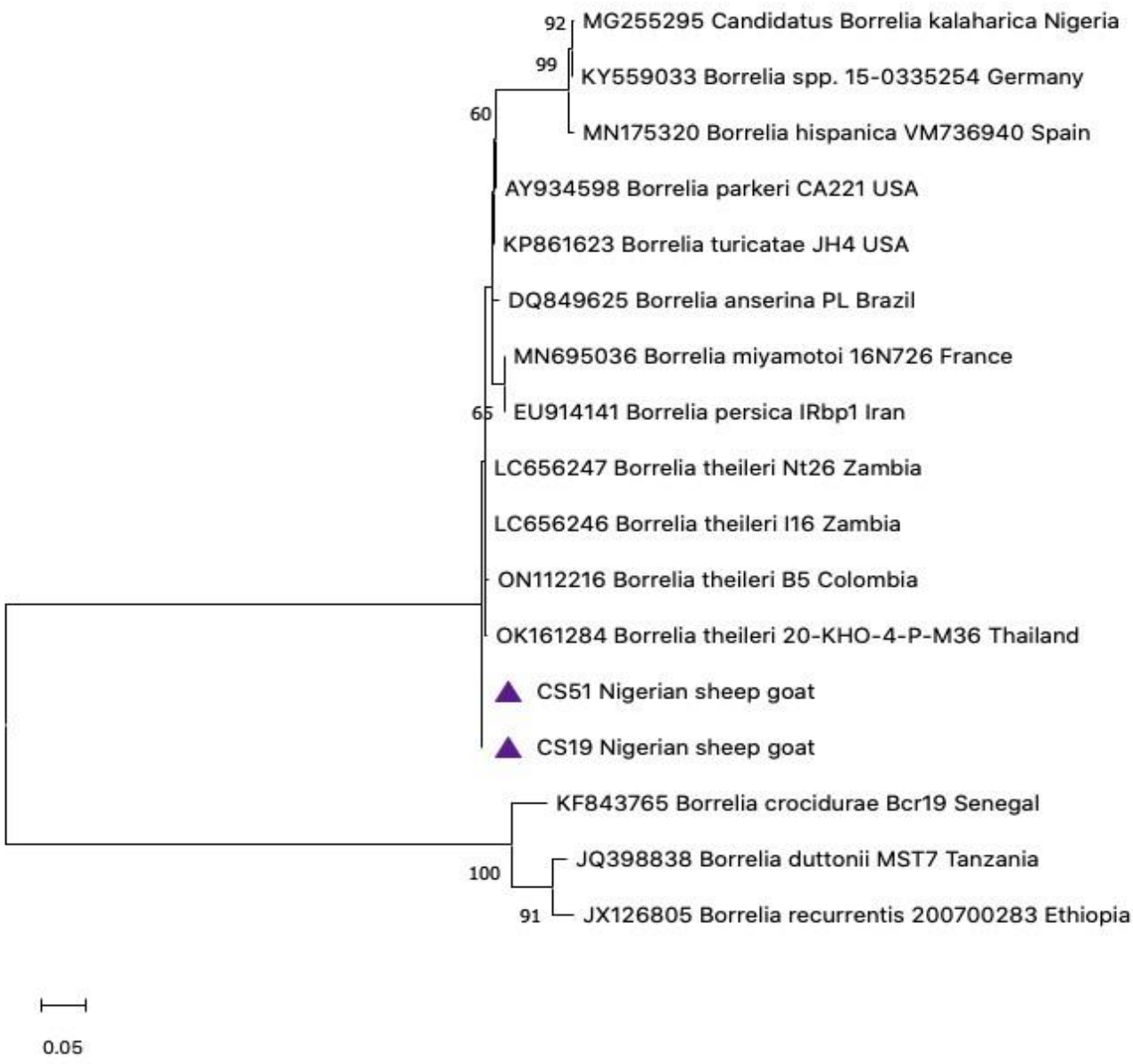


Figure 2.3.4(b): Phylogenetic analysis of Neighbor-joining of *Borrelia* 16S rRNA (622bp) goat sequences from Nigeria. The tree with the highest log likelihood (-133.32) is shown. Evolutionary distances were calculated using the maximum likelihood method and Tamura 3 models in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The triangles signify the goat *Borrelia* sequences from this study.

2.4 DISCUSSIONS

A total of 621 animals comprising of 350 cattle, 200 goats, and 71 dogs were investigated for the presence of *Borrelia* infection. In addition to this, 318 soft ticks comprising of 211 *Argas* and 107 *Ornithodoros* ticks were also screened and identified to the molecular level. Of the 621 animals investigated, borreliae infection was confirmed in 1.5% (3/200) of the goats from Shendam and Kanke LGAs in Plateau, with an overall prevalence of 0.5% (3/621) using the flagellin and 16S gene targets. None of the ticks from Nigeria harboured the spirochaetes. BLAST analysis and phylogenetic information revealed the identity of the *Borrelia* as *B. theileri* an agent of bovine relapsing fever (RF). Although the disease is mostly characterised by mild febrile symptoms in livestock and equids in tropical and subtropical regions globally (Uilenberg, 1995), it sometimes causes haemoglobinuria and anaemia in severe cases (Callow, 1967). This is only the second confirmed evidence of *Borrelia* described in Nigeria only after *C*. B. kalaharica using molecular method. However, historical evidence documented the presence of *Borrelia anserina* infection in blood smears of indigenous and imported chickens from Northern Nigeria (Barnes, 1975). The study also reported deaths in Muscovy ducks due to *Borrelia* infection as the ducks were bred in a village infested with *A. persicus* ticks the primary vector of the spirochaete.

The prevalence in goats from this study aligns with the other report of 0.9% (5/557) in cattle and sheep from Egypt (Abdullah et al., 2021), 0.4% (1/234) in cattle from Egypt (Abdullah. et al., 2021), and 0.5% (1/193) in racoon dogs (Kang et al., 2018). In addition to this, our data is also comparable to the prevalence reported in *Rhipicephalus* ticks from Mali 0.5% (1/184) (McCoy et al., 2014), Ethiopia 2.2% (9/404) (Kumsa et al., 2015), Brazil 0.25% (1/400) (Yparraguirre et al., 2007), Brazil 2% (1/50) (Cordeiro et al., 2018).

However, the infection level herein is lower than those reported in cattle from Cameroon 3.3% (42.1260) (Abanda et al., 2019), Zambia 4.1% (20/488) (Qiu et al., 2021), and Botswana 17.9% (40/225) (Sharma et al., 2000).

The low prevalence of *B. theileri* in the goat could in-part be attributed to the low spirochaetemia level typical of this species which makes them clear faster in the blood as previously described (Matton & Van Melckebeke, 1990).

Of the 318 soft ticks screened, 14% (31/211) *Argas* and 18.6% (20/107) *Ornithodoros* ticks produced positive amplicons. This is lower than the 49% (23/49 pooled ticks) reported in pooled *O. savignyi* ticks from Nigeria (Cutler et al., 2018). However, analysing pooled ticks makes it difficult to determine the exact rate of tick DNA yield as each of the pooled vials contained 5-20 ticks, whereas ticks in our study were assessed individually.

Of all amplified tick DNA templates, 10 of each species were randomly selected and sequenced, and the identity of the ticks were revealed as 100% and >97% homology to *Argas persicus* and *Ornithodoros savignyi* ticks respectively.

To the best of our knowledge, this is the first study to identify *A. persicus* ticks in Nigeria using molecular tools. Until now, the few reports of *Argas* ticks infesting both domestic and semi-wild birds were conducted using morphological tools (Table 2.1.3) (Barnes, 1975; Bunza et al., 2008; Fabiyi et al., 2017; Lawal et al., 2019; Leeflang & Ilemobade, 1977; Okaeme, 1988; Usman et al., 2012). In fact, this is the second identification of soft ticks in Nigeria to the molecular level, and only after the report of *O. savignyi* by (Cutler et al., 2018).

Argas persicus are considered the most important ectoparasites of birds in many regions, especially in countries where the traditional method of free-range breeding is still predominant (Pantaleoni et al., 2010) such as in Nigeria. *B. anserina* spirochaetosis transmitted by these ticks in many cases is a highly deadly septicaemic disease in birds, especially in the tropics and subtropics where poultry farming is a major source of livelihood, as well as a major source of protein for the population (Cutler et al., 2012). Around 75% of Nigerians depend on one form of agricultural farming as a source of livelihood of which poultry makes up the highest. In fact, poultry farming is estimated to account for more than 38% of non-crude oil foreign exchange earnings (Adeyemo & Onikoyi, 2012). *A. persicus*

ticks do not only vector *B. anserina* but, they can also transmit *Aegyptianella pullorum* (rickettsial infection) in birds (Walker et al., 2003) that was reported in (18/52) semiwild guinea fowl and (20/30) wild guinea fowl in northern Nigeria (Ayeni et al., 1983).

This present study also documents the first evidence of *O. savignyi* ticks in Plateau state an area not previously known to be endemic to the species, as well as in Borno state that is endemic to these ticks. Previous literatures on *Ornithodoros* species parasitising livestock and poultry are from the most arid north-eastern and north-western regions such as Borno (Cutler et al., 2018; Musa et al., 2014) and Sokoto states (Bunza et al., 2008). The human pathogenicity of *O. savignyi* is still sparse, albeit reports of infections have been documented in humans (Fingerle et al., 2016; Stete et al., 2018).

Instead, hard ticks such as *Rhipicephalus* and *Amblyomma* species, and *Argas* species are common ectoparasites of livestock and poultry in Plateau state. *Rhipicephalus* species are widely distributed in the state due to favourable environmental conditions such as precipitation (800mm) and high elevation level (Ogo et al., 2012), which aligns with the report of *B. theileri* in ruminants from this study.

These ticks could have most likely been introduced to Plateau state via camel trade routes from places like Borno state that have a large camel population (Abdussamad et al., 2015).

Heterogeneities observed between the Nigerian and Ethiopian tick sequences on the phylogenetic tree (Fig, 2.3.1c) could be because of geographical differences, albeit whilst the Nigerian ticks showed >97% similarity, the Ethiopian ticks had ~94% identity to *O. savignyi* (chapter 3). Some studies reported a positive correlation between *O. porcinus* species (a *O. moubata* sensu lato group) and their geographical constraining characteristics, as species from the same environmental niche aligned closely to one another (Bastos et al., 2009; Boshoff et al., 2014). The most recent taxonomic classification also noted significant divergences between *O. savignyi* from Yemen and the species circulating in Sudan based

on the 16S sequences (Bakkes et al., 2018). These could well be the reason for the division observed between the ticks from the two countries on the phylogenetic tree.

Ornithodoros species are known to be endemic in arid and semi-arid regions of Africa (Bakkes et al., 2018; Trape et al., 2013) such as Borno, Nigeria as shown in existing studies as well as in this study. However, their presence in Plateau state, an area with an average daily temperature of 18°C known to be the lowest in the country, as well as having one of the highest elevations (Musa. & Dung-Gwom, 2018) shows that the ticks can also thrive outside their natural ecological habitat.

The possible coexisting of three TBRF vectors and their agents of significant clinical, veterinary, and economic importance will only increase the burden on these three sectors if effective preventative and control measures against these ticks are not implemented.

CHAPTER 3: ETHIOPIAN TICK-BORNE RELAPSING FEVER STUDIES

3.1 Introduction

Vector-borne diseases such as the endemic louse-borne relapsing fever (LBRF) is well established in Ethiopia as shown in (chapter 1, section 1.2.6d). However, the significance of tick-borne relapsing fever (TBRF) borreliae in animals is still lacking, as available evidence is only on infections in the associated tick vectors. The tick fauna comprises of 47 species (Tadesse & Sultan, 2014) that are quite extensive and widely distributed depending on the climate, especially in the western agro-ecological regions (Kaba, 2022; Kumsa, Socolovschi, et al., 2012; Pegram et al., 1981). For instance, *Amblyomma lepidum*, *A. variegatum*, *R. decoloratus*, *R. mushamae* are widely spread in the wetter regions of Ethiopia (Pegram et al., 1981), with majority of tick infestations occurring in the western parts of the country (Kaba, 2022).

Ixodid ticks from the *Hyalomma*, *Rhipicephalus*, *Amblyomma*, sub-genus *Rhipicephalus* (*Boophilus*), and *Haemaphysalis* genera are the most common species of livestock and are of significant veterinary importance some of which are listed in (Table 3.1a) (Kumsa et al., 2015). *Rhipicephalus* and *Amblyomma* species are two of the most abundant of these five ixodid group (Beyene et al., 2015) just like in Nigeria. The most common diseases caused by these ticks are Anaplasmosis, babesiosis, theileriosis, and cowdriosis (Abdela et al., 2018; Abera et al., 2010; Irvin et al., 1996; Kemal et al., 2020; Teshale et al., 2016).

Asides infestation of livestock, *Rhipicephalus, Hyalomma,* and *Amblyomma* species are also important ectoparasites of domestic animals such as dogs, cats, camel, and horses (Table 3.1.b) (Kaba, 2022). Apart from the hard ticks, camels are also known hosts to *O. savignyi* the vector of *C*. B. kalaharica, and possibly sand tampan toxicosis in mammals (Bakkes et al., 2018; Mans et al., 2002), which may pose a zoonotic risk in humans who are in proximity to these animals.

Table 3.1 (a): Predominant ectoparasites of livestock from Ethiopia

Tick species	Infestation level in livestock	Tick specie vs total no of ticks harvested	Tick identification method	References
Amblyomma sp.		298/767		(Kumsa et al., 2015)
Rhipicephalus sp.	Cattle	404/767	Morphology	
Hyalomma sp.	(244/244)	57/767		
Haemaphysalis sp.	100%	8/767		
A. cohaerens		109/284		(Mediannikov et al., 2013)
R. decoloratus	Cattle	173/284	Morphology	
Rhipicephalus sp.	(unknown)	2/284		
A. cohaerens		2274/5507		
R. decoloratus		1132/5507		
A. variegatum	Cattle	774/5507		
R. evertsi evertsi	(179/330)	548/5507		
R. lununatus	(54.2%)	80/5507		
A. lepidum		64/5507		(Abera et al., 2010)
A. cohaerens		105/5507		
R. decoloratus		92/5507		
A. variegatum	Sheep	41/5507	Morphology	
R. evertsi evertsi	(79/330)	124/5507		
R. lununatus	(23.9%)	15/5507		
A. lepidum		0/5507		
A. cohaerens		51/5507		
R. decoloratus		146/5507		
A. variegatum	Goats	45/5507		
R. evertsi evertsi	(72/330)	115/5507		
R. lununatus	(21.8%)	1/5507		
A. lepidum		0/5507		

Amblyomma sp. *Rhipicephalus sp.* *Hyalomma sp.* *Boophilus sp.*	Cattle (496) (infestation level unknown)	3270/10,055 2295/10,055 686/10,055 3804/10,055	Morphology	(Abebe et al., 2010)
A. cohaerens *A. variegatum* *R. decoloratus* *R. evertsi evertsi*	Cattle (315/384) (82%)	159/1984 25/1984 124/1984 52/1984	Morphology	(N. T. Beyene et al., 2015)
Amblyomma sp. *Rhipicephalus sp.* *Hyalomma sp.* *Boophilus sp.*	Cattle (225/384) (59.4%)	738/1889 916/1889 235/1889 443/1889	Morphology	(Tadesse & Sultan, 2014)
A. cohaerens *R. decoloratus* *A. variegatum* *R. evertsi evertsi*	Cattle (289/408) (70.8%)	58.5% 50.5% 44.1% 12.9% (Total no of ticks is undisclosed)	Morphology	(Abdela et al., 2018)
R. evertsi *R. decoloratus*	Cattle (94/94) (100%)	58/202 101/202	Morphology	(Teshale et al., 2016)
R. evertsi *R. decoloratus*	Sheep (28/28) (100%)	26/202 17/202		
R. decoloratus *A. variegatum* *R. evertsi evertsi* *R. pulchellos*	Goat (23/160) (14.4%)	366/1518 971/1518 53/1518 21/1518	Morphology	

A. gemma	Sheep (44/152) (28.9%) Cattle (28/121) (23.1%)	107/1518 (Overall infection in all animals)		(Kemal et al., 2020)

Table 3.1 (b): Predominant ectoparasites in domestic animals from Ethiopian

Tick species	**Infestation level in animals**	**Tick specie vs total no of ticks harvested**	**Tick identification method**	**References**
A. variegatum *R. sanguineus* *R. pulchellos* *H. leachi*	Dogs (194/200) (97%)	17/200 16/200 11/200 35/200	Morphology	(Kumsa et al., 2019)
A. variegatum *H. leachi* Other parasites	Cats (124/137) (90.5%)	2/137 15/137 120/137		
A. variagatum *Rhipicephalus sp.* *Hyalomma sp.* *B. decolaratus*	Horses (455/1618) (39.04%)	38/917 342/917 55/917 32/917	Morphology	(Kumsa et al., 2012)
Amblyomma sp. *Rhipicephalus sp.* *Hyalomma sp.* *Boophilus sp*	Camels (374/384) (97.4%)	164/4417 3780/4417 414/4417 59/4417	Morphology	(Elias et al., 2020)
Amblyomma sp. *Rhipicephalus sp.* *Hyalomma sp.*	Camels (17/17)	968/16422 14040/16422 1248/16422	Morphology	(Zeleke & Bekele, 2004)
Boophilus sp	(100%)	164/16422		
Amblyomma sp. *Rhipicephalus sp* *Boophilus sp.*	Horses (72)	28/56 16/56 12/56	Morphology	(Ulsido, 2019)

In contrast, the distribution, veterinary and economic importance of Agasidae ticks in Ethiopia is still sparse, with only a handful of borreliae infection reported in soft and hard ticks, whilst no data on TBRF is available for animals and humans (Table 3.1c). In France, there was a case report of TBRF in a traveller that at the time only returned from Ethiopia. The *Borrelia* specie had a 100% identity to the classical old-world strains except for *B. recurrentis*, and a 98% identity to *B. burgdorferi* using the 16S sequences (Socolovschi et al., 2012). In addition to soft ticks being the predominant vectors for TBRF, species from the *Rhipicephalus* and *Amblyomma* genera have also shown competence as vectors for RF spirochaetes as listed in (Table 3.1c).

Table 3.1 (c): Tick borne relapsing fever species in ticks from Ethiopia

Tick species	**Tick sampling site**	**Borreliae in ticks**	**Borrelia in humans**	**Borreliae species**	**References**
A. persicus *Amblyomma sp.* *Rhipicephalus sp*	Ruminants Cattle	4/77 (5%) 1/21 (4.7%) 1/21 (4.7%)	NA	*B. anserina* *B. theileri* *B. theileri*	(Cutler et al., 2012)
A. cohaerens	Cattle	8/109 (7.3%)	NA	*Borrelia sp*.	(Mediannikov et al., 2013)
A. variagatum *A. cohaerens* *Amblyomma sp.*	Cattle Sheep	3/767 8/767 9/767	NA	*B. turcica*	(Kumsa et al., 2015)
R. decoloratus *R. pulchellus*	Cattle Sheep	4/767 5/767	NA	*B. theileri*/*B. lonestari group*.	

With the scarcity of literature on soft ticks and their importance as vectors for TBRF, the objective of this study was to:

- Identify and characterise *Ornithodoros* ticks to the specie circulating in the region.
- Investigate the possibility of TB borrelial species of veterinary and clinical importance coexisting with the endemic LBRF.

3.2 MATERIALS AND METHODS

3.2.1 Tick sampling

A total of 312 *Ornithodoros* soft ticks were manually collected from soils around cattle dwellings in Amibara district in the Eastern Afar Regional State of Ethiopia in 2020 by collaborator vets at the Addis Ababa University (Fig. 3.2.1). The minimum sample size of 176 was determined using the same parameters shown in section 2.2.1.

$z= 1.96$, $p= 6.1\%$ or 0.061, $q=1 - 0.061\ (0.939)$, $d=0.05^2$

$$= \frac{(1.96)^2 \times (0.061) \times (0.939)}{(0.05)^2}$$

$$= \frac{0.2200}{0.0025}$$

$= 88 + 88(100\%$ of $88)$

$= 176$ (minimum sample size)

Amibara district is 250 km away to the northeast of Addis Ababa, with an estimated elevation level between 550 and 1100 m above sea level, an average temperature of 29°C, and a yearly precipitation level between 400-600 mm. The state is comprised of 18 pastoral associations (PAs), which is the largest in Africa (Abdulatife & Ebro, 2015). The ticks were mainly concentrated in the dusty soil where these animals rest and sieved out. Like the Nigerian ticks, ticks here were physically visualised to ensure intactness before classifying into four developmental stages using their body length as previously described (chapter 2, section 2.2.1) (Table 3.2.1).

Figure 3.2.1: Livestock shelters in the Amibara zone 3 district of Eastern Afar state where soft ticks were collected from.

Table 3.2.1: Classification of ticks into developmental stages

Developmental stage	**Tick genus**	**Physical appearance**		**Total no of ticks (N=312)**
		Engorged	Not engorged	
Stage 1 (nymph <3mm)	*Ornithodoros sp.*	0	101	101
Stage 2 (nymph 3–6mm)	*Ornithodoros sp.*	15	86	101
Stage 3 (nymph 6.1–9mm)	*Ornithodoros sp.*	27	23	50
Stage 4 (adult >9mm)	*Ornithodoros sp.*	28	32	60

3.2.2 DNA extraction of ticks

After rinsing with phosphate-buffered saline (PBS), individual ticks were halved into equal parts with one part stored in 70% ethanol as backup. Halved ticks were placed in clear tubes containing homogenising beads (Matrix H) (MP biomedicals) and freshly made PBS before homogenising as described in (chapter 2, section 2.2.2). Homogenates were centrifuged briefly before lysing with lyses buffer and proteinase K as previously described (chapter 2, section 2.2.2). samples were then incubated overnight at 56 °C.

DNA was extracted using the same materials and protocols described in chapter 2. After DNA extraction, samples were quantified on nanodrop to determine the concentration and purity level.

3.2.3 Tick DNA Amplification for Species Identification

A 450bp tick DNA were amplified using tick-specific oligonucleotides listed in (Table 3.2.3) that was previously designed by (Black & Piesman, 1994) and protocols described in (chapter 2, section 2.2.4). DNA of an *Ixodes ricinus* and *Ornithodoros savignyi* tick were used as the positive template, while nuclease-free water was used as the negative template control. Successful amplicons were resolved on 1.5% agarose gel electrophoresis stained with SYBR safe

(Invitrogen®), while a 100bp DNA ladder was used to determine the sizes of the amplicons (either Invitrogen®; or New England BioLabs®) at 100volts for 80mins. All positive tick amplicons were subjected to PCR clean-up using the QIAquick® PCR Purification kit (Qiagen) that can isolate up to 10µg of DNA and between 100bp–10kb in sizes according to the protocol in (chapter 2, section 2.2.4). Once PCR was completed, samples were nano-dropped again to check the concentration before rerunning on 1.5% to view the purified bands as stated

above. Amplification was conducted using the Bio-Rad T100 thermal cycler, and amplicons were viewed using the Bio-Rad ChemiDocÔ MP imaging system. Amplified products at a volume of 10µl were each transferred into 0.5ml Eppendorf's, and tick 16S primers (forward and reverse) were each diluted to a final concentration of 3.2uM before submitting for Sanger sequencing to the DBS Genomics unit at Durham University.

Table 3.2.3: Primers and probes used in this study

Gene Target	**Nucleotide sequences** ($5^1 - 3^1$)	**Band Size**	**Reference**
16S-23S intergenic spacer region (IGS)	**First round**: F: GTATGTTTAGTGAGGGGGGTG R: GGATCATAGCTCAGGTGGTTAG **Nested**: F: AGGGGGGTGAAGTCGTAACAAG R: GTCTGATAAACCTGAGGTCGGA	750bp	(Scott et al., 2005)
Flagellin B	F: TAATACGTCAGCCATAAATGC R: GCTCTTTGATCAGTTATCATTC	770bp	(Assous et al., 2006)
RT-PCR 16S rRNA	F: AGCCTTTAAAGCTTCGCTTGTAG R: GCCTCCCGTAGGAGTCTGG P: [**FAM**] CCGGCCTGAGAGGGTGAACGG	148bp	(Parola, Julien, et al., 2011)
Tick 16S rRNA	F: CTGCTCAATGATTTTTTAAATTGC R: CCGGTCTGAACTCAGATCATGTA	450bp	(Black & Piesman, 1994)

3.2.4 Borrelial Investigation in Ticks

Ticks were screened for the presence of borreliae infection using the borrelia-specific RTPCR primers (Merck) designed to amplify a 148bp fragment of the 16S rRNA gene (Parola, Julien, et al., 2011) listed in table 3.2.3 above. A final PCR volume of 25 µl containing reaction buffer (1X), dNTPs (0.2 mM each), $MgCl_2$ 2.5 mM), 2 µl DNA template, each primer at (5µM), probe (2.5µM), and 0.15U taq polymerase. Cycling conditions were as follows: 95

°C for 10 mins, 40 cycles of 95 °C for 15 secs, and 60 °C for 1min as previously described (Cutler et al., 2018). DNA of *B. burgdoferi senso stricto* B31 strain was used as positive control and nuclease free water (Invitrogen) as negative control. Samples with a cycle threshold (CT) value of £36 were considered as positive. Amplification was conducted using the Agilent AriaMxÒ 1.2 Real-Time thermocycler.

All *Borrelia* screen-positive samples from the initial qPCR above were retested and subjected to further confirmation on nested conventional PCR using the intergenic spacer region (IGS), and flagellin B gene using the primers listed above in table 3.2.3.

Cycling conditions were as follows: **IGS first round**: 94 °C for 3 mins, 35 cycles of 94 °C for 30 secs; 56 °C for 30 secs; 72 °C for 60 secs, and a final extension of 72 °C for 7 mins; **nested second round**: 94 °C for 3 mins, 35 cycles of 94 °C for 30 secs; 60 °C for 30 secs; 72 °C for 60 secs, and a final extension of 72 °C for 7 mins (Scott et al., 2005). **FlaB**: 95 °C for 5 mins, 40 cycles of 95 °C for 30 secs; 56 °C for 30 secs; 72 °C for 90 secs, and a final extension of 72 °C for 10 mins (Assous et al., 2006).

Amplified borreliae products were viewed on agarose gel, purified, and quantified as previously stated in section 3.2.3. Amplified products at a volume of 10µl were each transferred into 0.5ml Eppendorf's, and both *Borrelia* primers (flagellin and 1GS nested forward and reverse primers) were each diluted to a final concentration of 3.2uM before submitting for Sanger sequencing to the DBS Genomics unit at Durham University.

3.2.5 Phylogenetic Analysis

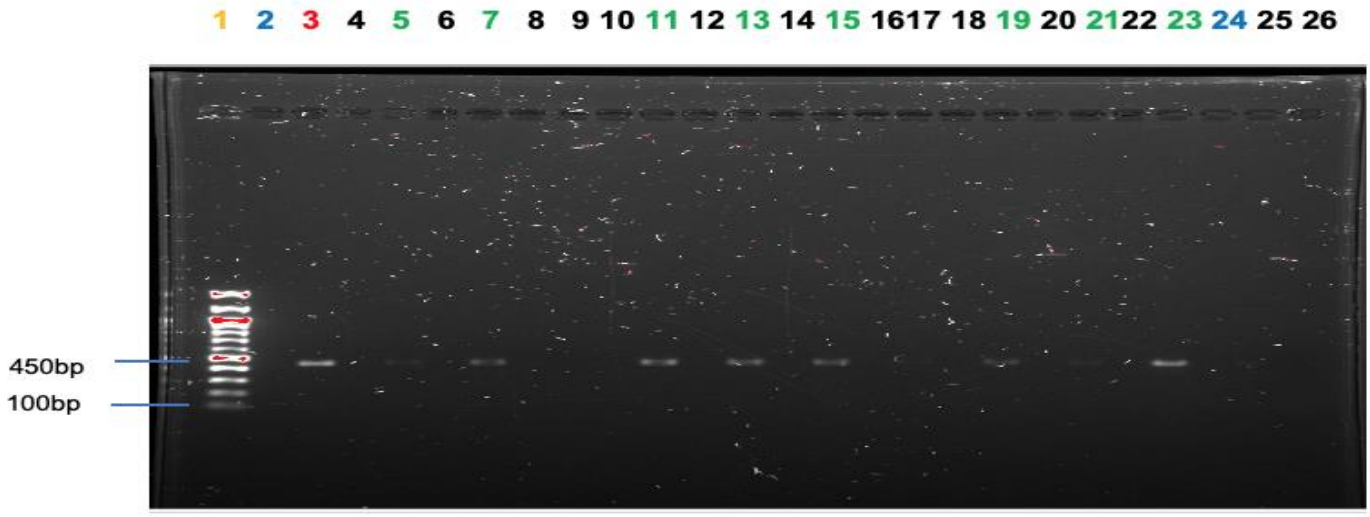
The *Borrelial* infection level in ticks was determined using descriptive statistics, and statistical significance based on the tick developmental stage was set at $p < 0.05$. All resulting sequences derived from this study were compared to those in the GenBank® using the basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST). Multiple sequences alignments were carried out using the MUSCLE program, and phylogenetic construction using the Neighbor-joining and Maximum Likelihood methods at a confidence test of 1000 bootstrap on MEGA 11. Sequence annotation was conducted using the Geneious Prime software v2022.2.2 https://manage.geneious.com).

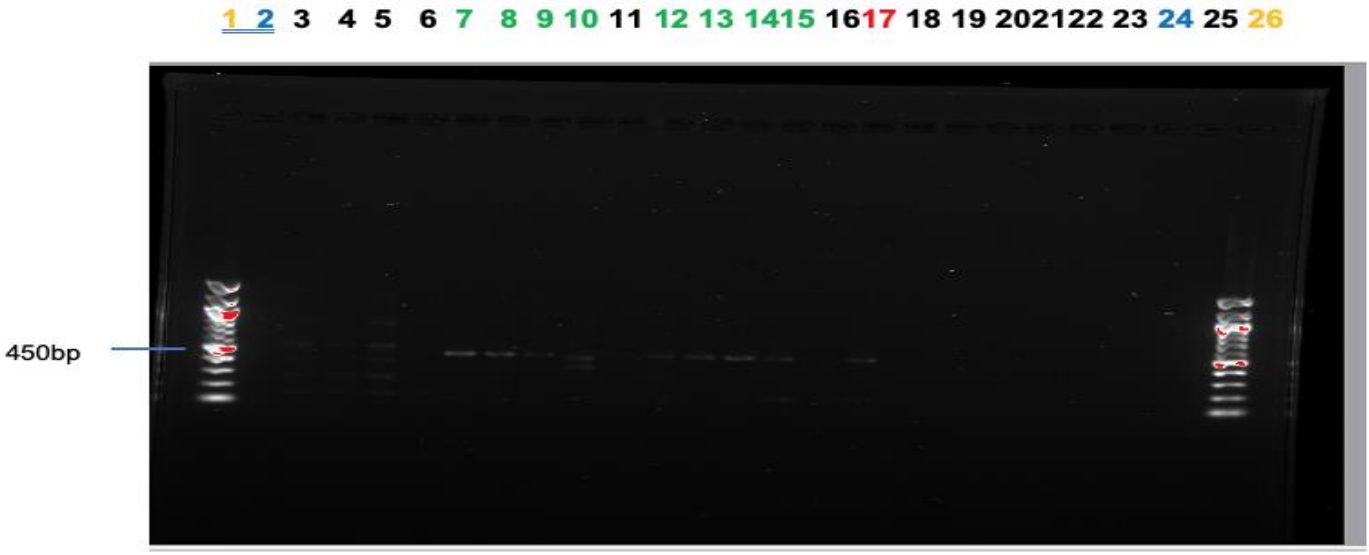
3.3 RESULTS

3.3.1 Tick Identification

Of the 312 soft ticks from Ethiopia analysed for specie identification, only 7% (22/312) yielded amplifiable products at 450bp using the tick 16S rRNA gene (Fig. 3.3.1a). Of these, a random subset of 10 samples consisting of ticks from all four developmental stages were sequenced. BLAST analysis of samples SC2, SC4, SC9, and SC80 16S sequences revealed the identities as >94% similarity to *Ornithodoros savignyi* KJ133578, KJ133577, and MF415646 from Sudan, Saudi Arabia (Mans et al., 2019), and Egypt (Bakkes et al., 2018) respectively, while SC8 had >93% which is consistent on the phylogenetic tree (Fig. 3.3.1b). BLAST analysis of all five sequences also revealed a 94% homology to the Nigerian sequences generated from this study which is also evident on the tree. Representative sequences were deposited into the GenBank under accession numbers OP688111, OP688113, OP688114, OP688116, and OP688117.

Tick DNA yield is comparable to the amplicon yield rate obtained for the Nigerian *O. savignyi* ticks.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 1617 18 19 20 2122 23 24 25 26
450bp
100bp


1 2 3 4 5 6 7 8 9 10 11 12 13 1415 1617 18 19 202122 23 24 25 26
450bp

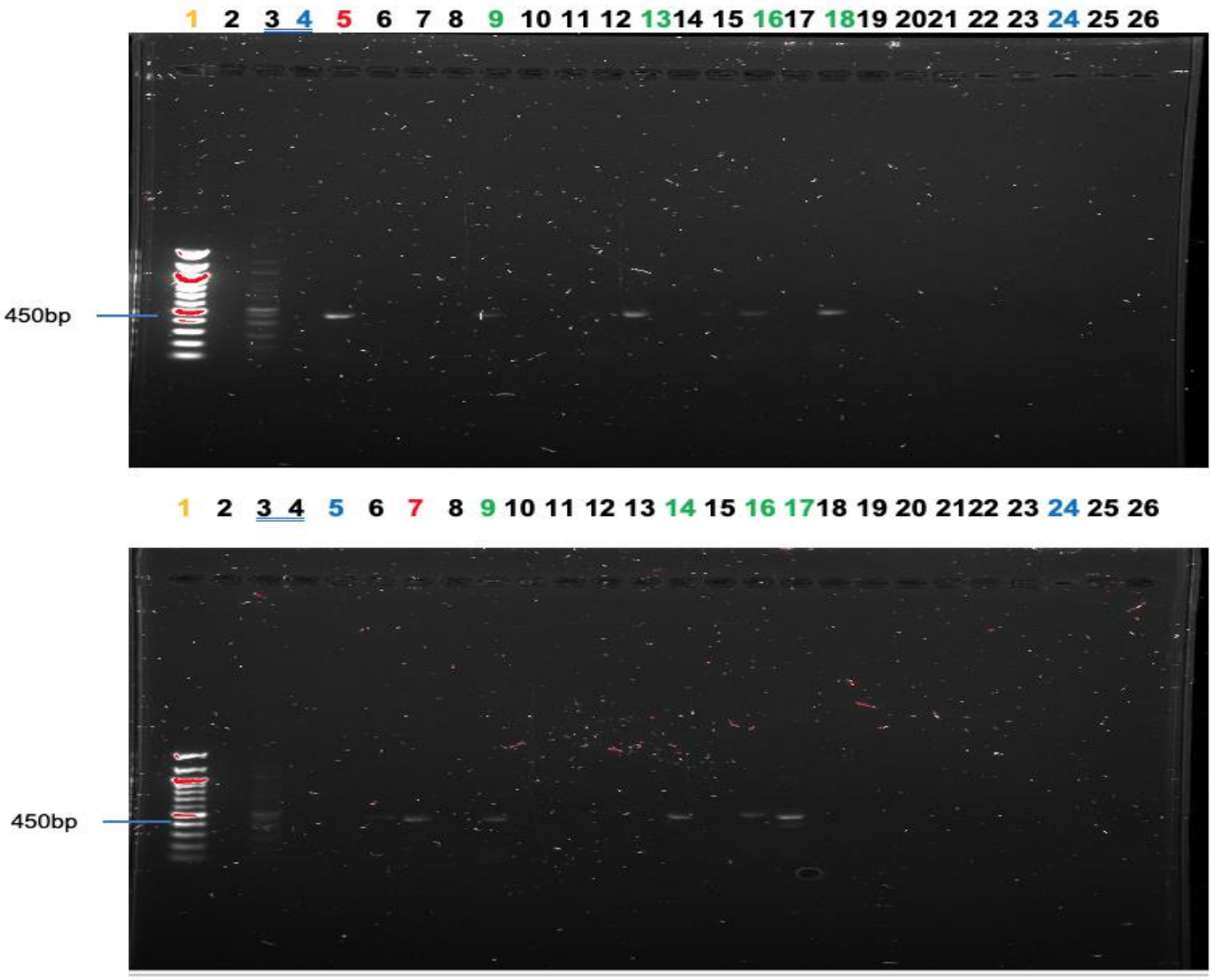


Figure 3.3.1 (a): *Ornithodoros savignyi* tick amplicons using 16S rRNA gene at 450bp viewed on 1.5% agarose gel. Positive control template in lanes with numbers highlighted in red, negative template controls in blue, DNA 100bp ladder in orange, amplified tick DNA lanes in l green, and ticks unsuccessfully amplified in black.

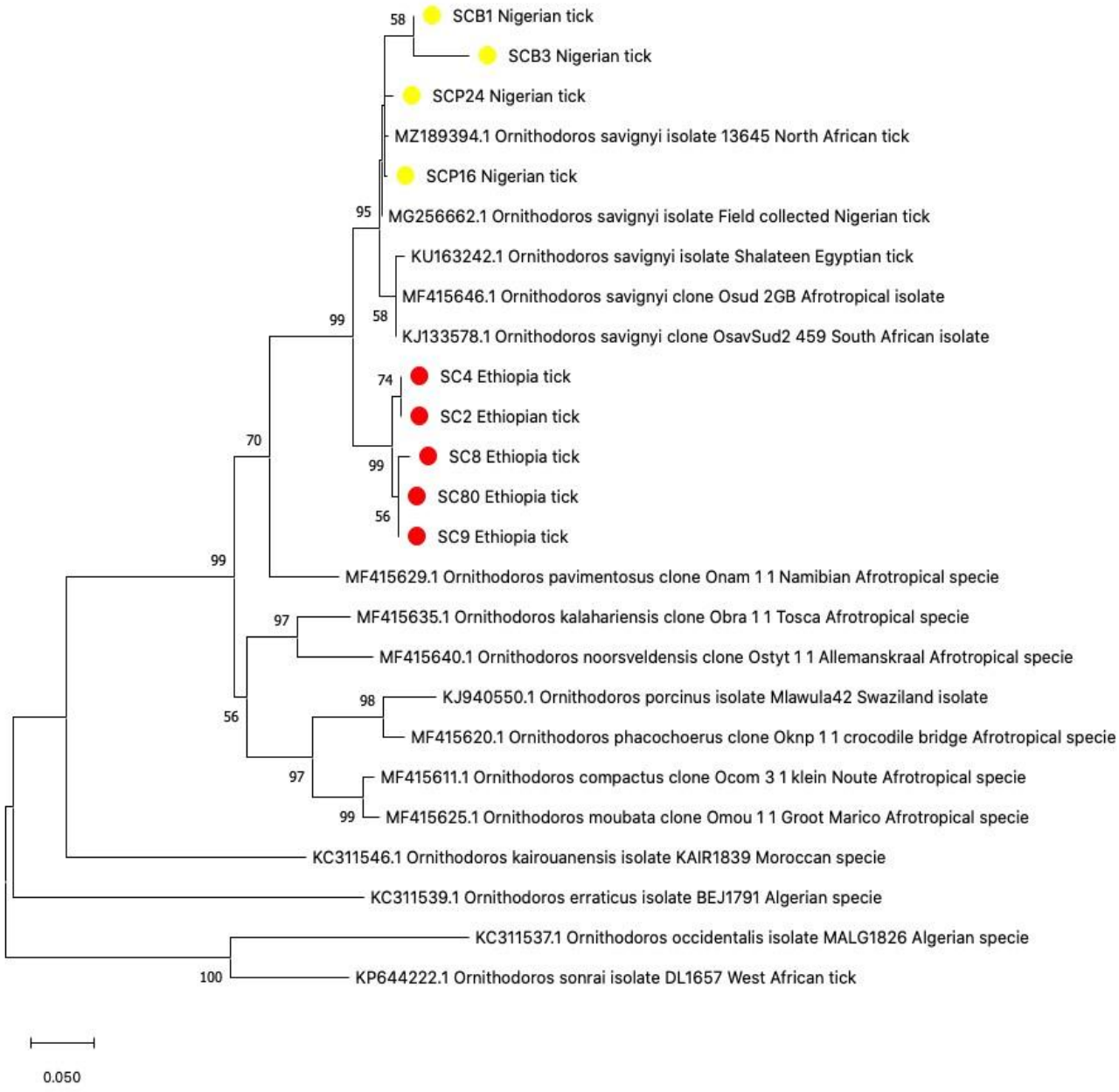


Figure 3.3.1(b): Phylogenetic analysis of Neighbor-joining tree of tick 16S rRNA sequences (450bp), comparing the Nigerian *Ornithodoros* ticks with other *Ornithodoros* species. The tree with the highest log likelihood (-3939.77) is shown. Evolutionary distances were calculated using the maximum likelihood method and the Hasegawa-Kishino-Yano model in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The red circles represent the Ethiopian ticks while the yellow circles represent the Nigerian ticks from this study.

3.3.2 Heterogeneities in tick Sequences

Just as shown in (chapter 2, section 2.3.2b) heterogeneities in tick sequences that caused the ticks to align separately on the phylogenetic tree were observed between the Nigerian and Ethiopian ticks. Whilst SC2, SC4, SC9, SC80 had identical sequences, few divergences were also observed in sample SC8 at positions (250-251bp; 356-357bp) (Fig. 3.3.2), albeit had very little impact on the phylogenetic information as all ticks aligned on the same branch within the tree.

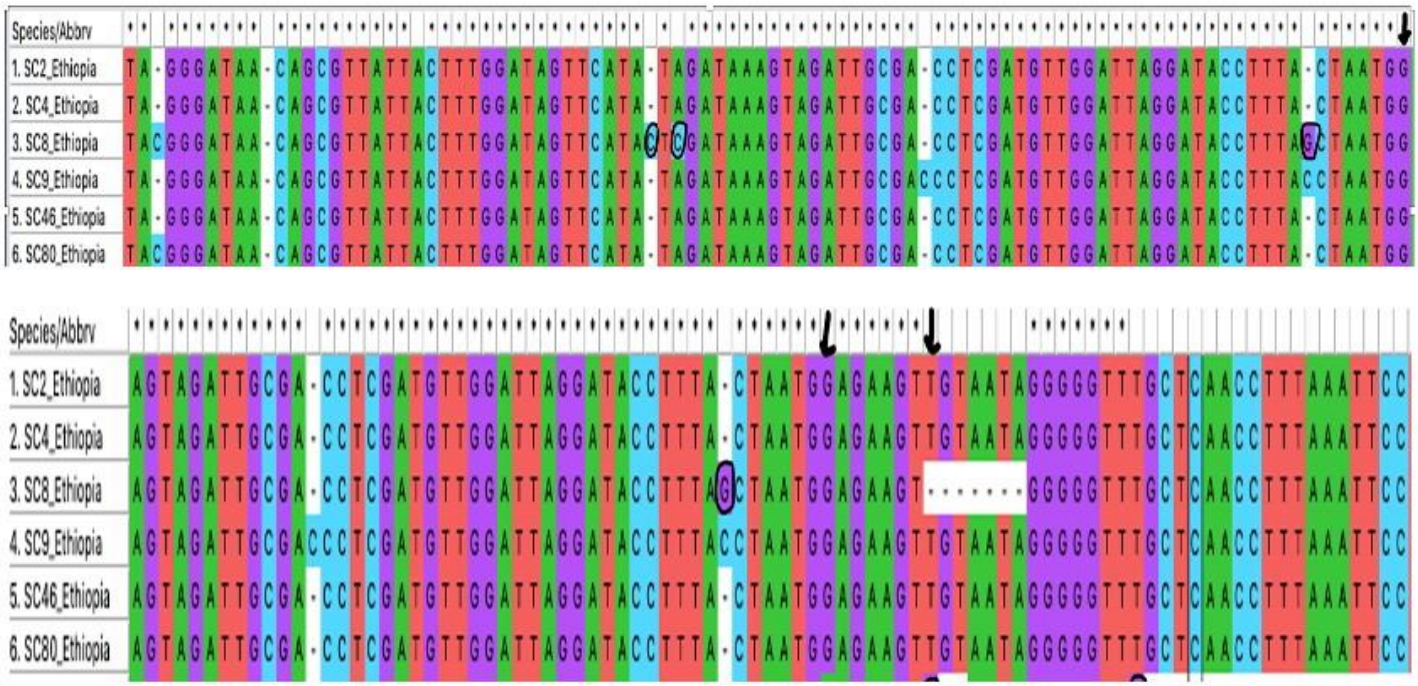


Figure 3.3.2: Divergences in 16S rRNA sequences at (450bp) in tick sample SC8 from Ethiopia, with the polymorphisms circled in black and location depicted with black arrows.

3.3.3 Polymerase Chain Reactions for *Borrelia* infection

A total of 312 of *O. savignyi* ticks collected from soils around cattle shelter were screened for the presence of *Borrelia* infection. Of these, 3.5% (11/312) of the ticks were positive for *Borrelia* after initial screening on 16S real-time PCR (Fig. 3.3.3a). Positive samples were subjected to additional confirmation on conventional PCR using the nested IGS and flagellin B gene target. Of these 11 positive samples, four samples SC2, SC4, SC7, and SC56 (consisting of 3 adults, and 1 stage 3 nymph) produced amplifiable products of 770bp (Fig. 3.3.3b) and 750bp (Fig.

3.3.3c) on the flagellin and IGS targets respectively. When comparing infection level in ticks, there were no difference between adults and nymphs, as well as engorged and non-engorged ticks (Table 3.3.3a)

BLAST analysis of the flagellin sequences revealed samples SC2, SC4, and SC56 had a 99-100% identity while SC7 had a 92.24% to *Candidatus* Borrelia kalaharica MG257489 from Nigerian ticks as evident on the phylogenetic tree (Fig. 3.3.3d) (Cutler et al., 2018). Whilst BLAST analysis of all four the IGS sequences revealed >99% and 97% identity to *Candidatus* Borrelia kalaharica MG257909 from Nigeria as shown on the phylogenetic tree (3.3.3e). Representative flagellin sequences were deposited into the GenBank under accession numbers OP839113 and OP839114, while the IGS sequences were deposited under accession numbers OP745098, OP745099, OP745100, and OP745101.

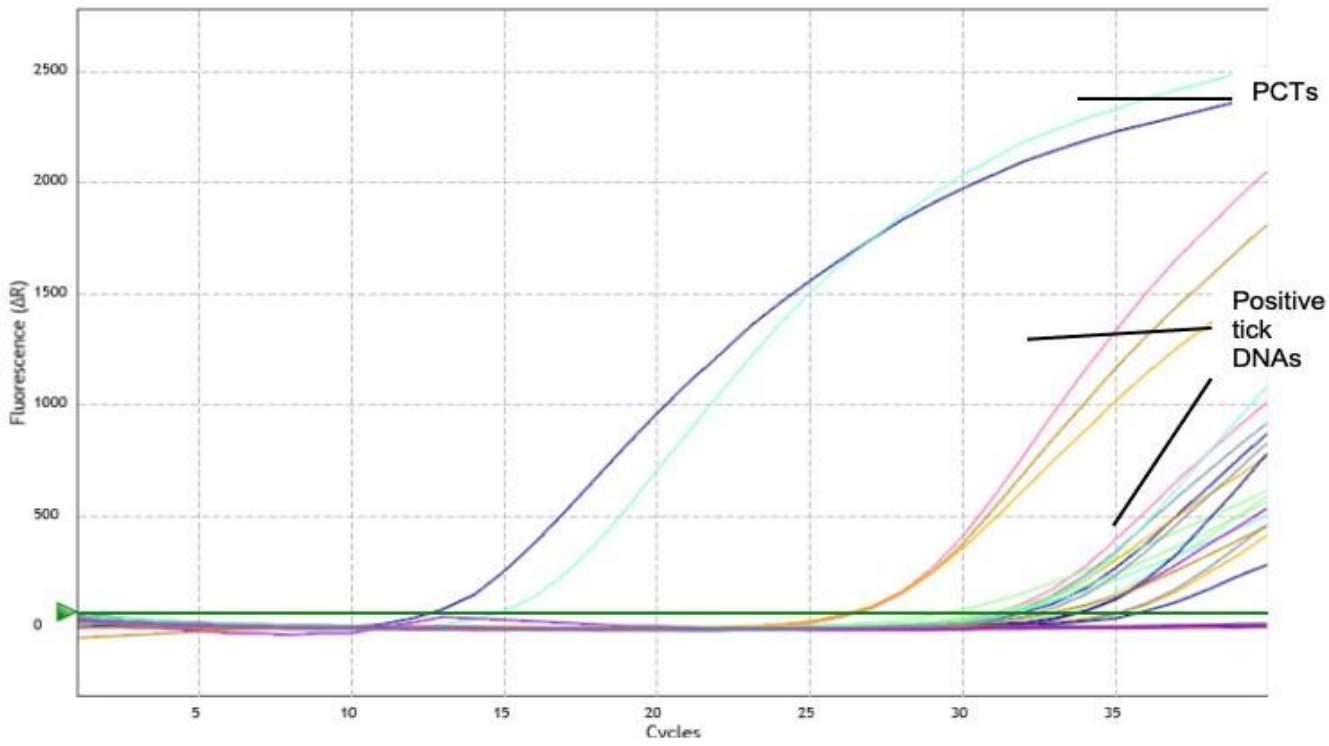


Figure 3.3.3(a): Screen-positive borrelial infection in ticks on real-time 16S *Borrelia* PCR.

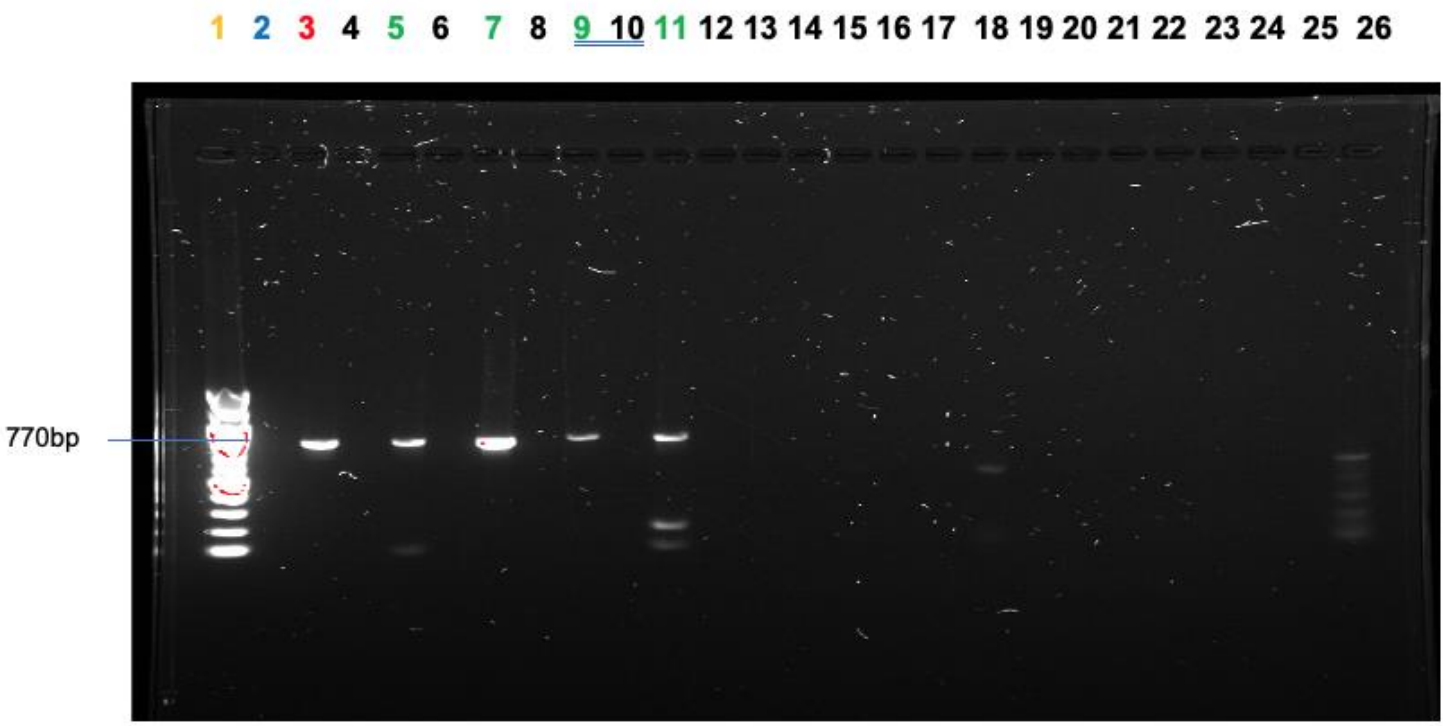


Figure 3.3.3(b): *Borrelia* confirmation in ticks on conventional PCR amplifying a 770bp of the flagellin gene viewed on 1.5% agarose gel. Highlighted number lanes are same as described in figure 3.3.1(a)

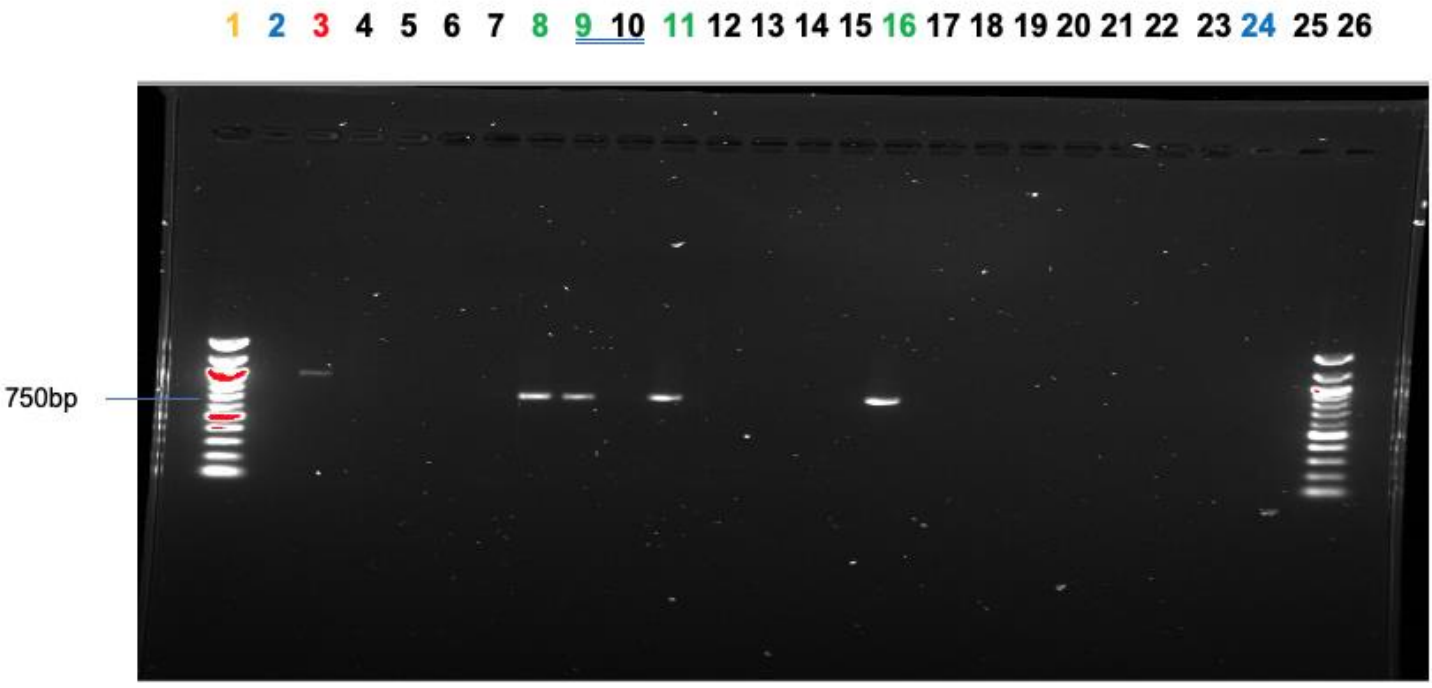


Figure 3.3.3(c): *Borrelia* confirmation in ticks on conventional PCR amplifying a 750bp of the intergenic spacer region (IGS) viewed on 1.5% agarose gel. Highlighted number lanes are same as described in figure 3.3.1(a).

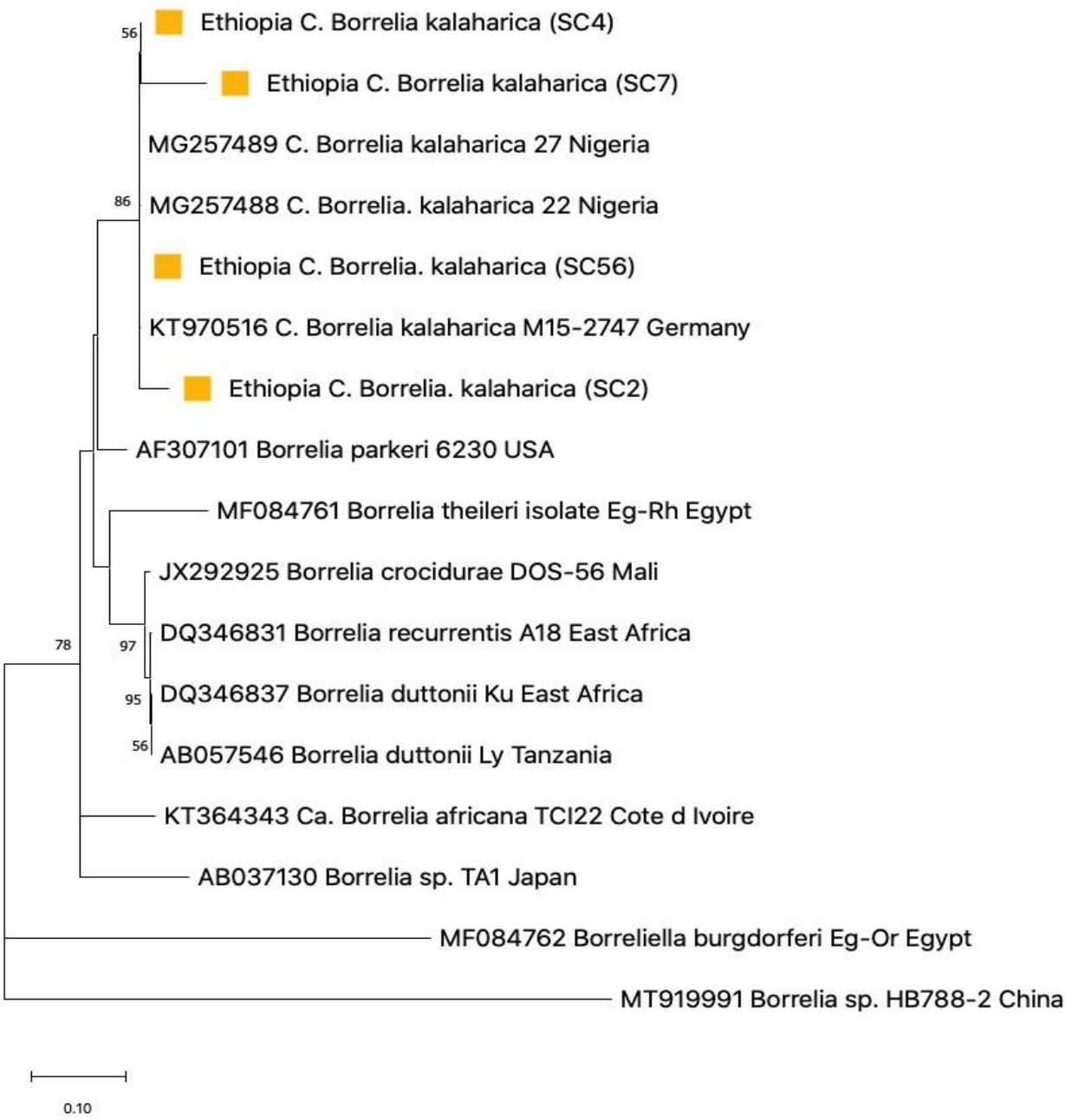


Figure 3.3.3(d): Phylogenetic analysis of Neighbor-joining *Borrelia* flagellin tick sequences (770bp) from Ethiopia. The tree with the highest log likelihood (-4282.93) is shown. Evolutionary distances were calculated using the maximum likelihood method and Tamura 3 models in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The square signifies the goat *Borrelia* sequence from this study.

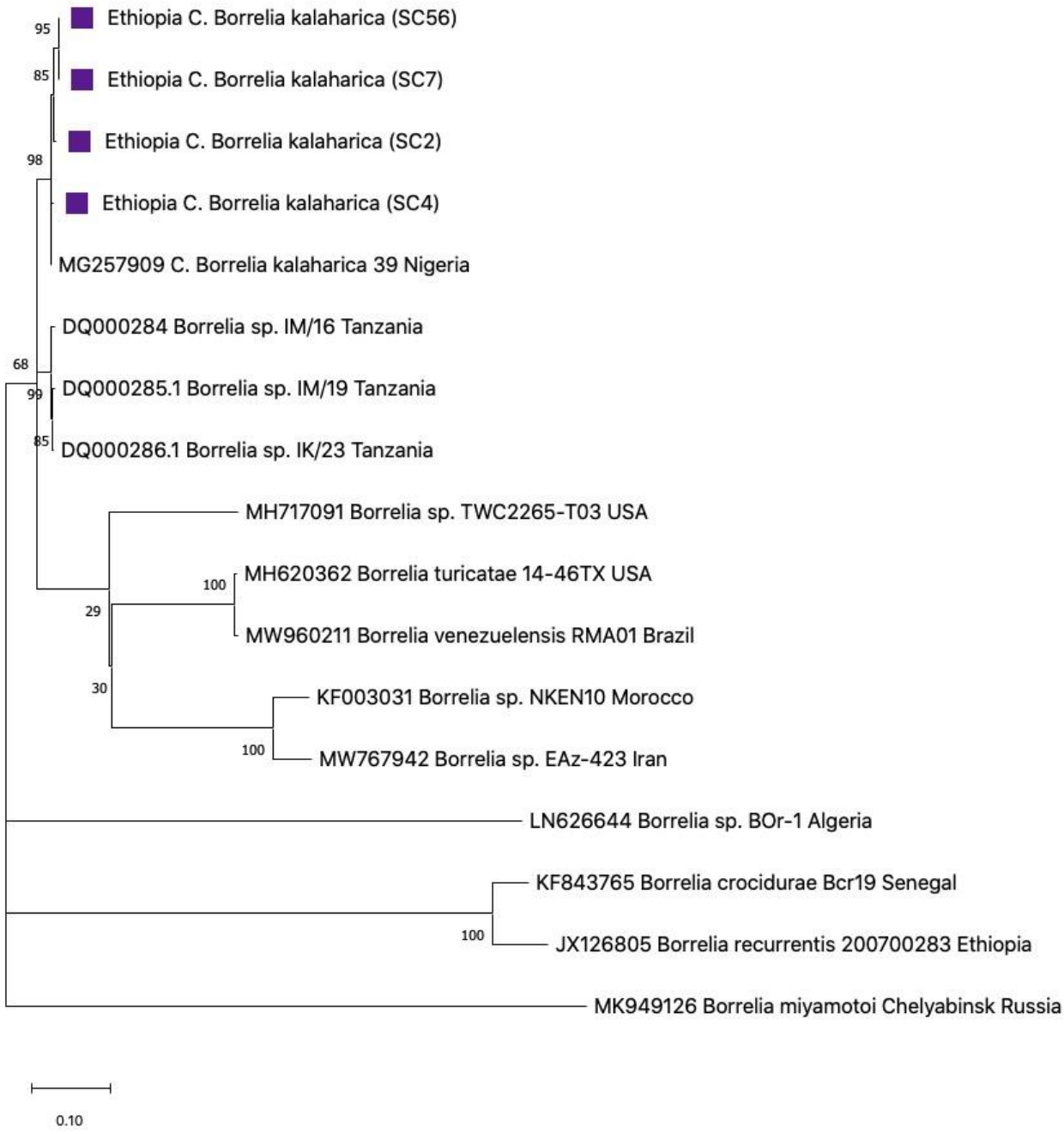


Figure 3.3.3(e): Phylogenetic analysis of Neighbor-joining *Borrelia* IGS tick sequences (750bp) from Ethiopia. The tree with the highest log likelihood (-5105.81) is shown. Evolutionary distances were calculated using the maximum likelihood method and Tamura 3 models in MEGA 11. Bootstrap values were >50% based on a test of confidence of 1000 replicates shown on branch nodes. The square signifies the tick *Borrelia* sequence from this study.

Table 3.3.3(a): Prevalence of *Borrelia* in ticks at different life stages

Stage/State	**16S RT-PCR**			**Flagellin**	**IGS**	
	Borrelia infection vs. total ticks screened	*Borrelia* positive (engorged)	*Borrelia* positive (not-engorged)	Amplified	Amplified	p-value
Adult ticks	10% (6/60)	36% (4/11)	18% (2/11)	27% (3/11)	27% (3/11)	p* 0.25
Nymphs	2% (5/252)	27% (3/11)	18% (2/11)	9% (1/11)	9% (1/11)	p**0.51
Total	11/312	7/11(64%)	4/11 (36%)	36% (4/11)	36% (4/11)	

p value of 0.05 considered as significant.

p* *Borrelia* infection level between adult and nymphs.

p** *Borrelia* infection between engorged and non-engorged ticks.

3.3.4 Heterogeneities in *Borrelia* Sequences

Analysis of the *Borrelia* flagellin sequences revealed polymorphisms in all samples, most especially in sample SC7 that has the lowest identity rate of all four sequences (Fig. 3.3.3a). Whereas all IGS sequences were homologous which is consistent on the phylogenetic tree.

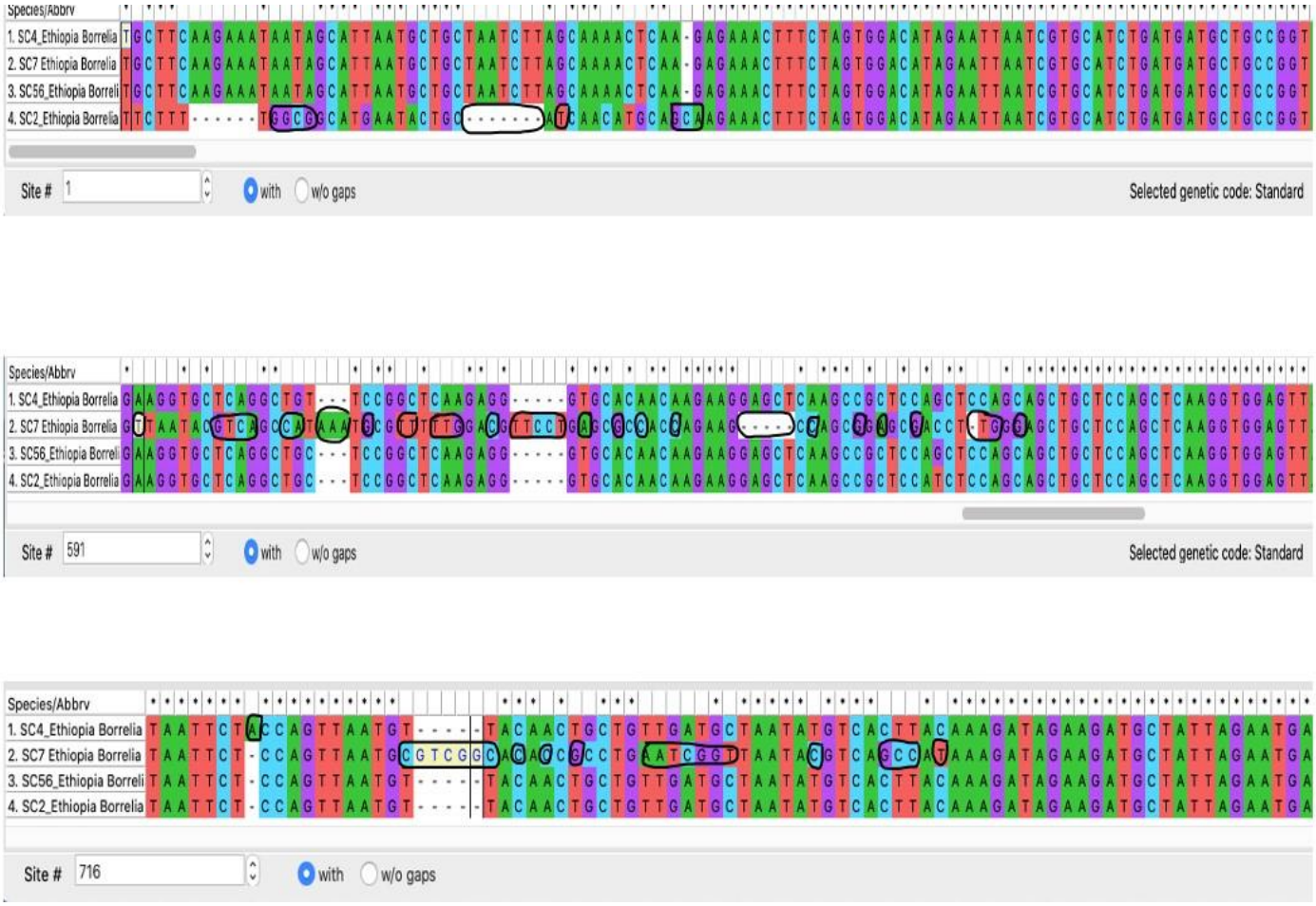


Figure 3.3.4 (a): Divergences in the borrelial flagellin sequences at 770 bp among the Ethiopian ticks circled in black, with SC7 having the most polymorphisms.

3.5 DISCUSSIONS

In this study, *Ornithodoros* species collected around cattle shelters were identified to the species level and investigated for the presence of tick-borne relapsing fever (TBRF). Molecular analysis of the tick 16S rRNA sequences revealed the identity as 94% similarity to *O. savignyi* from Sudan, Saudi Arabia, Egypt, and the Nigerian species from this study. However, the Ethiopian species formed a separate clade from all other Afrotropical *O. savignyi* species as evident on the phylogenetic tree (Fig. 3.3.1b). Noteworthily, the most recent taxonomic revision of Afrotropical *Ornithodoros species* revealed divergences in the 16S gene sequences of the Yemeni and Sudanese *O. savignyi* and suggested a possible sub-specie or new specie. The authors also documented differences in the sizes of the anterior and posterior eyes, and tarsus 1 in the Somali and Egyptian *O. savignyi* ticks compared to the Sudanese specie (Bakkes et al., 2018). Based on this revision, one can hypothesise that the Ethiopian ticks could be a sub-specie or a distinct species within the *O. savignyi* sensu strict group. Notwithstanding, there is a need for further taxonomic investigation of the *O. savignyi* group to clarify these sequence divergences. A similar report from Pakistan on *Ornthodoros* ticks reported a 93% sequence identity to *O. verrucosus* using the 16S gene. The low identity level was evident on the phylogenetic tree as the Pakistan ticks formed a sister clade from other *O. verrucosus* from Iran which made the authors suggest a different specie or sub-specie (Ali et al., 2022).

Historically, relapsing fever outbreaks had been reported in the neighbouring Somali during the 1920s and 1930s and was suspected to be tick-related imported from Ethiopia through migrants and animal routes (Clark, 1937), however, vector confirmation was conducted. This contributed to the controversy about the presence of soft ticks of veterinary and clinical importance in the country. All but one reports on TBRF spirochaetes such as *B. theileri* (the agent of bovine relapsing fever), *B. turcica* (Kumsa et al., 2015), and other *Borrelia* species closely related to the *B. theileri*/*B. lonestari* group (Mediannikov et al., 2013) in Ethiopia have

been in hard-bodied ticks. The only exception is that of borreliae infected *Argas persicus* described around human dwellings (Cutler et al., 2012).

The latest taxonomic revision of the *O. savignyi group* (*O. parvimentous*, *O. kalaharensis, O. noorsveldensis, O. savignyi*) that were previously misidentified as *O. savignyi* revealed their geographic restriction to the neighbouring Bushmanland, Kalahari Desert, Eastern Cape, and Northern Africa respectively (Bakkes et al., 2018). Furthermore, *O. savignyi* are major ectoparasites of camels and they widely distributed in regions with large camel populations or camel trades such as the middle East (Hoogstraal, 1956b). This could be suggested for Ethiopia that have one of the large camel populations in Africa (CSA, 2017).

In addition, the *O. savignyi* complex ticks stated above were exclusively sampled from burrows of small mammals based on the conventional dogma of the ticks predominantly parasitising the burrows of these animals, whilst infection in humans is viewed as accidental (Diatta et al., 2015a; Trape et al., 2013). Whereas *O. savingyi* of all stages are known to parasitise shelters and soil around domestic animals such as camels (Hoogstraal, 1956b) and livestock that serves as a major source of their blood meal. This is even more prominent in regions where traditional method of farming that involves free range breeding whilst the animal's dwell in shelters in proximity to humans is still predominant such as in Africa (Pantaleoni et al., 2010), thus posing a significant risk for zoonosis. One of such examples is the ticks collected in our study that were sampled from earth around livestock shelters in Ethiopia. Given the large population of livestock and camels present in Ethiopia, one can suggest that *O. savignyi* as well as their *Borrelia* pathogen could be widely distributed.

This study described the presence of *C*. Borrelia kalaharica a human RF agent in its *O. savignyi* vector with an overall prevalence of 1.3% (4/312) which is lower than the 5% (4/77) of *B. anserina* in *A. persicus* tick from Ethiopia (Cutler et al., 2012). Although, the pathogenic capacity of this species is still largely unknown, infection have been reported in tourists who visited areas around the Kalahari Desert (Fingerle et al., 2016; Stete et al., 2018) where the

ticks are endemic (Bakkes et al., 2018). The presence of a TBRF specie in a country where louse-borne relapsing fever (LBRF) is still endemic in some places could pose a diagnostic and public health concern. The gold standard for LBRF diagnosis is Giemsa blood smear microscopy that does not distinguish between the louse and tick-borne species (Cutler et al., 2009). The density of some TBRF spirochaetes such as *B. hispanica*, *B. crocidurae* in the blood during acute infection is estimated at <200 spirochaetes/µl (Cutler et al., 2009), whereas at least a volume of 10^4 to 10^5 spirochaetes/ul is required for its detection (Anda et al., 1996). In contrast, the level of *B. recurrentis* (LBRF) and the genetically related *B. duttonii* in the blood during an infection is known to be often 10 times higher which aides' easy detection (Cutler et al., 2009), hence the reason microscopy is still predominant in LB endemic regions in Africa including Ethiopia.

There is also the possibility of misdiagnosis which will only increase the disease burden on the patients and could hinder the prescription of appropriate and effective treatment. The study clearly shows that TBRF could pose a significant public health crisis in the region, especially when louse-borne relapsing fever is still very much endemic.

Like *O. savignyi*, this is the first report to prove the presence of TBRF in the country. Whether TBRF is endemic and has been coexisting with LBRF, or the infection in tick vectors is sporadic requires additional investigation, especially its clinical importance in humans since this was not investigated in this study.

CHAPTER 4: TICK-BORBE RELAPSING FEVER: SCREENINGS, KNOWLEDGE AND RISKS ASSOCIATED WITH TICK BORNE RELAPSING FEVER

4.1 Introduction

In Nigeria, the clinical evidence and significance of tick-borne relapsing fever (TBRF) is non-existent, albeit a handful of studies reported the presence of TBRF pathogens detected in ticks. One of such studies reported the presence of a *Borrelia. burgdorferi* sensu lato complex group in 0.4% (3/700) *Rhicephalus evertsi* ticks (Reye et al., 2012). However, phylogenetic information to further confirm the *Borrelia* specie was not successful using the 16S gene sequences which is known to be less sensitive for specie delineation. More recently, another study reported the presence of *Candidatus* Borrelia kalaharica in 6.1% of pooled *Ornithodoros savignyi* ticks investigated from Borno state, Nigeria (Cutler et al., 2018). For this study, we also reported the presence of a bovine relapsing fever strain in small ruminants (chapter 2; section 2.3.4).

Prior to the report of *C.* B. kalaharica, there were suspected cases of TBRF in Borno state reported by a local newspaper (daily trust) in December 2008, however, there was no follow up to identify the causative agent, nor confirm the suspicion. This may be attributed to the general poor level of awareness of TBRF in the region and country at large. The Northeast region where Borno state is located has the second highest poverty rate of (76.3%) in Nigeria only after the North-western region with (77.7%) (Khan & Cheri, 2016). This in part is attributed to the ongoing security crises facing the state, as well as desertification that is mostly caused by droughts and displacements (Amaza. et al., 2009).

Confirmation of TBRF is via thick and thin blood microscopy, serology (ELISA), and polymerase chain reaction (PCRs). As a result of socioeconomic challenges and poor awareness of the disease in the region and country at large, diagnosis will continue to be a challenge, as reported elsewhere in other countries in Africa such as in Tanzania (McConnell, 2003), thereby underestimating its true significance.

Nigeria has no known evidence of public awareness, surveillance, public health programs, nor community preventive measures designed for TBRF. Certainly, an important prerequisite for the successful implementation of preventive and control measures for any disease transmission is community participation (Achoki et al., 2009) as collaboration between the local community and relevant stakeholders is vital for the implementation of interventions.

To determine the clinical relevance of TBRF in Nigeria with a particular focus on two states in the north Plateau and Borno, we carried out a relapsing fever screening for patients presenting with febrile symptoms, as well as exploring the information on exposure to ticks, and lifestyle practices of the local population in relation to TBRF.

4.2 MATERIALS AND METHODS

4.2.1 Study locations

This study was carried out between April and May 2019 in Borno and Plateau states in Nigeria. Plateau is a mountainous state of about 30,913km^2 located in the North Central region of Nigeria, with Jos as it states capital. The altitude ranges to about 4000 feet above sea level and enjoys a more temperate weather with an average monthly temperature of 21°C to 25°C compared to the 28°C for the rest of the nation (Musa. & Dung-Gwom, 2018). In contrast, Borno a North-eastern Sahelian state has an estimated landmass of 69,435km^2 is one of the hottest states with an estimated elevation of 1060 feet above sea level, average annual temperature of 37°C (Mohammed. et al., 2019), and annual rainfall of 600 to 1200mm (Amaza. et al., 2009).

Participants were recruited from Plateau State Teaching Hospital (PSTH) (Jos-North), Jos University Teaching Hospital (JUTH) (Jos-East) both in Plateau State, and from the State Specialist Hospital Maiduguri (SSHM), Borno State. These hospitals serve as the focal healthcare point for the local communities in the states. Ethical approval was obtained from the above hospitals (appendix 6), and informed and signed consent were sort from all participants and the guardians of all minors (appendix 7).

4.2.2 Study participants and sample size

A total of 152 patients between the ages five and 60 who came for consultation with a temperature of >37.5°C and presenting with at least one other fever symptoms such as (Headache, vomiting, or general body aches) were randomly selected from JUTH (50), PSTH (52) hospitals in Plateau state, and SSHM (50) and recruited for this study. Two people from PSTH without febrile symptoms were included as controls. The minimum sample size 191 of was determined using the formula from section 2.2.1 and an attrition of 10%, based on the 13% prevalence in the clinical survey from Senegal (Parola et al., 2011).

z= 1.96, p= 13% or 0.13, q=1 - 0.13 (0.87), $d=0.05^2$

$$= \frac{(1.96)^2 \text{ X } (0.13) \text{ X } (0.87)}{(0.05)^2}$$

$$= \frac{0.4344}{0.0025}$$

= 174 + 17.4 (10% of 174)

= 191 (minimum sample size)

4.2.3 Inclusion and exclusion criteria

Patients showing symptoms of febrile illness and report of recurrent fever that could give signed or verbal consent were included. Patients who requested for incentives before agreeing to join the study were excluded. Children below 10 years were not included in the study.

4.2.3 Quality assurance

The research questionnaire used for this study was designed by the researcher. Questionnaires were only administered by the researcher to ensure consistency. The questionnaire employed was in English (the official language of Nigeria), however, a translator was available to translate the questions into the local dialect (Hausa) for those with language barrier, and the responses were filled in by the researcher on behalf of those patients. While the human samples were collected by trained laboratory scientists in all three study locations.

4.2.4 Blood sample collection and Questionnaire administration

Finger prick totalling 152 was obtained from each patient from the two states, and a 100µl whole blood was dropped on Whatman FTA™ card using individual sterile disposable blood lancets. Finger pricks were also collected on glass microscopic slides for thick smear

preparation. Each FTA card was allowed to dry and stored at room temperature according to manufacturer's guidelines pending analysis. Prepared smear slides were also left to dry at room pending analysis. Questionnaires on socioeconomic and demographic characteristics like level of education, occupation, household characteristics (mud/hut, wooden, cement), lifestyle practices (inhouse livestock rearing, domestic animals) were obtained from the participants in Plateau state. Knowledge about ticks, exposure to bites, febrile illness symptoms, duration of each episode, were also investigated.

It was impossible to interview and administer questionnaires to the participants in Borno state due to the ongoing security crises, as well as cultural/religious obstacles embedded in their sharia law practices. It was observed that majority of the patients that visited the hospital in Borno state during the time of study were females. For this study, 76% (38/50) of the participants were female, who could not give consent for recruitment without the approval of their men because of these sharia practices. The participants including the men only consented to give blood samples as they were in the hospital for blood tests but were not interested to be interviewed. The women who visited the hospital without their men refused outrightly to be included in the study.

4.2.5 DNA extraction of human finger prick

Individual deoxyribonucleic acid (DNA) was extracted from dried blood collected on Whatman FTA cards using a single one-hole puncher with the DNA blood and tissue extraction kit (Qiagen, Valencia, CA, USA) according to the manufacture's guidelines (appendix 5).

Each sample was resuspended in 180µl lysis buffer and 20µl proteinase K, and vortexed for 30secs. Samples were heated in the water bath at 56°C for 60mins whilst vortexing for 30secs every 10mins to ensure adequate breakdown of cell walls. 280µl of AL buffer was added into each sample and vortexed. Samples were incubated at 70°C for 10mins whilst

vortexing for 30secs every 3mins. Samples were centrifuged at a maximum speed of 20000g per minute for 30secs and the lysate transferred into a sterile spin column.

Samples were recentrifuged at a lower speed of 8000rpm, and the flowthrough discarded Samples were washed with 700µl AW buffer to remove non-specific binding materials and centrifuged for 1min at 6000g, and flowthrough discarded. 700µl of absolute ethanol (100%) was added into each sample and centrifuged for 1min at 6000rpm before discarding flowthrough. Samples were centrifuged at 20000g for 3mins after which the spin column was opened and incubated for 10mins at room temperature (RT). Each DNA was eluted with 50µl elution buffer and incubated for 5mins at RT Sample concentration and purity level were determined on nanodrop. Eluted DNAs were centrifuged at 20000g for 1min and stored at -20°C pending analysis.

4.2.6 Real-time PCR screening for Borrelia detection

All finger prick samples were investigated for the presence of *Borrelia* by using an optimised version of the *Borrelia*-specific real-time polymerase chain reaction (qPCR) primers (Merck™) designed to amplify a 148-bp fragment of the 16S rRNA of *Borrelia* as previously described (Kingry et al., 2018). A final PCR volume of 25µl containing 10x reaction buffer, dNTPs (0.2mM each), MgCl2 (2.5mM), 2µl DNA template, each primer at (5uM), probe (2.5uM), and 0.15U taq was used. Cycling conditions were as follow: 95°C for 10mins, 40cycles of 95°C for 15secs, and 60°C for 1min as previously described (Cutler et al., 2018). DNA of a *Borrelia.* The DNA of a *B*. *burgdoferi* B31 strain was used as the positive template, while nuclease free water (Invitrogen™) was used as the negative template control. Cycle threshold (CT) cut-off value was set at £36. Amplification was conducted as described in (section 4.2.7).

4.2.7 Microscopic examination for Malaria Plasmodium parasite

A hundred and fifty-two thick blood smears stained with Giemsa were prepared on glass microscope slides to investigate for the presence of *P. falciparum* parasites the endemic specie in Nigeria. Parasites were viewed under the Motic™ B1-220E-SP series microscope at a magnification of 10X as previously described (Nordstrand et al., 2007). Thick blood film method was adopted due to the large volume of 100µl whole blood collected, that is known to improve the sensitivity of parasite detection (as low as 50 parasites/µl) (Moody, 2002; Tek et al., 2010).

4.2.8 Real-time PCR screening for Malaria

All samples were screened for the presence of malaria parasite using the genus-specific 18S rRNA primers (Merck) (Mach 60 [5′-ACATGGCTATGACGGGTAACG-3′]; Mach 61 [5′-TGCCTTCCTTAGATGTGGTAGCTA-3′]) and probe (Mach 62 [5′-TCAGGCTCCCTCTCCGGAATCGA-3′]) designed to amplify a 84bp fragment of all four *Plasmodium* species (Lee et al., 2002). Cycling conditions were as follow; 95°C for 10 min, followed by 32cycles of 94°C for 30secs; 60°C for 30secs, and 72°C for 30secs as previously published (Gama et al., 2007). Cycle threshold (CT) cut-off value was set at £37.

Amplified products were read on FAM wavelength. Amplification was conducted using the AriaMx 1.2 Real-Time thermocycler (Agilent). DNA of a human *P. falciparum* was used as positive template and DNA non-febrile patients was used as negative template control.

4.2.9 Data Analysis

The data obtained from the questionnaires were analysed using frequency distribution graphs, and simple percentage method on graph pad prism version 9.4.1 (458).

4.3 RESULTS

Of the 152 participants recruited, only 102 of which 52 were from Plateau State Teaching Hospital (PSTH), and 50 from Jos University Teaching Hospital (JUTH) had the questionnaire successfully administered to. It was impossible to administer the questionnaires for the participants from Borno state as they cited cultural reasons.

4.3.1(a) Sociodemographic of study participants

Of the 102 patients, approximately 63% (64/102) of the participants were female, while 37% (38/102) were male, with the overall mean age of 41.8 years (Table 4.3.1a). Most of the participants are students 33.3% (34/102), followed closely by unemployed people 27.4% (28/102), 18.6% are civil servants (19/102), 13% (14/102) traders, and 4.9% (5/102) are farmers (Fig. 4.3.1a).

Table 4.3.1a: Mean age of Participants

Age Groups	Frequency	Min	Max	Midpoint	Σfx	Σfx^2	Mean age	Variance	SD
10 – 17yrs	22	10	16	18	396	7128			
18 – 25yrs	12	18	24	30	360	10800	41.8	230	15.17
26 – 35yrs	25	26	34	43	1075	46225			
>35yrs	43	35	43	56.5	2429.5	137267			
Total	102				4260.5	201419.8			

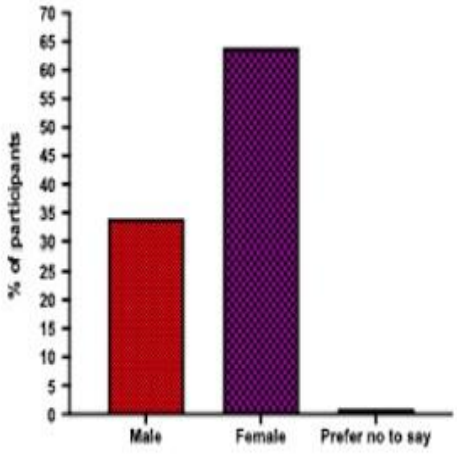
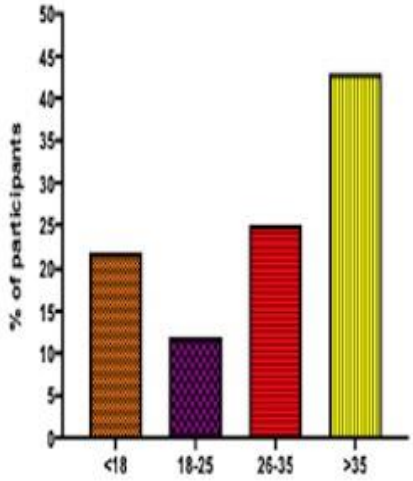
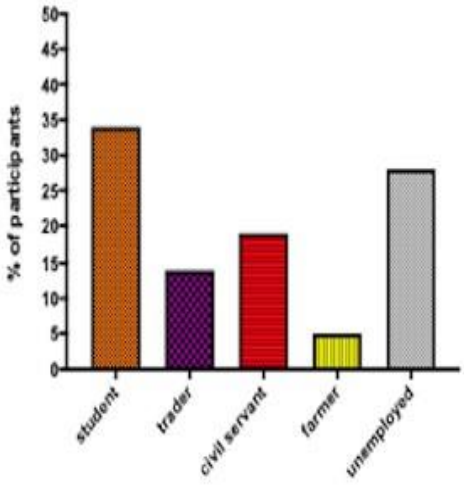


Figure 4.3.1(a): Gender



Age distribution



Occupation

4.3.1(b): Febrile symptoms of study participants

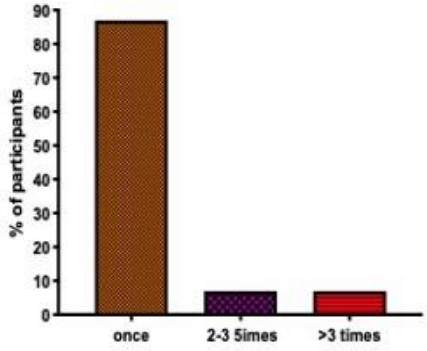
More than 80% (81/102) of respondents reported to have had fever in the past month, with an average duration of 1.9 days per episode, and an average recurrence of 1.1 times per year (table, 4.3.1b). All participants reported experiencing febrile symptoms, with headache, high temperature, and general body ache being the most common. About 20% (20/102) of participants had not been diagnosed of any illness nor received any form of treatment, while 80% (82/102) of participants had previously tested positive for malaria of which ~90% (74/82) were treated for malaria (Fig. 4.3.1b).

Table 4.3.1b: Mean duration of fever episode per month

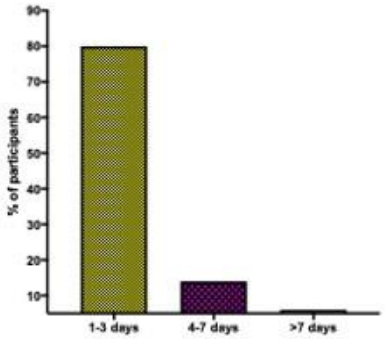
Fever duration	frequency (f)	Min	Max	Midpoint (x)	Σfx	Σfx^2	Mean (days)
once	87	1	1	1.5	130.5	195.8	1.9
2-3 times	8	2	2.5	3.3	26	84.5	
>3 times	7	3	3.5	4.8	33.3	157.9	
Total	102				189.8		

Table 4.3.1b: Mean number of fever episodes per year

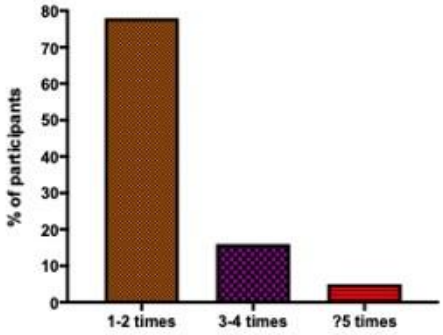
Episode (yearly)	frequency (f)	Min	Max	Midpoint (x)	Σfx	Σfx²	Mean (yearly)
1 - 2 times	78	1	1	1.5	117	175.5	1.1
3 - 4 times	16	2	2.5	3.3	52	169	
>5 times	5	3	3.5	4.8	23.8	112.8	
Total	99				192.8		



Fever episode monthly



Average duration per episode



Fever episode yearly

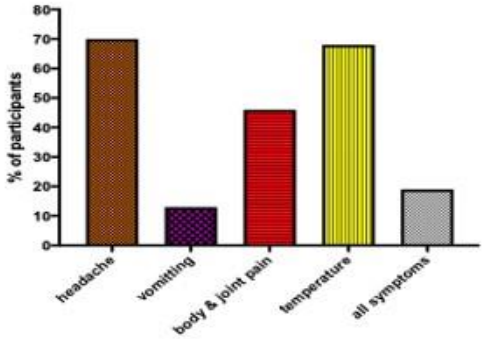
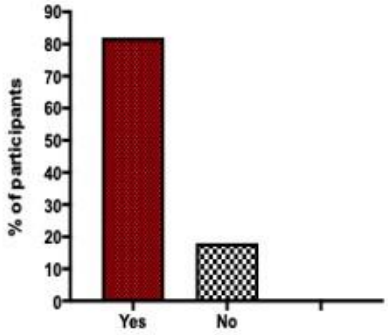
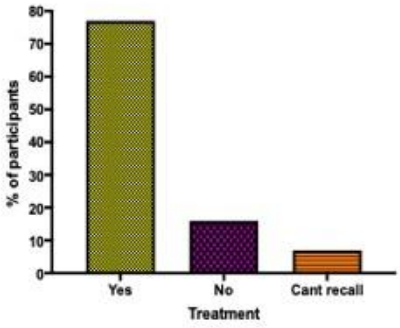


Figure 4.3.1(b): Presenting symptoms



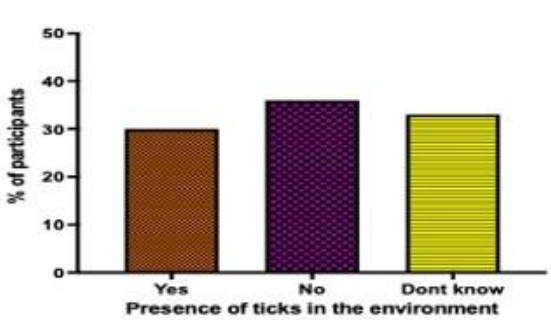
Malaria Diagnosis



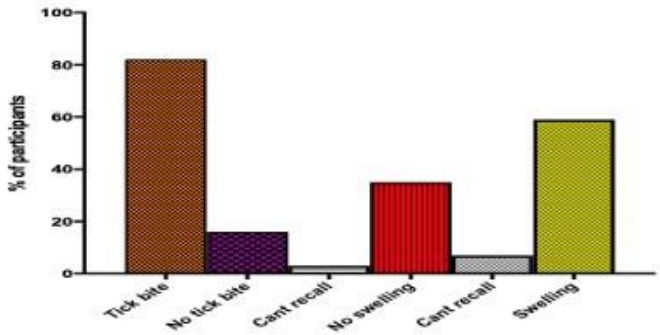
Treatment for Malaria

4.3.1(c): Knowledge of ticks and exposure to bites

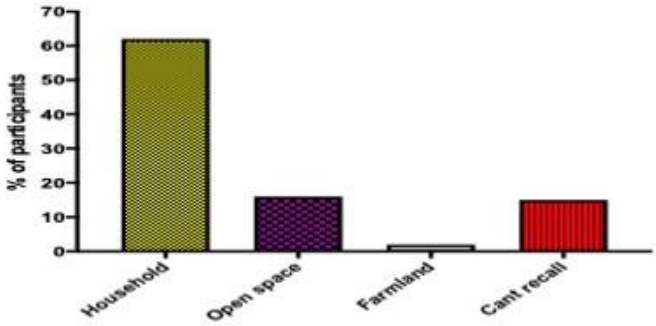
More than 29.4% (30/102) of respondents reported the presence of ticks in their immediate environment, 36.3% (37/102) reported no evidence of ticks, and 34.3% (35/102) had no prior knowledge about ticks. For tick locations, 60% (18/30) reported it in their households, 16.6% (5/30) in open spaces, 6.6% (2/30) in farmlands, and 16.6% (5/30) could not recall the precise location of the ticks. Of those who reported ticks in their environment, 80% (24/30) reported bites, 14% (4/30) reported no bites, and 6% (2/30) were unsure. 57% (14/24) reported swelling at the site of bite, while 43% (10/24) had to swellings. Only 22.5% (5/24) reported symptoms post tick bite, while the others could not recall whether their symptoms were associated with the bite (Fig 4.3.1c).



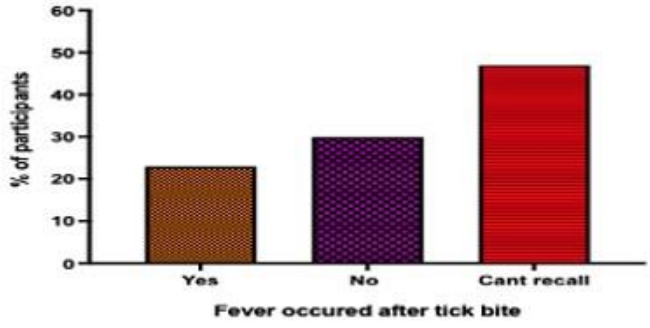
Exposure to ticks



Report of tick bites



Location of tick exposure



Onset of febrile symptoms after tick bite

Figure 4.3.1 (c):

4.3.1(d) Household characteristics of participants

A significant number of participants 91% (93/102) lived in cemented houses with concrete flooring, and only 5.8% (6/102) lived in traditional mud houses. About 70.5% (72/102) live in proximity to domestic animals or were involved in some sort of animal husbandry, with poultry farming being the most practice (Fig. 4.3.1d).

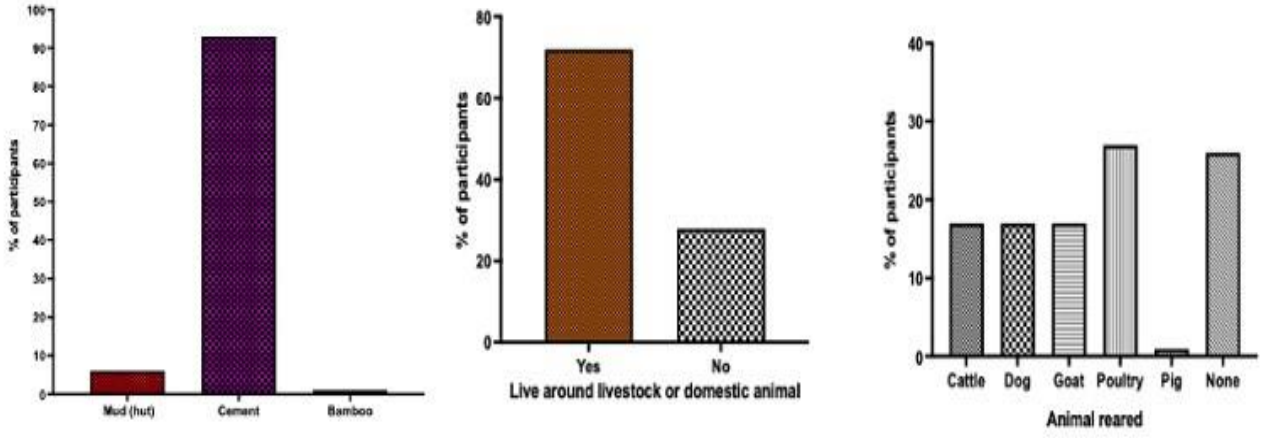


Figure 4.3.1 (d): Household characteristics Proximity to animals Range of animals

4.3.2 PCR screening for TBRF

All human DNA samples were free from *Borrelia* infection after initial screening using genus-specific 16S rRNA primers. Both positive controls of a *B. burgdoferi* B31 strain at a concentration of 1:10 (cq 13) and 1:100 (cq 15) amplified as expected.

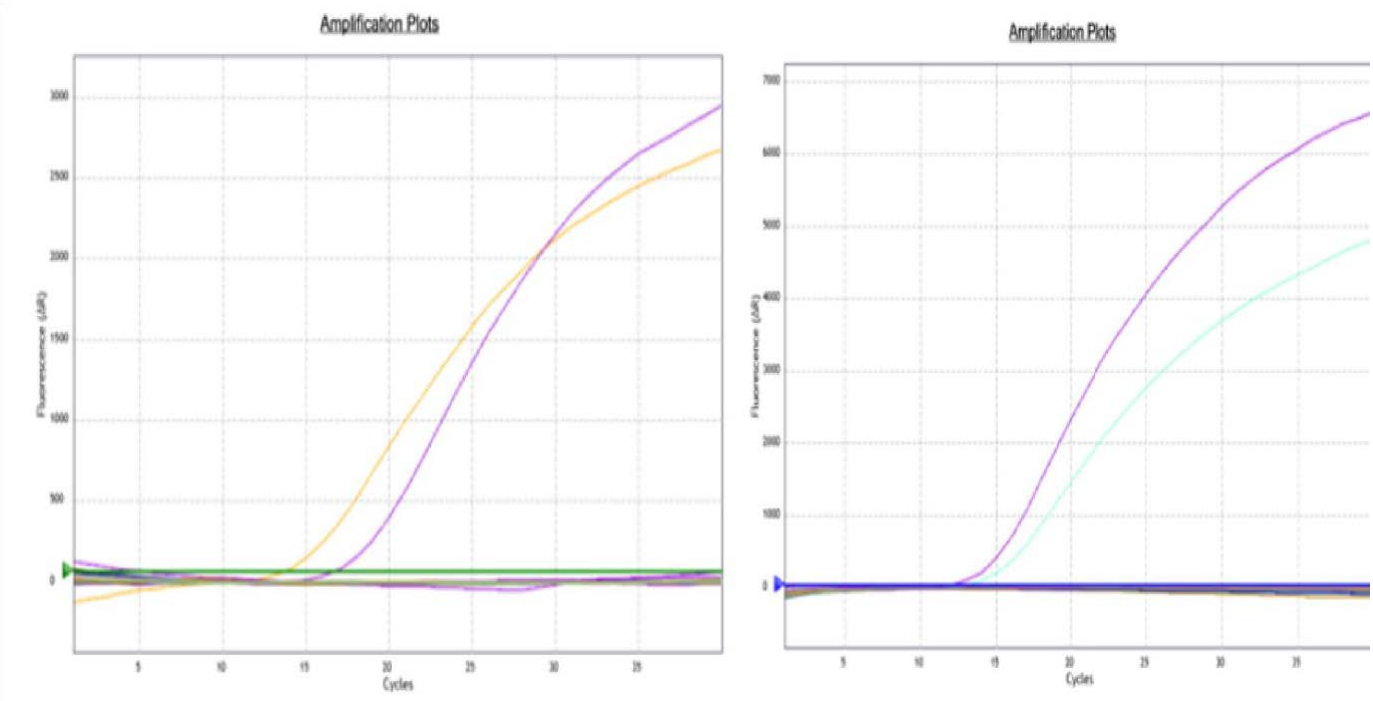


Figure 4.3.2: Borrelia screening of febrile patient's using 16S primers on RT-PCR.

4.3.3 Microscopic examination of blood films for Malaria parasites

All 152 thick smear Giemsa-stained slides were negative for Plasmodium parasites. This result can also be attributed to the fact that over 80% of the patients had already been diagnosed with malaria and had received some sort of treatment. It is possible that the level of parasitaemia in the blood had reduced significantly beyond the detection level. The method adopted for microscopic detection is known to have better sensitivity in detection low levels of blood parasitaemia.

4.3.4 PCR screening for Malaria parasites

All 152 patients screened for malaria were negative for *Plasmodium* parasites. This result is in correlation with that of the microscopic examination in section 5.3.2 which improves its validity.

4.4 DISCUSSIONS

In this study, 152 patients presenting with one or more febrile symptoms were recruited from Plateau State Teaching Hospital (PSTH) (Jos-North), Jos University Teaching Hospital (JUTH) (Jos-East) both in Plateau State, and from the State Specialist Hospital Maiduguri (SSHM), Borno State. The hospitals were chosen as they serve as the focal healthcare centres for the populace in the two states. A hundred and fifty-two finger prick samples were obtained, and data on knowledge, attitudes, and practices relating to ticks and their associated risk were obtained from 102 participants.

There was no evidence of tick-borne relapsing fever (TBRF) in the patients from two states. More than 90% (92/102) of the participants live in cemented houses, with only ~10% reported living in either traditional mud or bamboo houses. The predominant household of the participants would have made it more difficult for ticks to hide in the walls or crevices, or around small animal burrows that opens close to households as they are known for (Trape et al., 2013) without been detected. Only 29.4% (30/102) reported ticks in their proximity, 36.3% (37/102) reported no ticks, while 34.3% (35/102) had no prior knowledge about ticks. Overall, only 23.5% (24/102) of participants reported tick bites, majority of which occurred in their households or in open spaces, while a combined majority of 66% (67/102) had prior knowledge about ticks (section 4.3.1c).

Majority of the participants were students 33%, and a combined 41% (38/102) were employed of which only ~5% were famers, while (27%) were unemployed. The high unemployment rate observed could in part be linked to the demographic characteristics of the participants as >60% of responders were females. In fact, due to cultural and religious barriers, women in the northern part of Nigeria are known to be the most secluded when it comes to employment, access to productive resources, education, or even making decisions independently (Jackson, 1985; Rahman, 2008) as was the case for the Borno participants. We were unable to administer questionnaires to the Borno participants due to these barriers.

The obligation between men and women are defined in such that, men are placed as the sole providers for their household, while the women oversee domestic household responsibilities (Rahman, 2008).

This study also showed that none of the patients had malaria infection at the time of recruitment. Majority of the respondents (80%) had previously been diagnosed of malaria of which more than 75% had received treatment. This could account to why none of the samples screened were positive for malaria using both molecular and microscopic tools as shown in sections (4.3.3; 4.3.4). Only about 20% of the febrile patients had not received any form of diagnosis at the time of recruitment. Furthermore, due to unfavourable climatic condition in Plateau state, there has been a continued decline in malaria cases as the cold temperature is not suitable for the survival of the plasmodium pathogen. In fact, Plateau state has the lowest incidence of malaria in the country, especially during the coldest months between December and February where the temperature can be as low as 18°C. This was further corroborated by the work of (Nanvyat et al., 2018) that reported the lowest incidence of malaria in Plateau during the coldest months compared to the regions in the country.

Generally, for West Africa, the epidemiology of TBRF is still largely known, with few reports showing the soft tick vector from the genus *Ornithodoros* to be largely concentrated to the areas bordering the Sahelian and Saharan regions (Godeluck et al., 1996; Vial et al., 2006) such as Borno state in this study.

The absence of TBRF particularly for Borno state, an area endemic to *Ornithodoros* ticks cannot be ruled as an absence of infection in the entire region but, could be because of the small sample size employed in this study as stated in section 4.4.2. Notwithstanding, since the participants were recruited from the state capital, one may assume that they live in urban households as opposed to mud or bamboo houses in the rural areas where these ticks are resident and puts the occupants at risk of bites. It is also possible that the participants aren’t

involved in any major animal farming besides subsistent farming, that might expose them to these ticks.

On the other hand, the lack of clinical evidence of TBRF in Plateau state can be attributed to the fact that it is not an area where *Ornithodoros* ticks nor *Borrelia* had been reported prior to this study. In addition, based on the sociodemographic data, majority of the participants were less likely to be exposed to ticks as they lived in urban households. Furthermore, the climatic condition there is a temperate one with an average monthly temperature of 21°C to 25°C compared to the rest of the nation (Musa. & Dung-Gwom, 2018). Ecological factors such as relative humidity, warm temperature are major drivers for tick infestations/questing, particularly for soft ticks that tend to hibernate during the colder seasons (Latif & Walker, 2016), which means the climatic conditions in the state may be unfavourable for them to thrive. In addition, lower temperatures increase the likelihood of humans wearing additional clothing and reducing their outside time, which in turn will reduce their risk to tick exposure (Ostfeld & Brunner, 2015; Wikel, 2018). Nonetheless, with the continued negative consequences of climate change on the global temperature, tick-borne diseases such as this will continue to expand as reported in the study of (Gray et al., 2009).

Overall, the small sample size in this study may have been a barrier in establishing the true clinical picture of the disease in the region. Even more disappointing was the inability to obtain data on the socioeconomic and lifestyle practices of the Borno participants which makes it difficult to determine whether they are at risk to tick exposure or not.

In conclusion, this study reveals that most of the patients from Plateau state had prior knowledge about ticks. The lack of evidence of infection in all participants supports existing evidence that poor living conditions and lifestyle are drivers of domestic infestations of ticks, which in turns increases the likelihood of TBRF transmission. Given the small study size, there is clearly a need to further investigate the relevance of TBRF particularly in rural parts

of states such as Borno, Sokoto, Yobe, Katsina, etc. that share close borders with countries in the Sahelian belt such as Niger, Chad, etc., where soft ticks are known to be endemic.

CHAPTER 5: GENERAL DISCUSSIONS, LIMITATIONS, AREAS FOR FUTURE STUDIES, AND CONCLUSIONS

5.1 Overall Discussions

The epidemiology of tick-borne relapsing fever (TBRF) and their tick vectors in Nigeria and Ethiopia represents a clear knowledge gap as is the case in many parts of Africa (Diatta et al., 2015a; Nordstrand et al., 2007; Sarih et al., 2009; Souidi et al., 2014; Vial et al., 2006). This is based on increasing reports of borreliae infections in its associated tick vectors and small mammal hosts (Diatta et al., 2015a; Trape et al., 2013), especially in regions where the *Ornithodoros* ticks were not previously known to be present which was supported by the findings from this study. Despite the absence of clinical evidence of TBRF in both countries, one can to a large extent suggest the possibility of infections in the high-risk regions where these tick vectors are endemic and widely distributed, and where infested domestic animals are bred and kept in proximity to human shelters. There is no available comprehensive surveillance data of TBRF on its veterinary and clinical burden in both counties investigated in this study.

This is the first study conducted of TBRF borreliosis in domestic animals from Nigeria, with the only previous evidence of relapsing fever *B. theileri* infection in livestock conducted under controlled experimental settings (Trees, 1978). This animal spirochaete is vectored by hard-body ticks mostly from the *Rhipicephalus* genus that are widely distributed in many regions of the country such as Plateau state (Ogo et al., 2012). These ticks thrive best in environments with high altitudes and precipitation of ~800mm or more (Pegram et al., 1981) as is the case of Plateau state (Musa. & Dung-Gwom, 2018), hence, increasing the risk for relapsing fever borreliosis in animals particularly livestock.

We also documented the presence of *Ornithodoros savignyi* ticks in Plateau state which was not previously known to be endemic to the species. *Ornithodoros* species are known to be widely distributed in the semi-arid and arid regions such as Borno state, Nigeria as reported

in this study and in available literatures (Cutler et al., 2018; Trape et al., 2013). However, finding them in a region that is non-arid but, instead known to be the coldest in the country with the highest elevation shows the ticks can survive outside their natural ecological niche. The most likely scenario could be that the *O. savignyi* ticks were introduced from the endemic areas into Plateau state via animal trade routes such as camels that are known to hosts these ticks with little or no harm on the animals (Hoogstraal, 1956b).

We identified another Argasid tick *Argas persicus* using molecular methods that is the first for the country. These ticks have been previously identified in poultry and other semidomestic birds using entomological keys. They are one of the two most important ectoparasites of domestic birds in Nigeria only after *Bdellonyssus bursa* (the fowl mite) (Usman et al., 2012). They cause significant damage to the birds directly by causing physical harm such as paralysis through excessive sucking of blood and tissue fluid (Bunza et al., 2008). They also indirectly cause harm to birds through the transmission of deadly pathogens like *B. anserina* the agent of avian spirochaetosis, and *Aegyptianella pullorum* the agent of aegyptianellosis (Usman et al., 2012). Nigeria is a country where poultry farming outnumbers all other animal farming, with an estimated 75% of the population most of whom are in the rural areas practicing some form of poultry farming either for commercial or subsistent farming purpose (Adeyemo & Onikoyi, 2012). Poultry products such as milk and egg also serve as the main source of protein, continuous infestation of these ticks will only continue to deepen the veterinary and economic burden particularly for people whose livelihood depends on it.

As part of the Nigerian study, we also explored the knowledge, practices, and perceptions of the local population in relation to TBRF borreliosis, as well as screening for *Borrelia* infection in persons presenting with febrile symptoms. The data obtained suggests that knowledge about ticks is quite high with ~80% of respondent reporting bites just as observed

elsewhere (Kisinza et al., 2008). Notwithstanding, it is important to state clearly that majority of the study participants had some degree of financial freedom and literacy based on their household characteristics, occupation, and educational status. In addition, these participants were recruited from the major hospitals which shows they make better choices in terms of where they seek healthcare, which suggests that are fairly in average socioeconomic class.

Our study in Ethiopia is the first to describe the presence of *Ornithodoros* ticks in the region, more so, the identification of *O. savignyi* the vector of a human TBRF agent. Although, one can argue that the identity level of 93-94% is low which was evident on the phylogenetic tree and may suggest a distinct specie or a sub-specie within the *O. savignyi* group. A similar observation was reported in a *O. verrucosus* tick from Pakistan that had a 92.5% identity to existing species and formed a sub-clade from the other *O. verrucosus* on the phylogenetic tree (Ali et al., 2022). Notwithstanding, additional taxonomic revision by expert entomologists is required to resolve these discrepancies.

O. moubata the agent of *B. duttonii* is the endemic specie for East Africa that is of significant clinical importance in countries such as Tanzania, where TBRF is one of the leading causes of morbidity and mortality in children (Talbert et al., 1998), and abortion in women (Jongen. et al., 1997). The presence of another vector of TBRF will only aggravate the impact of the disease in the region especially in women and children.

In Ethiopia, hard tick from the *Rhipicephalus*, *Amblyomma*, and *Hyalomma* are the predominant ectoparasites parasitising both livestock and domestic animals in the endemic in the region (Kumsa et al., 2015, 2019; Olivieri et al., 2021; Teshale et al., 2016). In contrast, there has only been a handful literature on soft ticks such as *A. persicus* found around animal and human shelters (Cutler et al., 2012; Pader et al., 2012).

This is in fact surprising especially knowing that Ethiopia has the largest livestock and domestic animal population in Africa such as camels that are one of the preferred hosts for these *O. savignyi* ticks (Bakkes et al., 2018). Just as stated previously, *O. savignyi* is believed to have been distributed into regions of Africa via camel trades from the Middle East. There have been speculations regarding the presence of these ticks in the country, the data from this study can help address some of those uncertainties.

We also present the first evidence of *C*. B. kalaharica infection in the *O. savignyi* ticks which pose a significant clinical risk. The exact pathogenic capability of this borreliae is still unfolding, albeit available evidence shows it is capable for causing disease in humans (Fingerle et al., 2016; Stete et al., 2018). Historically, there were suspected outbreaks of TBRF in the neighbouring Somaliland which peaked yearly during the rainy seasons between April and September when the population were lower outside in numbers. The ticks were suggested to have been introduced into the country through human and animal migration from Ethiopia. However, there was no formal tick identification and the possibility of coexisting with LBRF was also not ruled out, hence, the existence of the disease could not be verified. Confirmation of the disease was not until the description of *C*. B. kalaharica in humans that visited countries around the Kalahari Desert region (Fingerle et al., 2016; Stete et al., 2018).

This data from our study together with existing data suggests that TBRF is endemic in the Eastern and Southern Africa region, and possible coexists with the endemic LBRF in Ethiopia. This will provide new insights for clinicians in the country when dealing with suspected cases of relapsing fever in relation to choosing appropriate diagnostic method, as many TBRF species are known to have low level of spirochaetemia in the blood unlike the LBRF specie (Cutler et al., 2009). With microscopy being the gold standard method for

diagnosis of RF in the region including Ethiopia (Cutler et al., 2010), this will be less sensitive for the TBRF species.

5.2 Conclusion

We have demonstrated the presence of tick-borne relapsing fever borreliosis in small ruminants from Nigeria. In addition, we also reported the first evidence of *Ornithodoros savigni* ticks in region where it was not previously known to be present due to less favourable conditions. Finally, we confirmed the presence and identity of the poultry tick *Argas persicus* using molecular tools.

The data generated from the Nigerian studies is of clinical, veterinary, and economic important particularly in regions where tick infestation is high, and for people whose main source of income is animal farming. This study can serve as a wakeup call to the relevant stakeholders in charge of controlling and preventing tick-borne diseases in animals and humans alike.

We also documented the presence of *Candidatus*. Borrelia kalaharica and its *Ornithodoros savignyi* tick vector in Ethiopia which could pose a public health and diagnostic problem in the country due to the established presence of louse-borne form of the disease.

Overall, our research will stir up other researchers and vets to carry out additional studies in humans and animals to identify the pathogenicity of these borreliae pathogens as well as the role of the tick vectors.

5.3 Study Limitations

There are several limitations that may have impacted the overall robustness of this study. One of such is the small human sample size on the knowledge about ticks and its associated risks from Nigeria which cannot be generalised for the entire country. This was even more limiting as we were unable to administer questionnaires to the participants from Borno state which further reduced our overall sample size. Notwithstanding, it was worth carrying out as there is no alternative data available.

Secondly, we could have included hard ticks of the *Rhipicephalus* and *Amblyomma* genus's that are established vectors for bovine relapsing fever to better understand the possible risk of infection within the animal population.

Thirdly, we could have attempted to go back to the field to sample more *Ornithodoros* ticks from Plateau state to determine whether they are widely distributed or not. This is particularly important as the discovery of *Ornithodoros savignyi* in the region is new and still unfolding as previously stated.

For the Ethiopia study, we did not investigate livestock and humans for the evidence of TBRF which could have given more robustness to our data to understand its clinical and veterinary significance in the country. Unfortunately, due to time and financial constraints, it was impossible to travel down to collect these additional specimens.

5.4 Areas of Future Research

Clearly, our results and limitations signify the need for further investigation of the disease in both countries. In addition, research into easily accessible and cost-effective commercial diagnostic kits for both veterinary and commercial use is needed.

More research is also needed to map out the exact geographical distribution and burden of TBRF fever in Nigeria and Ethiopia, and ultimately Africa.

Future large-scale studies on local knowledge and practices around ticks and the associated risks is needed in Nigeria.

Further taxonomic revision of the Afrotropical *Ornithodoros* that includes tick sampling from other known animal hosts such as livestock, domestic, and wild animals instead of just small mammals will be beneficial in expanding the knowledge regarding the genus. In addition to this, revision is also needed with the genus for the current species, which can provide better proffer answers to why the ticks from Ethiopia had low identity level.

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APPENDICES

Appendix 1.0: Publication to support this thesis

Adefolake A. Bankole, Bersissa Kumsa, Gezahegne Mamo, Ndudim I. Ogo, Nusirat Elelu, Winston Morgan, and Sally J. Cutler

Comparative Analysis of Tick-Borne Relapsing Fever Spirochaetes from Ethiopia and Nigeria

Appendix 1.1: Other collaborative publications to support thesis

N. Elelu; **A. A. Bankole**; H.P Daphe; M. Rabiu; S. D Ola-Fadunsin;

H.M. Ambali; S.J. Cutler

Molecular characterisations of *Rhipicephalus sanguineus* sensu lato ticks from domestic dogs in Nigeria.

Nusirat Elelu, Shola David Ola-Fadunsinl, **Adefolake Ayinke Bankole**, Mashood Abiola

Raji, Ndudim Isaac Ogo, Sally Jane Cutler

Prevalence of tick infestation and molecular characterization of spotted fever *Rickettsia massiliae* in *Rhipicephalus* species parasitizing domestic small ruminants in north-central Nigeria

pathogens

MDPI

Article

Comparative Analysis of Tick-Borne Relapsing Fever Spirochaetes from Ethiopia and Nigeria

Adefolake A. Bankole [1], Bersissa Kumsa [2], Gezahegne Mamo [2], Ndudim I. Ogo [3], Nusirat Elelu [4], Winston Morgan [1] and Sally J. Cutler [1,*]

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Abstract: Despite increasing reports of tick-borne diseases in Africa, remarkably, reports of tickborne relapsing fever (TBRF) in Nigeria are lacking. *Ornithodoros savignyi* from Nigeria have been reported with the relapsing fever *Candidatus* Borrelia kalaharica. Conversely, in Ethiopia, the agent of relapsing fever is the louse-borne relapsing fever (LBRF) spirochaete *Borrelia recurrentis* with no TBRF reported to occur. A total of 389 *Ornithodoros* ticks, Ethiopia (N = 312) and Nigeria (N = 77), were sampled, together with 350 cattle, and 200 goat sera were collected from Nigeria. Samples were screened for *Borrelia* spp. by RT-PCR. Reactive samples were confirmed, then sequenced using flagellin B, 16S rRNA, and 16S–23S intergenic spacer region. The prevalence of *Borrelia* spp. in livestock was 3.8% (21/550) and 14% (3/21) after final molecular confirmation. Of 312 ticks from Ethiopia, 3.5% (11/312) were positive for *Borrelia*, with 36% (4/11) by conventional PCR. Sequencing revealed that the borreliae in soft ticks was *C.* B. kalaharica, whilst that found in animals was *Borrelia theileri*. Soft ticks were confirmed by sequencing 7% (22/312) and 12% (9/77) of the Ethiopian and Nigerian ticks, respectively. Phylogenetic analysis revealed that these were *Ornithodoros savignyi*. This is the first evidence of *C.* B. kalaharica in Ethiopia and demonstrates the co-existence of TBRF in a country endemic to LBRF. Important, this might cause a diagnostic challenge given that LBRF is predominantly diagnosed by microscopy, which cannot differentiate these two spirochaetes. Furthermore, we report *B. theileri* in ruminants in Nigeria, which may also be of veterinary and economic importance.

Keywords: soft ticks; ruminants; Ethiopia; Nigeria; tick-borne relapsing fever

Citation: Bankole, A.A.; Kumsa, B.; Mamo, G.; Ogo, N.I.; Elelu, N.; Morgan, W.; Cutler, S.J. Comparative Analysis of Tick-Borne Relapsing Fever Spirochaetes from Ethiopia and Nigeria. *Pathogens* **2023**, *12*, 81. https://doi.org/10.3390/pathogens12010081

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ORIGINALARTICLE

WILEY

[1]. Introduction

Most tick-borne relapsing fever (TBRF) are zoonotically caused by the bacteria of the genus Borrelia that are majorly vectored by soft *Ornithodoros* ticks. The existing dogma is that these ticks inhabit the burrows of their preferred vertebrate hosts, such as rodents and hedgehogs, and feed indiscriminately upon them [1] serving to ensure the circulation and maintenance of these spirochaetes within the host-tick ecosystem. Many can also undergo transovarial transmission, but this is not believed to be sustainable over multiple generations [2]. However, it can provide a valuable demonstration of tick vector competence [2]. These ticks have secondarily adapted to domestic animal and human dwellings, often taking advantage of anthropogenic factors such as densely populated clusters of people and livestock [3].

Molecular characterisation of *Rhipicephalus sanguineus* sensu lato ticks from domestic dogs in Nigeria

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Abstract

Rhipicephalus sanguineus is the most widely reported tick in the world. Molecular characterisation is important to verify its taxonomic status in the different parts of the world. In this study, we provide information on the molecular characterisation of *R. sanguineus* tick of dogs collected from Nigeria. Ticks were collected from 62 of 93 sampled dogs. The collected ticks were subjected to morphological identification with the aid of appropriate entomological keys. Deoxyribonucleic acid (DNA) was extracted from the mostprevalenttickspecies(*R.sanguineus*)andwassubjectedtofurthermolecularcharacterisation protocols. The partial mitochondrial 16S rRNA gene sequences (~300 bp) were obtained from representative specimens. Data were statistically analysed using the chi-square (χ^2) test. Phylogenetic analysis was performed including different lineages of *R. sanguineus* (sl) from Africa, Asia, Europe and America, and other species belonging to the *R. sanguineus* 'tropical lineage' (*R. linnaei*) as well as *Rhipicephalus turanicus* and *Ixodes ricinus*. Results of this study showed that *R. sanguineus*= was the most abundant ticks of dogs with a prevalence of 61.8% (68/110; 95% CI 52.5–70.54), followed by *Amblyomma variegatum* (20.0%) and *Haemaphysalis leachi* (18.2%). The molecular analysis shows that they are genetically different from the temperate strains but closely related to those from other West African countries. There is a need to establish the vector competence of this common Nigerian dog tick.

KEYWORDS
16S rRNA gene, brown dog tick, endemic, Kwara State, North-central

1 | INTRODUCTION

Ticks are obligate blood-sucking arthropods and they are believed to be next in importance only to mosquitoes among other arthropods as vectors of bacterial, viral and protozoan disease agents (Abdullah et al., 2016; Ola-Fadunsin et al., 2021; Smith et al., 2011).

The *Rhipicephalus sanguineus* group (Acari: Ixodidae) includes 12 tick species, namely: *R. sanguineus*, *R. sulcatus*, *R. rossicus*, *R. schulzei*, *R. pumilio*, *R. pusillus*, *R. turanicus*, *R. leporis*, *R. guilhoni*, *R. moucheti*, *R. bergeoni* and R. *camicasi* (Nava et al., 2015). These groups of ticks have been documented to transmit several pathogenic bacteria such as *Rickettsia massiliae* reported in the Americas and *R. rickettsia* in Mexico (Eremeeva et al., 2006). They have also been associated with protozoan diseases such as *Babesia canis*, *Ehrlichia canis* and *Hepatozoon canis* (Dantas-Torres & Otranto, 2015; Dantas-Torres et al., 2018; Taylor et al., 2016).

Citation: Elelu N, Ola-Fadunsin SD, Bankole AA, Raji MA, Ogo NI, Cutler SJ (2022) Prevalence of tick infestation and molecular characterization of spotted fever *Rickettsia massiliae* in *Rhipicephalus* species parasitizing domestic small ruminants in north-central Nigeria. PLoS ONE 17(2): e0263843. https://doi.org/10.1371/journal.pone.0263843

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DataAvailabilityStatement: All relevant data are within the paper and its Supporting information files.

RESEARCH ARTICLE

Prevalence of tick infestation and molecular characterization of spotted fever *Rickettsia massiliae* in *Rhipicephalus* species parasitizing domestic small ruminants in north-central Nigeria

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Abstract

Ticks are of great menace to animal and human health. They serve as vectors to both animals and human pathogens including *Rickettsia* species. Tick-borne rickettsiosis in West Africa remains incompletely understood. We determined the prevalence of tick infestation among small ruminants and molecularly described a clinically significant spotted fever *Rickettsia massiliae* from *Rhipicephalus* ticks collected from North-Central, Nigeria. A total of 352 small ruminants comprising of 152 sheep and 200 goats that were brought for slaughter at the major small ruminant slaughterhouse in Ilorin were examined for the presence of ticks. The collected *Rhipicephalus* species were subjected to molecular studies to detect and characterize *Rickettsia massiliae*. Of the small ruminants examined, 21 sheep and 46 goats were infested with ticks representing 13.82% and 23.00% respectively. Eight and nine different species of ticks were detected in sheep and goats respectively, with *Rhipicephalus* (*Boophilus*) *decoloratus* being the most prevalent tick species in both sheep and goats. There was a significant difference ($p < 0.01$) in the prevalence of the different tick species collected in sheep and in goats. Based on the PCR amplification of the 23S-5S intergenic spacer (IGS), only 2 of the 142 *Rhipicephalus* tick samples screened for *R. massiliae* were positive (1.41%; 95% CI = 0.39–4.99). *Rickettsia massiliae* was detected from *Rhipicephalus turanicus* collected from sheep. Sequences obtained from the PCR carried out by amplifying *Rickettsia* 23S-5S IGS showed 99–100% close identity with members of the *R. massiliae* group. This study has for the first time confirmed the presence of spotted fever group *Rickettsia massiliae* from feeding ticks in Nigerian small ruminants. Further investigations to determine the possible pathogenic role of human *R. massiliae* infection in Nigeria would be beneficial.

Appendix 2.0: Ethical Approvals

Updated Ethical Approval for Research

University of East London

Pioneering Futures Since 1898

Dear Adefolake,

Application ID: ETH2223-0091

Original application ID: EXP 1819 01

Project title: Comparative analysis of tick-borne relapsing fever spirochaetes from Nigeria and Ethiopia

Lead researcher: Mrs Adefolake Bankole

Your application to Ethics and Integrity Sub-Committee (EISC) was considered on the 5th January 2023.

The decision is: **Approved**

The Committee's response is based on the protocol described in the application form and supporting documentation.

Your project has received ethical approval for 4 years from the approval date.

If you have any questions regarding this application please contact your supervisor or the administrator for the Ethics and Integrity Sub-Committee.

Approval has been given for the submitted application only and the research must be conducted accordingly.

Should you wish to make any changes in connection with this research/consultancy project you must complete 'An application for approval of an amendment to an existing application'.

Approval is given on the understanding that the UEL Code of Practice for Research and the Code of Practice for Research Ethics is adhered to.

Any adverse events or reactions that occur in connection with this research/consultancy project should be reported using the University's form for Reporting an Adverse/Serious Adverse Event/Reaction .

The University will periodically audit a random sample of approved applications for ethical approval, to ensure that the projects are conducted in compliance with the consent given by the Ethics and Integrity Sub-Committee and to the highest standards of rigour and integrity.

Please note, it is your responsibility to retain this letter for your records.

With the Committee's best wishes for the success of the project.

Yours sincerely,

Catherine Hitchens

Ethics, Integrity and Compliance Manager

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Older Ethical Approval for Research

8th August 2018

Dear Adefolake,

Project Title:	**What is the significance of Candidatus Borrelia kalaharica as a cause of febrile disease in Nigeria?**
Principal Investigator:	**Professor Sally Cutler**
Co-Researcher:	**Adefolake Bankole**
Reference Number:	**EXP 1819 01**

I am writing to confirm the outcome of your application to the University Research Ethics Committee (UREC), which was considered by UREC on **Wednesday 8 August 2018.**

The decision made by members of the Committee is **Approved**. The Committee's response is based on the protocol described in the application form and supporting documentation. Your study has received ethical approval from the date of this letter.

Should you wish to make any changes in connection with your research project, this must be reported immediately to UREC. A Notification of Amendment form should be submitted for approval, accompanied by any additional or amended documents: http://www.uel.ac.uk/wwwmedia/schools/graduate/documents/Notification-of-Amendment-to-Approved-Ethics-App-150115.doc

Any adverse events that occur in connection with this research project must be reported immediately to UREC.

Approved Research Site

I am pleased to confirm that the approval of the proposed research applies to the following research site.

Research Site	Principal Investigator / Local Collaborator
Community centres in Kwara state, Plateau state teaching hospital, and The Usman Dan-Fodio teaching hospital, Sokoto (In view) Nigeria.	Professor Sally Cutler

Appendix 2.2: Change project title - Mrs Adefolake Bankole

Change project title - Mrs Adefolake Bankole

Date	18 Dec 2022
Doctoral Researcher	Mrs Adefolake Bankole
Student ID	1346016
Doctoral Research Project	Comparative analysis of tick-borne relapsing fever spirochaetes from Ethiopia and Nigeria
Project type	MPhil/PhD - PhD
Project mode	Full Time
Project start	06 Feb 2017
School	Health, Sport & Bioscience

Change request form

Project title form

Please Note, if you have received Ethical Approval for your research you must also submit an Amendment to an approved Ethics Application. This can be done via the Ethics tab on your record and by starting a new application and choosing the 'Amendment to an application approved outside of ResearchUEL' option.

Failure to do this may result in a case of academic misconduct as your new research title will not have Ethical Approval.

Proposed new title:
Comparative analysis of tick-borne relapsing fever spirochaetes from Ethiopia and Nigeria

Reason(s) for proposed change:
The study has expanded and is now focused on both Nigeria and Ethiopia.

Researcher form

Did your research require Ethical Approval?
Yes

I confirm that I have completed an Amendment to an Approved Ethics Application form to change the title of my thesis

Having discussed the proposed change of title with my supervisory team, I am satisfied with the change proposed.
Yes

Supervisor form

Supervisor form

Did your student require Ethical Approval for their research?

Yes

I confirm that my student has completed an Amendment to an Approved Ethics Application form to change the title of their thesis

We recommend that the change in the registered title of the thesis progress as requested.
Yes

Notes
The new title is a better reflection of the work undertaken.

Research Degrees Leader form

Second approver form

Recommend this application for consideration at the School's Research Degrees Sub-Committee
Yes

Notes

HSB Research Degrees Sub-Committee report

Committee report

Comments
I approve the changes to the project title

Recommendation
Approve

Appendix 3: DNA Extraction Protocol for DNeasy Blood and Tissue kit (Qiagen®)

Procedure — using an electric homogenizer (ticks).

1. Place up to 50 mg insects(tick) in a 1.5 ml microcentrifuge tube.

2. Add 180 µl PBS and homogenize the sample using the Tissue-Ruptor, an equivalent electric homogenizer, or a disposable microtube pestle.

3. Add 20 µl proteinase K and 180 µl Buffer AL 1:10 Mix thoroughly by vortexing, and incubate at 56°C for 10 min. Ensure that ethanol has not been added to Buffer AL.

4. Add 200 µl ethanol (96–100%) to the sample and mix thoroughly by vortexing.

5. Pipet the mixture from step 4 into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000g for 1 min. Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000g. Discard flow-through and collection tube.

7. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000g to dry the DNeasy membrane. Discard flow-through and collection tube.

8. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not touch the flow-through. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000g.

8. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000g to elute.

https://www.qiagen.com/us/resources/resourcedetail?id=68f29296-5a9f-40fa-8b3d-1c148d0b3030&lang=en

Appendix 3.1: DNA Extraction Protocol for DNeasy Blood and Tissue kit (Qiagen®)

Procedure — (animal samples).

1. Add 20 µl Proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 100µl anticoagulated blood. Adjust the volume to 220 µl with PBS.

2. Add 200 µl Buffer AL. Mix thoroughly by vortexing, and incubate at 56°C for 10 min.

3. Add 200 µl ethanol (96–100%) to the sample and mix thoroughly by vortexing.

4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000g for 1 min. Discard flowthrough and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000g. Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000g to dry the DNeasy membrane. Discard flow-through and collection tube.

7. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not touch the flow-through. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000g

8. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1min, and then centrifuge for 1 min at ≥ 6000g to elute.

https://www.qiagen.com/us/resources/resourcedetail?id=aa250d94-fc4b-4e27-bb74-d32391ff8a48&lang=en

Appendix 4: QIAquick DNA purification kit protocol (Qiagen®) (~10ug DNA)

Procedure — (animal, tick, and human DNA).

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the colour of the mixture is orange or violet, add 10µl 3M sodium acetate, pH 5.0, and mix. The colour of the mixture will turn yellow.

2. Place a QIAquick column in a provided 2 ml collection tube or into a vacuum

manifold.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge at 17,900g for 30–60s until all the samples have passed through the column. Discard flow-through and place the QIAquick column back in the same tube.

4. To wash, add 750 µl Buffer PE to the QIAquick column centrifuge at 17,900g for 30–60s. Discard flow-through and place the QIAquick column back into the same tube.

5. Centrifuge the QIAquick column once more at 17,900g in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add 50 µl Buffer EB (pH 8.5) or water (pH 7.0–8.5) to the centre of the QIAquick membrane and centrifuge the column at 17,900g for 1 min.

8. If the purified DNA is to be analysed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

https://www.qiagen.com/us/resources/resourcedetail?id=e0fab087-ea52-4c16-b79f-c224bf760c39&lang=en

Appendix 5: DNA Extraction Protocol from Whatman FTA® cards using QIAamp™ kit

Procedure — (Human finger prick)

1. Use a disposable punch to remove a sample disc from the centre of each dried sample spot.

Place the disc in a clean RNase/DNase-free 1.5 mL microcentrifuge tube.

2. Add 280 µL of ATL buffer to the tube.

3. Add 20 µL of Proteinase K and vortex for 30 sec.

4. Heat for 60 min at 56 °C, vortexing for 30 sec every 10 min.

5. Centrifuge at 20,000 x g for 30 sec.

6. Add 300 µL of AL buffer and vortex for 10 sec.

7. Incubate at 70 °C for 10 min, vortexing for 10 sec every 3 min.

8. Centrifuge at 20,000 x g for 30 sec and transfer the lysate to a min-Elute column.

Centrifuge at 6000 x g for 1 min and discard the flowthrough.

9. Add 700 µL of AW2 buffer and centrifuge for 1 min at 6000 x g. Discard the flowthrough.

10. Add 700 µL of 100% ethanol and centrifuge for 1 minute at 6000 x g. Discard the flowthrough.

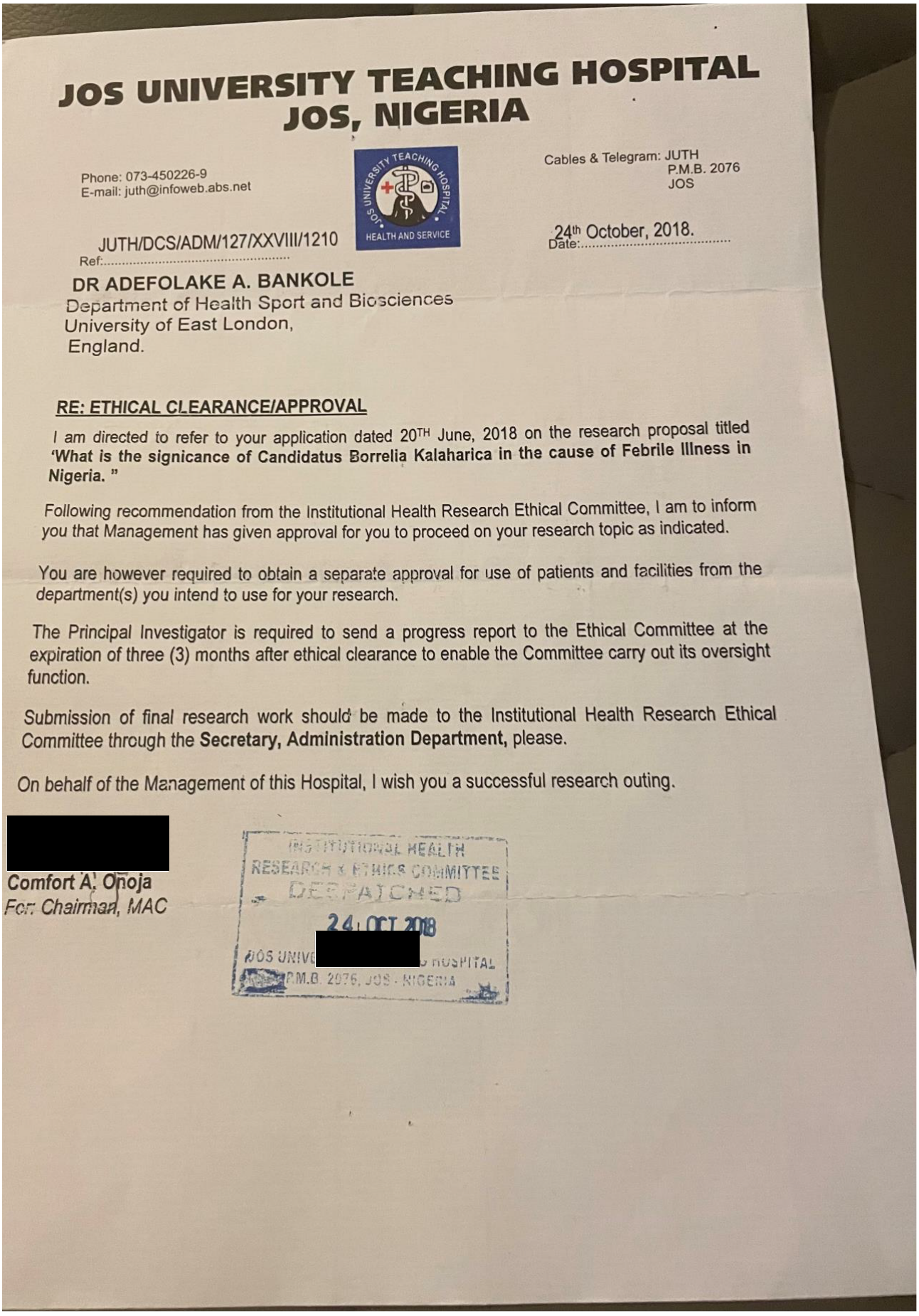
11. Centrifuge at 20 000 x g for 3 min, then open the column lid for 10 min at room temperature.

12. Add 50 µL of distilled water and incubate for 5 min at room temperature.

Centrifuge at 20 000 x g for 1 min. Store the flowthrough at 4 °C pending analysis.

https://www.sigmaaldrich.com/GB/en/technical-documents/protocol/genomics/dna-and-rna-purification/whatman-reliable-extraction-of-dna

Appendix 6: Ethical approval for recruitment of patients



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Cables & Telegram: JUTH
P.M.B. 2076
JOS

JUTH/DCS/ADM/127/XXVIII/1210
Ref:.....

24th October, 2018.
Date:.....

DR ADEFOLAKE A. BANKOLE
Department of Health Sport and Biosciences
University of East London,
England.

RE: ETHICAL CLEARANCE/APPROVAL

I am directed to refer to your application dated 20TH June, 2018 on the research proposal titled **'What is the signicance of Candidatus Borrelia Kalaharica in the cause of Febrile Illness in Nigeria. "**

Following recommendation from the Institutional Health Research Ethical Committee, I am to inform you that Management has given approval for you to proceed on your research topic as indicated.

You are however required to obtain a separate approval for use of patients and facilities from the department(s) you intend to use for your research.

The Principal Investigator is required to send a progress report to the Ethical Committee at the expiration of three (3) months after ethical clearance to enable the Committee carry out its oversight function.

Submission of final research work should be made to the Institutional Health Research Ethical Committee through the **Secretary, Administration Department,** please.

On behalf of the Management of this Hospital, I wish you a successful research outing.

[redacted]
Comfort A. Onoja
For: Chairman, MAC

INSTITUTIONAL HEALTH
RESEARCH & ETHICS COMMITTEE
DESPATCHED
24 OCT 2018
JOS UNIV[redacted]HOSPITAL
P.M.B. 2076, JOS - NIGERIA

PLATEAU STATE SPECIALIST HOSPITAL JOS

Ref. No. PSSH/ADM/ETH. CO/2015/006

Old Bukuru Road,
P.M.B. 2113,
Jos, Nigeria
Tel. 073-462180
Fax: 073-464031

Email: psshjos@yahoo.com

Date: August 14, 2018

Reg. No: NHREC/05/01/2010b

NOTICE OF EXPEDITED REVIEW AND APPROVAL

RE: What is the Significance of Candidatus Borrelia Kalaharica in the Cause of Febrile Illness in Nigeria

Name of Principal Investigator: Adefolake Bankole
Address of Principal Investigator: University of East London, School of Health, Sport and Biosciences
Date of receipt of valid application: August 01, 2018
Date of meeting when final determination of research was made: August 14, 2018.

This is to inform you that the research described in the submitted protocol, has been reviewed and *given expedited approval by the Health Research Ethics Committee.*

This approval dates from **14/08/2018** to **14/08/2019**. Note that no participant accrual or activity related to this research may be conducted outside of these dates. You may liaise with the Hospital records department for necessary cooperation / assistance.

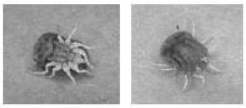
All informed consent forms used in this study must carry the HREC assigned number and duration of HREC approval of the study. In multiyear research, endeavor to submit annual report to the HREC early in order to obtain renewal of your approval and avoid disruption of research. *The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the HREC. No changes are permitted in the research without prior approval by the HREC except in circumstances outlined in the Code. The HREC reserves the right to conduct compliance visit your research site without previous notification.*

[redacted]

Dr. Bitrus Matawal, MBBS,
FWACSChairman, HREC PSSH

Appendix 7: Questionnaire on socio-demographic data and tick exposure and knowledge

QUESTIONNAIRE NUMBER -------



DATE ----------

Location ----------

UNIVERSITY OF EAST LONDON

DEMOGRAPHIC INFORMATION AND ASSOCIATED RISK FACTORS OF POSSIBLE CASES OF TICK-BORNE RELAPSING FEVER IN NORTHERN NIGERIA

HOSPITAL/HEALTH CENTRE SURVEY

Consent from Participants: This is a study on tick-borne relapsing fever in Nigeria, and we kindly request your permission to collect some information about your health and day to day lifestyle. Your participation is voluntary, and you are free to withdraw from the study anytime you want. All information obtained will be solely used for research purposes, and will be treated in the most confidential way and manner.

Thank you.

1. Participant Number _____
2. Age (Years) ☐ Under 18 ☐ 18-25 ☐ 26-35 ☐ Above 35
3. Gender ☐ Male ☐ Female
4. Occupation ☐ Civil servant ☐ Farmer ☐ Trader ☐ Student ☐ Unemployed ☐
5. Education ☐ Primary ☐ Secondary ☐ Tertiary
6. Type of household ☐ Cement ☐ Mud (hut) ☐ Wooden (bamboo) ☐ Others
7. Body temperature ☐ ≤37°C ☐ >37°C

QUESTIONNAIRE NUMBER -------

8. Did you experience any/all of the following symptoms?
   ☐ Headache ☐ Body and Joint pains ☐ Vomiting ☐ High temperature
9. How many times did you have fever in a month?
   ☐ Once ☐ 2 – 3 ☐ > 3 times
   times
10. How many times did you have fever in a month?
    ☐ 1 – 2 times ☐ 3 – 4 times ☐ ≥ 5 times
11. What was the average duration of each fever?
    ☐ 1-3 days ☐ 4-7 days ☐ > 7 days
12. Have you been diagnosed of malaria for the fever? ☐ Yes ☐ No
13. Do you recall ever been bitten by an insect/tick on any part of your body? ☐ Yes ☐ No
14. **IF YES** to question 9, did you notice any painless swelling around the point of insect/tick bite? ☐ Yes ☐ No
15. **IF YES** to question 9, where did the bite occur?
    ☐ Household ☐ Open space ☐ Farmland ☐ Bush ☐ Can't recall
16. Did the fever occur after the insect/tick bite?
    ☐ Yes ☐ No ☐ Can't recall
17. Do you rear or live around any livestock or domestic ☐ animal? Yes ☐ No
18. **IF YES** to question 13, what type of animal do you keep?
    ☐ Dog ☐ Goat ☐ Cattle ☐ Pig ☐ Poultry
19. Are ticks common in your environment (household, farmland, bush or open space)?
    ☐ Yes ☐ No ☐ Don't know

Consent form for study participants

Annexe 2

UNIVERSITY OF EAST LONDON

Consent to Participate in a Study Involving Adult Participants

Topic: What is the significance of ***Candidatus*** **Borrelia kalaharica** as a cause of febrile disease in Nigeria?

Program: PhD via Mphil

Researcher: Adefolake Bankole

Please tick as appropriate:

	YES	NO
I have the read the information leaflet relating to the above program of research in which I have been asked to participate and have been given a copy to keep. The nature and purposes of the research have been explained to me, and I have had the opportunity to discuss the details and ask questions about this information. I understand what is being proposed and the procedures in which I will be involved have been explained to me.		
Do you give consent for the administration of questionnaire		
I understand that my involvement in this study, and particular data from this research, will remain strictly confidential as far as possible. Only the researchers involved in the study will have access to the data. *(Please see below)*		
I understand that maintaining strict confidentiality is subject to the following limitations: The confidentiality of the information provided by participant is subject to legal limitations in data confidentiality (i.e. data may be subject to a subpoena, a freedom of information request or mandated reporting by some professions). There will be limitation on confidentiality where disclosure of imminent harm to self, and/or others may occur and may be reported to relevant authorities.		
Anonymity will be adopted in all publications related to this study		
Individual names of participants will not be published in any publications related to this study		
All research finding will be disseminated via thesis, journals and relevant scientific conferences.		
Do you give consent to be contacted by the researcher or her team for any future study		
It has been explained to me what will happen once the program has been completed.		
I understand that my participation in this study is entirely voluntary, and I am free to withdraw at any time during the research without disadvantage to myself and without being obliged to give any reason. I understand that my data can be withdrawn up to the point of data analysis and that after this point it may not be possible.		
I hereby freely and fully consent to participate in the study which has been fully explained to me and for the information obtained to be used in relevant research publications.		

Participant's Name (BLOCK CAPITALS)
……

Participant's Signature or thumb print
……

Researcher's Name (BLOCK CAPITALS)
……

Researcher's Signature
……

Date: ……