بسم الله الرحمن الرحيم

Sudan University of Science and Technology
College of Graduate Studies

Molecular Detection of Natural Clinical Infections of Cattle with *Theileria lestoquardi* and Sheep with *Theileria annulata* in Atbara - Sudan

الكشف الجزيئي عن العدوي السريرية الطبيعية في الأبقار بثاليريا لستكواردى وفي الاغنام بثاليريا أنيولاتا في عطبرة – السودان

A Thesis Submitted to the College of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Preventive Veterinary Medicine (M.P.V.M)

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الآية

قَالَ تَعَالَىٰ:

﴿ قُل لَّوْ كَانَ ٱلْبَحْرُ مِدَادًا لِّكَلِمَٰتِ رَبِّى لَنَفِدَ ٱلْبَحْرُ قَبْلَ أَن تَنفَدَ كَلِمَٰتُ رَبِّى وَلَوْ جِئْنَا بِمِثْلِهِۦ مَدَدًا ﴿١٠٩﴾ قُلْ إِنَّمَآ أَنَا۠ بَشَرٌ مِّثْلُكُمْ يُوحَىٰٓ إِلَىَّ أَنَّمَآ إِلَٰهُكُمْ إِلَٰهٌ وَٰحِدٌ ۖ فَمَن كَانَ يَرْجُوا۟ لِقَآءَ رَبِّهِۦ فَلْيَعْمَلْ عَمَلًا صَٰلِحًا وَلَا يُشْرِكْ بِعِبَادَةِ رَبِّهِۦٓ أَحَدًۢا ﴿١١٠﴾ ﴾

صدق الله العظيم

سورة الكهف: الآيات ١٠٩ – ١١٠

Dedication

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

To my father who was always my support

To my brother, sister and their children

To all my friends& colleagues

With love and respect

Acknowledgments

Firstly, praise and thanks Almighty Allah, for giving me the health and strength to complete this study.

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Abstract

Generally, in Northern Sudan cattle and sheep are grazed together in the same pastures, in such a situation the transmission of the *Theileria annulata* to sheep and *Theileria lestoqurdi* to cattle can occur.

The aims of this study were to evaluate the possibility of transmission of the *Theileria annulata* to sheep and *Theileria lestoqurdi* to cattle that showed clinical signs of theileriosis in the field.

The DNA extracted from sheep was investigated by PCR using sets of primers specific for *T.annulata*. Whereas the DNA extracted from cattle blood was investigated by PCR using sets of primers specific for *T.lestoquardi*.

Three out of 40 sheep (7.5%) were positive for *T. annulata*. These positive samples (3 samples) were rechecked for the infection with *T.lestoquardi* by using sets of primers specific for *T.lestoquardi*. The result of these samples was negative for *T.lestoquardi*. Thus, the presence of *T. annulata* in sheep is the main cause of the present pathogenic symptoms.

All blood samples extracted from cattle were negative for *Theileria lestoqurdi*. These results indicate that the transmission of the *Theileria annulata* to sheep with the appearance of clinical signs could happen under field conditions, while the transmission of *Theileria lestoqurdi* to cattle was not observed under the same conditions.

Therefore, breeding of sheep and cattle should be done separately to avoid the transfer of *Theileria* from cattle to sheep vice versa.

ملخص الاطروحة

بشكل عام في شمال السودان ، الأبقار والأغنام ترعي في نفس المكان ، فى هذة الحاله يمكن أن تنتقل الثاليريا أنيولاتا إلي الأغنام و الثاليريا لستوكاردي إلي الأبقار .

الهدف من هذه الدراسة هو تقيم إحتمالية إنتقال الثاليريا أنيولاتا إلي الأغنام والثاليريا لستوكاردي إلي البقر فى الحيوانات التى تظهر عليها علامات سريريه لمرض الثايليريا في الحقل. تم فحص الحمض النووي المستخرج من دم الأغنام بواسطة تفاعل البلمرة المتسلسل بإستخدام مجموعات من البادئات الخاصة بالثاليريا أنيولاتا في حين تم فحص الحمض النووي المستخرج من دم الأبقار بواسطة تفاعلالبلمرةالمتسلسل بإستخدام مجموعات من البادئات الخاصة الثايليريا لستوكاردي.

ثلاثة من اصل40 رأس من الأغنام (7.5%) كانت موجبة لثاليريا أنيولاتا ، وتم إعادة فحص هذه العينات الموجبة (3عينات)للكشف عن الإصابة بالثاليريا لستوكارديوكانت نتيجة هذه العينات سلبية بالنسبة لثايليريا لستوكاردي.بالتالي فإن وجود ثاليريا أنيولاتا في الأغنام هو السبب الرئيسي للأعراض المرضيةالحالية.

كانت جميع عينات الدم المستخرجة من الابقار سلبية بالنسبة لثايليريا لستوكاردي ،و تشير هذه النتيجة إلي أن انتقال الثاليريا أنيولاتا إلي الأغنام مع ظهور الأعراض السريرية المرضية .يمكن أن يحدث في ظل ظروف الحقل ، بينما لم يلاحظ إنتقال الثايليريا لستوكاردي إلي الأبقار تحت نفس الظروف.لذلك يجب تربية الأغنام والأبقار بعيداً عن بعض لتجنب إنتقال الثاليريا من الأبقار إلي الأغنام او العكس.

Introduction

Generally, parasitic diseases are a major threat to global animal health and causing an important loss in livestock compared with other infectious or metabolic diseases, particularly in the tropics area (Perry and Young, 1995). Among parasitic diseases, tick-borne diseases such as Theileriosis, Babesiosis, Cowdriosis and Anaplasmosis are the major health problems affecting the productivity of livestock in many developing countries including Sudan (De Castro, 1997).

Theileriosis is a tick-borne disease caused by species of protozoa belongs to the genus *Theileria* and affecting mainly cattle, sheep, goats, buffaloes and other wild ruminants (Losos, 1986).The disease is transmitted by *Hyalomma* (Dolan, 1989). Globally, the diseases have a serious economic impact in view of mortality, reduced milk yield, weight losses and abortions (Gharbi *et al*., 2015). Many factors such as locations, management systems and age of animals could be considered as factors that increased the infection with *Theileria* in northern Sudan (Salih *et al*., 2007a).

In Sudan, six species of *Theileria* have been reported; *T.annulata, T.mutans, T.lestoquardi, T.velifera, T. ovis*, and *T.parva* (FAO, 1983b). Among all these species *T.lestoquardi*, the causative agent of malignant ovine theileriosis, and *T. annulata,* the causative agent of tropical theileriosis, are considered as the most important species in Sudan (Latif *et al*., 1994). The previous study showed that around 14% of cattle in Northern Sudan are infected with *Tropical theileriosis* (Salih *et al*., 2009). Later, a serological study using an indirect fluorescent antibody assay (IFA) and ELISA suggests that the prevalence is much greater (over 30%) (Salih *et al*., 2007b). Moreover, they found that the prevalence of *T. lestoquardi* in northern Sudan about 23.4% by using indirect fluorescent antibody (IFA) test (Salih *et al*., 2003).

In the northern Sudan cattle and small ruminants are grazed together, which may lead to the transmission of *Theileria annulata* from cattle to sheep and *Theileria lestoquardi* from sheep to cattle (Taha *et a*l.,2013).

Although, the cross infectivity of *Theileria lestoquardi* and *Theileria annulata* in cattle and sheep, respectively, was reported. The link between the infection and the pathogenetic symptoms has not been investigated. In this study, PCR technique was used to detect the infection with *Theileria annulata* in sheep and *Theileria lestoquardi* in cattle in animal that showing clinical signs of theileriosis.

Objectives of the study:

1/ To estimate the natural prevalence of *Theileria lestoquardi* in cattle and *Theileria annulata* in sheep in Atbara locality using PCR.

CHAPTERONE

LITERATURE REVIEW

1. Etiology:-

Piroplasmida contains two main genera (Babesia and Theileria). The genus *Theileria* is distinguished by infection of Lymph nodes by sporozoites, maturation of schizonts into merozoites and subsequent infection of red blood cells to form piroplasms (Mans *et al*., 2015). Theileriosis is caused by the protozoan parasite of *Theileria* species, which are round ovoid rod like, or irregular shaped organism found in lymphocytes, histiocytes and erythrocytes (Bhatnagar *et al*., 2015).

Theileria annulata, Theileria lestoquardi and Theileria parva are considered as most important species of *Theileria* (Uilenberg, 1983). In Sudan we have just *Theileria annulata and Theileria lestoquardi* (Latif *et al*., 1994).

1.1 *Theileria annulata*:-

Theileria annulata is the causative agent of tropical theileriosis, which affects mainly cattle (Soulsby, 1982; FAO, 1983a; OIE, 2004).

1.2 *Theileria lestoquardi*:-

Theileria lestoquardi is the causative agent of malignant ovine theilerioses and it affects mainly sheep (Soulsby, 1982; Arnold and Dias, 1983; Uilenberg, 1983).

2.Taxonomy of *Theileria*:-

Classification according to the scheme set by Levine *et al*., (1980) *Theileria* is classified as follows:

Phylum: Apicomplexa

Class : Sporozoea

Subclass: Piroplasmia

Order: Piroplasmida

Family: Theileridae

Genus: *Theileria*

Specie: *Theileria lestoquardi***,** *Theileria annulata,Theileria parva***,** *Theileria mutans.*

3. Epidemiology:-

3.1 Malignant Ovine Theileriosis:-

Malignant Ovine Theileriosis has been reported in various countries throughout the world. It is prevalent in Eastern Europe, the Middle East, North Africa, Iran, Iraq and Sudan (Soulsby, 1982; Latif *et al.,* 1994). The disease occurs across a wide zone of the Sudan that extends from Northern Sudan, Khartoum, Gezira, Kassala up to Sennar in the South (FAO, 1983b).A high percentage of sheep disease reported in north Sudan(El Ghali and El hussein, 1995).

The disease is tied to many factors such as the vector distribution and climatic effects and the suitable climatic condition for vectors to survive (Haddadzadeh *et al*., 2004).

The disease flares up in the summer, in a pattern of seasonal outbreaks, whereas 63.5% of sheep admitted to Atbara veterinary hospital and Atbara veterinary research laboratory were diagnosed as suffering from theileriosis

during the summer season (El Ghali and El Hussein, 1995). Moreover, the presence of infected ticks leads to the epidemiology outbreak of malignant ovine theileriosis in the Rive Nile State (Ahmed *et al*., 2003).

3.2 Tropical Theileriosis:-

Tropical Theileriosis is prevalent in the Southern Europe (Portugal, Spain, Italy, Bulgaria, Greece, and Turkey), Middle East, India, China, Central Asia and the former USSR (OIE, 2004). It is also distributed in a wide belt of tropical and subtropical zones, Northern Africa (Dolan, 1989; d'Oliveira, 1997; El-Metenawy 2000).The distribution is determined by the presence of the tick vectors. Therefore, the incidence of the disease has a seasonal occurrence, which is modulated by the ecology of its vectors (Pipano and Hadani, 1974; Pipano, 1976).

T. annulata is an endemic disease in Sudan (Latif and Hassan, 1982; FAO, 1983a; Hassan; 1987). The disease occurs across a wide zone of the Sudan that extends from Northern Sudan, particularly in Khartoum, Gezira, Kassala up to Sennar in the South (FAO, 1983b). Antibodies to *T.annulata* and *T. parva* were detected in cattle in the Southern Sudan (Morzaria *et al*., 1981). Recently the disease was shown to represent 14.8% and 18% of the diseases of cattle diagnosed in Atbara Veterinary Hospital during 1991-1992 and 1992-1993 respectively, with most of the cases (52.2%) being diagnosed during the summer-March-June (El Ghali and El Hussein, 1995)

4. Life Cycle of Parasite:-

Theileria spp. have a complex life cycles in both vertebrate host and invertebrate (ticks) where the sexual reproduction takes place in the tick (Dolan, 1989) (Fig. 1).

4.1 Life Cycle of *Theileria spp*. in the vertebrate host:-

The sporozoite stage of the parasite is transmitted through the saliva of infected ticks (Walker, 1990). Sporozoites enter their mammalian host during tick feeding and they rapidly invade mononuclear leukocytes, where they mature into macroschizonts (Shahnawaz *et al*., 2011). Macroschizonts are the first stage noticed in the lymphoid cells of regional lymph nodes (Uilenberg, 1983). Macroschizonts inside the lymphoid cells stimulate their mitosis. During this division, macroschizonts undergo successive multiplication forming numerous microschizonts (Uilenberg, 1981).The microschizonts differentiate into merozoites, which increase in number. The lymphocyte ruptures and releases the merozoites (Mehlhorn and Schein, 1984). These merozoites invade erythrocytes and develop into piroplasms (Khattak *et al*., 2012).

4.2 Life Cycle of *Theileria spp*. in the tick vector:-

Ticks ingest the piroplasm during the blood meal from infected host. The piroplasms start to differentiate into macrogametes and microgametes in the gut. The fusion of the two gametes forms a zygote (Gauer *etal*., 1995). The zygote invades the epithelial cell of the tick gut and differentiation into motile kinetes (Shein, 1975). Then the kinetes migrate through to the haemolymph until and reach the salivary glands (Mehlhorn and Sehein, 1977).

The kinete invades the salivary gland and then differentiates into sporoblasts. When the tick starts feeding on a vertebrate host, the sporoblasts become mature sporozoites. The sporozoites are injected into the host through the saliva of the tick during the feeding process (Fawcett *et al*., 1985).

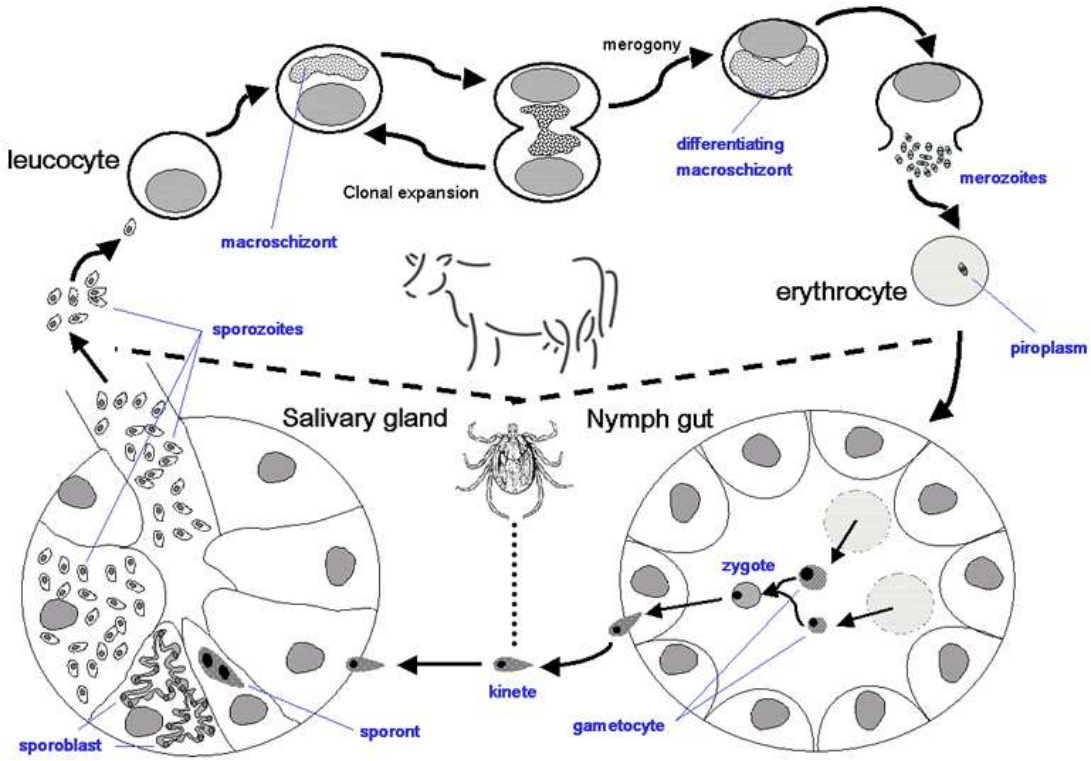

Figure 1:The life cycle of *Theileria* species**(www. Theileria. Org)**

5. Host Range:-

Theileria lestoquardi affects both sheep and goats (El Hussein *et al*., 2004)Malignant ovine theileriosis (MOT) was first described in the Sudan by Mason (1915),and the high morbidity and mortality in sheep rates have been reported in the Sudan (Salih *et al*., 2003; El Imam *et al*., 2016)

Theileria annulata infects cattle and water buffalo (*Bubalus bubalis*) causing tropical theileriosis (Robinson, 1982). Infection with *Theileria annulata* in cattle highly pathogenic, while it is mild in buffalo (losos,1986). In Sudan under normal conditions, indigenous zebu cattle (*Bosindicus*) are normally resistant to TBDs, but they may be severely affected or even die if stressed (Osman, 1976).

Attempts were done in vivo to infect sheep with *Theileria annulata* produced only macro and microschizonts in the lymphocytes and no piroplasm

develop in the erythrocytes of sheep (Leemans *et al.,* 1999a).In addition to that, detection of *T. lestoquardi* in cattle and *Theileria annulata* in sheep has been done by Taha (Taha, 2013; Jalali *et al*.,2016).

6. Transmission and Vector:-

The distribution of the common tick species correlates with the occurrence of tick–borne diseases of domestic animals (FAO, 1987).

The main tick species that known to infest animals in the Sudan are *Hyalomma anatolicum, H.marginatum rufipes, H.dromedarii, H. truncatum, H. impressum, H. impeltatum, Rhipicephalus evertsi evertsi, R. sanguineus group, R. appendiculatus, R. Pratextatus group, R annulatus, R. decoloratus, Amblyomma lepidum and A.variegatum (Hoogstral*, 1956**;** Osman *et al* 1982; Hyati, 2015; ElGhali and Hassan, 2009).

Generally**,** the most important species of tick that transmitted *T. annulata* in the field are *Hyalomma anatolicum. anatolicum* and *Hyalomma detritum* (Morel, 1989; Flach *et al*., 1995). In Sudan, *Theileria annulata*is mainly transmitted *by Hyalomma anatolicum (*Um Elhussan *et al* 1983; Walker *et al* 1983). Moreover, *Hyalomma anatolicum* to be the main vector responsible for the transmission of malignant ovine theileriosis in Sudan (Latif *et al*., 1994; Salih *et al* 2003). *Rhipicephalus bursa* was suggested to be as another vector responsible for transmission of *Theileria lestoquardi* (Soulsby, 1982).

Developmental stages of the *Theileria spp* occur in the tick and they pass trans-staidly through the stages of larva, nymph and adult, but there is no transovarian transmission. Consequently, larvae or nymphs can become infected and transmit infection like adults (Radostits *et al*., 2007).

7. Clinical signs:-

The occurrence of the disease varies depending on the parasite strain, the host's susceptibility and the number of inoculated sporozoites (Boulter and Hall, 2000). The acute form of tropical theileriosis is characterized by fever, enlargement of the superficial lymph nodes, pale visible mucous membranes and jaundice, depression and respiratory symptoms. The chronic form is characterized by anorexia, congestion of the visible mucous membranes, conjunctivitis, severe congestion of the eyes, excessive lacrimation, corneal opacity, nasal discharge, cough, and dyspnea (Mahmmod *et al*., 2011).

Malignant ovine theileriosis has three forms acute, sub-acute and chronic. Acute form may lead to death after a short course of fever and one or more of the following clinical symptoms that may appear, including; depression, swelling of superficial lymph nodes, pale mucous membranes, jaundice, edema of the throat, pulmonary involvement and may lead to respiratory failure and death (Latif *et al*, 1994; Osman, 1999 and Ahmed *et al*., 2016). Similar symptoms are noticed in the sub-acute and chronic forms, but they are less marked (Losos, 1986). Diarrhea and lacrimation may lead to corneal opacity or complete blindness (Norval *et al*., 1992). The animal becomes dull, recumbent and death may follow within two to three weeks of infection (Gill *et al.*, 1977; Uilenberg, 1981).

8. Pathological Features:-

The most prominent pathological features of tropical theileriosis are are enlargement and swelling of the superficial and internal lymph glands and spleen. The lesions are generally of hemorrhages of most of the internal organs, kidney infarcts liver degeneration, ulcers on the abomasal mucosa, which may extend to the intestines (Irvin and Mwamachi, 1983). Pulmonary congestion,

edema, hemorrhage and emphysema of variable extents are also observed in clinically infected cattle (Hassan *et al.*, 2012).

The pathological features of malignant theileriosis of sheep are also very similar to those described for tropical theileriosis of cattle. The main macroscopical lesions are hyperplasia and edema of lymph nodes, splenomegaly, a yellowish enlarged liver and the lungs are frequently edematous (Hooshmand-Rad and Hawa, 1973; Tageldin *et al*., 1992).

The infected sheep appear severe enteritis and hemorrhages on the serosal and mucosal surface along the small and large intestines. All infected animals revealed sever pneumonia associated with edema. The severe pulmonary involvements accompanied by emphysema and interstitial pneumonia may lead to respiratory failure and death. (El Imam *et al.*, 2016).

9. Diagnosis:-

9.1 Laboratory Diagnosis:-

Generally, laboratory diagnosis of theileriosis is more accurate and confirmatory. (FAO, 1984). The provisional diagnosis includes case history, clinical signs, postmortem findings and geographic distribution of disease and vector (OIE, 2000).

9.2 Microscopic Examination:-

The method used for detection of the parasite in mammalian hostsincludes preparation of blood, lymph node biopsy and postmortem impression smears that are stained with Giemsa's stain (Norval *et al.*, 1992; Forsyth *et al.*, 1999). Piroplasms appear in the red blood cells (erythrocytes forms) as round, ring, rod or comma-like shaped (Fischer and Say, 1989). Schizonts are usually detected in smears of lymph node biopsies (lymphocytic forms) ten to twelve days after infected ticks start feeding (Osman, 1999).

Macroschizont contains up to eight large irregular nuclei (Stagg *et al*, 1981), while microschizont contains up to 80 nuclei that appear as numerous small dense dots (Hooshmand-Rad and Hawa, 1973).

9.3 Serological Tests:-

The serological test used for the detection of antibodies produced by *Theileria* species (Uilenberg, 1981). Antibody detection depends on antigen antibody reaction; antibodies against tick- born disease can be detected by different serological tests (Burridge and Kimber, 1973).

9.3.1 Indirect Fluorescent Antibody Test (IFAT):-

The indirect fluorescent antibody test has been widely applied in epidemiological studies in different countries of Africa including Sudan (FAO, 1983b). This test is sensitive, fairly specific, and usually easy to perform (Darghouth *et al*., 1996). The indirect fluorescent antibody (IFA) test either based on the use of schizont or piroplasms antigens has been applied to detect circulating antibodies against *Theileria* species (Morzaria *et al*., 1981, Irvin and Morrison, 1987).Generally, the schizont antigen prepared from *Theileria* infected lymphoblastoid tissue culture cell lines, while the piroplasm antigen obtained from highly parasitaemic animals (Pipano and Cahana, 1969).

9.3.2 Enzyme-Linked Immuonosorbent Assay (ELISA):-

The Enzyme-Linked Immuonosorbent assay is a rapid, sensitive and specific test and a large number of animals can be tested in quite a short time (Gao *et al*., 2002;Manuja *et al*., 2000). ELISA has been extensively used to detect the antibodies against *Theileria* species in cattle. This test has been used also in serological surveys, the prevalence of the disease and to monitor vaccination (Bakheit *et al*., 2004, Salih *et al*., 2010). ELISA was initially generated from piroplasm antigens (Gray *et al*., 1980). Modern ELISAs have

been developed using recombinant proteins based on the surface molecules TaMS1 (Gubbels. 2000).

9.3.3 Other Tests:-

Other tests have been developed such as haemagglutination (IHA), complement fixation test (CFT) and capillary agglutination (CA), but they are not widely used (Duffus and Wagner, 1974; Brown *et al.*, 1990).

9.4 Molecular Techniques:-

The advances in molecular biology enabled genotypic characterization of the parasite and have also proved very useful for the identification and classification of many haemoparasite species of the *Theileria*/*Babesia* group (Caccio *et al*., 2000).

The techniques depend on the hybridization of known sequences of the parasites' nucleic acid strands by using a designed thermal program (Viljoen *et al* 2005). Although this method provided accurate detection of parasites (Zarlenga and Higgins, 2000), It is very expensive (Dolan, 1986).

9.4.1 Polymerase Chain Reaction (PCR):-

This test is more sensitive and specific than other conventional methods (d'Oliveira *et al*., 1995, Almeria *et al*., 2001).

PCR is the best method to detect carrier cattle that affected by *T. annulata* (d'Oliveria, 1995). The test is able to differentiate between *T. annulata* and *T. lestoquardi* in *Hyalomma* vector and in sheep and goats (Leemans *et al*., 1999b).

9.4.2 Reverse Line Blot (RLB):-

It is used to detect mixed infections that PCR cannot detect and has poor sensitive to detect subclinical infections, so a reverse line blot (RLB) assay hasbeen developed for detection of Theileria and Babesia parasites in small ruminant (Schnittger et al., 2004).

9.4.3 Loop-Mediated Isothermal Amplification (LAMP):-

Loop-mediated isothermal amplification of DNA (LAMP) has been successfully developed for the detection of some Theileria species. This technique is rapid and simple to run, cost effective, sensitive, and specific. Therefore, the respective development of LAMP for Theileria species can be of potential usefulness for application in diagnostics and epidemiological studies. (Salih et al., 2008; Liuet al., 2008).

10. Treatment:-

Many drugs have been used with varying success against theileriosis the Tetracycline drugs are effective only when they concurrently are administered with the infection (Brown et al., 1977). Primaquine (Primaquine Phosphate) is only effective against the piroplasm stage in the erythrocyte (Brown, 1990). Artificial Synthetic naphthoquinine from which parvaquone was derived (Clexon, Parvexon) are effective against the schizont stage (McHardy et al., 1985).

Parvaquone was followed by another naphthoquinine, buparvaquone (Butalex), which is effective against both schizont and piroplasm stages of Theileria species (McHardy et al.,1985).

Although the efficacy of the drugs buparvaquone and parvaquone is well established, animals can still die from pre-acute tropical theileriosis where treatment is often too late, due to lack of prompt diagnosis (Hashemi-Fesharki, 1988).

11. Control of Disease:-

11.1 Resistant Breeds.

Resistant livestock has been proposed as a sustainable method for controlling ticks-borne diseases in the developing countries (Glass *et al.* 2005). In endemic areas, the complementary control measures should also be introduced which will allow protecting cattle of high productivity (Young *et al*., 1988). In a

recent Sudanese study, indigenous Kenana and Friesian calves were experimentally infected with a lethal dose of *T. annulata*. Only two Kenana cattle required treatment, compared to complete mortality in Friesian calves (Bakheit and Latif ., 2002).

11.2 Acaricides:-

Chemical acaricides has been widely applied to control theileriosis and other tick-borne diseases (Jongejan and Uilenberg, 1994). It has been achieved mainly by application of acaricides either dipping or spraying, whereas dipping is considered the most effective method of acaricide application (Norval, 1989). Unfortunately, the extensive use of chemical acaricides leads to the development of resistance (Wharton, 1976). Tropical theileriosis in the Sudan is mainly controlled by using anti-theilerial drugs and acaricide application. It is recommended that live attenuate schizont vaccines developed from locally isolated *T.annulata* strains be used to control the disease (Abdelrahim, *et al* 2012).

11.3 Chemotherapy:-

The control of *Theileria* by using chemotherapy could be expensive in many developing countries in addition to the cost of diagnosis (Salih, 2008). In addition to that, the effective treatment requires an early diagnosis of the disease (El Hussein *et al*.,1993).

11.4 Farm management:-

Control can be implemented through roughcasting and smoothing of the outer and inner surfaces of the animal's buildings. This way of control is expensive, but leads to the control of the parasite from the farm (Gharbi *et al*.,2015)

11.5 Natural predators:-

Biological control in this aspect depends on using natural tick enemies such as parasites, pathogens and predators (Hassan *et al.*, 1992).

CHAPTER TWO

MATERIAL AND METHODS

2.1. Study Area:-

This study was carried out in Atbara which located in River Nile State in northeastern Sudan. It is located at the junction of the Nile and Atbara rivers between latitudes ($33^{o}.59^{'}$E) and longitudes ($17^{o}.43^{'}$N). Atbara is a hot desert climate. The annual mean temperature reaches over 30 °C (86 °F) during winter and 40 °C (104 °F) in summer (7 months of the year). The annual average r**a**infall is 60 mm, mostly from July to August.

2.2. Samples Collection:-

A total of 80 blood samples were collected from animals (40 samples from sheep and 40 samples from cattle) that showing clinical signs of theileriosis such as fever, enlagement of superficial lymph nodes, congestion of the eyes, lacrimation , dull. The blood collected on Whatman filter paper from the ear vein between January and July 2019 .The filter papers were individually sealed off in small polythene bags very carefully to avoid contamination

2.3. DNA Extraction:-

DNA was extracted from blood collected on filter paper using the guanidine chloride method according to the protocol.

Briefly, filter paper was cut into small pieces and put it in a 1.5ml Eppendorf tube. One ml STE-Buffer (Sodium Tris EDTA), 10µl proteinase k, 500ml Guanidine chloride and 150 µl NH_4 acetate were added to the tube and incubated at 37°C overnight. The tube was shaken for 30 minutes and then put in room temperature(25°C).The mixture was transferred into 15ml falcon tube contained pre-chilled 2ml chloroform, then vortexed, and centrifuged for 10 min at 6000 rpm.

The upper layer was collected into a new falcon tube and 10ml of cold absolute ethanol was added, shook and keep at- 20^{o} C overnight. The next day the mixture was centrifuged at 6000 rpm for 15-20 min and carefully the supernatant was discarded and the tube was inverted on a tissue paper for5 min. The pellet was washed two timeswith4ml of 70% ethanol, and air dried for 1-2 hours before resuspended in 200 µl of H_2O and kept at- 20^{o}C until used.

2.4. PCR Program for *T. lestoquardi*:-

Two primer pairs [Forward 5′- GTGCCGCAAGTGAGTCA-3′ and Reverse 5′- GGACTGATGAGAAGACGTGAG-3′] were used to amplify a 730 bp fragment of the 18S rRNA gene of *T.lestoquardi* according to the method described by (Allsopp *et al*. 1993). The positive control was prepared from *T.lestoquardia* culture (Central Laboratory, Ministry of Higher Education and Scientific Research, Sudan), while the PCR mixture was used without DNA template as a negative control. PCR was performed in a final reaction volume of 25 µl containing; 13µl of H_2O, 2µl of each primer, 5µl of genomic DNA and 5µl of Maxime PCR Premix (iNtRON Biotechnology, Korea).

The Maxime PCR Premix contained; 1x reaction buffer (10x), 2.5 U of iTaqTM DNA Polymerase (5 U/µl), 2.5 mM of each dNTPs and 1x Gel loading buffer. The amplification was performed with an initial denaturation at 94°C for 3 minutes, then 35 cycles consisted of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR products were visualized on 1.5% agarose gel stained with Ethidium Bromide.

2.5. PCR Program for *T. annulata*:-

Two primer pairs [Forward 5′-ACTTTG GCC GTA ATG TTA AAC-3′ and Reverse 5′-CTCTGG ACCAACTGTTTGG-3′] were used to amplify a 312bp fragment of the cytochrome gene of *T. annulata* according to the method described by Bilgic (Bilgic *et al*., 2010). The positive control was prepared from *T. annulata* culture (Central Laboratory, Ministry of Higher Education and Scientific Research, Sudan), while the PCR mixture was used without DNA template as a negative control. PCR was performed in a final reaction volume of 25 µl containing; 13µl of H_2O, 2 µl of each primer, 5µl of genomic DNA and 5 µl of Maxime PCR Premix (iNtRON Biotechnology, Korea). The Maxime PCR Premix contained; 1x reaction buffer (10x), 2.5 U of iTaqTM DNA Polymerase (5 U/µl), 2.5 mM of each dNTPs and 1x Gel loading buffer.

The amplification was performed with an initial denaturation step at 94°C for 3 minutes, then by 30 cycles consisted of denaturation 95°C for 50 seconds, annealing at 50°C for 50 seconds and elongation at 65°C for 1 min, and a final extension step at 65°C for 10 min. The PCR products were visualized on 1.5% agarose gel stained with Ethidium Bromid.

CHAPTER THREE

RESULTS

The DNA extracted from sheep blood were investigated by PCR using sets of primers specific for *T. annulata*. The results showed that 3 out of 40 samples (7.5%) were positive for *T. annulata* (Fig. 2).In order to confirm there is no mixed infection with *T. lestoquardi*, the positive sheep samples (3 samples) were screened using PCR. The result revealed that all samples (3 samples) were negative for *T. lestoquardi*. All these animals (3 sheep) showed typical clinical signs of theileriosis, which means that the infection with *T. annulata* can be pathogenic in sheep under field conditions.

The DNA extracted from cattle blood were investigated by PCR using sets of primers specific for *T.lestoquardi* . Our study showed that all cattle DNA were negative for *T. lestoquardi* (Fig. 3).

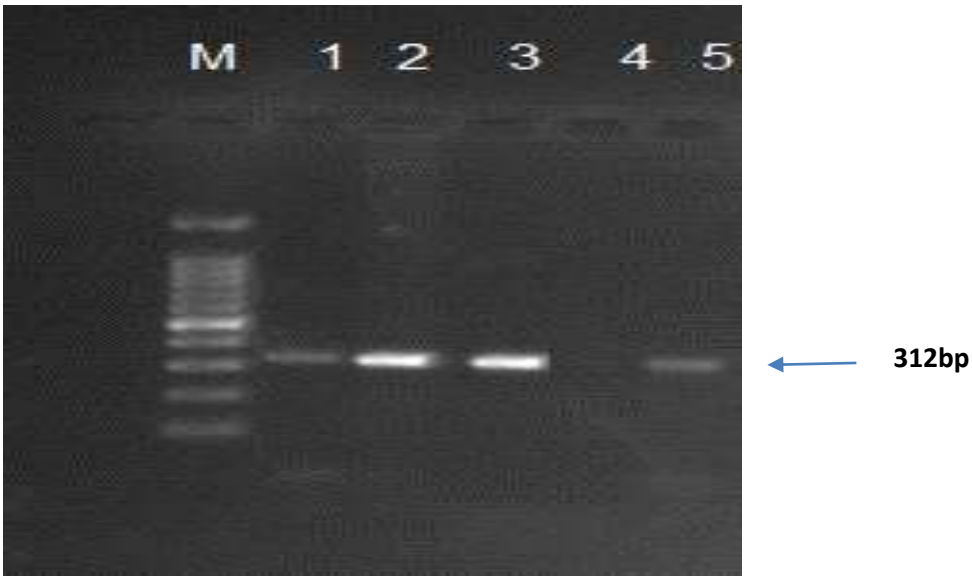

Figure 2: Agarose gel electrophoresis of the products amplified with PCR using the specific primers for *T. annulata*. M; 100 bp DNA ladder, Lane 1,2,5; positive samples, Lane 3; positive control, Lane 4; negative control.

Figure 3: Agarose gel electrophoresis of the products amplified with PCR using the specific primers for *T. lestoquardi*. M; 100 bp DNA ladder, Lane 1; positive control, Lane 2; negative control, Lane 3,4,5; samples.

CHAPTER FOUR

DISCUSSION

In northern Sudan *T. Lestoquardi* and *Tannulata* in sheep and cattle respectively, are widespread (Salih *et al*., 2003)**.**The Prevalence of these diseases is associated with the distribution of the vectors (Dolan, 1989). *Hyalomma anatolicum* is the proven vector of both species in northern Sudan (Ahmed *et al*., 2003).

Generally, the economic impacts of livestock diseases are estimated by the number of animals that died, retarded of growth, decline in productivity, failure of conception, abortions, sterility, and the cost of disease control and management (Gamal and El Hussein, 2003). Theileriosis is known to cause high mortality rates among sheep in Northern Sudan, and a highly fatal rate in cattle, especially in a high producing improved breed (Tageldin *et al*. 1992; El Ghali and El Hussein1995; El Hussein *et al*., 1991).

Several studies have been investigated the presence of the *T. lestoquardi* in cattle and *T.annulata* in sheep.They aimed to know the ability of the parasites to cross- transfer between the hosts as the two parasites species (*T. lestoquardi* and *T.annulata*) are transported by the same vector (*Hyalomma anatolicum)*. In addition to that, the herding and breeding of cattle and sheep are happening close to each other, especially in developing countries (Salih *et al*., 2009).

Although detection of the parasites *T. lestoquardi* in cattle and *T.annulata* in sheep in Sudan has been investigated in several studies. They did not link between the presence of the parasite with the appearance of the clinical signs.

The present results showed that 3 out of 40 sheep (7.5%) were positive for *T. annulata,* which indicates the susceptibility of sheep to this parasite. This result is in agreement with a previous study done by Taha *et al*., (2013) who found that infection of sheep with *T. annulata* in the filed was (7.8%).A similar finding was reported by Salih *et al*., (2003) who detected *Theileria annulata*

antibodies in Sudanese sheep with a prevalence rate of (9.3%). Our results are supported also by Taha (2009) who reported successful experimental transmission of *T. annulata* to sheep using infected ticks. Besides, the current results are analogous with those of Zaeemi *et al*. (2011) who found that the prevalence of *T. annulata* by using PCR-RFLP was (4.8%) in sheep in Iran.

The current results showed that all sheep samples that were positive for *T.annulata* (3 samples) were negative for *T.lestoquardi*. That means there is no mixed infection in these animals. In addition to that, these animals (3 sheep) showed clear clinical-pathological symptoms of theileriosis that mean *T.annulata* in sheep could be pathogenic under field condition . This observation has not been reported before.

In this study, *T. lestoquardi* was not detected in the blood sample of cattle (0%). This finding contradicts the previous study conducted by Taha, (2009) who detected *T. lestoquardi* in cattle (2.7%). This difference could be due to the type of study, since the study of Taha is an experimental study, while our study based on the collection of samples from the naturally infected animals.

On the other hand, our findings differ also from a previous study conducted in northern Sudan in which they revealed the transfer of *Theileria lestoquardi* to cattle (3.8%) (Taha *et al*., 2013).

The results mentioned here may indicate that the possibility of sheep to be infected with *Theileria annulata* with the development of disease symptoms was much higher than the probability of cattle to infect with *Theileria lestoquardi* under field condition. These general outcomes are in agreement with Leemans *et al*., (1998), who found a relatively higher prevalence of *T. annulata* in sheep compared to the prevalence of *T.lestoquardi* in cattle. The authors deduced that the previous infection of cattle with *T. annulata* led to the development of cross-immunity against *T.lestoquardi,* while immunity against *T. annulata* that developed in sheep due to the previous infection with *T. lestoquardi* was less marked.

CONCLUSION

Generally, sheep and cattle are raising together in Atbara in River Nile State, Sudan. In this situation, the opportunity of the transmission of *T. annulata* to sheep and *T. lestoquardi* to cattle under field conditions is possible. That makes Atbara the best area to conduct this study.

The present results showed that 3 out of 40 sheep (7.5%) were positive for *T. annulata* by using PCR screening, whereas these samples (3 samples) were negative results for *T. lestoquardi*. This result indicates that the pathological symptoms, which shown by sheep, are mainly due to *Theileria annulata* and this pathogen can be transmitted to the sheep and causes pathological symptoms. On the other hand, *T. lestoquardi* was not detected in the blood sample of cattle.

RECOMMENDATIONS

1- Ticks are the only carrier of *Theileria*, so they must be controlled completely. On the other hand, effective control measures mustalso be applied to reduce the prevalence rate of *T. lestoquardi* and *T. annulata* in sheep and cattle respectively.

2- Both species, sheep and cattle, must raise separately to avoid the cross-transmission of *T.annulata* to sheep and *T.lestoquardi* to cattle.

3- These results must be supported by further studies by using a large number of samples to document these results and make sure of it.

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