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Pathogenesis and susceptibility of sheep to *Theileria lestoquardi* and molecular detection of other ovine *Theileria* species in the Sudan.

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DEDICATION

Yes, gold never rust. The father of tick-borne diseases in the Sudan. Who is great by virtue of his first report on heartwater in the Sudan and of his early suspicion of camel susceptibility to infection. The economic outcome of his research, not only in the Sudan but in the African continent is never missed.

To

Dr. Gaafar Karrar I dedicate this thesis.

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ABSTRACT

The objectives of the present study were to detect and identify *Theileria* species infecting sheep in the Sudan, and to study the susceptibility of three sheep eco-types to natural *T. lestoquardi* infection. Molecular survey of 219 blood samples using Reverse Line Blot (RLB) hybridization assay was conducted to detect, discriminate and identify different *Theileria*/*Babesia* and *Ehrlichia* spp. infecting sheep. In addition, three sheep eco-types – Garag (16), Watesh (15) and Desert (14) from Malignant Ovine Theileriosis (MOT) free area were introduced into Atbara endemic area where they picked infection following natural exposure to the tick vector infestation. RLB detected three *Theileria* species; namely, *T. lestoquardi* 36/219 (16.3%), *T. ovis* 194/219 (88.6%) and *T. separata* 44/219 (20%). The latter two species are reported for the first time in the Sudan. Infected sheep had variable *T. lestoquardi* parasitaemia (5-24%) with morbidity rate of 92.9%, 43.8% and 53.3% in Desert, Garag and Watesh, respectively. Natural infection with *T. lestoquardi* caused obvious clinical symptoms in all sheep eco-types including pyrexia, inappetence, loss of body weight, anaemia and jaundice. Pulmonary emphysema and degenerative hepatic changes were commonly seen, together with lymphoid hyperplasia in spleen and lymph nodes. A high number of large monocytes were detected in dilated hepatic sinusoids, pulmonary blood vessels and lymph node and splenic sinuses. Peculiar large monocytes with more than one nucleus and with dark blue inclusion-like cytoplasmic bodies were occasionally seen in the lungs. Four distinct abnormal features in lymphocytes (designated as *Theileria* markers), which were differentiated infected from non-infected lymphocytes were detected. These can be used in early diagnosis of *T. lestoquardi* infection. The findings, also, indicated that

infection of erythrocytes by merozoites mainly occurred in the lungs, a new observation which may fill the gap in *T. lestoquardi* life cycle. Rosette phenomenon (surrounding the infected cells by 8-9 lymphocytes) and pitting phenomenon (vacuoles in schizont infected cell cytoplasm) were observed for the first time. Serum enzymes (AST, ALT and AP) were increased in all sheep eco-types. The clinical manifestations of infection, parasitological, haematological, serum biochemical findings and pathological changes were more prominent in the Desert eco-type, indicating their high susceptibility to natural *T. lestoquardi* infection.

المستخلص:

هدفت هذه الدراسةِ إلي الكشفِ والتعرف على أنواع الثايليريا المختلفة التي تصيب الضأن بالسودان إلي جانب دراسة قابلية ثلاث سلالات من الضأن لإصابتها طبيعياً بثايليريا ليستوكاردي. استعمل فحص لطخات الخطوط المتعاكسة (RLB) لمسح 219 عينة دم للكشف و التعرف علي أنواع الثايليريا و البابيزيا و الإهرليخيا التي تصيب الضأن. إضافة الي ذلك اختيرت ثلاث سلالات من الضأن القرج [(16) و الوتيش (15) و الصحراوي (14)] من المناطق الغير موبوءة بثايليريا الضأن الخبيثة و إدخالها لمنطقة عطبرة الموبوءة بالمرض حيث أصيبت نتيجة لتعرضها لغزو القراد. اكتشف فحص لطخات الخطوط المتعاكسة ثلاثة أنواع من الثايليريا وهي ثايليريا ليستوكاردي 219/36 (16.3%)، ثايليريا اوفس 219/194 (88.6%) و ثايليريا سبراتا 219/44 (20%). سجل النوعان الأخيران لأول مرة بالسودان. ظهر الطفيل داخل خلايا الدم بمعدل متفاوت 5-33% مع معدل نفوق بلغ 92.9% و 43.8% و 53.2% في كل من الضأن الصحراوي و القرج و الوتيش علي التوالي. تسببت العدوى الطبيعية لثايليريا ليستوكاردي في أعراض سريرية واضحة لكل سلالات الضأن و تضمنت حمى و فقدان الشهية و نقصان الوزن و فقر الدم و اليرقان. من الصفات الشائعة التي لوحظت، إنتفاخ الرئتين و تغيات تنكسية في الكبد إلي جانب تكدس خلايا الليمف داخل الطوحال و العقد الليمفاوية. لوحظت أعداد كبيرة من الخلايا الأحادية الليمفاوية كبيرة الحجم داخل تجاويف الكبد و الأوعية الدموية الرئوية و العقد الليمفاوية و تجاويف الطوحال. في حالات عارضة رصدت داخل الرئتين خلايا ليمفاوية أحادية غريبة بها أكثر من نواه و إحتوت علي جسيمات داكنة الزرقة. اكتشفت أربعة إختلافات غير عادية في خلايا الليمف (عرّفت بمؤشرات الثايليريا) و هي تميز الخلايا الليمفاوية المصابة من غير المصابة و التي يمكن أن تستخدم في التشخيص المبكر للإصابة بثايليريا ليستوكاردي. كما دلت نتائج البحث على أن إصابة خلايا الدم الحمراء

بالميروزويت تحدث داخل الرئتين و هذه الملاحظة الجديدة قد تسد الفجوة المعلوماتية في حلقة تطور الثايليريا ليستوكاردي. لوحظ لأول مرة ظاهرة التورد (إحاطة الخلايا المصابة ب8-9 خلايا ليمفاوية) و ظاهرة التجويف (فراغات سيتوبلازمية في خلايا الشايظونط). زادت إنزيمات مصل الدم (AST وALT وAP) في كل سلالات الضأن. و كانت الأعراض السريرية للمرض و التغيرات الطفيلية و الدموية و الكيميائية الحيوية في مصل الدم و التغيرات المرضية أكثر وضوحاً في سلالة الضأن الصحراوي. و هذا مايدل على قابلية هذه السلالة للإصابة بطفيل الثايليريا ليستوكاردي.

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LIST OF ABBREVIATIONS

S rRNA	Small subunit ribosomal RNA
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AST	Aspartate Amino-transferase
ATP	Adenosine triphosphate
BCG	Bromocresol green
bp	Base pair
C-	Negative control
C+	Positive control
CXCR6	Chemokine (C-X-C motif) receptor6
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E. cells	Eccentric cells
ECL	Enhanced Chemiluminescence
EDAC	Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylene Diamine Tetra Acetic Acid.
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate conjugate
G-CSF	Granulocyte colony-stimulating factor
GOT	Glutamic Oxaloacetic Transferase

H&E	Hematoxylin and eosin
Hb	Haemoglobin
IFA	Indirect fluorescent antibody
IgG	Immunoglobulin G.
IU/L	International unit per litre
L/L	Litre/litre
LAMP	Loop-mediated isothermal amplification
dUTP	Deoxyuridine triphosphate
mMPS	Major Merozoite Piroplasms Surface Antigen
MOT	Malignant Ovine Theileriosis
MPT	Malignant Pulmonary Theileriosis
OD	Optical Density
OIE	The office international des epizootics
Tp67	*Theileria parva* 67 antigen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
pmol	picomole
PTFE	Polytetrafluoroethylene
RBCs	Red Blood Cells
RDS	Respiratory distress syndrome
RESA	Ring-infected erythrocyte surface antigen
RFLP	Restriction fragment length polymorphism
RLB	Reverse Line Blot
rpm	Revolution per minute
SD	Standard deviation

SDS	Sodium Dodecyl Sulfate
SLAG-1	Sporozoites lestoquardi antigen-1
SPAG-1	Sporozoite Surface Antigen 1
SPSS	Statistical package for social science
SSPE	Saline Sodium Phosphate EDTA
TBF	Tick-borne fever
TcHSP7	*Theileria* China heat shock protein 70
UDG	Uracil DNA glycosylase
UV	Ultra violet
V/V	Volume/volume
W/V	Weight/ volume
WBCs	White Blood Cells

INTRODUCTION

Malignant Ovine Theileriosis (MOT) is a tick-borne protozoan parasite of sheep, caused by *Theileria lestoquardi* and transmitted by *Hyalomma anatolicum*. Sheep is considered as a very receptive host for *T. lestoquardi*, based on the infection usually it evolves as sub-acute and acute theileriosis even in indigenous sheep (Hooshmand-Rad and Hawa, 1973a, Tageldin *et al*., 1992, El Hussein *et al*., 1998). Globally, high morbidity and mortality rates among local sheep in Iraq (Hooshmand-Rad and Hawa, 1975), Iran (Hooshmand-Rad, 1977) and in exotic sheep (Gautam *et al*., 1975) were reported. So far, the disease occurs as a major constraint for sheep production in many areas of the world. In the Sudan, the disease was first reported in Khartoum State by Tageldin *et al.* (1992) and then in Northern Sudan by El Ghali *et al*. (1995). So far, the disease is widespread, with a prevalence rate of (23%) was recently reported (Salih *et al.,* 2003). Under endemically stable conditions in Northern Sudan, 100% mortality in outbreaks was reported (Latif *et al.*, 1994, El Ghali and El Hussein, 1995). To date, little is known about the susceptibility of sheep and their various breeds (Uilenberg, 1997) and about the mechanisms involved in the pathogenesis of *T. lestoquardi* infection (Leemans *et al*., 2001). In addition, there is a considerable lack of knowledge of many points of the host-parasite relationship in different sheep eco-types (Leemans *et al*., 1999a,b). Sheep from disease free zones when introduced to endemic areas, high morbidity and mortality rates are expected, consequently, improvement of livestock production in these zones is hampered. Accordingly, the disease is expected to be of a high economic importance, especially in countries including the Sudan where export of sheep and sheep products is a major component of

their national economy. Despite the importance of the disease, little is known about the susceptibility of different sheep eco-types to the disease. Therefore, information on the detection, identification, distribution and susceptibility of different sheep ecotypes to MOT are required as a baseline data to design suitable control measures to overcome an expected disaster.

Objectives of this study:

The overall objectives of the present study focused on detection and identification of different *Theileria* parasites infecting Sudanese sheep, and on a comparative study ot three indigenous sheep eco-types and their susceptibility to natural *T. lestoquardi* infections. The specific objectives included the followings

1. To detect, identify and to discriminate between different *Theileria* spp. infecting sheep by Reverse Line Blot (RLB) hybridization assay.
2. To study clinicopathological aspects of *T. lestoquardi* naturally infecting sheep.
3. To study susceptibility of three different sheep eco-types to *T. lestoquardi* infection.

CHAPTER ONE
REVIEW OF LETERATURE

1.1. Sheep ecotypes in the Sudan:

The Sudan is endowed with large livestock wealth. This was estimated as 140 million heads of cattle, camels, goats and sheep. Sheep represent about 36% of the total estimated numbers (Anon, 2004). They play an important social and economic role in the country and are a valuable strategic resource for both local and export purposes. They also play a major role in the maintenance of rural populations living under conditions of poverty and are of major cultural importance due to their traditional use in religious and celebrations.

1.1.1. Sheep phenotypes:

Based on geographic distribution of the sheep and on phenotypic appearance, the Sudanese sheep have been classified by Bennett *et al.* (1948), McLeroy (1961a; 1961b), and Wilson and Clarke (1975). The precise phenotypic classification was reviewed by McLeroy (1961b). There are five basic ecotypes (Desert, Nilotics, Arid equatorial, Arid upland and West African), and three fused (crossed) ecotypes (crosses of Desert X Nilotics (Nilodesert), Desert X Arid upland and Nilotics X Arid Equatorial have been reported in the Sudan). Also, many other migrant animals have originated that are difficult to be categorized in respect of their final ecotype classification. However, at that time only two ecotypes-Desert and Nilotic-were of particular importance from a numerical standpoint and they can be said to have truly originated within the present confines of the nation (McElroy, 1961b) and later Abualazayium (2004) assumed that Sudanese

sheep descended from two original breeds: the Ovilongeps; the ancestors of Nilotic sheep, and the Oviplatre; the ancestors of Desert sheep of North Sudan. The latter author mentioned that according to numerical point of view, only three populations are important; Desert; Nilodesert and Nilotics populations which comprised 65%, 18% and 12%, respectively of the total sheep population.

1.1.2. Sheep genotypes:

Mitochondrial DNA was usually used to assess the origins and genetic relationships among different groups of livestock. Hiend leder *et al*., (1998) used one African sheep breed within the group of sheep from different areas to investigate the origins and phylogenetic relationships of domestic sheep. The study revealed that there are two main haplotypes (A and B) of mitochondria. Accordingly, African sheep were classified as B haplotype carriers like the groups of European sheep; where as A haplotype was found mainly in Asian breeds (Hiendleder *et al.,* 1999). A contrast study using 11 different Sudanese sheep types showed that 90% of the Sudanese tested sheep carried type B haplotype, whereas only 10% carried A haplotype. Also, the same group of animals were subjected to genetic diversity study using microsatellites, and the results revealed distinct demarcations of West African sheep population from all other populations in the Sudan, and a relatively slight distance of Desert (Dongla) from other member of the Desert populations. The structure modelling of these genotype classifications of Sudanese sheep, clustered into West African, Arid upland and Nilotic populations in one group, and divided the populations of the Desert and Nilodesert in two mixed clusters with incomplete demarcation (Nahid, G., 2010, unpublished data).

1.2. Tick-borne diseases of small ruminants:

In fact three genera have been described to infect small ruminants including *Ehrlichia, Babesia* and *Theileria*. Historically, infections caused by the species of last genera were described as *piroplasma* species (reviewed in Neitz and Jansen, 1956), because of the pear-shaped parasitic stages that can be found in the erythrocytes of a wide mammal host species. Piroplasmosis is a major constraint of small ruminant production in Asia, Africa and Southern Europe (Mehlhorn and Schein 1984). A number of these parasites are highly pathogenic to sheep and goats. These piroplasms causing agents have been somewhat neglected but they increasingly attract attention due to their economic importance being higher than cattle-infecting piroplasms in some countries e.g. China, Iran (Luo and Yin 1997, Schnittger *et al*., 2000a).

1.2.1. The genus *Ehrlichia*:

A number of these parasites (*Ehrlichia ruminnantium* and *Ehrlichia phagocytophilum*) are highly pathogenic for small and large ruminants, and the diseases emerging from these infections are referred to as *Ehrlichiasis*. *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*), the causative agent of heartwater, is transmitted by *Amblyomma* spp. ticks. *Amblyomma variegatum* ticks, which are only found in sub-Saharan Africa and the Caribbean is the major vector, except in certain areas of southern Africa where *A. hebraeum*, are the major vectors of *E. ruminantium* (Uilenberg, 1983, Walker and Olwage, 1987, Burridge *et al*., 2002, Faburay *et al*., 2007). *A. lepidum* is also an important vector of heartwater, especially in eastern Sudan (Jongejan *et al*., 1984). The disease is endemic in sub-Saharan Africa and in some islands in the Caribbean (Camus *et al*., 1996). In the

Sudan, the disease was recorded for the first time in sheep and goats in Kassala Province by Karrar (1960), in Kosti by Karrar (1966) and at Umbenin by Jongejan *et al.* (1984).The disease is fatal and is a serious constraint to animal production (Dumler *et al*., 2001).

Other Ehrlichia species that infect small ruminants is *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) causes tick-borne fever (TBF) in domestic ruminants and is mainly transmitted by the tick *Ixodes ricinus* (Ogden, *et al*., 2003). In Europe, *E. phagocytophilum* is the most widespread tick-borne infections in animals and has become one of the most prevalent tick-borne diseases of ruminants in some regions (Stuen and Bergström, 2001, Barandika *et al*., 2007; Stuen, 2007; Torina *et al*., 2007), and more recently, has been reported to infect sheep in China (Zhan *et al*., 2010). In Europe for instance, the disease has for decades been one of the main scourges for the Norwegian sheep (Stuen, 2003 Stuen *et al*., 2009). The infection in cattle and sheep are characterized by high fever, reduced milk yield, inclusions in circulating neutrophils, leucopenia, abortions and reduced fertility (Pusterla *et al*., 1997). On the other hand, less pathogenic *Anaplasma* species infecting sheep and goats is *Anaplasma ovis,* it causes severe disease in sheep and goats (Shompole *et al*., 1989) and is distributed throughout the tropics and subtropics (Visser *et al*., 1991).

1.2.2. The genus *Babesia*:

Babesiosis belongs to the complex of several tick-borne diseases that are widespread in tropical and subtropical areas of the world (Uilenberg, 2001), and causes diseases in livestock with high morbidity and mortality, thereby resulting in high economic losses worldwide (Mehlhorn *et al*. 1994, Ahmed *et al.,* 2002a). Ovine babesiosis is one of the important

haemoparasitic tick-borne diseases of sheep caused by *Babesia ovis* (Shayan *et al.,* 2008), *Babesia motasi* (Hashemi-Fesharki and Uilenberg, 1981, Levine, 1985), *Babesia crassa* (Friedhoff, 1997, Hashemi-Fesharki, 1997), *Babesia foliata*, *Babesia taylori*, *Babesia* sp. China (Bai *et al*., 2002) and *Babesia* sp. Xinjiang (Liu *et at*., 2007). Among all sheep *Babesia* species, however, *Babesia* sp. China infects both sheep and goats (Bai *et al*., 2002), and *B. ovis* was considered as highly pathogenic to small ruminants, especially in sheep, and causes severe disease. In general, the disease caused by the two *Babesia* species is characterised by fever, anaemia, icterus, haemoglobinuria and mortality in susceptible hosts ranges from 30 to 50% in natural infection.

Microscopic examination of Giemsa's stained blood smears remains the most appropriate method for the diagnosis of acute babesiosis, but this conventional diagnostic method does not permit its use in epidemiological investigations (Almeria *et al*., 2001). Serological methods are frequently employed in determining subclinical infections in epidemiological studies. However, these methods lack specificity due to cross reactivity with other *Babesia* species (Passos *et al.,* 1998), false positive and negative results were commonly observed in these tests. Recently, more sensitive and specific molecular techniques have been used as preferred methods for diagnosis of field babesiosis (Alhassan *et al.,* 2005; Aktas *et al.,* 2005b).

1.2.3. The genus *Theileria*:

The tick transmitted obligate intracellular parasites of the genus *Theileria* form the family Theileriidae in the order Piroplasmida (Levine, 1985). This order belongs to the phylum Apicomplexa, subkingdum

Protozoa. The phylum Apicomplexa also contains other orders: Plasmodium, *Eimeria*, *Toxoplasma*, *Neospora*, *Sarcocystis* and *Cryptosporidium*.

1.2.3.1. Taxonomy:

Since the first description of *Theileria* piroplasms - by Koch 1898 (sited in Leemans 2001) - in blood of diseased cattle around Dar Es Salaam, the taxonomy of *Theileria* species has been the subject of controversy (Neitz and Jansen, 1956, Uilenberg, 1981a, Irvin and Morrison, 1987). This controversy has been mainly due to the fact that the life cycle of the parasite was not adequately understood as the parasites are very small and their morphological details remained indefinable by light microscopy. The status of some species such as *T. annulata* and *T. parva* has since long been established, but speciation of other *Theileria* parasites, including those of low pathogenicity in cattle, sheep and goats, is a complex. At the genus level, the piroplasmids have also been the subject of changes as far as their classification is concerned. Based on biological data, morphological features, biochemical properties and molecular biological relationship, *Babesia equi*, a piroplasmic parasite of equids, has been reclassified as *Theileria equi* (Katzer *et al.,* 1998, Mehlhorn and Schein, 1998). Development in lymphocytes has been reported for *Babesia microti* (Mehlhorn *et al.,* 1996) and the 18S rRNA gene sequence evidence suggested that this organism should be removed from the genus *Babesia*.

1.2.3.2. Life cycle:

The majority of protozoan parasite life cycles are of a complex and dynamic nature (Kennedy, 1976). *Theileria* species can infect a vast range of domestic and wild animals but are of particular importance in domestic

livestock. They are transmitted by various numbers of tick vectors of the family Ixodidae. The parasites have a typical apicomplexan lifecycle involving several differentiation and multiplication stages in their mammalian hosts and their vector ticks. Comprehensive and precise reviews of the life cycle of the parasite were given by Mehlhorn and Schein (1984, 1993) and Norval *et al.* (1992). Generally, the life cycle of the parasite involves both the transmitting invertebrate tick vectors, in which sexual reproduction and sporogony takes place, and the vertebrate host in which asexual reproduction by schizogony and merogony occurs.

1.2.3.2.1. Life cycle in the invertebrate host:

Specific *Theileria* species are transmitted by specific tick species (Mehlhorn and Schein, 1993). Consequently, the distribution of a particular parasite species is directly related to the distribution range of its vector tick(s). Unlike transmission of *Babesia* species, which is often transovarial, transmission of the *Theileria* species in the tick is always transstadial. The major characteristic that distinguishes *Theileria* species from true *Babesia* species is that the latter develop exclusively in the erythrocytes (Mehlhorn *et al.,* 1994). The parasite infecting erythrocyte is the stage that is infective to the ticks. Lysis of infected erythrocytes occurs in the gut of the tick host and the piroplasms begin to develop to ray bodies from the 2nd to the 4th day of tick feeding (Mehlhorn and Schein, 1984, 1993). From the 5th day of tick feeding, the ray bodies divide and develop to 4 nuclei and thorn, give rise to uninucleate gametes which are considered as the microgametes (Norval *et al.*, 1992,). Spherical stages, which do not divide, are also found in the tick gut and are considered to be the macrogametes (Mehlhorn and Schein, 1984). Syngamy of gametes occurs on about 6th day of tick feeding and the

spherical zygote formed invades the gut epithelium and develops eventually into motile kinete (Berger *et al.,* 1971). Fully differentiated kinetes penetrate the gut and appear in the haemolymph of tick stage usually 17-20 days after repletion. While the infected larvae or nymphae are moulting to nymphae or adults, the kinetes migrate from the haemolymph to the salivary glands and colonize the type III acini, especially the 'e' cell (Norval *et al.,* 1992, Fawcett *et al.,* 1984) and/or type II salivary gland acini (Mehlhorn and Schein 1993). Inside the salivary gland and within these acini cells, the kinetes undergo a multiplicative process of sporogony leading to the production of infective sporozoites stage (Mehlhorn and Scheith, 1984).

1.2.3.2.2. Life cycle in the vertebrate host:

When the infected ixodid nymphs or adults feed on a susceptible vertebrate host, they inject the saliva containing sporozoites - the infective stage of *Theileria* spp. - into the host. Within a few minutes or even seconds, these sporozoites enter most often peripheral blood mononuclear cells (Fawcett and Doxsey 1982, Jura *et al.,* 1983). Once the sporozoite inside the host cell, the host cell membrane initially surrounds the parasite rabidly and disintegrates. The vertebrate host cell entry of *T. parva* and *T. annulata* sporozoites is likely to be dependent upon interaction between molecules of the parasite surface coat and those of the host cell surface as determined by the activity of antibodies that inhibit invasion (Musoke *et al.,* 1984, Williamson *et al.,* 1989). Monoclonal antibodies to sporozoite stage-specific surface protein of *T. parva* and *T. annulata*, known as p67 and SPAG-1, respectively neutralise sporozoite infectivity for host cells *in vitro* (Musoke *et al.,* 1984, Williamson *et al.,* 1989). To complete the life cycle, the *Theileria* sporozoites eventually transform into trophozoites and it

differentiate into a multinucleated schizont (the pathogenic stage) and this modifies the phenotype and behaviour of the lymphocytes. Development of the schizont causes activation and proliferation of the host cells. By dividing at the same time as the transformed lymphocytes, the parasites multiply by clonal expansion of the infected cell population (Musoke *et al.,* 1992). Those multiplicative processes, generally referred to as schizogony and merogony, producing merozoites that infect the erythrocytes and develop into piroplasms. After some time, depending on the *Theileria* species involved, usually eight to ten days after infection in the case of *T. annulata*, micro-schizonts can be observed, and soon unicellular merozoites develop. The merozoites, which are set free following disruption of the host cells, then invade erythrocytes where they transform into piroplasms. Again depending on the parasite species involved, these erythrocytic stages may multiply by schizogony (Conrad *et al.,* 1985). The parasite infected erythrocyte is the stage that is infective to the ticks.

1.2.3.3. *Theileria* species:

Worldwide, cattle bovine theileriosis has been extensively studied, but a paucity of information exists concerning ovine theileriosis. In fact, 6 *Theileria* species have been described to infect small ruminants including *T. ovis, T. recondita*, *T. separata* (Van Vorstenbosch *et al*. 1978, Uilenberg 1981a Alani and Herbert 1988a,b), *Theileria lestoquardi* (Morel and Uilenberg, 1981a), *Theileria luwenshuni* and *Theileria uilenbergi* (Yin *et al.,* 2007). According to the species phathogenecity and the rate of mortality, *Theileria* species infecting small ruminants were categorized in non pathogenic and pathogenic *Theileria*.

1.2.3.3.1. Non pathogenic *Theileria* species of sheep:

There is considerable confusion in the taxonomy of non pathogenic theileriasis of small ruminants (Uilenberg, 1995). However, at least three non pathogenic *Theileria* species (*T. ovis, T. recondita* and *T. separata*) do exist in sheep/goats (Uilenberg, 1995).

1.2.3.3.1.1. *Theileria ovis*:

Theileria ovis represents a single species; some authors (Uilenberg, 1981a, Leemans, 1997) suggested a complex of different species. Naturally it is transmitted by *Rhipicephalus bursa* in Turkey (Aktas *et al.,* 2006) whereas in Spain the parasite was detected in sheep came from areas where *R. bursa* is not found, while the predominating tick found on domestic ruminants was *R. turanicus* but the role of this tick species as a possible vector of benign theileriosis in the Mediterranean basin cannot be ruled out (Ferrer and Castellá, 1999). In Britain, *Haemaphysalis punctata* is probably the vector tick (Lewis *et al.,* 1981) and in India experimental transmission of *T. ovis* to susceptible sheep was performed by *Rhipicephalus haemaphysaloides*, while adults *Haemaphysalis bispinosa* failed to transmit the infection (Gill *et al.,* 1980). *T. ovis* was detected in the salivary gland of the vector *R. evertsi evertsi* (Mehlhorn *et al.*, 1979) and experimental transmission by *Rhipicephalus evertsi mimeticus* was successful (Nietz, 1972). The parasite was distributed in Turkey with prevalence range (54.0 to 67.9%) in sheep and 1.6% in goats (Aktas *et al.,* 2005a, Altay *et al.,* 2005) but in Iran it was reported as 7% (Bami *et al.,* 2009), whereas in Spain, the disease was detected in 18.9% of sheep (Ferrer and Castellá, 1999).

1.2.3.3.1.2. *Theileria separata*:

Haematoxenus separatum and *Theileria ovis* are synonyms. It was described by Uilenberg and Andreasen (1974) as a non-pathogenic parasite of sheep in Tanzania but later renamed as *T. separata* (van Vorstenbosch *et al.,* 1978). The parasite was morphologically characterized by veil in infected erythrocytes which were often separated from piroplasms and marginal in distribution. It is transmitted by *R. e. evertsi* and distributed in Asia and tropical Africa. In reference to the bibliography by Hassan and Salih (2009) and search in different internet, the parasite has not been reported in the Sudan.

1.2.3.3.1.3. *Theileria recondita*:

Theileria recondita was first reported in Germany and was found well distributed in Western Europe (Uilenberg, 1980). The parasite invades lymphocytes and erythrocytes of sheep but not reported to infect goats or red deer (Enigk *et al.,* 1964 cited in Alani and Herbert, 1988b). Lewis *et al,* (1981) thought that *Hae. punctata* was the probable vectors. Later, Alani and Herbert (1988b) confirmed transmission by the adult ticks, but the nymphs collected from field failed to do so. The latter authors could mechanically transfer the infection by syringe passage of blood from and to splenectomised/non-splenectomised sheep. In contrast to other *Theileria* species infecting small ruminants, little information is available concerning the biology, morphology and transmission of *T. recondita*.

1.2.3.3.2. The pathogenic *Theileria* species of sheep:

The pathogenic *Theileria* species of small ruminants are stimulating a surge of veterinary researcher’s interest and the animal health vigilance, so

recent, interest has arisen in sheep-infecting *Theileria* parasites (Brown *et al.*, 1998). *Theileria luwenshuni* and *Theileria uilenbergi* are the main disease threatening agents in China (Yin *et al.,* 2007); and *Theileria lestoquardi* (Morel and Uilenberg, 1981) is the causative agent of Malignant Ovine Theileriosis. Both of them are considered as the highly pathogenic *Theileria* spp. to sheep and goats.

1.2.3.3.2.1. The Chinese *Theileria*:

Not long than a decade ago, it was assumed that only *T. lestoquardi* is pathogenic for small ruminants, and thus ovine theileriosis in China was first reported to be caused by *T. lestoquardi* (Luo and Yin 1997) but later biological and phylogenic studies revealed that the causative agent is divergent from *T. lestoquardi* (Schnittger *et al.,* 2000a). However, a number of previously identified parasites have been recently described as also pathogenic for small ruminants, among which is a *Theileria* species causing a fatal disease of small ruminants (Luo and Yin 1997, Schnittger *et al.,* 2000a,b, Bai *et al.*, 2002, Yin *et al.*, 2002a,b). Much has been described regarding *Theileria* sp. (China) in the last few years regarding its phylogenetic characterization (Schnittger *et al.*, 2000a,b, 2003) and transmission (Yin *et al*. 2002a,b). The Chinese *Theileria* is phylogenetically most closely related to *Theileria buffeli* and *Theileria sergenti* (Schnittger *et al*. 2003), which have the common feature of exhibiting a less marked leukocytic phase and not being able to transform their host cells (Gao *et al*., 2002). Initially, the *Theileria* sp. that were identified in China were thought to be only one species, as they are similar from a biological point of view and may occur in mixed infections. Further phylogenetic analysis revealed that they were, in fact, two species rather than one, designated later as *T.*

uilenbergi and *T. luwenshuni* (Schnittger *et al.,* 2000a,b, Yin *et al.,* 2004, Gao *et al.,* 2002). They were transmitted by the ixodid tick *Haemaphysalis qinghaiensis* and caused a disease that exerted limitations on the development of the livestock industry, mainly in exotic animals northern and western parts of China (Luo and Yin 1997, Ahmed *et al.*, 2002, Yin *et al*,. 2002a,b). Apart from China, the occurrence and economic impact of this disease and in other parts of the world are unclear, though the description of a very similar parasite in Spain may suggest an impact in southern Europe (Nagore *et al.*, 2004).

1.2.3.3.2.2. Malignant ovine theileriosis:

The disease was first described in details from Sudanese sheep exported to Egypt (Mason 1914 cited in Leemans, 2001), and then, a similar disease was recorded as being of common occurrence in Egyptian sheep (Littlewood, 1916, cited in Leemans 2001). The parasite was first described as *T. ovis* (du Toit, 1918 cited in Leemans, 2001), then as *T. hirci* (Dschunkovsky and Urodschevich, 1924) and eventually designated as *T. lestoquardi* (Morel and Uilenberg, 1981). In the Sudan, the disease was first reported in Khartoum State by (Tageldeen *et al.,* 1992) and then in northern Sudan by El Ghali *et al.* (1995).

1.2.3.3.2.2.1. Transmission:

The ticks of the genus *Hyalomma* had been suspected to be responsible for transmission of *T. lestoquardi* (Mazlum, 1970), and their role as a vector was later demonstrated by transmission of *T. lestoquardi* from stage to stage through *H. anatolicum* (Hooshmand-Rad and Hawa, 1973b). This tick was associated with an outbreak of ovine theileriosis in the Sudan

(Tageldin *et al*., 1992, Latif *et al*., 1994). *H. anatolicum* appears to be the only reported vector for the transmission (Uilenberg, 1997), however, *Rhipicephalus* spp. (Sisodia and Gautum, 1983), *H. excavatum* group (Hooshmand-Rad and Hawa, 1973b, Hashemi-Fesharki, 1997), *Hyalomma impeltatum* (El-Azazy *et al*., 2001), and *R. sanguineus* (Razmi *et al*., 2003) have been suspected to be incriminated in the natural transmission of the parasite. Reports differ as to whether *T. lestoquardi* infections can be easily transmitted mechanically from one animal to the next through inoculation of infected blood as in the case of *T. annulata* infections of cattle (Sergent *et al*., 1945, cited in Leemans 2001). Whereas several researchers in India successded in the transfer of infection to susceptible sheep (Sisodia and Gautam, 1983), while other authors (Hawa *et al*., 1976, 1981 and Hooshmand-Rad, 1985) reported on difficulties in the mechanical transmission of the parasite.

1.2.3.3.2.2.2. Distribution:

Malignant theileriosis of sheep has been recorded in many countries: Turkey (Sayin, 1997), Iran (Hashemi-Fesharki, 1997, Spitalska *et al*, 2005), Irag (Latif, 1977), India (Sisodia, 1981), Sudan (Tageldin *et al*. 1992), Saudi Arabia (El-Metenawy, 1999 and El-Azazy 2001) but the parasite has not been reported from Jordan (Sherkov 1977) and from Israel (Pipano 1991). More precise data are needed on the geographic distribution of the disease and its epidemiology (Uilenberg, 1997).

1.2.3.3.2.2.3. Symptoms:

Neitz, 1956, Hooshmand-Rad and Hawa, 1973a, El Hussein *et al*., 1998, Leemans *et al*., 1999a described the symptoms of malignant

theileriosis of sheep and goats. The symptoms include generalized enlargement of superficial lymph nodes, high fever, listlessness, anorexia, emaciation, intermittent diarrhoea or constipation and loss of conditions as the most prominent symptoms of *T. lestoquardi* infections. Pale and icteric mucous membranes were occasionally noticed. Marked fall in WBCs, RBCs counts often result in leukopenia that last for several days, anaemia for more than 3 weeks and fall in blood PCV were reported.

1.2.3.3.2.2.4. Clinical signs and pathology:

The disease in sheep is highly pathogenic (Leemans *et al*., 1999a,b), in most cases leading to high morbidity and mortality rates even in indigenous sheep (Hooshmand-Rad and Hawa, 1973a, Tageldin *et al*., 1992, El Hussein *et al*., 1998). Clinical signs of the infected animals include leukoproliferation in the early stage of the disease, which is later followed by a leuko-destructive phase leading to leukopenia and anaemia (Irvin and Morrison 1987). The reported prominent macroscopic lesions of *T. lestoquardi* infection to sheep are hyperplasia and oedema of lymph nodes, splenomegaly, a yellowish enlarged liver and the lungs are frequently oedematous. However, the typical haemorrhagic ulcers of the abomasums seen in *T. annulata* infection of cattle were notably absent (Hooshmand-Rad and Hawa, 1973a).

1.2.3.3.2.2.5. Pathogenesis:

Little is known about the mechanisms involved in the pathogenesis of *T. lestoquardi* infection (Leemans *et al*., 2001). The pathogenicity is primarily attributed to infection of the host's leukocytes by macroschizonts, which proliferate synchronously with their host cells and their multiplication

and invasion of host's lymphoid organs accompanied by severe clinical signs and death in per-acute form of the disease. Severe tissue destruction and pulmonary oedema leading to respiratory failure are the main cause of death (Uilenberg 1981b; Irvin and Morrison 1987). *Theileria* schizont-infected cells are known to be immortalized and the transformation is known to be an entirely reversible mechanism (Dobbelaere and Heussler, 1999). However, *T. lestoquardi* appear to transform mainly major histocompatibility complex class II-positive cells (Ahmed *et al*., 1999, Preston *et al*., 1999). It was later established that *Theileria* manipulates the molecular and signal transduction pathways of the host cells by different means (Dobbelaere and Kuenzi 2004, Dessauge *et al*., 2005). Furthermore, schizont-infected cells undergo a wide range of phenotypic alterations including production of a number of cytokines, surface receptors, adhesion molecules, and presentation of infection-associated antigens (Ahmed *et al*., 1999; Dobbelaere and Heussler 1999) and tumour necrosis factor alpha was also involved in the pathogenesis of the disease (Ahmed, 2002). Cytokine is a potent inducer of fever and may play a role in anaemia, muscle wasting and necrosis (Ahmed, 2002).

1.2.3.3.2.2.6. Immune responses:

Theileria lestoquardi immune response has not been a subject of specific studies (Leemans *et at.,* 2001). So far, little is known about the susceptibility of sheep and goats and of the various breeds of each species (Uilenberg, 1997). The phenotype of ovine cell lines infected with *T. lestoquardi* was studied by cytometric analysis and the results revealed that, this parasite infects the same cell types in sheep as *T. annulata* in cattle, notably monocytes/macrophages and B cells (Leemans, *et al*., 2001). In

experimental study of sheep infected with *T. lestoquardi* cell lines, the parasite antibodies were detected 15 days after inoculation, raised to 32-64-fold by 30 days later, and substantial antibody titres were still observed on day 90 post infection (Leemans *et al.*, 1997). However, it is known that animals that survive infection are resistant to challenge infection, and indigenous sheep and goats acquire immunity at an early age (Hooshmand-Rad and Hawa, 1973a). However, the mechanisms involved in the pathogenesis of *T. lestoquardi* infection and the sheep host immune response has not been adequately studied.

1.2.3.3.2.2.7. Diagnosis:

Routine field diagnosis of *T. lestoquardi* infection is based on the combination of the host specificity, transmission mode, vector competency, epidemiololgical data, clinical signs or pathological findings with morphological demonstration of the parasite stages in blood/or organ smears. The provisional diagnosis includes case history, clinical signs, postmortem findings and geographic distribution of the disease and vector (Anon, 2000). In the last decade, a considerable progress was made in the development of diagnostic tests for tick and tick borne-diseases, but their cost has limited their use mainly to international research and development projects, and to a few national veterinary laboratories (Minjauw and McLeod, 2003).

1.2.3.3.2.2.7.1. Microscopic examinations:

In developing countries, including the Sudan, most veterinary laboratories are able to perform direct diagnosis, but few are equipped for the more sophisticated indirect methods. The direct method involves

identifying the parasite in Giemsa's-stained blood smears or lymph-node biopsy samples. This method requires only a light microscope and can be performed in a very simple field laboratory. It allows identification of all major tick-borne parasites and is the method of choice for the early treatment of their associated diseases including *T. lestoquardi* infection. The major problems associated with direct microscopy are its low sensitivity when parasitaemia is low, and the level of expertise required for differentiating species on the basis of morphology. The method is good for clinical diagnosis, very subjective for determining the prevalence of pre-immunity and/or carrier status and detection of mixed infections, therefore, of little use for epidemiological surveys.

1.2.3.3.2.2.7.2. Serology:

The representative serological antibody detection tests, commonly used in identification of *T. lestoquardi* are indirect fluorescent antibody (IFA) test; and the enzyme-linked immunosorbent assay (ELISA). Tests based on antibody detection would be of little value, since the clinical signs of *T. lestoquardi* as with other pathogenic *Theileria*, appear before antibodies can be detected. In addition, maternal immunity would produce false positive results. Furthermore, lack of determination of antibodies in carriers may be the result of long-term infection (Burridge *et al*., 1974, Leemans *et al*., 1999a). *T. lestoquardi* and *T. annulata* exhibit astonishing similarities with regard to serology and morphology (Brown *et al*., 1998), beside the capability of their *H. anatolicum* vector to transmit both species in the Sudan (Mustafa *et al.*, 1983), however, differential diagnosis for between these species is so difficult and very subjective.

1.2.3.3.2.2.7.2.1. Indirect fluorescent antibody test:

The indirect fluorescent antibody (IFA) test either based on the use of schizont or piroplasms antigens to detect circulating antibodies against bovine *Theileria* species has been applied (Morzaria *et al*., 1977, Irvin and Morrison, 1987). IFA test based on *T. lestoquardi* schizont antigen has been developed and used either to follow up immunization or in epidemiological survey on theileriosis of sheep (Hawa *et al*., 1981, Leemans *et al*., 1997). In the Sudan detection of antibodies against *T. lestoquardi* infection using IFA test was applied in epidemiological survey (Salih *et al*., 2003, Taha *et al.,* 2003). However, the vast limitations of IFA test hinder its routine use in large scale epidemiological investigations. These limitations are mainly due to its tediousness, the subjectivity of the results obtained, and the absence of means of standardization, thus making the test unsuitable for automation. A major obstacle for the use of IFA test might be the reported cross-reactivity between other sheep and cattle tick borne-diseases (Leemans *et al*., 1997). Therefore, the use of this test is not always satisfactory for diagnosing infections in regions where animals are infected with different piroplasms (Papadopoulos *et al*., 1996, Leemans, *et at*., 1997).

1.2.3.3.2.2.7.2.2. Enzyme-Linked Immunosorbent Assay:

Enzyme-Linked Immunosorbent Assay (ELISA) for serological detection of antibodies against *Theileria* species in cattle have been extensively used (Bakheit *et al*., 2004, Salih *et al*., 2010). To date, very few works in ELISA diagnosis of small ruminant infections were reported. Development of *Theileria* piroplasms antigen for ELISA has been proved as an effective technique for detection of antibodies in sheep infected with *Theileria* sp. (China) and hence to constitute a valid additional

serodiagnostic technique for use in epidemiological investigations (Gao *et al*., 2002). Later, Miranda *et al*. (2006) used *Theileria* China heat shock protein 70 (TcHSP70), developed a recombinant indirect ELISA for diagnosis of *Theileria* sp (China) and the test resulted in 94.3% and 89.5% sensitivity and specificity, respectively in comparison with the merozoite homogenate ELISA. Recently in China, clone-9 gene was partially recombinantly expressed was used for development of an indirect ELISA for the detection of circulating antibodies in sera of *T. uilenbergi*-infected sheep and was evaluated by testing 101 field samples collected from an endemic area in China (Abdo, 2010). In fact, the new recombinant protein-based ELISA for the diagnosis of *T. lestoquardi* is documented (Bakheit *et. al.,* 2006). This test was based on an immunogenic *T. lestoquardi* schizont surface protein (Clone-5) and it results, however, demonstrated a satisfactory performance with 94.6 and 88%, sensitivity and specificity, respectively, when countertested with the standard IFA test. Furthermore, the test was validated by testing field samples collected from Northern Sudan (Bakheit *et. al.,* 2006), and currently it's field applications in Sudan is going on (Salma K., 2010, personal communication).

1.2.3.3.2.2.7.3 Molecular biology techniques:

The advances in molecular biology enabled genotypic characterization, and have proved very useful for the identification and classification of many haemoparasite species of the *Theileria*/*Babesia* group (Caccio *et al*., 2000), the *Ehrlichia*/*Anaplasma* group (Arens *et al*., 2003) or the Rickettsia group (Christova *et al*., 2003). These molecular techniques offer high sensitivity and specificity compared to morphological examination methods and serological tests (Papadopoulos *et al*., 1996).

1.2.3.3.2.2.7.3.1. Polymerase chain reaction:

Recently, polymerase chain reaction (PCR) is the most commonly used molecular technique to detect ovine theileriosis in epidemiological studies (Aktas *et al.,* 2005a, Altay *et al*., 2008). PCR has been developed using specific primers to amplify the *T. lestoquardi* fragment of the gene coding for a 30-kDa merozoite surface gene to study and to diagnose the parasite in both sheep and goats and in tick vectors (Kirvar *et al*., 1998). However, this technique is more sensitive and specific than other conventional methods (d'Oliveira *et al*., 1995, Almeria *et al*., 2001) in addition; this PCR is able to differentiate between *T. annulata* and *T. lestoquardi* in *Hyalomma* vector and in sheep and goats (Leemans *et al*., 1999b). On other hand, its sensitivity is too poor to detect subclinical infections and very subjective, especially in mixed infections.

1.2.3.3.2.2.7.3.2. Reverse line blot:

In order to overcome these limitations of PCR, a reverse line blot (RLB) assay was developed for the detection and discrimination between different piroplasm species (Gubbels *et al*., 1999). RLB based on amplification of a fragment of the 18S, 16S ribosomal DNA from virtually all species of *Theileria*/*Babesia* and of *Ehrlichia* respectively. The oligonucleotide probes used in this assay is reacted with their corresponding species and almost did not cross-react with other species. The important aspect of this diagnostic method is reliable, sensitive and specific for the identification and discrimination between different sheep tick-borne diseases. However, this test is a powerful tool and practical assay, since it is able to detect extremely low parasitemia rates and simultaneously identify *Theileria* and *Babesia* species (Gubbels *et al*., 1999). Later, various RLB

assays have been developed for detection of rickettsial and protozoan species infecting cattle and small ruminants (Georges *et al*., 2001, Bekker *et al*., 2002, Sparagano *et al*., 2003, Schnittger *et al.,* 2004). Further more, the small subunit ribosomal RNA gene (18S RNA gene) has been successfully carried out to improve detection, identification and classification of previously known *Theileria* and *Babesia* species (Sparagano *et al*., 2006), and several novel unknown *Theileria* and *Babesia* species (Altay *et al*., 2007, Oosthuizen *et al*., 2009). The disadvantages of this molecular method, however, are expensive and require sophisticated laboratory equipment, complex protocol and involve hybridization to achieve higher sensitivity. Therefore, development of more simple and applicable PCR-based systems suitable for routine diagnosis and useful for detecting mixed infections is needed.

1.2.3.3.2.2.7.3.3. Restriction fragment length polymorphism:

Restriction fragment length polymorphism (RFLP) of the PCR products allowed differentiation between *T. lestoquardi* and *T. annulata* (Spitalska *et al*., 2004) and between three different *Theileria* species (*T. annulata, T. lestoquardi* and *T. ovis*) and seems to be useful for differentiation of other species such as *T. separata* and *Theileria* spp. China (Bami *et al*., 2009). The high sensitivity and specificity of PCR–RFLP method was recently proved and appeared as a very powerful tool to detect extremely low parasitemia rates and discriminate between ovine *Theileria* species in mixed infections (Bami *et al*., 2009). Such an assay is therefore superior to PCR-based methods designed to detect a single species in situations including the routine screening of samples in a veterinary diagnostic laboratory.

1.2.3.3.2.2.7.3.4. Loop-mediated isothermal amplification:

Loop-mediated isothermal amplification (LAMP) has been successfully developed for detection of sheep theileriosis (Liu, *et al.,* 2008). The technique revealed a rapid method with high specificity and efficiency under isothermal condition using a set of four specifically designed primers that can recognize six distinct sequences on the target gene. This method has, also, been developed for detection of *Babesia* spp. (He *et al*., 2009). Recently, in the Sudan, the LAMP was optimized to detect *T. lestoquardi* in sheep blood (Salih, D., 2010, personal communication), and their applications in epidemiological investigations is currently in progress (Bakheit M, 2010, personal communication). Since early detection of infection is important if treatment is to be successful, the development of cheaper and faster diagnostic tests could have a significant impact on the control of malignant theileriosis of sheep and goats, as yet no diagnostic tests are available to detect an early infection at the field level.

1.2.3.3.2.2.8. The relationship between *T. lestoquardi* and *T. annulata*:

Many authors studied the relationship between *T. lestoquardi* and *T. annulata* and concluded that they exhibit a strong serological cross-reactivity (Leemans, *et al*, 1997), similarities with regard to morphology (Allsopp, *et al*., 1994, Brown, *et al*., 1998), share the same vector (Uilenberg *et al.,* 1980) and their geographic distribution tends to overlap (Hooshmand-Rad and Hawa, 1973, Neitz, 1957). At least two immunogenic schizont proteins with an approximate molecular weight of 73 and 42 kDa are shared between *T. annulata* and *T. lestoquardi* (Spooner *et al*., 1988, Namavari *et al*., 2008). Phylogenetically, *T. lestoquardi* is more closely related to *T. annulata* than it is to *Theileria parva* (Katzer *et al*., 1998). However, phylogenetic analysis

have been conducted to test the relationship of *T. lestoquardi* to other *Theileria* species. These were based on small subunit ribosomal RNA (srRNA) and major merozoite piroplasms surface antigen (mMPSA) gene sequences from *T. lestoquardi.* Various different *Theileria* species and *Babesia* species indicated that *T. lestoquardi* is phylogentically more closely related to *T. annulata* than other sheep and cattle *Theileria* and *Babesia* spp. (Schnittger *et al*., 2003). Also, in RLB study, targeting the 18S rRNA gene using *T. lestoquardi* and *T. annulata* probes their sequences linked showed the two species having important species-specific probes between them (Sparagano *et al*., 2006). On the other hand, the srRNA sequences of *T. lestoquardi* were found to be strikingly similar to that of *T. annulata* with an identity value of 99.7% (Schnittger *et al.*, 2000a). However, *T. lestoquardi* is closely related to *T. annulata* (Katzer *et al*., 1998). Thus, they suggested that *T. annulata* evolved relatively recently from a common ancestor with *T. lestoquardi.*

1.2.3.3.2.2.9. Treatment:

Little is known about the efficacy of the theilericidal drugs used for treatment of *T. annulata* and *T. parva* infection of cattle against *T. lestoquardi* infection in sheep and goats. The chemotherapeutic efficacy of a number of compounds including parvaquone (Clexon) and buparvaquone (Butalex) have been effectively tested for the treatment of the disease (Hooshmand-Rad, 1989). Although some of these drugs are likely to be effective (El Hussein *et al*., 1993, Hashemi-Fesharki, 1997), but they are not easily and quickly eliminated from the body of animals (McHardy, *et al*., 1985) which can constitute a public health/hazard if milk and meat of treated animals are consumed by humans. From an economic point of view, such

drugs may be too expensive and in most cases are unaffordable for treatment of these animal species. To overcome the disadvantages of the chemical treatment, however, medical plant for the treatment of natural ovine malignant theileriosis was tried. The therapeutic effect of the alkaloids of the plant (*Peganum harmala*) has been investigated for the treatment of malignant theileriosis of sheep (Mirzaiedehaghi *et al.,* 2006). A Chloroform extract was prepared from the stem and leaves of this plant and was administered intramuscularly to *T. lestoquardi* naturally infected sheep at a dosage rate of 5 mg/kg body weight for 5 days. The parasite examination and the therapeutic effect of the extracted plant were followed. In 12–20 days after the commencement of the treatment schizonts and piroplasms were not detected in 65/100 (65%) of the infected sheep, in addition fever and other clinical signs disappeared (Mirzaiedehaghi *et al*., 2006).

1.2.3.3.2.2.10. Control:

Following the successful cultivation of *T. lestoquardi* schizont infected ovine cell (Hooshmand-Rad and Hawa, 1975) and the discovery that the parasite, like *T. annul*ata, could be attenuated while their immunogenicity is maintained following prolonged culture in vitro. Immunization of sheep with such cells provided sheep with solid immunity (Gill *et al*., 1978), later this immunoprophylaxis trial has been successfully carried out in Iraq and in Iran (Hawa *et al.,* 1981, Hooshmand-Rad, 1985, Hashemi-Fesharki *et al.,* 1997). In the last decade, the molecular characterization of sporozoites lestoquardi antigen-1 (SLAG-1) protein can be used for inclusion in a sub-unit vaccine against *T. lestoquardi* infection of sheep and goats (Skilton *et al.,* 2000). Recently, detection and identification of parasite vacuolar H+ATpase as potential molecular marker of attenuated

T. lestoquardi-infected cell line can possibly be used in vaccine trials (Ali *et al*., 2008). Many parts of the Sudan are affected by Malignant Ovine Theileriosis. Eradication is not a practical proposition due to environmental, managerial and resource constrains. Accordingly, controlling and living with tick-borne disease is the only practical option. Currently, *T. lestoquardi* as well as *T. annulata* are controlled mainly by applications of acaricides to tick- infested hosts and treatments of infected animals are practiced on household.

More resent in the Sudan, attenuation of schizonts infected lymphocytes cell lines by serial passage for production of *T. lestoquardi* vaccine was recently undertaken (Bukhari, A. 2010, personal communication).

1.2.3.3.2.2.11. Economic impact:

Due to the economic loss they cause, the most prominent representatives of *Theileria* parasites are the cattle-infecting species *T. parva* and *T. annulata*, which are the causative agents of East Coast fever and tropical theileriosis, respectively (Mehlhorn and Schein, 1993). Sheep which are severely infected usually die unless they are treated. Indigenous sheep are at risk in situation where they are subjected to intensive tick control, or when they are moved from disease free to endemic areas and heavy losses due to *T. lestoquardi* infection are reported (Hooshmand-Rad, 1974, Tageldin *et al*., 1992). Case-fatality rate can vary from 40 to 100% during outbreaks (Freidhoff, 1997). In the Sudan, the disease is widely distributed in sheep grazing areas of the country with a prevalence rate reaching 23% (Salih *et al.,* 2003). Under endemically stable conditions in Northern Sudan 100% mortality in outbreaks were reported (Latif *et al*., 1994, El Ghali and

El Hussein, 1995). Globally, high morbidity and mortality rates among local sheep in Iraq (Hooshmand-Rad and Hawa, 1975), in Iran among lambs (Hooshmand-Rad, 1977) and in exotic sheep (Gautam *et al*., 1975) were reported. The high economic importance of the disease is expected, especially in countries where export of sheep and sheep products is a major component of their foreign income. Animal which recover from *T. lestoquardi* may suffer from weight loss, produce low milk yield and may experience reduce fertility and delay in reaching maturity. These animals also remain carrier and may disseminate infection. But the extensive studies on economic impact as well as these types of production losses in the Sudan have not been yet undertaken.

CHAPTER TWO
MATERIALS AND METHODS

2.1. Detection of sheep tick-borne pathogens:

An epidemiological study for detection, identification and discrimination among different sheep theileriosis, babesiosis and ehrlichiosis in the Sudan was carried out as follow.

2.1.1. Blood samples collection:

Blood spots on filter paper from 219 apparently healthy sheep belonging to 13 flocks from six distinct geographic localities (Table 2.1, Map 2.1) in the Sudan were collected.

2.1.2. Deoxyribonucleic Acid extraction:

DNA was extracted from blood spots using the QIAamp DNA Extraction Kit (QIAGEN, Southern Cross Biotechnologies), following the manufacturer's protocol, briefly: from dry blood spots, 5 circles (diameter) were punched out using a paper puncher, placed in a 1.5 ml tube and was labeled. In each tube 180 µl of buffer ATL, 20 µl of proteinase K (20 mg/ml) were added and the tubes were incubated at 56° C for 60 min. Each tube was thoroughly vortexed, 200 µl buffer AL added, vortexed and was incubated at 70°C for 10 min. A volume of 200 µl ethanol was then added to the sample and was mixed. The whole mixture was transferred into QIAamp mini column, 500 µl buffer AW1 were added to the column, centrifuged for 1 min at 12000 rpm and the flow through was discarded. To remove the residual AW buffers, the column was placed in a new collection tube and was centrifuged for 1 min at 12000 rpm.

Table 2.1. Localities where blood samples were collected from sheep in 2008-2009.

Locality	No. of samples	No. of flocks	E (degree/second)	N (degree/second)
Atbara	57	3	34º 02′	17º 40′
Khartoum North	36	1	32 32	15 48
Kosti	30	3	32 40	13 10
Medani	35	2	33 30	14 31
Damazin	33	2	34 18	11 52
Nyala	28	2	24 55	12 05
Total	219	13		

Map 2.1. Localities(●) where blood samples were collected in Sudan, in 2008-2009. Modified from International Committee of the Red Cross (ICRC), 2004.

Each column was then placed in 1.5 ml tube, 100 µl buffer AE was directly added onto the column, centrifuged for 1 min at 12000 rpm and was incubated at 25ºC for 2 min. The DNA was eluted and stored at -20°C.

2.1.3. Reverse Line Blot hybridization assay:

In this study, the use of Reverse Line Blot (RLB) Hybridization assay for amplification of a fragment of the 18S, 16S ribosomal DNA from virtually all species of *Theileria*/*Babesia* and *Ehrlichia* respectively, are described for differentiation of various tick-borne diseases infecting sheep in the Sudan. The procedure of RLB is described in details in Gubbels *et al*., (1999).

2.1.3.1. Preparation of the membrane:

Briefly, a Biodyne C blotting membrane (Pall Biosupport) orientation was marked, activated at 25°C by incubation in freshly prepared 10 ml solution of 16% EDAC (Sigma), washed in demineralized water and was placed on a support cushion in clean MN45 miniblotter apparatus (Isogen). The screws were tightened turned hand and the miniblotter was put in vertical position. The residual water was removed from the slots by aspiration (vacuum). The specific Oligonucleotide probes (Table 2.2) containing an N-terminal (TFA)-C6 aminolinker (Isogen) were diluted to give a concentration of 100 to 800pmol/150 µl in 500mM NaHCO3 (pH 8.4) solution. Each slot was filled with 150 µl diluted probes. The first and the last slots were filled with drawing ink. The miniblotter was incubated at room temperature for 2 min. The probe solution was removed by aspiration as they were applied. The membrane was removed from the blotter with a pair of forceps, placed in a washing tray and was inactivated by 9 min in 100 ml freshly made 100mM NaOH solution at 25ºC.

Table 2.2. Genus and species-specific RLB probes used.

Oligonucleotide probe	Sequence (5˜–3˜)
Ehrlichia/*Anaplasma* catch-all	GGG GGA AAG ATT TAT CGC TA
Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagoctophilum	TTG CTA TAG AGA ATA GTT AG
Anaplasma bovis	GTA GCT TGC TAT GAG AAC A
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Ehrlichia chaffiensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia sp. Omajienne	CGG ATT TTT ATC ATA GCT TGC
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Theileria/*Babesia* catch-all	ATT AGA GTG TTT CAA GCA GAC
Theileria catch-all	TAA TGG TTA ATA GGA RCR GTT G
Babesia catch-all1	ATT AGA GTG TTT CAA GCA GAC
Babesia catch-all2	ACT AGA GTG TTT CAA ACA GGC
Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia canis	TGC GTT GAC GGT TTG AC
Babesia divergens	ACT RAT GTC GAG ATT GCA C
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G
Babesia leo	ATC TTG TTG CCT GCA GCT T
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia occultans	CCT CTT TGG CCC ATC TCG
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C
Babesia vogeli	AGC GTG TTC GAG TTT GCC
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria separata	GGT CGT GGT TTT CCT CGT
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T

Then, it was washed in 100 ml 2xSSPE (20x SSPE is 3.0M NaCl, 0.2M $NaH2PO_4$, and 0.02M EDTA [pH 7.4])/0.1% Sodium Dodecyl Sulfate (SDS) solution at 60° for 5 min under gentile shake in shaker (Labtec, Germany), placed in plastic tray, 20mM EDTA (pH 8) were added and was stored at 4°C until used.

2.1.3.2. Polymerase Chain Reaction:

For the amplification of the V4 variable region of the parasite 18S rRNA gene the following PCR protocol was performed. In a 0.2 ml PCR tube, 12.5 µl UDG-mix (Platinum® Quantitative PCR SuperMix, Invetrogen™ , USA), 0.25 µl of each RLB-Reverse and forward primers (RLB F_2 5˜-GAC ACA GGG AGG TAG TGA CAA G-3˜ and biotinlabeled RLB R_2 5˜-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3˜), 7.0 µl water and 5 µl DNA were added. Amplification was performed in automated thermocycler according to the *Babesia*/*Theileria* touchdown PCR programme (Table 2.3).

2.1.3.3. Blotting of the Reverse Line Blot membrane:

Subsequently, the membrane was placed perpendicular to its previous orientation into the miniblotter. Hybridization of the RLB membrane with the PCR products was proceeded as follows: Twenty five microlitres of PCR products were diluted in 130 2xSSPE/0.1% SDS to a final volume of 155 µl, denatured for 10 min at 99.9°C and subsequently chilled on ice to prevent annealing of the denatured strands. In the meantime, the RLB membrane was rinsed at 25°C in a 2x SSPE/0.1% SDS solution. The denatured samples were loaded onto the slots of the miniblotter and were incubated at 42°C for 60 min.

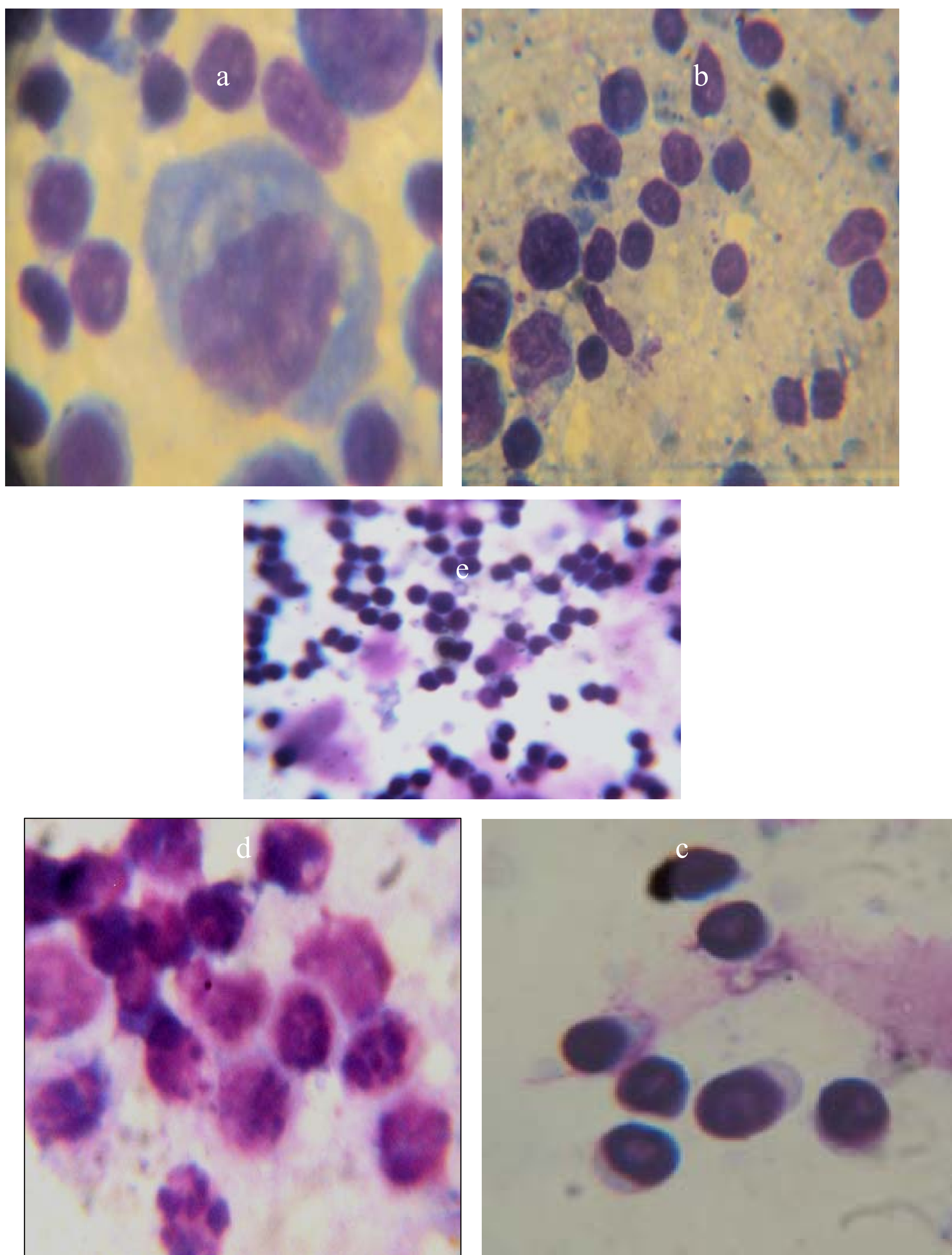


Plate 3.1. Photomicrograph of lymph node biopsy smears (a-e) showing four different *Theileria* markers (a,b,c,d) shortly before detection of schizonts and normal lymphocytes (e) (Giemsa's stain x 100).

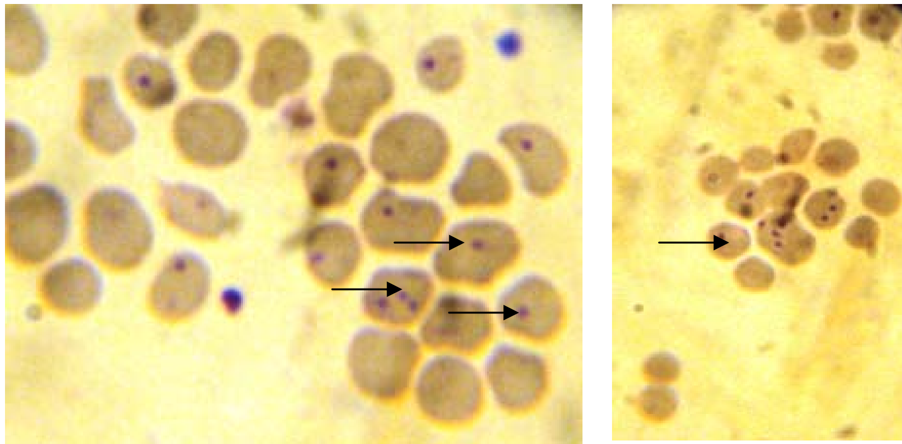


Plate 3.2. Photomicrograph of thin blood smear showing multiple *T. lestoquardi* piroplasms inside the erythrocytes (Giemsa's stain x 100).

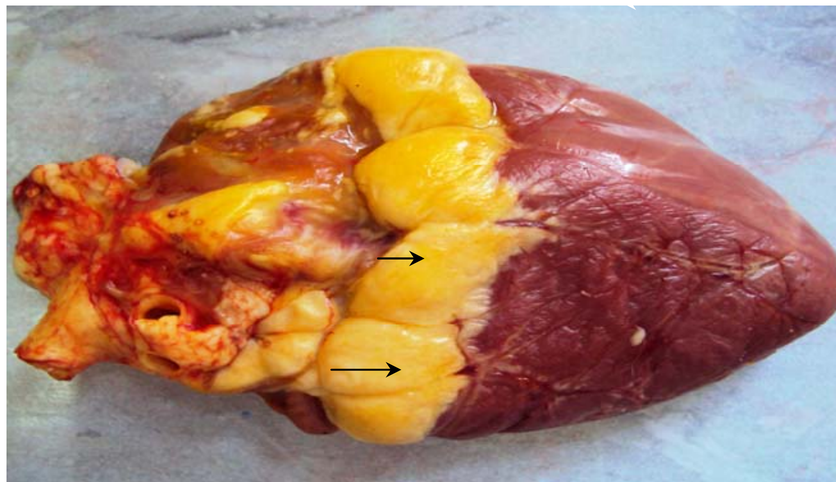


Plate 3.3. Photograph of hert of (animal No. 33) showing yellow fat (arrows), indicating jaundice during the sub-acute course of *T. lestoquardi* infection.

Table 2.3. The *Babesia*/*Theileria* touchdown PCR thermocycler programme.

Number of cycles	Time (min/second)	Temperature (°C)
1cycle	3min	37
1 cycle	10min	94 *
	20	94
2	30	67
	30	72
	20	94
2	30	65
	30	72
	20	94
2	30	63
	30	72
	20	94
2	30	61
	30	72
	20	94
2	30	59
	30	72
	20	94
40	30	57
	30	72
1	7 min	72
Kept at 4°C for ∞		

To activate the uracil DNA glycosylase (UDG), thereby degrading previous PCR amplicon contamination possibly present

* To inactivate the UDG.

Thereafter, the PCR products were aspirated and the membrane was washed twice in preheated 2xSSPE/0.1% SDS in water path (Labcon, Germany) at 50°C for 10 min under gentle shaking. Subsequently, the membrane was treated with 12.5 µl (1:4000) diluted peroxidaselabelled streptavidin conjugate (Roche Diagnostic GmbH, Germany) in 2x SSPE/0.1% SDS at 42°C for 30 min under gentle shaking. The membrane was washed twice in preheated 2x SSPE/0.1% SDS at 42°C for 10 min under gentle shaking and twice for 5 min each time in a 2xSSPE solution at 25°Cunder gentle shaking in a shaker (Heidolph, Germany). The membrane was placed in 10 ml (5 ml each of ECL1 and ECL2) of Enhanced Chemiluminescence (ECL) detection fluid (Perkin®, USA) and incubated for 1 min at 25°C, placed between two clean overhead sheets in an exposure cassette (Hyper cassette TM, UK) and was exposed to X-ray film (Kodak Scientific images film, USA) for 10 min. The X-ray film was developed and fixed in developer and fixer (AXIM, South Africa).

2.1.3.4. Stripping of the Reverse Line Blot membrane:

The PCR products were stripped from the membrane by two wash steps for 30 min each time at 80°C in a 1% SDS solution. The membrane was rinsed in 20mM EDTA solution (pH 8.4) for 15 min and was stored in fresh Ethylene Diamine Tetra Acetic Acid (EDTA) at 4°C until used.

2.2. Malignant Ovine Theileriosis:

The experiment was design to study the limit of resistance, susceptibility and pathogenesis of *T. lestoquardi* infecting three different

sheep eco-types originating from known disease free districts, transferred to an endemic focus and maintained under natural ticks challenge.

2.2.1. Selection of *T. lestoquardi* free sheep:

A total of 45, males, 4 to 5 months old, apparently healthy sheep and clinically free from any abnormalities were purchased. Sixteen Garage eco-type from Upper Nile (Renk), 14 Watesh from White Nile (Kosti) and 15 Desert from Kordofan (Umrwaba) States. The animals were transported by truck and were maintained in Khartoum for two weeks. They were sprayed with a chemical acaricide twice a week, using Amitraz (1:1600) diluted in tap water. Amprolium Hydrochloride (Amprocidia, Jordan) at dose 0.5-2 mg per head as anticoccidia and Oxytetracycline antibiotic injections (Norbrook, Irland) at dose 1.5-4 ml/50 kg were daily given for 5 consecutive days. The animals were also de-wormed with Alvenax drench (star laboratories LTD, Lahore, Pakistan) at dose of 1 ml per 3kg body weight and given tonic (Multivitamin and minerals) twice a week. They were fed on fresh grasses and concentrate rations. Serum from each sheep was tested for antibodies against *T. lestoquardi* using the indirect fluorescent antibody (IFA) test (section 2.2.8.1). The sero-negative animals were considered susceptible to *T. lestoquardi* and were subsequently used in the natural infection.

2.2.2. Selection of *T. lestoquardi* endemic focus:

Atbara town is a known endemic focus for *T. lestoquardi*. In this town, cattle, sheep and goats are kept under small-scale farming systems and cultivation of crop is practiced along the main water canal, 5-10 kilometers North Atbara town. In these small-scale animal farms, 6-10 cattle and/or 10-20 sheep and goats are kept in each small farm. Livestock movement is

restricted in terms of space and time. Cattle are kept in homestead until midday, after which they are allowed restricted grazing chiefly around the cultivated farm areas. Sheep and goats on the other hand, graze on grass and agricultural residues along small water canals, tethered in fields left uncultivated or browse bushes and trees and then returne to homestead just before sun set. The livestock kept in this farm system are obviously exposed to ticks throughout their lives in a situation where tick controls by regular acaricide spraying or by other control alternatives are rarely practiced. Therefore, incidence of malignant ovine theileriosis regularly reported in Atbara (Atbara Veterinary Office Record, 2010). The local sheep (Baladi eco-type) were invariably expected to *H. anatolicum* infestations and *T. lestoquardi* infections. A farm with a history of recently diagnosed malignant ovine theileriosis and abundant *Hyalomma anatolicum* was selected, and all the sheep kept were tested for *T. lestoquardi* infections. Eleven 6-12 months old, apparently healthy sheep and positive to *T. lestoquardi* (piroplasms and/or schizonts) were selected and were subsequently used as control animals in the present study.

2.2.3. Maintenance of animals:

The test animals were transported by truck to the selected endemic area and were kept on a private farm at Atbara (North 34º 02′, East 17 º 40′) together with the control selected group. They were housed in a pen constructed with brick and shaded with trees. Animals were neck-tagged for identification and were fed with fresh Berseem (clover) and Sorghum (biclover) offered twice daily. In addition, all animals were allowed to daily graze outdoors on crop residues for three hours. Drinking water was offered *ad libitum*.

2.2.4. Clinical studies:

Throughout the study periods, all the experimental animals were daily monitored for clinical signs for 12 weeks from day of entry (day zero) to the endemic area and the following parameters were recorded: Daly rectal temperature, pulse and respiratory rates, general condition, appetite, faeces consistency, presence of cough, conjunctival and nasal discharge and the size of superficial lymph nodes. The total numbers of infesting ticks and body weights were weekly recorded.

2.2.4.1. Parasitological evaluation of the infection:

The natural infection of sheep with *T. lestoquardi* piroplasms and schizonts were detected in sheep blood by the conventional diagnostic methods including:

2.2.4.1.1. Thin blood films:

Capillary blood samples were obtained by pricking the ear vein with a needle; standard thin blood films (McCosker, 1975) were prepared in duplicate slides, air dried and fixed in absolute methanol for 2-3 min. The slides were then stained in 10% Giemsa's stain (solution 1: 9 in buffer distilled water at pH 7.2) for 45 min, washed and air dried. They were thoroughly examined under light microscope using oil immersion. The numbers of parasitized cells in 2000 red blood cells were counted on each slide and the results were expressed in percentage (parasitaemia)

2.2.4.1.2. Lymph node biopsy smears:

Examination of biopsy smears was carried out whenever there was an enlargement of any of the superficial lymph nodes, (parotid, pre-scapular, or pre-crural lymph nodes). The enlarged lymph node was immobilized between two fingers, punctured by a 1.8 gauge needle and lymph node tissues were aspirated with a disposable syringe fixed to the needle. The syringe was, then, withdrawn and expressed forcibly onto a microscope slide and the aspirates were spread. The smear was air dried, fixed and stained with Geimsa's stain as mentioned above. The results were expressed in percentages (parasitosis).

2.2.4.2. Blood examination for haematological changes:

Different haematological values of the test animals as the result of natural infection were determined and were compared with those haematological changes of the control animals.

2.2.4.2.1. Blood collection:

Weekly, 5 ml whole blood was withdrawn from jugular vein of each animal at 8:00 a.m., immediately was positioned 1 ml in 2 ml eppendorf tube containing anticoagulant (EDTA) and 4 ml in plain tube (no additives). The blood samples anticoagulant were immediately used for the determination of the haematological values On the other hand, the blood without anticoagulant was processed for serum collection, kept at - 20ºC and later used for biochemical analysis.

2.2.4.2.2. Blood values:

For the determination of haemoglobin (Hb) concentration, packed cell volume (PCV), RBCs, and WBCs count were determined as described by Schalm's Veterinary Haematology (Jain, 1986) with modifications suggested by Dcie and Lewis (1991).

2.2.4.2.2.1. Determination of packed cell volume:

Blood with EDTA samples were drawn into capillary tubes (Hawksley and Sons ltd., England), its one end was sealed off and centrifuged at 1200 rpm for 5 min using a microhaematocrit centrifuge (Hawksley and Sons ltd., England). The packed cell volume (PCV) was measured as a percentage of the whole blood using microhaematocrit scale provided by the manufacturer.

2.2.4.2.2.2. Determination of haemoglobin concentration.

The principle of the test was based on the amount of the globin (protein) that contain haem which gives the red colour to the blood, can be colourmetrically determined, and the optical density of the blood is proportional to the haemoglobin concentration (Hb). Hb was measured using a colourmeter (CIBA Corning, England). In this method Drabkin solution (0.05 potassium cyanide, 0.2 potassiumfricyanide and 1.0 gm sodium bicarbonate per one litre of distilled water) was used. The colourmeter was calibrated by adjusting the zero reading using the Drabkin solution as blank and the optical density of the standard (spinreact S. A. U., Santa Coloma, Spain) was read. A volume of 20µl of EDTA treated whole blood was added to 0.4 ml of Drabkin solution (1/20 dilution) in a test tube, mixed well, allowed to stand for 10 min and the optical density was read. The Hb

concentration was calculated refer to standard's reading and the results were expressed in gram/decilitre (g/dl) as follow:

$$\text{The Hb of the test blood} = \frac{\text{Test OD}}{\text{Standard OD}} \text{ X concentration of the standard.}$$

OD = Optical Density.

2.2.4.2.2.3. Red blood cells count:

Red blood cells (RBCs) were counted in an improved Neubauer Haemocytometre (Hawksley and Sons Ltd., England) using Hymen's solution (32 gm trisodium citrate and 10 ml of 40% formalin made up to one litre with distilled water) as described by Dacie and Lewis (1984). The blood sample was pipetted to the 0.5 mark and the diluent was drawn up to 101 mark. The pipette was held horizontal and rotated for three minutes to ensure uniform dilution. The fluid in the lower capillary stem was discarded off the counting chamber and microscopically examined. The RBCs count was expressed in million/microlitre (x10^{12} cells/L).

2.2.4.2.2.4. White blood cells count:

White blood cells (WBCs) were counted in an improved Neubauer Haemocytometre (Hawksley and Sons Ltd., England) using Turk's solution (2% glacial acetic acid tinged with gentian violet) as described by Dacie and Lewis (1984). The blood sample was pipetted to the 0.5 mark and mixed with the diluent and the process continued as previously described. The WBCs count was expressed in thousands/microlitre (X10^{9} cells/L).

2.2.5. Macro-pathological findings:

A necropsy was performed on test sheep that died and/or sacrificed within 30 min of death. The carcass was laid on its left side and a ventral midline incision from tip of the jaw to the pubis was made, the skin was dorsally reflected and the thoracic, abdominal and pelvic cavities were opened. All the viscera were examined and abnormalities were recorded. The brain was removed after ventral disarticulation of the atlanto-occipital joint.

2.2.6. Micro-pathological findings:

Examination of impression smears and histopathological sections were conducted to study the microscopic alterations and lesions of the disease.

2.2.6.1. Impression smears:

Impression smears from kidney, liver, lungs, spleen, heart, lymph nodes, intestine and brain were made on microscope slides and stained with Giemsa’s stain.

2.2.6.2. Histopathological findings:

For histopathological study, specimens were collected, fixed and processed using standard methods.

2.2.6.2.1. Specimen collection and fixation:

For histopathology, tissues samples were taken from Brain, kidney, liver, lungs, spleen, heart, lymph nodes, intestine and rumen as soon as the animal died and/or sacrificed (Bncroft *et al*., 1996). Briefly, tissue samples

cuts (1 cm^3) from each animal were collected in tissue containers, fixed in 10% neutral buffered formalin (100 ml formaldehyde, 900 ml tap water, 4gm sodium monophosphate, 6.5gm sodium diphosphate), labeled and were kept till used.

2.2.6.2.2. Specimen processing:

Small pieces 2-3mm thickness were trimmed prior to processing. The tissues were passed through series of alcohol solutions for complete dehydration, then through a clearing agent (xylol) and were embedded in paraffin. Five to six micrometer thick slices on a microtome sections were cut, floated on water, picked up on slides, dried and were stained with Hematoxylin and eosin (H&E) for light microscopy.

2.2.7. Biochemical analysis:

The liver function tests were conducted to assess the performance of the liver during the disease. However, the biochemical determination of serum constituents will help in monitoring the health status of sheep infected with *T. lestoquardi*. The total protein, albumin concentration, total bilirubin, urea, aspartate aminotransferase, alanine aminotrasferase and alkaline phosphatase were calourimetrically measured using Minder BS-300 biochemical analyzer (Shenzben minray Bio-Medical Electronic Co.LTD, Hamdurg, Germany).

2.2.8. Confirmatory tests:

2.2.8.1. Indirect fluorescent antibody test:

Indirect fluorescent antibody (IFA) test was used to detect the antibody titre to confirm susceptibility of the experimental sheep, using *T. lestoquardi*

schizont antigen. The procedure followed in materials preparation and running of the test were essentially as described by Leemans *et al*., (2001). The schizont antigen slides were prepared from *in vitro* cultures of *T. lestoquardi* schizont-infected cells which were kindly provided by Prof. A. M. El Hussein, National Research Central, Ministry of Science and Technology, Soba, Sudan. The cells were washed twice by centrifugation in PBS and were re-suspended in 10 ml PBS to a concentration of $5x10^6$ cell/ml. Fifty microlitre of the suspension were transferred into the 12 wells of each clean PTFE (polytetrafluoroethylene) coated multi-test microscopic slides. The cell suspension was pipetted into a well and immediately the excess was aspirated. The slides were allowed to air-dry for one hour, fixed in cold acetone for 10 min and allowed to dry at room temperature. They were then wrapped in aluminum foil, labeled and stored at -20ºC for later use. The conjugate, rabbit anti-sheep FITC labeled antibody was obtained from Sigma® (USA), and the positive and negative control sera were kindly provided by Dr. K. M. Taha, Veterinary Research Laboratory, Atbara, Sudan. The conjugate titration was carried out to determine it's working dilution. This was taken as the dilution that did not lose the titre of the positive control serum (C+) and at the same time did not react with the control negative serum (C–) at a concentration greater than 1:10. All the test animal's sera were examined for antibodies to *T. lestoquardi* antigen. The thawed sera (test and controls sera) and the conjugate were diluted 1/80 in PBS. The antigen slides were thawed and were placed in humidity chamber (moist filter paper in Petri dishes). To each labeled slide, 50µl per well of diluted test sample and C- and C+ were added. The slides were incubated in a moist chamber at room temperature for an hour. Slides were singly washed in PBS using a wash bottle then

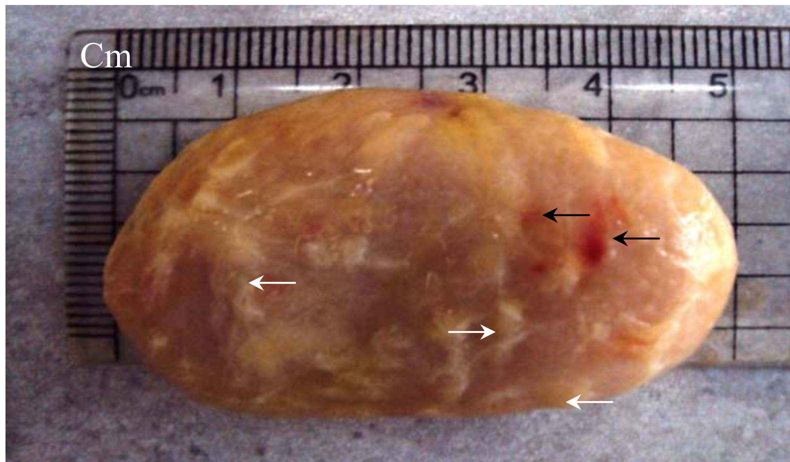
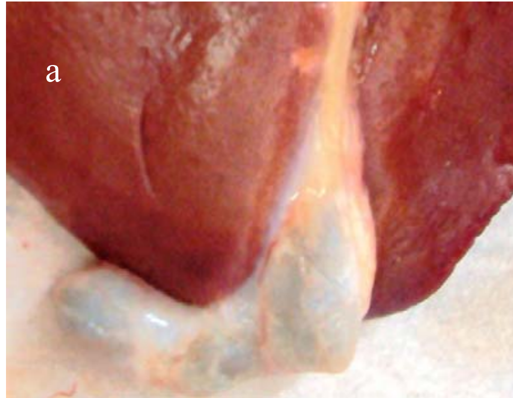


Plate 3.4. Photograph of prescabuler lymph node of (animal No. 40) showing remarkable nodular masses (white arrows) and haemorrhagic foci (black arrows) in *T. lestoquardi* chronic infection of sheep.



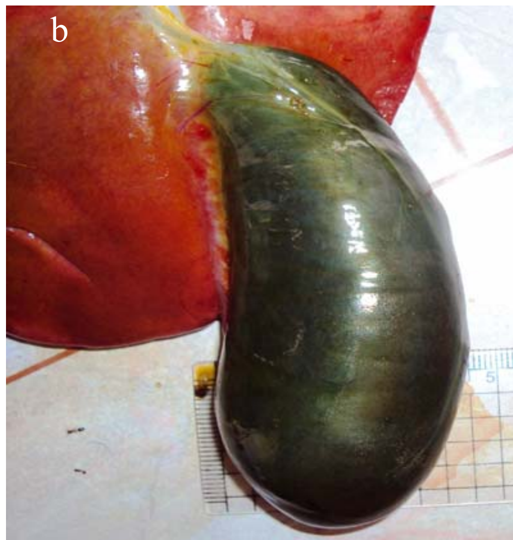


Plate 3.5. Photograph of gall bladder of nun-infected (a) and *T. lestoquardi* infected animal (b).

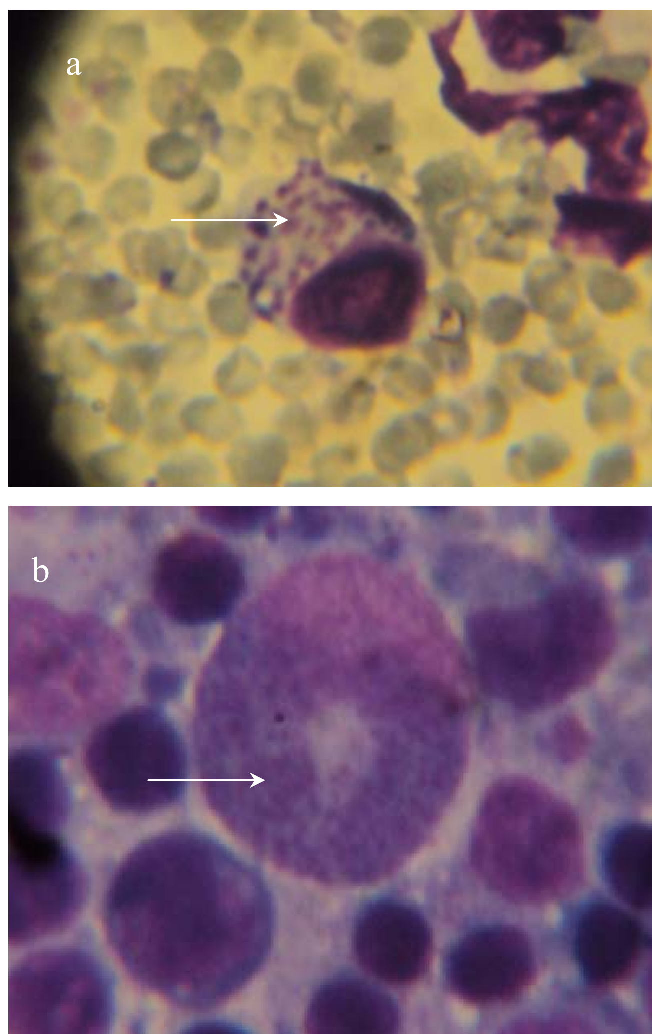


Plate 3.6. Photomicrograph of lung impression smears of (animal No. 43) showing large lymphocyte filled with 30 (a) and 300 (b) (arrow) *T. lestoquardi* merozoites (Giemsa's stain x 100).

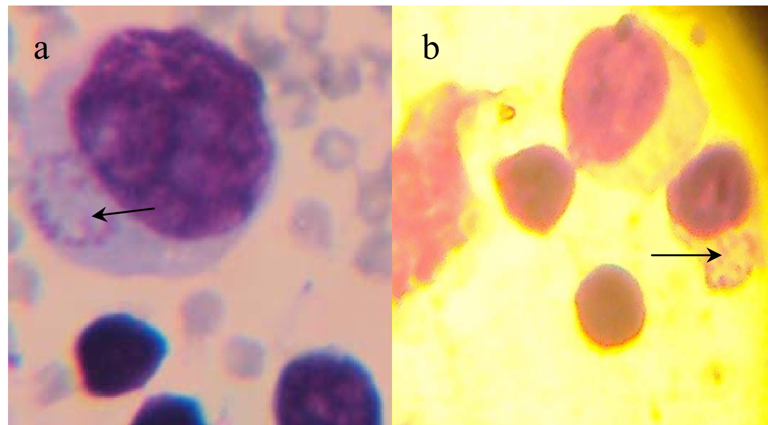


Plate 3.7. Photomicrograph of lung impression smears of (animal No. 43) showing large lymphocyte with ring stage (a) and outward evagination (b) (Giemsa's stain x 100).

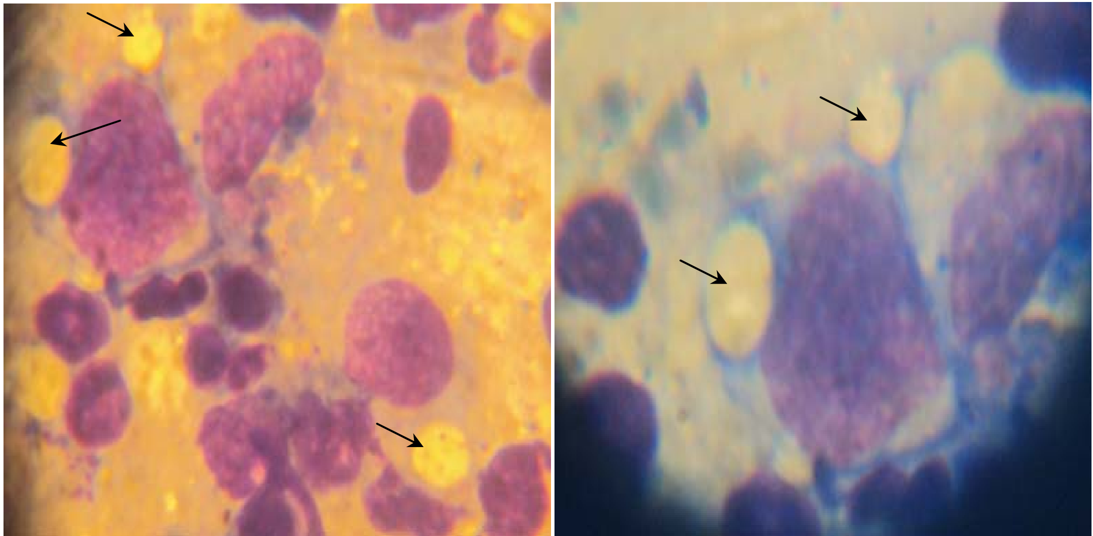


Plate 3.8. Photomicrograph of lung impression smears of (animal No. 43) showing large lymphocyte with circular outlets (arrows) (Giemsa's stain x 100).

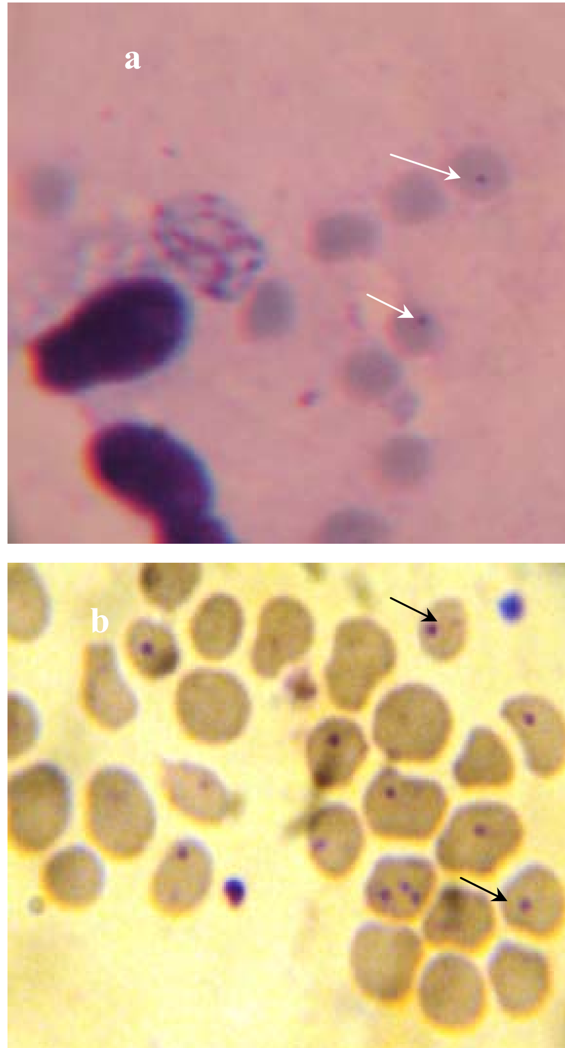


Plate 3.9. Photomicrograph of lung impression smears (a) showing RBCs infected with one and small *T. lestoquardi* piroplasm. Peripheral blood smears (b) showing RBCs infected with 1-3 large *T. lestoquardi* piroplasms (Giemsa's stain x 100).

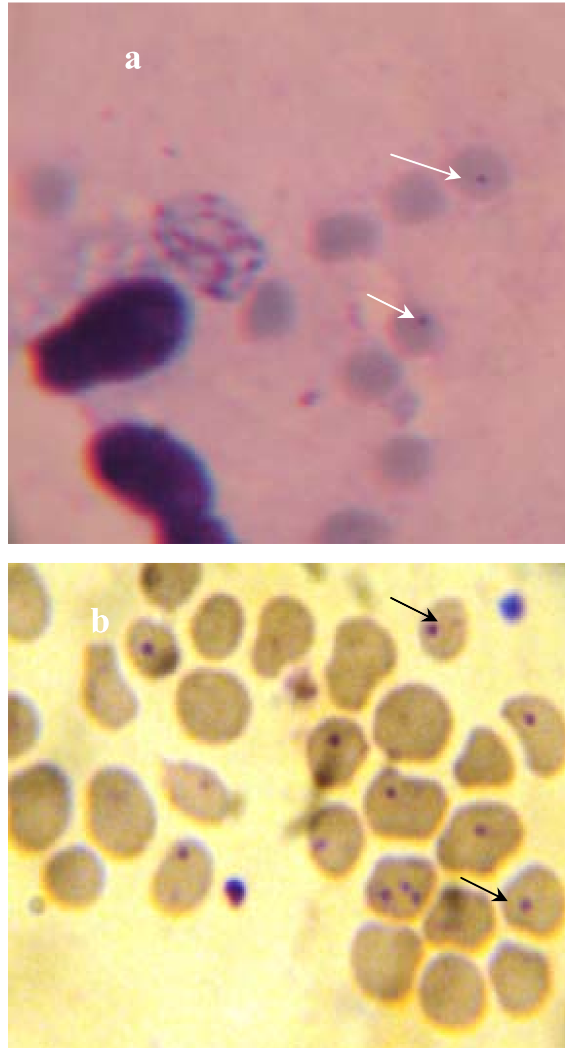


Plate 3.9. Photomicrograph of lung impression smears (a) showing RBCs infected with one and small *T. lestoquardi* piroplasm. Peripheral blood smears (b) showing RBCs infected with 1-3 large *T. lestoquardi* piroplasms (Giemsa's stain x 100).

placed in a staining jar and washed in PBS for 30 min with constant agitation. The slides were placed inverted onto a filter paper to drain excess PBS. A volume of 10µl of the diluted conjugate was added to each well of the slides and they were incubated at 37ºC for 30 min in dark, washed with PBS as described above and were air dried. The slides were mounted with glycerol: PBS (3:1) and cover slips were placed over the wells of the slides. Examination of the slides was carried out in a dark room using Olympus Vanox incident-light excitation fluorescent microscope (Japan). The result was considered positive when bright fluorescence from the intracellular schizonts were detected.

2.2.8.2. Molecular detection of infection:

All animals (test and control) were subjected to conventional PCR and/or LAMP-PCR test for further confirmatory tests of *T. lestoquardi* infectivity.

2.2.8.2.1. Polymerase chain reaction:

The DNA extracted from all the control animal blood were tested to *T. lestoquardi* infectivity by conventional PCR.

2.2.8.2.1.1. Primers:

T. lestoquardi SPEC. F: GTGCCGCAAGTGAGTCA.
T. lestoquardi SPEC. R: GGACTGATGAGAAGACGATGAG.

2.2.8.2.1.2. Reaction condition:

The reaction was performed in a total volume of 50 µl as follow: 25 µl Green master mix, 6 µl H_2O, 2 µl of each primers and 5 µl genomic DNA.

Thermocyler program as follow: 94 C for 3 min., then 35 cycles consisted of 94ºC for 1 min., 55 ºC for 1 min., and 72ºC for 1 min., final extension step 72ºC for 7 min., and hold on 4ºC.

2.2.8.2.1.3. Detection of the reaction product:

The amplified fragments were separated by electrophoresis on 1.5% agarose gel. For this purpose, 1.5 g agarose (Invitrogen, UK) was dissolved in 100 ml of 1X Tris/boric acid/EDTA (TBE; 89 mM Tris, 89mM boric acid, 2 mMEDTA, pH 8.0). The solution was boiled in a microwave oven for 1-2 min, then left on the bench to cool down. Subsequently, 10 µl of Ethidium bromide (Merck, Darmastadt) was added, carefully mixed and poured in a gel tray. The comb was inserted in melted gel in order to make wells, then the gel was left on the bench for about 30 min. to solidify. The PCR products were prepared by aliquoting 1.5µl of 6X loading buffer (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) in 96 wells plate. A volume of 5µl of each PCR products were mixed well with the aliquoting 1.5 µl loading buffer, then the samples were loaded onto the agarose gel. A volume of 5µl of 100bp DNA-ladder (Roth, Germany) was also loaded. The gel was run at 70V for 1h, transferred to a UV-illuminator for examination and documentation.

2.2.8.2.2. Loop-mediated isothermal amplification:

The DNA extracted from all the test animal blood were tested for *T. lestoquardi* infectivity by LAMP-PCR.

2.2.8.2.2.1. Primers:

L. F3: AGATACCAAGGAAACTGAAGG.
L. B3: TGTATCCTTAGGTTTTTCATGTTC.
L. FIP: CAGGAGAAATAGGAGTTTCAGGTTCCAAAGGATAAGAA AGATGAAAAGG.
L. BIP: GTATCGCACCAGAACCTCAACACAGTTTCTTCTTTATC CTGATC.

2.2.8.2.2.2. Reaction condition:

The reaction was performed in a final volume of 25 µl which contained 12.5 µl 2 x LAMP reaction buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ and 0.2% Tween 20], 125µM each deoxynucleoside triphosphate, 0.8 M betaine (Sigma, Germany), 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, Germany), 40 pmol each FIP and BIP, 20 pmol LF and LB, 5pmol each F3 and B3, and 2 µl of target DNA. The mixture was incubated at 63°C for 45 min using a conventional heating block (Stuart, Scientific, UK) and then heated at 80°C for 3 min to terminate the reaction.

2.2.8.2.2.3. Detection of the reaction product:

An aliquot of 5 µl of LAMP product was subjected to electrophoresis on a 1.5% agarose gel in a Tris-acetic acid-EDTA (TAE) buffer at 90 V for 1h, then visualized under UV light after staining with ethidium bromide. In addition, LAMP amplicons were detected directly by the naked eye by addition of 1.0 µl of 1:10-diluted SYBR Green I (Roche Diagnostics, Germany) to the mixture and observation of the solution color. The solution turned green in 1 min in the presence of a LAMP amplicon, while it remained orange when no amplification occurred.

2.2.9. Statistical analysis:

Data collected from laboratory studies and clinical examinations were analyzed using statistical package for social science (SPSS) software programme. All data were presented as mean plus or minus standard deviation (SD). Data from control and test cells were compared by Student t test for paired samples. P values of 0.05 or less were considered statistically significant. Levels of significance are denoted as $P < 0.05$. For multiple comparisons, analysis of variance (ANOVA) followed by post hoc analysis with Tukey test was used.

CHAPTER THREE

RESULTS

3.1. Detection of sheep tick-borne pathogens:

The *Theileria* and *Babesia* genus-specific primers were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene. The PCR amplicons were analyzed using the RLB. These PCR products were hybridized onto the membrane and were observed for reaction to specific oligonucleotide probes. The total number of positive cases and the *Theileria* species that infect the Sudanese sheep are shown (Table 3.1). Primers RLB-F2 and RLB-R2 amplified bands corresponding to the hypervariable V4 region of *Theileria* and *Babesia* species are shown (Fig. 3.1). Four *Theileria* species were detected in sheep blood, these were: *Theileria annulata* 36/219 (16.3%), *T. lestoquardi* 36/219 (16.3%), *T. ovis* 194/219 (88.6%) and *T. separata* 44/219 (20%). All probes bound only to their respective target species, except probes positive to *T. lestoquardi* that 100% contemporaneously reacted with *T. annulata*. *T. lestoquardi* was detected in two localities (Atbara and Khartoum North). *T. ovis* was widely detected in all the localities, whereas, *T. separata* was detected in four localities: Damazin 23/219 (10.5%), Khartoum North 17/219 (7.8%), Kosti 2 (6.7) and Medani 2 (5.7). Also, mixed *Theileria* spp. infections were detected in 74 (33.8%) of the positive samples.

3.2. Malignant Ovine Theileriosis:

3.2.1. Clinical manifestations:

Naturally *T. lestoquardi* infected sheep developed variable clinical signs few days after tick attachment.

Table 3.1. Molecular detection of *Theileria* species infecting sheep in different localities in the Sudan during 2008-2009.

Locality	No. of samples	No. of flocks	*T. ann.* (%)	*T. lest.* (%)	*T. ovis* (%)	*T. sep.* (%)
Atbara	57	3	19 (33.3)	19 (33.3)	57 (100)	0 (0)
Khartoum	36	1	17 (47.2)	17 (47.2)	36 (100)	17 (47.2)
Kosti	30	3	0 (0)	0 (0)	16 (53.3)	2 (6.7)
Medani	35	2	0 (0)	0 (0)	32 (91.4)	2 (5.7)
Damazin	33	2	0 (0)	0 (0)	26 (78.8)	23 (69.7)
Nyala	28	2	0 (0)	0 (0)	27 (96.4)	0 (0)
Total	219	13	36 (16.4)	36 (16.4)	194 (88.6)	44 (20.1)

T. ann. = *T. annulata*.

T. les. = *T. lestoquardi*.

T. sep. = *T. separata*.

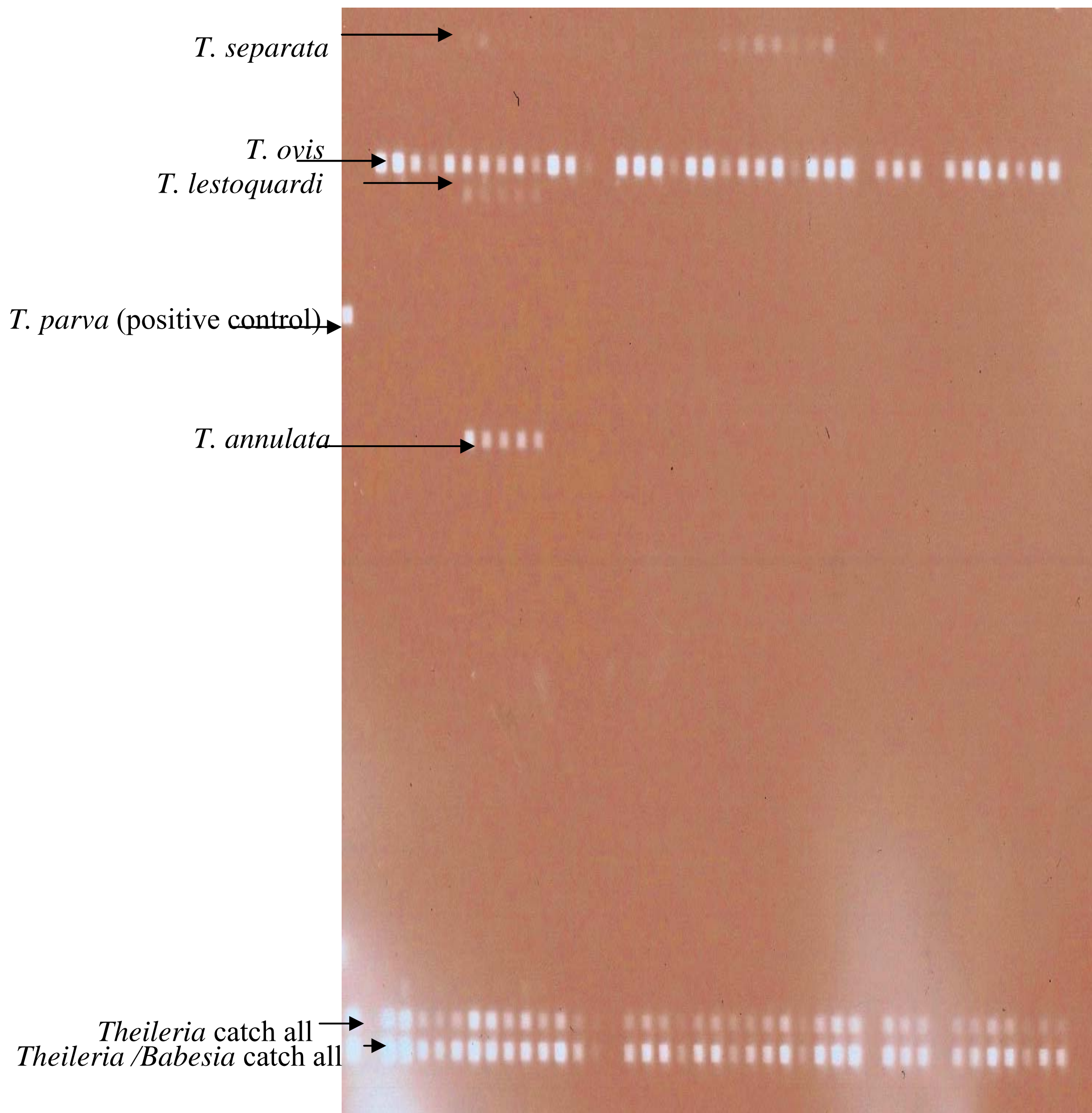


Fig. 3.1. X-ray film of plotting RLB membrane, showing the positive tick-borne parasites of sheep in the Sudan in 2008-2009.

The three eco-types (Garag, Watesh, Desert) were infested with variable number of ticks (mean 7.2, 2.7, 5.5, respectively). On the other hand, the control animals were infested with relatively low number (mean 1.8). The mean total number of ticks were significantly ($P<0.05$) related to the mean number of days with fever. Post-infections, 2.0 kg live body weight loss were recorded in Desert sheep, while others lost 1.2-1.3 kg and slight increases (0.4-0.6 kg) were recorded among control animals.

The clinical sings were manifested by fever, lymph nodes appeared enlargeme, Schizonts parasitosis and piroplasms parasitaemia. The parotid and maxillary lymph nodes were considerably enlarged within 4-5 days and reached a maximum size in 9-10 days later. In addition, lacrimation, nasal discharges and oedematous swelling of the face and the eyelids were rarely observed. There is frequent urination and accelerated pulse and respiratory rates. During the sub-acute and chronic courses of the disease, chest percussion revealed lung's dull sound. Diarrhoea was occasionally observed and their frequencies significantly coincided with the first day of fever and/or schizonts. All animals had an apparently normal appetite but in a few days after the onset of fever, they ceased eating and later on they became progressively emaciated and went into coma at the terminal phase of the disease. The severity of symptoms and duration of the clinical courses of the disease were considerably variable within the eco-types and among individual within the groups. The morbidity of natural *T. lestoquardi* infection to the three eco-types is illustrated in (Table 3.2). Test animals showed a high morbidity rate (average 62.2%) being highest in Desert sheep (92.9%). Nevertheless, death and/or sacrifice of the recumbent animals occurred in all infected sheep.

Table 3.2. Morbidity of natural *T. lestoquardi* infection in sheep ecotypes.

Sheep eco-type	Number	Morbidity (%)
Garag	16	7 (43.8)
Watesh	15	8 (53.3)
Desert	14	13 (92.90
Total	45	28 (62.2)

Days from appearance of symptoms to death/sacrifice of Garag, Watesh and Desert sheep ranged between 5-17/ (9.6), 8-10/ (9.0) and 5-33/ (21.6) days respectively. All sheep eco-types developed clinical signs after the natural infection with *T. lestoquardi* while severe clinical manifestation was observed in the Desert sheep.

3.2.1.2. Parasitological findings:

The classification of the host reactions to natural *T. lestoquardi* infection were based on the mean fever duration and/or maximum body temperature, schizonts infected cell (parasitosis) and piroplasms infected erythrocyte (parasitaemia).

The Primary reaction course and response of different sheep eco-types to natural *T. lestoquardi* infections are summarized in table (3.3). The mean maximum body temperatures 40.8ºC, 40.7ºC and 41.2ºC were respectively recorded in Garag, Watesh and Desert eco-types. Moreover, fluctuations in daily rectal temperature were observed in all infected animals but the mean durations of fever ranged between 4 and15 days.

Schizonts were detected in superficial lymph node 2-3 days after the rise in rectal temperature, persisted for a mean of 2 days and disappeared till the death/sacrifice of the animals. The percentage of schizonts infected cells (parasitosis) in all *T. lestoquardi* infected sheep were detected relatively low. Moreover, schizonts (irrespective to their size) were hardly seen in thin blood smears made from peripheral blood.

During the routine detection of the schizonts in the lymph node biopsy smears, remarkable changes in lymphocyte morphologies were noticed. The size, shape, cytoplasm and the nucleus of these lymphocytes displayed distinct abnormalities (Plate 3.1 a-d).

Tab 3.3.

Plate 3.1.a-d

The deformed lymphocytes possessed relatively irregular, oval, elliptical and amoeboid shapes. The abundant cytoplasm was faintly blue stained. Other characteristic findings included the presence of large, irregular, kidney shaped or cleaved nuclei partially occupying centre or laterally displaced to the side of the cell. Different sizes and shapes of fine to condense chromatins interspaced the prominent nucleoli giving the pattern of dark-gray diffuse cloudy effaced the moon light. In contrast, a typical lymphocyte was smaller it compact nucleus filling the entire cytoplasm (Plate 3.1 e). At least 4 distinct characteristic abnormality findings have obviously differentiated the deformed lymphocyte from the typical one, and cordially were arbitrated the term (*Theileria* markers). These abnormalities were clearly visible within 1-2 days after the rise in body temperature and coincided with the later detection of schizonts and/or piroplasms. *T. lestoquardi* intra-erythrocytic piroplasms were demonstrated in the blood smears taken from different sheep ecotypes and erythrocyte multiple infections with more than one piroplasm (i.e., 2-4 piroplasms in one erythrocyte) were commonly observed (Plate 3.2). In most cases, daily increases in piroplasm levels occurred in a dramatic matter for 5-7 days and were slightly reduced before death. The parasitaemia (6-8%) in the recovered animal (No. 40) fluctuated for 4 weeks after which they gradually decreased for 6 weeks before they disappear. In all infected animals, piroplasms parasitaemia were detected in variable percentages (5-24%) while animals (No. 38, 12, 13) showed 1%, 2% and 33% parasitaemis, respectively.

Plate 3.2

3.2.1.3. Haematological values in *T. lestoquardi* infection:

The haematological values in *T. lestoquardi* naturally infected sheep and the control sheep were recorded as follows:

3.2.1.3.1. Packed cell volume:

The packed cell volume (PCV) values in *T. lestoquardi* naturally infected and control sheep are illustrated in table (3.4). There were significant reductions ($P<0.05$) in the mean PCV in all infected sheep (Garag, Watesh, Desert) with a mean value of 9.9, 5.9 and 15.9 respectively. The infected Desert eco-type showed a highest drop in PCV value (47.6%). On the other hand, no significant change noticed in the control sheep.

3.2.1.3.2. Haemoglobin concentration:

The haemoglobin concentration (Hb) in *T. lestoquardi* naturally infected and control sheep are demonstrated in table (3.5). The mean Hb concentrations slightly decreased (0.9) in Watesh, while significant reductions (1.7, 2.9) were respectively reported in Garag and Desert infected sheep. Throughout the experimental period, however, the Hb concentrations in control sheep did not significantly change.

3.2.1.3.3. Red blood cells count:

The erythrocyte counts before and after *T. lestoquardi* infection in the experimental and in the control sheep are demonstrated in table (3.6). The RBCs count of the three sheep eco-types was reduced post *T. lestoquardi* natural infection and a negligible increase was observed in the control group.

Table 3.4. Packed cell volume in *T. lestoquardi* naturally infected and control sheep.

Sheep eco-type	n	Pre-infection Mean ± sd	Post-infection Mean ± sd	fall Mean ± sd	(%)
Garag	7	27.4 ± 2.6	17.6 ± 5.6	9.9 ± 5.4	36.1
Watesh	8	28.8 ± 3.7	22.3 ± 2.9	5.9 ± 2.7	20.5
Desert	13	33.4 ± 1.7	17.4 ± 3.4	15.9 ± 3.8	47.6
Baladi (control)	11	27.6 ± 2.9	27.8 ± 2.8	$0.3^{(+)}$ ± 0.8	

Mean (V/V).
sd. = standard deviation.
n = number of animal.
$^{(+)}$ increased.

Table 3.5. Haemoglobin concentration in *T. lestoquardi* naturally infected and control sheep.

Sheep		Pre-infection	Post-infection	fall	
eco-type	n	Mean ± sd	Mean ± sd	Mean ± sd	(%)
Garag	7	10.4 ± 0.6	8.7 ± 1.4	1.7 ± 1.4	16.3
Watesh	8	10.5 ± 1.0	9.6 ± 1.3	0.9$^{(+)}$ ± 0.7	8.6
Desert	13	12.3 ± 1.2	9.4 ± 2.1	2.9 ± 1.8	23.5
Baladi (control)	11	10.3 ± 0.8	9.9 ± 1.1	0.4$^{(+)}$ ± 0.8	3.8

Mean (g/dl).
sd. = standard deviation.
n = number of animal.
$^{(+)}$ = non-significant.

Table 3.6. Red blood cells count in *T. lestoquardi* naturally infected and control sheep.

Sheep eco-type	n	Pre-infection Mean ± sd	Post-infection Mean ± sd	fall Mean ± sd	(%)
Garag	7	4.9 ± 0.8	4.6 ± 0.7	0.3 ± 0.2	6.1
Watesh	8	5.6 ± 1.0	5.2 ± 0.9	0.4 ± 0.4	7.1
Desert	13	6.8 ± 0.4	4.8 ± 0.8	2.0 ± 1.3	14.7
Baladi (control)	11	6.1 ± 0.7	6.2 ± 0.8	$0.1^{(+)}$ ± 1.4	

Mean (number of RBC x 10^6 cells/µL).
sd. = standard deviation.
n = number of animals examined.
$^{(+)}$ = increased.

3.2.1.3.4. White blood cells count:

The white blood cells count in *T. lestoquardi* naturally infected and control sheep are illustrated in table (3.7). All infected animals showed an increase in WBCs counts. A significant increase (P<0.001) in WBC count was observed in Desert sheep with a mean count decrease of 58.4% for pre-infection value. Throughout the study period, the white blood cells count in control sheep fluctuated between 7-8 ($x10^3$ cells/µl).

3.2.1.4. Pathological findings:

3.2.1.4.1. Macroscopic findings:

The post-mortem finding of the *T. lestoquardi* naturally infected animals and pathological lesions involving the organs were categorized according to the different courses of the disease. The most prominent alterations were seen in the Desert sheep compared with Garag and Watesh. These sheep developed the following macroscopic alterations:

3.2.1.4.1.1. Acute:

The Post-mortem legions of acute *T. lestoquardi* infected sheep (Numbers. 1, 5, 34, 36,) that died or were sacrificed 6-9 days post infection shown in the form of severe enteritis and congestion of the digestive system with scattered areas of petechial haemorrhages on the serosal and mucosal surface of small and large intestines.

The lungs were diffusely congested and accompanied by pneumonie with clear to yellowish-brown hydrothorax. The livers were relatively enlarged, congested and the gall bladders were slightly distended with green bile. Jaundice was not seen in the acute course of the disease. The superficial and mesenteric lymph nodes were variably enlarged.

Table 3.7. White blood cell counts in *T. lestoquardi* naturally infected and control sheep.

Sheep		Pre-infection	Post-infection	increase	
eco-type	n	Mean ± sd	Mean ± sd	Mean ± sd	(%)
Garag	7	7.6 ±1.7	9.6 ± 3.6	2.0 ± 3.1	26.3
Watesh	8	8.3 ±3.1	11.5 ± 1.8	3.2 ± 2.3	38.6
Desert	13	8.9 ±1.9	14.1 ± 2.3	5.2 ± 4.6	58.4
Baladi (control)	11	8.1 ± 2.3	7.8 ± 1.4	0.3* ± 1.1	3.7

Mean ($x10^3$ cells/µl).
sd. = standard deviation.
n = number of animal.
* = non-significant.

The spleens looked normal or slightly enlarged. Only one animal (No. 35) that died 6 days post infection of *T. lestoquardi* infection showed no macroscopic changes.

3.2.1.4.1.2. Sub-acute:

The post-mortem examination of sheep (Nos. 8, 20, 28, 33, 43, 45) which died/sacrifice10-20 days post infection, revealed remarkable enlargement of liver while gall bladders were distended with thick viscid greenish bile. Icterus was evident by the diffuse yellowish discolouration of the body fat and fluids (Plate 3.3). The blood was rather thin and watery. The spleens were congested and extremely enlarged. The gastrointestinal tract was diffusely congested and showed variable catarrhal changes. Mesenteric lymph nodes were extremely enlarged and fragile.

3.2.1.4.1.3. Chronic:

Three sheep (Nos. 37, 39, 40) which died, sacrificed or recovered 33, 30 and 42 days post *T. lestoquardi* infection respectively, showed the following post-mortem findings: All animal revealed emaciation and severe icterus accompanied by yellowish discolouration of visceral organs and body fats. The subcutaneous, subserous and intramuscular connective tissues were oedematous and yellow in colour. The superficial and internal lymph nodes were firm, oedematous and had haemorrhagic foci on the surface (Plate 3.4). Viscous yellowish to brown-reddish fluid filled the abdominal and thoracic cavities. Livers were enlarged, showed evidence of fatty change and the gall bladders were markedly distended. Notably, the gallbladder in animal (No. 37) was filled (50 ml) with thick dark-greenish bile (Plate 3.5).

Plate 3.3

Plate 3.4

Plate 3.5

The spleens were congested and extremely enlarged with prominent splenic pulps and contained scattered foci of capsular haemorrhages. The hearts were flabby and showed petechial haemorrhages in the atrio-ventricular and epicardial surfaces. The kidneys were severely congested and the fat around the kidneys were relatively depleted and gelatinous. The lymph node near the hilus was markedly enlarged. Multiple petechial haemorrhagic spots were observed on mucosa of both small and large intestines.

3.2.1.4.1.4. Lung involvement in pathogenesis of *T. lestoquardi* infection:

The most prominent and remarkable macroscopic lesions during the different courses of the disease were obviously seen in the lungs, and these are demonstrated. Animals that died or sacrificed post *T. lestoquardi* infections revealed severe pneumonia. The pneumonia was associated with oedema, prominent interstitial emphysema and accumulation of creamy-grayish frothy exudates that extended along the pulmonary bed, trachea bifurcation and trachea. In chronic courses, the lungs lobules were specially noncollapsed with rubbery texture (interstitial pneumonia) and multiple haemorrhagic foci were diffusely scattered.

Dark-brown foamy fluids filling the interlobular septae and bronchi were constantly seen. In addition, the pulmonary lymph nodes were markedly enlarged and oedematus.

3.2.1.5. Microscopic findings:

The results of impression smears and histopathological changes from different organs during the course of the disease are shown below.

3.2.1.5.1. Impression smears:

The microscopic examinations of bone marrow aspiration and impression smears taken from different animal organs (internal/superficial lymph nodes, liver, heart, spleen, intestines, kidney, brain and lungs) post *T. lestoquardi* infections revealed variable cell types. The morphologies of these cells varied with the different courses of disease. During the acute course, few schizonts were detected in internal lymph node (mesenteric, hepatic and pulmonary) impression smears and none in lungs or in other visceral organs. Comparatively, the number of these schizonts was extremely lower than the schizonts detected in superficial lymph nodes. During the sub-acute course; relatively uniform, large size parasitized lymphocytes were detected in the lungs. Approximately 30-300 merozoites were seen in the cytoplasm of each parasitized lymphocyte (Plates 3.6 a,b). Their nucleli were laterally displaced to almost one third of an abundant cytoplasm and stained basophilic dark-blue. These types of lymphocytes were not detected in the peripheral blood smears, superficial or in internal lymph node biopsies as well as in impression smears from other major organs such as liver, kidney, spleen and heart. In addition, numerous other forms of schizonts parasitized cells were scattered throughout the lung tissues and their merozoites contents were well differentiated and arranged in a ring form. Approximately, 9-12 merozoites were seen in each ring form, and subsequently they were designated as ring stage. In several cases, the ring stage inside the infected cell was pushed to the apical end of the cell cytoplasm by outward evagination (Plate 3.7). The extracellular ring stages were detected in the peripheral blood, all organs examined and even in bone marrow aspiration, but not in brain impression smears.

Plate 3.6

Plate 3.7

However, enlarge cells with a circular outlet cytoplasm and ring stages around, were occasionally noticed in lung smears in all animals (Plate 3.8). Both, circular outlet and ring stage were more or less equal in diameters. In addition, intra-erythrocytic piroplasms were detected in the lung impression smears with relative parasitaemia ranging between 3-6%. Comparatively, each RBC was infected with only one piroplasm and it was relatively smaller than those detected in the peripheral blood smears (Plate 3.9). On the other hand, more schizonts were detected in internal lymph nodes compared with the superficial lymph nodes. In general, the *T. lestoquardi* schizonts steadily increased in number in lymph nodes and in other organs during the disease courses (Table 3.8). The results of lungs impression smears during the chronic course of *T. lestoquardi* infection revealed plenty of infected lymphocytes.

Massive numbers of schizonts parasitized cells were predominantly detected in the lungs throughout the chronic course in all infected animals. Schizonts infected cells were different in sizes and were differentiated into numerous merozoites (Plate 3.10). In all animals, however, lymphocytes containing schizonts were closely encircled with more than 8-9 monocytes and were predominately noticed in rosette shaped (Plate 3.11 a,b). Some of these parasitized lymphocytes were seen ruptured leaving empty circles (Plate 3.12 a,b). Relatively, numerous schizonts were detected in lung impression smears. Some of these schizonts show obvious deformities. Large and small empty cytoplasm punctures were noticed in these schizonts (Plate 3.13 a,b). This phenomenon we termed as (schizont pitting). Lung impression smears from recovered animals showed different cell types. Examples of these cells are the ubiquitous neutrophils which disseminated throughout the pulmonary bed (Plate 3.14).

Plate 3..8

plate 3.9

Table 3.8

plate 3.10

plate 3.11

pate 3.12

plate 3.13

plate 3.14

This pulmonary neutrophillia, however, was not detected in the entire infected animals during the disease courses except in the recovered animal. In addition, eccentric cells, designated as (E. cell) were also seen (Plate 3.15 a-d). These heterogeneous (E. cells) resemble the typical lymphocyte but with some disparities. They have abundan basophilic cytoplasm with round to oval nuclei and reddish-blue and fine to slightly condensed chromatins. Condensed reddish-blue inclusion body-like structures; 5-6 in numbers; clumped or splintered in the cytoplasm were also clearly seen. These cells represented approximately 6% of the lung lymphocytes. Notably, the same cells were noticed in different mitotic division stages (Plate 3.16 a-d). Trials to characterize E. cell were made. Four lung impression smears slides, in which the E cells were previously seen, were subjected to IFA test. Briefly, to partially dissolve the Giemsa's stain, the slides were rinsed in absolute alcohol and air dried. The slides were covered with diluted *T. lestoquardi* positive serum (1:80 in PBS), incubated at 37ºC for 30 min. Then, they prepared and examined following the previous procedures for IFA test (section 2.2.8.2). Antigen-antibody complex inside (schizont antigens coated by antibodies) and around the E. cell's cytoplasm (surface schizont antigens) indicated by the fluoresce emitting greenish light were seen (Plate 3.17 a,b). Fluorescence pattern observed for E cells in these slide resembled that seen in schizonts infected cells from established cells culture lines.

3.2.1.5.2. Histopathological findings:

The most important histological alterations in *T. lestoquardi* infected sheep were the following:

The most prominent histological alterations are seen in the lungs and showed emphysema, congestion and collapse (Plate 3.18 a-c).

Plate 3.15

Plate 3.16

Plate 3.17

Plate 3.18

Congestion was common but oedema was seen in one case. Alveolar wall appeared thickened and pneumocytes looked cuboidal with distinct nuclei On the other hand, many section showed large mononuclear cells (manly lymphocytes and macrophages) in alveoli and also in blood vessels (Plate 3.19). Interstitial thickening was slightly seen but not marked in many section and infiltrated with round cells. In only one section suppurative bronchopneumonia was diagnosed.

Spleen section showed lymphoid hyperplasia with prominent white pulp and periarterial lymphocytic sheath (PALS) and Haemosiderin deposition (Plate 3.20). Mononuclear cells (MNCs) were seen in splenic sinuses predominantly macrophages.

The microscopic lesions of the lymph nodes in many sections were lymphoid hyperplasia (Plate 3.21a,b) especially in interfollicular and paracortical tissue. Lymphoid follicles were distinct but appeared sometimes with proliferating lymphocytes. Medullary sinuses contained large lymphocytes and macrophages and medullary cords were thickened.

In some section of the heart, the muscle cells were widely separated, or closely packed with each other (Plate 3.22 a,b). In few sections focal proliferation of interstitial cells was seen with presence of prominently large mononuclear cells.

The liver in some sections of the live showed marked sinusoidal congestion with dilated central veins and infiltration of portal trials with mononuclear cells. In the congested sinusoids large mononuclear cells were seen (Plate 3.23). Many section showed widened sinusoids thickened hepatic cords and large monocyte cells in sinusoids. Some of these cells appeared to have more than one nucleus or appear to show cytoplamic granules.

Plate 3.19

Plate 3.20

Plate 3.21

plate 3.22

plate 3.23

In two cases capsule was obviously thickened and other two showed distinct cytoplasmic vaculations indicative of fatty change, which was mainly perilobular.

Most section of the kidneys appeared normal though in all glomerular tuft appear cellular. In few cases the glomerular tuft was either highly cellular or with lobulated tuft or shrunken tuft and widened Bowman's capsule (Plate 3.24). In two cases there were few focal areas of interstitial mononuclear cells infiltration. In a number of sections the tubular epithelial cells particularly in medulla sloughed into the lumen.

The sections of the stomach appeared normal but mucosal oedema in few cases and in some mononuclear cells infiltration at the base of gastric glands or in submucosa was seen. In one section parietal cells were not distinct and chief cells predominately seen.

The section of the pancrease, rumen and the intestine appeared normal, but hypercelllarly of lamina proprie was commonly observed in the small intestine.

Vaculations with gliosis and satillitiosis were the prominent histological changes noticed in the brain sections. In addition only one small area of haemorrhage was seen (Plate 3.25).

3.2.1.6. Biochemical changes:

The serum biochemical constituents from naturally infected *T. lestoquardi* and control sheep are demonstrated in table (3.9 a-g). Alanine aminotransferase (ALT) activities increased in all test sheep. However, Desert eco-type showed the highest increase (31.1%) in ALT activities, followed by Garag (27.3%) and only 3.1% increase was noticed in the Watesh eco-type.

Plate 3.24

Plate 3.25

Table 3.9 a-g. Mean serum biochemical constituents in natural *T. lestoquardi* infected and control sheep.

(a) Alanine aminotransferase.

Sheep eco-type	N	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	Changes remark	Changes %
Garag	7	9.9 ± 4.2	12.6 ± 10.3	2.7	(+)	27.3
Watesh	8	9.6 ± 3.9	9.9 ± 1.2	0.3	(+)	3.1¤
Desert	13	10.3 ± 1.5	13.5 ± 7.5	3.2	(+)	31.1
Baladi (control)	11	10.8 ± 4.9	11.2 ± 8.3	0.4¤	(+)	3.7

(b) Alkaline phosphatase.

Sheep eco-type	n	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	Changes remark	Changes %
Garag	7	43.8 ± 24.3	30.0 ± 5.7	13.8	(-)	31.5
Watesh	8	43.0 ± 10.0	48.0 ± 12.7	5.5	(+)	11.6
Desert	13	30.7 ± 4.0	42.5 ± 26.7	11.8	(+)	38.4
Baladi (control)	11	41.6 ± 12.9	41.9 ± 10.7	0.3¤	(+)	0.7

(c) Aspartate Amino-transferase.

Sheep eco-type	n	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	Changes remark	Changes %
Garag	7	99.5 ± 57.3	93.0 ± 8.5	6.5¤	(-)	6.5
Watesh	8	90.8 ± 51.1	125.0 ± 82.0	34.2	(+)	37.7
Desert	13	92.7 ± 12.7	224.3 ± 35.2	131.6	(+)	142.0
Baladi (control)	11	87.5 ± 4.6	90.1 ± 23.5	2.6¤	(+)	2.9

Mean (µkal/L).
sd. = standard deviation.
n = number of animals examined.
(+) = increased.
(-) = decreased.
¤ non-significant.

(d) Albumin concentration.

sheep eco-type	N	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	remark	%
Garag	7	2.7 ± 0.6	2.1 ± 0.1	0.6¤	(-)	22.2
Watesh	8	2.5 ± 0.5	2.3 ± 0.6	0.2¤	(-)	8.0
Desert	13	2.5 ± 0.2	2.2 ± 0.7	0.3¤	(-)	12
Baladi (control)	11	2.6 ± 0.5	2.6 ± 0.2	0.0¤		0.0

Mean (/gm100 ml).

(e) Total bilirubin.

Sheep cco-type	N	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	remark	%
Garag	7	0.2 ± 0.2	0.6 ± 0.1	0.4	(+)	200
Watesh	8	0.1 ± 0.1	0.7 ± 0.4	0.6	(+)	600
Desert	13	0.2 ± 0.1	1.9 ± 0.2	1.7	(+)	850
Baladi (control)	11	0.1 ± 0.1	0.2 ± 0.2	0.1	(+)	100

Mean (µmol/L).

(f) Total protein.

Sheep eco-type	N	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	remark	%
Garag	7	6.6 ± 11	5.9. ± 0.1	0.7¤	(-)	10.6
Watesh	8	5.7 ± 0.8	6.0 ± 0.9	0.3¤	(+)	5.3
Desert	13	6.5 ± 0.4	5.9 ± 2.4	0.6¤	(-)	9.2
Baladi (control)	11	5.8 ± 1.4	5.4 ± 2.7	0.4¤	(-)	6.9

Mean (g/L).

(g) Urea concentration.

Sheep eco-type	N	Pre-infection Mean ± sd	Post-infection Mean ± sd	Changes Mean	remark	%
Garag	7	25.3 ± 8.4	41 ± 21.2	15.7	(+)	62.1
Watesh	8	25.0 ± 8.6	34 ± 1.4	9	(+)	36.0
Desert	13	22.3 ± 3.8	44 ± 11.1	21.7	(+)	97.3
Baladi (control)	11	24.6 ± 7.7	23.7± 12.6	0.9¤	(-)	3.7

Mean (mmol/L).

sd. = standard deviation. n = number of animals examined. (+) = increased.
(-) = decreased. ¤ non-significant.

The activities of alkaline phosphatase (AP) during infection increased in Desert (38.4%) and in Watesh (11.6%) while it decreased in Garag (31.5%). Aspartate Amino-transferase (AST) activities significantly increased in Desert (142%) and in Watesh (37.7%) but no significant changes were noticed in Garag.

Albumin concentrations slightly decreased with no significant differences among eco-types. On the other hand, highest serum bilirubin was noticed in the Desert sheep. Serum total proteins were not significantly affected and fluctuated within the normal levels during the experimental period. The urea concentrations in Garag, Watesh and Desert sheep eco-types variably increased (62.1%, 36%, and 97.3%, respectively). On the other hand, control animals did not show significant changes in all serum biochemical constituents. The pattern of the parasitaemia and the serum biochemical constituents in one recovered animal from *T. lestoquardi* infection were illustrated in (Fig. 3.2).

3.2.2. Confirmation of sheep infections by molecular diagnostic tools:

At the end of the experiment, the DNA was extracted from blood of all (test and control) animals and was subjected to conventional PCR and LAMP-PCR tests for further confirmation of *T. lestoquardi* infection. The results and documentations of *T. lestoquardi* by LAMP are shown in (Fig. 3.3). The comparative results of blood film, lymph node biopsies and molecular detections of *T. lestoquardi* infection are demonstrated in table (3.10).

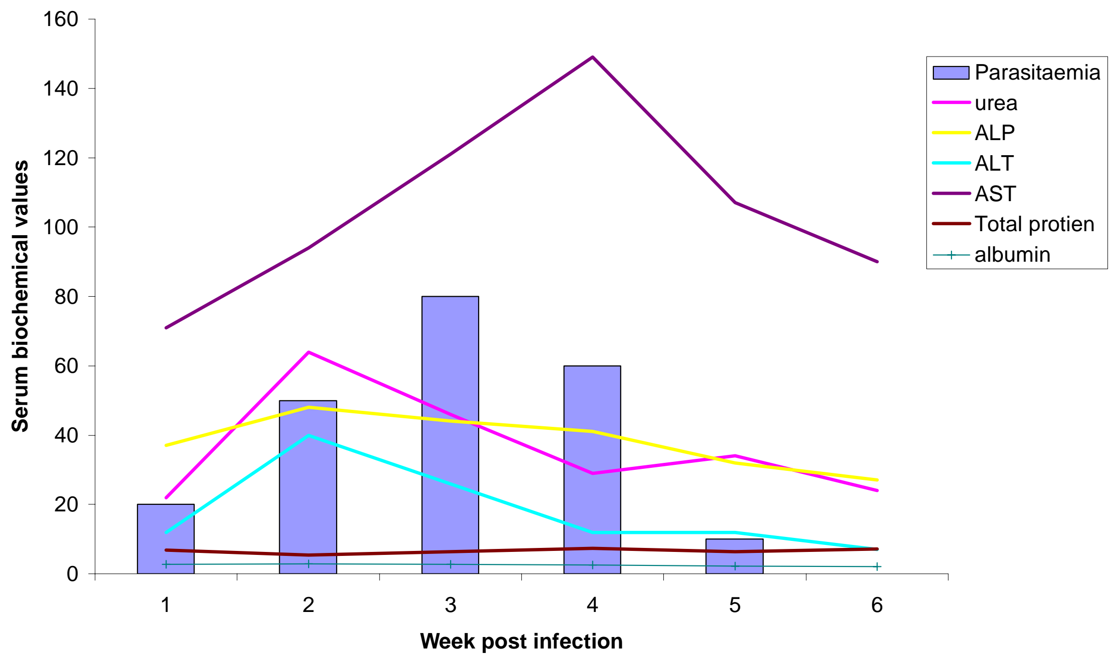


Fig. 3.2. Parasitaemia and serum biochemical constituents in one recovered animal from *T. lestoquardi* infection.

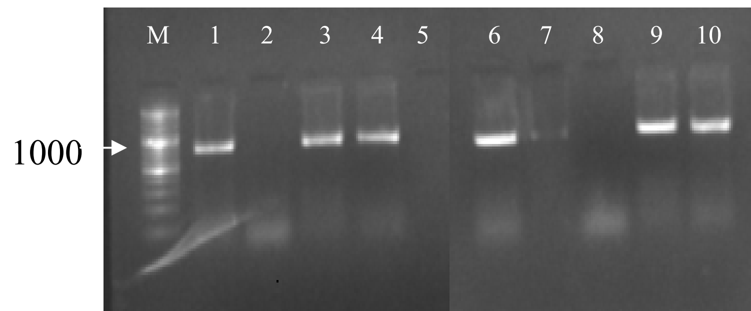


Fig. 3.3. Documentations of *T. lestoquardi* by LAMP using *T. lestoquardi* specific primers. Lane $_{M,}$ standard size marker, L_1 positive control, L_2 negative control, L $_{3\text{-}10}$ test samples.

Table 3.10. Confirmatory detection of *T. lestoquardi* infectivity of sheep by microscopy and molecular diagnostic methods.

Eco-type	No. of sheep	B.F	Lymph node biopsy	PCR	LAMP
Garag	16	8	8	6	7
Watesh	15	8	8	8	8
Desert	14	14	13	13	13
Control	11	5	1	9	11
Total	56	35	30	36	39

B. F. = blood film.

CHABTER FOUR
DISCUTION

Bovine theileriosis has been extensively studied, but a paucity of information exists concerning ovine theileriosis. Recently, interest has arisen in *Theileria* spp. infecting sheep.

Identification of *Theileria* spp. is based on morphology, host specificity, transmission mode, vector competency, and epidemiological data. Moreover, identification of *Theileria* spp. by conventional microscopy examination of blood and/or lymph node biopsy smears is time consuming and their results are subjective, particularly when mixed infections occur. Frequently serological methods are employed in determining subclinical infections. As different species share similar morphology and vector competency data are not always available and differential diagnosis for a particular species is difficult. False positive and negative results are commonly observed in serological tests due to cross-reactions or weak specific immune response. Furthermore, lack of determination of antibodies in carriers may be the result of long-term infection (Burridge *et al.,* 1974, Leemans *et al*., 1999a).

Recently, PCR-based molecular techniques are increasingly used. Results presented here showed that RLB based on amplification of a fragment of the 18S, 16S ribosomal DNA from virtually all species of *Theileria*/*Babesia* and of *Ehrlichia* is reliable, sensitive and specific test for identification and discrimination among different sheep tick-borne disease pathogens. RLB assay is a powerful tool and practical assay, since it is able to detect extremely low parasitemia rates and simultaneously identify *Theileria* and *Babesia* species (Gubbels *et al*., 1999, Schnittger *et al*., 2004).

This is the first report in which RLB has been used to detect, identify and to discriminate between different ovine *Theileria* parasites in the Sudan. The cross reaction of *T. lestoquardi* probe with *T. annulata* agree with what had been previously reported by many authors (Nagore *et al.,* 2004, Sparagano *et al*., 2006 and Altay *et al.,* 2007). It is known that *T. lestoquardi* and *T. annulata* have more features in common and they exhibit a strong serological cross-reactivity (Leemans, *et al*, 1997), similarities with regard to morphology (Allsopp, *et al*., 1994, Brown, *et al*., 1998), share the same *H. anatolicum* vector (Hooshmand-Rad and Hawa 1973b, Uilenberg *et al.,* 1980), but occur in separate host species. Both species parasitize the same cell phenotypes of their respective hosts (Leemans *et al*., 2001). In addition, *T. annulata* is capable of infecting and transforming ovine and caprine peripheral blood monocytes in vivo (Leemans *et al*., 1999a) and in vitro (Leemans *et al*., 1999b). In most cases their geographic distribution tends to overlap (Hooshmand-Rad and Hawa, 1973b). Moreover, sequence analysis of small subunit ribosomal RNA gene and sporozoites surface antigen has confirmed that *T. lestoquardi* was phylogenetically closely related and strikingly similar to that of *T. annulata* with an identity value of 99.7% (Schnittger *et al.*, 2000b). Thus, the present study confirmed the existence of cross-reaction between *T. lestoquardi* and *T. annulata* and also pointed to a closer antigenic relationship. Therefore, the current RLB results demonstrated that at least three distinct *Theileria* species (*T. ovis, T. lestoquardi* and *T. separata*) were found infecting sheep in the Sudan.

Infection with *T. lestoquardi* was expected, since this parasite had been previously reported in the Sudan (Salih *et al*., 2003 and Taha *et al*., 2003). While detection of *T. separata* and *T. ovis* in field samples from apparently healthy sheep are reported for the first time in the Sudan. In this

respect, the differentiation between malignant and benign *Theileria* spp. by conventional microscopic examination is very difficult. Therefore, the prevalence and surveillance of *T. lestoquardi* in the Sudan by conventional microscopy examination of blood smears taken from apparently healthy sheep is not reliable and subjective. Thus, sensitive and specific laboratory tests which can clearly differentiate and discriminate among pathogenic and non pathogenic ovine *Theileria* species are essential. The RLB assay described here will be a useful technique for both, detection and discrimination among different *Theileria* species in mixed infections of field samples. The disadvantages of this molecular method are that, they are expensive and require sophisticated laboratory equipment, complex protocol and involve hybridization to achieve higher sensitivity. RLB test was used in the Sudan with unsuccessful results (Salih, D., 2010 personal communication).

T. ovis is considered to be widely distributed in Africa, Asia and in Europe, partially corresponding with that of *T. lestoquardi*, whereas *T. separata* has been reported from more limited area, including several countries in Southern and Eastern Africa (Uilenberg 1981b). The high prevalence of *T. ovis* in the present investigations was not a surprising, since high prevalence of this *Theileria* spp. was detected in Spain (18.9%) prevalence (Ferrer and Castellá, 1999), Turkey with a prevalence range 54.0 to 67.9% (Aktas *et al.,* 2005a, Altay *et al.,* 2005) and Iran (7%) (Bami *et al.,* 2009). Therefore, further studies involving of *Theileria* spp. infecting sheep in the Sudan are needed to clarify their morphological, biological, serological, molecular relationships and interactions with the abundant causative agent of malignant theileriosis.

Sheep in the Sudan were classified on phenotypic bases into eight eco-types (McLeroy 1961b). There is an insufficient knowledge of relative susceptibility of sheep to Theileriosis worldwide (Uilenberg, 1997) and susceptibility of different sheep eco-types to *T. lestoquardi* infections in the Sudan is unknown. The routine spraying by chemical in addition to the use of domestic chickens as natural predators in Atbara, however, could be the result in low tick infestations. In spite of, low tick burdens, the detection of *T. lestoquardi* few days after natural tick infestations may indicate that the vectorial capacity of *H. anatolicum* in the endemic area is high. In addition, the naiive sheep showed high sensitivity to natural infections when introduced to the endemic area could be attributed to dose delivered and/or to the high susceptibility of animals.

Loss of condition could be due to *T. lestoquardi* infection. In fact, *T. lestoquardi* infected animals had an apparently normal appetite but in a few days after the onset of the fever they ceased eating and later on they became progressively emaciated. These findings are in agreement with other reports on experimental or tick induced infections (Hooshmand-Rad and Hawa, 1973a, Sisodia and Gautam, 1983, Brown *et al*., 1998, Leemans 2001).

The infectivity of many tick-borne diseases causal agent can be fatal to small ruminant under certain circumstances, depending on infective dose, breed, age, etc (Uilenberg, 1997). In this respect, the present result suggests that Desert sheep could be highly susceptible to *T. lestoquardi* infections.

Elevation of body temperature, or fever, is associated with many disease states. Since the hypothalamus is the control centre for thermal regulation, alterations that influence that centre must occur (Thomson, 1978). It is generally considered that chemicals (called pyrogens) can influence and alter the control centre. Certain bacteria and parasites contain,

secrete or excrete pyrogens with the result that infection with such agents causes fever. There is a tendency to think that fever means infection, but this is not always so. The pyrogens are released from body tissues and fluids when either or both are injured. Neutrophils release pyrogens and are one of their main sources. There is some argument as to whether pyrogens are released from damaged tissues or from the neutrophils that arrive in response to the damaged tissue. Immune mechanisms may induce release of pyrogens but, again probably by damaging tissue or drawing neutrophils to a site of injury or just with antigen-antibody complexes (Thomson, 1978). The marked thermal reactions of the Desert sheep to the infection could plausibly attribute to severe tissue damage and neutrophillia. This supported by the fact that, the severity of symptoms and duration of the clinical course of *T. lestoquardi* infection in addition to the post-mortem alterations and neutrophils infiltrations were more noticeable in this eco-type, than in the others. However, the detailed explanation of mechanisms that cause fever awaits full elucidation.

When the infected ixodid nymphs or adults feed on a susceptible vertebrate host, they inject the saliva containing sporozoites - the infective stage of *Theileria* spp. - into the host. Within a few minutes or even seconds, these sporozoites enter most often peripheral blood mononuclear cells (Fawcett and Doxsey 1982, Jura *et al.,* 1983). Then, in natural *Theileria* spp. infections, parasites initially invade lymphoid cells and, at a later stage, the erythrocytes (Soulsby, 1982). To complete the life cycle, the *Theileria* sporozoites eventually transform into trophozoites and it differentiate into a multinucleated schizont and this modifies the phenotype and behaviour of the lymphocytes. By dividing at the same time as the transformed lymphocytes, the parasites multiply by clonal expansion of the infected cell

population (Musoke *et al.,* 1992). The detection of clearly visible *Theileria* markers before the identification of schizonts infecting lymphocytes could be attributed to early transformation of infected lymphocytes into blast cells which divide synchronously along with the parasite they contain (Leemans, 2001). Examination of lymph nodes biopsy smears taken at weekly basis from animal that eventually recovered (7 weeks post *T. lestoquardi* infection), showed the above mentioned 3-4 obvious different morphological changes (*Theileria* markers). Moreover, no distinct lymphocyte abnormalities were seen in all animals prior to their exposures to natural infections. If proven unique to *Theileria,* the finding of *Theileria* markers observed in this study may contribute in early diagnosis of malignant ovine theileriosis. As reported, observation of these *Theileria* markers coincided with the later confirmation of schizont(s) in lymph node biopsy and/or piroplasm detection in blood smears.

T. lestoquardi are well established to have a schizogony reproductive cycle, usually in lymphocyte (Dolan, 1989) and detection of typical schizonts in lymph node smears is crucial for diagnosis. In the present study, schizonts infected cells in lymph node smears were detected in low numbers and persisted for a mean of 2 days and disappeared till the death/sacrificed of the animals. *T. lestoquardi* infection in sheep is apparently associated with low level of schizont parasitosis (Hooshmand-Rad and Hawa, 1973a,b, El-Hussein, 1997, Leemans *et al*., 1999a). According to Hawa *et al*., (1981), low duration of detectable schizonts (1-3 days) was recorded. On the other hand, schizonts were hardly seen in thin blood smears made from peripheral blood. On probability point of view, the detection of one out of the four distinct *Theileria* markers probably is easier than detection of typical

schizonts which persist only for two days. Thus, diagnosis of *Theileria* based on observation of *Theileria* markers would be advantageous.

The multiplication processes of schizonts and merozoites are generally referred to as schizogony and merogony, producing merozoites. These merozoites are set free following disruption of the host cells, and then invade erythrocytes where they transform into piroplasms and usually referred to as parasitaemia (Norval *et al.*, 1992, Mehlhorn and Schein, 1993). The erythrocyte infections with more than one piroplasm observed in the present study supporting the previous observation by Conrad *et al,* (1985).

The two animals that showed piroplasms in their blood and benign clinical course of *Theileria* infections and were negative in LAMP confirmatory test are assumed to be infected with non-pathogenic *T. ovis* and/or *T. separata*. *Theileria ovis* was highly prevalent in Atbara with 100% (57/57), whereas *T. separata* was not detected in any of the blood samples.

Under general field conditions, the most satisfactory diagnosis is made by the demonstrations of the schizonts in material obtained from superficial lymph nodes. The form in the erythrocytes may be difficult to see at times, and in the early part of the infection they may be very few. Differential diagnosis between *T. lestoquardi* and other benign *Theileria* spp. (*T. ovis, T. separata*) is not always easy, if not impossible. Notably, the confusion between *T. ovis* and *T. lestoquardi* is historically reported, since the latter parasite was first described as *T. ovis* (du Toit, 1918 cited in Leemans, 2001), then as *T. hirci* (Dschunkovsky and Urodschevich, 1924) and eventually designated as *T. lestoquardi* (Morel and Uilenberg, 1981). However, the results of the present study clearly point to the confusion in

field identifications of *T. lestoquardi* and *T. ovis*, due to the existence of mixed infections of both pathogenic and non-pathogenic *Theileria* spp.

An interesting finding of this study was the high parasitaemia which were detected in all *T. lestoquardi* infected animals. Similar finding were previously noticed (Tageldin *et al*., 1992), however, low parasitaemia were reported in field cases in Atbara (El-Hussein, 1993) and 1.8-5% parasitaemia were noticed in Garag sheep experimentally infected *T. lestoquardi* strain isolated from the same area (Osman, 1999). The very high parasitaemia (33%) reported may be attributed to the low (11.6 kg) body weight of this particular animal in addition to the loss of 1.5 kg (12.9%) of it's body weight post infection. Very high parasitaemia (5.5-32%) in *T. lestoquardi* infection was previously reported in experimentally infected sheep (Leemans *et al.,* 1999a). The high parasitaemia reported by these researchers may be attributed to high dose of stabilates under experimental conditions. But the parasitaemia (33%) reported here, in fact, is the first report high parasitaemia due to natural infection of sheep.

The high parasitaemia reported in the Desert sheep, may be attributed to their high susceptibility to the natural infection. From a numerical standpoint of the Sudanese sheep, only two ecotypes-Deserts and Nilotic- are of particular importance (McElroy, 1961b, Abualazayium, 2004). Desert sheep are the most valuable eco-type and play an important economic role in the country as it the main eco-type for export to neighbouring countries. High losses due to malignant theileriosis with mortality up to 100% were reported in this eco-type (Tageldin *et al*., 1992). Warranting and more attention to this particular sheep eco-type if they raised for export in the vast areas of North Sudan.

Leukoproliferation at early stage of the *T. lestoquardi* infections, which is later followed by a leuko-destructive phase leading to leuckopenia and anaemia were similar to previously those reported for *T. annulata* infection in cattle (Irvin and Morrison 1987). Sheep infected with *T. lestoquardi* also manifested anaemia due to erythrocytes destructions, but the precise mechanism of this anaemia is still unknown. Many studies tried to clarify these mechanisms of the development of anaemia (Shiono, *et al*., 2004). Some possible factors concerning the anaemia's pathogenesis such as surface morphological changes in RBC and an increase in osmotic fragility (Yagi *et al.,* 1989), abnormal RBC clearance (Yagi *et al*., 1991), changes in membrane glycolipid components (Watarai *et al*., 1995) and oxidative injuries (Shiono *et al*., 2001, 2003, Yagi *et al*., 2002) have been reported, but the precise mechanism is not yet clearly elucidated. An accelerated destruction of RBC in anaemic sheep may be attributed to the binding of autoantibody (IgG) to parasitized RBC that results in phagocytosis during the development of anaemia (Shiono, *et al*., 2004) or to the cytokine tumor necrosis factor (TNFα1) which is a potent inducer of fever, may play a role in anaemia (Ahmed, 2002). It is, thus, very much desired to explore the pathogenesis and the mechanism of this anaemia. The reductions in PCV, Hb concentration and RBCs counts reported in the present study, however, were shown to be correlated with the corresponding levels of parasitaemia. On the other hand, remarkable reductions in these values were noticed in the Desert sheep. These transient reactions were probably related to the course of the disease and/or to its susceptibility.

The clear macroscopic alterations of the Desert sheep during different courses of the disease may be due to variations in immune responses to the infection. The slight distention of gall bladders with green bile during acute

T. lestoquardi infected sheep had been previously noticed (Osman, 1999), while their remarkable distension during the chronic course may be attributed to the heavy destruction of infected RBCs. The disease severity and their pathological changes were similar to the previous reports in the Sudan for both natural outbreaks (Tageldin *et al*., 1992) and experimental infection of malignant ovine theileriosis (Osman, 1999). The typical haemorrhagic ulcers of the abomasi seen in *T. annulata* infection of cattle were notably absent in this study, this will support the previous observations (Hooshmand-Rad and Hawa, 1973a).

The hepatization and rubbery texture of the lungs observed in addition to the accumulations of excessive fluids and exudates in the chest cavity may enhance the labour respiration. Serious tissue destructions and pulmonary oedema, suggest that emphysema and interstitial pneumonia lead to respiratory failure and provides direct evidence for death of sheep suffering from *T. lestoquardi* infections. These findings were previously reported by many workers (Uilenberg 1981b, Irvin and Morrison 1987).

During the early of infection few schizonts can be detected in superficial lymph nodes and later schizogonies shift to invade the internal lymph nodes. This may support the earlier discussion on *Theileria* markers and early detection of *Theileria* infection. In the present study, the differentiation between micro and macro-schizont were not considered. The terms micro and macro-schizont that were previously reported in many *Theileria* spp. are confusing. In fact, in schizogony the schizont nuclei divided to merozoites. The number of merozoites are related to the division of the schizont nuclei and consequently to the size of the merozoites yield. The number of schizont nuclei in culture remains relatively constant, indicating the rate of division of the host cell (Irvin *et al*., 1982). The

confusion noticed here is supporting a proposal that the terms macroschizonts and microschizonts are better avoided (Mehlhorn and Schein, 1984). In simultaneously while the schizonts shift to internal lymph nodes, parasitized lymphocytes with approximately 30-300 merozoites were detected in the lungs. These parasitized lymphocytes might rupture and the merozoites might infect new lymphocytes that proliferate in the lungs. In fact, these types of schizont infected lymphocytes were not detected in the peripheral blood smears, superficial or in internal lymph node biopsies as well as in impression smears from other major organs such as liver, kidney, spleen and heart. This assumption could further add evidence to explain the massive schizogonies in the lungs and not in other organs. Infection of lymphocyte may be extremely important in the pathogenesis of *T. lestoquardi* infections. In addition, numerous other forms of schizonts parasitized cells with 9-12 merozoites were scattered throughout the lung tissues. This schizonts stage was designated as (ring stage). The ring stage inside the infected cell was pushed to the apical end of the cell cytoplasm by a progressive, outward evagination. Nevertheless, enlarged cells with a circular outlet were occasionally noticed in all animals' lungs. The equal diameters of both circular outlet and ring stage in addition to its outward pushing that was clearly seen, may suggest the budding process. Furthermore, extracellular ring stages were detected in the peripheral blood, all organs examined and even in bone marrow aspiration, but not in brain impression smears. The detection of the ring stages in the peripheral blood may disseminate the ring stages leading to spread throughout the reticuloendothelial system in the internal lymph nodes, kidneys, heart and mucosae and generating new intra-lymphocytes schizogonies that may later accumulate in the lung tissues. It is assume that the Schizonte infected

lymphocytes remain intact during passage through the heart and become sequestered within lung capillaries where the membrane eventually disintegrated liberating merozoites into the lung circulation to initiate the erythrocytic phase of the *Theileria* cycle. A large number of merozoites were also seen arrested in alveolar capillaries suggesting that many merozoites lie free in the pulmonary and can contact and infect RBC. The lung is a major portal of entry both for non-pathogenic antigenic material as well as a range of viral, bacterial and parasitological pathogens. In addition, the red blood cells during the pulmonary circulation are stagnantly filling the lungs' alveoli for vital physiological exchange of gases. This stagnation may ease the penetration of the merozoites. So far, as proposed previously (Baer, *et al*., 2007) for malaria merozoites, the release of merozoites into the lung microvasculature rather than into larger blood vessels is advantageous, because the low macrophage density and the reduced blood velocity with reduced shear forces will enhance the ability of merozoites to invade erythrocytes. In the present study, there is evidence that the infectivity of the RBCs may take place in the lung. Intra-erythrocytic piroplasms were detected in the lung impression smears with relative parasitaemia ranging between 3-6% and RBC was infected with only one piroplasm and it was relatively smaller than those detected in the peripheral blood smears. The majority of protozoan parasite life cycles are of a complex and dynamic nature (Kennedy, 1976). Earlier workers had believed that hepatocytes release Plasmodium merozoites into the sinusoidal blood where erythrocytic invasion take place. But, recently, this hypothesis was mitigated by Baer, *et al*. (2007). Using intravital microscopy and fluorescent parasites, these authors studied the mode and dynamics of *Plasmodium yoeli* parasite release from the liver, a critical stage in the malaria life cycle. Their results showed

that the majority of hepatocytes containing merozoites (merosomes) exit the liver intact and contain 100–200 merozoites. Merosomes survived the subsequent passage through the right heart undamaged and accumulated in the lungs where it effectively cleared the blood from all large parasite aggregates. The study concludes that merosome packaging protects hepatic merozoites from phagocytic attack by sinusoidal Kupffer cells, and that release into the lung microvasculature enhances the chance of successful erythrocyte invasion. Like the *Plasmodium*, *Theileria* parasites have a typical apicomplexan lifecycle involving several differentiation and multiplication stages in their mammalian hosts and their vector ticks (Norval *et al*., 1992, Mehlhorn and Schein, 1993). The present study proposes similar to *P. yoelli*, red blood cell invasion takes place in the lungs.

The present study represents the first report of parasitized cells which were closely encircled with more than 8-9 monocytes in a rosette shape. Some of these rosettes were seen ruptured leaving empty circles. The animals' innate immunity is usually sensitized during the acute course of infections, progress and reaches maximum level to control the disease in the chronically infected and/or recovered animals. The monocytes that were closely encircling the schizonts infected lymphocytes detected, however, were presumably (T cells) rather than (B cells) initiating the animals' adaptive defense mechanisms. It is thought that, T cells (cellular immunity) are usually found in direct contact with the antigens while the B cells (humoral immunity) are found remote from antigen source. The lung is a major portal of parasitological pathogens entries. In fact, lung is populated by large numbers of memory T cells which mainly reside within the bronchial lamina propria, in the alveolar walls and the interstitium (Campbell *et al*., 2001). There are more T cells in the lung than in the

peripheral circulation, and in a normal lung it has been calculated to contain ten times more T cells than neutrophils (Pabst *et al*., 1987). The functions of these cells aggregated in the lungs are thought to be important in host defense (Wardlaw and Hamid, 2002) and are involved in initiating and regulating the immune response to pathogens. However, lung T cells are also involved in inflammatory conditions affecting the lung (Inui *et al.,* 2001, Brightling *et al*., 2002). It has therefore a sophisticated immune system which involves barrier, innate and specific immune components. Moreover, chemokine receptors CXCR6 is a key receptor involved in constitutive migration of T cells to the lung (Day *et al*., 2009). Furthermore, cytotoxic T-cell secretions can destroy the infected cell, hence the name killer cells. More recent suggestions (Anon, 2010), state that, if lymphocytes encounter an antigen trapped by the antigen-presenting cells of the lymphoid organs, lymphocytes with receptors specific to that antigen stop their migration and settle locally to mount an immune response.

Schizonts aggregate in the lungs are highly susceptible to phagocytosis and must therefore avoid contact with macrophages and/or others killing cells. Released merozoites have also a short life span and must infect erythrocytes immediately after release into the lungs. As previously suggested, acute danger of closely encircled elimination is presented in the form T cells. The resident T cells of the lung that comprises by far the largest population of tissue apoptosis of the body. This strategic position of T cells makes it difficult for free merozoites to exit the schizonts infected lymphocytes without being trapped and/or killed by these surveillance cells of the immune system. The present study also proposed that, each merozoite must pit out from schizonts to infect the red blood cells. The pitting theory, thus, allows the safe passage of merozoites through the gauntlet of lung T

cells. This assumption, however, may give a robust evidence to explain the appearance of empty cytoplasm punctures phenomenon. The above proposed pitting theory is not reported in *Theileria* parasites but pitting of malaria parasites was documented (Anyona *et al*., 2006). The growth of *T. lestoquardi* schizonts in the lymphocytes leads to membrane insertion of parasite antigens such as *T. lestoquardi* schizont surface protein Clone-5 ((Bakheit *et. al.,* 2006), and also induces profound modifications to the lymphocyte membrane integrity resulting in schizonts infected lymphocyte deformity. The schizont surface proteins then become the sites for deposition of immunoglobulins or complement. All these alterations of infected lymphocyte surface are signals to macrophage to attack and destroy these lymphocytes by phagocytosis or perhaps lysis (Anyona *et al*., 2006). Certainly, the removal of large numbers of infected lymphocytes as demonstrated earlier in the lungs is one of the mechanisms that contribute to development of leukopenia reported here in early sage of the infection. A competing mechanism of parasite stages clearance through the process of pitting with infected lymphocytes salvage as suggested in the present study, might act as a host mechanism of attenuating leukopenia. The current hypothesis is in accord with *Plasmodium* piroplasms pitting from the infected RBCs (Anyona *et al*., 2006). It was speculated that the spherocytes reflect intraerythrocytic removal of malaria parasites with a concurrent removal of RBC membrane through a process analogous to pitting of intraerythrocytic inclusion bodies (Anyona *et al*., 2006). In fact, animal's spleen can remove intraerythrocytic parasites while leaving the host erythrocyte intact, in a pitting process as previously reported (Conrad and Dennis, 1968, Schnitzer *et al*., 1972). Further evidence of pitting in human infected with *Plasmodium falciparum* malaria came from demonstration of

circulating RBCs containing abundant ring-infected erythrocyte surface antigen but no intracellular parasites ((Angus *et al*., 2004, Newton *et al*., 2001). It also been demonstrated that, because of the rigidity that is associated with infected RBCs, trophozoites may be pitted out of infected erythrocyte by the shear pressure of the tight spleen capillary bed (Shelby *et al*., 2003). Whether by action of macrophages or capillary bed pressure, the product of pitting is creation of surface area depleted RBCs (Jain, 1993) that are free of parasites. Pitting and re-circulation of RBCs devoid of malaria parasites could be a host mechanism for parasite clearance while minimizing the anaemia that would occur were the entire parasitized RBC removed (Anyona *et al*., 2006). The trigger mechanisms for infected lymphocytes salvage as opposed to destruction are unknown. However, the current study suggests based on micro-pathological evidence, merozoites pitting off the schizont to infect new cells or removal of parasite particles with a concurrent removal of infected lymphocytes is possible and plausible.

In fact, respiratory distress syndrome (RDS) denotes impaired pulmonary functions of which pneumonia is the main cause (Hierholzer *et al*., 1998). Activated neutrophils have been implicated in the pathogenesis of RDS (Tate and Repine, 1983). It adheres to vascular endothelium and migrates into lung interstitium and alveolar spaces (Weiland *et al*., 1993) causing endothelial and epithelial cell damage (Knight *et al*., 1992) through production of reactive oxygen intermediates (ROI) and the release of lysosomal proteases (King *et al*., 1995). A variety of chemical mediators of inflammation as well as cytokines have been demonstrated to prime or activate neutrophils *in vitro*, including granulocyte colony-stimulating factor (G-CSF). G-CSF protein is a cytokine produced by a variety of cell types, including macrophages, fibroblasts, endothelial cells, and bronchioepithelial

cells (Hierholzer *et al*., 1996). This protein also is essential for neutrophils production and has been shown *in vitro* and after systemic administration to enhance functional activities of mature neutrophils, including phagocytosis. Moreover, it is produced in the lungs of rats subjected to haemorrhagic shock (Hierholzer, *et al*., 1996). It was documented that G-CSF cause neutrophils recruitment and neutrophil-mediated injury (Hierholzer *et al*., 1998). Therefore, it resulted in increased wet-to-dry ratios, an increase in bronchoalveolar fluid and accumulation of neutrophils into widened interstitial and alveolar spaces. Decades ago, pulmonary neutrophillia infiltrations were implicated in the pathogenesis of RDS (Tate, and Repine, 1983). The current study indicates that *T. lestoquardi* infections are accompanied by severe pulmonary involvements. Hitherto, the complications of pulmonary oedema in respiratory distress syndrome in *Theileria* infected animals are not studied and the magnitudes of these clinical complications are also not undertaken. In fact, causes of the protracted pulmonary complications in severe *T. lestoquardi* infection are currently unclear. Presumably, *Theileria* and *Plasmodium falciparum* exhibit astonishing similarities with regard to parasite infectivity and pathological changes that involve the lungs. Therefore, pulmonary oedema as a result of increased vascular permeability is a cardinal feature of the impairment of lung function and RDS in severe *P. falciparum* malaria (Maguire *et al*., 2005). In addition, the occurrence of prolonged impairment of gas transfer demonstrated in patients with *Plasmodium vivax* infection would also be consistent with this hypothesis (Anstey *et al*., 2007). In comparison, *T. lestoquardi* infection results in severe tissue destruction and pulmonary oedema leading to respiratory failure (Uilenberg 1981b, Irvin and Morrison, 1987) as in human malaria (Taylor *et al*., 2006). RDS in human is a major

prognostic determinant in both African and Western adults and is associated with a high fatality rate of 60% to 70% in the absence of ventilatory support (Robinson *et al*. 2006, Bruneel *et al*, 2003). However, alarming increase in sheep deaths due to lethal *T. lestoquardi* infections could be attributed to lungs involvement rather than to any often organ. Based on this pulmonary manifestations, the present study proposes, Malignant Pulmonary Theileriosis (MPT) as a more descriptive nomenclature than Malignant Ovine Theileriosis especially in view of the fact that this disease is lethal to both ovine and caprine (Hooshmand-Rad and Hawa, 1973a), therefore, is more confusing than the proposed MPT.

The current study did not deal with the treatment of *T. lestoquardi*, however, the possibility to propose a symptomatic treatment respect to the pulmonary oedema in view of our results could not be excluded. Usually animals suffering from severe MOT are given medication accompanied with blood expander such as Glucose and/or mineral infusions. These blood infusions excessively exaggerate the pulmonary oedema and interfere with breathing. Therefore, diuretics such as (Lasix) in addition to anti-inflammatory drugs such as corticosteroids and bronchodilators have presumably beneficial effects. Previously, it was postulated that vitamin E reduces transendothelial migration of neutrophils and prevents pulmonary oedema (Rocksen *et al*., 2003). Saprophytic bacteria involve in catarrhal pneumonia was isolated in sheep infected with *T. lestoquardi* (Leemans, 2001) thus, combinations of long acting tetracycline and buparvaquone (Butalex) injections were also proposed.

Macrophages are tissue cells that derive from circulating blood monocytes. Normally, they are diffusely scattered and found in increasing numbers in organs such as lungs (alveolar macrophages). In these sites, they

act as filters for particular matter. These cells constitute the critical mainstay and heart during antigenic infection leading to eliminations of infected cells. The E cells detected in the present study are probably macrophages. An early transformation of infected lymphocytes into blast cells which divide synchronously along with the parasite they contain are reported (Leemans, 2001). The large size of the E cell, however, resembles the macrophages. Since, when monocytes reach the extravascular tissue, they undergo transformation into larger macrophages now capable of substantial phagocytosis. The phenotypes of ovine cell lines infected with *T. lestoquardi* are monocytes/macrophages and B cells (Leemans *et al*., 2001). The condense inclusion body-like splintered in the E cell's cytoplasm may be fragments of schizont infected cell. In fact, macrophages when activated have increased content of lysosomal enzymes, more active metabolism, and greater ability to kill ingested organism. Furthermore, schizont-infected cells undergo a wide range of phenotypic alterations including production of a number of cytokines, surface receptors, adhesion molecules, and presentation of infection-associated antigens (Ahmed *et al*., 1999, Dobbelaere and Heussler 1999). Such cytokine can induce macrophages to fuse into cells containing antigens. Then, initiating the process of phagocytosis (and ultimately kill) microbes coated by antibody and/or complement. Consequently, they are important effector elements in humoral and cellular immunity which facilitate spontaneous recovery. These E cells when subjected to IFA test, schizont antigens coated by antibodies were seen by the fluoresce emitting greenish light. These emitting add a clue evidence for detection of antigen-antibody complex inside and around the E. cell's cytoplasm. Certainly, however, the E. cells are macrophages engulfing schizont infected lymphocytes and the 5-6 inclusions body-like are

fragmented nuclei. Discussion concerning cells involves in immunity as such are diversified. Nevertheless, this study could not find obvious explanations for all observations, thus precise studies are needed.

Metastases of schizont infected cells and large phagocytic cells in addition to remarkable inflammatory reactions that observed in the lungs were similar to these previously reported in MOT out breaks (Tageldin 1992) and experimental infection of sheep (Osman 1999). It is well established that lymphocytes are the target cells for *Theileria* spp. where they undergo their first schizogony reproductive cycle (Dolan, 1989). This would lead to the evident transformation of the parasitized lymphocytes with consequent hyperplasia and proliferative changes (Dolan, 1989, Norval *et al*., 1992). The microscopical appearance of different cells infiltration and pulmonary involvement are coincided with the previous macroscopical findings. In fact, the pathological and histopathological changes observed in the different organs and tissues may be caused by proliferation of schizont-infected macrophages, which subsequently stimulate a severe uncontrolled proliferation of uninfected T lymphocytes, (Branco *et al*., 2010) as observed in this study macrophages and lymphocytes were seen in large numbers in liver sinusoid, lung alveoli, and in pulmonary blood vessels.

The increase in all liver enzymes (ALT, AP, AST) may be attributed to degenerative and necrotic changes of the hepatocytes and to the level of schizonts parasitosis (moderate) in the liver. Respect to the remarkable increases in activity of these enzymes in the Desert eco-type could be attributed to severity of *T. lestoquardi* infections due to the high susceptibility of this eco-type. In fact, plasma activities of liver enzymes are considered as sensitive indications of liver damage in sheep. The gradual changes of these enzymes during the progressive infection of *T. lestoquardi*

infections may be used as reliable diagnostic tools for the differentiation of the various stages of the parasite, indicating the presence of cell necrosis and liver lesion in billiary system. Further research is therefore required to determine the effect of *T. lestoquardi* on the cellular integrity and related plasma enzymes activities in sheep.

The rise in urea concentrations is mostly attributed to histopathologicl alterations that occurred in the renal parenchyma due to the presence of the parasite and appears to have a strong correlation with the level of parasitaemia. The increase in bilirubin concentration in blood circulating is probably due to the destructive action of the blood piroplasms. This finding was further supported by the significant reduction in Hb, PCV and RBCs count. In addition, the Desert sheep showed retention of bile up to 50 ml, consequently bilirubin could be too high. Often, bile constitutes the primary pathway for the elimination of bilirubin. A decrease in total protein level may be caused by liver disease associated with a reduction in protein synthesis or related to fluctuation in the diet protein supplements.

The results of molecular confirmation (PCR and LAMP) certainly proved that all animals were infected with *T. lestoquardi.* Therefore, *T. lestoquardi* is very lethal disease that causes high mortality among Sudanese sheep population if they are exposed to infected ticks in endemic area such as Atbara.

Remarks:

1. This is the first report in which RLB has been used to detect, identify and to discriminate between different ovine *Theileria* parasites in the Sudan.
2. *T. separata* and *T. ovis* in field samples from apparently healthy sheep are reported for the first time in the Sudan.
3. Four distinct characteristic abnormalities (*Theileria* markers) differentiated the infected lymphocytes from non-infected are advantageous in MOT diagnosis.
4. Budding of the ring stage to liberate merozoites to invade the erythrocytes in the lungs was suggested to fill the gap in the previous unknown part of *T. lestoquardi* life cycle.
5. Rosette phenomena (8-9 T cells encircled the schizont infected-lymphocytes) were reported for the first time.
6. Parasitaemia (33%) is the first report of high parasitaemia under natural infection of sheep.
7. Merozoite must pit out from schizont-infected cell to infect the RBCs in the lungs is proposed.
8. Malignant Pulmonary Theileriosis (MPT) is proposed as descriptive nomenclature than Malignant Ovine Theileriosis.
9. Three native sheep eco-types were ranked according to *T. lestoquardi* susceptibility bases for the first time.
10. New line of symptomatic treatment respect to the pulmonary oedema is proposed.

Conclusion and recommendations:

The present study focused on natural *T. lestoquardi* infection and it pathogenesis and diagnosis in three indigenous sheep eco-types. The current investigations, give clear evidence that native Sudanese sheep are highly susceptible to *T. lestoquardi* infections.

Malignant Ovine Theileriosis is widely spread in River Nile and Khartoum States. The disease therefore, is considered a real threat to sheep industry and exportation. In this respect, Desert sheep have to be brought to Khartoum for finishing and subsequently shipped through River Nile State (endemic areas) for exportations. Although, sizeable sheep populations in the country are at risk due to animals' movements and/or vectors tick disseminations. Little has been done concerning MOT. High abundance of *H. anatolicum* with renowned (high) vectorial capacities represents a major obstacle for future planning of livestock improvement in endemic areas. This is due to the fact that introduction or crossing with Desert (more valuable eco-type) would be at the expense of the natural resistance of Baladi eco-type against the infections. The parasitological, haematological, pathological and biochemical investigations currently studied concluded that, the Desert eco-type are highly susceptible to *T. lestoquardi* infections than Garag and Watesh. The radical solution of these problems does not seem to be an easy or a simple task. The classical approach for tick control by the expensive use of chemical acaricides was not feasible and successful in many African countries due to the rising cost of chemical and the tendency of ticks to develop resistance against various compounds. Extensive research, however, is essentially required for establishing more effective reliable control measures. In this respect ecologically based strategies have further advantages as a possible alternative for chemicals control. Exploitation of

natural enemies such as domestic chickens could be attempted in the endemic farms with the least financial cost. In the Sudan, the Nile from Wadi Halfa to Khartoum and the White Nile from Khartoum to Renk represents the natural barrier isolating *T. lestoquardi* free zones from endemic areas. Therefore, sheep from endemic areas not allowed crossing this barrier to settle in and/or transit thought Kordofan and to Darfur States. Nevertheless, immunization of sheep against *T. lestoquardi* with a candidate tissue culture vaccine would hopefully be a solution for this problem. The present study concludes that Desert Sudanese sheep eco-type are highly susceptible to MOT and require special care regarding diagnosis, treatment, vector control and other measures when introduced to endemic areas.

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Table (3.3): Mean (± sd.) of primary reactions of the three sheep eco-types to natural infection with *T. lestoquardi*.

Sheep eco-type (n)	Fever		Detectable schizonts/10^3cells		Paras
	Mean duration (days)	Maximum temp. (°C)	Mean duration (days)	Maximum	Mean dura (days)
Garag (7)	4.4 ± 2.9	40.8	2.0 ± 1.3	4	9.8 ± 6.
Watesh (8)	12.0 ± 16.6	40.7	1.8 ± 2.1	8	8.3 ± 1.
Desert (13)	15.5 ± 11.7	41.2	2.1 ± 1.6	11	6.4 ± 9.

(n) = number of animals.
temp. = temperature.
sd = standard deviation

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Table (3.8): The Mean numbers of *T. lestoquardi* schizont infected cells in impression smears from lymph nodes and lung during disease course.

n = number of animals examined.

Disease			Mean numbers of schizonts ($/10^3$ cells) ± sd.					
course	duration		Superficial lymph nodes			Internal lymph nodes		
	(days)	n	prescapular	parotid	Submandibular	mesenteric	hepatic	pu
Acute	6-9	4	39.8 ± 21.4	26.8 ± 12.7	25.5 ± 17.0	3.8 ± 1.7	6.0 ± 2.9	12
Subacute	10-20	3	9.7 ± 2.1	12.7 ± 4.0	13.0 ± 7.0	101 ± 16.7	70 ± 34.2	10
Chronic	21-33	2	4.0 ± 2.8	4.0 ± 1.4	2.5 ± 2.1	93.0 ± 2.8	71.0 ± 8.5	83
Recovered	42	1	4	5	7	63	42	

sd. = standard deviation.