بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of *Theileria Lestoquardi* in Sheep in Animal Production Research Stations, Sudan (APRS).

الكشف عن الثايليريا ليستوكاردي في الضأن في محطات بحوث الإنتاج الحيواني- السودان

A Thesis Submitted to the College of Graduate Studies in Fulfillment of the Requirements for Master Degree in Parasitology.

By:

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B.V.M. University of Gezira, 2012

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February 2019

Dedication

To my mother who granted me all the beautiful things in life

To the souls of my father

To my brother, sister and their children

To my dear wife Omslama Altash

To my dear daughter Aya

With love and respect

Abdelmonim Magzoub

Acknowledgements

Thanks first and last to Almighty Allah for supporting and giving me uncountable good think in my life.

I am deeply indebted to my supervisor, Prof: Ahmed ElGhali Ahmed for his advice and in reviewing this thesis.

My appreciation is extended to my co-supervisor Dr: Sara Basher Taha who helped me in this study.

Also, I am greatly indebted to Dr: Diaeldin Ahmed Salih, Mohammed Tom Elhaj and all staff in Entomology and Tick Department, CVRL.

I would like also to extend my deepest gratitude to Dr: Mohammed Osman Hussien and staff of Central Laboratory.

Special thanks for my best friend Dr: Yasir Juma who helped me to finish this work.

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المستخلص

أجريت دراسة مقطعية في شهري مايو وديسمبر 2016 وذلك لتقدير معدل الإصابة بالثايليريا في الضأن والتقصي حول عوامل المرض المرتبطة به من العوامل الأتية: السلالة, العمر, الجنس, حالة الجسم, الشهر من السنة والمنطقة وذلك في قطيع الضأن بمحطتي الهدى والنهود لأبحاث الضأن- السودان.

تم جمع 400 عينة بواقع 202 عينة من محطة الهدى و 198 عينة من محطة النهود وتم تشخيص الإصابة بواسطة مسحة الدم وإختبار التألق المناعي وتفاعل البلمرة المتسلسل.

أظهرت الدراسة أن نسبة إنتشار الإصابة بالثايليريا في الضأن بنسبة 13.4%, 22.0% و 12.8% لكل من مسحة الدم وإختبار التألق المناعي وتفاعل البلمرة المتسلسل على التوالي.

في التحليل الفردي لمعرفة عوامل الخطر المرتبطة بالإصابة بإستخدام مربع كاي وجدت علاقة معنوية تحت قيم معنوية أقل من أو يساوي 0.05 بين حدوث المرض وكل من عوامل الخطر التالية: حالة الجسم وحدوث الإصابة بالثايليريا وفق لإختبار مسحة الدم (القيمة المعنوية = 0.012) أما إختبار التألق المناعي فكانت هنالك علاقة معنوية مع السلالة (القيمة المعنوية = 0.005), حالة الجسم (القيمة المعنوية = 0.003) والمنطقة (القيمة المعنوية = 0.005) والشهر من السنة (القيمة المعنوية = 0.000) في حين أن نتائج تفاعل البلمرة المتسلسل قد وجد علاقة معنوية بين مرض الثايليريا في الضأن وكل من سلالة الحيوان (القيمة المعنوية = 0.051) والمنطقة (القيمة المعنوية 0.005).

في محطة الهدى وجد نوعين من القراد هما *R. e. evertsi* و *Hy. anatolicum* بينما وجد في محطة النهود بالإضافة للنوعين أعلاه نوعان من جنس *Hy. dromedarii* و *Hy.impeltatum*.

Abstract

A cross sectional study was conducted in May and December 2016 to investigate the prevalence of *T. lestoquardi* infection in sheep and to assess the association between the occurrence of the infection with risk factors (ecotype, age, sex, body condition score , season and location) in Elhuda and Elnuhud Animals production research stations, Sudan.

Four hundred sheep were selected randomly from Elhuda and Elnuhud (202 samples from Elhuda and 198 samples from Elnuhud). Whole blood samples were examined by blood smear (n≡400), polymerase chain reaction (PCR) (n≡200), and blood for serum (n≡400) to detect the antibodies using indirect fluorescent technique (IFAT).

The result showed that *Theileria* spp infection is common in sheep and the prevalence rate was estimated to be 13.4 % by blood smear, and *T. lestoquardi* estimated to be 22.0% and 12.8% by IFAT and PCR respectively.

The subsequent risk factors revealed that association with sheep *T. lestoquardi* in the univariate analysis under significant level of P-value ≤ 0.05: Concerning blood smear Significance association was observed for body condition score (BCS) (p-value =0.012) while IFAT recorded significant association with ecotype (p-value =0.005), BCS (p-value =0.003), Stations (p-value =0.005) and season (p-value =0.000) beside PCR which was found positively associated with ecotype (p-value =0.051) and Stations (p-value = 0.005).

The ticks found in Elhuda research station were *R. e. evertsi* and *Hy. anatolicum*, while in Elnuhud in addition to these two species *Hy. dromedarii* and *Hy. impeltatum* were also found.

Introduction

World sheep population outnumber one billion head in 2010 and act as animated socioeconomic part in the communities of the world. Around 205 million of these sheep originate in Africa, predominantly in dry (arid) and semi- arid areas of sub-Saharan and offer up to 30% of agricultural gross domestic product in developing countries (FAO, 2010).

The Sudan hopes to meet the challenge of economic development by improving its rich agricultural potential. The animal wealth of the country is among its major resources together with agricultural products form the backbone of its economy. The importance of improving livestock program in the Sudan is handi-capped by certain factors such as the vastness of the country, the way livestock is scattered, the poor pasture potential, lack of proper transportation and the lack of a satisfactory marketing system (Ockerman and Aziz, 1985).

Sudanese sheep have been classified by Mcleroy (1961) basing on physical features and ecological distribution into eight ecotypes local groups: Sudan desert (65%), Sudan Nilotic (12%), Arid Upland(1%), Arid Equatorial(1%), West African Fulani(1%), Fusion of desert x Nilotic(18%), Fusion of desert x Upland(1%) and fusion of Nilotic x arid equatorial (1%). The sheep population in the Sudan is estimated as 40 million heads according to (FAO, 2013).

Parasites play an important role in every ecosystem, as one of the regulating mechanisms of population dynamics for species within that system (Begon, 2007). Parasitic infections in the tropics are responsible for unlimited damages in the meat industry than some other infectious or metabolic disease (Perry *et al.*, 1995).

Theileriosis is a tick -borne disease which cause wide range of damage, it's most economically important infections of domestic ruminants in sub-Saharan

Africa. Little is known about theileriosis in sheep, while *Theileria* infection in cattle has been extensively studied (Gao *et al*., 2002).

In the Sudan, sheep are considered as very receptive host for *T. lestoquardi* as infection usually evolves into subacute and acute theileriosis even in indigenous sheep (Tageldin *et al*., 1992; El Hussein *et al*., 1998; Tageldin *et al*, 2005; El Imam *et al*., 2015). Malignant ovine theileriosis (MOT) was first described in the Sudan by Mason (1915), high morbidity and mortality rates have been reported in the Sudan (Salih *et al*., 2003; El Imam *et al.*, 2015).

Poor animal health is often a consequence of parasitic diseases, Malignant ovine theileriosis regard as one of these parasitic diseases which cause major constrains to livestock development in the Sudan.

Formerly conventional identification of *Thieleria* spp was done by microscopic examination depending on morphology. Molecular and serological aspects of ovine theileriosis are poorly understood and they are sensitive and precise techniques. In this study, we used molecular identification (PCR) technique beside serological and conventional methods to fill the gaps in our knowledge about *Theileria lestoquardi* in Elhuda and Elnuhud sheep research stations in the Sudan.

Objectives of the study:

1- To survey the prevalence of ovine theileriosis among sheep of Elhuda and Elnuhud sheep research stations to contribute in the control of the disease.

2- To identify tick species infesting sheep in and around the Elhuda and Elnuhud stations.

3- To determine the prevalence of *Theileria* spp, among the sheep of the two stations.

4- To determine *T. lestoquardi* antibodies and DNA among sheep of the two stations.

5- To study the impact of ecological zone, sheep ecotypes, seasons, age and sex on the prevalence of the disease.

Chapter One

Literature review

1.1 Theileriosis:

1.1.1 Definition:

Theileriosis is a tick-borne disease of cattle, sheep, goats, buffalo and infrequently wild ruminants caused by species of protozoa belonging to the genus *Theileria*, The parasite is transmitted by a species of tick known as *Hyalomma anatolicum* (Soulsby, 1982; Losos, 1986).

Malignant ovine theileriosis (MOT) is an endemic disease caused by *T. lestoquardi* and causes great loss among sheep. It seems as a fever, anaemia, emaciation and low activity, abortion and death may occur in severe cases.

1.1.2 Classification:

According to Levine *et al* (1980) *Theileria* classified as follows:

Sub-Kingdom. protozoa.
Phylum: Apicomplexa.
Class: Sporozoa.
Sub-class: Piroplasmina.
Order: Piroplasmida.
Family: Theileriidae.
Genus: *Theileria*.

1.1.3 Life cycle of the parasite:

1.1.3.1 Life cycle in mammals:

The mature sporozoites get into the blood stream and invade the lymphocytes during tick feeding. Macroschizont is the first stage seen in the lymphoid cells of regional lymph nodes , Macroschizonts usually contain about eight large irregular reddish–purple nuclei (Stagg *et al*.,1981) with pale surrounding cytoplasm when stained with Geimsa stain (Losos,1986). Presence of macroschizonts inside the

lymphoid cells stimulates their mitosis. During this division, macroschizonts undergo successive multiplication forming numerous microschizonts, each containing many small dense nuclei. These disintegrate liberating merozoites which are released into the blood stream by rupture of the lymphoid cells and enter the erythrocytes giving rise to piroplasms (Uilenberg, 1981).

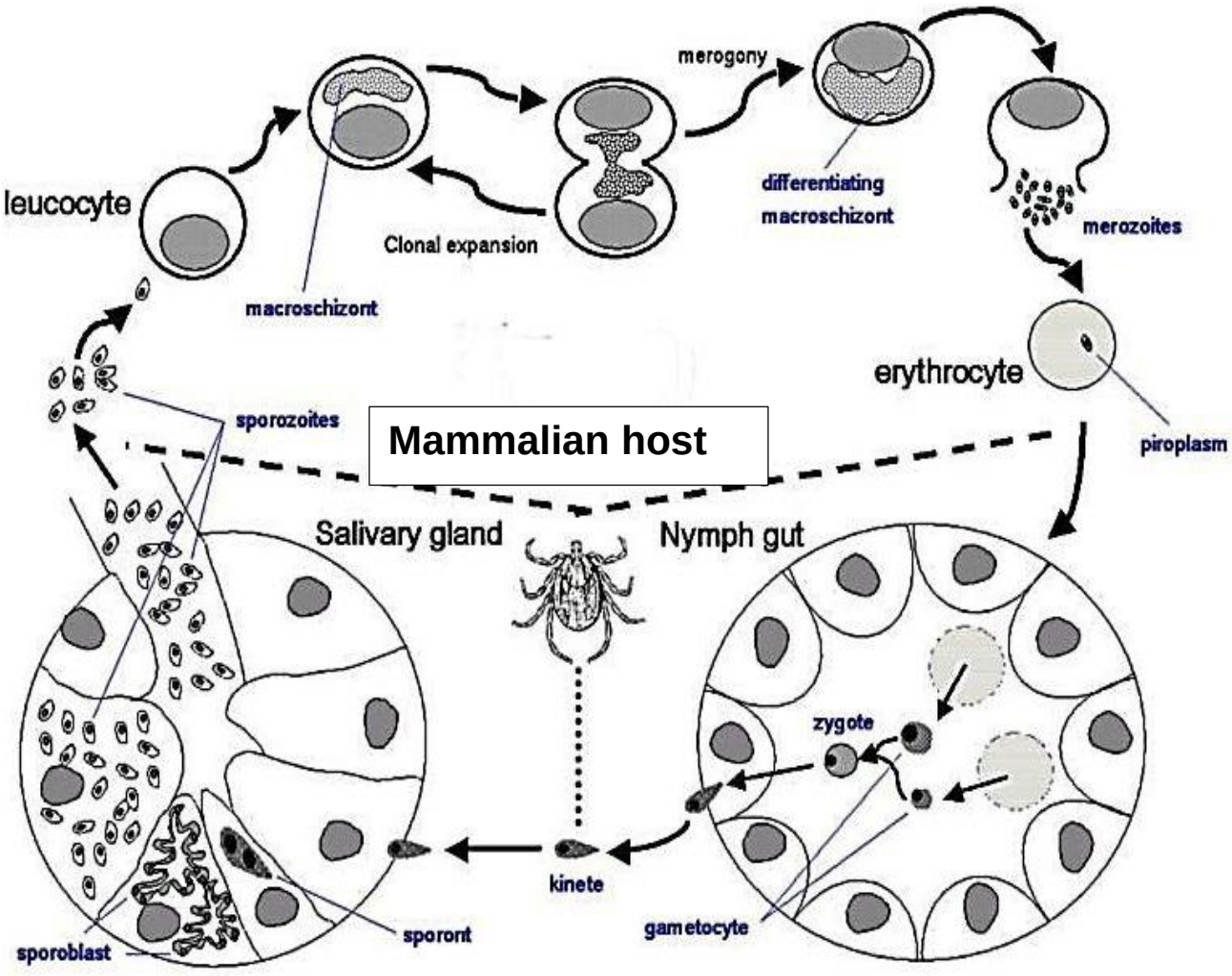

Figure (1): The life cycle of *Theileria.*

(Adopted from: www. Theileria. Org)

1.1.3.2 Life cycle in ticks:

The ticks ingest piroplasm (infected erythrocytes) which start to discriminate into macrogametes and microgametes. The gametes unite to form zygote in the lumen of the tick's gut. In the gut epithelium the zygote develops the kinete that invades type III acini of the salivary gland (Schein, 1975). After moulting to the next stage, the parasite begins to develop and the acini undergo marked hypertrophy (Irvin *et al*., 1981).

With the onset of tick attachment and feeding on a new host, there is a rapid increase in acinus hypertrophy and the parasite undergoes multiple divisions to form sporont giving rise to active sporozoites (Fawcett *et al*., 1985).

1.2 The epidemiology of malignant ovine theileriosis (MOT):

1.2.1 Geographical distribution of ovine theileriosis:

Malignant ovine theileriosis has been reported in several countries in the world, it is widespread in Eastern Europe, Middle East, North Africa, Iran, Iraq and the Sudan (Dzhunkovskii and Urodshevich, 1924; Hooshmand- Rad and Hawa, 1973; Soulsby, 1982; Tageldin *et al*, 1992; Latif *et al*, 1994). Its economic importance is gradually and increasingly becoming evident (Hawa *et al.*, 1981), and this may be due to high mortality rates which may reach 100% among infected animals (Soulsby, 1982).

In the Sudan, the disease was firstly described by Mason (1915) and then reported in Khartoum State and Western parts of the country (Nagwa, 1986; Tageldin *et al.*, 1992; Latif *et al.*, 1994).

In Northern Sudan, it was reported by El Hussein *et al.*, (1993), ElGhali and EI Hussein (1995) and Ahmed (1999) as causing serious losses among sheep. The disease flares up in summer, in a pattern of seasonal outbreaks, where 63.5% of sheep admitted to Atbara Veterinary Hospital and Atbara Veterinary Research

Laboratory were diagnosed as suffering from theileriosis during the summer season (ElGhali and ElHussein, 1995).

In across sectional study conducted in River Nile State, 1905 blood smears from sheep were examined for the presence of *Theleria* piroplasms. Out of 800 samples from resident sheep 177 (22.1%) found infected, while in 1105 samples from slaughterhouses 197 (17.8) were infected. Thirteen out of 210 (6.2%) *Hyalomma anatolicum* ticks were found infected with *Theileria* sporoblasts (ElGhali, *et al.*, 1994).

In latter study, the prevalence of *T. lestoquardi* antibodies in Sudanese sheep from nine geographical parts in Sudan was determined using indirect fluorescent antibody test (IFAT). Out of 315 samples examined, 51(16.2%) were found positive and ranged between 23.4% in River Nile State and 10% in Kasala and Darfour provinces indicating widespread distribution of the infection. It was also reported the presence of antibodies reactive to *Theileria annulata*, in sheep sera (Salih *et al.*, 2003).

1.2.2 Risk factors associated with ovine theileriosis:

1.2.2.1 Age and sex factor:

Concerning age and sex susceptibility to infection, many studies showed no statistical significance between sex; age and *Theileria* infection (Razmi and Yaghfoori, 2013).

1.2.2.2 Environmental factor:

Many studies showed that environmental temperature affects the number of ticks and then represent as limiting factors on the geographical distribution of malignant ovine theileriosis. Areas with a mean annual temperature of 20-25°C are the most suitable areas for development of ixodid ticks and *T. lestoquardi* infection (Hashemi-Fesharaki, 1997; Haddadzadeh *et al.*, 2004; Razmi *et al.*, 2006).

1.2.2.3 Seasonal Factor:

Many studies revealed that the infection of ovine theileriosis in definite season did not vary, for example, Ahmed *et al.* (2003) in Sudan reported that, the incidence of *Theileria* infection did not vary much with season. This might be attributed to continuous transmission of *Theileria* spp (Asmaa *et al.*, 2016).

1.2.3 Transmission:

According to Mazlum, (1970), *Hyalomma* genus had been suspected to be responsible for transmission of *T. lestoquardi* and later Hooshmand-Rad and Hawa (1973) demonstrated the transmission of *Theileria lestoquardi* by *H. anatolicum* from stage to stage. *H. anatolicum* appears to be the only proven vector for *T. lestoquardi* (Uilenberg, 1997).

In the Sudan this tick was associated with an outbreak of sheep theileriosis (Tageldin et al., 1992), and in other reports from Sudan *H. anatolicum* ticks were demonstrated to transmit *T. lestoquardi* to sheep (Latif *et al*., 1994).

1.2.4 The Clinical signs of MOT:

T. lestoquardi regarded as very pathogenic to sheep even in indigenous breeds with high morbidity and mortality rates (Tageldin *et al*., 1992).

The symptoms of acute malignant theileriosis in sheep or goats include high fever, anorexia, emaciation, lethargy, diarrhea or constipation, enlarged superficial lymph nodes and pale and icteric mucous membranes (Soulsby, 1982).

Ovine theileriosis is a tick-borne haemoprotozoan disease in sheep and goats caused by several *Theileria* species and among them *Theileria lestoquardi* is considered as the most pathogenic one. Ticks of the genus *Hyalomma* act as vectors for this parasite (Salih *et al.*, 2003). The disease is manifested by pyrexia, leucopenia, edema of the throat, nasal and ocular discharge, salivation, reduced appetite, paleness of mucus membranes and rough body coat (Naz *et al.*, 2012),

while *Theileria ovis*, *Theileria separate* and *Theileria recondita*, cause subclinical infection in small ruminants (Altay *et al.*, 2005).

1.2.5 The pathological features of MOT:

The main macroscopical lesions are hyperplasia, oedema of lymphnodes, splenomegaly, a yellowish enlarged liver and the lungs are frequently oedematous (Tageldin *et al*., 1992).

El Imam *et al*. (2015) recorded in an experimental study severe enteritis with scattered areas of petechial hemorrhages on the serosal and mucosal surface along the small and large intestines. In most animals, the superficial lymph nodes, liver and spleen are enlarged and their gall bladder was distended. Heart showed petechial hemorrhages and kidneys were congested.

All infected animals (100%) revealed severe pneumonia associated with edema and frothy exudates. Comparatively, the most remarkable microscopic lesions in infected sheep were obviously seen in the lungs which exhibited emphysema, congestion, collapse and proliferation of large mononuclear cells (El Imam *et al.*, 2015).

1.2.6 Diagnosis of Malignant Ovine Theileriosis (MOT):

Diagnostic tests currently used for detection of ovine theileriosis infection include conventional, serological and molecular methods.

1.2.6.1 Conventional methods:

Conventional diagnosis of *Theileria* parasites has mainly been based on microscopic examination of blood and lymph node smears for the presence of the parasites which could be differentiated from other blood parasites by morphological and staining properties in acutely infected animals, this method is the method of choice for early and rapid treatment of the disease (Morzaria *et al.*, 1999).

1.2.6.2 Serological methods:

There have been a variety of serological tests described for *Theileria* piroplasm antigens. These include coaglutination (Cawdery *et al.,* 1968), capillary tube agglutination (CA) (Ross and Löhr, 1972), indirect immunofluorescent antibody (IFA) test (Burridge, 1971), indirect hemagglutination assay (IHA) (Duffs and Wagner, 1974) and the Enzyme-linked immunosorbent assay (ELISA) (Katende *et al.*, 1998). IFA and IHA were the best assays for field work and CF, IFA, IHA were most suitable for experimental work, with respect to their sensitivity (Duffs and Wagner, 1980).

1.2.6.2.1 Indirect immunofluorescent test (IFAT):

IFAT was applied by Salih *et al.* (2003); Taha *et al.*, (2003) in epidemiological surveys for *T. lestoquardi,* but false positive and negative results due to cross-reactions or weak specific immune response are some disadvantages that are commonly observed in this test (Leemans *et al.*, 1997).

1.2.6.2.2 Enzyme linked immunosorbent Assay (ELISA):

An enzyme-linked immunosorbent assay (ELISA) has been developed for the serological detection of *T. lestoquardi* using recombinant protein to minimize the chance for cross-reactivity. This ELISA is based on the newly discovered clone 5 surface protein of *T. lestoquardi* and was applied in field samples collected from northern Sudan (Bakheit *et al.*, 2006).

1.2.6.3 Molecular techniques:

Detection of *Theileria* infections in carrier animals has always been a challenge when using parasitological and serological methods. Until recently, experimental tick transmission of the parasite from infected animals to susceptible animals was the definitive method of determining a carrier state. However, this approach is expensive and time consuming and can also be intermittent. The advent of molecular diagnosis has led to the discovery of molecular techniques ranging from

the classical single polymerase chain reaction (PCR) to more sophisticated techniques based on the use of DNA probes. Their use in diagnosis has improved the sensitivity and specificity that previous diagnostic tests lacked over the years (Dolan, 1986).

1.2.6.3.1 Polymerase chain reaction (PCR):

Several molecular techniques for detection of ovine *Theileria* species were developed. PCR has been developed using specific primers to amplify the *T. lestoquardi* fragment of the gene coding for a 30-kDa merozoite surface protein from ticks, sheep and goats (Kirvar *et al.,* 1998).

1.2.6.3.2 Reverse Line Blot (RLB):

Mixed infections cannot be detect by PCR and has poor sensitive to detect subclinical infections so, a reverse line blot (RLB) assay has been developed for detection of *Theileria* and *Babesia* parasites in small ruminant (Schnittger *et al.,* 2004).

1.2.5.3.3 Loop-mediated isothermal amplification (LAMP):

Theileria species such as *T. annulata, T. luwenshuni and T. uilenbergi* has been successfully detected by loop-mediated isothermal amplification of DNA (LAMP) (Salih *et al.,* 2008; Liu *et al.,* 2008). This technique is rapid and simple to run, cost effective, sensitive, and specific. Therefore the respective development of LAMP for *T. lestoquardi* can be of potential usefulness for application in diagnostics and epidemiological studies.

1.2.6 Treatment and control of theileriosis:

Ticks control aims to keep the incidence of the ovine theileriosis within manageable limits. To carry out the control of tick-borne diseases there are three major approaches (Dipeolu *et al.,* 1992). De-ticking and application of chemical acaricides, these approaches are faced some problems namely, the costs of labour, acaricides, resistance and effects on the non-target organisms in ticks which make

the use of acaricides a less attractive option. Chemotherapy of sick animals is used at wide range. Drugs can be used during the period of initial exposure in a method known as chemo- immunization. However, the method can be expensive and relies on challenge occurring during the period of drug cover that may create problem under a pastoral system (Ilemobade, 1991).

Controlling of malignant ovine theileriosis in general largely depends on the control of ticks and control of animal movement. Control of tick population by chemical acaricides is carried out by dipping or spraying with acaricides because efficient methods of immunization and chemotherapy of malignant ovine theileriosis are still yet to be developed. This method of control is very costly, less efficient in controlling diseases and ecologically undesirable (Latif and Pegram, 1992). Biological control in this aspect depends on using natural tick enemies such as parasitoids, pathogens and predators (Hassan *et al.,* 1992) in addition to host resistance and anti-tick vaccines. The latter is expected to reduce the reliance on acaricides and other methods (Willadsen *et al*., 1995). It should be noted that reliance on any single method of tick control often causes problems or lead to breakdown in the control system.

Tetracylines were the first compounds to be used in the control of theileriosis, their effect was suppressive only in the early stages of *Theileria* infection (Brocklesby and Bailey, 1962).

ElGhali *et al*. (1994) reported that, Oxytetracycline at 5 mg/kg Bwt showed an efficacy of 70% when used for treatment of moderate cases and overall efficacy of 52.9%. Oxytetracycline at 10 mg/kg Bwt showed an efficacy of 61.1% when used for treatment of moderate cases and overall efficacy of 55%. Diminazine aceturate efficacy when used for treatment of moderate cases was 47.1% and overall efficacy was 40%. They concluded both drugs are recommended to be used against this disease when other drugs are not available.

In addition, study conducted by Taha *et al.* (1997) the overall recovery rates were 60% and 44% for the combination of oxytetracycline and diminazine aceturate and oxytetracycline and cholorguine phosphate, respectively. These results indicated that both drugs regimens can be used for treatment of malignant ovine theileriosis.

Naphthoquinone compound, menoctone was subsequently discovered as theileriacidal, but it was too expensive to synthesize other effective derivatives of this compound were developed: parvaquone, buparvaquone which were very effective and safe with a wide therapeutic index (Hudson *et al.*, 1985).

In field conditions, halofuginone appears to be the most active compound against early stages of the disease as it is only active against the schizont stage, unlike parvaquone and buparvaquone, which are active against the schizont and piroplasm stages (Njau *et al.*, 1985).

1.3 The ticks:

1.3.1 Tick Classification:

Ticks are classified under the phylum Arthropoda, class: Arachnida, Order: Acarina, suborder: Ixodoidea, families: Ixodidae, Argasidae, and Nutalliellidae which are distributed worldwide (De Lafuente and Kocan, 2006).

Barker and Murrell (2004) reported that there are 899 tick species which parasitize vertebrates including Argasidae (185 species) Ixodidae (713 species) and Nuttalliellidae (one species). Family Ixodidae (hard ticks) contains 684 species under many genera. These include *Amblyomma* (102 species) *Aponomma* (24 species) *Dermacentor* (30 species) *Haemaphysalis* (155 species) *Hyalomma* (30 species), *Ixodes* (254 species), *Cosmiomma* (1 species), *Nasomma* (1 species), *Rhipicephalus* (75 species), *Anomalohimalaya* (3 species), *Rhipicentor* (2 species), and *Margaropus* (3 species). Hard ticks have scutum or dorsal shield covers the entire upper surface of male and relatively a small area just behind the head in the

female (Soulsby, 1982) while soft ticks lack scutum and the dorsum is covered by a leathery integument.

All ticks are blood sucking parasites found in most parts of the world (Oleg Kozhukhov, 2007) and there are four stages in the life cycle of ticks which are egg, larva, nymph, and adult (Bowman, 1999)

1.3.2 The distribution of ticks in the Sudan:

In the Sudan, tick fauna is composed 64 species and subspecies of both Argasid and Ixodid ticks (Hoogstraal, 1956).Ticks occupy a wide range of ecological niches that form the climate of the country. According to Hoogastraal (1956), most Sudanese tick collections were made from Equatoria region, the distribution of *A. lepidum* in the Sudan is generally concentrated in the eastern parts of the country (Osman and Hassan, 2003). This tick is absent from Northern and Khartoum provinces. It is present together with *A. variegatum* in Darfur, Kordofan, Baher Elgazal and Equatoria provinces (Osman, 1978; Abdalla, 2007; Gaafer, 2008).

Karrar *et al.* (1963) reported that *H. dromedarii* was the main tick species of camels together with *Amblyomma lepidum*, *H. impeltatum*, *Rhipicephalus sanguineus sanguineus*, *Rhipicephalus praetextatus*, *H. a. excavatum*, *H. truncatum* and *H. rufipes*.

Hoogstraal (1956) recorded *A. exornatum*, *A. lepidum*, *A. variegatum* and *R. decoloratus* in western Sudan and *R. annulatus* in Kordofan. He also reported the presence of *H. dromedarii*, *H. excavatum*, *H. impeltatum*, *H. impressum*, *H. marginatum*, *H. rufipes*, *H. scupense*, *R evertsi evertsi*, *R. praetextatus* and *R. sanguineus*. Although Osman *et al*. (1982) later recorded various species of *Amblyomma*, *Rhipicephalus* and *Hyalomma* in Kordofan, the dominant tick species was *H. impeltatum.* Osman (1997) also argued that the unusual distribution of *A. lepidum* and *A. variegatum* on sheep and goats in the Nuba Mountains required further study. In a subsequent study, cattle in Kadogli and Dilling in Kordofan

State were found to be infested with *A. lepidum, A. variegatum, H. rufipes, H. truncatum, R. annulatus, R. decoloratus, R. evertsi evertsi, R. praetextatus* and ticks of the *R. sanguineus* group (Sowar, 2002). In ElObeid, the predominant tick species on horses were *H. anatolicum*, whilst *H. dromedarii*, *R. evertsi evertsi* and *R. sanguineus* were also present (Salim, 2008). Salih *et al*. (2004) recorded *A. lepidum* on cattle at several localities in western Sudan, whilst *A. variegatum* was found in ElObeid and Nyala, and *H. dromedarii* in all localities sampled.

In Darfour, Osman (1978) found that *H. rufipes*, *H. truncatum* and *R. sanguineus* were the dominant species. He also recorded *H. turanicum, R.annulatus, Rhipicephalus cuspidatus* and *R. sulcatus* for the first time in Darfour and *R. guilhoni* and *Rhipicephalus turanicus* for the first time in the Sudan. In Southern Darfur, ticks reported on cattle were *H. truncatum, H. rufipes*, *H. impeltatum*. *Rhipicephalus annulatus, R. saguineus, R. longus, R. e. evertsi* and *R. praetextatus*.

In Gezira State, Hyati (2015) reported *Rhipicephalus e. evertsi* as dominant ticks, follow *H. anatolicum.* He also identify *H. rufipes, R. sanguineus, R. decoloratus, Amblyomma lepidum, Hyalomma impeltatum and Hyalomma dromedarii.*

In Northern Sudan, *Hyalomma dromedarii* were found to be the predominant (89%) tick species infesting the camels. Other tick species found in very low numbers were *Hyalomma impeltatum* (7.7%), *Hyalomma anatolicum* (3.3%), *Hyalomma truncatum* (0.29%*), Hyalomma marginatum rufipes* (0.25%), *Rhipicephalus praetextatus* (0.30%) and *Rhipicephalus sanguineus* group (0.09%). (ElGhali and Hassan, 2009).

Chapter Two

Materials and Methods

2.1 Area of the study:

2.1.1 El-Huda Research Station:

EI-Huda station at approximately 14°15'N latitude and 320°50'E longitude, at an altitude of about 250 m, about 90 km north-west of Wad Medani and about 150 km south of Khartoum, concerns with three subtypes of Sudanese desert sheep (Shugor, Dubasi and Watish) and comprises a number of 250 heads (APRC, 2016) (Figure:2).

2.1.2 El-Nuhud Research Station:

El-Nuhud station located in West Kordofan State, latitude and longitude are 12°42'N, 28°25 59.99'E, which found in savannah geographical zone and concerns with two subtypes of Sudanese desert sheep (Hamari and Kabashi) with a number of 300 head (APRC, 2016) (Figure: 2).

2.2 Samples collection:

202 sheep were randomly chosen from Elhuda and 198 sheep from Elnuhud research station each samples collected at two months (May and December 2016). Samples collected were whole blood and blood for serum. The tubes were then labeled indicating animal number, station and date of collection. The whole blood was then used for blood smears preparation and PCR. The sera were used for detecting of antibodies using indirect fluorescent technique (IFAT). The blood and serum samples were stored at −20 ◦C until used.

2.3 Blood examination:

Blood smear were air dried, fixed by absolute methyl alcohol for 2-3 minutes and stained with 10% Geimsa’s stain solution for 45 minutes. Stained blood smear

were then examined for piroplasm using light microscope. Thirty five microscopic field per slide were examined for detection of piroplasm.

2.4 Indirect florescent test (IFAT):

2.4.1 Antigen preparation:

The schizont antigen was prepared from a local *T. lestoquardi* cell line at low passage (<20 passage) according to the method described by FAO (1984) in 12-well Teflon-coated multispot slides (Highveld Biological, USA). Antigen-coated slides were individually wrapped in tissue paper and packed in aluminum foil with five slides in each packet. The slide packets were labeled and stored in airtight, waterproof plastic containers at −20 ◦C until used.

2.4.2 Conjugate:

Rabbit anti-sheep immune gammaglobulin (IgG) conjugated to fluoresein isothiocyanate (FITC) were obtained from Nordic Immunological Laboratory, The Netherlands. The conjugate was used at dilution of phosphate buffer saline (PBS) that gives no loss of titre of positive control serum in the IFAT, but which at the same time gives a reaction not greater than 1/10 with the negative control. This was achieved by dilution 1/80. Evans blue at a concentration of 0.01% was added to the conjugate as a counterstain.

2.4.3 Control sera:

Positive control (C +ve) serum was obtained from Razzi institute, Iran. Negative control (C −ve) sera was obtained from BDSL, UK. Control sera (C −ve and (C +ve) were diluted directly to 1/80.

2.4.4 Test sera:

Tested sera were diluted in PBS 1/80 for screening the antibodies to *T. lestoquardi* according to FAO (1984).

2.4.5 Indirect florescent antibody IFAT procedure:

The materials preparation and running of the test usual's described by Burridge *et al*. (1974) and FAO (1984).

2.5. Polymerase chain reaction (PCR):

2.5.1 DNA isolation

DNA extraction was carried out by following method previously described by Shahnawaz *et al*. (2011). Blood samples were suspended in 500 µL of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 30 mM DTT, 0.5% SDS) supplemented with 0.4 mg/mL proteinase K (Fermentas, USA). The samples were kept overnight in a heating block set at 55ºC. After the lysis, samples were heated at 95ºC for 10 min. Then equal volume of phenol: chloroform: isoamylalcohol (25:24:1 v/v/v), was added, vortexed for 30 seconds and centrifuged at 12000×g for 10 min. The aqueous phase was transferred to new clean tube and equal volume of ice cold isopropanol was added. The DNA was pelleted by centrifugation at 12000×g for 15 min, the pellet washed with 70% ethanol and dried at 65ºC for 5 min. The DNA was finally resuspended in 50 µL sterile distilled water. The isolated DNA was quantified on agarose gel before its further analysis.

2.5.2. Oligonucleotide design and PCR amplification:

A set of oligonucleotide primer [fwd 5'- GTGCCGCAAGTGAGTCA-3' and rev 5'-GGACTGATGAGAAGACGATGAG-3'] was used to amplify the 730 bp sequence from 18S rRNA gene of *T. lestoquardi* as previously described by Taha *et al*. (2011). PCR was performed in a final reaction volume of 25 µl. PCR reaction mixture contained 10X buffer A [500 mM KCl, 100 mM Tris-HCl, (pH 9.1 at 20) and 0.1% TritonTMX- 100], 250 ng genomic DNA, 20 pM of each primer, 0.16 mM of dNTPs, 2.5 U Taq DNA polymerase (Vivantus, UK) and 2.5 mM Magnesium chloride.

Theileria lestoquardi positive DNA sample (+ve control) was obtained from *T. lestoquardi* culture [prepared in the Central Laboratory, Sudan] and negative control sample (PCR mixture without DNA) were amplified during every PCR.

DNA amplification was carried out in a DNA thermal cycler (Gene Amp® PCR system 2700 Applied Biosystems Inc., UK).The thermo-profile used by Taha *et al*. (2011) was modified for the present study which consists of an initial denaturing step of 3 minutes at 94ºC was followed by 35 cycles consisting of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min, and elongation at 72ºC for 1 min, and final extension was carried out at 72ºC for 7 min. The amplified PCR products were visualized on 1.5% agarose gel following electrophoresis and staining with ethidium bromide. PCR was used to examine 200 samples those were positive using blood smears and/ or IFAT.

2.6. Tick collection:

Tick collection was carried out from surrounding area of the both stations. A pair of blunt metal forceps was used for collection. The ticks of each animal were separately preserved in vials containing 70% ethanol and labeled indicating animal, body condition score, age, gender, ecotype, district (station) and date of collection. The ticks were identified under a dissecting microscope according to Hoogstraal (1956) and Walker *et al.* (2003).

2.7. Data analysis:

The collected data were coded and entered into an Excel spreadsheet (Microsoft Excel, 2007). Statistical analysis was performed using statistical package for the social sciences (SPSS), version 16 software. Percentage was used to calculate prevalence. Data were statistically analyzed using Chi-squared test to calculate degree of association between risk factors and prevalence of *Theileria lestoquardi* infection, 95% confidence interval (CI) and $p \leq 0.05$ was considered for statistically significant difference.

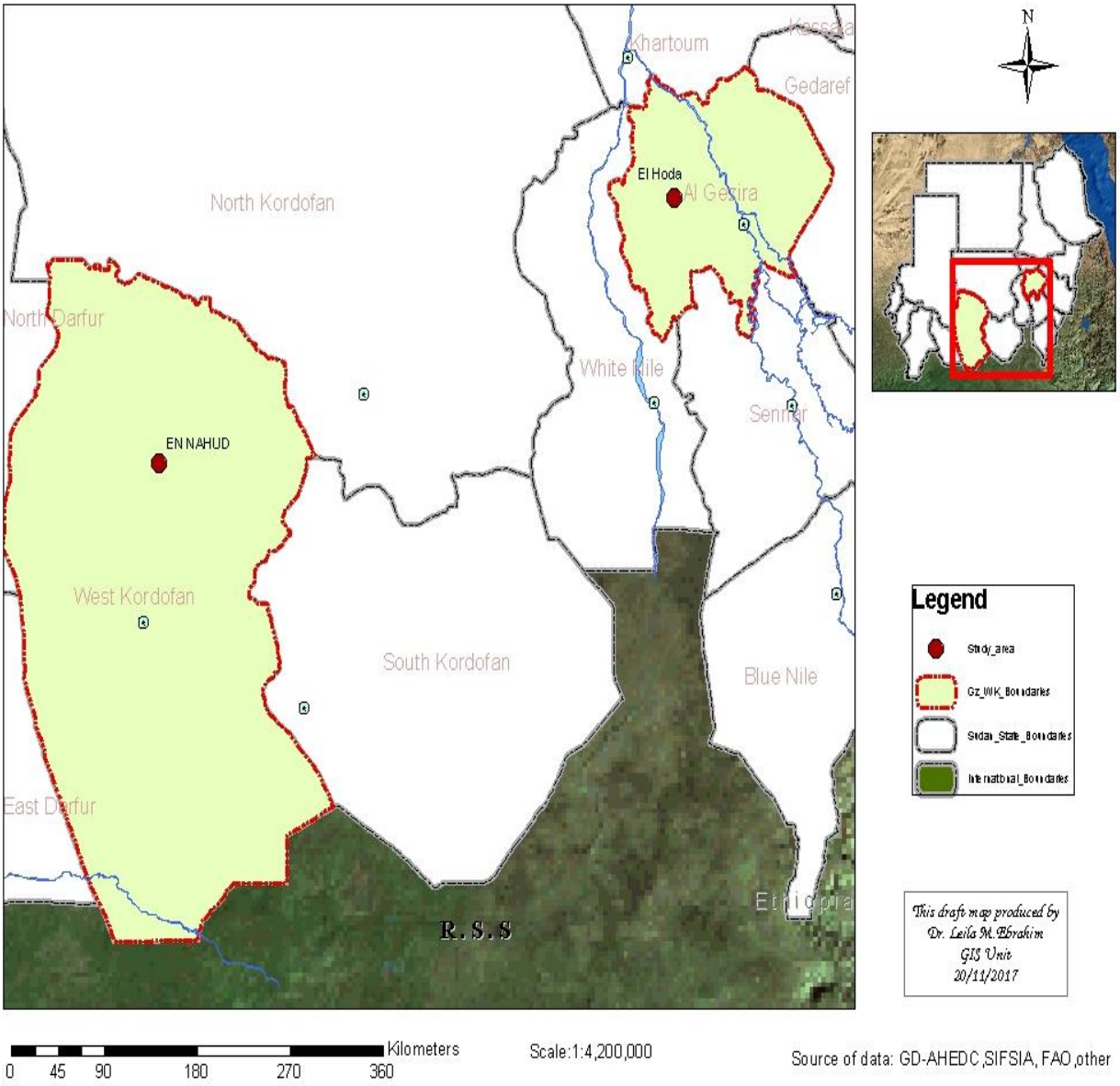

Figure: 2: Study area map.

(Source of data: GD-AHEDC, SIFSIA, FAO, other)

Chapter Three

Results

3.1 Prevalence rate of sheep Theileriosis:

Piroplasms were determined in 31 out of 202(15.3%) animals by microscopic examination in Elhuda, while 23 out of 198 (11.6%) animals were detected in Elnuhud, with an overall prevalence of 13.4% (Table 3.1).

On the other hand, IFAT results showed that *T. lestoquardi* antibodies were detected in 56 out of 202 (27.7%) in Elhuda and 32 out of 198 (16.2%) in Elnuhud Station with an overall prevalence of 22.0% (Table 3.2).

An overall prevalence of *T. lestoquardi* was 12.8% (26/200) as detected by PCR with 20 out of 103 (19.4%) and 6 out of 97 (6.2%) in Elhuda and Elnuhud stations respectively (Table 3.3).

3.2 Risk factors analysis:

3.2.1 Risk factors of *Theileria* species piroplasm:

As shown in (Table 3.4) there were differences in prevalence rate in different categories, but the only significant difference was in the body condition score (BCS) where the sheep of poor body condition had got the higher *Theileria* spp. piroplasm prevalence rate (P= 0.012).

3.2.2 Risk factors for *Theileria lestoquardi* antibodies:

When using IFAT there were differences in the different categories of each risk factor (Table 3.5). The significant differences were shown in sheep ecotypes where prevalence rate as higher in Shugor ecotype, While in these animals the body condition score as the poor ones harbour more infection, in season (month) where significant more infection happened in December and in the station where sheep in Elhuda station showed more prevalence rate (P between 0.00 and 0.005).

3.2.3 Risk factors for *Theileria lestoquardi* DNA detection:

(Table 3.6) showed that the only significant differences were in sheep ecotype where Shugor showed the higher prevalence rate (P= 0.051) and in the station where the sheep of Elhuda showed the higher prevalence rate (P= 0.005).

3.3. Ticks:

The types of ticks that infested sheep in Elhuda Station were *R. e. evertsi* and *H. anatolicum*, while in Elnuhud in addition to these two species *H. dromedarii* and *H. impeltatum* were also found (Table 3.7, Table 3.8 and Fig.2).

Table 3.1: Detection of *Theileria* species piroplasm in sheep (n≡400) in Elhuda and Elnuhud research stations by Blood Smear (B.S):

Station	Blood Examined	Positive	Prevalence (%)
El-huda	202	31	15.3%
El-nuhud	198	23	11.6%
Total	400	54	13.4%

Table 3.2: Detection of *T. lestoquardi* antibodies in sheep (n≡400) in Elhuda and Elnuhud research stations by indirect immunofluorescent Test (IFAT):

Station	Serum Examined	Positive	Prevalence (%)
El-huda	202	56	27.7%
El-nuhud	198	32	16.2%
Total	400	88	22.0%

Table 3.3: Detection of *T. lestoquardi* DNA in sheep (n≡200) in Elhuda and Elnuhud research stations by polymerase chain reaction (PCR):

Station	Blood Examined	Positive	Prevalence (%)
El-huda	103	20	19.4%
El-nuhud	97	6	6.2%
Total	200	26	12.8%

Table.3.4: Summary of statistical analysis for risk factors associated with *Theileria* spp infection (n≡400), using the Chi-squared test pertains to blood smear results in Elhuda and Elnuhud research stations, Sudan:

Risk factor	**No. tested**	**No. positive (%)**	**Df**	**X^2**	**p- value**
Ecotype					
Shugor	102	15 (14.7%)			
Dubasi	80	14 (17.5%)	4	2.320	0.677
Watish	20	3 (15.0%)			
Hamari	138	16 (11.6%			
Kabashi	60	6 (10.0%)			
Age					
Less than ≤1	38	7 (18.4%)			
Between 1-3	291	38 (13.1%)	2	0.878	0.645
Above >3	71	9 (12.7%)			
Sex					
Female	351	45(12.8%)	1	1.133	0.287
Male	49	9 (18.4%)			
BCS					
Poor	21	21 (33.3%)	2	8.874	0.012*
Moderate	248	27 (10.9%)			
Good	131	20 (15.3%)			
Month					
May 2016	200	26 (13.0%)	1	0.086	0.770
December 2016	200	28 (14.0%)			
Location					
Elhuda	202	31 (15.3%)	1	1.192	0.275
Elnuhud	198	23 (11.6%)			

***= means significant association at p≤ 0.05**

Table.3.5: Summary of statistical analysis for risk factors associated with *T. lestoquardi* infection (n≡400), using the Chi-squared test pertains to IFAT results in Elhuda and Elnuhud research stations, Sudan:

Risk factor	No. tested	No. positive (%)	Df	X^2	p- value
Ecotype					
Shugor	102	36 (35.3%)			
Dubasi	80	15 (18.8%)	4	15.048	0.005*
Watish	20	5 (25.0%)			
Hamari	138	22 (15.9%)			
Kabashi	60	10 (16.7%)			
Age					
Less than ≤1	38	15 (23.7%			
Between 1-3	291	64 (22.0%)	2	4.765	0.092
Above >3	71	9 (21.1%)			
Sex					
Female	351	73 (20.8)	1	2.414	0.120
Male	49	15 (30.6)			
BCS					
Poor	21	11 (52.4%)	2	11.931	0.003*
Moderate	248	50 (20.2%)			
Good	131	27 (20.6%)			
Month					
May 2016	200	29 (14.5%)	1	13.112	0.000*
December 2016	200	59 (29.5%)			
Location					
Elhuda	202	56 (27.7%)	1	7.788	0.005*
Elnuhud	198	32 (16.2%)			

***= means significant association at p≤ 0.05**

Table.3.6: Summary of statistical analysis for risk factors associated with *T. lestoquardi* infection (n≡200), using the Chi-squared test pertains to PCR results in Elhuda and Elnuhud research stations, Sudan:

Risk factor	No. tested	No. positive (%)	Df	X^2	p- value
Ecotype					
Shugor	57	13 (22.8)			
Dubasi	35	5 (14.3)	4	9.416	0.051*
Watish	11	2 (18.2)			
Hamari	68	5 (7.4)			
Kabashi	29	1 (3.4)			
Age					
Less than ≤1	15	0 (0.00)			
Between 1-3	148	18 (12.2)	2	4.765	0.092
Above >3	37	8 (21.6)			
Sex					
Female	172	21(12.2)	1	0.679	0.410
Male	28	5 (17.9)			
BCS					
Poor	23	6 (26.1)	2	4.525	0.104
Moderate	112	11(9.8)			
Good	65	9 (13.8)			
Month					
May 2016	100	10(10.0)	1	1.592	0.207
December 2016	100	16(16.0)			
Location					
Elhuda	103	20 (19.4)	1	7.733	0.005*
Elnuhud	97	6 (6.2)			

***= means significant association at p≤ 0.05**

Table.3.7: Number and gender of ticks in Elhuda research station.

Species	***Hyalomma anatolicum***		***Rhipicephalus evertsi evertsi***	
Gender	**Male**	**Female**	**male**	**Female**
December 2016	**7**	**13**	**43**	**34**

Table.3.8: Number and gender of ticks in Elnuhud research station.

Species	***Hyalomma impeltatum***		***Hyalomma dromedarii***		***Hyalomma anatolicum***		***Rhipicephalus evertsi evertsi***	
Gender	**male**	**female**	**Male**	**female**	**Male**	**female**	**Male**	**Female**
May 2016	**20**	**32**	**13**	**8**	**16**	**10**	**7**	**12**
December 2016	**12**	**19**	**9**	**10**	**18**	**12**	**90**	**47**

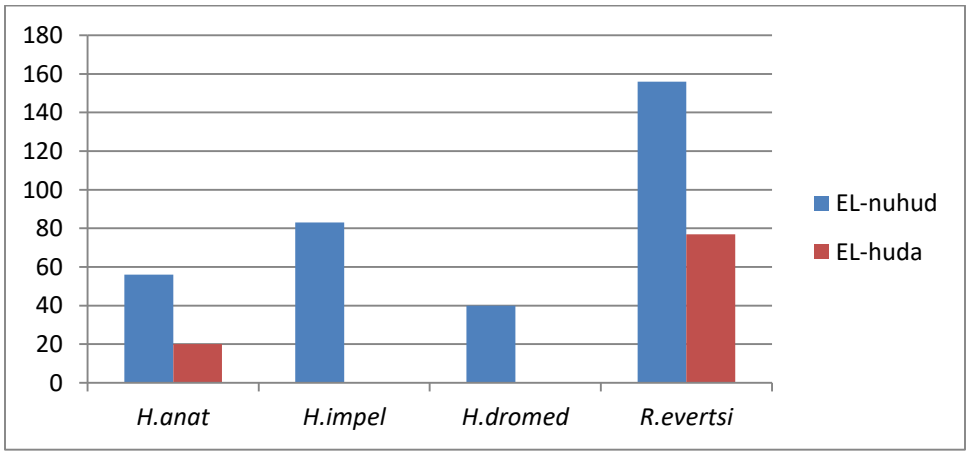

Figure: 3: Frequency distribution for Species of ticks in Elhuda and Elnuhud research stations.

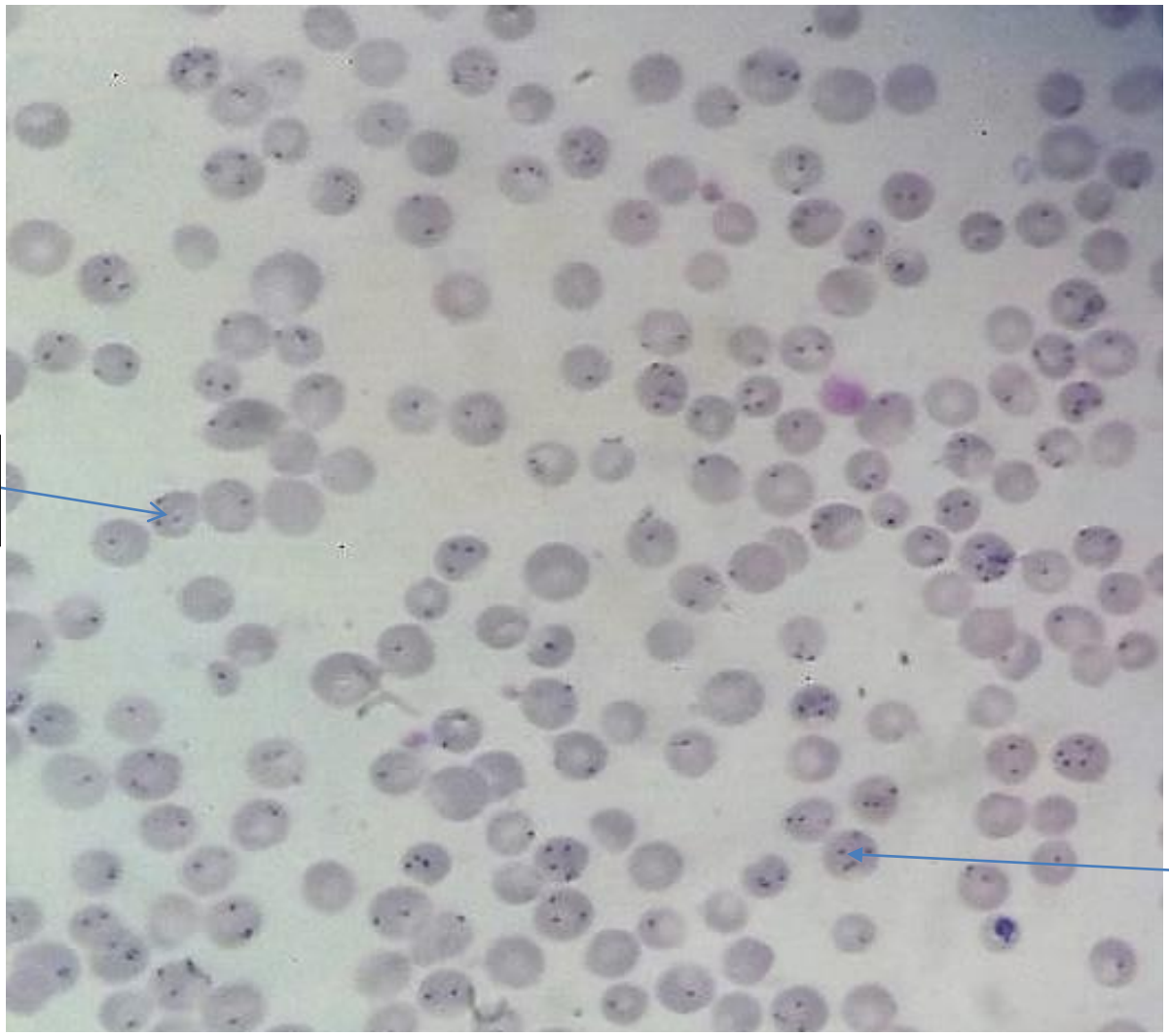

Figure: 4: *Theileria piroplasm* infection in erythrocyte.

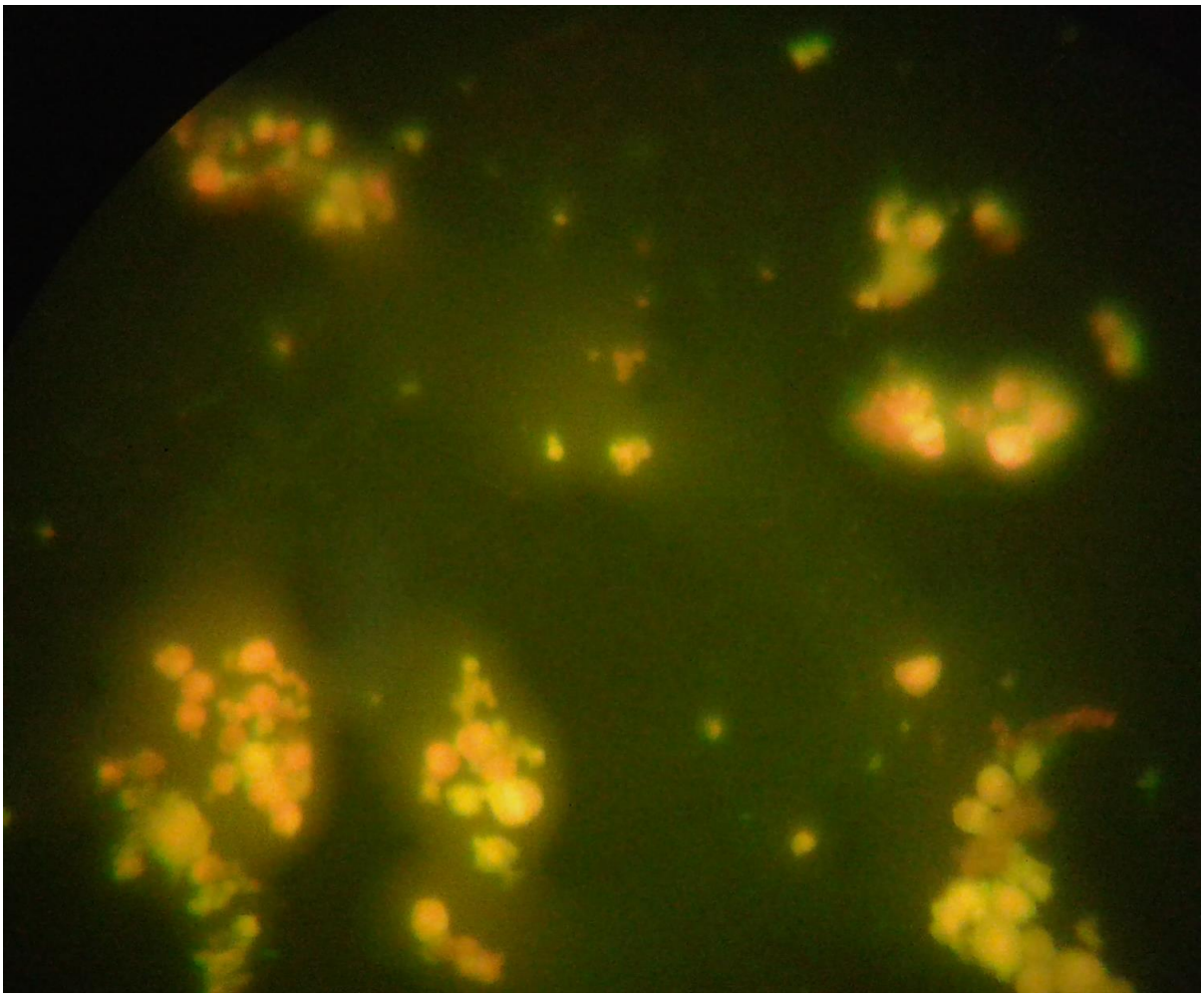

Figure: 5: Detection of *Theileria lestoquardi* Antibodies using schizont at antigen by IFAT.

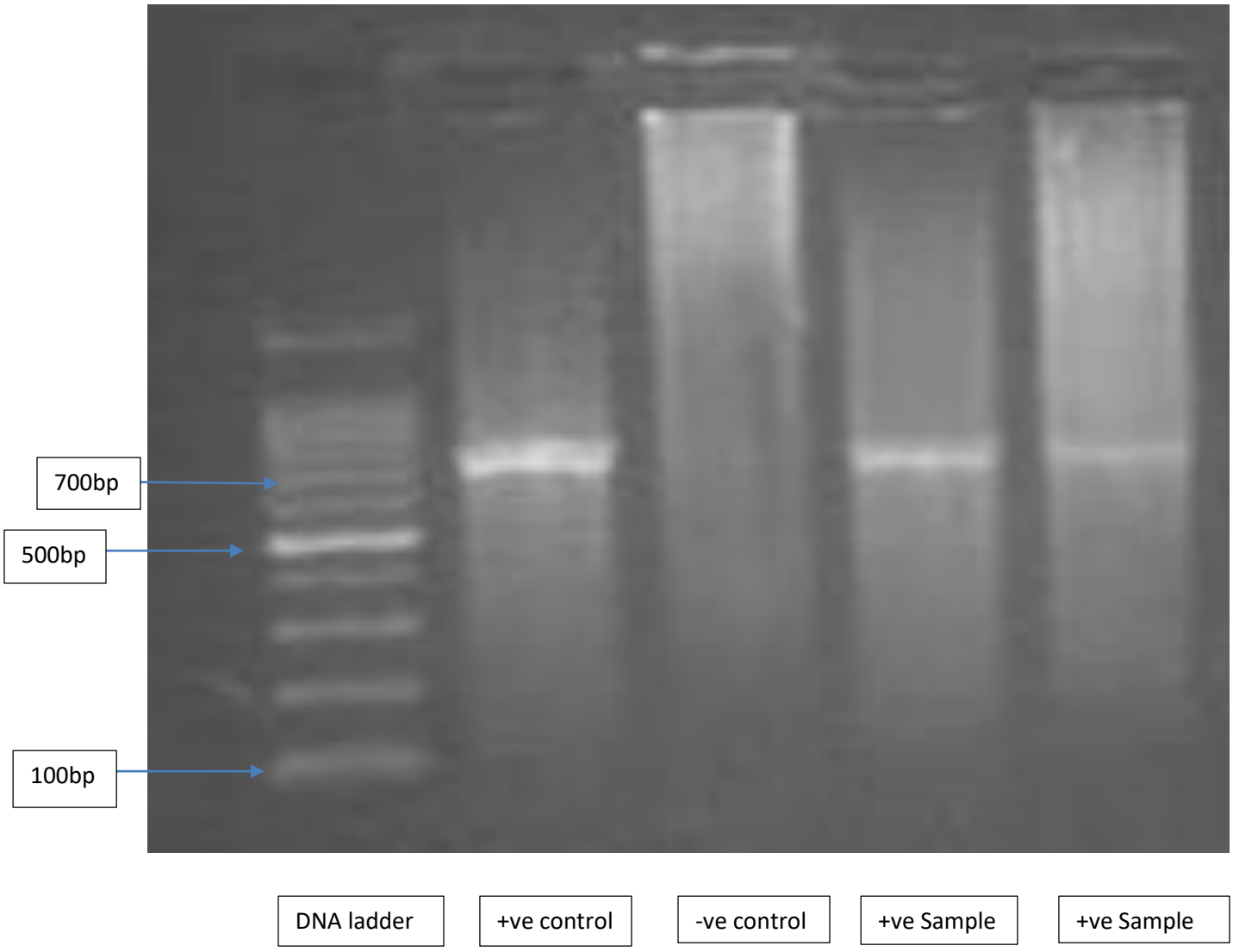

Figure .6. Agarose gel electrophoresis of amplified DNA from *Theileria lestoquardi*. Lanes.

Chapter Four

Discussion

Ovine theileriosis is a significant disease of small ruminants in tropics and subtropics regions (Criado *et al.,* 2009). *Theileria lestoquardi* is the most pathogenic among the various species causing malignant ovine theileriosis, it's a severe lympho proliferative disease with high morbidity and mortality rate (Naz *et al.,* 2012).

In the present study, the prevalence rate of *Theileria* spp piroplasm detected by microscopic examination was 13.4% and 22.0% by IFAT whilst PCR was 12.8%. PCR is more sensitive than IFA test. Regarding to blood examination, in this study our results are similar to Aktas *et al.* (2005) who detected 15.5% prevalence rate of *Theileria* spp and contradicted with Yaghfoori *et al.* (2013) in Iran, who reported 46%, while 76 % were positive by using semi-nested PCR. In Pakistan, the Prevalence was 22% by microscopic examination and 35% by PCR (Durrani *et al.,* 2011). Also, Aktas *et al.* (2005) detected *Theileria* spp in 15.5% of blood smears, while 41.2% by PCR, and whereas Ahmed *et al.* (2003) recorded the prevalence rate of *Theileria* infection in resident animals was 22.1% and from pre slaughtered animals was 17.8% in River Nile State in the Sudan.

In Egypt, Asmaa *et al.* (2016) showed that the prevalence of *Theileria* spp by blood smears examination under light microscope was 15.56%. These variations in the prevalence rate may be attributed to the locations differences and sampling size or to agro climate condition which affect victors of population dynamics.

The prevalence rate of *T. lestoquardi* in the current study (12.8%) regard low when compared with previous study carried out in the Sudan, while Hassan *et al.* (2018) who reported that the prevalence rate of *T. lestoquardi* 20.6% in sheep in Khartoum state in the Sudan.

Data concerning risk factors involved in the spread of *T. lestoquardi* was collected. Statistical significant was observed between animal ecotype and *T. lestoquardi* infection (P = 0.051), the highest prevalence was in Shugor (22.8%), Watish (18.2%), Dubasi (14.3%), Hamari (7.4%) and Kabashi (3.4%). Our finding contradicted with Hassan *et al.* (2018) who recorded the highest prevalence rate of *T. lestoquardi* in Dubasi, Kabashi followed by Baladi and Hamari ecotype.

When the age of infected and non –infected animals was compared, the results indicated that the animals over three year were more prone to *T. lestoquardi* infection (21.6%) followed by animal between 1 – 3 year (12.2%) and finally animal under one year with 0.00%.This is in line with Hassan *et al.* (2018) who found that 93% of seropositive animals belonged to older one because young animal had less exposure time to ticks infestation, also this observed by Taha *et al.* (2003)who reported that goats less than one-year-old showed the lower prevalence rate of antibodies compared to those older than four years of age which showed the highest rate.

A similar observation was reported previously (Naz *et al.*, 2012; Durrani *et al.*, 2012) during studies in small ruminants from Multan and Lahore districts in Pakistan, respectively.

Results indicated that the gender of sheep is important factor regarding *T. lestoquardi* infection. It was observed that males were more likely to have *T. lestoquardi* infection (17.9%) as compared to females (12.2%) but statistically this difference was not significant (P =0.410). This is contrasting with Rehman *et al.* (2010) and Naz *et al.* (2012) who reported that in their studies gender does not affect the incident of ovine theileriosis. On other hand, Bell-Sakyi *et al.* (2004) in Ghana and Dhaim and A'aiz (2014) in Iraq showed that *Theileria* infection did not affected by animal gender.

According to our results, the prevalence of *T. lestoquardi* infection in different body condition score of sheep was 13.8% in good body condition, 9.8% in moderate body condition score and 26.1% in poor body condition score. Although there was no association between the body condition score and *T. lestoquardi* infection (P =0.104), the rate of infection is higher in sheep with poor body condition. This is disagreed with the results obtained by Ahmed (1999) who reported that 22% out of 800 apparently healthy sheep showed patent theilerial Piroplasmosis in River Nile State, Northern Sudan.

In our survey, two month (May and December) have been investigated, but no significance effect of season on the *T. lestoquardi* infection rate was observed (P=0.207). Our results is consistent with Ahmed *et al.* (2003) in the Sudan as they observed that, incidence of *Theileria* infection did not vary much with seasons. These finding supports the general understanding of continuous transmission of *Theileria* spp. throughout the year. Also Asmaa *et al.* (2016) in Egypt support the fact that the vector found to be active throughout most of the year even in small number.

In our survey, two stations have been investigated for the prevalence of *T. lestoquardi* infection. The highest prevalence of infection was recorded in Elhuda station (19.4%) and the lowest one recorded in Elnuhud (6.2%). Significant association between *T. lestoquardi* infection and district have been reported, (P =0.004).This finding could be attributed to the fact that, most of ecotype exist in Elhuda research station were obtained from resident animals, from markets, where these ecotype are mainly raised along the Nile, therefore, favorable microhabitat for survival and reproduction of the dominant tick vector *Hyalomma anatolicum* exists (Ahmed, 1999).

When Ticks data was analyzed separately it was seen that the main prevalent ticks in Elhuda was *R. e. evertsi* and *H. anatolicum* like in Elnuhud. These results

confirmed that ticks in particular *H. anatolicum* acts as a vector for transmission of *T. lestoquardi*. Similar trend of tick infestation and its association with theileriosis has previously been reported by Hayati (2015).

The presence of carrier animals, susceptible animals and the vector (*H. anatolicum)* may lead to sporadic cases or outbreak of MOT in the two stations.

The number of male ticks in Elnuhud station was higher than the number of females in all tick species except *Hyalomma impeltatum* in which the number of females was higher than the number of male ticks. This finding agrees with the reports of Abdisa (2012) and Badaso *et al.* (2014) who reported similar results.

This high number of male ticks may be attributed to the fact that fully engorged female tick drops off the host to lay eggs while males tend to remain on the host up to several months to continue feeding and mating with other females on the host before dropping (Solomon *et al*., 2001). Similar results were obtained in Elhuda station where the male number was higher than females concerning *Rhipicephalus evertsi evertsi* unlike *Hyalomma anatolicum.*

Conclusion

The prevalence rate of *T. lestoquardi* in sheep in Elhuda and Elnuhud research stations was 13.4%, 22.0% and 12.8% by blood smear, IFAT and PCR respectively.

Body Condition Score of sheep was found positively associated with *T. lestoquardi* concerning blood smear whereas ecotype, Body Condition Score, District and Season were found positively associated with *T. lestoquardi* concerning IFAT beside ecotype and district were found positively associated with *T. lestoquardi* in relation to PCR test.

Recommendation

1. Additional surveys should be conducted to further investigation for the epidemiology and control of *T. lestoquardi* infection.
2. Effective control measures must be applied to reduce *T. lestoquardi* and consequently sheep heath and production.

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