DISTRIBUTION AND ABUNDANCE OF TICKS ON CATTLE AND ASSOCIATED TICK-BORNE PATHOGENS FROM KILOMBERO AND IRINGA DISTRICTS IN TANZANIA

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A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE.

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EXTENDED ABSTRACT

Ticks are a major group of arthropod vectors that transmit pathogens that cause devastating diseases in humans and animals. The information on tick infestation and related tick-borne pathogens in Tanzania is insufficient. Therefore, this study was conducted to determine tick prevalence and degree of infestation on cattle as well as associated tick-borne pathogens, in Kilombero and Iringa districts of Tanzania. A repeated cross-sectional study was conducted to collect ticks on cattle in wet and dry seasons from January to August 2021. Out of 740 cattle examined, 304 were infested with ticks. In total 1,780 ticks were counted on one side of the animal's body and doubled, whereby a total of 3,560 ticks were recorded. A total of 1,889 tick were collected from the infected cattle including 109 more tick observed while collecting ticks based on the animal's posture when restrained on ground. Thereafter, ticks were identified morphologically using published morphological keys under a stereomicroscope and confirmed using polymerase chain reaction (PCR) and sequencing of the mitochondrial CO1 and 16S rRNA genes. The tick-borne pathogens were detected using PCR. Fisher's exact test was performed to detect the difference between the proportion of hard tick species and the study areas and season. One-way ANOVA was performed to compare mean tick burden between variables (including cattle age groups, body condition score and frequency of tick control). Out of 1,889 ticks, 1,377 fit in the genus *Rhipicephalus*, 459 in the genus *Amblyomma* and 53 in the genus *Hyalomma*. The most prevalent tick species identified were *Rhipicephalus microplus* (48.1%), *Rhipicephalus evertsi* (16.4%), and *Amblyomma lepidum* (16.4%). The sequencing results of the mitochondrial DNA fragments indicated high nucleotide identity (96-100%) with sequences in GenBank and Barcode of Life Database (BOLD) (OM974109-OM974112 and OM978262-OM978265). Seasonality results indicate no statistically significant difference in the prevalence of tick infestation on cattle during the

dry (41.05%) and wet (41.11%) seasons. The DNA of *Anaplasma* spp. and *Theileria/Babesia* spp. were detected in (70.33%, n=64) of all tick pools. The detection rate of both *Anaplasma* and *Theileria/Babesia* spp. was high in *Amblyomma lepidum* (25.00%, n=16) followed by *Rhipicephalus evertsi* (23.44%, n=15) tick pools. The results showed high tick prevalence and abundance on cattle suggesting increased risk of tick-borne disease transmissions and reduced animal production and productivity. Therefore, tick infestation in the study areas highlight the need for strategic tick control approaches.

DECLARATION

I, Walter Simon Magesa, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor is concurrently being submitted for a higher degree award in any other institution.

Walter Simon Magesa **Date**

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(1st Supervisor)

Deceased

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This dissertation is dedicated to my Almighty God for free life, my parents (Mr. Simon Magesa and Mrs. Rosemary Magesa) for their unconditional love, help and support as well as my brothers and sisters, for their tolerance and motivation throughout the course of my study.

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LIST OF ABREVIATIONS AND ACCRONYMS

ANOVA	Analysis of Variance
atp6	adenosine triphosphate synthase subunit 6
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Database
bp	base pair
CA	California
CCHF	Crimean Congo hemorrhagic fever
CDC	Center for Disease Control and Prevention
CO1	Cytochrome c Oxidase subunit 1
CVMBS	College of Veterinary Medicine and Biomedical Sciences
Cyt B	Cytochrome B
ddH_2O	double distilled water
DNA	Deoxyribonucleic Acid
dNTPs	dinucleotide Triphosphates
DVOs	District Veterinary Officers
ECF	East Coast fever
EDTA	Ethylene Diamine Tetra-acetic Acid
HALI	Health for Animal and Livelihood Improvement
ITS2	Internal Transcribed Spacer 2
LEOs	Livestock Extension Officers
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium Chloride
mM	millimole
mtDNA	mitochondrial Deoxyribonucleic Acid

nad2	nicotinamide adenine diphosphate dehydrogenase 2
NCBI	National Center for Biotechnology Information
NCRs	Non-Cording Regions
OIE	World Organization for Animal Health
PCGs	Protein Coding Genes
PCR	Polymerase Chain Reaction
rDNA	ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
SE	Standard Error of the Mean
spp	species
SUA	Sokoine University of Agriculture
TBDs	Tick-borne Diseases
TBE	Tris-borate Ethylene Diamine Tetra-acetic Acid
TBPs	Tick-borne Pathogens
tRNA	transfer Ribonucleic Acid
UC	University of California
UK	United Kingdom
URT	United Republic of Tanzania
µl	microliter
µM	micromole

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background

Ticks are one of the most important arthropod vectors and reservoirs for a wide variety of pathogenic agents such as viruses, bacteria, fungi, protozoa and nematodes, which can cause diseases in human, livestock and wild animals (Kerario *et al*., 2017; Chiuya *et al*., 2021). Ticks are considered the second only after mosquitoes as worldwide vectors of medical and veterinary importance (Ćakić *et al*., 2014; Wikel, 2018; Tan *et al*., 2021). Ticks are obligate blood-feeding ecto-parasites of mammals, birds and reptiles throughout the world, with different species of relevance regionally. All stages of the tick developmental cycle (larva, nymph and adult) are parasitic on vertebrates. Ticks transmit diseases that lead to extensive economic loses to resource-poor farming communities especially in tropical and subtropical regions where almost 80% of the world's cattle population is reared (Rehman *et al.,* 2017; Wikel, 2018).

Tick-borne diseases (TBDs) such as East Coast fever, bovine babesiosis, anaplasmosis and cowdriosis attribute to over 70% of all cattle deaths in Tanzania consequently over TSh. 72 billion is lost yearly (Kerario *et al.,* 2017; Silatsa *et al*., 2019b; Raboloko *et al*., 2020). The burden of ticks and TBDs on the economy and livelihood of those involved in the livestock industry in Africa remains significant (Raboloko *et al*., 2020). Many factors have been identified to explain the continuous increase in the incidence of ticks and TBDs (including; inadequate monitoring and surveillance programs targeting ticks and tick-borne diseases, deforestation and human encroachment on wildlife habitats, tick resistance to acaricides and climate change) (Nchu *et al.,* 2020). For effective control of ticks and TBDs, knowledge on identification of tick species, their abundance and distribution is needed.

Livestock farming is one of the major occupations in Tanzania and contributing to food security and source of income to farmers. Tanzania has approximately 33.4 million cattle, 21.3 million goats and 5.7 sheep (URT, 2021). Nearly 90% of agricultural households keep livestock of different kinds (URT, 2021). About 95% of cattle populations in the country are reared under traditional agro-pastoral and pastoral husbandry systems (URT, 2021). The grazing land for these animals is no longer sufficient due to the increased number of cattle, other domestic animals and human population. Most of the indigenous livestock are widely grazed in grasslands and woodlands and consequently exposed to high risk of tick infestation (Mamiro *et al*., 2016; Kerario *et al*., 2017).

Ixodid ticks of the genera *Rhipicephalus, Hyalomma* and *Amblyomma* are the most important and widely distributed species found in many parts of Tanzania (Cumming, 1999; Kerario *et al*., 2017). The species included in the three genera are *Rhipicephalus appendiculatus, Rhipicephalus evertsi, Rhipicephalus bequarti, Rhipicephalus compositus, Rhipicephalus hurti, Rhipicephalus interventus, Rhipicephalus kochi, Rhipicephalus lunulatus, Rhipicephalus praetextatus, Rhipicephalus pulchellus, Rhipicephalus simus, Hyalomma albiparmatum, Hyalomma impeltatum, Hyalomma marginatum rufipes, Hyalomma truncatum, Ixodes* spp., *Amblyomma variegatum, Haemaphysalis leachi* and *Haemaphysalis silacea* (Lynen *et al*., 2007).

Rhipicephalus appendiculatus is a vector of *Theileria parva* which causes East Coast fever (ECF) in cattle (Swai *et al*., 2006), *Rhipicephalus* ticks also transmits pathogens causing Boutonneuse fever, Lyme disease, and Q fever (Kwak *et al*., 2014). *Amblyomma variegatum* is of great veterinary importance because it is a competent vector of *Ehrlichia ruminantium*, that cause cowdriosis (Heartwater) and triggers the growth of severe dermatophilosis, caused by *Dermatophilus congolensis* (Kerario *et al*., 2017). *Amblyomma*

variegatum has also been proven to be a vector of Crimean Congo hemorrhagic fever (CCHF) virus (Gonzalez *et al*., 1991; Akuffo *et al*., 2016). CCHF virus has been isolated from adult *A. variegatum* from cattle in Senegal (Nemes *et al*., 2004), Nigeria and other countries in close proximity to Tanzania like Uganda and Kenya (Hoogstraal, 1979; Ikpeze *et al.*, 2011). *Rhipicephalus microplus* is known to be a good vector of highly pathogenic *Babesia bovis*. In addition, *R. microplus* and *R. decoloratus* are of extreme veterinary importance as they are vital carriers of *Babesia bigemina* and *B. bovis*, that causes bovine babesiosis. The two are also carriers of *Anaplasma marginale* causing bovine anaplasmosis. *Rhipicephalus evertsi* transmits *B. bigemina* in cattle (Kerario *et al*., 2017). *Hyalomma rufipes* is also known to transmit *A. marginale* (Kererio *et al*., 2017), and in addition, the species is a good vector of *Theileria annulata* and *Babesia occultans*. *Hyalomma rufipes* has been reported to be vector for the viruses of Bunyaviridae family including; Tete and Matruh in Egypt and CCHF virus in Senegal and Nigeria, and Dugbe virus in Nigeria (Hoogstraal, 1979). This tick is also a vector of *Rickettsia conori* causing boutonneuse fever (Hoogstraal, 1979).

Factors that may have a considerable impact on the tick distribution and abundance include: the increase in the human population and domestic animal hosts, environment variations in vegetation and change in climatic conditions, agricultural activities, increase in tick control programs and their successfulness (Kerario *et al*., 2017; Rehman *et al*., 2017; Nchu *et al.*, 2020).

Studies aiming at quantifying and identifying tick species in Tanzania are still limited. Published studies related to tick species composition have been conducted in Ngorongoro (Lynen *et al*., 2007), Iringa, Maswa (Kwak *et al*., 2014), Mvomero (Emmanuel *et al*., 2012), Rufiji (Mamiro *et al*., 2016), Mara, Singida, and Mbeya (Kerario *et al*., 2017).

Nevertheless, their data are limited to morphological characteristics. This study sought to provide molecular information on tick species collected at selected areas of Kilombero and Iringa districts of Tanzania. Molecular data on Ixodidae ticks are of value to farmers and other stakeholders as information about the distribution, burden and species diversity present in the area and their seasonal variation. Data from this study will contribute to the national livestock sector development goal and be used to formulate rational control strategies of ticks and TBDs in Tanzania.

1.1.1 Classification of ticks

Ticks are members of the phylum (Arthropoda) of the animal kingdom. However, within this phylum, ticks and their allies are grouped into subphylum Chelicerata based on the presence of anterior pair of Chelicerae used for grasping, piercing, cutting and other functions associated with food gathering and feeding (Cupp, 2019). Ticks are thus more closely related to spiders and scorpions than insects and are therefore placed into class Arachnida within subclass Acari. Acarines are characterized by the extreme fusion of body segments, in contrast to the known three body segments head, thorax and abdomen in insects (Oliver, 1989). Ticks comprising the suborder Ixodida are joined with a related suborder of mites and placed within the order Parasitiformes. Within the suborder Ixodida, there are three tick families including Ixodidae, Argasids and Nuttalliellidae making up the superfamily Ixodoidea (Oliver, 1989).

Ticks thus belong to three different families; the majority of tick species belong to the two main families: Ixodidae (Hard ticks) and the Argasidae (soft ticks) (Hoskins, 1991). Argasid ticks are often called soft ticks because they do not have hard plates on their bodies while, Ixodids with these plates are called hard ticks (Cupp, 2019). The two main families of ticks not only have different life cycles but also, they have many morphological

features that clearly distinguish them. The third family is Nuttalliellidae presented by only a single species *Nuttalliella namaqua* which is found in Namaqualand in South Africa and some parts of higher rainfall areas of Tanzania (Oliver, 1989). It is of minor medical and veterinary importance (Hoskins, 1991).

Ixodid ticks of all life stages possess a sclerotized scutum and an apically located gnathosoma. The other important structures on the ventral side are anus, anal grooves, adanal plates, and respiratory spiracular plates in the nymphs and adult ticks (Walker *et al*., 2003). They slowly feed for several days to weeks because their body wall needs to grow before it can expand to take a very large volume of blood meal (Jongejan and Uilenberg, 1994). In contrast, soft ticks do not possess a scutum, their prognathous mouthparts are located anterioventrally and they have a leathery integument that can expand rapidly allowing nymphs and adults to engorge within few hours (Cupp, 1991; Estrada-Peña, 2015). Ixodid ticks secrete excess water derived from a blood meal back to the host via their salivary glands, while soft ticks use their specialized ultrafiltration organ on the coxae (Estrada-Peña, 2015).

The Ixodidae is the dominant tick family, with respect to number of species and their medical and veterinary importance. The taxonomic situation of the Ixodidae has been studied in depth, and there is almost agreement on the systematic position of the families and genera. There are at least 900 species arranged in two major groups namely Prostriate and Metastriate, consisting of 5 subfamilies and 13 genera (Oliver, 1989). The genus *Ixodes* have the anal groove surrounding the anus anteriorly hence are called prostriate ticks. For the genera *Rhipicephalus, Hyalomma, Amblyomma, Haemaphysalis,* and *Dermacentor* the anal groove surrounds the anus posteriorly thus they are called metastriate ticks. The family Ixodidae comprises approximately 80% of all tick species,

including the species of greatest economic importance (Jongejan and Uilenberg, 1994). The family Ixodidae contains the genera *Ixodes*, *Rhipicephalus, Hyalomma*, *Amblyomma, Haemaphysalis,* and *Dermacentor* (Guglielmone and Nava, 2014; Estrada-Peña, 2015).

At least 79 tick species have been identified and documented in East Africa of which some have little or no economic importance (Cumming, 1999). The basic information on distribution of various tick species in Tanzania was provided in the extensive tick surveys conducted in 21 regions of Tanzania between 1955 and 1961 (Mamiro *et al*., 2016; Kerario *et al*., 2017). Previous studies showed that *Amblyomma variegatum* and *Rhipicephalus appendiculatus* represents the most catholic species in Tanzania (Lynen *et al*., 2007).

1.1.2 Ixodid tick feeding and life cycle

For the development and adoption of effective tick control strategies, understanding the ticks feeding biology and their life cycles is very important. The feeding of ticks makes them of importance in the health of domestic animals, humans and wildlife. During blood feeding ticks may transmit pathogens to their host, injuring the skin, causing irritation and pain and sometimes causing poisoning (Kiszewski *et al*., 2001; Latif and Walker, 2016).

1.1.3 Life cycle of Ixodid ticks

All ticks have four life cycle stages, the embryonated egg, motile larva, nymph and adult. All Ixodid ticks are oviparous and have a single nymphal stage in contrast to the several nymphal instars of the Argasids (Oliver, 1989; Estrada-Peña, 2015). Ixodid tick lifecycles are classified into 3-, 2- or 1- host tick based on whether the moulting of larvae to nymph and nymph to adult occurs off or on host. Ixodid ticks exhibits questing behaviour when seeking host by crawling onto vegetation, waiting until activated by sensing host vibrations or other stimuli, then spreading their first pair of legs containing Haller’s organ, and

waiting to attach to the host as it passes by (Estrada-Peña, 2015). This type of ambush and the behaviour of waiting on vegetation is called questing (Latif and Walker, 2016).

Three-host tick life cycle may take six months to several years making it slows (Walker *et al*., 2014). Most Ixodid ticks require three individual hosts in their life cycle. In this life cycle each active stage repeats the pattern of host-seeking, feeding and off-the-host moulting in the environment (Hoogstraal, 1979). Larvae and nymphs of Ixodids feeding on mammals usually do so on small to medium sized host species, while the adults engorge on large mammals. *Rhipicephalus appendiculatus, Hyalomma impeltatum, H. impressum, H. nitidum, H. truncatum* and *Amblyomma variegatum* are some of the examples of three-host ticks (Hoogstraal, 1979). *Rhipicephalus appendiculatus,* as the most important tick species transmits the protozoan *Theileria parva* that cause ECF to cattle (Laisser *et al*., 2017; OIE, 2020). In Tanzania among the TBDs, ECF is the major cause of cattle deaths and costs the government a huge financial resource for its control (Laisser *et al*., 2017; Kerario *et al*., 2018). The genus *Hyalomma* can be either three or two-host tick depending on the available host species (Hoogstraal, 1979). Some few Ixodids have evolved a two-host or one-host feeding behavior thus, no longer require multiple hosts (Oliver, 1989; Jongejan and Uilenberg, 1994). On the other hand, climatic conditions and diapause may delay development, host seeking behavior or even the onset of oviposition, so that only one life stage can be completed each year. Furthermore, the duration of the life cycle can further be extended up to 3 years due to environmental limitations (Walker *et al*., 2014).

A two-host life cycle is the one in which the larvae and nymph feed on the same individual host and the adult feeds on another host after several days or longer following the nymph engorgement and detachment. Some species of ixodid ticks undergoes two-host life cycle including, *Hyalomma anatolicum excavatum*, *Hyalomma detritum* and *Rhipicephalus*

evertsi. The cycle is almost similar to one-host cycle except that the larvae and nymphs feed on the same individual host (Walker *et al*., 2014). Upon finding a host the adults feed and the female drops from the host after engorgement. The female deposits many eggs (~2,000-20,000) in a single batch after several days or longer before dying. Ixodid tick larvae then undergo a brief inactive period once hatched, during which the body hardens and food reserves from the previous stage are digested. The larvae disperse once ready for a blood meal and finally position themselves on edges of grass and other types of protruding vegetation along their respective host trails and pathways. This positioning ensures adequate host contact, which is signaled by sensory organs that distinguish a hierarchy of stimuli, including host odors, carbon dioxide, warmth, moisture, interrupted light, and mechanical forces. When on host, larval attachment occurs, and feeding begins (Cupp, 2019). The host skin provides a warm, humid and optimum microenvironment for the fed larvae on the host skin. The developmental process is often quick, (Oliver, 1989; Walker *et al*., 2014; Cupp, 2019).

A one-host life cycle is the one in which all stages remain on the host after the larvae attachment. It is a less common however, occurs in all the *Rhipicephalus (Boophilus)* and in other genera. In this life cycle, eggs are laid on a physical environment and the female adult dies, larvae hatch after several weeks of development and crawl onto vegetation for questing. They grab onto the host using their front legs and then crawl over the skin to find a suitable place to attach (Jongejan and Uilenberg, 1994; Cupp, 2019). The nymph feeds on the same host and remains attached until it moults into adult. The adults have their blood meals and change position for mating on the same host. Therefore, all the three feedings of any individual one-host tick occur on the same individual host (Latif and Walker, 2016). The one-host tick life cycle is usually rapid, for example, *Rhipicephalus* (*Boophilus*) may take three weeks for the feedings on one host and two months for egg

laying and larval development (Hoogstraal, 1979; Walker *et al*., 2014). In general, the length of tick life cycles are quite variable since they are regulated by seasons, as well as complex interactions among photoperiod, temperature, moisture, and availability of suitable hosts and mates, acting on the genetic variability of each tick species (Oliver, 1989).

1.1.4 Reproduction

Mating in Ixodid ticks takes place on the host, except Ixodes which may mate while on the vegetation (Walker *et al*., 2014). Male ticks remain on the host and tend to mate with many females while they are feeding (Walker *et al*., 2014). However, newly moulted adult metastriate ticks are sexually immature thus, gametogenesis only begins during blood feeding. Female ticks secrete multiple pheromones that regulates mating in ticks by attracting fed males. The males then transfer a sack of sperms to the females. The female ticks mate only once, before they are ready to fully engorge with blood. Once have enough sperms to fertilize their eggs and fully engorged, the females drop off from the host, lay eggs in a suitable physical environment then dies (Kiszewski *et al*., 2001; Nejash, 2016; Latif and Walker, 2016).

1.1.5 Host and habitat

Ticks have specific species of hosts to which they are adapted. The presence of a suitable maintenance host for the reproduction of adult ticks therefore, determines the survival of a particular tick population (Lindsay, 1999). Host resistance, behaviour response such as grooming and tick attachment success are among other factors influencing the survival of ticks (Randolph, 1994). Tick abundance mainly depends on host composition, density and abundance. However, the most important component in the physical environment for tick survival is climate of which temperature and moisture are the primary factors influencing

tick host seeking behaviors in an environment (Randolph, 1997; Kamani *et al*., 2017). These climatic factors, along with the abundance and availability of host, determine the seasonality of tick abundance as the host seeking behaviors are consistent with the most favorable climatic conditions (Randolph, 1994, 1997; Estrada-Peña, 2015; Kamani *et al*., 2017).

When seeking for the host especially during high temperature periods, ticks usually lose water and become dehydrated. Ticks thus reabsorb water periodically from a humid atmosphere, like from moist leaf litter in the lower vegetation (Randolph, 1994; Estrada-Peña, 2015). Since many tick species lay eggs in the soil, the soil type and its properties like water retention can thus determine the survival and development of their larvae (Cumming, 1999). The tick population could consequently be affected by other factors such as predators (including birds), frequency of habitat disturbance such as field fire, droughts, and floods (Estrada-Peña, 2015).

1.1.6 Distribution and abundance of ticks in Tanzania and their influential factors

In many parts of the Tanzania where cattle are raised, *Rhipicephalus appendiculatus, R. microplus, R. decoloratus* and *Amblyomma variegatum, A. lepidum and A. gemma* are the most widely distributed ticks (Lynen *et al*., 2007; Kwak *et al*., 2014). Under favorable condition an extensively studied *R. appendiculatus* was shown to complete its life cycle in three months, but occurrence becomes seasonal where there is a noticeable dry season (Randolph, 1994). The pattern of seasonal occurrence is regulated by the unfed adult ticks, which enter diapause and do not engage in host seeking until the start of the rainy season. Several overlapping generations are completed annually in areas where the rainfall is evenly spread through the year. However, there is no clear pattern of seasonal abundance evident (Randolph, 1994).

In a study conducted in Rufiji district, *Rhipicephalus microplus* and *R. evertsi* have been observed on cattle during dry and rainy season (Mamiro *et al*., 2016). However, earlier studies showed that, *R. microplus* has extended its distribution range and is now present in all northern regions of Tanzania except in extremely cold and dry areas, and that high suitability is currently recorded for most of the previously non occupied areas. *Rhipicephalus microplus* occur in areas with an estimated mean rainfall of 58 mm (Mamiro *et al*., 2016). Its steady spread in Tanzania is assisted by its higher reproductive potential and most favorable climates which enable it to compete successfully against *R. decoloratus* where they occur together (Walker *et al.*, 2014). *Rhipicephalus decoloratus* has been previously reported in Singida, Mbeya and Mara regions. Its relatively high abundance in these regions is explained by its preference for highlands and sub-highlands with an annual rainfall of more than 800 mm (Kerario *et al.*, 2017).

Previous studies indicate that among *Amblyomma* species, *A. variegatum* is the most widespread species in Tanzania, covering the subhumid and low to high altitude areas of the country (Kwak *et al.*, 2014). In contrast, adults of *Amblyomma lepidum* in Tanzania are most abundant between October and February. Infestations begin either shortly before or after the onset of the rainy season, but there may be considerable variation in the timing of the peak (Walker *et al*., 2014). On the other hand, *A. gemma* is most commonly found in the arid or semi-arid bushland or wooded and bushed grassland areas with shorter drought periods and bimodal rainfall (Lynen *et al*., 2007).

The other ixodids that characterize tick population in Tanzania include; *Haemaphysalis leachi, H. silacea, Hyalomma albiparmatum, H. impeltatum, H. marginatum rufipes, H. truncatum, H. turanicum, Ixodes* spp., *Rhipicephalus bequarti, R. compositus, R. sanguineus, R. pravus, R. hurti, R, interventus, R. kochi, R. lunulatus, R. praetextatus,*

R. pulchellus, R. simus, R. muhsamae, Amblyomma hebraum and *A. marmoreum* (Lynen *et al*., 2007; Kwak *et al*., 2014; Walker *et al*., 2014; Mamiro *et al*., 2016; Kim *et al*., 2018). Their abundance varies with time, habitat and agro-ecological zones due to interaction of diverse factors such as host diversity and resistance, climate, absence of control measures and managerial activities that may affect the host behavior (Kerario *et al*., 2017).

1.1.7 Seasons and other eco-climatic conditions affecting distribution of Ixodid ticks

Hard tick abundance and distribution are affected with time, seasons, habitat, agro-ecological zones. Moreover, various diverse factors like host diversity and resistance, climate change, absence of control measures and improper host management that may affect host behavior are also known to affect tick distribution (Alanazi *et al.,* 2019). Knowledge about tick distribution and species composition provides significant information on tick population dynamics, disease transmission dynamics and estimation of resistance of different hosts (Salih *et al*., 2004, 2008). Information on tick distribution can be an indicator for the presence of tick-borne diseases circulating in a given agro-ecological zone (Sorvillo *et al*., 2020).

The seasonal activity of ticks is characterized by several cycles of ascending and descending movements in the vegetation, regulated by temperature and loss of water. The energy reserves in ticks, their water retention ability and relative humidity and temperature are therefore some of the factors regulating the questing activities and survival of ticks in the field (Porretta *et al.*, 2013; Walker *et al*., 2014; Estrada-Peña, 2015; Randolph *et al*., 2016). In the field, the density of questing ticks could be estimated by dragging a white piece of blanket or flannel flag over the low vegetation (Perret *et al.,* 2004; Swai *et al.*, 2006).

Ticks may dehydrate during questing but, they rehydrate it by descending at intervals to the layer of dead leaves or grass covering the soil where they can reabsorb water vapor from the atmosphere and once they are hydrated, they climb the vegetation again (Lindsay, 1999; Perret *et al*., 2004). Microclimate, diversity and the availability of the host shapes the abundance of ticks, a feature called "phenology" (Perret *et al.*, 2004). Each tick stage succeeds definite time of the year, given the specific combination of climate and seasonal changes in the host abundance (Estrada-Peña, 2015).

Various studies showed that climate regulates tick questing activities. Most ticks are inactive at the lower layer of vegetation before they begin questing. With combination of climate and photoperiod the questing process is triggered (Oliver, 1989; Estrada-Peña, 2015). Photoperiod may act on the moulting stages, activating or delaying moulting until more favorable conditions are available.

Many species of ticks are adapted to seasonal variation in climate within their geographical range. To the questing ticks, prolonged dry environmental conditions cause serious danger especially to the questing larvae which are susceptible to drying out fatality (Oliver, 1989). The larvae are cutaneous respires in contrast to nymphs and adults which have specialized spiracles to breathe through (Latif and Walker, 2016). The survival of many tick species is improved if they have a seasonal cycle which reduces these risks. To overcome the adverse effects of prolonged dry seasons, some species of Ixodides have developed a behaviour called diapause, a suppression of the host seeking activity in unfed ticks and delays of the engorgement of the on-host ticks (Oliver, 1989).

Behavioral diapause has been recorded in *Ixodes, Hyalomma, Haemaphysalis, Amblyomma, Rhipicephalus and Dermacentor* (Oliver, 1989; Lindsay, 1999; Porretta *et al*., 2013). For example, *R. appendiculatus* has developed a diapause mechanism which reduces the activity of some parts of the life cycle so that the reproduction of adults is at the beginning of the single wet season when humidity is highest (Lindsay, 1999).

1.1.8 Tick identification

Accurate Ixodid tick species identification from parasitized hosts or from the surrounding vegetation is an important factor in the detection and diagnosis of TBDs and is a prerequisite for control and likely eradication.

1.1.8.1 Morphological identification of ticks

The taxonomic key for Ixodid uses important morphological characteristics arranged to facilitate identification of the ticks (Hoskins, 1991). This simplified key is useful primarily for identifying adult ticks that are of veterinary importance in Africa (Walker *et al*., 2014; Cupp, 2019). The best method for morphological identification of ticks is the dichotomous key which provides a series of choices between two or more features of ticks at each stage. If each choice is made correctly the key can lead down to a single species. This type of key is one of the best methods for identifying tick species from a wide variety that may be found on domestic and wild animals in Africa (Latif and Walker, 2016). Ixodid ticks infesting cattle in Africa are of many sizes and share some morphological features useful for their identification. In the genus Ixodes (prostriate), the anal grove passes to the anterior of the anus, while, in all other genera of ixodid ticks (metastriate), the anal grove passes posterior to the anus, or is absent (Walker *et al*., 2014; Cupp, 2019).

Amblyomma and *Hyalomma* are the two genera of large ixodid ticks (approximately 6-7 mm). They have long mouthparts, which project to the anterior of the body and large eyes. These two genera have pale rings on most segments of their legs. *Amblyomma* spp. have very long mouthparts, elongated second segment of palps, they have ornate conscutum and scutum, eyes and festoons are present. *Amblyomma* males may not or have ventral plates but when present very small and have banded legs (Latif and Walker, 2016).

Hyalomma have long mouthparts, their second segment of palps is elongated. The scutum is pale to dark brown in color, they have convex eyes and festoons. Males have adanal, sub-anal and accessory anal plates. They have coxae of first pair of legs with long prominent posteriorly directed spurs and banded legs. Males of *Hy. albiparmatum* are very similar to *Hy. truncatum* except that, they have a central festoon in the form of a large white parma. Whereas, in *Hy. truncatum* the central festoon is not well defined adequately to form a parma. *Hy. albiparmatum* adults feed on cattle, sheep and goats while the immature stages of the same feed on small mammals (Madder *et al.*, 2013). The second group is the genus *Ixodes* of medium size ticks (approximately 3-5 mm) with long mouthparts that are sexually dimorphic, but no eyes, no festoons, no adanal plates on males and plain dark legs. Their coxae I have a large single spur. The anal groove passes anterior to the anus (Walker *et al*., 2014; Latif and Walker, 2016). The third group is the genus *Rhipicephalus*, is also of medium size ticks (approximately 3-5 mm), but with short mouthparts and eyes. Its coxae I have large and equal paired spurs. The fourth group, a genus of medium size ticks is *Dermacentor*. The last group is the small sized ticks (less than 3 mm), (*Boophilus), Margaropus* and *Haemophysalis*, all have anterior and short mouthparts and their eyes are small for (*Boophilus) and Margaropus* or absent for *Haemophysalis* (Cupp, 1991, 2019; Walker *et al*., 2014; Latif and Walker, 2016).

The dichotomous key commonly used for the identification of ticks found on domestic animals of Africa is a useful guide for discriminating Ixodid ticks. However, for those unfamiliar with the taxa, the Metastriate becomes particularly problematic to discriminate and might be misidentified. This problem is often broadened during molecular surveys for pathogens or genetic analysis, as the entire immature specimens are often destroyed. Therefore, an alternative method for quick and precise identification is needed, particularly for confirmation of specimen identification post processing (Anderson *et al*., 2004).

1.1.8.2 Molecular identification of ticks

Besides morphological identification, confirmation of tick species in some studies have been done using nucleic acid amplification methods targeting barcoding genes as molecular markers including; Mitochondrial Cytochrome c Oxidase I (CO1) subunit, Internal Transcribed Spacer 2 (ITS2), Mitochondrial Cytochrome b (Cyt B), 16S rDNA, and 12S rDNA genes. Mitochondrial and nuclear DNA has been widely used to conduct phylogenetic studies (Jizhou *et al*., 2014a; Rehman *et al*., 2017). Some studies have confirmed that mitochondrial DNA (mtDNA) provides useful markers for species identification, genetic characterization and studies of phylogenetic relationships of organisms at different taxonomic levels (Chitimia *et al*., 2010; Caetano *et al.*, 2017; Silatsa *et al*., 2019a; Kanduma *et al*., 2020). There has been efforts to standardize the molecular methods for identification of Ixodid ticks but, no one gene has been formally designated as an admitted DNA marker to deal with problems of classification and phylogenetics in Ixodid ticks (Gou *et al.*, 2018).

Similar to other arthropods, tick mitochondrial genome has a circular, double-stranded DNA structure. The length averages 14 – 16 Kilobases, characterized by low molecular weight, high copy quantity and genetic conservation. The differences in length of

mitochondrial genomes between ticks may be influenced by gene rearrangement and the length of non-cording regions (NCRs) (Liu *et al.*, 2013; Li and Liang, 2018). The genome contains a total of 37 genes, including 13 protein-coding genes (PCGs), 2 ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes. Among the 13 PCGs, 9 PCGs (cox1-3, nad2, nad3, nad6, atp8, atp6, cytb) are located in the majority strand (J strand) and 4 PCGs (nad5, nad4, nad4L, nad1) are located in the minority strand (N strand). To date, 63 complete ticks mitochondrial genomes are available in National Center for Biotechnology Information (NCBI) database, and these genomes have recently become an increasingly important genetic resource and source of molecular marker in phylogenetic studies of ticks (Wang *et al*., 2019).

The mitochondrial cytochrome c oxidase subunit 1 (CO1) gene is one of the most common markers used for tick molecular systematics. Fragments of this gene are normally used to infer phylogenies, mainly the region near the 5' end, which is used by the DNA Barcoding Consortium. The CO1 is one of the building blocks of the cytochrome c oxidase protein. The protein is the last enzyme in the electron transport chain, reducing oxygen and pumping protons across the inner mitochondrial membrane (Pentinsaari *et al*., 2016). The CO1 gene appears to be an informative molecular marker and thus is a potential tool for species identification in Ixodidae. It could be insufficient to use a single mitochondrial gene (CO1) for DNA taxonomy thus, there is a need for an integrated approach to combine nuclear and mitochondrial genes, morphological characters, and ecological information (Gou *et al*., 2018).

1.1.9 Tick-borne disease of cattle

Approximately 80% of the world cattle population are affected by the tick-borne diseases that are widely distributed in the tropical and subtropical countries (Asmaa *et al*., 2014;

Raboloko *et al*., 2020). In Tanzania, TBDs cause over 72% of the annual cattle deaths (Emmanuel *et al.,* 2012). East Coast fever (ECF) caused by *Theileria parva*, babesiosis caused by *Babesia bigemina* and *B. bovis*, anaplasmosis caused by *Anaplasma marginale* and heart water caused by *Ehrlichia ruminantium* are the most important TBDs of cattle in Tanzania (Kerario *et al.,* 2018; Nchu *et al*., 2020). Only three TBDs namely theileriosis, babesiosis and anaplasmosis have been considered for discussion in this study.

1.1.9.1 Theileriosis

Theileriosis is caused by an obligate intracellular parasite of the genus *Theileria*. It affects both wild and domestic animals, mostly the bovines (OIE, 2020). There are about 15 species within the genus *Theileria*, that are capable of infecting cattle and other domestic ruminants such as sheep, and goats (Moumouni *et al*., 2015; Spickler, 2019). However, *T. parva* that causes ECF and *T. annulata* that causes tropical theileriosis are the only two species commonly known to be pathogenic and of economic importance in cattle (OIE, 2020). Cattle become infected when theileria sporozoites enter into the body via tick saliva during blood feed (Namgyal, 2020). The sporozoites then invade lymphocytes and develop into schizonts which induce division of infected cells resulting in lymphocytosis.

The schizonts develop into merozoites and invade red blood cells to develop into piroplasm (Watts *et al*., 2016). Piroplasm then infects ticks as they blood feed on cattle. In ticks the piroplasm form gamonts which develop into gametes. The gametes fuse to form zygote and latter form motile kinete. Sporogony then occurs in the sporoblast to form sporozoites in the tick salivary glands. The sporozoites are then secreted from the salivary glands into the feeding site, infecting the cattle and the cycle continues (Spickler, 2019). The incubation is usually 7-12 days for ECF in experimentally infected animal and 1-3 weeks for tropical theileriosis (Spickler, 2019). East Coast fever in cattle is characterized

by fever, peripheral lymphadenopathy, anorexia, respiratory distress, and in some animals, nasal discharge, and diarrhea (Fry *et al*., 2016). *Theileria* spp. are transmitted by several ixodid ticks of the genera *Rhipicephalus, Hyalomma, Amblyomma* and *Haemaphysalis*. *Theileria parva* is the cause of tropical theileriosis, naturally transmitted by *R. appendiculatus* and *R. zambesiensis* and can also experimentally be transmitted by *R. evertsi* and *R. pulchellus* (Lorusso *et al*., 2016). *Theileria annulata* is transmitted by *Hyalomma* spp. (Fry *et al*., 2016; Spickler, 2019; OIE, 2020).

1.1.9.2 Babesiosis

Babesiosis in cattle is caused by an intraerythrocytic protozoan parasite of the Phylum Apicomplexa, and genus *Babesia* (OIE, 2021). Two species of *Babesia* (namely *B. bovis* and *B. bigemina*) are known to infect cattle in tropical and subtropical countries (Spickler, 2019). Cattle become infected through a bite from an infected tick that secretes *Babesia* sporozoites into the blood circulation (Bock *et al*., 2004). Sporozoites invade red blood cells, transform into trophozoites, and then grow and divide into two round or pear-shaped merozoites (Namgyal, 2020). The merozoites rupture cells and subsequently infect new red blood cells (Bock *et al*., 2004). The incubation period is often 2–3 weeks or longer after tick infestation. (OIE, 2021). *Babesia. bovis* and *B. bigemina* are transmitted by *R. microplus* as the principal vector (Bock *et al.*, 2004; Namgyal, 2020). Transmission of the pathogen in ticks occurs both transovarially and transstadially. However, transstadial transmission does not occur at all stages (Gray *et al.*, 2019).

1.1.9.3 Anaplasmosis

Anaplasmosis in the bovine is caused by a Gram-negative, obligate intracellular parasite *Anaplasma marginale* that belongs to the order Rickettsiales, family Anaplasmataceae, genus Anaplasma (Kocan *et al.*, 2004; Selmi *et al*., 2019). There are six known species

belonging to this genus, namely *Anaplasma bovis*, *A. ovis*, *A. marginale*, *A. centrale*, *A. phagocytophilum* and *A. platys* (Selmi *et al*., 2019). *Anaplasma marginale* mostly occurs in tropical and subtropical regions, and it has a significant economic effect through decreased livestock productivity (Felsheim *et al*., 2010; Namgyal, 2020). *Anaplasma centrale* causes benign infection with some degree of anemia (OIE, 2018). Once susceptible cattle are infected with *Anaplasma*, the organism multiplies in the bloodstream and attaches to the animal's red blood cells. The animal's immune system destroys the infected red blood cells in an attempt to fight off the infection. Following the process, uninfected blood cells are also destroyed (Kocan *et al*., 2010; OIE, 2018). When the number of blood cells being destroyed exceeds that being produced by the body, the animal becomes anemic. In cattle, red blood cells are the only known site of infection with *A. marginale,* and the clinical presentation of the disease is marked by severe anemia and jaundice without hemoglobinemia (Aubry and Geale, 2011; Namgyal, 2020). To develop the clinical signs, it takes 2 to 6 weeks after the animal has been infected (OIE, 2018; Namgyal, 2020). Several ixodid ticks from the genera *Rhipicephalus*, *Hyalomma*, *Ixodes* and *Dermacentor* are capable of transmitting *A. marginale*. Transmission of the pathogen in ticks occur only transstadially (Kocan *et al*., 2010). Transmission in cattle can occur mechanically by biting flies and blood contaminated fomites. Cattle of all ages are susceptible to *A. marginale* infection but, older ones (<3 years) are more severely affected. Cattle that survive the infection become a lifelong carrier of the pathogen (Whittier *et al*., 2009).

1.1.10 Tick-borne diseases transmission

Ixodid ticks are important vectors for tick borne diseases (TBDs) including rickettsiosis, babesiosis, anaplasmosis, heartwater, East Coast fever, Ehrlichiosis and CCHF Worldwide (Kerario *et al*., 2017; Kim *et al*., 2018). Ixodids of the genera *Amblyomma*, *Rhipicephalus*

and *Hyalomma* are the most important and widely distributed species in many parts of Tanzania (Cumming, 1999; Kerario *et al*., 2017). *Amblyomma gemma* adults usually feed on domestic animals such as cattle and camels, but large herbivores such as giraffe and buffaloes are the preferred host of this tick species. The tick was considered insignificant to the health of domestic animals. However, recent studies have shown that in arid areas it is primarily responsible for the maintenance and transmission of a number of tick-borne viruses in cattle and human (Sang *et al*., 2006).

Amblyomma variegatum (commonly known as Tropical bont tick) is an important vector of the rickettsia *Ehrlichia ruminantium* that causes heartwater in cattle, sheep and goats (Madder *et al.,* 2013). *Amblyomma variegatum* has also been implicated as vector of Crimean Congo hemorrhagic fever virus (CCHFV), Dugbe virus, yellow fever virus, the *Ehrlichia bovis* causing bovine ehrlichiosis and the protozoans *Theileria mutans* and *Theileria velifera* causing benign bovine theileriosis as well as *Anaplasma* spp., causing anaplasmosis (Ikpeze *et al.*, 2011; Madder *et al.,* 2013). This tick has also been associated with dermatophilosis caused by the bacteria *Dermatophilus congolensis.* The wound caused by this tick and the immunosuppression that occurs after blood feeding, facilitate entry of the bacteria into the host skin (Ambrose *et al.*, 1999). Dermatophilosis results in a loss of milk production, poor quality hides, weight loss and occasionally death in cattle (Ambrose *et al.*, 1999).

Amblyomma lepidum like *A. variegatum* transmits the rickettsia *Ehrlichia ruminantium,* which causes heartwater in cattle, sheep and goat (Collins *et al*., 2022), and the protozoans *Theileria mutans* and *Theileria velifera* which cause benign bovine theilerioses (Tatchell and Easton, 1986; Walker *et al.*, 2014).

Rhipicephalus appendiculatus (the Brown ear tick) transmits *Theileria parva* that causes East Coast fever in cattle (Kanduma *et al*., 2016), and different strains of *Theileria parva* that cause Corridor disease. The tick also transmits *Anaplasma bovis* causing bovine anaplasmosis, *Rickettsia conorii* causing tick typhus in human and Nairobi sheep disease virus. There is also remarkable loss of growth of cattle even without any disease transmission (Jongejan and Uilenberg, 1994; Kanduma *et al*., 2016).

Rhipicephalus (Boophilus) microplus (Asian blue tick) transmits *Babesia bovis* and *B. bigemina* that causes bovine babesiosis (Baron *et al*., 2018; Silatsa *et al*., 2019b). *Babesia bovis* infection is acquired by the adults of one generation of ticks and transmitted transovarially by the larvae to the next generation. It also transmits *Anaplasma marginale* causing bovine anaplasmosis and *Borrelia theileri* that causes spirochaetosis (Fyumagwa *et al*., 2009; Madder *et al*., 2013; Silatsa *et al*., 2019b; Rojas *et al*., 2021).

Rhipicephalus (Boophilus) decoloratus (African blue tick) is a one-host tick. The tick transmits *Babesia bigemina* to cattle (Baron *et al*., 2018). This infection is transmitted only by the nymphal and adult stages of the tick after it has passed transovarially from one generation to the next. *Rhipicephalus decoloratus* also transmits *Anaplasma marginale* to cattle and *Borrelia theileri*, causing spirochaetosis to cattle, sheep, goats and horses (Madder *et al*., 2013; Latif and Walker, 2016).

Rhipicephalus evertsi evertsi (the red legged tick), may play a role in the transmission of *T. parva* to cattle. Studies showed that it can transmit *B. bigemina* transovarially to cattle. In addition to that, stage to stage transmission of *Theileria separata* to sheep can also occur. It also transmits *Borrelia theileri* that causes spirochaetosis in cattle, horses, sheep and goats. The saliva of engorged female tick contains a toxin that causes paralysis, mostly in

lambs but it may also affect calves and adult sheep (Madder *et al*., 2013; Walker *et al*., 2014).

Hyalomma rufipes (the bont-legged tick) is a two-host tick which serves as both reservoir and vector of CCHF virus, of which additional to its transmission to human through tick bite it can be also be acquired through crushing of engorged tick or secondarily through contact with body fluids. However, domestic animals or patients with CCHF (Alsarraf *et al*., 2017; Sorvillo *et al*., 2020) are the principal source of human infection with CCHF virus (Chitimia et al., 2019; Spengler *et al*., 2019).

Hyalomma spp. transmits *Anaplasma marginale* causing bovine anaplasmosis, *Rickettsia conorii* causing tick typhus in humans and *Babesia occultans* that causes benign babesiosis in cattle (Chitimia *et al*., 2019; Bellabidi *et al*., 2020). The feeding of adults of this tick on cattle causes large lesion at the attachment sites, leading to the formation of severe abscesses due to secondary bacterial infections (Madder *et al*., 2013). On the other hand, *Rickettsia conorii* have been successfully isolated from *Hyalomma albiparmatum* a three-host tick (Heisch *et al*., 1962).

Although *Hyalomma* species are not usually involved in the transmission of main pathogenic species of *Babesia* such as; *B. bigemina*, *B. bovis* and *B. divergens,* recently few genetically distinct Babesia like *Babesia* U spp., *Babesia* spp. Kashi 1 and 2, *Babesia* spp. Kayseri 1, *Babesi*a spp. CS58, *Babesia* spp. Hy, *B. beliceri* and *B. ocultans* have been recorded from *Hyalomma* spp. (Kumar *et al.*, 2020).

1.1.11 Tick control methods

Tick control is treatment that reduces exposure of livestock to the target ticks within a specific area and time (Walker, 2011). Tick control is critical for the mitigation of the direct and indirect effects of ticks on livestock productivity (Jongejan and Uilenberg, 1994). There are many tick control approaches such as the use of chemical acaricides, selection of genetically-resistant breeds and anti-tick vaccines, but there is no single approach that could be considered as a stand-alone solution (Willadsen, 2006).

1.1.11.1 The use of acaricides in tick control

The conventional method for tick control is the treatment of animals by dipping or spraying with acaricides. Spraying can be performed using motorised spray-races or hand-sprays. Of the tick control methods, direct application of chemical acaricides to host animals still remain to be the most commonly used method for controlling ticks on livestock (Jongejan and Uilenberg, 1994). Acaricides are efficient and cost effective when used correctly. However, acaricides can have some negative impacts, such as the potential resistance of ticks to acaricides, residues in food products including milk and meat as well as the harmful effects on human, animals and the environment (Willadsen, 2006). An acaricide is acceptable when has an efficacy of ≥ 95% as per the government of Tanzania regime (Emmanuel *et al*., 2012). The prolonged use and malpractices during acaricide application have been associated with the development of resistance in ticks (Emmanuel *et al.*, 2012) and the development of resistance is most common in *R. microplus* (Malan, 2015).

1.1.11.2 Host resistance to ticks

Selection of genetically-resistant host breeds could help in the control of ticks. Host acquired resistance to ixodid ticks has been recognized as a possible means of biological

tick control. Such immunologically mediated resistance is acquired through subsequent repetitive infestations by ticks (Jongejan and Uilenberg, 1994). An improved acquired immunity is expressed in terms of minimized number of on-host ticks, minimized engorgement weights, and minimized egg and larval production resulting in significantly decreased tick populations. Acquiring such host resistance varies with on-host tick species, host breed and depending on natural selection of the host animal exposed to the query tick species over several subsequent generations (Jongejan and Uilenberg, 1994; Walker, 2011). Previous studies showed that the Tanzania shorthorn zebu is comparatively more resistant to ticks as well as to ECF (Nchu *et al*., 2020). Differences in the ability of cattle to become resistant against ticks, between or within cattle breeds, have been recognized, considering that the ability to acquire resistance is heritable (Willadsen, 2006).

1.1.11.3 Anti-tick vaccines

Anti-tick vaccines minimize the number of engorging female ticks, their weight and capability to lay eggs. That means a good vaccination effect is expected to have a decreased larval infestation in a following generation (Willadsen, 2006). The likelihood of ant-tick vaccine being more complex could be due to a possibility that the method also affects the biology of tick-borne pathogen transmission (Willadsen, 2006). In Tanzania anti-tick vaccines are not readily available to the farmers. However, in countries where the vaccines are available, the existing vaccines have relatively less impact on tick control which could be due to a mixture of scientific and commercial reasons. In addition, pesticides are readily available, effective, well known to farmers, and are actively promoted by the suppliers (Namgyal, 2020). However, if vaccines of greater efficacy could be achieved this would lead to greater adoption. Many years have passed since the release of the first *R. microplus* commercial vaccine based on a recombinant antigen, Bm86 (Willadsen, 2006). This has been followed by several investigations on related vaccines

including BD86, Hd86, Hm86, He86 Hdr86, Haa86 vaccines (Kumar *et al.,* 2020). Renewed interest in this strategy has been triggered by the increasing trend in acaricide resistance together with advancement in bioinformatics (Nchu *et al.,* 2020). Despite the fact that, the Bm86 vaccine uses an antigen from *R. microplus*, studies showed that it is also efficacious against *R. annulatus*, *R. decoloratus* and *Hyalomma dromedarii* but not *R. appendiculatus* (Willadsen, 2006). At present, there are no commercially available anti-tick vaccines in developing countries including Tanzania and thus dependency on acaricide increases leading to the development of acaricide resistant-tick populations (Kumar *et al.,* 2020; Nchu *et al.,* 2020).

1.1.11.4 Ethnoveterinary medicine and practices

Ethnoveterinary medicine and practices are based on traditional knowledge that is passed from one generation to the next. The practices are widely spread across tropical and subtropical African regions and are particularly preferred by small-scale farmers in rural areas (Kerario *et al*., 2018; Nchu *et al*., 2020). However, cultural practices associated with ethnoveterinary practices are not well-documented and thus prone to easy vanishing. In East Africa, out of 47 plant species that have been documented as useful for tick control, only 14 have been scientifically validated. Experiments aiming at validating the anti-tick activities of a variety of ethnoveterinary plants are recommended and should be documented (Nchu *et al*., 2020).

With regards to all these methods, use of acaricides still remains as a primary method of tick control in livestock due to their efficient use and availability (Kerario *et al*., 2018). However, the challenge of increasing costs and resistance toward different acaricides could become a major future threat to the use of acaricides. An integrated approach involving combination of two or more tick control methods is therefore recommended (De Castro,

1997; Willadsen, 2006). Developing good understanding of farmers about TBDs and effectiveness of the proposed tick control methods could facilitate successful implementation of tick control programs (Kerario *et al*., 2018; Namgyal, 2020).

1.2 Problem Statement and Study Justification

Ticks and TBDs are still major problems in livestock and human health in Tanzania. Ticks harbor and transmit zoonotic pathogens to livestock, wild animals and humans. As the transfer of ticks between livestock, wild animals and humans may largely influence general tick-borne pathogen transmission; there is a need for studies on distribution, infestation and seasonal variation of ticks as vectors. However, there are still limited studies especially in Kilombero and Iringa district (Kwak *et al*., 2014). The existing information on ticks infesting cattle was reported in Ngorongoro, Iringa municipality, Maswa, Mara, Singida, Mbeya, Mvomero and Rufiji, which were based on morphological characteristics. This is the first study in the Kilombero and Iringa district in which molecular methods were used to confirm the identified tick species to support morphological identification. This study aimed at providing baseline information on the current distribution, abundance and seasonal variation of Ixodid ticks infesting cattle in the selected areas using molecular data.

1.3 Research Objectives

1.3.1 General objective

The overall objective of this study was to establish the distribution, abundance and seasonal variation of Ixodid ticks infesting cattle and pathogens in Kilombero and Iringa districts, Tanzania.

1.3.2 Specific objectives

i. To determine prevalence of tick infestation in cattle in Iringa and Kilombero Districts.

ii. To establish seasonal dynamics of Ixodid ticks in terms of distribution, abundance and burden in cattle in the study areas.

iii. To determine occurrence of tick-borne pathogens in ticks collected from cattle in the study areas.

1.3.3 Research questions

i. What is the hard tick distribution and abundance in cattle in Kilombero and Iringa districts?

ii. What are the hard tick species prevailing in the study area?

iii. What tick-borne pathogens are present in the identified tick species?

CHAPTER TWO

MANUSCRIPT ONE

Distribution and Molecular Identification of Ixodid Ticks Infesting Cattle in Kilombero and Iringa Districts, Tanzania

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Abstract

Background: Hard ticks infesting cattle are vectors of Tick-borne diseases that causes major public health problems and considerable socioeconomic losses to livestock industry in tropical and subtropical countries. A repeated cross-sectional study was carried out by collecting ticks on cattle during the wet and dry seasons from January to August 2021 in order to determine hard tick prevalence, distribution and abundance on cattle, in Kilombero and Iringa Districts of Tanzania. The collected ticks were identified morphologically using published morphological keys, under a stereomicroscope and confirmed by polymerase chain reaction (PCR) and sequencing.

Results: Out of 740 examined cattle, 304, (41.08%) were infested with ticks. In total 1,780 ticks were counted on one side of the animal's body and doubled, where by a total of 3,560 ticks were recorded. A total of 1,889 tick were collected from the infected cattle including 109 more tick observed while collecting ticks based on the animal's posture when restrained to the ground. Out of 1,889 ticks, nine species from three genera were identified morphologically, 1,377 fits in the genus *Rhipicephalus*, 459 in the genus of *Amblyomma* and 53 in the genus *Hyalomma*. *Rhipicephalus microplus* was predominantly prevalent 909, (48.1%), followed by *Rhipicephalus evertsi* 310, (16.4%), *Amblyomma lepidum* 310, (16.4%), *Rhipicephalus appendiculatus* 140, (7.4%), *Amblyomma gemma* 120, (6.4%), *Hyalomma rufipes* 50, (2.6%), *Amblyomma variegatum* 29, (1.5%), *Rhipicephalus decoloratus* 18, (1.0%) while the least common was *Hyalomma albiparmatum* 3, (0.2%). Tick diversity was highest in Iringa than Kilombero District. Ticks were widely distributed in different parts of the host body, the distribution was highest on zone 4 (n = 1,060, 56.11%) which includes (groin, flank, abdomen and around inner thigh of the hind legs) and least on zone 2 (n = 14, 0.74%) which includes back surface of the body. *Amblyomma lepidum* and *R microplus* species were distributed in all the five body zones and recorded with the highest proportions on zone 4, *A. lepidum* (n = 209, 67.42%) and *R. microplus* (n = 714, 78.55%). The nine tick species identified morphologically were identified by molecular method, however during sequencing two species (*Rhipicephalus appendiculatus and R. decoloratus*) had poor quality sequences and were excluded from the analysis. The sequencing results indicate high nucleotide identity (96-100%) with sequences available in GenBank and Barcode of Life Database (BOLD). The phylogenetic analysis of partial mitochondrial COI and 16S rRNA gene sequences of ticks confirmed the morphological identification. Tick prevalence was higher in wet season (n=148, 41.11%).

Conclusion: The results showed high burden of tick infestation on cattle and this could reduce animal production and possibly increase the risk of tick-borne diseases. Therefore, it is necessary to explore the epidemiological and molecular aspects of various tick species in other regions of Tanzania.

Keywords: Ticks, Distribution, Prevalence, Burden, Cattle

Background

Ticks are one of the most important arthropod vectors and reservoirs for a wide variety of pathogenic agents such as viruses, bacteria, fungi, protozoa and nematodes, which can cause diseases in human, livestock and wild animals [1]. Ticks transmit diseases that lead to extensive economic loss to resource-poor farming communities especially in tropical and subtropical regions where almost 80% of the world's cattle population are reared [2,3]. Tick-borne diseases (TBDs) such as East Coast fever, Babesiosis, Anaplasmosis and Ehrlichiosis contribute to more than 70% of all cattle deaths in Tanzania resulting in more than TSh. 72 billion loss annually [4]. In addition to the transmission of infectious diseases, ticks are associated with great loss in milk and meat production as well as reduction in hide quality [4].

Several ecological factors influence the tick prevalence and adaptation in different parts of the country. Abiotic factors such as soil moisture, humidity, soil pH, temperature and natural disasters and biotic factors e.g., host availability, vegetation cover, predators, parasites of ticks and the relations between individual tick species all affects the availability and diversity of ticks. The presence or absence of ticks is primarily dependent on humidity and moisture content of a local microclimate. Moreover, environmental conditions are continuously changing due to global warming, which may alter the distribution patterns and vectorial capacity of ticks [5].

Tanzania as an agricultural country where livestock related activities contribute only 7.4% to the Gross Domestic Product (GDP) and the growth rate of the sector

is only about 2.6% per annum [6]. About 65.7% of the households are involved in agricultural activities, 64.9% are engaged in crops only, while 33.3% engaged in crops and livestock and 2% in livestock production only. The country has approximately 33.9 million cattle, 24.5 million goats and 8.5 million sheep. About 90% of agricultural households keep livestock of different kinds. Almost 95% of cattle populations in the country are reared under traditional agro-pastoral and pastoral husbandry systems. The National Livestock Policy recognizes that apart from contributing to the GDP, the livestock sector has a role to play in ensuring food security, source of income, providing farmers with employment and investment opportunities, providing draught power and manure for sustainable agriculture, and satisfying cultural roles [6]. The grazing land for the animals is no longer sufficient due to increased number of cattle, other domestic animals and human population. Most of the indigenous cattle are thus widely grazed in grasslands and woodlands and hence exposed to high risk of tick infestation [1,7]. The climatic condition of Tanzania is greatly favoring the development and survival of several tick species.

Ixodid ticks of the genera *Rhipicephalus* (*R. appendiculatus, R. microplus, R. decoloratus*) and *Amblyomma* (*A. variegatum, A. lepidum and A. gemma*) are the most important and widely distributed tick species found in many parts of the country where cattle are raised [8,9]. These tick species are important vectors of TBD pathogens reported in Tanzania and bordering countries.

When compared to the studies in other countries, Tanzania's data on tick epidemiology and genetic diversity is limited and insufficient [10]. Studies related to tick infestation and species composition have been conducted in Ngorongoro [8], Iringa, Maswa [9], Mvomero [11], Rufiji [7], Mara, Singida, and Mbeya [1]. However, their information is limited to morphological characters. Studies have successfully demonstrated that mitochondrial DNA provides useful markers for studies on phylogenetic relationship of ticks.

Tick problems have been reported in Kilombero and Iringa Districts. There is insufficient information on molecular characterization of ticks, tick prevalence, distribution and infestation on cattle in Kilombero and Iringa Districts. Therefore, this study aimed to determine tick prevalence, distribution and infestation on cattle, at Kilombero and Iringa Districts of Tanzania.

Material and Methods

Description of the study area

The study was conducted in Kilombero and Iringa Districts in Morogoro and Iringa regions respectively in the Southern Highlands of Tanzania. Kilombero district is one of the six administrative districts in Morogoro region and is located between 8 ° 00'–16 ° S and 36 ° 04'–36 ° 41' E, with elevation ranging from 262 to 559 m above sea level and covering an area of 14,246 km² in the region [12]. The climate is marked by a rainy season from November to May and a dry season from June to October with annual rainfall ranging from 1200 to 1800 mm. Average annual temperatures in Kilombero district range from 26–38 °C. The cattle population was about 157,000 [6]. The sampling villages in Kilombero district were Merera, Idunda, Sagamaganga and Lufulu (Fig. 1). Iringa district, one of the seven administrative districts in Iringa region is located between 7 ° 46'23.14" S and 35 ° 41' 56.83" E and covers an area of 20,576 km² with an elevation ranging from 900 to 2300 m above sea level. The climate is marked by a rainy and cooler season from November to May and a dry and cool season from June to September. It generally receives annual rainfall of about 500-1600 mm. The average annual temperature in Iringa district ranges from 20-25 °C. The lowland zone in Iringa district is characterized by low mean annual rainfall of about 500–600 mm, and temperatures of about 20–25 °C [6]. The land is mostly occupied by the National parks, forests, rocky mountains and water bodies. The district had the third largest number of cattle in the region and they were nearly all indigenous. The population of cattle was about 150,810. Most of the animals were indigenous reared under traditional system, largely free grazing and tethering. The sampling villages in Iringa District were Magombwe, Kisanga, Kitisi and Malizanga (Fig. 1).

Sample size estimation

Sample size was estimated by using CDC Epi info software version 7.2.4.0 adapting the following formula: $n = ((Z_{\alpha\beta})^2 \times pq) \div d^2$, where; n = required sample size; $Z_{0.033/2} = Z_{0.0165} = 1.96$ at 96.7% confidence interval; α = probability of type 1 error (0.05 sided); p = estimated prevalence of cattle with ticks (30%) [1]; q = Power (1 – p); d = Margin of error for expected confidence level (3.3%). Based on this software, the minimum required sample size was 739 cattle of all ages and sexes present in the study areas.

Study design and sampling method

A repeated cross-sectional study design using qualitative and quantitative methods of data collection and analysis was adopted. Tick sampling was performed both in wet (January and May) and dry (July and August) seasons of the year 2021. The two districts were purposively selected as they are bordering the wildlife conservation areas and existing previous engagements with the villages during the past health for animal and livelihood improvement studies. The eight study villages (four from each district) were also purposively selected. Three herds located approximately 5-10 km apart and having at least 40 cattle were conveniently selected from each village with the assistance of Livestock Extension Officers (LEOs) providing the information on areas where pastoralists have settled with their animals.

Tick collection and counting

During field visit twenty cattle were randomly selected from each herd and manually restrained by the help of herdsmen to allow physical examination and tick inspection [13]. During tick inspection the animal's body was marked into five body zones; zone 1 (Head, ears, flap of skin on lower surface of neck and ventral surface of thorax), zone 2 (Back surface of the body), zone 3 (Side of main body and area between forelegs and body), zone 4 (Groin, flank, abdomen and around inner thigh of the hind legs), zone 5 (Perineum including areas between anus and genital organs). The animals were then visually inspected for ticks all over the body sites. Additional information on animal health status (body condition score), age, sex was

recorded and frequency of tick control and tick control methods used in each household were also obtained from the herds owner and recorded [1]. Tick burden on each animal was obtained by counting the number of ticks from one side of the body and the results were multiplied by two to represent the tick burden on the whole body of the animal as described by Rehman [3]. Ticks were then removed from different prediction sites of the animal's body marked as zone 1-5 using blunt ended forceps [14]. The forceps were used to grip the tick firmly over its scutum and mouthparts as closely to the host skin as possible, then pulled strongly and directly out of the skin. All ticks from each zone were pooled together and transferred into respective empty 15 ml falcon tube and kept in a dry shipper (MVE vapor shippers, Borngasse 20 35619 Braunfels, Germany) to immobilize the ticks until sorting at the Health for Animal and Livelihood Improvement (HALI) project molecular diagnostic laboratory at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture.

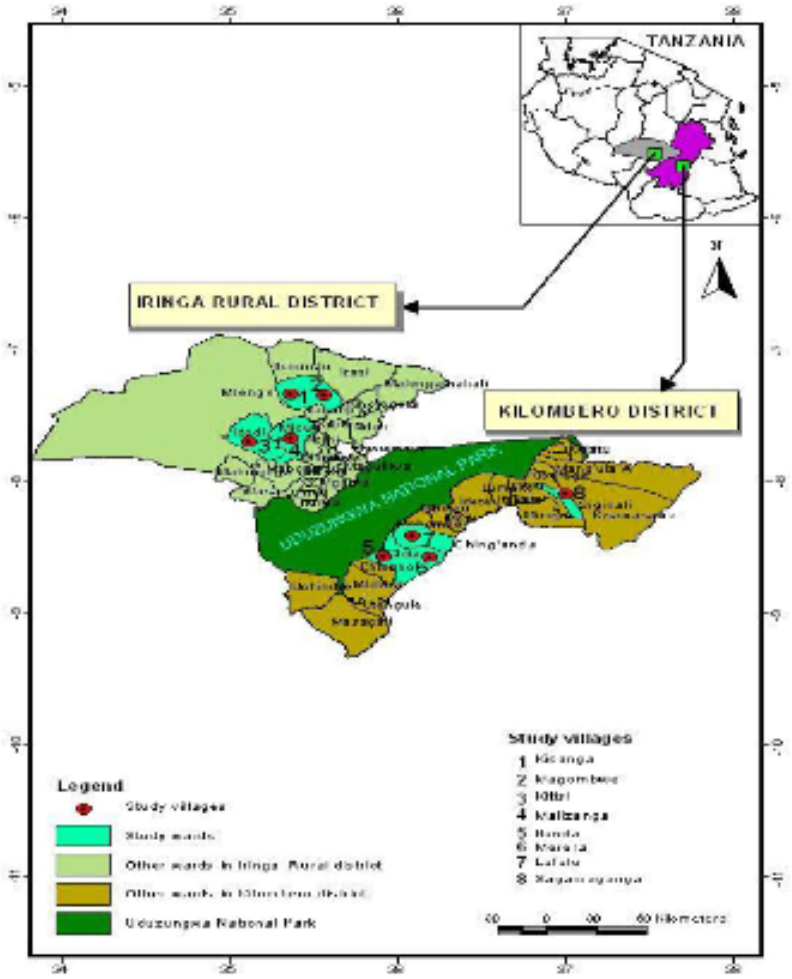

Fig. 1. A map of Tanzania showing study areas. The red dots indicate the study villages both in Kilombero and Iringa districts from which ticks were sampled.

Morphological identification of ticks

The preserved ticks were taken from -80°C (Ultra-low temperature freezers, Thermo Fisher Scientific, Marietta, OH 45750, USA), rinsed with 70% ethanol then distilled water followed by brief drying on paper towel. The ticks were sorted by sex and genera based on the presence or absence of banded legs, coloured or patterned scutum and conscutum, presence or absence of festoons and eyes and shapes of the mouthparts using magnifying hand lens. The sorted ticks were then identified using published morphological keys for African ticks [15,16]. The ticks were further morphologically identified to species on a stereomicroscope (Brunel Stereomicroscope Ltd, UK) by experienced laboratory personnel at the Parasitology laboratory in the Department of Veterinary Microbiology, Parasitology and Biotechnology at Sokoine University of Agriculture.

DNA extraction and molecular identification of ticks

The DNA was extracted from 42 ticks, (1-5 ticks from each species) randomly selected from the nine tick species initially identified by morphological characters. The DNA extraction was performed using Quick-DNA Minprep Plus Kit (D4068, Zymo Research, CA, USA) according to manufacturer's instructions. The extracted DNA was eluted in 50 µl of DNase/RNase-free water and stored at -

80°C for subsequent use for PCR [17-19]. The tick species were confirmed using conventional PCR targeting Mitochondrial Cytochrome c Oxidase subunit 1 (CO1) or 16S rRNA as DNA barcoding genes for selected members of tick species and those which were found difficult to identify to species level morphologically [13,20,21].

The fragment of CO1 gene was amplified using primer sets Cox1-F- (5'- GGA ACA ATA TAT TTA ATT TTT GG-3') and Cox1-R- (5'-ATC TAT CCC TAC TGT AAA TAT ATG -3') amplifying approximately 820 bp [22]. The 16S rRNA gene was amplified using primer sets T16S-F- (5'-TTA AAT TGC TGT RGT ATT-3') and T16S-R- (5'-CCG GTC TGA ACT CAS AWC-3') amplifying approximately 455 bp [23]. The PCR amplifications were performed in a total reaction volume of 25 µl containing 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1.0 µl (10 µM) of each forward and reverse primer, 0.10 µl of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 17.15 µl of Molecular grade water and 2 µl of template DNA in a thermal cycler (SimpliAmp thermocycler, Applied Biosystems, Thermo Fisher Scientific Inc). The cycling conditions for CO1 were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 54 °C for 1 min, 72 °C for 1 min and then 72 °C for 5 min [21]. For the 16S rRNA, the cycling conditions were as follows: 94 °C for 5 min, followed by 45 cycles of 94 °C for 30 s, 50 °C for 45 s, 72 °C for 45 s and then 72 °C for 7 min [23]. A negative control with ddH_2O in place of DNA was included in each run. The obtained PCR products were separated on a 1.5 % (w/v) agarose gel in 1x TBE buffer (SERVA Electrophoresis, Heidelberg, German) stained with gel red (Phonex Research Products, Candler, USA) and viewed under UV transilluminator.

The PCR products of CO1 and 16S rRNA fragments were then sequenced using forward and reverse primers used to generate the PCR products. The sequencing reactions were performed in the DNA Master cycler pro-384 (Eppendorf) using BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) following the protocols supplied by the manufacturer. The fluorescent-labelled fragments were purified using the BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA). The samples were run for electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

Data management and statistical analysis

All data collected in this study were stored in a computer, using Microsoft excel software version 2109 where they were sorted and checked for completion before doing statistical analysis. The age of cattle was grouped into calves (≤ 6 months), juveniles (7 to 24 months) and adults (> 24 months) [1,24]. Cattle health status was obtained using Body Condition Score (BCS). The BCS was categorized into poor (BCS 1 and 2), average (BCS 3) and good (BCS of 4 and 5) [1,24]. The prevalence of tick was determined by dividing the number of infested cattle by total number of cattle examined and was expressed as percentage. Descriptive statistics on tick prevalence data was performed using Epi Info software version 7.2.4 (CDC, Atlanta, USA) to compare the difference in tick species proportions between the study areas and seasons. Fisher's exact test was performed to detect the difference between the proportion of hard tick species and the study areas and seasons. The tick species count was used as dependent variable while district and season were used as independent variables. One-way ANOVA was performed to compare mean tick burden between variables (e.g., Cattle age groups, BCS and frequency of tick control). The *p*-value (0.05) was considered statistically significant in all statistical tests.

The obtained DNA sequences were compared with the sequences on GenBank database using Basic Local Alignment Search Tool (BLAST) to obtain sequence similarities (https://blast.ncbi.nlm.nih.gov (accessed on 23 December 2021)). The quality of sequencing chromatogram was checked using Sequence Scanner Version 2.0 software (Applied Biosystems, Foster City, CA). The reverse complement and forward nucleotide sequences delimited by reverse and forward primers sequence were aligned to obtain a consensus nucleotide sequence using Bioedit version 7.2.5 (Ibis Biosciences, Carlsbad, CA). The consensus nucleotide sequence was used in BLASTn to search for nucleotide identity in comparison with available nucleotide sequences at GenBank database. The nucleotide sequences were then analysed using Molecular Evolutionary Genetics Analysis (MEGA) X software [25] and aligned using Clustal-W to determine the similarity between the sequences. In addition, representative CO1 and 16S rRNA tick sequences from previous studies were downloaded from GenBank for phylogenetic analysis. Multiple sequence alignments were performed and the neighbor-joining (NJ) method was used to construct the phylogenetic tree in MEGA X [25-28]. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. The confidence values for individual branches of the resulting trees were determined through bootstrap values with 1000 replicates to statistically support the nodes on the tree.

Ethical approval

The ethical approval for this research was obtained from Ethical Committee of the Sokoine University of Agriculture, permit number - SUA/ADM/R.1/8A/734_15/02/2021. The permission to carry out this study in the respective study sites was granted by the local government through District Veterinary Officers in Kilombero and Iringa Districts. Animal owner's consents were sought verbally prior to data collection. The study was conducted with full approval from household owners, district councils of the study areas and the Sokoine University of Agriculture.

Results

A total of 740 cattle were examined for tick infestation, (45.68%, n=338) from Kilombero district and (54.32%, n=402) from Iringa district. An overall tick prevalence of (41.08%, n=304) was recorded in the study areas. The tick prevalence in Kilombero district was (41.42%, n=140) and (40.80%, n=164) in Iringa district, both were nearly equal to the overall tick prevalence and no significant difference observed ($p > 0.05$) (Table 1). In total 1,889 ticks were collected from the infected cattle whereas, after counting

ticks on one side of the animal's body and doubled a total of 3,560 ticks were recorded. Season wise, tick infestation prevalence was (41.11%, n=148) during the wet season and (41.05%, n=156) during the dry season (Table 1). Tick infestation prevalence was highest in male (44.02%, n=92) than female cattle (39.92%, n=212). Based on cattle age groups, tick infestation prevalence was highest in calves (age ≤ 6 months) (51.61%, n=16), followed by adults (age > 24 months) (40.91%, n=216) and juvenile (age 7 to 24 months) (39.78%, n=72). With cattle health status, tick infestation was highest on cattle with average health condition (41.78%, n=216), and least in poor (39.82%, n=45) and cattle with good health condition (39.09%, n=43). Moreover, tick infestation prevalence was almost equal between the tick control frequency categories (Table 1). Therefore, there was no statistically significant difference in tick infestation prevalence between the two seasons, cattle sex, age groups, health categories and tick control frequencies observed in this study $p > 0.05$.

Of all tick species identified, *Rhipicephalus microplus* had the highest prevalence (48.1%, n=909), followed by *Rhipicephalus evertsi* (16.4%, n=310) and *Amblyomma lepidum* (16.4%, n=310) while, *Hyalomma albiparmatum* had the lowest prevalence (0.2%, n=3) (Table 2). Season wise, *Rhipicephalus microplus* was recorded with the highest proportions during the wet season 59.8% and 35.2% during dry season, followed by *A. lepidum* which had a higher proportion 19.3% during dry and 13.8% during wet season and *R. evertsi* had a higher proportion 20.8% during dry compared to 12.4% during wet season. The least tick proportion was observed in *H. albiparmatum* with a proportion of 0.3% during wet and 0% during dry season (Table 3). In general, there was statistically significant difference between the seasons on the proportion of *R. microplus*, *R. evertsi*, *R. appendiculatus*, *H. rufipes*, and *A. lepidum* ($p < 0.05$) (Table 3). On the other hand, *Amblyomma gemma*, *A. variegatum* and *R. decoloratus* had a higher proportion during wet than dry season however, the difference was not significant ($p > 0.05$) (Table 3).

For the case of mean tick burden, a higher overall mean tick burden of 12.07 ± 0.91 was observed in Kilombero compared to 11.40 ± 1.00 in Iringa district however, the difference was not statistically significant (CI = 95%, p = 0.640). Mean tick burden on cattle was higher during wet than dry season however the difference was not statistically significant (p = 0.436) (Table 4).

Table 1. Number of cattle examined, infested cattle and tick infestation prevalence with respect to district, season, cattle sex, age, animal health status and tick control frequency

Variables		Number of cattle examined	Number of cattle infested	Prevalence (%)	p- value
District					
	Iringa	402	164	40.80	0.864
	Kilombero	338	140	41.42	
Season					
	Dry	380	156	41.05	0.987
	Wet	360	148	41.11	
Cattle sex					
	Female	531	212	39.92	0.308
	Male	209	92	44.02	
Cattle age group					
	Adult	528	216	40.91	0.240
	Calf	31	16	51.61	0.217
	Juvenile	181	72	39.78	0.789
Cattle health status					
	Average	517	216	41.78	0.603
	Good	110	43	39.09	0.911
	Poor	113	45	39.82	0.702
Tick control frequency					
	Weekly	135	56	41.48	0.844
	Biweekly	294	119	40.48	0.909
	Monthly	154	65	42.21	0.901
	Occasionally	133	54	40.60	0.884
	Unknown	24	10	41.67	0.987

Table 2. Sex ratio and prevalence for the identified tick species collected on cattle from Kilombero and Iringa districts. Male (M); Female (F).

Tick species	Males	Females	M: F	Total ticks	Prevalence (%)
Amblyomma gemma	88	32	2.6:1	120	6.4
Amblyomma lepidum	247	63	3.9:1	310	16.4
Amblyomma variegatum	14	15	1:1.1	29	1.5
Hyalomma albiparmatum	3	0	3:0	3	0.2
Hyalomma rufipes	32	18	1.8:1	50	2.6
Rhipicephalus appendiculatus	45	95	1:2.1	140	7.4
Rhipicephalus decoloratus	0	18	0:18	18	1.0
Rhipicephalus evertsi	188	122	1.5:1	310	16.4
Rhipicephalus microplus	63	846	1:13.4	909	48.1

Table 3. The proportion and counts of hard ticks from Kilombero and Iringa Districts during dry and wet season.

	Season			District		
	Dry (%)	Wet (%)	*p*-value	Iringa (%)	Kilombero (%)	*p*-value
Tick genera						
Amblyomma	235 (51.2)	224 (48.8)	0.021	447 (97.4)	12 (2.6)	0.000
Hyalomma	32 (60.4)	21 (39.6)		53 (100.0)	0 (0.0)	
Rhipicephalus	630 (45.8)	747 (54.3)		501 (36.4)	876 (63.6)	
Tick species						
A. gemma	50 (5.6)	70 (7.1)	0.219	119 (99.2)	1 (0.8)	0.000
A. lepidum	173 (19.3)	137 (13.8)	0.001	306 (98.7)	4 (1.3)	0.000
A. variegatum	12 (1.3)	17 (1.7)	0.576	22 (75.9)	7 (24.1)	0.014
H. albiparmatum	0 (0.0)	3 (0.3)	0.252	3 (100.0)	0 (0.0)	0.252
H. rufipes	32 (3.6)	18 (1.8)	0.021	50 (100.0)	0 (0.0)	0.000
R. appendiculatus	121 (13.5)	19 (1.9)	0.000	137 (97.9)	3 (2.1)	0.000
R. decoloratus	6 (0.7)	12 (1.2)	0.246	14 (77.8)	4 (22.2)	0.055
R. evertsi	187 (20.8)	123 (12.4)	0.000	306 (98.7)	4 (1.3)	0.000
R. microplus	316 (35.2)	593 (59.8)	0.000	44 (4.8)	865 (95.2)	0.000

Table 4. Number of cattle infested, Total number of ticks, Mean tick burden per cattle ± standard error of mean (SE) with respect to district, season, cattle sex, age, animal health status and tick control frequency

Variables		No. of cattle	Tick counts	Mean tick burden ± SE	Std Dev	*p*-value
District						
	Iringa	164	1870	11.40 ± 1.00	12.76	0.625
	Kilombero	140	1690	12.07 ± 0.91	10.80	
Season						
	Dry	156	1746	11.19 ± 0.99	12.33	0.436
	Wet	148	1814	12.26 ± 0.92	11.40	
Cattle sex						
	Female	212	2286	10.78 ± 0.73	10.66	0.039
	Male	92	1274	13.85 ± 1.47	14.14	
Cattle age group						
	Adult	216	2654	12.29 ± 0.85	12.44	0.403
	Calf	16	160	10.00 ± 2.98	11.91	
	Juvenile	72	746	10.36 ± 1.18	10.02	
Cattle health status						
	Average	216	2394	11.08 ± 0.75	11.01	0.221
	Good	43	624	14.51 ± 2.21	14.44	
	Poor	45	542	12.04 ± 1.95	13.09	
Tick control frequency						
	Weekly	56	338	6.11 ± 0.77	5.75	0.000
	Biweekly	119	1 516	12.77 ± 1.01	11.02	
	Monthly	65	970	14.83 ± 2.20	17.74	
	Occasionally	54	550	10.15 ± 0.95	6.99	
	Unknown	10	186	18.60 ± 2.91	9.19	

Among the ticks collected from Kilombero district, *Rhipicephalus microplus* was the most abundant tick species (97.4%, n=865) while *A. lepidum* and *R. evertsi* were the most abundant species of all ticks collected on cattle from Iringa district each (30.6%, n=306). In addition, *Hyalomma albiparmatum* (100%, n=3) and *H. rufipes* (100%, n=50) were only recorded in Iringa district while none was recorded in Kilombero. For *H. rufipes*, the difference in proportion between the two districts was statistically significant ($p < 0.05$). The other six tick species including, *A. gemma, A. lepidum, A. variegatum, R. appendiculatus, R. decoloratus* and *R. evertsi* were all recorded with significantly higher proportions in Iringa district compared to Kilombero district ($p < 0.05$) (Table 3). *Rhipicephalus microplus* was the only species recorded with significantly higher proportion in Kilombero district than in Iringa district.

With regards to cattle sex, age group, health status and tick control frequency, a significantly high mean tick burden of 13.85 ± 1.47 was recorded in male as compared to female cattle 10.78 ± 0.73, ($p < 0.05$) (Table 4). The highest mean tick burden 12.29 ± 0.85 was recorded in adult cattle (>24 months) as compared to calf (≤ 6 months) and juvenile (7 to 24 months) which had mean tick burden of 10.00 ± 2.98 and 10.36 ± 1.18 respectively. In general, there was no statistically significant difference in mean tick burden between the cattle age groups ($p = 0.403$) and between the cattle health status groups ($p = 0.221$). Based on tick control frequency categories, a significant low mean tick burden (6.11 ± 0.77) was recorded on cattle reported with weekly tick control frequency ($p < 0.001$) (Table 4).

With regard to tick distribution, there was high tick species diversity in Iringa than Kilombero district (Table 5). However, in Kilombero district, *R. microplus* was highly distributed in all sampled villages as compared to Iringa district. Among the five predilection sites on cattle's body, ticks were distributed in all the five body

zones. Tick distribution was highest on zone 4 (56.11%, n = 1,060) which includes (groin, flank, abdomen and around inner thigh of the hind legs), followed by zone 5 (23%, n = 451) and least on zone 2 (0.74%, n = 14) which includes back surface of the body. *Amblyomma lepidum* and *Rhipicephalus microplus* species were distributed in all the five body zones and recorded with the highest proportions on zone 4 (including, *A. lepidum* (67.42%, n=209) and *R. microplus* (78.55%, n=714) (Table 6).

Table 5. The distribution of the tick species collected on cattle from villages in Kilombero and Iringa Districts

	Iringa				Kilombero			
Tick species	**Kisanga**	**Kitisi**	**Magoombwe**	**Malizsnga**	**Lufulu**	**Idunda**	**Merera**	**Sagama**
A. gemma	45	23	5	46	0	0	1	0
A. lepidum	88	6	202	10	0	0	4	0
A. variegatum	2	9	9	2	0	0	2	5
H. albiparmatum	0	0	1	2	0	0	0	0
H. rufipes	27	7	14	2	0	0	0	0
R. appendiculatus	4	25	0	108	0	0	0	3
R. decoloratus	6	0	8	0	1	1	0	2
R. evertsi	104	32	154	16	0	0	4	0
R. microplus	2	3	39	0	119	299	293	154

Name shortened: Sagama (Sagamaganga).

Table 6. The distribution of hard tick species identified with respect to cattle body zones

	Cattle body zones (Tick predilection site on cattle)				
Tick species	**Zone 1**	**Zone 2**	**Zone 3**	**Zone 4**	**Zone 5**
A. gemma	6	0	36	59	19
A. lepidum	11	3	60	209	27
A. variegatum	0	4	12	10	3
H. albiparmatum	1	0	0	2	0
H. rufipes	0	0	2	3	45
R. appendiculatus	103	0	4	27	6
R. decoloratus	0	2	1	6	9
R. evertsi	4	0	2	30	274
R. microplus	12	5	110	714	68
Total ticks n (%)	137 (7.25)	14 (0.74)	227 (12.02)	1,060 (56.11)	451 (23.88)

For molecular identification of tick species, a total of 42 representative ticks, (1-5 ticks) from each species were randomly selected for molecular analysis. The nine tick species identified morphologically were also identified by molecular method however, during sequencing two species (*Rhipicephalus appendiculatus and R. decoloratus*) had poor quality sequences and were excluded from the analysis. The CO1 gene was successfully amplified from 92.86% (n=39) of the selected-on host ticks. The 16S rRNA gene was successfully amplified from 100% (n=8 including 3 of the samples with unreliable CO1 results and 5 more samples that were successfully amplified by CO1. The amplification of approximately 455 bp sequence of 16S rRNA produced the expected amplification products. The nucleotide sequences of Tanzanian ticks obtained from this study were submitted at the GenBank and provided with accession numbers (OM974109 - OM974112 and OM978262 - OM978265).

Based on the CO1 gene sequences, *Amblyomma gemma* from this study (GenBank accession no. OM974111) was 100% identical to *A. gemma* isolate sequence from Kenya (BOLD: ARAK131-13). The *A. lepidum* sequence (GenBank: OM974112) from this study had the closest identical (99.57%) *A. lepidum* isolate sequence from Kenya (GenBank: KP987775). *Hyalomma albiparmatum* sequence from this study (GenBank: OM974110) had the closest identical (96.08%) *H. albiparmatum* isolate sequence from Israel (GenBank: KU130576), whereas, the *H. rufipes* sequence from this study (GenBank: OM974109) had the closest identical (99.74%.) *H. rufipes* isolate sequence from France (GenBank: KX000643)

Based on the 16S rRNA gene sequences, *R evertsi* sequence from this study (GenBank: OM978262) had the closest identical (100%) *R. evertsi* isolate sequence from Zambia and Tanzania (GenBank: LC634571 and MN961124) respectively. *Amblyomma variegatum* from this study (GenBank: OM978264) had the closest identical (99.47%) *A. variegatum* isolate sequence from Ethiopia (GenBank: MN150175). Lastly, the 16S rRNA gene sequence of *R. microplus* from this study (GenBank: OM978265) had the closest identical (100%) *R. microplus* isolate sequence from Uganda and Colombia (GenBank: KY688461 and MN650726) respectively.

Phylogenetic analysis based on mitochondrial CO1 and 16S rRNA nucleotide sequences of the identified tick species was performed to determine the genetic relationship between the nucleotide sequences obtained in this study and reference sequences obtained from GenBank. Alignment of CO1 gene nucleotide sequences obtained from each tick species in this study showed that the sequences were 100% identical. Similarly, the 16S gene nucleotide sequences obtained from each tick species were found to be 100% identical. Therefore, a single sequence from each tick species was selected for

phylogenetic analysis. In both mitochondrial CO1 (Fig. 2) and 16S rRNA phylogenetic trees (Fig. 3), four major clusters were observed with all the nodes strongly supported by high bootstrap values.

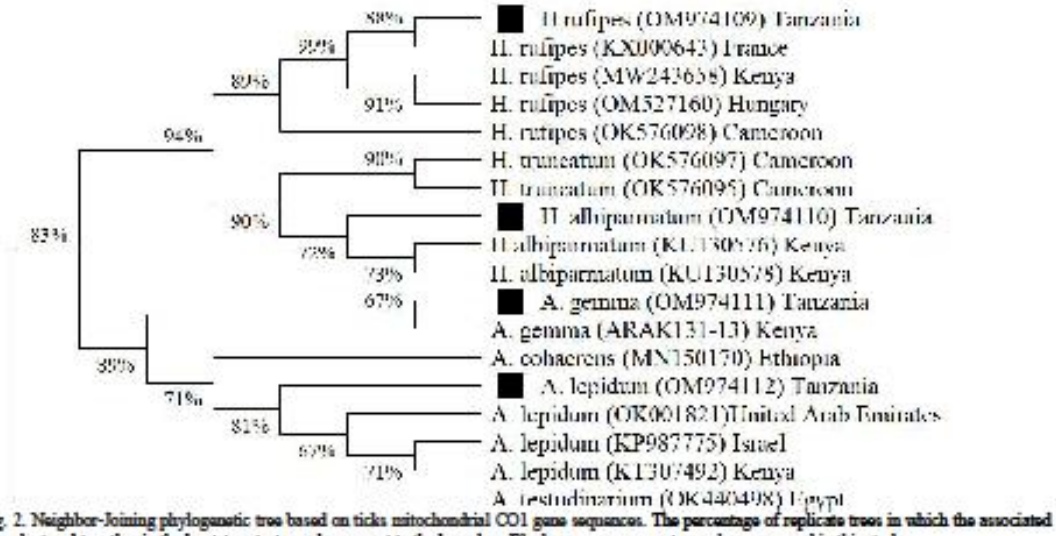

Fig. 2. Neighbor-Joining phylogenetic tree based on ticks mitochondrial CO1 gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Black squares represent samples sequenced in this study.

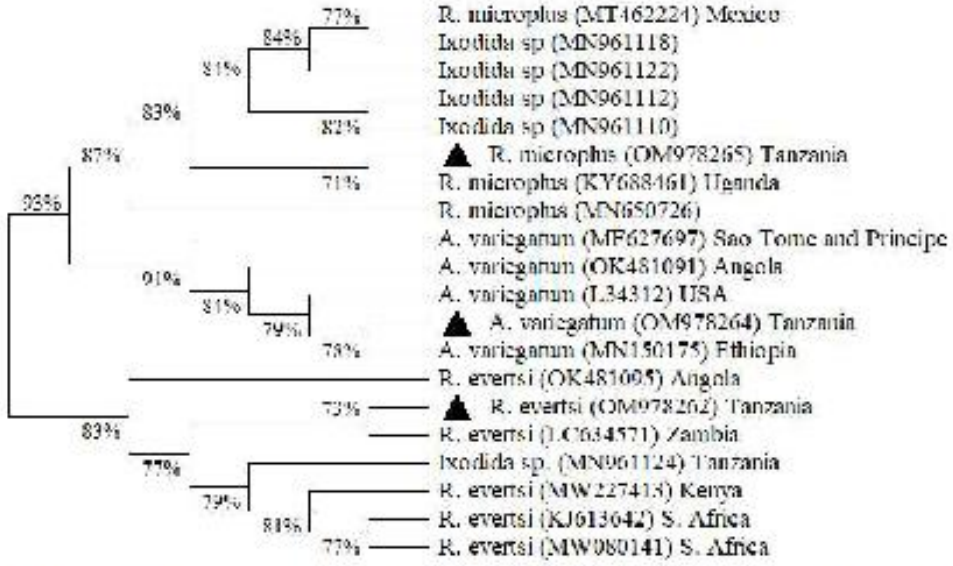

Fig. 3. Neighbor-Joining phylogenetic tree based on ticks 16S rRNA gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Black triangles represent samples sequenced in this study.

Discussion

In this study ticks were prevalent in the study areas and a high overall tick prevalence was reported. The presence of tick species and the high tick infestation prevalence in the study has been reported in other areas of Tanzania and may be attributed to unrestricted cattle movement from one area to another for water and pasture and cattle trade which is a common phenomenon across the country. Similar findings on tick prevalence was reported in the previous studies [29]. The small difference in tick prevalence between the two districts could be due to similar agroecological setting and animal husbandry practices including strategies and awareness of the farmers on application of acaricides through hand spray [24,30]. In this study season had no significant effect on tick prevalence, suggesting that cattle are susceptible to tick infestation during both wet and dry seasons. In the current study, cattle sex had no significance effect on tick prevalence on cattle. However, the slightly higher prevalence on males may signify the male cattle are less resistant to ticks than female, and this could be attributed to testosterone in males, which reduce innate and acquired resistance to tick feeding [31].

The findings from this study suggest that male cattle could be more likely challenged by tick infestation as a result of high tick prevalence. Age group had no significance effect on tick prevalence on cattle. Although cattle age group was not significantly associated with tick prevalence, it was observed that calves were slightly more but not significantly susceptible to tick infestation than other age groups. In this study, animal health condition had no significance effect on tick prevalence on cattle, suggesting an equal tick challenge by tick infestation to all cattle health groups. This could be due to the fact that all animals from the health categories walk for long distance to be grazed in the field and kept together at home, as a result all groups were equally susceptible to tick infestation. Similar findings among cattle health groups was reported in previous studies [1].

In this study tick control frequency had no significance effect on tick prevalence on cattle. Although the use of acaricides was not significantly associated with tick prevalence in this study, cattle from herds where acaricides were used on bi-weekly basis had the least tick infestation prevalence. Ticks were found in all cattle herds suggesting that acaricide resistance could occur. Finding from this study suggest an equal challenge by tick infestation to all tick control frequency groups.
A significantly higher proportion of *R. microplus* recorded in Kilombero District (95.16%, n=865) as compared to Iringa District, could be due to favourable climatic condition in Kilombero, which is marked with annual rainfall ranging from 1 200 to 1 800 mm and average annual temperatures ranging from 26–38 °C compared to 500 to 1 600 mm and 20-25°C in Iringa district. In addition, the study area lies along Kilombero valley in the lowland ranging from 270-300 m asl. The households are also scattered and dispersed in the study areas which may limit the interaction between the animal herds resulting in less distribution of other ixodid ticks. Similar findings have been reported in other part of Tanzania [7,32]. Previous studies in Sudan have shown that this tick occurs in humid localities with steppe areas that have hot dry seasons [15]. *Rhipicephalus microplus* and *R. decoloratus* are important vectors of *Babesia bigemina* and *Babesia bovis* that cause bovine babesiosis. Moreover, they are vectors of *Anaplasma marginale* that causes bovine anaplasmosis. However, *R. microplus* in terms of control management is known to be more resistant to many acaricides. These two species rarely occur together due to interspecies competition in spite of their similarity in temperature and rainfall requirements [1].

The tick species in this study were present both in dry and wet season except *H. albiparmatum* which was only found during the wet season. *Ambiyomma lepidum, H. ruffpes, R. appendiculatus* and *R. evertsi* were both prevalent on cattle during the dry season while, *A. gemma, A. variegatum, R. decoloratus* and *R. microplus* were more prevalent on cattle during the wet season. The results show that these ticks can maintain themselves under certain condition and perhaps occur seasonally. Findings from this study have showed a clear seasonal variation demonstrated by *R. microplus*, with significantly high infestation occurring during the wet season [7]. This suggests that environmental factors such as rainfall, temperature and relative humidity have great influence on the population of ticks as observed in the previous reports from Tanzania [33,34]. The absence of significant difference in proportions of the other three species including, *A. gemma, A. variegatum* and *R. decoloratus* between the seasons suggests that their activities are less affected by weather parameters [35]. The few numbers of *H. albiparmatum* collected indicate that climatic conditions and other unknown factors in Kilombero and Iringa districts are not favourable for this tick species to be able to establish itself and adopt the climatic conditions.

The small difference in tick abundance observed between the two districts could be due to similar agroecological setting and animal husbandry practices including strategies and awareness of the farmers on application of acaricides through hand spray [24,30]. Likewise, such mean tick burden of Ixodid ticks was reported from different parts of the country in Mara region (35.80 ± 4.30), Singida (12.9 ± 2.10) and Mbeya (7.0 ± 0.40) [1].
The higher mean tick burden per animal during the wet season could be due to high humidity and low temperature range that facilitate the growth and survival of ticks at their different stages of life. Similar findings have been reported in the previous studies [36,37]. The higher tick infestation in male cattle may signify the male cattle are less resistant to ticks than female, and this could be attributed to testosterone in males, which reduce innate and acquired resistance to tick feeding [31]. The findings from this study suggest that female cattle are less likely challenged by tick infestation as a result of low mean tick burden. Similar finding has been reported in the previous studies [38]. These findings are not consistent with studies reported in Mara, Singida and Mbeya regions, Tanzania [1], Pakistan [3] and Eastern Ethiopia [39].

Age group had no significant effect on mean tick burden per animal ($p > 0.05$). Finding from this study suggest an equal challenge by tick infestation to both calves, juvenile and adult cattle. Calves have lower but not significant mean tick burden per animal than adults and juveniles. Adult and juvenile cattle were grazed in grasslands and bushy areas located far away from the households. The calves usually do not graze with adult cattle rather graze near the house, thus reducing their chance of contacting ticks. Similar findings have also been reported in Ngorongoro, Tanzania [37], Central Nigeria [1,36] where calves were grazed separately from adults.

In the present study no significant effect of cattle health status was observed on mean tick burden per animal ($p > 0.05$). This could be due to the fact that all the animals walk long distance to be grazed in the field and kept together at home, as a result all groups are equally susceptible to tick infestation. Similar findings among cattle health groups was reported in Mbeya region, Tanzania [1]. This was not consistent with previous studies in Ethiopia [40]. The significantly lower mean tick burden observed on the cattle reported with weekly tick control frequency could be attributed to the frequent application of acaricide to control ticks on cattle by hand spraying. A similar observation was reported in Mara and Mbeya regions, Tanzania [1]. However, when using hand spraying method, the acaricides do not reach the hidden parts of the animal body, as a result not all ticks are killed [41]. Acaricide application and other means of tick control

like rotational grazing, hand picking, pasture burning and many others are economical methods for controlling ticks and TBDs on cattle and reduces tick burden on large scale. However, their practicability is still not promising [11].

The tick species found in this study had a great diversity and widely distributed in Iringa District as compared to Kilombero District. This could be attributed by the average annual temperature in Iringa which ranges from 20-25°C, a low mean annual rainfall of about 500–600 mm and the elevation that ranges from 718–945 m asl in the sampled areas which favours the reproduction of these tick species [8]. Furthermore, the area was characterized with trees, short shrubs and grass cover which could be favourable for the survival of the ixodid ticks [8]. In addition, extensive livestock grazing practices put more pressure on the land resources which results in the need of continuous movement of large number of livestock in search of water and pasture. This often brings livestock to share the pasture with wild animals in the wildlife-livestock interface ecosystem bordering the conserved area of Ruaha National Park [42-44]. Previous studies have also reported the presence of ixodid ticks in cattle and wild animals in Iringa Municipality [9]. Ixodid ticks have been reported in livestock and wild animals such as zebra, buffalo, elephant, leopard, antelope and warthog [9,45]. These findings suggest that these ticks could be predominantly found on animals that live in and around wildlife-livestock interface bordering the Ruaha National Park. As a result, the above factors could have sponsored the great diversity and distribution of the Ixodid tick species in the study area. The lower numbers of *H. albiparmatum* has been previously reported in some parts of Tanzania [8,34].

The highest number of tick on zone 4 which include groin, flank, abdomen and around inner thigh of the hind legs may be attributed to that fact the external genitals and inguinal/groin region of the body are highly supplied with blood, thinner and short hair skin. Ticks usually prefers thinner and short hair skin for infestation as this help easy penetration of mouth parts into richly vesicular areas for blood feeding [46]. The higher proportion of ticks in these predilection sites could be due to high supply of blood and are difficult to reach by hand spray which was the method of application of acaricide for tick control as was reported in all the study areas. Similar findings have been reported in previous studies [24,40]. Findings from this study were similar and confirm the report of other investigators [24,39,47]. In general, most of ticks in this study were observed to infest sites with shorter hair and thinner skin. These sites could facilitate penetration of tick mouth parts and allow better access to the blood circulatory system for feeding [48]. Moreover, distribution of ixodid ticks in different predilection sites may involve complex intrinsic behaviours that are under chemical control. Different pheromones which emanate from the anus, coxal glands and female genital aperture control other behaviours such as aggregation, clasping and attachment during mating attraction and potential mate recognition in males, mounting and copulation [33,49]. Furthermore, a variety of factors such as host diversity, interaction between tick species, time and season, and inaccessibility of grooming determines the attachment of ticks on the host's skins [40].

The higher number of males than females observed in *Amblyomma* and *Hyalomma* spp. could be due to their preference on the selected animal body zones (including; fore flank, groin, udder, scrotum), forming clusters with a few females resulting in a concentration on more males than females on the attachment site. The higher number of females than males of *Rhipicephalus* spp. could be due to the observed difficulty in collecting male ticks from the host animal because of their smaller sizes [13,34,36].

In this study the mitochondrial CO1 and 16S rRNA genes were successfully amplified and these makes the first barcoding sequences of Ixodid ticks reported from Kilombero and Iringa districts, Tanzania. Similar findings have been reported in the previous studies from Brazil [50], Republic of China [22], Tanzania [29], Uganda [23], and Malaysia [51]. The small sequence differences observed in this study could be due to intraspecific variations and in some cases, it could be due to low sequence quality in few of the samples, especially when nucleotides with weak signal were present in a sequence [22,52]. The observed low nucleotide sequence identity of 96.08% compared to the respective reference sequence in the GenBank for *H. albiparmatum* in this study most likely reflects the presence of intra-species genetic variation between ticks from the same species adopted to various geographical regions of different countries as described by previous studies [53-55]. Interestingly, the registered sequence data of mitochondrial CO1 barcoding gene of *A. gemma* were not available on GenBank, however it was available on BOLD (www.barcodinglife.org). The lack of corresponding mitochondrial CO1 gene sequences in GenBank for *A. gemma* and intra-species genetic variation could be considered one of the limitations of the molecular approach for tick species identification [55]. The evolutionary and phylogenetic analyses of ticks using DNA barcoding system could be utilized to discriminate ticks within existing classification systems. The traditional taxonomic traits like lifecycle and morphological characters should be considered in addition to one or several genes when a new species or subspecies is to be designated.

Conclusion

This study reports the abundance and distribution of Ixodid ticks on cattle in Kilombero and Iringa districts. *Rhipicephalus microplus* was the most abundant species and have a wide geographic range in Kilombero with a higher proportion during the wet season. It showed a higher infestation prevalence on cattle in the study area. *Rhipicephalus evertsi* and *Amblyomma lepidum* were the most abundant species and have a wide geographical range in Iringa with higher proportions during the dry season. Morphological and molecular identification of ticks has greatly expanded the understanding of the geographical distribution and phylogenetic relationship of the tick species. Therefore, it is necessary to explore the epidemiological and molecular aspects of various tick species in other parts of Tanzania. This study will be useful in the investigation and designing control strategies for tick control.

Abbreviations

BOLD: Barcode of Life Database; CO1: Cytochrome c Oxidase subunit 1; rRNA: ribosomal Ribonucleic Acid; SUA: Sokoine University of Agriculture; TBDs: Tick Borne Diseases

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by W.S.M, E. K, I. H, J. S. N, and R. K. The first draft of the manuscript was written by W. S. M and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset generated during the current study are available at the NCBI GenBank database (Accession numbers: OM974109, OM974110, OM974111, OM974112, OM978262, OM978264 and OM978265).

Declarations

Ethics approval and consent to participate

The ethical approval was obtained from Ethical Committee of the Sokoine University of Agriculture, permit number - SUA/ADM/R.1/&A/734_15/02/2021. The permission to carry out this study in the respective districts was granted by the local government through District Veterinary Officers in Kilombero and Iringa Districts. All owners of the animals were informed of the study and their verbal consent was obtained before commencement of data collection. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors have no conflict of interest related to this work.

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CHAPTER THREE

MANUSCRIPT TWO

Occurrence of Tick-Borne Pathogens in Ticks Collected from Cattle at Iringa and Kilombero Districts, Tanzania

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Abstract

Background: Ticks and tick-borne diseases (TBDs) undermine livestock production and productivity in Tanzania. Cattle in Tanzania are challenged by several tick species and TBDs which are known to be endemic in cattle such as *anaplasmosis, theileriosis* and *babesiosis*. However, there is insufficient information on tick and associated tick-borne pathogens (TBPs) in Tanzania. Therefore, this study was conducted to determine the occurrence of TBPs including *Anaplasma, Theileria* and *Babesia* spp., in ticks from Kilombero and Iringa districts of Tanzania.

Method: During January through August 2021, a repeated cross-section study was carried out and a total of 1,889 ticks were collected from 304 randomly selected cattle. The ticks were morphologically identified using morphological keys and a stereomicroscope. The identified tick species were grouped into 91 pools of 1-30 ticks according to species, sex, and collection site for analysis. The TBPs were then detected from the tick pools using polymerase chain reaction (PCR) targeting *Anaplasma* and *Theileria* or *Babesia* spp. Chi square/**Fisher's** Exact test was performed to determine the difference between the proportion of pathogen positive tick pools in Iringa and Kilombero districts and seasons.

Results: Overall, *Anaplasma* and *Theileria/Babesia* spp. were detected by PCR in 70.3% (n=64) tick pools. The detection rate of both *Anaplasma* and *Theileria/Babesia* spp. was highest in *Amblyomma lepidum* (25.%, n=16) followed by *Rhipicephalus evertsi* 23.4% (n=15) tick pools. Tick pools from Iringa district had a significantly higher proportion of pathogen positive samples 91.0% (n=61) as compared to Kilombero 12.5% (n=3), ($p < 0.001$). The co-infection rate of *Anaplasma* spp. and *Theileria/Babesia* spp. in all tick pools was 33.0% (n=30). A significant higher co-infection rate of 43.3% (n=29) was recorded in tick pools from Iringa district ($p < 0.05$).

Conclusion: The results showed a high TBPs detection rate on tick pools collected on cattle from the study areas which could increase the risk of TBDs transmission on livestock and cause increased loss in milk, meat production and animal productivity. Therefore, the ticks and TBPs in the study areas highlight the need for strategic tick control approaches.

Keywords: Ticks, Cattle, Tick-borne pathogens, Tanzania

Introduction

Background

Ticks are obligate blood-feeding ecto-parasites of mammals, birds and reptiles worldwide, with different relevant species regionally [1]. An increasing emerging tick-borne zoonoses has been witnessed in the recent decades worldwide. Ticks and TBDs are major problems in livestock and human health in Tanzania. Ticks transmit diseases that leads to extensive economic loses to poor-resource farming communities especially in subtropical regions where almost 80% of the world's cattle population are reared [2]. TBDs such as East Coast fever, babesiosis, anaplasmosis and ehrlichiosis attribute to more than 70% of all cattle deaths in Tanzania resulting to more than TSh. 72 billion loses annually [3]. The burden of ticks and TBDs on the economy and livelihood of those involved in the livestock industry in Africa remains significant. Many factors have been identified to explain the continuous increase in the incidence of ticks and TBDs (including; inadequate monitoring and surveillance programs targeting ticks and TBDs, deforestation and human encroachment on wildlife habitats, tick resistance to acaricides and climate change) [4,5].

Tanzania has approximately 33.9 million cattle, 24.5 million goats and 8.5 million sheep, about 90% of agricultural households keep livestock of different kinds. Approximately 95% of cattle populations in the country are reared under traditional agro-pastoral and pastoral husbandry systems [6]. The grazing land for these animals is no longer sufficient due to increased number of cattle, other domestic animals and human population. Thus, most of the indigenous cattle are extensively grazed in grasslands and woodlands and hence exposed to high risk of tick infestation [3,7]. Ixodid ticks of the genera *Rhipicephalus, Hyalomma* and *Amblyomma* are the most important and widely distributed species found in many parts of Tanzania [3,8]. The species included in the three genera are *Rhipicephalus appendiculatus, R. evertsi, R. bequarti, R. compositus, R. hurti, R. interventus, R. kochi, R. lunulatus, R. praetextatus, R. pulchellus, R. simus, Hyalomma albiparmatum, H. impeltatum, H. marginatum rufipes, H. truncatum, Ixodes* spp., *Amblyomma variegatum, A. lepidum, A. gemma, Haemaphysalis leachi* and *Haemaphysalis silacea* [9-12].

The extreme changes in climate and land use patterns including pastoralism, tourism and illegal grazing practiced have increased the interaction between domestic

with wild animals that could increase the risk of TBDs transmission. Therefore, the occurrence and development of TBPs are increasingly linked to physical environmental changes and vector-host interactions [13-15]. Tick and TBDs have been recently reported by smallholder farmer from Southern Highlands of Tanzania. Such reports identified the presence of ticks and associated TBDs in cattle at the wildlife-livestock interface. This study aimed to investigate using molecular approach the occurrence of TBPs of both veterinary and zoonotic potential in ticks infesting cattle at Kilombero and Iringa Districts.

Material and Methods

Description of the study area

The study was conducted in Kilombero and Iringa districts in Morogoro and Iringa regions of Southern Highlands of Tanzania respectively. Kilombero district is located between 8° 00'–16° S and 36° 04'–36° 41' E, with elevation ranging from 262 to 550 m above sea level and covering an area of 14,246 km^2 in the Morogoro region [16]. Average annual temperatures in Kilombero district range from 26–38 °C [17]. Sampling villages in Kilombero district were Merera, Idunda, Sagamaganga and Lufulu villages. Iringa district is located between 7° 46'23.14'' S and 35° 41' 56.83'' E and covering an area of 20,576 km^2 with an elevation ranging from 900 to 1800 m above sea level. The average annual temperature in Iringa district ranges from 20-25°C [17]. Sampling villages in Iringa district were Magombwe, Kisanga, Kitisi and Malizanga villages.

Sample size estimation

Sample size was estimated base on CDC Epi info software version 7.2.4.0. Probability of type 1 error (0.05 sided) and 30% estimated prevalence of cattle with ticks were used [3]. A total of 740 cattle (402 from Iringa and 308 from Kilombero) of all ages from the study areas were investigated in this study.

Ethical approval

The permission to carry out this study was granted by the District Veterinary Officers of Kilombero and Iringa Districts. The ethical approval for this research was obtained from Ethical Committee of the Sokoine University of Agriculture. Permit number - SUA/ADM/R.1/8A/734 _19/02/2021. Animal owner's consents were sought verbally prior to data collection.

Study design and sampling method

A repeated cross-sectional study design using qualitative and quantitative methods of data collection and analysis was adopted. Tick sampling was performed from January through August 2021. The two districts were purposively selected as they are bordering the wildlife conservation areas. The eight study villages (four from each district) were selected due to previous engagement in the past livestock studies. Three herds located approximately 5-10 km apart and having at least 40 cattle were conveniently selected from each village with the assistance of Livestock Extension Officers (LEOs) providing the information on areas where pastoralists have settled with their animals. Fifteen to twenty cattle from each herd were randomly selected and examined for tick infestation by the help of herdsmen.

Tick collection

After a verbal concern was given by the herd owner, the animals were visually inspected for ticks all over the body sites including head, ears, neck, belly, flank, back, legs, perineum and tail. Ticks were then removed from different predilection sites of the animal's body using blunt ended forceps. The collected ticks were placed into respective empty 15 ml falcon tube labeled with unique identification number and transferred into a dry shipper to immobilize the ticks until transported to the Parasitology laboratory at the Department of Veterinary Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture.

Sorting of ticks into pools

The preserved ticks were taken from -80°C, rinsed with 70% ethanol then distilled water followed by brief drying on paper towel. Ticks were sorted by sex and genera based on the presence or absence of banded legs, colored or patterned scutum and conscutum, presence or absence of festoons and eyes and shapes of the mouthparts using magnifying hand lens [10,18]. The ticks were further morphologically identified to species using a stereomicroscope (Brunel Stereomicroscope Ltd, UK) and published morphological keys for African ticks by experienced laboratory personnel at the Parasitology laboratory and confirmed by molecular methods [10,18]. The sorted ticks were grouped into pools of 1-30 ticks according to species, sex and collection site. The tick pools were placed into a cryo vial tubes 2.0 ml (Quanta Biosciences, Gaithersburg, USA). Multiple cryo vial tubes were used for engorged ticks as such ticks could not fit into a single tube. The tick pools were then preserved at -80°C for later genomic DNA extraction. Petri dishes and forceps used in the tick identification were cleaned after every batch of ticks using distilled water, followed by wipe with 1% virkon and 4% bleach, then distilled water and lastly wiped with 70% ethanol to avoid cross-contamination.

Total genomic DNA extraction

The frozen tick pools (n=91) were thawed and crushed using motor and pestle followed by addition of 0.5 ml Tri-reagent in each 1.5 ml microcentrifuge tube, vortexed for 5 secs and centrifuged at 2000 × g for 5 min. The motor and pestle were rinsed using distilled water, followed by wipe with 1% Virkon and 4% bleach, then distilled water and lastly wiped with 70% ethanol and left to dry for 5 minutes between each sample to avoid cross-contamination [15]. For each sample 200 µl supernatant was used for DNA extraction. Quick-DNA Minprep Plus Kit (D4068, Zymo Research, CA, USA) was used to extract DNA from the homogenized tick pools for pathogen screening as per manufacturer's instructions. The extracted DNA was eluted in 50 µl of DNase/RNase-free water and stored at −80°C for subsequent use for PCR [19-21]. Extraction control using distilled water was included in each extraction process to monitor for the possibility of cross-contamination.

Detection of host Mitochondrial (Cyt B) gene

The success of DNA extraction process from tick pools was confirmed by determining the DNA concentration and purity. The obtained DNA were quantified using a NanoVue Plus Spectrophotometer (Thermo Scientific, Marlborough, England, UK). The DNA yield (ng/µl) were determined from the concentration of DNA in the elute, measured by absorbance at 260 nm (A260/A280). In addition, a quality control Cyt B PCR and gel electrophoresis were performed. A portion of mitochondrial Cyt B gene was amplified by conventional PCR with a set of universal primers and reaction conditions. The primers

CytB_Foward- (5′-GAG GMC AAA TAT CAT TCT GAG G-3′ and CytB_Reverse- (5′-TAG GGC VAG GAC TCC TCC TAG T-3′) that preferentially amplifies a 457-bp region of the Cyt B gene within the mitochondrial DNA of vertebrates and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) were used [22]. The mixture was amplified in a thermal cycler (SimpliAmp Thermal cycler, Applied Biosystems, Thermo Fisher Scientific Inc) with an initial denaturation at 94°C for 2 min, followed by 50 cycles of 94°C for 50s, 52°C for 50s, 72°C for 60s, and a final extension step at 72°C for 10 min.

Molecular detection of tick-borne pathogens

For screening of the DNA extracts from tick pools for TBPs of the genera Anaplasma, Theileria and Babesia, PCR was performed using genus-specific primers in a thermal cycler (SimpliAmp thermocycler, Applied Biosystems, Thermo Fisher Scientific Inc). The primer pairs Ana WS-F (5'-TAGTGGCAGACGGGTGAGTA-3') and Ana WS-R (5'-AATTCCGAACAACGCTTGCC-3') designed for this study were used to amplify 424-bp fragment of 16S rRNA gene of *Anaplasma* spp., The primer pair THB-F (5'-GAGGTAGTGACAAGAAATAACAATA-3') and THB-R (5'-TCTTCGATCCCCTAACTTTC-3') were used to amplify 540-bp of 18S rRNA fragment of *Theileria/Babesia* spp. [23].

The PCR amplifications were performed in a total reaction volume of 25 µl containing 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1.0 µl (10 µM) of each forward and reverse primer, 0.10 µl of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 17.15 µl of Molecular grade water and 2 µl of template DNA in a thermal cycler (SimpliAmp thermocycler, Applied Biosystems, Thermo Fisher Scientific Inc). The cycling conditions were as follows: 94 °C for 2 min, followed by 50 cycles of 94 °C for 30 s, 52 °C for 50 s, 72 °C for 1 min and then 72 °C for 7 min. Positive and no-template controls were included in all the runs. The obtained PCR products were separated on a 1.5 % (w/v) agarose gel in 1x TBE buffer (SERVA Electrophoresis, Heidelberg, German) stained with gel red (Phonex Research Products, Candler, USA) and viewed under UV transilluminator.

Data management and statistical analysis

Tick pools data were stored using Microsoft excel software version 2109 and checked for completion before doing statistical analysis. The pathogen detection rate in tick pools was determined by dividing the number of pathogen positive tick pools by total number of tick pools tested and was expressed as percentage. Descriptive statistics on pathogen detection rate was performed using Epi Info software version 7.2.4 (CDC, Atlanta, USA) to compare the difference in proportion of pathogen positive tick pools between the districts and seasons. Fisher's exact test was performed to detect the difference between the proportion of pathogen positive tick pools in Iringa and Kilombero districts and seasons. The pathogen detection rate in tick pools was used as dependent variable while district and season were used as independent variables. The p-value (0.05) was considered statistically significant in all statistical tests.

Results

The ticks identified from this study were all from the family Ixodidae. Nine tick species from three genera *Rhipicephalus* 72.9% (n=1,377), *Amblyomma* 24.3% (n=459) and *Hyalomma* 2.8% (n=53) were identified from the 1,889 tick that were all collected from 304 randomly selected cattle from Iringa and Kilombero districts. Of the 91 tick pools tested, female 46.15% (n=42 and male 53.85% (n=49) comprises 9 tick species. *Amblyomma gemma, A. variegatum, A. lepidum, Rhipicephalus microplus, R. appendiculatus, R. decoloratus, R. evertsi, Hyalomma rufipes and H. albiparmatum* were the tick species identified prior to processing for DNA extraction and used for screening of *Anaplasma spp* and *Theileria/Babesia* spp., (Table 3.1).

The extracted total DNA from the 91 tick pools had an average DNA yield of 1,427.1 ± 120.1 ng/µl and 1.80 ± 0.02 purity. Host DNA was successfully amplified from all 9 randomly selected tick pools. The amplification of the host mitochondrial Cyt B gene yielded the expected amplification products. This experiment showed that DNA extraction protocol was efficient and eluted the DNA that could be used for subsequent PCR amplification of TBPs. Eight out of nine tick species identified contained *Anaplasma* spp. similarly eight out of nine tick species contained *Theileria/Babesia* spp. (Table 3.1).

The 16S rRNA gene of *Anaplasma* spp. (Figure 3.1) was successfully amplified in 56.04% (n=51) while, the 18S rRNA gene of *Theileria/Babesia* spp. (Figure 3.2) was amplified in 47.25% (n=43) tick pools. Appearances of the DNA band of the target sizes were the criterion for the detection of the pathogens in the tick pools. Overall, pathogens were detected by PCR in 70.33% (n=64) tick pools. The detection rate of both *Anaplasma* and *Theileria/Babesia* spp. was high in *Amblyomma lepidum* 25.00% (n=16) followed by *Rhipicephalus evertsi* 23.44% (n=15) tick pools and least in *Rhipicephalus decoloratus* and *Hyalomma albiparmatum* tick pools each 1.56% (n=1). *Anaplasma* spp. detection rate was high in *Rhipicephalus evertsi* and *Amblyomma lepidum* tick pools each 25.48% (n=13) while *Theileria/Babesia* spp. detection rate was high in *Amblyomma lepidum* 25.58% (n=11) followed by *Rhipicephalus evertsi* 20.93% (n=9) tick pools (Table 3.1).

Tick pools from Iringa district showed a significantly higher proportion of pathogen detection 91.04% (n=61) than Kilombero 12.50% (n=3), ($p < 0.001$) (Table 3.1). Tick pools from Iringa district had a significantly higher mono-infection rate of *Anaplasma* spp. 31.34% (n=21) as compared to Kilombero district. The mono-infection rate of *Theileria/Babesia* spp. was higher in tick pools form Iringa 16.42% (n=11) than Kilombero 8.33% (n=2), however the difference was not statistically significant ($p = 0.502$). The co-infection rate of *Anaplasma* spp. and *Theileria/Babesia* spp. was 32.97% (n=30) of all the tick pools. A significant higher co-infection rate of 43.28% (n=29) was recorded in tick pools from Iringa district as compared to 4.17% (n=1) in Kilombero district, ($p < 0.05$) (Table 3.1). Pools of tick collected during the wet season showed a significantly higher pathogen detection rate 95.31% (n=61) compared to tick pools collected during the dry season 4.69% (n=3), ($p < 0.05$).

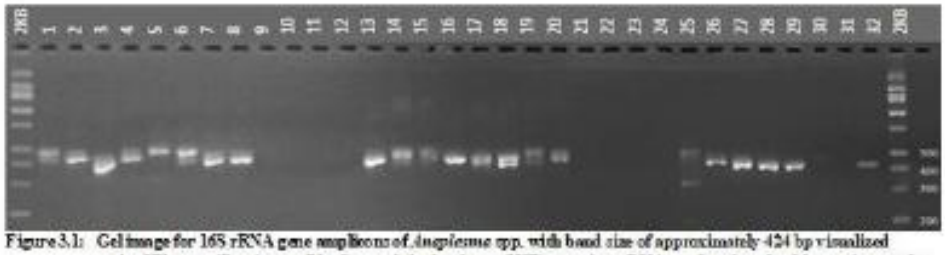

Figure 3.1: Gel image for 16S rRNA gene amplicons of *Anaplasma* spp. with band size of approximately 424 bp visualized under UV trans-illuminator. The first and the last lanes (2KB) contains a DNA marker, lane 1 – 30 contain samples, while 31 contain negative control used in DNA extraction and 32 the positive control

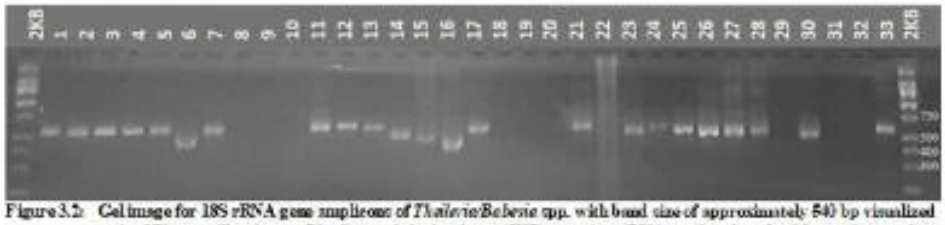

Figure 3.2: Gel image for 18S rRNA gene amplicons of *Theileria/Babesia* spp. with band size of approximately 540 bp visualized under UV trans-illuminator. The first and the last lanes (2KB) contains a DNA marker, lane 1 – 30 contain samples, while 31 and 32 contain negative controls used in DNA extraction and PCR Mix preparation respectively, and 33 contain the positive control.

Table 3.1: Pathogen detection frequencies showing mono and co-infected tick pools collected on cattle from Kilombero and Iringa districts

Varisble	Tick species	No. of pools	No. of positive pools	No. of Mono and Co-infected tick pools		
				Anaplasma spp.	*Theileria/ Babesia* spp.	Co-infection
District						
Iringa	*R. evertsi*	17	15	6	2	7
	R. microplus	6	5	3	0	2
	R. decoloratus	1	1	1	0	0
	R. appendiculatus	5	5	0	3	2
	H. rufipes	6	6	0	1	5
	H. albiparmatum	1	1	0	1	0
	A. variegatum	3	2	0	0	2
	A. lepidum	16	16	5	3	8
	A. gemma	12	10	6	1	3
Subtotal n (%)		67	61 (91.04)	21 (31.34)	11 (16.42)	29 (43.28)
Kilombero	*R. microplus*	18	2	0	2	0
	R. evertsi	1	0	0	0	0
	R. appendiculatus	1	0	0	0	0
	R. decoloratus	1	0	0	0	0
	A. lepidum	1	0	0	0	0
	A. gemma	1	0	0	0	0
	A. variegatum	1	1	0	0	1
Subtotal n (%)		24	3 (12.50)	0 (0)	2 (8.33)	1 (4.17)
Grand total tick pools n (%)		91	64 (70.33)	21 (23.08)	13 (14.29)	30 (32.97)
p-value			0.000	0.001	0.502	0.000

Discussion

In this study, diverse tick species were identified including *Amblyomma*, *Rhipicephalus* and *Hyalomma* genera which has been previously reported in Iringa and other parts of Tanzania [24]. . The DNA of TBPs were detected in 70.33% (n=64) of all tick pools. The 16S rRNA gene of *Anaplasma* spp. (Figure 3.1) was detected in almost half 56.04% of the tick pools, and the 18S rRNA gene of *Theileria/Babesia* spp. (Figure 3.2) was also detected in almost half 47.25% of the tick pools. *Anaplasma* spp and *Theileria/Babesia* spp were detected in almost 25% of *Rhipicephalus evertsi* and *Amblyomma lepidum* tick pools (Table 3.1). A higher multiple infection rate 43.28% was observed in tick pools from Iringa district (Table 3.1). Tick pools from the wet season showed a significantly higher pathogen detection rate 95.31%.

The overall high pathogen detection rate in tick pools during the study suggest that cattle from the study areas in which the tick were collected may serve as reservoir for the detected pathogens. This study provides molecular evidence of the presence of TBPs that are of veterinary importance in tick collected on cattle in Kilombero and Iringa districts. Finding from this study corroborate findings from previous studies in Tanzania, Kenya and Italy, which have demonstrated the existence of *Anaplasma*,

Theileria and *Babesia* spp. from various ecologies [15,24,25]. This study confirms the occurrence of *Anaplasma* and *Theileria/Babesia* spp. in the tick pools which is an evidence of livestock pathogens in the Ixodid ticks collected from cattle from Iringa and Kilombero districts.

Anaplasma and *Theileria/Babesia* spp. DNA was detected in all tick pools of a wide range of tick species except in *H. albiparmatum* for *Anaplasma* spp., and *R. decoloratus* for *Theileria/Babesia* spp. *Anaplasma* and *Theileria/Babesia* spp DNA were detected in *R. microplus* while in *R. decoloratus* only *Anaplasma* spp DNA was detected. Since all the ticks were collected from cattle (on-host ticks), the higher TBPs detection rate *in Rhipicephalus evertsi* and *Amblyomma lepidum* tick pools suggest that the two could had the pathogens from the hosts taken during blood feeding. Because the DNA was extracted from the whole ticks in pools, detection of DNA from the tick pools thus does not necessarily mean the tick can transmit a particular pathogen especially if the tick is not a biological vector of that pathogen unless DNA extraction was from the tick's salivary glands only.

Rhipicephalus microplus is known to be a good vector of highly pathogenic *Babesia bovis*. Moreover, *R. microplus* and *R. decoloratus* have great veterinary significance as they are important vectors of *Babesia bigemina* and *Babesia bovis*, which causes bovine babesiosis [10,11]. The two are also vectors of *Anaplasma marginale* which causes anaplasmosis in cattle. *Rhipicephalus evertsi* transmits *B. bigemina* in cattle [3,11]. *Amblyomma variegatum* and *A. lepidum* transmits the protozoans *Theileria mutans* and *Theileria velifera* causing benign bovine theilerioses and *Anaplasma* spp., causing anaplasmosis, they also transmit the bacterium *Ehrlichia ruminantium* that causes heartwater in cattle, sheep and goats [10-13]. *Rhipicephalus appendiculatus* transmits *Theileria parva* that causes East Coast fever in cattle and different strains of *Theileria parva* that cause Corridor disease, it also transmits *Anaplasma bovis* causing anaplasmosis in cattle [10,11].

Because, *R. microplus* and *R. decoloratus* are known biological vectors of *Babesia bovis* and *B. bigemina* that cause babesiosis in cattle, *Anaplasma marginale* that cause anaplasmosis and *Borrelia theileri* that cause spirochaetosis in cattle [10,11]. The TBPs DNA detected in *R. microplus*, *R. decoloratus*, *R. evertsi*. *A. lepidum* and the other tick pools suggest that the ticks play an important role in the epidemiology of TBPs and the disease they cause. Such TBDs cause approximately 72% of cattle deaths in Tanzania [5]. Detection of TBPs DNA in tick does not mean that ticks are capable transmitting the infection. However, because of the fact that both biological and mechanical transmissions have been described [26], there is a high possibility of a susceptible host to contact the infection especially with *A. marginale*, because of the mechanical nature of transmission [5].

The higher pathogen detection rate in *Rhipicephalus evertsi* and *Amblyomma lepidum* tick pools in this study could be attributed to the high number of tick pools tested. *Rhipicephalus evertsi* is known to transmit *Babesia bigemina* in cattle [3]. The lower pathogen detection rate in *Rhipicephalus decoloratus* and *Hyalomma albiparmatum* in this study could be due to their fewer number of tick pools tested. Both *Anaplasma* and *Theileria/Babesia* spp. DNA were detected in *Hyalomma rufipes* tick pools. *Hyalomma rufipes* is known to transmit *A. marginale*, that cause anaplasmosis in cattle. In addition, it also transmits *Theileria annulata*, *Babesia occultans*, *Rickettsia conorii* and Crimean Congo hemorrhagic fever virus (CCHFV) [12-14].

The significantly higher pathogen detection rate on tick pools from Iringa district could be contributed to great tick species diversity recorded in Iringa district since *A. lepidum* and *R. evertsi* which were the most predominant tick species in Iringa exhibited higher infection rates of both *Anaplasma* spp. and *Theileria/Babesia* spp. The area is characterized with Miombo woodlands, short vegetation which might be favorable for the survival of the ixodid ticks [9]. Furthermore, Iringa is located at a higher elevation with annual rainfall range of about 500-1600 mm and has low temperature range of about 20–25°C and low humidity as compare to Kilombero. This difference in climatic conditions could be among the factors that might have led to high tick species diversity in Iringa as a result high pathogen detection rate. Moreover, continuous movement of large number of livestock in search of water and pasture often brings livestock to share the pasture with wild animals in the wildlife-livestock interface ecosystem bordering Ruaha National Park [27,28].

Because, the DNA was extracted from tick pools, the significantly higher co-infection rate of *Anaplasma* and *Theileria/Babesia* spp. recorded in tick pools from Iringa district in this study could also be due to mono-infection of different ticks in the same pool. These findings suggest that cattle from this area are at high risk of contacting the infection. The interaction between livestock and wild animals might have contributed to the high pathogen detection rate on tick pools from Iringa considering the richness of the competent vectors [24]. Either livestock or wildlife around the study areas could be the original host of these pathogens or forementioned tick species [15].

The lower pathogen detection rate in tick pools from Kilombero might be attributed to fewer number of tick pools which was due to lower tick species diversity and distribution in the area. Distribution limits of ticks are variable and are influenced by several factors, including climate, vegetation, host density, host susceptibility, and host gazing habits [29].

The climatic conditions in the area including, average annual rainfall of 1200 to 1800 mm and average annual temperatures range of 26–38 °C and other factors might be unfavorable for other tick species while favors *R. microplus* which was the predominant species in Kilombero. *Theileria/Babesia* spp. DNA was detected in *R. microplus* tick pools from Kilombero. The high number of *R. microplus* tick pools compared to other tick species in Kilombero in current study was due to their high abundance and distribution in the area. The rapid expansion of *R. microplus* in Kilombero was probably attributed to the shorter life cycle and higher egg production capacity [30]. Additionally, their ability to develop resistance to most available acaricides might also have favored their expansion as compared to more susceptible tick species [20]. This

tick species is of great interest as it is well known to be a vector of highly pathogenic *Babesia bigemina* and *B. bovis*, causing babesiosis in cattle [31]. This species is also a vector of *Anaplasma marginale*, which causes anaplasmosis in cattle [3]. However, in this study *Anaplasma* spp. was not detected in any of the tick pools from Kilombero.

Livestock in the study areas of Kilombero district rarely share habitats and grasslands near the Udzungwa Mountain National Park with limited livestock-wildlife interactions. The limited livestock-wildlife interaction due to forests, valleys, and mountains could have attributed to lower TBPs transmission as a result the lower pathogen detection rate. Moreover, the effects of climatic conditions on the activity of ticks and occurrence of TBPs have been identified on other studies [15].

The TBPs in this study were not sequenced to identify the pathogens to species therefore, there is a need for subsequent studies to further characterize the pathogens using other housekeeping genes in addition to 16S and 18S rRNA. Further studies concentrating on understanding the association between climatic conditions and the occurrence of similar and other zoonotic TBPs could be useful in the early warning systems [32]. Information on tick species diversity and distribution would also help to improve the understanding of disease dynamics which is prerequisite for control measures of ticks and TBDs.

Conclusion

This study demonstrates the presence of *Theileria*, *Babesia* and *Anaplasma* spp in the tick pools obtained from cattle that could increase the risk of transmission of TBDs and decrease livestock production and productivity in Kilombero and Iringa districts. The PCR method has allowed the detection of the pathogens in tick pools from the study areas where limited information of the TBPs exists. **However, detection of pathogen's DNA does not** imply a transmission competence of the tick vector concerned since they could have been obtained from the infested cattle.

The finding from this study shows that ticks from Iringa district harbor more pathogens which could stimulate further extensive epidemiological investigations and characterization.

Abbreviations:
Cyt B: Cytochrome B; PCR : Polymerase Chain Reaction; TBDs: Tick-Borne Diseases; TBPs: Tick-Borne Pathogens

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All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Waltar Magesa, Isihaka Haji, Jahashi S. Nzalawaha and Rudovick Kazwala. The first draft of the manuscript was written by Waltar Magesa and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials:
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The ethical approval was obtained from Ethical Committee of the Sokoine University of Agriculture, permit number - SUA/ADM/R.1/&A/734_15/02/2021. The permission to carry out this study in the respective districts was granted by the local government authorities in Kilombero and Iringa Districts. All owners of the animals were informed of the study and their verbal consent was obtained before commencement of data collection. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interests related to this work.

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CHAPTER FOUR

4.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

The distribution and abundance of Ixodid tick species infesting cattle in Tanzania varies greatly from one area to another. This study aimed to establish the distribution, abundance and seasonal variation of Ixodid ticks infesting cattle and pathogens in selected areas of Kilombero and Iringa districts of Tanzania and quantifying and identifying Ixodid tick species using morphological and molecular techniques.

Tick infestation in animals generally varies with host and geographic factors. The host (cattle) factors such as age, sex, body condition and breed can influence the susceptibility of animals to tick infestation (Asmaa *et al*., 2014). Ticks also mostly dependent on temperature and rainfall for their development and activities (Estrada-Peña, 2015). Thus, the distribution, abundance and seasonal variation of Ixodid ticks infesting cattle and pathogens in the study areas were determined.

In this study, *R. microplus* was found to be the most abundant and have a wide geographic range in Kilombero district, and it had a higher proportion during the wet season. It has the highest infestation prevalence in the area. *Theileria/Babesia* spp. was detected in *R. microplus* tick pools obtained on cattle from Kilombero. *Rhipicephalus microplus* is an important vector for babesiosis and anaplasmosis in cattle (Baron *et al*., 2018; Silatsa *et al*., 2019b). It is one of the most predominant tick species infesting livestock in Tanzania (Kerario *et al*., 2017). *Rhipicephalus evertsi* and *A. lepidum* were the most abundant and have a wide geographical range in Iringa district. They have higher proportions during the dry season and also have higher infestation prevalence in the area. Pathogen detection rate

was higher in tick pools from Iringa whereby *R. evertsi* and *A. lepidum* have shown the highest pathogen detection rates.

The small difference on mean tick burden between Iringa and Kilombero district could probably be due to similarities in agroecological setting and animal health practices in the two districts. The higher tick infestation 14.53 ± 1.71, observed in Iringa district during the dry season could be attributed to the higher tick species diversity and the significantly higher number of *Amblyomma lepidum* and *Rhipicephalus evertsi* observed in the area during the dry season. Furthermore, the difference in climatic conditions, low temperature range (20-25°C), relative humidity and higher elevation (900-2300 m) above sea level in Iringa district could be the factors that have resulted in high tick infestation during the dry season. The tick distribution and abundance in Iringa district could also be related to factors such as hosts available, microclimate, grazing habits, cattle management and other unknown factors which affect the development and growth of ticks. Ixodid ticks have been reported from previous studies conducted on cattle and wild animals from Iringa (Kwak *et al*., 2014; Kim *et al*., 2018). These findings corroborate with the previous studies, thus suggest that ticks in Iringa are mostly found on cattle that live in and around wildlife-livestock interface bordering the Ruaha National Park (Kwak *et al*., 2014; Kim *et al*., 2018). However, for better understanding, future studies should conduct sampling throughout the year, targeting a wider range of hosts including other domestic and wild animals (Kemal *et al*., 2016). The significantly higher mean tick burden 17.30 ± 1.55 in Kilombero during the wet season could be contributed to the high abundance of *R. microplus* in the area. The higher temperature (26-38°C) and rainfalls (1200-1800 mm) in Kilombero compared to (20-25°C) and (500-1600 mm) in Iringa during the wet season could have favoured the developmental activities of the ticks in the area. This study indicates that season coupled with hand spray of acaricides on cattle, environmental and

climatic changes could have an impact on tick infestation (Okello-Onen *et al*., 1999; Kerario *et al*., 2017). A similar observation was reported in Nigeria (Lorusso *et al*., 2013) and Zambia (Simuunza *et al*., 2011).

The high prevalence of tick infestation observed in male 44.02% compared to female cattle 39.92% despite the higher number of female cattle examined during this study, suggest that female cattle are less likely challenged by tick infestation. Lower tick infestation in female cattle may signify that female cattle are more resistant to ticks than males, and this could be attributed to testosterone, which reduce innate and acquired resistance to tick feeding (Mapholi *et al*., 2014). Findings have been reported in other studies such as those of Hughes and Randolph (2001). The equal challenge by tick infestation to juvenile and adult cattle observed in this study could be attributed to the fact that adult and juvenile cattle were grazed together in grasslands and bushy areas located far away from the households. Similar findings have also been reported in previous studies in Tanzania (Swai *et al*., 2005) and Central Nigeria (Lorusso *et al*., 2013; Kerario *et al*., 2017).

The lower mean tick infestation 9.61 ± 0.85 observed in Tarime cattle breed compared to 14.79 ± 4.41 in other indigenous breeds in this study, could be due to the fact that Tarime breed is more resistant to ticks and TBDs than other indigenous (*Bos indicus*) cattle breeds (Laisser *et al*., 2016). The fact that these cattle were grazed at early stage in life could have increased the frequency of contact with ticks at an early stage of life. This reflects the confidence that Tarime cattle are naturally resistant to ticks and TBDs similar to reports in previous studies in Lake zone, Tanzania (Chenyambuga *et al*., 2010; Laisser *et al*., 2016). Similarly lower mean tick infestation among other indigenous cattle breeds was reported in previous studies (Sajid *et al*., 2009; Piper *et al*., 2010; Asmaa *et al*., 2014). Although the mechanism of resistance acquired by indigenous cattle breeds is not fully understood

however, it could be attributed to less exposure to ticks and it could be related to frequency of contacts with the parasites at an early stage of life, which could help in establishment of pre-immunity against ticks (De Castro *et al*., 1991; Mapholi *et al*., 2014; Rehman *et al*., 2017).

The weak variation in mean tick infestation observed between cattle with different body conditions in this study could be due to the fact that the animals walk long distance for grazing in the field and kept together at home and as a result all groups are equally exposed to tick infestation. Similar findings among cattle health groups was reported in Mbeya region, Tanzania (Kerario *et al.*, 2017).

The lower mean tick burden 6.05 ± 0.77, reported on cattle with weekly tick control frequency compared to 14.91 ± 2.20, on cattle with monthly acaricide application and 18.60 ± 2.91, on cattle with unknown frequency of acaricide application could be attributed to frequent control of ticks through hand spraying of acaricides. When using hand spraying method, the acaricides might not reach the hidden body parts of the animal, as a result not all ticks are killed (Kerario *et al.,* 2018). A similar observation was reported in Mara and Mbeya regions, Tanzania (Kerario *et al*., 2017).

The findings of high prevalence of *R. microplus* in this study is in accordance with studies reported from Singida and Mbeya regions of Tanzania (Kerario *et al.,* 2017). This study reports the presence of *H. rufipes* which was not recorded in the previous study in Iringa (Kwak *et al*., 2014). The tick species identified in this study were more abundant and distributed in Iringa as compared to Kilombero district. Previous studies have reported these Ixodid ticks in cattle and wild animals in Iringa municipality (Kwak *et al*., 2014). The higher abundance and distribution could be attributed to the climatic conditions in the

study areas which favor the reproduction of the tick species. The area is also characterized with trees, short shrubs and grass cover which might be favorable for the survival of ixodid ticks (Lynen *et al*., 2007). The higher proportion and wide distribution range of *Rhipicephalus microplus* in the surveyed areas in Kilombero district (Table 5), could be attributed to the environmental factor such as climate, temperature and relative humidity in the area (Khajuria *et al*., 2015). Similar findings has been reported in various part of Tanzania (Copland *et al*., 1986; Mamiro *et al*., 2016) and Zimbabwe (Sungirai *et al*., 2018). Previous studies have shown that this tick occurs in humid localities with steppe areas that have hot dry seasons (Walker *et al*., 2014).

The mitochondrial COI and 16S rRNA genes have been used in species identification and developing tick phylogeny in Tanzania (Damian *et al*., 2021), Uganda (Muhanguzi *et al*., 2020), and Malaysia (Low *et al*., 2015; Ernieenor *et al*., 2020). Both CO1 and 16S rRNA have been reported to be suitable markers for tick species identification as compared to 12S rRNA and ITS2 (Jizhou *et al*., 2014b; Roy *et al*., 2018).

The lack of corresponding CO1 gene sequences in GenBank for *A. gemma* and intra-species genetic variation could be considered as one of the limitations of the molecular approach for tick species identification (Kumsa *et al*., 2016). Thus, for improvement in sensitivity for the *A. gemma* ticks, further improvement of the database is needed. The tick DNA sequence-based analysis for *R. appendiculatus* and *R. decoloratus* with CO1 and 16S rRNA in this study did not provide conclusive results. This was due to the sequences obtained were not of good quality after the purification and sequencing of the PCR product and therefore were excluded from the analysis. However, it is believed that the former could be a useful alternative to the latter for identification of tick species (Takano *et al*., 2014). Similar findings have been reported in previous studies as described by Black and

Piesman (1994). Failure to obtain clear sequences from the *R. appendiculatus and R. decoloratus* from this study was surprising considering that CO1 and 16S rRNA are conserved regions across *Rhipicephalus* spp. However, these results could be due to co-amplification of different copies of the CO1 and 16SrRNA gene which could be due to intraspecific variation as it has been reported for various species of Ixodid ticks (Kanduma *et al*., 2016). It is therefore recommended to consider a whole mitochondrial genome analysis or the use of different DNA barcoding genes from multiple conserved regions such as 12S rRNA, 18S rRNA, CytB and ITS2 for their identification (Kanduma *et al*., 2020).

In reference to this study, there is a worldwide development of DNA barcoding databases, such as the Barcode of Life Database (BOLD) system (Ratnasingham and Hebert, 2007). Therefore, further studies for updating the DNA database would be beneficial to the public health for control of ticks and TBDs (Takano *et al*., 2014).

The overall high pathogen detection rate 70.33% (Table 3.1), on tick pools in this study suggest that cattle from the study areas in which the tick were collected may serve as reservoir for the detected pathogens. Findings from this study agree with findings from previous studies in Tanzania, Kenya and Italy, which have revealed the existence of *Anaplasma, Theileria* and *Babesia* spp. from various ecologies (Georges *et al*., 2001; Kim *et al.,* 2018; Oundo *et al*., 2020; Chiuya *et al*., 2021). The higher pathogen detection rate in *Rhipicephalus evertsi* and *Amblyomma lepidum* tick pools (Table 3.1), in this study could be attributed to the high number of tick pools tested and the larger number of ticks in their particular pools. Moreover, the interaction between livestock and wild animals might have contributed to the high pathogen detection rate on tick pools from Iringa considering the richness of the competent vectors at the wildlife-livestock interface ecosystem around

Ruaha National Park. Similar findings have been reported from previous studies in Kenya (Okal *et al*., 2020) and Botswana (Raboloko *et al*., 2020). *Rhipicephalus evertsi* is known to transmit *Babesia bigemina* in cattle (Kerario *et al.,* 2017). Both *Anaplasma* and *Theileria/Babesia* spp. DNA were detected in *Hyalomma rufipes* tick pools. This tick species is known to transmit *A. marginale*, that cause anaplasmosis in cattle. Moreover, it transmits *Theileria annulata* and *Babesia occultans* (Ikpeze *et al.,* 2011).

4.2 Conclusions

This study reports the presence and distribution of ticks in cattle. It indicates the current problem of ticks in cattle in Kilombero and Iringa districts as ticks were abundant and widely distributed in all the study areas. *Rhipicephalus microplus*, *R. evertsi* and *Amblyomma lepidum* were among the most predominant ticks. This study reports high pathogen detection rate in tick pools in from study area. Findings from this study reflect the presence of TBDs in the study areas that require further study on TBDs distribution and characterization. Molecular data on ticks collected from the study area will become a reference for tick taxonomy confirming morphological identification as well as provide some data that could be used in their systematic classification. Our findings on the presence and diversity of tick species in the two districts will help in contributing to future studies and can be used as a baseline for subsequent tick studies in the study area and other parts of Tanzania.

4.3 Limitations of the study

This study only reported tick infestation in cattle and not any other domestic animals in the study area. The study was limited to only eight months and could not cover several seasons of the year to include quite a few dry and wet seasons for good comparison of the seasonality findings. *Rhipicephalus appendiculatus* and *R. decoloratus* were successfully

amplified by PCR but their sequence analysis was not successful due to bad sequence quality. Because of the limited resources and shorter duration of the study, only three TBDs were detected and discussed. The PCR detection of Tick-borne pathogens DNA in the tick pools from the study areas were not confirmed by sequencing.

4.4 Recommendations

The tick species identified in the study areas deserve serious attention at all levels in order to minimize the spread of tick infestation and tick-borne pathogens to improve animal health and livelihood. Further longitudinal studies using larger sample sizes of livestock and wildlife that could contribute to improve ticks and TBDs control in the study area and to determine whether the tick species identified in the current study are stably or momentary in place are highly recommended. Furthermore, studies on tick species diversity and distribution would help to improve the understanding of disease dynamics which is perquisite for control measures of tick and TBDs in the study area and other parts of Tanzania. Even though there are some limitations to this study, as mentioned above, the findings from this dissertation have led to the development of the following recommendations.

i. To conduct active tick surveillance in domestic animals. This will enhance the understanding of the diversity and distribution of tick species infesting cattle national wide. It will also provide more information on predominant tick species infesting cattle and other domestic animals in the study areas, and subsequently, tick prevention and control efforts can be targeted towards a particular tick species.

ii. To conduct tick surveillance in wildlife and environment to determine the tick species, present on wildlife and the role that these wildlife play in the maintenance and spread of ticks in the study areas. This will also inform the risk of TBDs at the interface of wildlife, domestic animals, and humans.

iii. To understand tick distribution and seasonality in cattle and other domestic animals, and the variations among different regions in Tanzania. This information can be used to determine the timing of tick treatments in different parts of the country.

iv. To determine the pathogen diversity across tick species identified from the study area to understand the potential vector role these ticks could be playing in the transmission of TBDs. Such information will help in informing the risk of potential TBDs that can be transmitted to other animals and humans.

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APPENDICES

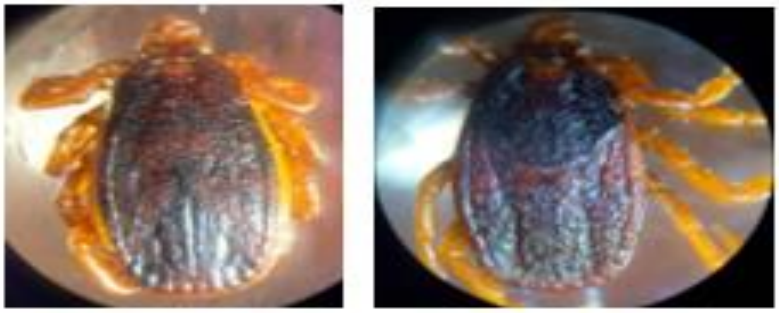
Appendix 1: Images of adult Ixodid tick from cattle in the study areas


A. gemma (male left and female right)

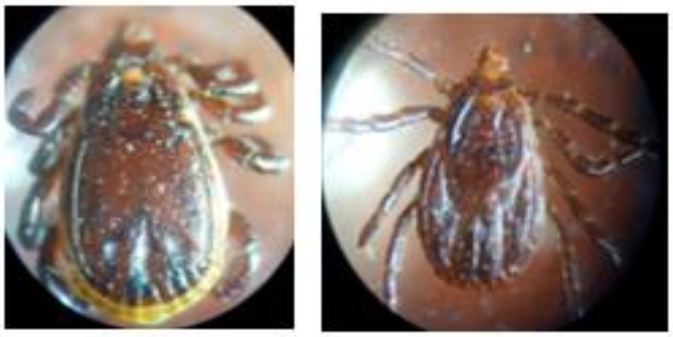

A. lepidum (male left and female right)


A. Variegatum (male left and engorged female right)

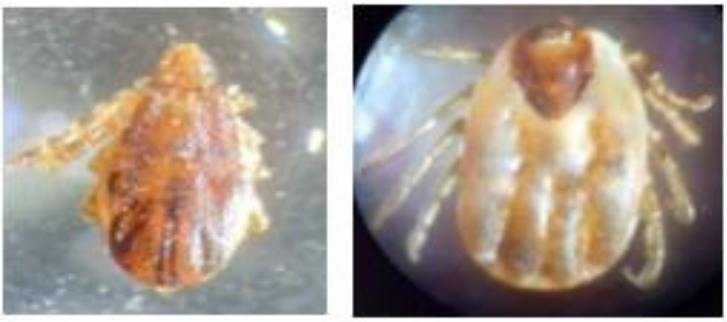

H. rufipes (male left and female right)



R. evertsi (male left and female right)



R. appendiculatus (male left and female right)



R. microplus (male left and female right)

R. decoloratus (female) *H. albiparmatum* (male)

Appendix 2: Informed concern form

Principal Investigator: Walter Simon Magesa MSc

Name of Organization: Sokoine University of Agriculture (SUA), Morogoro, Tanzania

Title: DISTRIBUTION AND ABUNDANCE OF TICKS ON CATTLE AND ASSOCIATED TICK-BORNE PATHOGENS FROM KILOMBERO AND IRINGA DISTRICTS IN TANZANIA.

Introduction

We are from Sokoine University of Agriculture (SUA) and we are studying the distribution, abundance of ticks on cattle and associated tick-borne pathogens infesting cattle at Kilombero and Iringa districts. The study is financially supported by HALI project from SUA. The purpose of this study is to generate baseline information on distribution and abundance of Ixodid ticks on cattle and associated tick-borne pathogens in Kilombero and Iringa Districts that will complement on designing appropriate control measures for ticks and TBDs in the respective study areas and Tanzania at large. There will be no money or anything offered to take part in this study. Ticks will be collected from cattle and the surrounding vegetation. No human samples will be collected and no administration of any drug will be done. The data collected will be used for establishing seasonal dynamics of Ixodid ticks in terms of distribution, abundance and burden in cattle and environment. Information obtained from this study will also aid in to determine occurrence of tick-borne pathogens in the study areas. Furthermore, it will contribute to the national livestock sector development goal and will be useful for rational control strategies of ticks and TBDs in Tanzania.

Confidentiality

The information that we will collect from this research project will be kept confidential and will be stored in a file, which will not have the participants name on it, but a number assigned to it. Which number belongs to which name will be kept under lock and key, and will not be divulged to anyone except the scientists and representatives of the SUA.

“I have read, understand this information and agree to take part in this study”

Name: Interviewee.....Signature.....Date.....

“I agree to abide to the above condition”

Name: Interviewer.....Signature.....Date.....

Appendix 3: Herd Enrolment form

Data Recorded by: _____

Date: (d/mm/yyy) _____ Time: _____

Herd ID: _____ Phone Number: _____

1. **District:**

Iringa ( ) Kilombero ( )

Ward: _____ Village: _____

2. **GPS Coordinates:**

Latitude: _____ Longitude: _____ Elevation: _____

3. **Estimated number of cattle in the Herd:**

1-20 ( ) 21-100 ( ) 101-500 ( ) >500 ( )

4. **History of the Acaricide use:**

Yes ( ) No ( )

5. **What measures are taken to prevent ticks? (Check all that apply)**

Dipping ( ) Hand spray ( ) Hand picking ( ) None ( ) Other ( ) (If other please specify)

6. **Frequency of tick control**

Weekly ( ) Biweekly ( ) Monthly ( ) Occasionally ( )

Individual Animal Data

Herd ID: _____ Animal ID: _____

1. Breed of cattle sampled: _____
2. Animal Sex: Male ( ) Female ( )
3. Animal Age: _____ Months
4. Body Condition Score: 1-5 ( )
5. Total tick count on the animal: _____

Appendix 4: Research clearance permit

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY.

SOKOINE UNIVERSITY OF AGRICULTURE
OFFICE OF THE VICE-CHANCELLOR

P.O Box 3000, CHUO KIKUU, MOROGORO, TANZANIA.
Phone: +255 (023) 2640006/7/8/9, Direct Line: +255 (023) 2640015,
E-mail:vc@sua.ac.tz, Website: https://www.sua.ac.tz

Please refer to:
Our Ref: SUA/ADM/R.1/8A/734 **Date:** 25th February, 2021

The Regional Administrative Secretary,
Morogoro Region,
P.O. Box 650,
MOROGORO.

The Regional Administrative Secretary,
Iringa Region,
P.O. Box 858,
IRINGA.

RE: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by University Act No. 7 of 2005 and SUA Charter, 2007 which became operational on 1st January 2007 repealing Act No. 6 of 1984. One of the mission objectives of the University is to generate and apply knowledge through research. For this reason the staff and researchers undertake research activities from time to time.

2. To facilitate the research function, the Vice Chancellor of the Sokoine University of Agriculture (SUA) is empowered to issue research clearance to staff, students, research associate and researchers of SUA on behalf of the Tanzania Commission for Science and Technology.

3. The purpose of this letter is to introduce to you **Mr. Walter Simon** a bonafide **MSc. (One Health Molecular Biology)** student with Registration number **MOH/D/2019/0021** of

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

SUA. By this letter **Mr. Walter Simon** has been granted clearance to conduct research in the country. The title of the research in question is **"DETERMINATION OF DISTRIBUTION, ABUNDANCE AND HOST PREFERENCE OF HARD TICKS INFESTING CATTLE AT KILOMBERO AND IRINGA RURAL DISTRICTS, TANZANIA".**

4. The period for which this permission has been granted is from **February, 2021** to **July, 2021**. The research will be conducted in **Morogoro (Kilombero District) and Iringa (Iringa Rural District).**

5. Should some of these areas/institutions/offices be restricted, you are requested to kindly advice the researcher(s) on alternative areas/institutions/ offices which could be visited. In case you may require further information on the researcher please contact me.

6. We thank you in advance for your cooperation and facilitation of this research activity.

Yours sincerely,

Prof. B. Chove
FOR: VICE-CHANCELLOR

c.c. Director, DPRTC, SUA. - To note in file.
c.c. Student – **Mr. Walter Simon**

VICE CHANCELLOR
SOKOINE UNIVERSITY OF AGRICULTURE
P. O. Box 3000
MOROGORO, TANZANIA

Appendix 5: Research permit Kilombero District

THE UNITED REPUBLIC OF TANZANIA
PRESINDENT'S OFFICE
REGIONAL ADMINISTRATION AND LOCAL GOVERNMENT

Telegraphic Address: "REGCOM"
Phones: 2934306/2934305
Fax No: 2601308/2604988
Website: www.morogoro.go.tz
Email: ras.morogoro@tamisemi.go.tz
In Reply please quote:

Regional Commissioner's Office,
Boma Road
P. O. Box 650,
67117 MOROGORO

Ref. No: AB. 175/245/01/95

10 March, 2021

District Administrative Secretary,
KILOMBERO.

Re: RESEARCH PERMIT

Please refer to the above mentioned subject.

2. I am introducing to you **Mr. Walter Simon** who is a bonafide **MSc. (One Health Molecular Biology)** student with Registration Number **MOH/D/2019/0021** of SUA and who is at the moment required to conduct research in our Morogoro Region.

3. The title of the research is **'Determination of distribution abundance and host preference of hard ticks infesting cattle at Kilombero District'**

4. The permit is granted **February,2021 to July, 2021.**

5. Please provide necessary assistance to enable the accomplishment of the research.

6. Thank you for your cooperation.

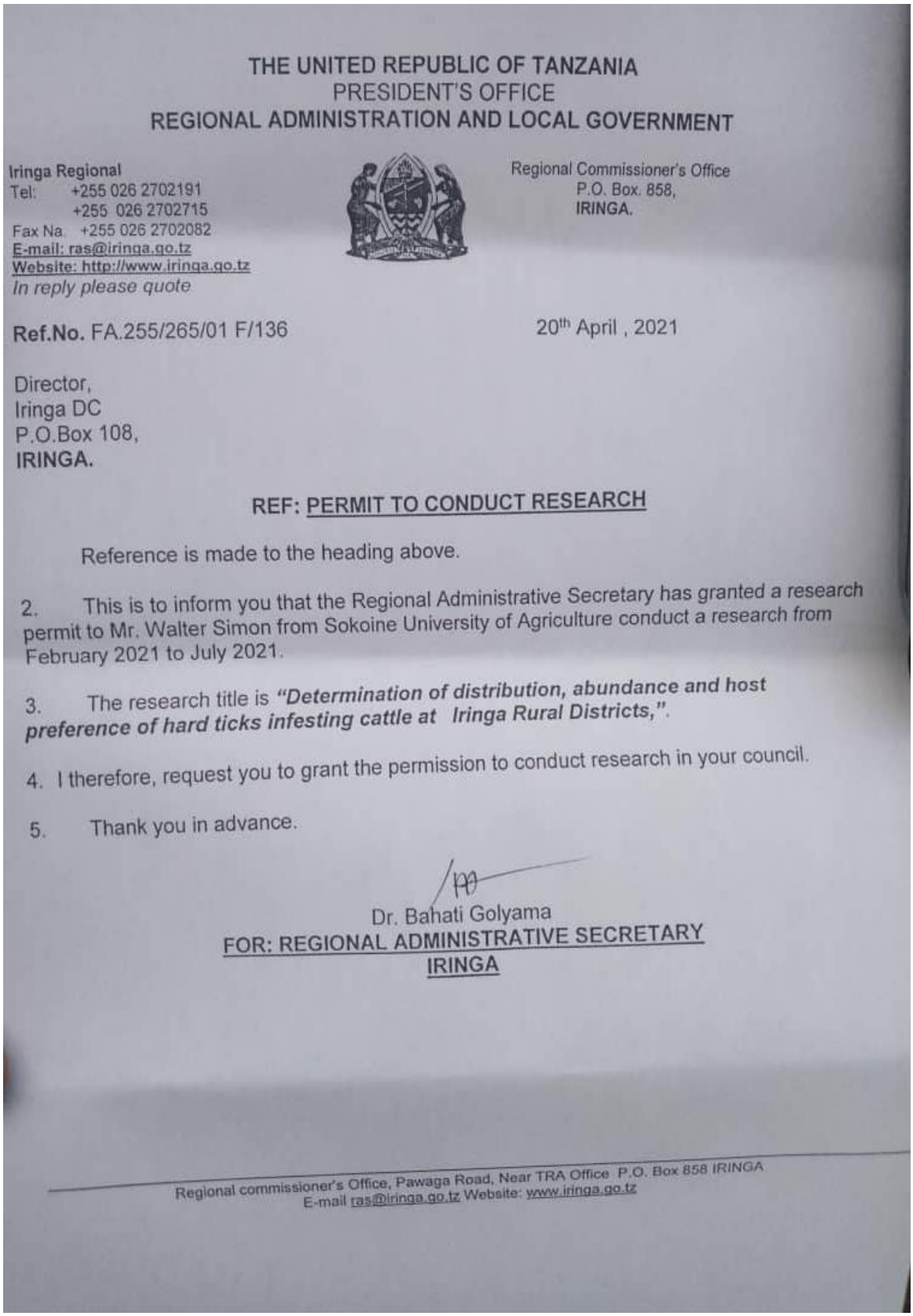
Erick Ulomi

FOR; **REGIONAL ADMINISTRATIVE SECRETARY**

Copy: Director,
DPRTC,
Sokoine University,

" **Mr Walter Simon - Researcher**

Appendix 6: **Research permit Iringa District**



THE UNITED REPUBLIC OF TANZANIA
PRESIDENT'S OFFICE
REGIONAL ADMINISTRATION AND LOCAL GOVERNMENT

Iringa Regional
Tel: +255 026 2702191
+255 026 2702715
Fax Na. +255 026 2702082
E-mail: ras@iringa.go.tz
Website: http://www.iringa.go.tz
In reply please quote

Regional Commissioner's Office
P.O. Box. 858,
IRINGA.

Ref.No. FA.255/265/01 F/136

20th April , 2021

Director,
Iringa DC
P.O.Box 108,
IRINGA.

REF: PERMIT TO CONDUCT RESEARCH

Reference is made to the heading above.

2. This is to inform you that the Regional Administrative Secretary has granted a research permit to Mr. Walter Simon from Sokoine University of Agriculture conduct a research from February 2021 to July 2021.

3. The research title is ***"Determination of distribution, abundance and host preference of hard ticks infesting cattle at Iringa Rural Districts,"***.

4. I therefore, request you to grant the permission to conduct research in your council.

5. Thank you in advance.

Dr. Bahati Golyama
FOR: REGIONAL ADMINISTRATIVE SECRETARY
IRINGA

Regional commissioner's Office, Pawaga Road, Near TRA Office P.O. Box 858 IRINGA
E-mail ras@iringa.go.tz Website: www.iringa.go.tz