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Molecular study of some blood parasites in Camels in Al-Diwaniyah province

A Thesis

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Certification

I certify that this thesis entitled **"Molecular study of some blood parasites in camels in Al-Diwaniyah province"** was prepared under my supervision at the College of Veterinary Medicine/Al-Qadissiyah University in partial fulfillment of the requirements for the degree of Master Science / Parasitology.

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/ / 2018

The recommendation of the Department in the view of the available recommendation, I forward this thesis for debate by the examining committee**.**

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We, the examining committee, after reading this thesis entitled (**Molecular study of some blood parasites in Camels in Al-Diwaniyah province)** and examining the Student **Zainab Hussein Chyad Al-Naily** in its contents, find it adequate as thesis, for the Degree of Master Science / Parasitology.

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Dedication

To the great Prophet and his family infallible.

To the spring tenderness and perfume My mother

To my lover who made my life happy , accompanied me and giving me a helping hand My husband .

To my dear and my strength in my life My brothers and sisters .

To my big home / Iraq I dedicate this work.

Zainab

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رقم الايداع في دار الكتب والوثائق الوطنية لسنة

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Abbreviation

Abbrev.	Terms
µm	micrometer.
A	*Anaplasma*
B	*Babesia*
CATT	Card Agglutination Test Trypanosomosis
CFT	Complement Fixation Test
DNA	Deoxy ribose Nucleic Acid
ELISA	Enzyme Linked Immune Sorbent Assay.
IFAT	Immune Fluorescents Antibody Test
Ig	Immunoglobulin.
IHA	Indirect Haemagglutination Assay
IL	Inter Lukine
MHCT	micro hematocrit concentration technique
NCBI	National Center Biotechnology Information
NCBI	National Center for Biotechnology Information.
PCR	Polymerase Chain Reaction.
PCV	Packed Cell volume
Spp.	Species.
T	*Trypanosoma*
Th	*Theileria*
VSGs	variable surface glycoproteins
W1 buffer:	Washing buffer1.
W2 buffer	Washing buffer2.

Summary

Due to the economic importance of the camels and because of the scantly of studies related to then in Iraq, our study aimed to investigate some of the blood parasites , such as *Trypanosoma spp*, *Babesia spp*, *Theileria spp* and *Anaplasma spp* by molecular methods .

The current study was conducted during the period from September 2017 to March 2018 and collected 200 random blood samples from camels included (125) females and (75) males and two groups of ages (150) sample older than 1 years and(50) sample less than 1year) some of them appear on it clinical signs and some did not show any symptoms from the slaughterhouse of Al- Diwaniya province.

This study was designed to diagnose these parasites firstly by geimesa stain method in parasitology laboratory of Veterinary Medicine / Qadissiya University . The results were positive sample (167/200)(83.5%) and the percentage each of *Trypanosoma spp* 76(38%), *Babesia spp*106(53%), *Theileria spp* 93(46.5%) and *Anaplasma spp* 132 (66%) .

The study showed that the prevalence of *Trypanosoma spp* in females (57/125) (45.6%) while males (75/19) (25.3%) and in all ages.

The prevalence of *Babesia spp* was in the female (76/125), (60.8%) while in males (30/75) (40%) and in all ages.

Theileria spp also recorded the prevalence in female (125/ 69) (55.2%) and in males (75 /24) (32%) and in all ages.

Anaplasma spp has the prevalence in female (75/37) (49.33%) and in males (125/95) (76%) and in all ages.

The highest incidence in months (September, October and March) (88.3%, 96.2% and 75%), respectively, according to the study time.

The results revealed that it is possible to infect the animal with more than one parasite, with 80 cases of mix infection of 167 positive samples such as (*Trypanosoma* &*Anaplasma*), (*Babesia* &*Theileria*), (*Trypanosoma , Babesia*

&*Theileria*), (*Anaplasma* & *Theileria*),(*Anaplasma* & *Babesia*), (*Anaplasma*, *Babesia* &*Theileria*) and (*Trypanosoma* ,*Anaplasma* , *Babesia* &*Theileria*) 6(%7.5) ,19(23.75%), 7(8.755), 8(10%), 16(20%), 15(18.75%) and 9(11.25%) respectively.

Secondly the diagnosis of blood parasites in 90 blood samples positive microscopically use molecular techniques, which included polymerase chain reaction (conventional PCR monoplex and multiplex) in the zoonotic diseases laboratory of Veterinary Medicine / Qadissiya University then DNA sequencing analysis and detecting genetic relationships (phylogenetic analysis).

The results were as follows *Trpanosoma spp , T. evansi Anaplasma marginale , Anaplasma central Theileria spp , Theileria annulata, Babesia bovis* and *Babesia bigemina* 10(11.11%), 7(7.77%), 14 (15.55%), 15(16.66%), 16(17.77%), 9(10%), 8(8.9%) and 11(12.22%) respectively.

Recorded our strains in NCBI-Gen bank 3strain for *B. bovis* (MH2125388), 3 strain for *T. annulata* (MH508088), 2 strain *A. marginale* (MH551232) 3 strain *Theileria spp* (MH482934), 2 *T. evansi* (MH697863) , 2strain *Typanosoma spp* (MH571705) and 2 strain *A. centrale* (MH588232) .

Then reading of phylogenetic tree of these parasites and comparison among it between them which infect camels.

Chapter One

Introduction

Introduction

one-humped and two-humped camels make up nearly 27 million animals (FAO,2014). The camel is considered to be one of the best farm animals that can tolerate the harsh conditions in the arid parts of the world because of their unique adaptive physiological characteristics Therefore, it has an important economic role and breeding, which requires good management and control programs of diseases (Kamani *et al*.,2008 ; Swelum *et al*.,2014 ;Karimi *et al* .,2014)

Camel is an important multi-use animal used for transportation and production of milk, meat and wool since ancient times (Kamani *et al*.,2008). Many diseases and parasites can infect the camels and thus affect their health and cause them many like anemia ,wasting, fever and death in heavy infection, therefore, camel medicine has a long history in the world and Iraq (Hilali *et al*.,2004 ;Barghash *et al*.,2014)

As we have pointed out that camels have special characteristic. Many research have proved its ability to resist animals diseases, however, it's possible to infestation of camels with many pathogens (Eyob and Matios ,2013; Karimi *et al* .,2014) . These diseases are transmitted from camel to camel by vectors such as hard ticks and number of species of haematophagous biting flies including Tabanus, Stomoxys, and Lyperosia and Haematobia which transmitted blood parasites (Gutierrez *et al*.,2005;Salim *et al*.,2010;Nourollahi *et al*.,2012).

Trypanosoma evansi is the main cause of the surra disease in camels and is one of the most important blood parasites that causes severe losses of economic, so it is considered the most important economic aspect in the camel breeding fields in the world (Desquesnes *et al*.,2013; Fong, 2017;Sazmand and Joachim ,2017). Surra disease transmitted by Tabanus and Stomoxys caused high morbidity and mortality(Salim *et al*.,2010; Sazmand *et al*.,2011). The acute form of the Trypanosomiasis is almost

always fatal during a few weeks, while the common chronic form is evidenced by anemia, emaciation, frequent fever, edema, conjunctivitis, abortions, enlargement of the lymph nodes and lacrimation (Ahmed ,2008; Sazmand *et al*.,2011). So far there is no vaccine available for Trypanosomiasis (Desquesnes *et al*.,2008).

Theileriosis is an important blood parasitic disease of animals inducing a variety of clinical appearance fever, superficial lymph node swelling, lacrimation, a sudden loss of condition and loss of appetite ranging from a subclinical to a fatal disease depending on the animal species, host, age and the species of the parasite (Ismael *et al*.,2014 ; Youssef *et al*.,2015; Sazmand *et al*.,2016). *Theileria* in camels transmitted by hard tick bite(McKeever,2009; Hamed *et al.*, 2011; Alim *et al*.,2012). Tropical theileriosis caused by *Theileria spp* has a broad distribution extending from North Africa to China (Qablan *et al.*,2012).

Babesia spp are suspected of infecting camels but research published so are limited (Swelum *et al*.,2014). *Babesia* infections have effect on domestic animals and some wildlife species (Uilenberg ,2006; Al-Khalifa *et al.*, 2009).

Abd Elmaleck *et al*.,(2014) are the first to record the infection of camels with *Babesia spp* specially *Babesia caballi* in Egypt and in Iraq Jasim *et al*.,(2015). *Babesia spp* is a hemoprotozoal that is transmitted by Anocentor nitens ticks (Abd-Elmaleck *et al*., 2014). The typical signs of babesiosis are fever, anemia, icterus, gastro-intestinal stasis and hemoglobinuria and *Babesia* DNA was identified in infected camels (Qablan *et al*.,2012; Swelum *et al*.,2014).

Anaplasmosis is an arthropod borne disease of ruminants caused by species of the genus *Anaplasma* (Kocan *et al*.,2004) *A. marginale* is the

more virulent, characterized by a hemolytic anemia and is responsible for large economic losses (Wernery and Kaaden,2002; Hairgrove *et al*.,2015). while *A. centrale* is capable of making a moderate degree of anemia, but clinical outbreaks in the fields are very rare, It is used as a live vaccine for animals against the pathogenic (Carelli *et al*.,2008). *A. marginale* can be differentiated from *A. centrale* by the characteristics and location of the inclusion bodies in the infected RBCS(Maghaddar, 2002;Al-Khaledi, 2008).

The most common methods for diagnosis of blood parasites in camels are the traditional methods such as tracking the clinical signs on the animal and checking the blood electron microscopy and then DNA extraction for is confirm diagnosis by the use of technologist (PCR) with analysis sequence(Qablan *et al*.,2012; FAO,2014)

There are no control programs to prevent blood parasites on a large scale(Hairgrove *et al* .,2015). Molecular tools and material increasingly have become an completed part of studying the epidemiology and diagnosis of blood parasites (Ganjali,2016) .

Aims of study

The study detects some blood parasites in the local Iraqi camels in Al-Diwaniyah province by molecular diagnosis in Iraq .

1. Parasitic study about blood parasites in camels(camelus dromedar), based on microscopic examination .
2. Molecular diagnosis by using a polymerase chain reaction technique (PCR).
3. Phylogenetic tree to local blood parasites in Iraqi camels .

Chapter Two

Literature Review

2-1-The *Trypanosoma spp*

Surra disease, caused by *Trypanosoma evansi* is one of the more important animals diseases and severe pathogenic protozoan disease for camel in tropical and semitropical regions (Omer *et al* .,2004; Barghash *et al*.,2014). The name surra called by local Indians that meaning emaciated (Al-Rawashdeh *et al*., 2000).

Trypanosoma spp of the susceptible animals, including camels (dromedary and bactrian), horses, buffalo, cattle and pigs are subdivided into two sections: the Stercoraria and the Salivaria, based on the mode of development in their insect vectors and vertebrae hosts, which are further divided into subgenera and species on the basis of morphological differences (Wernery & Kaaden ,2002 ;Basaznew *et al*., 2012). Surra is enzootic in Africa, the Middle East, many parts of Asia, and Central and South America also reported a prevalence of blood parasites in camels in Egypt (Abdel-Rady, 2008; Laha & Sasmal ,2008; Desquesnes *et al*., 2009). The animals suffer stress, malnutrition and pregnancy act are more susceptible to disease (Chau *et al* ,.2016).

Trypanosoma evansi is transmitted mechanically by insect various (tabanid) and other flies, and it can readily become epidemic when introduced into a new area (Paim *et al* ., 2011). The morbidity and mortality rates in a population of camels with no immunity can be high (Kassa *et al* .,2011). In addition to causes economic losses because decreased productivity in working animals, reduced weight gain, decreased milk yield, and the cost of treatment due to infected animals suffer from illness, anaemia and deaths, (Eyob & Matios, 2013).

2-1-1- Classification

Trypanosoma evansi is a salivarian trypanosoma and unicellular flagellated kinetoplastid protozoa belonging to *Trypanosoma,* it is the causative agent of trypanosomasis (Laha & Sasmal ,2008; Desquesnes *et*

al., 2009) . It is long slender *trypanosoma* with a prominent undulating membrane and long free flagellum (Abera *et al* .,2015) .

Kingdom: Protista

Subkingdom: Protazoa

Phylum: Sarcomastigophora

Subphylum : Mastigophora

Class: Zoomastigophorea

Order: Kinetoplastidae

Suborder: Trypanosomatina

Family: Trypansomatidae

Genus: *Trypanosoma*

Species : *Trypanosoma evansi*

Trypanosoma brucei

T. equiperdum

2-1-2-Morphology

Trypanosoma evansi is similar in shape in all mammals just different in size, *Trypanosoma* have a leaf-like or rounded body containing a vesicular nucleus ,and a varying number of sub pellicular microtubules lying beneath the outer membrane, There is a single free flagellum arising from a kinetosome or basal granule (Laha &sasmal,2008; Abera *et al* .,2015). An undulating membrane is present in some genera and the flagellum lies on its outer border, Posterior to the kinetosome is a rod-shaped or spherical kinetoplast containing DNA. Members of this family were originally parasites of the intestinal tract of insects, and many are still found in insects (Taylor e*t al*., 2007). Members of the genus *Trypanosoma* are characterized by four developmental stage (amastigote, promastigote, epimastigote and trypomastigote stages) in their life cycle and can be differentiation between them according to morphological feature under microscopic examination (Raoofi *et al*

.,2009). In some species only trypomastigote forms are found in the vertebrate host; in others, presumably more primitive species, both amastigote and trypomastigote forms are present (Desquesnes *et al*.,2013). In the trypomastigote form, the kinetoplast and kinetosome are close to the posterior end and the flagellum forms the border of an undulating membrane that extends along the side of the body to the anterior end (Desquesnes *et al*.,2009).. In the epimastigote form, the kinetoplast and kinetosome are just posterior to the nucleus and the undulating membrane runs forward from there and the promastigote form, the kinetoplast and kinetosome are still further anterior in the body and there is no undulating membrane (Radostits *et al*.,(2007). In the amastigote form, the body is rounded and the flagellum emerges from the body through a wide, funnel-shaped reservoir (Taylor *et al*., 2007).

2-1-3- Modes of transmission

Trypanosoma evansi is mechanically transmitted by hematophagous biting flies including *Tabanus spp*. and *Musca spp* also *Lyperosia*, *Stomoxys* and Atylotus genera ,There are more than 20 different species of Tabanus have been shown experimentally to transmit *Trypanosoma evansi* (Enwezor *et al*.,2005; Desquesnes *et al*.,2013 ; Nasir, 2015).

Trypanosoma evansi is transmitted in several ways, via biting insects, sucking insects, and vampire bats, contact healthy animals with affected , transmission can also be vertical, horizontal, iatrogenic, and per-oral with various epidemiological significances, depending on the season, the location, and host species (Desquesnes *et al*., 2013 ; Zakian *et al* ., 2017).

There are other secondary rote of infection like placenta or through infected meat and transmitted from animal to animal through sexual intercourse (Taylor *et al* .,2007)

2-1-4- Life-cycle

Replication of the *Trypanosoma spp* occurs by longitudinal binary fission both in the host and in the vector with the flagellum and kinetoplast both in the host and in the vector with the flagellum and kinetoplast dividing together (Omer *et al*., 2004; Liu-Liu *et al*., 2005). *Trypanosoma evansi* cannot undergo growth and differentiation in the insect vector because it lacks the genes necessary for mitochondrial development (Schnaufer *et al* ., 2002; Jilo *et al*.,2017)

The life cycle of *T. evansi* began from ingested by flies *Stomoxys* and *Hippoboscids* transmit *T. evansi* mechanically through their mouthparts when they feed on more than one host within a short interval because the *trypanosoma* remain infective for only a short period and then multiple in mid gut for 10 days and migrate to salivary glands in epimastigote form and return multiple in salivary glands convert to metacyclic form (infective form) injected by fly during bite to become trypomastigote in blood and lymph of the host (Dawood ,2007; Nasir , 2015).

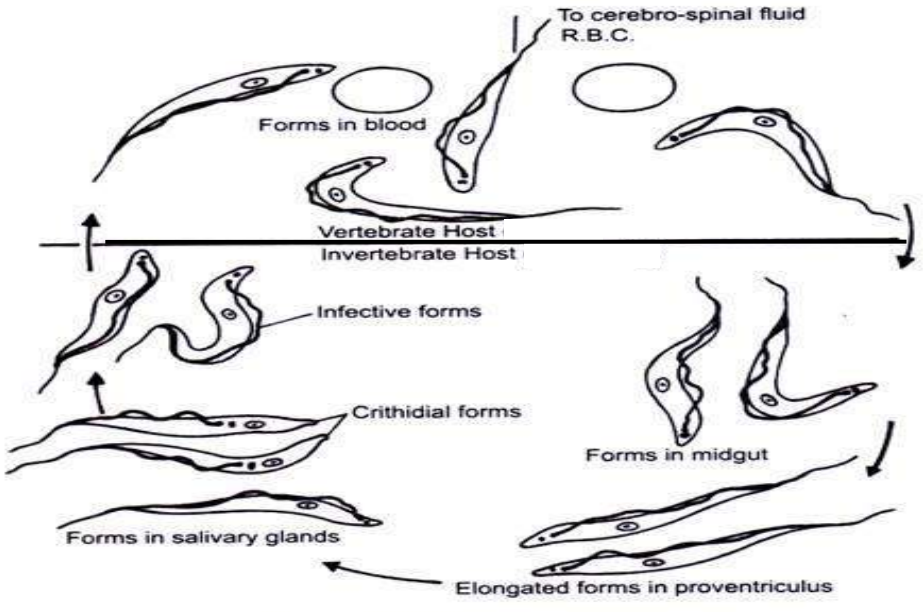


Figure 2-1 life cycle of *Trypanosoma spp*

http://cdn.biologydiscussion.com/wp

2-1-5- Clinical finding and Pathogenesis

Trypanosoma evansi can infect a variety of hosts and causes a species-specific pathology but in camels, the disease is manifested by elevation of body temperature with initial peaks of fever up to 41 °C which is directly associated with parasitaemia, Infected animals show isolate itself from flock with progressive anemia, marked depression, dullness, loss of appetite ,conjunctivitis , fall of hair ,abdominal and behind legs odema and often rapid death (Juyal, 2002; Ahmed ,2008; Desquesnes *et al*.,2013 ; Kingsley ,2014) .

Surra can cause an acute, sub-acute or chronic disease, with the severity of the clinical signs differing between individual animals, as well as between species and can attack camels at any age, even fetuses therefore cause abortion (Tadesse *et al*.,2012).

The main pathological sings the edema in the lower parts like abdomen ,legs and genital organs occur due to the presence of prekallikrein enzyme, which is found in inactive form but when there is *Trypanosoma* infection the enzyme will be activated and lead to increase the blood vessel permeability and lead to edema (Kohler-Rollefson *et al*., 2001; Kingsley ,2014)

Anemia is a major component of pathology of Surra occur due to red blood cells lysis by complement due to adhesion of parasite with R.B.Cs and phagocytosis (hemolytic and haemophagocytic) (Juyal, 2002; Maudlin *et al* .,2003). Poisoning effect of the parasite on the bone marrow leads to decrease the production of red blood cells (Bornstein & Younan, 2013).

The nervous signs in camel *Trypanosoma* occur due to manifestation of depression of cerebral cortical function in various degrees and other nervous signs reported, such as circling movement, incoordination and dullness, appear to be the results of brain tissue

disturbance or damage by the parasites, Evidence of *Trypanosoma evansi* being found in the cerebrospinal fluid has been presented (Reid ,2002; Brun, *et al.*,2010). When attached to healthy camels with camels suffering from mortality or abortion lead to a outbreaks due to trypanosomiasis (Derakhshanfar *et al.*, 2010; Zakian *et al.*, 2017) .

2-1-6- Diagnosis

Diagnosis surra disease for individual animals still rely on clinical signs and conventional parasitological techniques like Giemsa-stained slides and micro haematocrite concentration technique (MHCT), therefore likely that the diagnosis for single animals is often missed as the general clinical signs of *T. evansi* infection are not sufficiently pathognomonic for diagnosis (Adam, 2015; El-Naga & Barghash, 2016).

Surra disease is generally not easy to diagnose based on clinical examination and contraceptives given by the animal owner in the field (Adam,2015) classic laboratory tests difficult to detect the disease and to use suitable treatment because of this, they relied on accurate diagnosis of molecular diagnosis (Mastra, 2011;OIE, 2012).

The chronic form trypanosomiasis is more common in camel, It is associated with secondary infections due to immunosuppressant caused by *T. evansi* infection that may cause difficulty in clinical diagnosis (Ahmed ,2008; Eyob and Matios,2013).

The parasites can be revealed in blood 13 to 16 days after an infective fly has taken a meal of blood to confirm infection and diagnosis (Abdel-Rady, 2008).

2-1-7-1- Parasitological diagnosis

Parasitological diagnosis is mainly carried out by the direct microscopic methods for detecting the parasite are required (Abdel-Rady,

2008). The organism may be difficult to find, especially in mild or subclinical cases, and parasitemia is often intermittent in chronically infected animals (Enwezor *et al*.,2005). In early infection or acute cases, when the parasiteaemia is high, examination of wet blood films and thick smears stained blood smears or lymph node materials might reveal the *trypanosoma* ,direct microscopic examination should be performed as soon after collection as possible because *Trypanosoma* are rapidly immobilized and lysed, they are killed by direct sunlight but can survive for a few hours if kept in a cool, dark place (Chappuis *et al*.,2005).

Atarhouch *et al* .,(2003) and Zayed *et al*., (2010) are reported that a tendency of infection rate to increase with age due to the mainly fact of larger scale movement, which increases the risk of infection, by the adult camels than the younger.

2-1-7-2-Serological diagnosis

Serological tests are used to detect specific humoral antibodies and circulating antigen, e.g. Immuno Fluorescents Antibody Test (IFAT) useful when screening a small number of samples , Enzyme Linked Immuno Sorbent Assay (ELISA) and the Card Agglutination Test for Trypanosomiasis (CATT) (Browen and Torres,2008; Zayed *et al*.,2010).The Complement Fixation Test (CFT) and the passive haemagglutination test (Coetzer and Tustin,2004).

Enzyme Linked Immuno Sorbent Assay test (ELISA) has two parts, the antibody (Ab) detection and antigen(Ag) detection part. ELISA using variable surface glycoproteins from a *T. evansi* RoTat 1.2 clone successfully differentiated *T. evansi* from *T. brucei*. Protocols are available for equines, camelidae and water buffaloes (Maudlin *et al* .,2003; Browen and Torres,2008) .

In camels several changes in the hematology and biochemistry of the blood in haemoparasitic diseases like trypanosomiasis and dipetalonemiasis may take place specially the Packed cell volume (PCV) determination is often a reliable indicator of *trypanosoma* infection However, camels with sub clinical disease can have parasitaemia without any evidence of anemia (Ahmad *et al.*, 2004).

2-1-7-3- Molecular diagnosis

Molecular tests include the polymerase chain reaction(PCR), real-time PCR and nested PCR(Smuts, 2009; El-Naga and Barghash, 2016).

Molecular diagnostic techniques, several diagnostic assays based on the detection of trypanosomal DNA by PCR have been developed. PCR could offer a very precise method for detecting infection and discriminating between infected and non-infected animals PCR is reported to be more sensitive than conventional parasitological techniques in a level , number of hosts and has the advantage that it can identify parasites at the species level the analytical sensitivity of such tests is high but in experimental situation they have not always high diagnostic sensitivity (Gutierrez *et al*., 2004; Sawitri ,2016).

PCR techniques based on amplification of specific nucleic acid sequences are becoming more frequently used for the diagnosis of trypanosomiasis because they are essentially antigen detection tests, this is because the tests are designed to demonstrate the presence of the parasite rather than the host response to the parasite and therefore *T. evansi* can be distinguished from *T.brucei* and *T.equiperdum* using probes based on kinetoplast minicricle DNA (Smuts, 2009; Pruvot *et al.*, 2010)..

2-1-8- Immune response

The surra disease interferes with immune system and causes immunosuppression consider the way for easy bacterial and viral infections may complicates clinical diagnosis (Holland *et al*. 2003; Jittapalapong *et al*. 2009 ;Singla, *et al*., 2010). The chronic case is more associated with secondary infection thus causing weakness of immunity (Ahmed ,2008) .

The immune system is designed to save a host from pathogens, it can sometimes be respond inappropriately or result in immune mediated disease with clinical signs (Stijlemans *et al.,* 2007). *Trypanosoma* utilizes a variant surface antigen coat to avoid their mammalian hosts immune system, , the whole surface, including the flagellum, is covered by 5x10 dimers of variable surface glycoproteins (VSGs) that make up approximately 15-20% of the protein content of the cell and up to 95% of the external surface protein (Field and Carrington ,2009; Singla, *et al*., 2010). VSG's are able of inducing protective immunity, and the immune response to possess for each variant, although rapid and highly efficient in break down any *trypanosoma* that particular antigen is invariably very late to affect *trypanosoma* which hold changed their surface antigenic identity (Desquesnes, *et al*.,2013).

Trypanosoma evade the immune system as a result of their elevation rate of endocytosis , that allows ingestion and rotation of the entire surface coat every 12 minutes with any attached antibody complexes , these complexes are extracted and clean VSGs recycled to *trypanosoma* surface, so slowing host recognition (Pays , 2006 ; Bisser, *et al*., 2006).

Both IgM and IgG antibodies play a key role in the control of parasitaemia which produced in response to *trypanosoma* infection, IgM is produced early in infection followed by the more specific IgG (Naessens, 2006). Gamma-globulin (IgM) an increase in during both acute and chronic *Trypanosoma evansi* infections in camels has been reported ,but this is not protective, as the majority of the antibodies are auto antibodies (Pays , 2006). Also leukocytosis, neutrophil and eosinophilia have been increased these changes occur as a result of an increase in the activity of the mononuclear phagocytic system, the eosinophilia observed is a feature of parasitic infections and is associated with immediate-type hypersensitivity reactions (Enwezor *et al.*,2005; Gutierrez *et al.*, 2005). IL-6 secreted by activated macrophages resptors are only present on directly activated B-cells which result in an increase in IgM and IgG antibodies (Pays ,2006). Due to increased level of IL-10, CD4/Th-cells activated and secrete IL-4, IL-10 and IL-13 which are responsible for T-cell dependent B-cells activation (Naessens ,2006) .

The most *Trypanosoma spp* live free in the blood of the vertebrate host and possessed no intracellular stages, most times they can be a direct target for antibody mediated destruction and antibodies are therefore of major importance in the control of parasitaemia in the infected host, if *trypanosoma* are located in areas which cannot be targeted by antibodies such as the brain they are protected from this humeral immune response (Pays ,2006). Humoral immunity is rapid and strong but because of antigenic variation less effective (Desquesnes, *et al.*,2013). The majority of the antibodies are auto antibodies during the acute phase of the trypanosomiasis , lymph nodes and spleen are uncommonly reactive this may status for the generalized lymphoid tissue hyperplasia special of *Trypanosoma evansi* infections, however in the

last stages the immune system becomes drain of lymphoid cells (Bisser, *et al*., 2006).

2 -1-8- control and prevention

Tsetse-transmitted trypanosomiasis, the losses are due to reduced productivity, mortality and cost of treatment, Control of surra can be difficult as there is no vector specificity and a wide range of hosts (Bengaly &Sidibe , 2002). Complete fly control is not practical due to the complexity of the biology of fly vectors (i.e., mainly Tabanids) and the difficulty in application, usage of fly repellents to livestock so that, more research is necessary to identify , effective repellents and nature friendly to enhance trapping and baiting techniques for target fly species (Mihok *et al*., 2006). Control of camel trypanosomiasis include parasite control through vector control and treated affected animals with trypanocidal drug is the frequent method of control of *T. evansi* have therapeutic and prophylactic role (Abera *et al*.,2015). In the case of surra with the absence of a vaccine against *Trypanosoma* due to a major repertoire of variable surface antigens, disease control is fundamentally based on the use of trypanocides drug and preventive control methods to keep animals from infection , Animals should be protected away from places where the flies found in the hot part of the day and remain the camels far off the tabanus present region (Zeleke & Bekele,2001; Elhaig *et al* ., 2013).

Using of insecticides such as sprays for the inside walls of animals houses include chlorinated hydrocarbon insecticides, organophosphates such as Malathion with treating infected animals by use drugs (suramin, prothridium diminazeneaceturate and isometamidium chloride) and discard chronic cases (Eyob and Matios ,2013)

2-2- *Babesia spp*

Babesia species are tick-borne hemoprotozoan parasites that infects red blood cells leading to anemia in the host (Aktas *et al*., 2012). Numerous different species exist by varying host specificity and are found all over Asia, the Middle East, Europe, Africa and North America (Altay *et al*., 2008; Heidarpour Bami *et al*., 2009; Razmi *et al*., 2013). Infection occurs in domestic animals, including cattle, camel, horse, sheep, goats, pigs and dogs (Bock *et al*., 2004; Aktas *et al*., 2007).

Bovine babesiosis is a major tick-borne disease of cattle caused by six *Babesia* species that have an important effect on livestock health and productivity, two species, *Babesia bovis* and *B. bigemina* have the highest impact (Ghirbi *et al*., 2008). *B. bigemina* can cause massive destruction of the red blood cells leading to severe anemia and hemoglobinuria, this appear red urine (due to hemoglobin in urine) and the disease can kill cattle through a week (Uilenberg, 2006 ; Adham *et al* .,2009).

Babesia bovis is more dangerous than *B. bigemina* because it is less sensitive to some babesiacidal compounds, Thus causing a problem in treating infected animals(Abera, 2015). Animals that survive a *Babesia* infection generally become carriers of the parasite and serve as reservoirs for transmission (Chaudhry *et al*., 2010).

Ovine babesiosis is one of the most important disease in sheep with high mortality and morbidity, resulting in high economic losses globally, It's especially where ticks are located , *Rhipicephalus bursa,* is present (Aktas *et al*., 2007; Altay *et al.*, 2008). Sheep that recover from babesiosis become asymptomatic carriers (Aktas *et al*., 2005).

Camels were infected with *Babesia caballi* for the first record in the first record in Egypt (Abd-Elmaleck *et al*., 2014). *Babesia caballi* is a hemoparasitic protozoan that is transmitted naturally in New World by Anocentor nitens ticks (Abdelrahim *et al*.,2009 ; Abd-Elmaleck *et al*.,

2014). Very few reports are available about camel piroplasmosis recently in the one-humped camel zone, such as Iraq (Jasim *et al* .,2015) and Iran (Khamesipour *et al*.,2015) .

2-2-1-Classification

Babesia is a protozoan parasite found to infect vertebrate animals, mostly livestock mammals (Aktas *et al*., 2012) . The parasite is classified as follows according to (Schmidt & Roberts , 2006)

Kingdom: Animalia

Subkingdom: protozoa

Phylum: apicomplexa

Class: Aconoidasida

Order:Piroplasmoirda

Family: Babesiidae

Genus: *Babesia*

Species*:* *Babesia bovis*

Babesia ovis

Babesia bigemina

Babesia caballi

Babesia canis

Babesia cati

Babesia microti

Babesia motasia

2-2-2-Morphology

Babesia species enter red blood cells (erythrocytes) at the sporozoite stage within the red blood cell, the protozoa become cyclical and develop into a trophozoite ring (Abdullah and Mohammed, 2014). The trophozoites moult into merozoites, which have a tetrad structure

coined a Maltese-cross form (Herwaldt *et al*.,2003) . This tetrad morphology is seen with Giemsa staining of a thin blood smear which is unique to *Babesia*, and distinguishes it from *Plasmodium falciparum*, a protozoan of similar morphology that causes malaria (Ahmed *et al*., 2002). Trophozoite and merozoite growth ruptures the host erythrocyte leading to the release of vermicules, the infectious parasitic bodies, which rapidly spread the protozoa throughout the blood (Shayan & Rahbari ,2007). *Babesia* appear as reddish violet particles inside the blood cells (Swelum *et al*.,2014) . It appears as a single or double pyriform and the parasite has other forms depending on its type, round or oval or ring (Bai *et al* .,2002)

2-2-3-Modes of transmission

The disease (Babesiosis) is transmitted from sick to intact animals by about 900 species of ticks are record and belong to (2)major families include Ixodidae that referred to hard ticks (Barker & Murrell,2004) . These parasites are responsible for transmitting several diseases such as protozoa ,bacteria Rickettsia (Nava *et al* .,2009) through two ways :-

Transoverian transmission the protozoan passes transovarially (via the egg)from one generation of tick to the next , some species of *Babesia* can be transmitted from a female tick to its offspring before migrating to salivary glands for feeding (Ravindran *et al*., 2006; Altay *et al*., 2007; Ghirbi *et al*., 2010). .

Transtadial transmission the parasite persist from one stage to the next of the ticks feeding on different hosts *Babesia* species are spread through the saliva of a tick when it bites . Its nymph stage, a tick bites into the skin for a blood meal. *B. microti*, the most common species in humans , though, has not been shown to transmit trabsovarially (Ibrahim *et al*.,2010).

The tail is favored area for ticks parasiting due it near ground during the animals sleeping that facility the reach of ticks to it , while the present of ticks on ears due to the apparent of blood vessels (Esmaelinejad *et al*., 2014) . The ticks are spread in different parts of animals body such as ears, between thigh and scrotum the main site of ticks is perineum region (Altay *et al*., 2007).

2-2-4-Life-cycle

The life cycle of *Babesia spp* within the vertebrate hosts is only multiplication by simple fission with highly destruction (70%) of RBCs (Duh *et al*., 2008; Silva *et al*., 2010). The life cycle of *Babesia spp* occur when the sporozoites are usually injected in the blood stream of vertebrate host with amount of saliva during the blood meal of infected ticks ,the sporozoites invading erythrocytes then differentiate in to trophozoites that divided asexually and form the merogony into two or sometime four merozoites , the merozoites then exit from erythrocytes and invading new other one the replicative cycle is continuing in the host some of the merozoites are stop division and transform in to gamonts or pregametocytes the gamogony and sporogony take place in the ticks when the ticks feed on an infected animals when they differentiate in the gut to form gametes that fuse forming a diploid zygote, the zygotes then transform to ookinetes this ookinetes migrating through the hemocoel after that invade multiple ticks tissue such as the salivary gland in the sporogony process occur and produce about 100000 sporozoites in the salivary glands tissues and transform to vertebrate body host during ticks blood meal and invade the red blood cells and this called transtadial transmission (Schnittger *et al.,* 2012; Albdere ,2015) .

In invertebrate host (ticks), the life cycle rate of development is depending on environmental temperature and occur very rapid in 28c High humidity and rainfall accommodate ticks carrying *Babesia* (Laha *et*

al,. 2015) . inside the gut of ticks , after 24-48 hrs the ray bodies (gametes) fused to each other to form motile zygote (ookinet) which are cigar shape penetrate the epithelial cells of gut ,then started to divided and form clap shape bodies (kinetes) and detected in gut 3-4 days after tick feed the zygot will be rapture and liberates the kinetes that will enter varies oragans like ovaries and salivary glands , in the ovaries the kinetes are stay in the yolk of ovum and then found in the intestines of larval stages , so the infection occur by the infectious larve , nympha and this called ovarian transmission (Alain *et al*,. 2009)

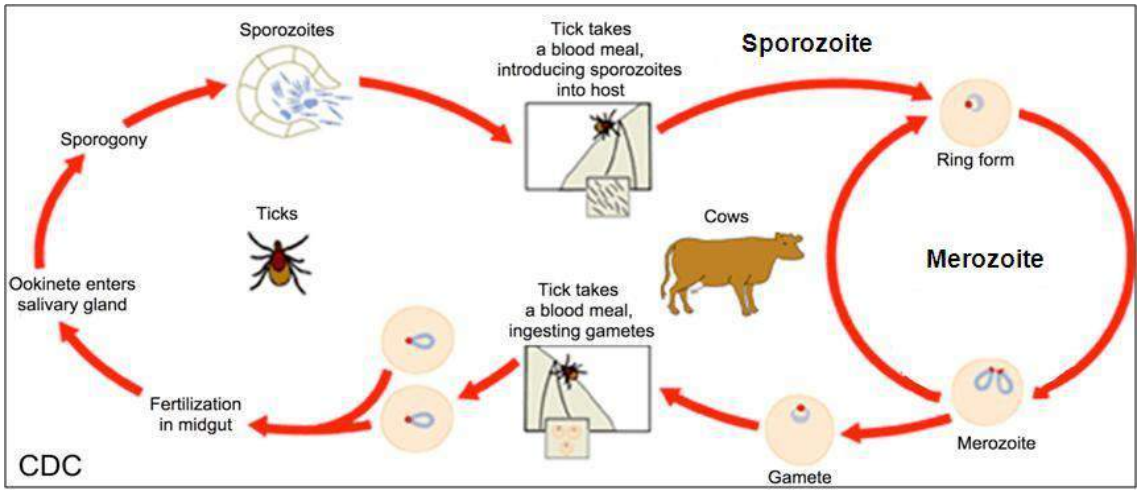


Figure 2-2 life cycle of *Babesia spp*

http://fullmal.hgc.jp/bb/icons/lifecycle.jpg

2-2-5-Clinical finding and pathogenesis

The clinical signs depend on (parasite species ,strain and host) for example the parasites species , *B .bovis* predilecation site is the capillaries of brain and kidney of the host and produce nervous sings while *B. bigemina* remain in blood stream so there are no nervous sings while the susceptibility of host affected by many factors like age ,breeds the local breed more resistant than foreign and environmental stresses which affect the parasite inside ticks or ticks themselves (Radostitis *et al*.,2007). The incubation period 1-2 weeks. and the important clinical

signs fever 40-41c(El Moghazy *et al.*,2014). The acute phase of the disease is characterized by hemolytic anemia (Talkhan *et al.*, 2010; Ziapour *et al.*, 2011).

Also ataxia, pale mucous membrane due to anemia, malaise, weakness ,muscle trembling ,grinding of teeth ,the feces are dry and bloody stained ,dehydration causes the eyes to become sunken in their sockets , falls of body temperature to subnormal level before a few hours of death , Haemoglobinuria and the prescapular and prefemoral lymph nodes are enlarge in size (Zobba *et al.*,2008). the hypoglycemia are associated with *Babesia* infection(Esmaeilnejad *et al*., 2014).

High pathogenicity when the animals infected with *Babesia* number of RBCs, packed cell volume (PCV) and hemoglobin concentration was decreased and the anemia occurred (Rubino *et al*., 2006). Anemia occurs due to mechanical destruction of R.B.Cs by the binary fission of trophozoites (Zobba *et al.*, 2008) .Hemolytic anemia occur that during acute stage appear as normocytic and then become macrocytic with the appearance of reticulocytes and rise of main corpuscular volume (MCV) (Sulaiman *et al*., 2010) .Leukocytes count fall slightly and then rise due to rise of lymphocytes (Rahbari *et al.*, 2008 ; Esmaeilnejad *et al.*, 2014).

Convert of inactive prekallikrein enzyme to active prekallikrein which lead to blood vessels dilatation that lead to slow blood flow and clot formation that lead to blockage of capillary blood vessels especially in central nerves system and appearance nervous sings particularly in *B.bovis* and *B.canis* (Schetters *et al*., 2009).

Other cause to anemia are due to decrease number of R.B.Cs lead to decrease of O2 that causing tissues anoxia and damage of organs especially liver and kidney (Turgut, 2000). Kidney damage occur due to

precipitation of immune complex in the glomeruli and glomerulonephritis occur (Rosenfeld and Dial, 2010).

2-2-6- Diagnosis

Babesia identification in infected animals cannot depend on the clinical signs because there is many other disease that has the same clinical signs, there for it is important to identify the parasites in the blood of the infected animal (Abdullkadim, 2009;Perez-Llaneza *et al*., 2010). Animal with piroplasmosis become carrier after infection and the value of these animals in the population is important in the epidemiology of Babesiosis (Swelum *et al*.,2014).

2-2-6-1- Parasitological Diagnosis:

The diagnosis of *Babesia spp* traditionally works by using Giemsa staining of suspicious blood smears and the morphology of the piroplasms in the RBC is decisive for the diagnosis the Giemsa staining blood smears may be accompanied with some technical problems lead to false morphological diagnose . Diagnosis of *babesia spp* in acute case by microscopic examination of Giemsa stained thin blood smears when the number of the parasites is high in the blood (schnittger *et al*., 2004; Aktas *et al*., 2007). while the diagnosis of *babesia spp* in chronic infection using thick blood smears to detect it is characterized by presence of a little number of the parasite(Abdullkadim, 2009; Schneider *et al*.,2011).

2-2-6-2 - Serological diagnosis

Serological techniques are frequently employed in determining subclinical infections despite lacking the sensitivity and specificity for detecting carrier state, especially when establishing the infection status (Durrani *et al*., 2006; Iseki *et al*., 2010).

The important method for serological diagnosis and survey of Babesiosis are the Indirect fluorescence antibody test (IFAT), it’s the

most widely used test for the detection of antibodies to *Babesia spp* (OIE, 2005;Shayan and Nabian ,2008; Swelum *et al*.,2014) and the enzyme linked immune sorbent assay (ELISA) , it's test number of serum samples in a short period of time with high sensitivity for there more ELISA is a reliable technique for the determination of low grade or subclinical case (Terkawi *et al*., 2011).

2-2-6-3- Molecular diagnosis :

Both *Babesia caballi* and *Theileria equi* were molecular confirmed in camels from Iraq (Jasim *et al*.,2015) using PCR. Therefore, equines are supposed to play an important role in the epidemiology of camel piroplasmosis because they are usually found to be infected with the same piroplasms species (Salim *et al* .,2013; Hosseini *et al*.,2017)

There are several molecular techniques are used for detection of *Babesia spp*. which include polymerase chain reaction (PCR) amplified fragment length polymorphism (AFLP) restriction fragment length polymorphism (RFLP) also Real - time PCR (Blears *et al*., 2000; Guy *et al* ., 2004; Parida *et al*., 2008; Quan *et al*., 2002).

The diagnosis of the parasite depends on DNA in the blood and body tissue therefore it has high sensitivity in the determination of *Babesia spp*, but it's sensitivity depends on the amount of the DNA that to exam it in the blood (Hilpertshauser *et al*., 2006; Iqbal *et al*., 2011).

DNA sequencing is the process that is used to determine precise order of nucleotides that found in a DNA molecule the advent of the rapid sequencing methods has a great accelerated medical and biological research and discovery the information of DNA sequences is becoming necessary for essential biological research and also in several fields such as biotechnology, diagnostic, biological systematics and forensic biology the rapid speed of sequencing attained with modern DNA sequencing or

genome of several types and species of life that include human genome and the sequences of DNA of the animal, plant and microbial species (Pettersson *et al*., 2009 ; Gallina *et al*., 2006; Ibrahim *et al*., 2017).

2-2-7- Immune response

The immunity in Babesiosis is mainly humeral nature and the parasite is able to activate the cellular immunity (Rahbari *et al*., 2008).

The infection with *Babesia* species activate the production of antibodies both of Immunoglobulin M (IgM) and Immunoglobulin G (IgG1,IgG2) are produced at the same time and they are specific to *Babesia*. and the (IgG) remand for a longer time in addition to that *Babesia* specific antibody (Ab) are act on the surface proteins of erythrocytes stage and they involve the opsonisation of the infected erythrocytes (Normine *et al*., 2003; Brown *et al*., 2006).).

The macrophage is activated during *Babesia* infection that release soluble factors which have been responsible for killing this parasite (Homer *et al*., 2000).

Also, Hamer *et al*., (2000) referred to the role of the cellular immunity is recognized by the function of T cell and the cytokines in fatal Babesiosis. The interferon gamma (I FN-Y) which is produced from the CD4 T helper cell act to activation the phagocytosis process and stimulate B cell to produce (IgG2) (Normine *et al*., 2003; Brown *et al*., 2006).

Bock *et al*., (2004) pointed to that the innate immunity also has an important role which means present of many factors that prevent *Babesia* infection such as the age of host, it's specificity and the genetic factors as the most *Babesia spp*. has a high specify in the infected of vertebrate host.

Goff *et al*., (2002) reported the monocytes, macrophages and neutrophils act as the first defines line during the infection by using

antimicrobial agent such as reactive oxygen intermediates and reactive Nitrogen intermediates in addition to phagocytosis process. The spleen has an important role in the infected RBC destruction and produce some of substance such as interferon gamma (I FN-Y) and interleukin-12 (I L-12) by the Natural killer cell (Goff *et al.*, 2006). The small ages are usually present in a high innate immunity in comparative with the old age due to the forming of (I L-12), (I FN-Y) and Nitric oxide (NO) while the forming of this cytokines is usually late in old age in additions to not forming of (NO) (Delfin *et al.*, 2003). The acquired of the young animal to the colostrum that contains the antibodies also make them more resistant (Ziapour *et al*., 2011) pointed to that the sheep are more infected in age about 6-12 month and the more infected age of the goat is about 1-2 year. While Emre *et al* .,(2001) referred to that the lamps in age about 2-4 month are more infected from the other ages. On the other hand, Aktas *et al* .,(2007) and Razmi *et al* .,(2003) referred that there is no significant relation between the animals ages and *Babesia ovis* infection rate confirming the presence of an endemic stability state.

2-2-8- control and prevention

In the endemic area the control of the disease depends on the mantines on the enzootic stability by using the roles of ticks control, chemoprophylaxis drugs and animals vaccination. (Zintl *et al*., 2003).

Also the using of resistant animals breed and Arcades are useful in disease control (Radostits *et al*., 2008).

The use of the chemical substance in the endemic area to control on the ticks may lead to future ticks burden (Dewaal and Combrink, 2006) . On the other hand, Homer *et al* (2000) mentioned the removal of the ticks within 24 hours of attachment may decrease the infection

because there is a correlation between the transmission of sporozoites and the attachment time.

Astrila, Brazil and Colombia use other roles in ticks control as using vaccines that are prepared from some ticks proteins, so this method gives good results and reduced from the environmental contamination (Delafuent *etal.*, 2007).

2-3- *Theileria* spp

Theileriosis is considered to be the second most important hemoprotozoal disease following Trypanosomosis affecting dromedary camels and domestic ruminants in the tropical and subtropical regions of the world (Mazyad, and Khalaf,2002 ; Sivakumar *et al.*,2014).

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

Ovine Theileriosis or Malignant Small Ruminant Theileriosis is a parasitic disease of sheep and mainly transmitted by *Hyalomma anatolicum* (Smith and Sherman, 2011). Sheep are considered a very receptive host for *T. lestoquardi*, as infection usually evolves into sub-acute and acute theileriosis even in indigenous sheep (Tageldin *et al*, 2005; El Imam *et al*., 2015).

Theileria is one of the most devastating blood parasites affecting cattle in Saudi Arabia, causing lethal infections in exotic cattle (El Imam *et al*., 2015). The thileriosis in cattle caused by the parasite *Theileria* (an obligate intracellular blood parasite) and transmitted by ticks, but very little is known about this disease in camels (Duh *et al*., 2008; Silva *et al*., 2010). *Theileria spp*. that have been reported in camels include *Theileria camelensis* and *T. dromedarii* (El- Metenawy, 2000) .

Disease is transmitted by the tick species *Hyaloma dromedarii* in camels, its main host, but it is also found on the skin of cattle, sheep, goats, and horses (Hamidinejat *et al*.,2008).

The first reported it in camels in Russia in 1917. So far, two species of *Theileria* have been reported in the world *T. camelensis* and *T. dromedarii* (Borji *et al* .,2009). As there is insufficient information on the microschizont stage of this parasite, and since it has been observed only its piroplasm stage in erythrocytes (Elghali and Hassan,2010).

2-3-1-classification

The genus *Theileria* belongs to the phylum Apicomplexa, which includes *Babesia*, *Toxoplasma, Neospora*, *Plasmodium* and others (Radostits *et al*., 2007).

Kingdom : Animalia

Subkingdom : protozoa

Phylum : Apicomplexa

Class : Aconoidasida

Order : Piroplasmoirda

Family : Theileriidae

Genus : *Theileria*

Species *: Theileria parva*

T.annulata

T.ovis

T.camelensis

T. equi

2-3-2- Morphology

There are various developmental stages of different shapes and forms of *Theileria spp.* which infected cattle ,baffuloes ,goats ,sheep and camels were in the form of ring form, slender spine-like form, an elongated structure or round form measuring 3.75 μm in diameter, and enclosing centrally located nucleus surrounded by a cloud-like dispersed

cytoplasm (Hamed *et al*.,2011). *Theileria spp* occur inside R.B.Cs has two forms the first form is (Erythrocyte form) which the parasite present in blood inside R.B.Cs and its take several shapes like ring , comma, rod and oval shape while the second form is (lymphocyte form) which the parasite present in lymph node and called a koch‘s blue bodies which represented the schizont of parasite and appear in two form macroschizont which consist of 8-12 nuclie and microschizont which consist of 50-100 nuclei(Telford *et al* ., 2002; Shaw,2003; Mans *et al*., 2015).

2-3-3- Mode of Transmission

Theileria spp transmitted only by Trans-stadial Transmission from tick’s stage to stage because ticks are often present on camels in large numbers (McKeever,2009; Hamed *et al*., 2011; Alim *et al*.,2012). The tick is infected when a larva is fed, or a nymph, and the parasite is transferred to the next developmental stage of the tick (Abdigoudarzi, 2013) . Also via vertical transmission (Zakian *et al*., 2014).

2-3-4- life cycle

The *Theileria* spp. life cycle is very complex and have several differentiation steps includes three typical phases: schizogony, gametogony and sporogony (Nazifi *et al*.,2011). Like all intracellular parasites(shaw,2003; Mans *et al.*, 2015). The life cycle in vertebrate host began after infection through tick’s bite the parasite was developed in lymphocytes and in this stage called (macroschizont) appears as roundish cells with pale blue cytoplasm and variable in numbers of red nuclei which refer to Koch’s blue bodies(Alim *et al*.,2012).The merozoites invade new host’s cells , the microschizont appear after two weeks of infection is distinguished by numerous spherical nuclei the microschizont

invade erythrocytes that which the infective stage to the ticks(Uilenberg, 2006; McKeever,2009 ; Mans *et al.*, 2015).

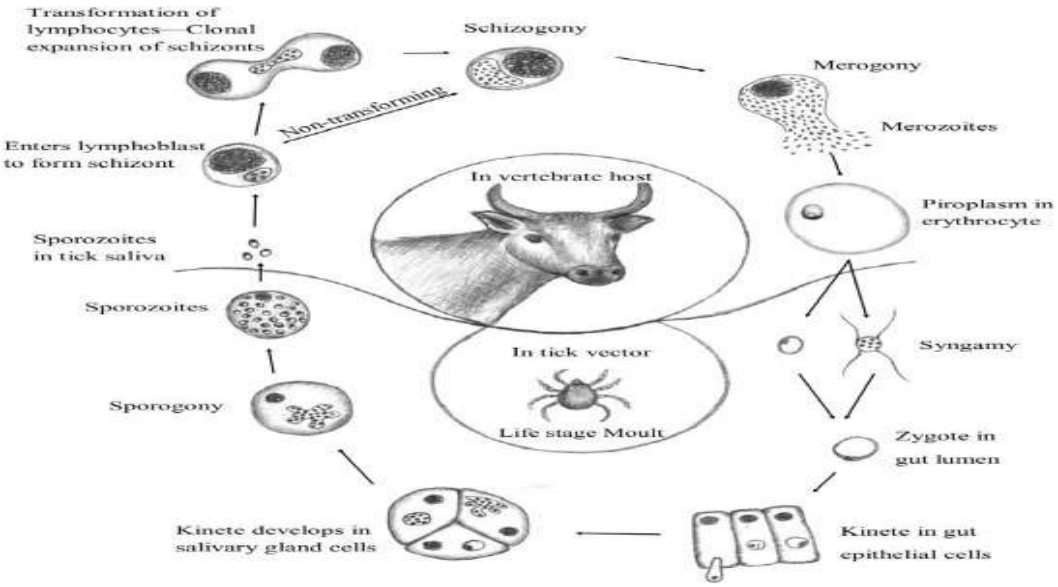


Figure 2-3 life cycle of *Theileria spp*

https://ars.els-cdn.com/content/image

2-3-5- Clinical Signs and pathogenesis

The most prominent clinical signs of camels infected with *Theileria* infections include generalized enlargement and swelling of the superficial lymph nodes especially superficial cervical lymph nodes were noticed, high fever, listlessness, anorexia, emaciation, intermittent diarrhoea or constipation and loss of condition (Shaikh,2004; Tageldin *et al.*, 2005; Hamed *et al.*,2011). Initially, infected animals have an apparently normal appetite, but in a few days after the onset of fever they stop eating and later on they become gradually emaciated (Qablan *et al.*,2012; El Imam *et al.*, 2015). Infected animals suffer from lacrimation (ocular watery discharge) , abortion, hemolytic anemia and infertility(El-Fayoumy *et al.*, 2005; Hamed *et al.*, 2011; Ismael *et al.*,2013).

The pathogenesis of *Theileria* caused by macroscizonts and effect the lymphocytes and reticular-endothelial ,the clinical sings and severity

of the disease is closely related to the degree of leukopenia arrested of maturation of these cells in bone marrow due to toxic effect of parasite and increase the numbers of infected lymphocytes (von Schubert *et al.*, 2010; Ismael *et al*.,2013). and production of a number of cytokine which may induce fever and play a role in anemia, muscle wasting and necrosis (Dobbelaere and Baumgartner, 2009).

On other hand, the hepatization rubbery texture of the lungs, and accumulation of excessive fluids and exudates in the chest cavity (Tageldin *et al.,* 2005). These fluids may weaken the host respiration are caused serious tissue destruction and pulmonary edema indicate that emphysema, congestion and collapse lead to a respiratory failure(El Imam, 2010).

2-3-6- Diagnosis

The diagnosis of *Theileria* parasites is based on the clinical symptoms and demonstration of morphological features of piroplasms inside red blood cells in stained blood smears(Minjauw and McLeod, 2003) .But in mix infection of *Theileria* with other pathogens, including *Babesia, Anaplasma* and gastrointestinal nematodes gave approximately the same picture as infection with *Theileria* alone, with no significant difference (Ismael *et al*.,2013). Therefore microscopic , serological, and molecular methods are used for identification of *Theileria* parasites(Liu *et al*., 2010).

2-3-6-1- Parasitological Diagnosis

The light microscopy was used for determine the presence of heamoprotozoal parasites during the acute phase of the disease give the best result (Chaudhrib and Gupta, 2003) . The direct method involves identifying the parasite in Giemsa's-stained blood smears or lymph-node biopsy samples, This method is reliable for diagnosing clinical acute

cases, but it is very subjective in pre-immunity and long-lasting carrier hosts, where low parasitaemia occur and schizont infected leukocytes cannot be detected (Ismael *et al*.,2013). Thus, a level of expertise is required for differentiating mixed *Theileria* spp. infection on the basis of morphology (Garcia-Sanmartin *et al.*, 2006).

2-3-6-2- Serological diagnosis

Serological diagnostic tools for the major tick-borne protozoan diseases of livestock were reviewed and include The Indirect Fluorescent Antibody Test (IFAT) and ELISA (Bakheit *et al.*, 2007).

The Indirect Fluorescent Antibody Test (IFAT) based on schizont or piroplasms antigen to detect the circulating antibodies against *Theileria* has been developed (Salih *et al.*, 2003; Taha *et al.*, 2003).

The Enzyme-Linked Immunosorbent Assay (ELISA) for serological detection of antibodies against *Theileria* spp. infecting sheep have been documented (Miranda *et al.* 2006; Abdo, 2010).

2-3-6-3-Molecular Diagnosis

Molecular techniques include sequencing, PCR would allow direct, specific and sensitive detection of parasites, and rapid, simultaneous detection and differentiation of different *Theileria* infecting a given animal (Schnittger *et al.*, 2004). polymerase chain reaction (PCR) has been developed for detecting *Theileria spp.* infection with high sensitivity and specificity (Altay *et al* ., 2008 ; Ranjbar .,2012). Sequencing the 18Sr DNA gene is one of the best molecular methods for determining the strains of this parasite, and many researchers have used it worldwide, The 18S small subunit ribosomal RNA (18S rRNA) gene has been shown to be an effective marker for investigation in *Theileria* spp (Ahmed *et al* .,2006 ; Niu *et al*.,2012).

2-3-8-Immune response

Cell-mediated immunity Innate and adaptive immune responses are simultaneously act to protect host against *T. annulata.* (Glass *et al* .,2012*) T. parva* sporozoites can bind to and infect B and T lymphocytes of both CD4 and CD8 T-cell lineage with similar efficiency, whereas *T. annulata* was found to infect monocytes and B lymphocytes but not T lymphocytes (Mckeever ,2009; Boulter and Hall, 2000).

Humoral immune responses occur following infection with *T. annulata*. These antibodies recognize surface epitopes of the sporozoite and have been shown to block the invasion of mononuclear cells by sporozoites and subsequently to inhibit transformation of the infected cells (Ghosh *et al*.,2007). Efforts to detect antibodies directed to surface molecules of the schizonts, piroplasms and cell surfaces of the infected mononuclear cells and erythrocytes have failed (Nene *et al*.,2016 ; Di Giulio *et al*.,2009). As is the case with *T. parva,* the role of antibodies in protective immunity may be confined to the neutralization of sporozoites (Pipano and Shkap,2000 ; Tindih *et al*.,2012) .

2-3-8- control and prevention

The control measures against tick-borne diseases, it is essential to detect the prevalence of tick borne pathogens in target populations of animals by using insecticide through dipping of animals (Oura *et al*., 2004). In addition, immunoprophylaxis trials of cell line vaccines have been successfully carried out in Iraq, Iran and in Sudan (Ahmed *et al*., 2013).

Quarantine and tested of in ported animals and slaughter of carrier and emaciated animals (Gharbi *et al*.,2011). Treated the infected animals by theilericidal drugs lick menoctone , chlotetracyclin or oxytetracycline Mirzaiedehaghi, 2006; Derakhshanfar and Mirzaei, 2008).

2-4- *Anaplasma spp*.

Anaplasmosis is tick-borne diseases ,an infectious non contagious considered to be one of the most important in domesticated and wild ruminant causing significant economic losses in tropical and subtropical areas (Kocan *et al*.,2000 ; Ismail *et al*., 2010; Nazifi *et al*, 2011)

The Arabian camels (Camelus dromedarius) show certain characteristics that enable them to survive malnutrition, thirst, drought and depend on secondary resources in extreme climatic conditions (Bekele *et al,* 2011). Although, large numbers of ticks are often found on camels , but it bears many diseases (Hamed *et al,* 2011).

The causative agent of Anaplasmosis in cattle and wild ruminants is *Anaplasma marginale* while in sheep and goats is *A. ovis* (Boes, and Durham, 2017).

Anaplasma centrale which appears to be less pathogenic than *Anaplasma marginal* and for which *Anaplasma* inclusions were more often found in the center of erythrocytes rather than in a marginal location (Kocan *et al*., 2003).

The disease occur sporadically or as outbreaks leading to substantial significant economic and veterinary interest worldwide (Alsaad, 2007).

The main characteristics of Anaplasmosis are fever, progressive anemia, digestive disturbance and emaciation (Radostitis *et al*.,2000), With all the causes of the disease , the clinical signs and economic losses research on the effects of *Anaplasma* in camels is still low (Al-Ani ,2004)

2-4-1-classification

Anaplasmosis are defined intra erythrocytic parasites belonging to the order Rickettsiales (Radostits *et al,* 2007).Classification according to (Kocan *et al*., 2004).

Kingdom : Protista

Phylum : Protozoa

Order : Ricketsiales

Family: Anaplasmataceae

Genus: *Anaplasma*

Species**:** *A. marginale*

A. centerale

A. phagocytophilum

A. ovis

A. bovis

A. platys

2-4-2- Morphology

Anaplasma stay in host blood cells and located in central or marginal of red blood cells according to species (Kocan *et al*., 2003 ; Capucille, 2011).

Anaplasma appear as black particles within the red blood cells in blood-stained with geimesa stain , *A. marginale* were observed at the periphery of infected RBCs and appeared as reddish-violet pleomorphic ordot-like forms (0.2-0.4 μm diameter) within erythrocytes (Ismael *et al*.,2014). *Anaplasma marginale* appear under microscopical examination of stained blood film as a spherical granules near periphery of infected erythrocytes (Maghaddar, 2002; Al-Khaledi, 2008). while *Anaplasma centrale* is intra erythrocytic tick-borne pathogen located in central of erythrocytes (Kocan *et al*., 2003; Liu *et al*., 2005).

2-4-3-Mode of Transmission

The important natural vectors for transmission are ticks from the family Ixodidae and flies in the family Tabanidae (Mahran, 2004; Radostits *et al*, 2007 ;). Of the ticks, the one-host *Boophilus* spp. are of

major importance in tropical and subtropical regions which act as a mechanical transmission can also occur through the contaminated mouthparts of blood sucking diptera of the genera Tabanus, Stomoxy and mosquitoes (Amanda *et al*.,2006; Nazifi *et al,* 2011;).The mechanical transmission by biting insects appears to be the major mode of *A. marginale* transmission (De la Fuente *et al*., 2001; OIE, 2012) . In addition to mechanical and biological transfer, *Anaplasma marginale* can be transfer from cow to the calf through the placenta during pregnancy (Kocan *et al*.,2010; Da Silva *et al*.,2014).

Ticks can transmit pathogens either transracially when a tick becomes infected and then transmits the pathogen during a successive life stage or intrastadially when a tick becomes infected and transmits the pathogen within the same life stage (Radostits *et al*, 2007; Nazifi *et al*, 2011).

Biological transmission of *A. marginale* occurs between Anaplasmosis carriers to susceptible animals , the first recognized mode of horizontal transmission of bovine Anaplasmosis is mediated by tick vectors and approximately 20 species of ticks have been incriminated as vectors worldwide (Kocan *et al*., 2004 ; Mohammed *et al*.,2007) .

2-4-4-Life cycle

The evolution of the *Anaplasma* parasite life cycle in ticks is complex and coordinated with the tick feeding cycle (Al-Khaledi, 2008). The tick take the infected red blood corpuscles through blood absorption which is a source of *Anaplasma spp* in the intestinal and tick cells, After the development of *Anaplasma spp* in the cells of the intestines of ticks, many tick tissues become infected, such as salivary glands, during which the parasite is transmitted to host during feeding(Futse *et al*., 2003; Amanda *et al*.,2006; Nazifi *et al.*,2011). *Anaplasma spp.* develops through gaps that are surrounded by membranes or colonies in all

infected tick tissues(Kocan *et al* .,2010). The first form *Anaplasma* is observed in the colonies of the vegetative form, which is divided by bilateral fission, to form large colonies (Kocan *et al* .,2003). Infected erythrocytes are disrupted and release bodies which can then invade other erythrocytes, These bodies form vacuoles within the cytoplasmic membranes of the red blood cells and then undergo binary fission to form dense blue-purple round/cube shaped inclusion bodies (Rikihisa,2011) . This amplifies infection within the host and increases the probability of transmission when insects blood feed (Scoles *et al*., 2005). The second round of replication in the salivary gland acinar cells, apparently dependent on resumption of tick feeding on a mammalian host, is followed by transmission via the saliva (Futse *et al*., 2003) .

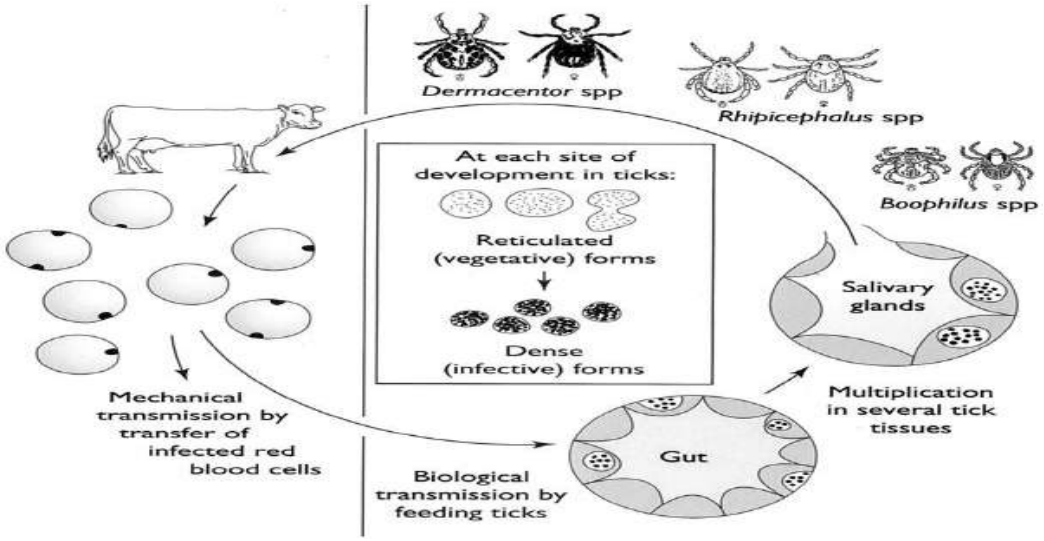


Figure 2-4 life cycle of *Anaplasma spp* (Kocan *et al*., 2003)

2-4-5- Clinical Signs and pathogenesis

The incubation period is the time from which the organism is inserted into the susceptible animal and depend on the infective dose and ranges from 7 to 60 days, with an average of 28 days (Kocan *et al*., 2010) . An anemia results from extra-vascular hemolysis by reticulo-endothelial system (liver and kidneys) which removal and destruction

of infected and non-infected RBCs by erythrophagocytosis at 8-10 times than the normal rate producing fatal hemolytic anemia and jaundice without hemoglobinemia and hemoglobinuria and this is the key feature of bovine anaplasmosis (Kocan *et al*.,2000 ; Mahran,2004) . During the erythrophagocytosis process , IgG and IgM molecules were elicited against A. marginale antigens and act as opsonizing antibodies which attract phagocytic cells and enhance the proces (Melendez , 2005 ;Mohammed *et al*.,2007).

Cardiovascular sings profound anemia resulting in tachycardia and dyspnea , Congestion of mucous membranes which may be jaundiced (Maghaddar,2002).

Gastrointestinal signs are anorexia , mucoid diarrhoea, rumenal atony, and excess salivation. does not occur haemoglobinuria but cause neurological signs such as shivering, fasciculations, ataxia, seizures and syncope (Kholer-Rollefson *et al* .,2001). Anaplasmosis effect on reproductive which cause abortion, infertility and agalactia (Amanda *et al*.,2006 ; Kocan *et al*., 2003).

Other symptoms like general paralysis, rough hair coat and weight loss Signs are often hardly exacerbated by exercise with caution needed not to overly stress animals during clinical examination which can lead to collapse and death (Kocan *et al*., 2010) .

2-4-6 – Diagnosis

Diagnosis of camels clinically affected with anaplasmosis infection the observed clinical signs included fever, anorexia, diarrhoea, emaciation, pale mucous membranes, lacrimation and anaemia that persisted up to 87 days in some cases(Hekmatimoghaddam *et al*., 2012). However, not all animals showed the typical clinical picture may be showed mild manifestation and mixed infections of *Anaplasma spp* with other pathogens, including *Theileria*, *Babesia*, gastrointestinal nematodes

and *Balantidium coli*, gave approximately the same clinical picture as infection with *Anaplasma* therefore must be depend on other methods for give confirmed diagnosis(Ismael *et al*.,2014)

2-4-6-1-Parasitological examination

Microscopically examination for presence of *Anaplasma spp* and other blood protozoa by using Giemsa stained blood films were thin or thick for diagnosis (Tejedor-Junco *et al* 2011).

Giemsa-stained blood smears can be actually used as a suitable method to detect *Anaplasma* in the animals clinically suspected acute anaplasmosis, but it is not applicable for the determination of pre-symptomatic or carrier animals (Carelli *et al.* , 2007).

Diagnosis of *A. marginale* under the microscope depends on the location of inclusion bodies and morphological identification which appear as dense, rounded approximately (0.3-1.0) μm in diameter marginally within the erythrocytes while *A. centrale* is more located in central of erythrocytes (Kocan *et al*., 2003; Liu *et al*., 2005; Abdalla *et al*.,2017).

Blood obtained from the jugular vein or other large vessels because *Anaplasma spp* don't accumulate in capillaries (OIE , 2008) .

2-4-6-2-Serological examination

Anaplasma infections usually continue for the all life of the animal and cannot easily be detected in blood smears after acute parasitemia , therefore use of serological tests have been developed with the aim of detecting infected animals (Ozlem and Ferda , 2011**)**. These serological tests include card agglutination test (CAT) ,enzyme linked immunosorbent assay (ELISA) , complement fixation (CF) test and the immunofluorescent-antibody test (IFA) which have been the most commonly to detect *Anaplasma*-infected animals in the field (OIE , 2008)

Competitive Enzyme-Linked Immuno Sorbent Assay(C-ELISA) uses a monoclonal antibody (MAb) specific for a recombinant antigen termed MSP5 , it has certain very sensitive and specific for the detection of *Anaplasma spp.* infected animals (Reinbold *et al.*, 2010).

2-4-6-3-Molecular Diagnosis

Several molecular techniques were used to detecting the different species of *Anaplasma* most of these techniques depend on the major surface proteins (MSPs) (De la fuente *et al* 2007), the heat-shock gene groEL (park *et al* .,2005) , the 23S rRNA (Dahmani *et al* .,2015) . Targeted the msp4 and msp2 genes, which are involved in host-pathogen and tick-pathogen interactions and have been used as markers for the genetic characterization of *A. marginale* strains (Reinbold *et al*., 2010)

2-4- 8- Immune response

During acute infection with high levels of *Anaplasma* parasite are planned after the development of primary immune response, but the emergence of Antigenic Variants of the parasite result in persistent infection characterized by sequent parasitemic cycles of low level of parasitemia (Palmer *et al*.,2000), continuous approximately 5-8 days of every 5-6 weeks intervals in which a new Major Surface Protein 2 (MSP2) replicate and then controlled by variant specific immune response designated Variants arising in three sequential parasitemic cycle (Kocan *et al*., 2003; De la Fuente *et al* 2007).

During the erythrophagocytosis process , IgM and IgG molecules were elicited against *A. marginale* antigens and act as opsonizing antibodies which attract phagocytic cells and enhance the process (Melendez , 2005).

A. marginale is responsible for severe hemolytic anemia associated with fever, weight loss, abortion, reduce milk production, and,

in some cases cause death of the infected mammalian (Kocan *et al*., 2003) while *A. centrale* causes asymptomatic or mild form of infections in cattle and is utilized for extensive vaccination of cattle against *A. marginale* infection in endemic areas (Kocan *et al*., 2003) . It is well documented that immunization with *A. centrale* does not prevent infection with *A. marginale* **,** but the severity of the disease is reduced and death is prevented because they share immunodominant epitopes that may play a role in the protection induced by *A. central* , the live *A. centrale* vaccine is available commercially and wide use in Africa , Australia , Latin America (Kocan *et al*., 2003; Abdalla *et al*.,2017) .

2-4-8-Control and prevention

There are many methods described by Radostits *et al* (2007) for the prevention and control of anaplasmosis from spread in flock ,These methods depend on treatment of infected camels with a single injection of long-acting oxytetracycline at a dose of 20 mg/kg BW intramuscularly. also include arthropods control , detection and elimination of persistently infected animals , chemoprophylaxis and vaccination (Kochan *et al* ,2000). Also imidocarb is used for the treatment of acute anaplasmosis infection (Potgieter and Stoltsz , 2004).

The introduction of the disease into herds by carrier animals should be prevented by serological testing , notice should be also given to preventing relating to illness caused by medical examination or treatment (Kocan *et al*., 2010). Sterilization and disinfection of surgical instruments and tools should be performed after each use on each animal, as it is a source of disease transmission (Radostits *et al*., 2007).

In many tropical and sub-tropical areasuse vaccination against *Anaplasma* in areas infested by several tick vector species and include vaccination with live *A. centrale* use vaccine to avoid heavy losses (Kocan *et al*., 2004; Kocan *et al*., 2010).

Chapter Three

Materials and Methods

3- Materials and Methods

3-1 Materials :

3-1-1-Equipments and Instruments

Table (3-1): show the Instruments and Equipment which were used in the study:

No.	Instruments and equipment	Manufacturers/ country
1	Autoclave supply	Arnold and Sons/ USA
2	Anticoagulant vacuum tube	Vapco /Jordan
3	Centrifuge	Memmert/ Germany
4	Deep freezer	GFL/ Germany
5	Distillatory	GFL/ Germany
6	Digital camera	Sony/ Japon
7	Disposable Gloves (large, meddle)	China
8	Electrophoresis power	Shandod Scientific/ UK
9	Eppendorf centrifuge	Memmert/ Germany
10	Eppendorf tubes (different size)	Bioneer /UAE
11	Gel documentation system (Vision)	SCIE-PLAS/ UK
12	High Speed Cooling centrifuge	LG /Korea
13	Microscope	Olympus / Japan
14	Microscope slides	China
15	Magnetic Stirrer with hotplate	Stuart /UK
16	Micropipette tips (different sizes)	Eppendorf / Germany
17	Nanodrop spectrophotometer	Thermo /USA
18	Sensitive Balance	Ohaous/ Swiss
19	Sticks	China

20	Thermocycler PCR	USA
21	UV Transilluminator	USA
22	Vortex	CYAN /Belgium
23	Water Bath	Mammert /Germany

3-1-2- . Chemicals and Reagents

Table (3-2) show The Chemicals and Reagents were used throughout this study:

No.	Chemicals and Reagents	Company
1	Absolute ethyl alcohol	England
2	Absolute Methanol alcohol	Spin
3	Cotton	China
4	Giemsa stain	Stock /Ireland
5	oil immersion	England
6	Xylene	England

3-1-3-Molecular identification

Table (3-3) Chemicals and reagents for DNA extraction and polymerase chain reaction.

No.	Name	Source
1	Colum – pure Blood Genomic DNA Mini Kit (Extraction Kit)	Abm / Canada
	Proteinase K	
	PBS solution	
	Buffer CL	
	Cw1 solution (concentrate)	
	Cw2 solution (concentrate)	
	Elution buffer (CE)	
	Buffer TBP	
	Collection tube 2ml	
2	DNA ladder	Kappa Bio systems , South Africa
3	Master Mix	Kappa Bio systems
4	Nuclease free water	Bio-Rad USA
5	Primers	IDT, Canada
6	Bromophenol blue dye	Kappa Bio systems , South Africa
7	Ethidium bromide	Kappa Bio systems , South Africa

3-1-4- Primers

Specific nine primers that used for detection specific sequence genes of blood parasite which provided by IDT(Canada) as following:

Table(3-4):Primers used in this study with their Sequence and PCR Size:

Primer	Sequence (5'-3')		product Size (bp)	source
Anaplasma marginale major surface protein 1b (SI3) gene	F	GCTCTAGCAGGTTATGCGTC	265bp	EL-Naga and Barghash (2016)
	R	CTGCTTGGGAGAATGCACCT		
Anaplasma centrale *Anaplasma platy* *Anaplasma marginale* ribosomal RNA gene	F	GTGGCAGACGGGTGAGTAATG	857bp	EL-Naga and Barghash (2016)
	R	CATGTCAAGAAGTGGTAAGGT		
Babesia bovis glutamine-dependent carbamoyl phosphate synthase (CPSII)	F	TTTGGTATTTGTCTTGGTCAT	446-453bp	EL-Naga and Barghash (2016)
	R	ACCACTGTAGTCAAACTCACC		
Babesia bigemina 18ribosomal RNA gene	F	TAGTTGTATTTCAGCCTCGCG	689bp	Bilgic *et al* 2010
	R	AACATCCAAGCAGCTAHTTAG		
Theileria annulata cytochrome b	F	ACTTTGGCCGTAATGTTAAAC	312bp	EL-Naga and Barghash (2016)
	R	CTCTGGACCAACTGTTTGG		
Theileria spp. 18ribosomal RNA gene	F	AGTTTCTGACCTATCAG	1100bp	EL-Naga and Barghash (2016)
	R	TTGCCTTAAACTTCCTTG		
Trypanosoma spp. 18ribosomal RNA gene	F	CCGGAAGTTCACCGATATTG	480-757bp	Njiru *et al*.,2005
	R	TGCTGC GTTCTTCAACGAA		
Trypanosoma evansi VSG gene	F	GCGCGGATTCTTTGCAGACGA	257bp	Salim *et al*., (2011)
	R	TGCAGACACTGGAATGTTACT		
Trypanosoma brucei *Brucei gene*	F	GAATATTAAACA ATGCGCAG	164bp	Shah *et al* ., 2004
	R	CCATTTATTAGCTTTGTTGC		

3-Methods

3-2-1-Blood samples collection

Total of 200 blood samples were collected from camels, some of these camels were suffered from weakness, anaemia , liver pale and other asymptomatic camels in the house slaughter in Al-Diwaniyah province during the period from September – 2017 to the end of march – 2018 and data included animal's age (>1 year to more than 2-7 year and <1 less than one year)and animal's sex (male and female) .

These samples were collected from the jugular vein of each camel and kept at clean sterile tubes containing ethylene di-amine tetra acetic acid (EDTA) in the Diwaniyah slaughterhouse and transported immediately in an ice pack to the laboratory of the Veterinary Medicine College /Al-Qadissiya University to make the slides and then put in deep freeze under (20-) c for DNA extraction.

3-2-2-Microscopic examination:

Blood smears prepared on slides were stained with Giemsa stain to detect blood-borne protozoa were identified based on morphological features described by Soulsby (1982).A drop of blood was taken and put on the glass microscopic slide and spread by another slide then left in air to dray fixed by absolute methanol for 5 minute then stained with Giemsa stain for 30 mints then washed by tab water and left to dry after drying they examined under the oil immersion lens of light microscope (Swelum *et al*., 2014).

3-2-3- Polymerase chain reaction (PCR)

The conventional PCR technique was performed to detection of as in the following steps:

3-2-3-1- Genomic DNA Extraction

Genomic DNA from camel blood samples were extracted by using Column-pure blood Genomic DNA Mini Kit ,Applied Biological Materials (Abm) Canada , and done according to company instructions as in the following steps:

1. Take 100µl of blood in to 2 ml centrifuge tube then add 100µl from PBS solution to a final volume of 200µl .
2. vortex gently and let stand 1 min at room temperature .If >100µl of blood used add 2 volumes of buffer TBP mix and let stand for 1 min until complete lysis .
3. spin 800rpm for 1 min. discard the supernatant carefully .wash the precipitate with 500µl TE buffer two time .spin 8000 rpm for 1min during each wash . the final precipitate appear white .
4. Add 20µl of proteinase K. mix well .Add 200µl of buffer CL. vortex gently . incubate at 56 c for 10 min.
5. Add 200µl of 100 % ethanol to the mixture and mix thoroughly .
6. Transfer entire tube components into a column that is in a 2 ml collection tube . let stand at room temperature for 1-2 min . spin at 10000 rpm for 2 min discard flow – through in the collection tube.
7. Add 500µl of cw1 solution .spin at 10000 for 1 min .
8. Add 500µl of cw2 solution .spin at 10000 for 1 min .
9. Discard the flow through .spin at 10000 for 1min .
10. Place column into a clean 1.5 ml eppendorf tube .add 50µl CE buffer into the center of the column’s membrane . incubate at room temperature for 3 min .to increase recovery yield .
11. Spin at 10000 rpm for 1 min to elute DNA from column .

12. For long term storage keep aliquots of purified DNA at -20°C until use .

3-2-3-2- Genomic DNA Profile

Nano drop spectrophotometer device (THERMO. USA) used to checked the extracted DNA concentration (ng/µL) and the DNA purity the reading of absorbance from 260 -280 nm as following steps:

1. opening the Nano drop software, choose the suitable application (Nucleic acid, DNA).
2. cleaning the measurement pedestals several times, take dry wipe and carefully pipette 2µl of free nuclease water on to the surface of the lower measurement basis for blank the system.
3. The sampling arm was drop and clicking OK to start the Nanodrop, then cleaning off the pedestals and add 1µl of DNA sample for measurement.

3-2-3-3- Agarose Gel Electrophoresis:

Prepare two agarose gel (1% and 1.5%) concentrations agarose of 1% concentration was used in the electrophoresis after DNA extraction process, while 2% agarose was used after PCR product detection, then the next steps were followed: of

A- Casting of the Agarose Gel:

• The gel was assembled to a casting tray and the comb was positioned at one end of the tray.

• The agaroose solution was poured into the gel tray and it was allowed to cool at room temperature for 30 min.

• removed the comb carefully and the gel was replaced in an electrophoresis chamber after that the chamber was filled with TBE - buffer until the buffer reached 3-5 mm on the surface of the gel.

B- Loading and Running DNA in Gel Agarose:

- loaded in the wells DNA (9 µl) of the 1% agarose gel which mixed with (5µl) bromophenole blue (loading buffer)
- The cathode was connected to the well side of the unit and the anode to the other side .
- The gel was run at 100 V until the bromophenole blue tracking dye migrated to the end of the gel.
- The DNA was observed by staining the gel with ethidiume bromide and viewed with UV trans illuminator.

3-2 -3-4-Protocol:

Table(3-5): Protocol of mono plex PCR reaction mixture volume

No	PCR reaction mixture	Volume (Total 25 µ)
1	master mix(2x)	12.5 µ
2	Forward Primer	1.25 µ
3	Reverse Primer	1.25 µ
4	DNA template	5 µ
5	PCR grade water	5 µ

Table(3-6): Protocol of Multiplex PCR reaction mixture volume

No	PCR reaction mixture	Volume (Total 25 µ)
1	master mix(2x)	12.5 µ
2	Forward Primer (1)	0.75 µ
3	Reverse Primer(1)	0.75 µ
4	Forward Primer (2)	0.75 µ
5	Reverse Primer(2)	0.75 µ
6	DNA template	5 µ
7	PCR grade water	4.5 µ

3-2-3-5-PCR Thermo cycler Conditions

PCR reactions were done for each thermo cycler condition by using conventional PCR thermo cycler system as following table(3-7):

Table (3- 7): PCR amplification program

Gene	Initial Denaturation	Cycling conditions			Final extension	Hold	Cycle No.
		Denaturation	Annealing	Extension			
Anaplasma marginale	94°C/280sec	94°C/120sec	62/60sec	72°C/60sec	72°C/60sec	4°c	40
Anaplasma centrale	94°C/280sec	94°C/120sec	60 /60sec	72°C/60sec	72°C/60sec	4°c	40
Babesia bovis	94°C/280sec	94°C/120sec	59/60sec	72°C/60sec	72°C/60sec	4°c	40
Babesia bigemina	94°C/280sec	94°C/120sec	58/60sec	72°C/60sec	72°C/60sec	4°c	40
Theileria annulata	94°C/280sec	94°C/120sec	56/60sec	72°C/60sec	72°C/60sec	4°c	40
Theileria sp.	94°C/280sec	94°C/120sec	56/60sec	72°C/60sec	72°C/60sec	4°c	30
Trypanosoma spp.	95°C/120sec	95°C/30sec	58°C/30sec	72°C/60sec	72°C/60sec	4°c	35
Trypanosoma evansi	94°C/280sec	94°C/120sec	58/60sec	72°C/60sec	72°C/60sec	4°c	40
Trypanosoma brucei	94°C/280sec	94°C/120sec	58/60sec	72°C/60sec	72°C/60sec	4°c	40

3-2-3-6- PCR product analysis

1- prepare by mix 1.5 g Agarose gel and 4ml of 1X TBE and completed the volume to 100 ml distal water after that heated on hoot belt device at 100 °C then, left to cool 50°C.

2- Then (5µ) of ethidiume bromide stain were added into agarose gel

3- Agarose gel solution was teeming in tray after fixed the comb in suitable situation, left to solidified for 15 minutes at room temperature, after that the comb was removed gently from the tray and 10µl of PCR product were added in to each well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill gently by 1X TBE buffer. Then electric current was performed at 80- 100 volt and 80 mA for 1hour.

5- PCR products were visualized by using UV Trans illuminator to show bands of product.

3-2- 4 -1: DNA sequencing Analysis.

DNA sequencing technique was carried out for Phylogenetic relationship analysis study of genes (major surface protein 1b (SI3) gene, ribosomal RNA gene, glutamine-dependent carbamoyl phosphate synthase (CPSII), 18S ribosomal RNA gene, cytochrome b , 18ribosomal RNA gene, VSG gene and 18ribosomal RNA gene) for (*A. marginale ,A. central, B. bovis, B. bigemina , T. annulata, Theileria spp, Trpanosoma evansi* and *Trypanosoma spp*) respectively in camels with NCBI-Gen Bank Global. PCR product carried out the DNA sequencing by (suol – South Korea).

3-2-4-2- Nucleotide sequences analysis and gene bank submissions.

Nucleotide sequence sets of (major surface protein 1b (SI3) gene, ribosomal RNA gene, glutamine-dependent carbamoyl phosphate synthase (CPSII), 18ribosomal RNA gene, cytochrome b , 18ribosomal RNA gene and VSG gene) genes were striped then confirmed and compared with other world sequences by using NCBI- Blast and analysis of these nucleotides sets of amino acids from forward and reverse translations of nucleotides by using of Open Reading Frame (ORF) program of any sequence in this study by using NCBI- ORF online. Then these nucleotide sequences sets and amino acids submitted directly through NCBI- Gene Bank in program Bank It under ID-Submission.

3-2- 4 -3. Phylogenetic analyses.

Nucleotide and amino acids sets were prepared for identity score of isolates blood parasites in this study with other world parasite by using NCBI-Blast program online, and alignment between genes of hem protozoan in this study and other world strains by using NCBI-Clustl W2 program online. Then all world strains identity with Iraq isolates range between (100%) in identity score was analysis to building phylogenetic tree by using neighbor method to designed tree with the MEGA 4software (Tamura *et al*., 2007). Phylogenetic trees were inferred with distance, parsimony and maximum likelihood methods and the reliability of the trees were determined by 1000 data set bootstrap resembling.

3-2-5- Statistical analysis

Statistical analysis for this study was achieved by using Chi Square tests (X^2) at $p \leq 0.05$ was used to analyze differences in the blood parasites rate among samples, sex and different studied ages (Leech *et al*., 2011).

Chapter Four

Results and Discussion

4-1- Result of Microscopic Examination :

Out of 200 camels blood samples which collected from the province of Diwaniyah slaughterhouse at from September - 2017 to the end of march – 2018 . the result show camels can be infected with *Trypanosoma spp* 76 (38%), *Babesia spp* 106 (53%), *Theileria spp* 93(46.5%), and *Anaplasma spp* 132 (66%) in both male and female and in all age of camels as in table (4-1).

Table 4-1 show the infection of camels with some blood parasites

Total No. of animals examined	*Trypanosoma spp*		*Babesia spp*		*Theileria spp*		*Anaplasma spp*	
	+	%	+	%	+	%	+	%
200	76	38	106	53	93	46.5	132	66

4-1-1- *Trypanosoma spp*

The results show that of the total 200 samples of camels blood examined microscopically found that 76(38%) samples infected with *Trypanosoma spp* as in figure (4-1) .

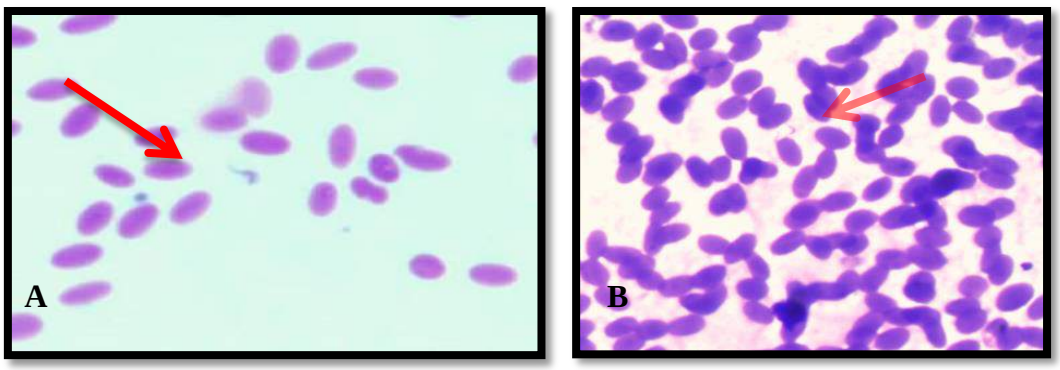


Figure 4-1

A -*Trypanosoma spp*. Show between a camel red blood cells (thin smear) 100x

C- *Trypanosoma spp*. Show between a camel red blood cells (thick smear) 100x

Many researcher recorded Trypanosomiasis in camels like Salim *et al*., (2011)recorded (365/687)(53.12%) in camels in Sudan , Abd-

Elmaleck *et al*.,(2014) recorded 3(3.06%) from 98 camels in Egypt and Barghash *et al*.,(2014) diagnosed *T. evansi* 52(20.9%) in Egypt.

According to the sex of animals, the number of infected females (57/125)(45.6%) and the number of infected males (19/75) (25.3%) of the total 200 samples. The results show there was significant difference at $p < 0.05$ between males and females as in table (4-2)

Table 4-2- The prevalence of *Trypanosoma spp* infection in camel according to the microscopic examination and sex

Sex	No .of samples	*Trypanosoma spp* infection	
		+	%
Males	75	19	25.3 b
Females	125	57	45.6 a
Total	200	76	38

Different letter = significant difference at $p \leq 0.05$

The results of the present study are similar to the results recorded by Joshua *et al*., (2008) recorded (7/45)(15.6%)females camels of Nigeria, Borji *et al*.,(2009) recoded (6/90)(6.66%) in female camels in Iran, Barghash (2010) in domestic animals in Egypt, Al-shabbani (2012) recorded (104/116)(89.65%)in Al-Diwaniyah province of Iraq by using Serological Finding by Card Agglutination Test (CATT), Barghash *et al*.,(2014) recorded 135(81.2%) out of 174 camels in Egypt, El-Naga and Barghash (2016) recorded (162 /241)(67.2%) in Northern West Coast of Egypt , Wakil *et al*. (2016) recorded 122 (60.4%) in camels in Maiduguri, Nigeria and Al-Amery *et al*.,(2017) recorded(18/70)(25.71%) in females camels in AL-Najaf province of Iraq. These recorded the highest infection of females more than males suspected to infection from males.

But disagree with Mahran (2004) recorded (23%)in male camels at Shalatin city, Red sea, Oryan *et al* (2008) recorded (23%) in male camels in Iran, These reported the higher infection was found in males more than females .

The differences could be due to the several effects such as the number of males of camels were using for meat slaughter while females of camels were nearly old age because using breeding and milk production , therefore stress during gestation and milk production performance them more susceptible *Trypanosoma spp*.

According to the age of animals, the number of infected less than (<1)year (14/50)(28%) and the number of infected camels more than (>1)year (62/150)(41.33%) as in table (4-3). There are no significant difference at $p \leq 0.05$. In this study show *Trypanosoma* can infected camels in all age with the increasing of animal age .

Table 4-3- The prevalence of *Trypanosoma spp* infection in camel according to the microscopic examination and age

Animals age	No.	*Trypanosoma spp* infection	
		+	%
< 1	50	14	28 [a]
> 1	150	62	41.33[a]
Total	200	76	38

Similar letter = no significant difference at $p \leq 0.05$

The results of this study are in agreement with Borji *et al*.,(2009) recorded (2/55)(3.6%), (3/41)(7.3%) and (9/165)(5.4%) in aged(>5) (5-8)(>8)year respectively of camels in Iran, Al-shabbani (2012)recorded (103/174)(59.2%)in more than 7 year in camels in Al-Diwaniyah province of Iraq by using Serological Finding by Card Agglutination Test (CATT) , Barhash *et al*.,(2014) recorded high infection (6/12)(50%) in

camels 10 years of age in Egypt , El-Naga and Barghash (2016)recorded (64/84)(76.2%) in camels in Egypt and Al-Amery *et al*.,(2017) recorded (22/40) (55%)in age 1< year camels in AL-Najaf province of Iraq. These found that the highest prevalence rate at all age especially in camels more 1 year .

Atarhouch *et al* .,(2003) and Zayed *et al*., (2010) are reported that a tendency of infection rate to increase with age due to the mainly fact of larger scale movement, which increases the risk of infection, by the adult camels than the younger.

(Ziapour *et al*., 2011)reported that the expected of the young animal to the colostrum that contains the antibodies make them more resistant .

But disagree with Joshua *et al*.,(2008) recorded (11/38)(28.9%)in young camels in Nigeria .

This could be attributed to the owners and nomads who preferring to graze the animals in open fields' because the open field increases the risk of infection because animals become more exposed to vector bites and small aged animals take protection via the colostrum and from mother's immunity and different age animals groups were breed together permitting transmission of infection from adults animals (carriers) to susceptible young animals and other reason such as differences in diagnostic techniques , location and climatic conditions

4-1-2- *Babesia spp.*

The results show that of the total 200 samples of camels blood examined microscopically found that 106(53%) samples infected with *Babesia spp* as in figure (4-2) .

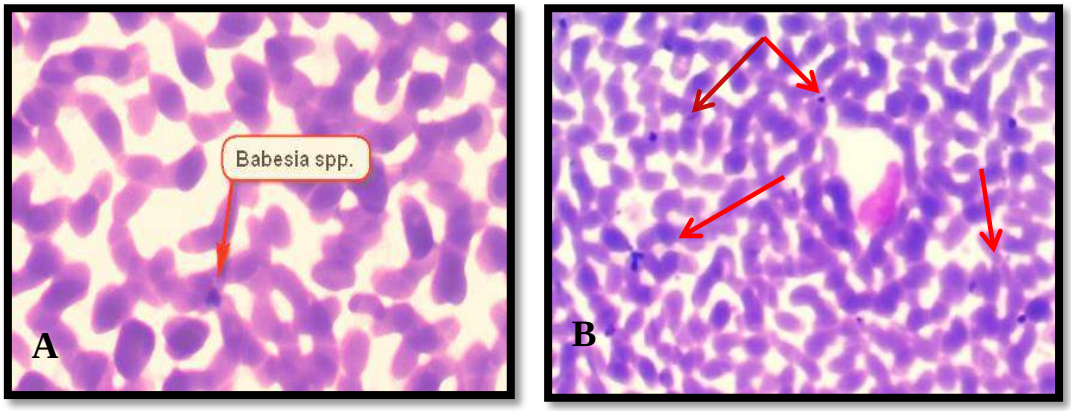


Figure 4-2

A- *Babesia spp*. Show inside a camel red blood cells (thin smear) 100x

B- *Babesia spp*. Show inside a camel red blood cells (thick smear) 100x

Many research recorded *Babesia* infection in domestic animals like Ranjbar and Afshari (2009)who recorded (3.54%)of 113 camel in Iran, Abd-Elmaleck *et al*.,(2014) recorded (46/98)(46.9%) from camels in Egypt, Swelum *et al*., (2014)recorded (34/258)(13.17) in camels in Saudi Arabia, Jasim *et al*.,(2015) recorded (24/241)(9.95%) in camels from Basra in south of Iraq.

According to the sex of animals, the number of infected females (76/125) (60.8 %) and the number of infected males (30/75) (40%) of the total 200 samples. The results show there was significant difference at $p \leq 0.05$ between males and female as in table (4-4).

Table 4-4- The prevalence of *Babesia spp* infection in camel according to the microscopic examination and sex

Sex	No .of samples	*Babesia spp* infection	
		+	%
Males	75	30	40 ^{B}
Females	125	76	60.8 ^{A}
Total	200	106	53

Different letter = significant difference at $p \leq 0.05$

The results of the present study are similar to the results recorded by El-Naga and Barghash (2016) recorded (47/241)(19.5%) in females camels in Egypt and Ibrahim *et al*.,(2016) recorded(16/34) (47.1) in females camels in Sudan and Abdalla *et al*.,(2017)who recorded (95/176) (54%) in female camel in Somalia.

But disagree with result of Al-Amery *et al*.,(2017) recorded (31/90)(34.44%)in males camels in AL-Najaf of Iraq.

Khamesipour *et al*.,(2014) recorded (5/74)(6.76%)in males and (3/58)(5.17%) in females camels in Iran and Faham *et al*.,(2015) recorded (3/58) (5.17%) and (5/74) (6.76%)in female and male of camel in Iran they are no significant difference between male and female.

The differences could be due to the several effects such as the time of study, number of samples, location, stress during gestation and milk production performance them more susceptible to *Babesia spp* .

According to the age of animals, the number of infected camels less than (<1) year (20/50)(40%) and the number of infected camels more than (>1)year (86/150)(57.33%). The results show there was significant difference at $p \leq 0.05$ as in table (4-3). In this study show *Babesia spp* can be infected camels in all age.

Table 4-5- The prevalence of *Babesia spp* infection in camel according to the microscopic examination and age

Animals age	No.	*Babesia spp* infection	
		+	%
< 1	50	20	40^{B}
> 1	150	86	57.33^{A}
Total	200	106	53

Different letter = significant difference at $p \leq 0.05$

The results of the present study are similar with Faham *et al*.,(2015) in camel in Iran, Ibrahim *et al*.,(2016) in camels in Sudan and

Al-Amery *et al*.,(2017) recorded (27/40)(67.5%) 1< year in camels in AL-Najaf of Iraq. which did reported infection in different age.

But disagree with result of Ziapour *et al*., (2011) who recorded (28%) in domesticated animals(>1) in north of Iran, El Moghazy *et al*.,(2014) reported the infection 17/79(21.5%) and (33/79)(41.8%) in calves (<1) year and adult in buffalo from Benha in Egypt.

The small age animals more susceptible to infection through bits of ticks , because innate immunity protect it until 1-6 months but if infected may be due to transmission via the placenta .

4-1-3 *Theileria spp*

The results show that of the total 200 samples of camels blood examined microscopically found that 93 (46.5%)samples infected with *Theileria spp*. as in figure (4-3) .

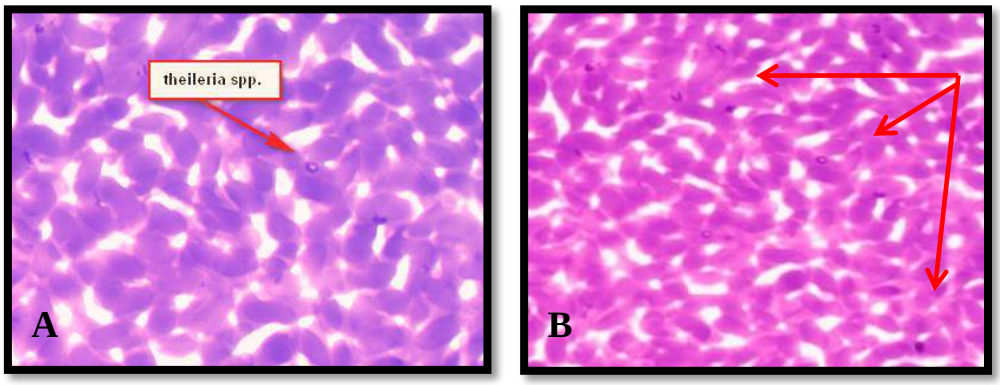


Figure 4-3

A- Show *Theileria spp*. inside a camel red blood cells (thick smear) 100x

B- Show *Theileria spp*. inside a camel red blood cells (thick smear) 100x

Many research recorded *Theileria* infection in camels like, Ranjbar and Afshari (2009) diagnosed (6.20%) *Theileria spp* in camels in Iran, Hekmatimoghaddam *et al*., (2012) diagnosed (15.79%) in camels of Iran, Abd-Elmaleck *et al*.,(2014) recorded (9/98)(9.18%) from camels in Egypt, Jasim *et al* .,(2015) recorded (14/241)(5.8%) in camels from

Basra in Iraq, Osman *et al*.,(2015) recorded (9/100)(9%) by blood smear and (11/100)(11%)by indirect fluorescent antibody technique camels are positive in Egypt

According to the sex of animals, the number of infected females (69/125) (55.2%) and the number of infected males (24/75) (32%) of total 200 samples. There was significant difference at $p \leq 0.05$ between males and females camels as in table (4-6).

Table 4-6- The prevalence of *Theileria spp.* infection in camel according to the microscopic examination and sex

Sex	No .of samples	*Theileria spp* infection	
		+	%
Males	**75**	**24**	**32^{B}**
Females	**125**	**69**	**55.2^{A}**
Total	**200**	**93**	**46.5**

Different letter = significant difference at $p \leq 0.05$

The results of the present study agree with Sallemi *et al*.,(2017) who recorded (46/69)(66%) in females cattle more than males in Northern Tunisia .

The results of the present study disagreement with to the results recorded by both of Al- khalidy (2008) which recorded (52.29%)from 114 male and (54.44%) from 202 female cows of Al-Diwaniyah province in Iraq and Ismeal *et al*.,(2014)recorded (67/173)(38.72%) in males and females of camels of Saudi Arabia, which recorded no difference between males and females. They pointed out the absence of the importance of the sex of the animal in the infection to *Theileria spp*.

while Joshua *et al*.,(2008) recorded (9/113)(8%)in males camels of Nigeria and Moezi *et al*.,(2016) recorded (120/310)(38.7%) in male more than females camels of Iran and Al-Amery *et al*.,(2017) recorded (27/90)(30%) in males camels in AL-Najaf of Iraq

The reason is that the transmission of the disease is one, it's tick of genus *Hyalomma* and that both males and females graze in the same place, activity of ticks in the period of study and when the proportion of female infection is higher due to the number of samples as the number of females in this study more than males because they are used for slaughter.

According to the age of animals, the number of infected camels less than (<1) year(18/50)(36%),and the number of infected camels more than(>1)year (75/150)(50%). There are no significant difference at $p \leq 0.05$. In this study show *Theileria spp* can infected camels in all age as in table(4-7).

Table 4-7- The prevalence of *Theileria spp* infection in camel according to the microscopic examination and age

Animals age	No.	*Theileria* infection	
		+	%
< 1	50	18	36^{A}
< 1	150	75	50^{A}
Total	200	93	46.5

Similar letter = no significant difference at $p \leq 0.05$

The results of the present study are similar to the results recorded by El-Fayoumy *et al*.,(2005) who recorded (56/125) (44.8%) in camels (<1 year age)in North Coast of Egypt, Hamed *et al* .,(2011) recorded (15/224) (6.75%) in camels (3-6month) of upper Egypt and El Moghazy *et al*.,(2014) reported the infection (11/19) (57.89%) in calves (<1) year from Benha in Egypt.

Ismeal *et al*.,(2014) recorded (67/173)(38.73%) in camels aged (>1) of Saudi Arabia .

Osman *et al*.,(2015) recorded (19/100)(19%)in camels age (1-9)years of Egypt by using both direct smear and indirect fluorescence

antibody technique (IFAT), Moezi *et al.*,(2016) recorded (150/310)(48.39%) camels >2 years in Iran.

Al-Amery *et al.*,(2017) recorded (25/90)(62.5%) in (<5) year camels in AL-Najaf of Iraq, which recorded infection in differ stage of animals ages.

This variations may ascribe to different reasons, including location, population density of animals, environment condition and hygienic measures. furthermore, time of study, the ecological , climatic factors and number of samples. Cases of young age might be under stress because they were weakness and emaciation and more susceptible to infection

4-1-4-*Anaplasma spp*

The results show that of the total 200 samples of camels blood examined microscopically found that (132)(66%) samples infected with *Anaplasma spp*. as in figure (4-4) .

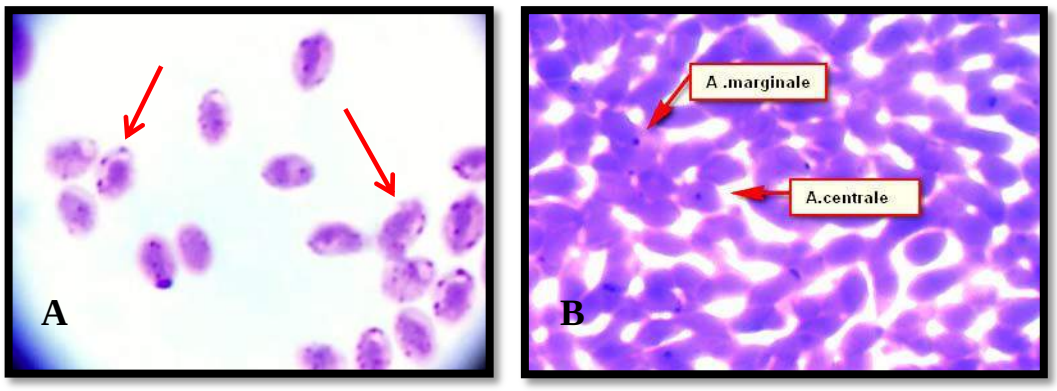


Figure 4-4

A- Show *Anaplasma spp*. inside camel red blood cells (thin smear) 100x

B- Show *Anaplasma spp*. inside camel red blood cells (thick smear) 100x

Many research recorded *Anaplasma spp*.infection in camels like Alsaad, (2009) recorded 11% from 62 Arabian one-humped camels from Mosul in Iraq and Ismeal *et al.*,(2016) recorded (72/96) (75%) from camels in Saudi Arabia.

According to the sex of animals, the number of infected females (95/125)(76%) and the number of infected males (37/75)(49.33%) of the total 200 samples. There was significant difference at $p \leq 0.05$ between males and females as in table (4-8).

Table 4-8- The prevalence of *Anaplasma spp.* infection in camel according to the microscopic examination and sex

Sex	No .of samples	*Anaplasma spp* infection	
		+	%
Males	75	37	49.33%^{B}
Females	125	95	76%^{A}
Total	200	132	66%

Different letter = significant difference at $p \leq 0.05$

The results of the present study are similar to the results recorded by Al- khalidy (2008) which recorded 52.29% from 114 male and 54.44% from 202 female cows of Al-Diwaniyah province in Iraq ,

But disagree with result of El-Naga and Barghash (2016) recorded (39/331) (11.8%) from camels in Egypt , these recorded infection in females more than males and Al-Amery *et al.*,(2017) recorded (26/90)(28.88%) in males camels in AL-Najaf of Iraq.

There is no difference between males and females because both sexes graze in the same field and the carrier of the same disease, habit and activity of the vector which live near the animals house. But the higher percentage of Anaplasmosis in females animals may be due to the true that contaminated needles and instruments are usually used for injection and given drugs in treated or in case decrease milk production .

According to the age of animals, the number of infected camels less than (<1)year (28/50)(56%) and the number of infected camels more than (>1)year (104/150)(69.33%) as in table (4-9).There are no

significant difference at $p \leq 0.05$. In this study show *Anaplasma spp* can infected camels in all age .

Table 4-9- The prevalence of *Anaplasma spp* infection in camel according to the microscopic examination and age

Animals age	No.	*Anaplasma* infection	
		+	%
< 1	50	28	56^{A}
> 1	150	104	69.33^{A}
Total	200	132	66

Similar letter = no significant difference at $p \leq 0.05$

The results of the present study are similar to the results recorded by El-Naga and Barghash (2016) recorded (105/152)(69.1%), (63/84) (75%) and (55/95) (57.9%) in ≤ 6 , 12 - 6 and > 12 years of camels in Egypt .

But disagree with result of Al- khalidy (2008),who did record 14(8%)in cows (1>) of Al-Diwaniyah province in Iraq and sajid *et al*., (2014) recorded (22/390)(5.64%)in aged (1>) cattle and buffalo in Pakistan.

While both of, Ait Hamou *et al*.,(2012) in cattle of North Central Morocco, Agaar and Jassem, (2014) recorded (13/79)(16.45%) in cattle (>2)years in Wassit province in Iraq, Al-Amery *et al*.,(2017) recorded (20/40)(50%) camels more than 7 years in AL-Najaf of Iraq. These recorded high infection in (>1) aged animals more than (<1)age .

This difference might be expounded by the more exposure of adults to tick vectors . Moreover, the calves are less susceptible to the infection because temporarily preserved (maternal antibodies) from the colostrum and mother's immunity, barring short-term protection.

4-1-5 -Mix infection

In this study, there were many cases of mix infection of blood parasites in camels as in table (4-10) and figure (4-5).

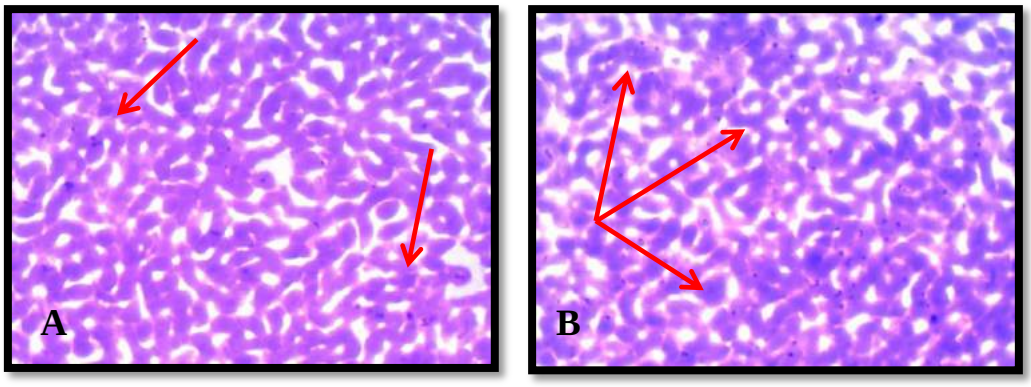


Figure 4-5

A- show mix between *Anaplasma* and *Babesia* (thick smear) X100

B-show mix between *Anaplasma* , *Babesia* and *Theileria* (heavy infection)

Table 4-10- show mix infection

Mix infection	Positive +	Percentage %
Trypanosoma &Anaplasma	6	7.5^{B}
Babesia &Theileria	19	23.75^{A}
Trypanosoma ,babesia & Theileria	7	8.75^{B}
Anaplasma ,Babesia & Theileria	15	18.75^{AB}
Trypanosoma ,babesia ,Theileria&Anaplasma	9	11.25^{AB}
Anaplasma & Babesia	16	20^{AB}
Theileria &Anaplasma	8	10^{AB}
Total	80	100

similar letter = no significant difference at $p \leq 0.05$

Different letter = significant difference at $p \leq 0.05$

The high percentage (23.75%) of mix infection between *Theileria* and *Babesia* the result similar with many researchers like Joshua *et al*.,(2008) recorded (11/38)(28.9%) between *T.evansi* and *Theileria camellensis* in camels of Nigeria, Swelum *et al* .,(2014) recorded

(6/258)(2.32%) mix infection between *Babesia* and *Theileria* and (10/258)(3.87) between *Trypannosoma* , *Babesia* and *Theileria* in camels of Saudi Arabia .

Faham *et al*.,(2015) recorded (48/98)(48.9%)mix infection between *Trypanosoma* , *Babesia* and *Theileria* in camel in Iran, Ismeal *et al*.,(2016) who recorded (5/72)(7%) mix infection between *Anaplasma* and *Theileria*, (3/72)(4.1%)*Anaplasma* and *Babesia* and (2/72)(2.77%) *Anaplasma* and *Trypannosoma* in camels in Saudi Arabia and Al-Amery *et al*.,(2017) recorded (53/160) (43.62%) between *Trypanosoma*, *Babesia*, *Theileria* and *Anaplasma* camels in AL-Najaf of Iraq.

The reason of occurrence mixed infections in the same animal probably trigger the low health condition of the affected animal, either through anemia caused by Trypanosomiasis, and Anaplasmosis or through effects due to interaction among different combinations of these diseases. In cases of mixed infections, stress creating from chronic diseases, malnutrition and the ability of *Trypanosoma* cause immunosuppression in the effected animals could lead to increased virulence of other blood parasites. Also stress resulting from chronic cases . In addition to exposure animals to blood sucking insects and ticks that transmission to blood parasites and also bad hygiene measures.

4-1-6- The prevalence of blood parasites according to the months of study:

The results of present study showed the higher rate in month October (46.1%) but the lower rate in month January (0.5%) in Al-Diwaniyah province, as in table (4-11)

Table (4-11) show the prevalence of blood parasites according to the months of study:

Month	No. of sample	Positive No.	Percentage(%)
September	**60**	**53**	**31.7^{A}**
October	**80**	**77**	**46.1^{A}**
November	**15**	**12**	**7^{A}**
December	**10**	**4**	**2.3^{B}**
January	**5**	**1**	**0.5^{C}**
February	**10**	**5**	**3^{B}**
March	**20**	**15**	**9^{A}**
Total	**200**	**167**	**83.5**

similar letter = no significant difference at $p \leq 0.05$

Different letter = significant difference at $p \leq 0.05$

The results of this study show the increase prevalence of blood parasites in the October and decrease in January with significant difference at $p \leq 0.05$ These results correspond to each of Borji *et al*.,(2009) recorded (7/100)(7%) and (6/60)(10%) in Spring and autumn of camels in Iran and also sajid *et al*.,(2014) recorded (9/209)(4.31%), (12/209)(5.74%) and (11/209)(5.26%) in spring ,Summer and autumn respectively in cattle and buffalo in Pakistan.

The distribution of the blood parasites diseases may be due to season of vectors and the climate condition that effect on distribution vectors especially hard ticks.

4-2-Result of Molecular Diagnosis by using Polymerase Chain Reaction (PCR) technique:

Out of 90 positive blood samples microscopically were examined by PCR technique to detect the prevalence of some blood parasites that can infect camels in the Al-Diwaniyah province and obtain on the result as table (4-13) . There are significant difference at $p \leq 0.05$

Table 4-12- show the diagnosis some blood parasites by PCR

Blood parasite	No. of positive by PCR	Percentage %
Trpanosoma spp	10	11.11%^{AB}
T. evansi	7	7.77%^{AB}
T .brucei	0	0%^{B}
Anaplasma marginale	14	15.55%^{A}
Anaplasma central	15	16.66%^{A}
Theileria spp	16	17.77%^{A}
Theileria annulata	9	10%^{AB}
Babesia bovis	8	8.9%^{AB}
Babesia bigemina	11	12.22%^{AB}
Total	90	100%

similar letter = no significant difference at $p \leq 0.05$

Different letter = significant difference at $p \leq 0.05$

4-2-1- Diagnosis *Trypanosoma spp* by PCR

In this study, 10 (11.11%) *Trypanosoma spp* ,7(7.77%) *Trypanosoma evansi* and 0(0%) *Trypanosoma bruci* were diagnosed in camel blood by using PCR technique as in table 4-12 and figure (4-6) , (4-7).

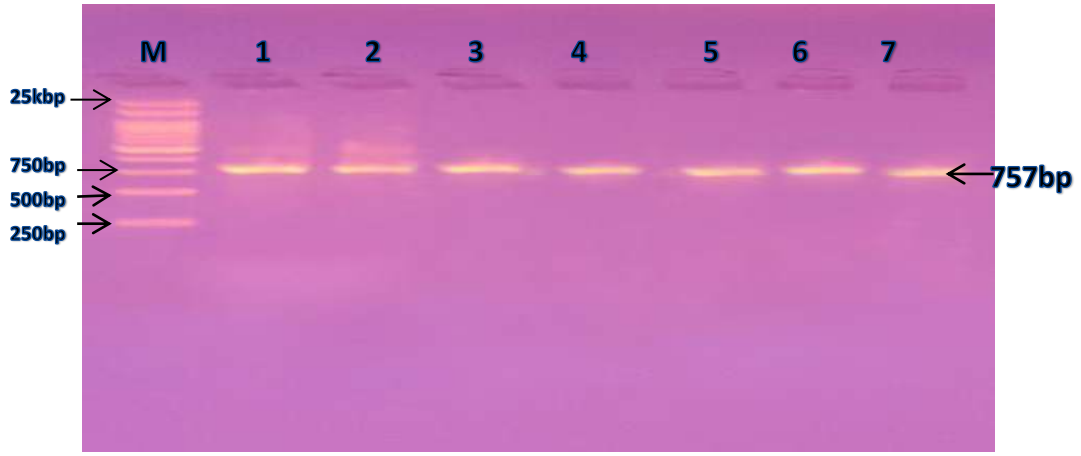


Figure (4-6): Agarose gel electrophoresis image that show PCR product that amplify fragment of small subunit ribosomal RNA gene in *Trypanosoma spp*. from camel blood samples. Where M: Marker (250bp-25kbp) and lane (1-7) some positive samples for *Trypanosoma spp*. at 757bp product size.

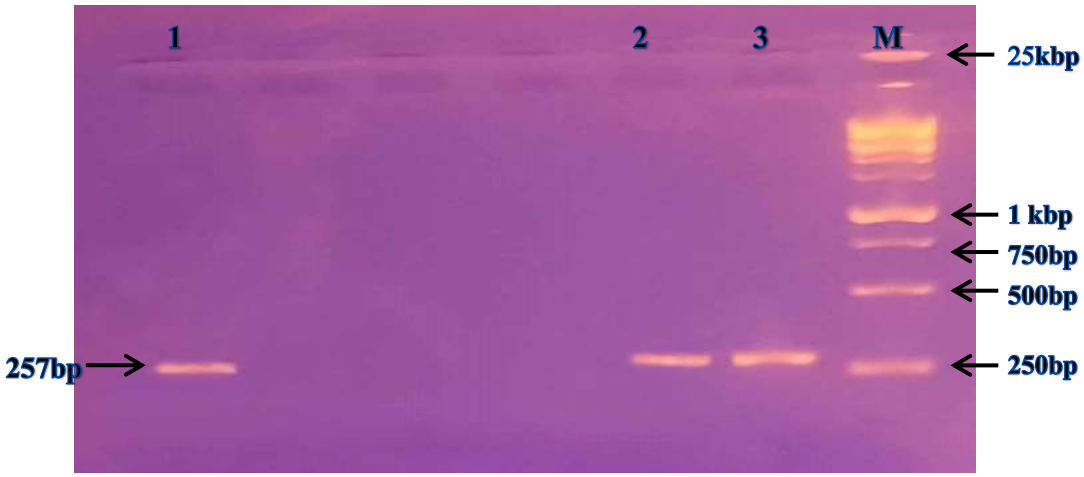


Figure (4-7): Agarose gel electrophoresis image that show PCR product that amplify fragment of small subunit ribosomal RNA gene in *Trypanosoma evansi* from camel blood samples. Where M: Marker (250bp-25kbp) and lane (1-3) some positive samples for *Trypanosoma evansi* at 257bp product size.

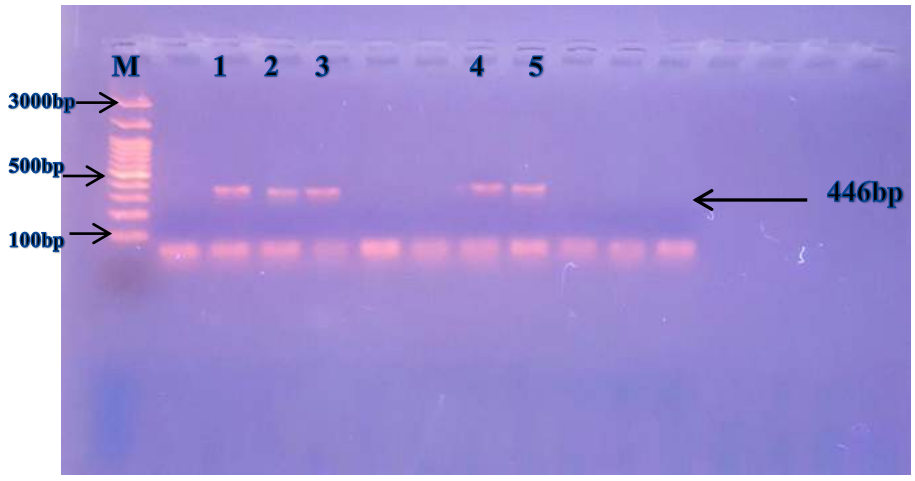
Many researchers diagnose *Trypanosoma spp* by PCR like Salim *et al*., (2011) recorded (117/205)(57.1%) *T. evansi* in camels in Sudan, Barghash *et al*.,(2014) diagnosed *T. evansi* 164(65.9%) and 186(74.7%) by PCR in camels in Egypt and Sawitri *et al*.,(2016) the result indicated that (33/44)(61.4%) samples were positive for *T. evansi* from blood cattle in Central Kalimantan PCR products of 470bp fragment . El-Naga and Barghash,(2016) recorded 194 (87.39%) *T. evansi*. but, disagree with him

because recorded 28 (12.61%) *T. brucei* in camels in Egypt while we didn't recorded *T. brucei* (0%).

The vitiation in percentages of researchers with our percentage can be explained by the following reasons, such as that the parasite *Trypanosoma* has the ability to change from the same itself by (VSGS) thus making it difficult to identify and may amount of parasite very little in size in sample compare with the researchers that the parasite was in part in the sample more than the presence in the sample quantity taken in this study. The genetic variation of *Trypanosoma* have a role in differenced the result between PCR and microscopic examination .

4-2-2- Diagnosis *Babesia spp* by PCR

In this study, 8 (8.9%) *Babesia bovis* and 11 (12.22%) *Babesia bigemina* were diagnosed in camel blood by using PCR technique as in table 4-12 and figure (4-8), (4-9).



Figure(4-8) : Agarose gel electrophoresis image shows PCR product that amplify fragment of small subunit ribosomal RNA gene in *Babesia bovis* from camel blood samples. Where M: Marker (100bp-3000bp) and line (1-5) some of positive samples for *Babesia bovis* at 446bp product size.

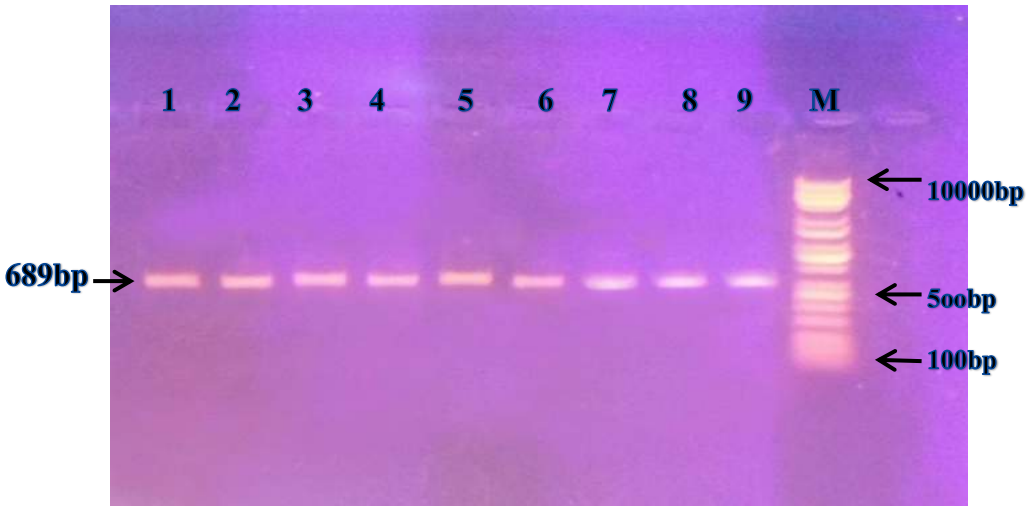


Figure (4-9): Agarose gel electrophoresis image shows PCR product that amplify fragment of small subunit ribosomal RNA gene in *Babesia bigemina* from camel blood samples. Where M: Marker(100bp-1000bp) and line (1-9)some of positive samples for *Babesia bigemina* at 689bp product size.

Many researchers diagnose *Babesia spp* by PCR like Qablan *et al*.,(2012) which recorded (6/100)(6%) *B. caballi* in camels of Jordanian dromedaries, Khamesipour *et al*.,(2015) recorded (8/122)(6.55%) *Babesia spp* in camels of Iran, Ibrahim *et al*.,(2016) recorded(24/34) (70.6) by PCR in camels in Sudan . Ganjali (2016) diagnosed *B. caballi* in camels in Iran, Jasim *et al*.,(2016) identified (39.47%) *B. caballi* in camels in south of Iraq, El-Naga and Barghash,(2016) recorded 36 (59.1%) *B. bovis* and 25 (40.9%) *B. bigemina* in camels in Egypt and Abdalla *et al*.,(2017)who recorded (96/176) (54.5%) *Babesia spp* in camel in Somalia.

The differentiations in percentages may be detected of *Babesia spp* which occur during the early stage of the disease and carrier animals cases usually difficult to determine the parasite because parasitemia is low . In addition to not all positive samples microscopically for *Babesia spp* diagnosed by PCR technique

4-2-3- Diagnosis *Theileria spp* by PCR

In this study, 16(17.77%) *Theileria spp* and 9 (10%) *Theileria annulata* were diagnosed in camel blood by using PCR technique as in table (4-12) and figure (4-10) .

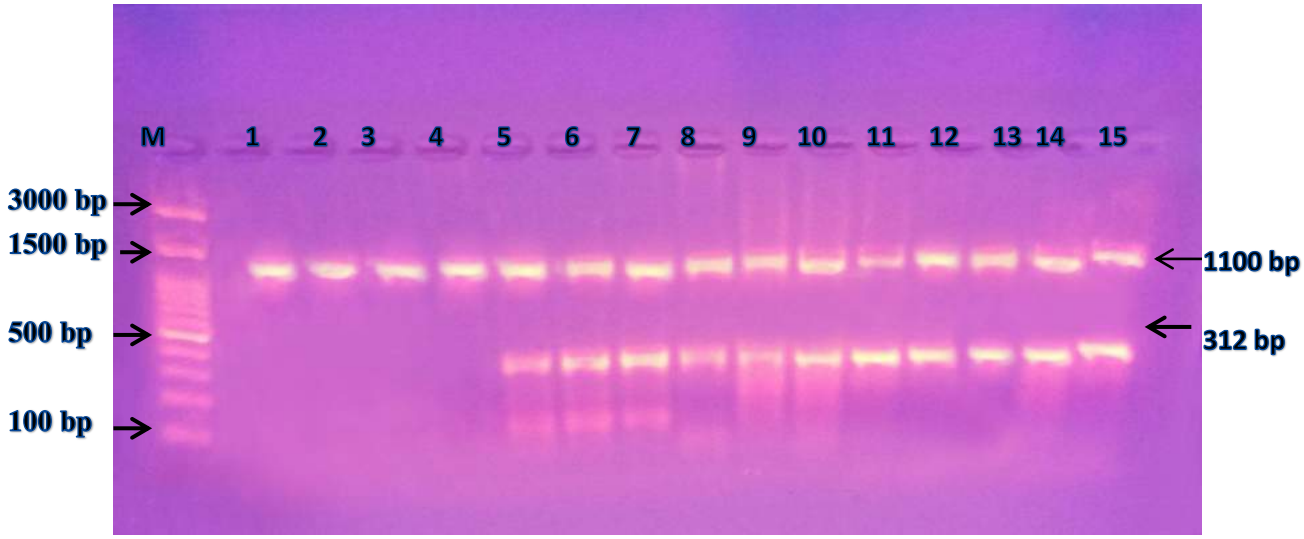


Figure (4-10): Agarose gel electrophoresis image shows PCR product that amplify fragment of small subunit ribosomal RNA gene in *Theileria spp* and *Theileria annulata* from camel blood samples. Where M: Marker (100bp-3000bp) and line (1-15)some of positive samples for *Theileria spp* at1100bp and *Theileria annulata* at 312bp product size.

Many researchers diagnose *Theileria spp* by PCR such as, Qablan *et al*.,(2012) which recorded (4/100)(4%) *T. equi* in camels of Jordanian dromedaries, Jasim *et al*.,(2016) identified (23.68%) *Theileria equi* in camels in south of Iraq, El-Naga and Barghash,(2016) recorded 238 (100%) *T. camelensis* but disagree with him because didn't record *T. annulata*(0.0%) in camels in Egypt.

Our results and the results of the researchers show that the possibility of infection of camels and other domestic animals more than species of *Theileria* , however, found a difference in the proportions of the results, it is due to primers , method and type of PCR Technique ,also

the product size of *T.annulata* is small camper with product size of *Theileria spp* therefore not appear in all line positive samples.

4-2-4- Diagnosis *Anaplasma spp* by PCR

In this study, 17(18.88%) *Anaplasma marginale* and 15(16.66%) *Anaplasma centrale* were diagnosed in camel blood by using PCR technique as in table (4-12) figure (4-11) and figure (4-12).

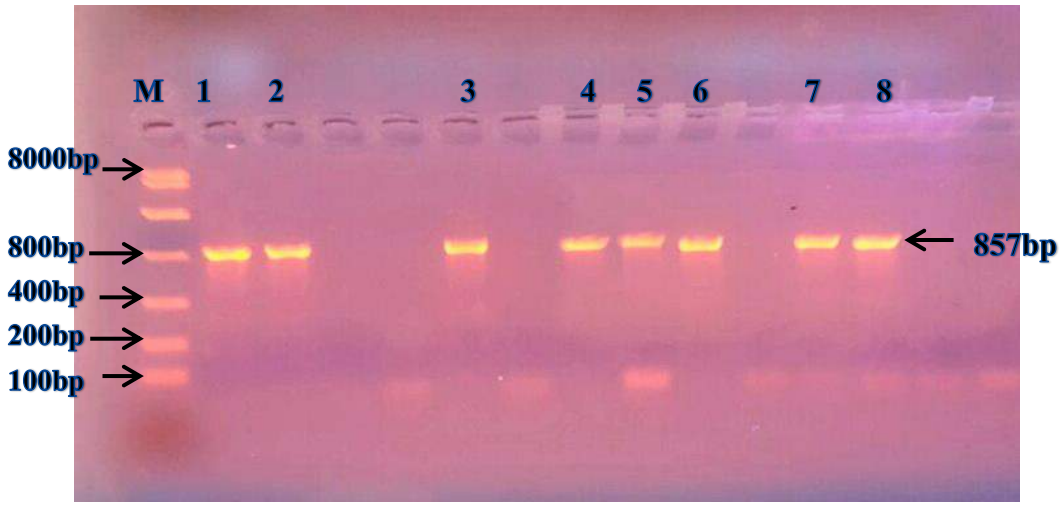


Figure (4-11): Agarose gel electrophoresis image shows PCR product that amplify fragment of small subunit ribosomal RNA gene in *Anaplasma marginale* from camel blood samples. Where M: Marker (100bp-8000bp) and line (1-8) some of positive samples for *Anaplasma margenale* at 857bp product size.

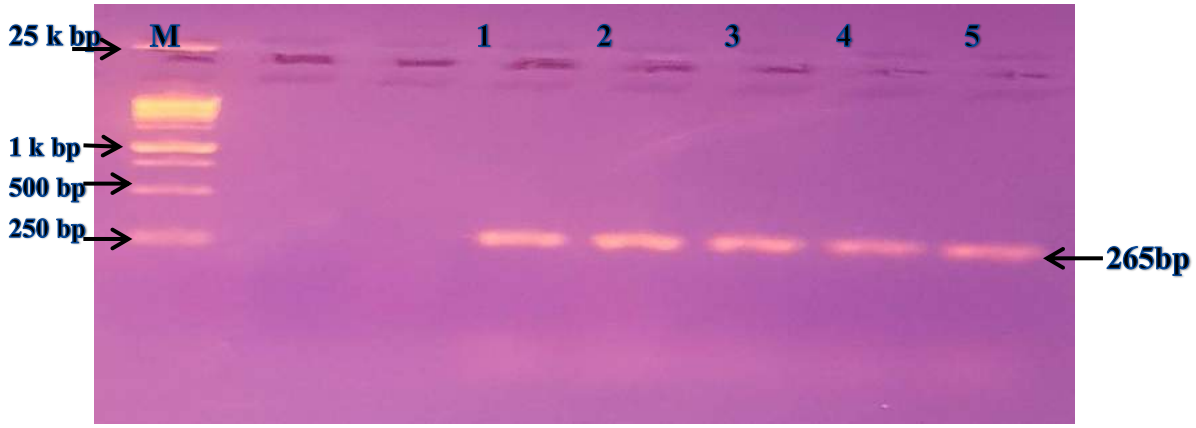


Figure (4-12): Agarose gel electrophoresis image shows PCR product that amplify fragment of small subunit ribosomal RNA gene in

Anaplasma centrale from camel blood samples. Where M: Marker (250bp-25kbp) and line (1-5) some of positive samples for *Anaplasma centrale* at 265bp product size.

Many researchers diagnose *Anaplasma spp* by PCR like El-Naga and Barghash,(2016) recorded 51(22.9%) *A. marginale* and 172 (77.13%) (*A. marginale* and *A. centrale*) in camels in Egypt and Abdalla *et al*.,(2017)who recorded (13.2%) *Anaplasma spp* in camel in Somalia.

The results of this study and the other studies show the prevalence of Anaplasmosis and the possibility of infection of the animal more than species of *Anaplasma* and were near relatively to the results of the microscopic examination, although not all samples of the positive microscopy examined molecularly by PCR technique. In addition to *Anaplasma* may be present in camels, cattle and sheep .

PCR method can be used for investigation on distribution of blood parasites in each of hosts and vectors because they are highly-accurate and specific. On the other hand ,The difference between the results of microscopy and molecular examination is due to the fact that in most cases chronic infections, the amount of DNA were low and *Trypanosoma* parasite has ability on genetic variation so the difference in results .

4-3- Results of DNA Sequencing

Twenty four purified PCR products samples were analyzed by the use of sequencing method in order to obtain the nucleotide sets of the (major surface protein 1b (SI3) gene) gene (265bp) to *A. marginale*, (ribosomal RNA gene) gene (857bp) to *A. central,* (glutamine-dependent carbamoyl phosphate synthase CPSII)gene (446-453bp) to *B. bovis*, (18ribosomal RNA gene)gene (689bp) to *Babesia bigemina*, (cytochrome b gene) gene (312bp) to *Theileria annulata*, (18ribosomal RNA gene) gene (1100bp) to *Theileria spp*, (18ribosomal RNA gene) gene (480-727bp) to

Trypanosoma spp and (VSG gene)gene (257bp) to *T. evansi* that isolated from camels of AL-Diwaniya province slaughterhouse. They were conducted as peaks and the set of nucleotides for each one of the strain with symbol code which represented the host ,region ,governorate and the country and recorded our strains as in table (4-13) .

Table (4-13): Summarized the sequencing of blood parasites by NCBI-Blast

Genes	Species	Date	Areas	Accession No.
major surface protein 1b (SI3)	*A. marginale*	4/JUL/2018	Al-Diwaniyah	MH551232 MH551233
ribosomal RNA gene	*A.centrale*	13/JUL/2018	Al-Diwaniyah	MH588232 MH588233
glutamine-dependent carbamoyl phosphate synthase CPSII	*B. bovis*	18/JUN/2018	Al-Diwaniyah	MH508091 MH508092 MH508093
18ribosomal RNA gene	*Theileria spp*	20/JUN/2018	Al-Diwaniyah	MH482935 MH482934 MH482936
Cytochrome b gene	*T annulata*	20/JUN/2018	Al-Diwaniyah	MH508088 MH508089 MH508090
18ribosomal RNA gene	*Trypanosoma spp*	11-JUL-2018 15-JUL-2018	Al-Diwaniyah	MH571705 MH595480
VSG gene	*T .evansi*	10/JUL/2018	Al-Diwaniyah	MH697863 MH697864

4-4- Result of phylogenic analysis

The multiple sequence alignment results are considered a very important step in the present study for phylogenetic analysis ,which clear the substitutions that occurred during evolutionary relationships between the different sequences sets . The alignment of multiple sequence can be

obtained by using (clustal W2) program online which is arranged three our many sequences sets each together in computationally manner .

4-4-1 The phylogenetic tree of *Trypanosoma spp*

The phylogenetic tree analysis was based on the (480-757bp) *Trypanosoma spp* 18ribosomal RNA gene and (257bp) *Trypanosoma evansi* VSG gene partial sequence that is used for *Trypanosoma* species typing detection so the phylogenetic tree was constructed by using using neighbor joining bootstrap 1000 radiation tree .

The results of analysis showed that the maximum homology of the nucleotide sequences between the local *Trypanosoma spp* isolated strains and Iraqi strain ranged from (99-100%) . while the maximum homology of the nucleotide sequences between the Iraqi *Trypanosoma spp* isolated strains and world strains ranged from (93-99 %).

Our strains(MH571705 *Trypanosoma spp* ZHA-1 small subunit ribosomal RNA gene partial sequence Iraq) was nearest from Indian strain while was forest from China strain as in figure (4-13).

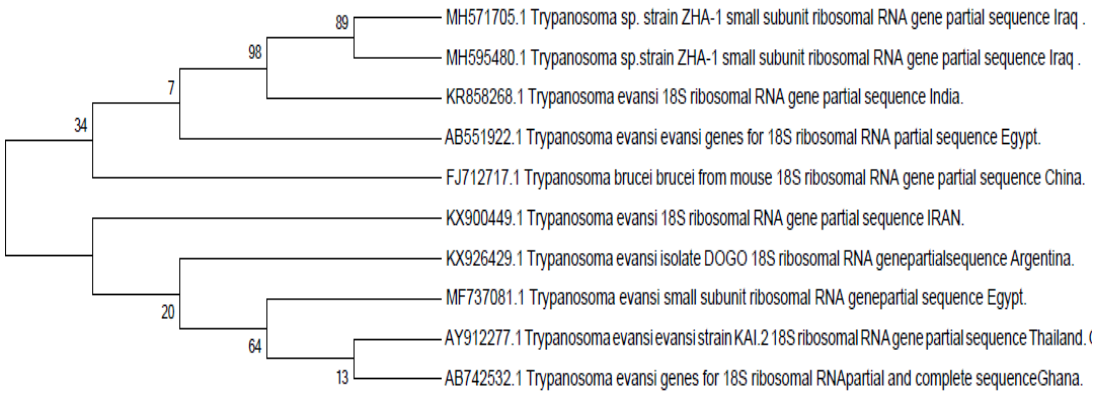


Figure 4-13 show the Phylogenetic tree of 18s rRNA of *Trypanosoma spp* partial sequence of local and global sequences using neighbor joining bootstrab 1000 radiation tree figure Evolutionary relationships .

4-4-2 The phylogenetic tree of *Theileria spp*

The phylogenetic tree analysis was based on the (1100bp) *Theileria spp* 18ribosomal RNA gene and (312bp) *Theileria annulata* cytochrome b gene partial sequence that is used for *Theileria spp* species typing detection so the phylogenetic tree was constructed by using neighbor joining bootstrap 1000 radiation tree.

The results of analysis showed that the maximum homology of the nucleotide sequences between the local *Theileria spp* isolated strains and Iraqi strain ranged from (99-100 %). while the maximum homology of the nucleotide sequences between the Iraqi *Theileria spp* isolated strains and world strains ranged from (93-99%).

Our strains(MH482936 *Theileria spp* small subunit ribosomal RNA gene partial sequence Iraq) was nearest from Indian strain while was forest from Brazilian strain as in figure (4-14).

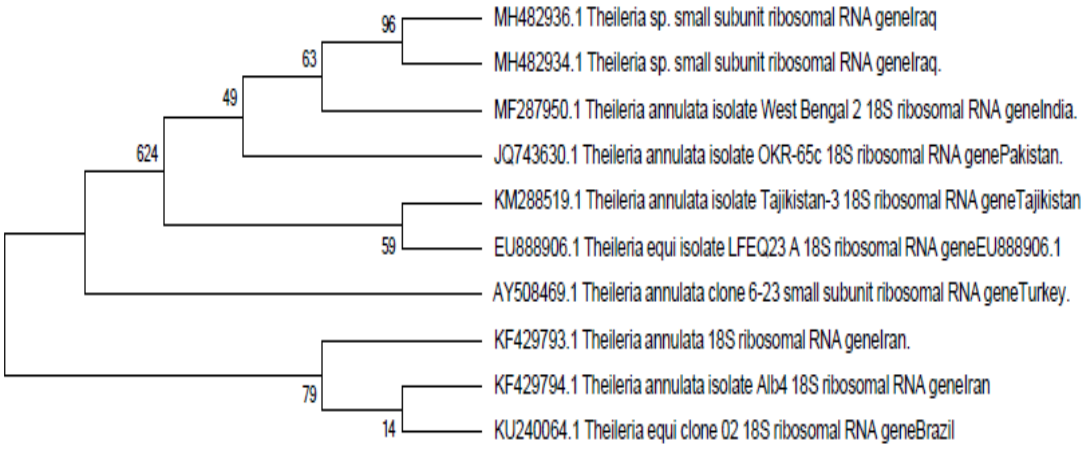


Figure 4-14 show the Phylogenetic tree of 18s rRNA of *Theileria spp* partial sequence of local and global sequences using neighbor joining bootstrab 1000 radiation tree figure Evolutionary relationships .

4-4-3 The phylogenetic tree of *Anaplasma spp*

The phylogenetic tree analysis was based on the (265bp) *Anaplasma marginale* major surface protein 1b(S13) gene and (857bp) *Anaplasma centrale* ribosomal RNA gene partial sequence that is used for *Anaplasma* species typing detection so the phylogenetic tree was constructed by using neighbor joining bootstrap 1000 radiation tree.

The results of analysis showed that the maximum homology of the nucleotide sequences between the local *Anaplasma spp* isolated strains and Iraqi strains ranged from (99-100%). while the maximum homology of the nucleotide sequences between Iraqi *Anaplasma spp* isolated strains and world strain ranged from (93-99 %).

Our strains(MH551232 *Anaplasma marginale* small subunit ribosomal 16srRNA gene partial sequence Iraq) was nearest from Taiwan strain while was forest from Thailand strain and Malaysian strain as in figure (4-15).

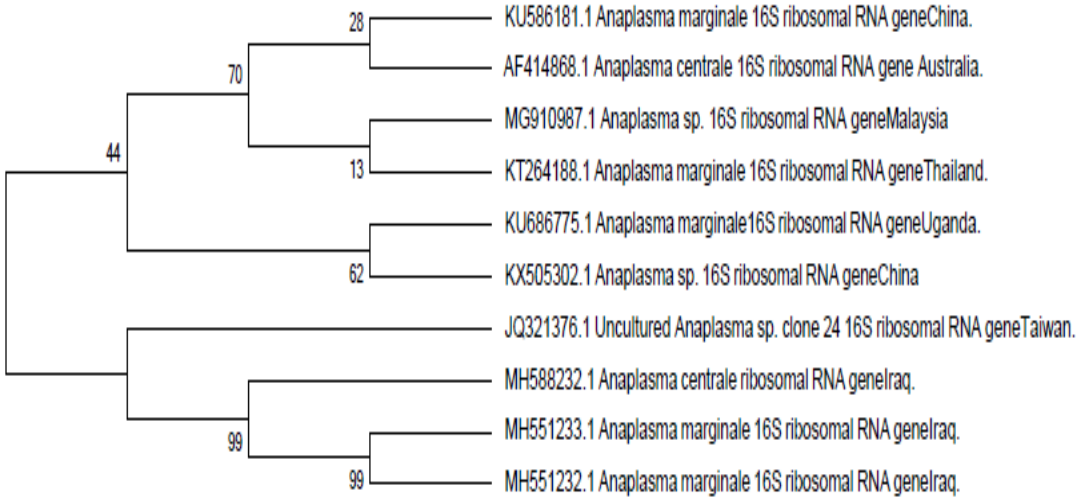


Figure 4-15 show the Phylogenetic tree of 16s rRNA of *Anaplasma spp* partial sequence of local and global sequences using neighbor joining bootstrab 1000 radiation tree figure Evolutionary relationships.

Chapter Five

Conclusion and Recommendations

Conclusions:

1. Depending on the results, showed that (*Trypanosoma spp ,Babesia spp , Theileria spp* and *Anaplasma spp*) were infected in camels in Al-Diwaniyah provinces according to Giemsa stain method and PCR technique.
2. Blood parasites can infect camels in all ages and both sex .
3. Parasitic blood infections increase in October according to time of study.
4. There are significant difference between sex, age.
5. There is a mixed infection for more than parasite in the same animals
6. Molecular and phylogenetic diagnostics are more accurate in determining the species of parasites

Recommendations

1. Using the PCR in a different study for detection of the parasite duo to it is faster, high sensitivity and specific method.
2. Using the phylogenetic study to detect the different parasites in camels in Iraq because it is high sensitivity in the determining of parasite strain and to any strain it belongs and knows the identity and similarity degree with the other world strain and this aids in determining the type of drug that can use against this parasite.
3. Following the scientific methods that would develop the production of livestock to raise the economic level of the country through the production of vaccines and the use of preventive treatments to prevent the spread of these diseases
4. For new research studies are directed to know if these parasites have the potential transmission to humans and study to detect the pathogenic effects on humans because farmers and owners are in continue contact with the animal

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والبابيزيا) و(الانابلازما والثليريا) ,(الانابلازما والبابيزيا) ,(البابيزيا ,الثليريا والانابلازما) و (التريبانوسوما ,الثليريا , البابيزيا والانابلازما)وبالنسب 6(7.5%) ,19(23.75%) ,7(8.75%) , 8(10%) ,16(20%) , 15(18.75%) و9 (11.25%) على التوالي .

وثانيا تشخيص الطفيليات الدموية في 90 عينة دم موجبة مجهريا باستخدام التقنيات الجزيئية والتي تضمنت تفاعل السلسلة البلمرة الاعتيادي (conventional PCR Monoplex and Multiplex) في مختبر الامراض المشتركة في كلية الطب البيطري/جامعة القادسية ومن ثم استخدام طريقة تحليل ترتيب النيوكليتيدات (sequencing analysis) وكذلك تم تحديد العلاقات الوراثية التطورية (التحليل الفيلوجيني) (phylogenetic analysis). وكانت النتائج كالاتي *Trpanosoma spp , T. evansi , Anaplasma marginale* , , *Babesia bovis, Theileria annulata , Theileria spp Anaplasma central* و *Babesia bigemina* وبالنسب التالية 10(11% .) ,7(7.77%),14(15.55%), 15(16.66%),16(17.77%),9(10%),8(8.9%) و11(12.22%) على التوالي .

وتم تسجيل العتر التالية في بنك الجينات الدولي 2 *A. marginale* , 3 *T annulata* , 3 *B. bovis* , 2 *T. evansi* , 2 *Typanosoma spp, A.centrale* , 3 *Theileria spp,* وارقام التسجيل (MH551232 , MH508088, MH508091, MH588232, MH571705, MH697863, MH482935) على التوالي . .ثم قراءة شجرة النشوء والتطور الوراثي لهذه الطفيليات والمقارنة فيما بينها التي تصيب الإبل.

الخلاصـــة

نظرا للأهمية الاقتصادية للابل وبسبب قلة الدراسات التي تتعلق بها في العراق هدفت الدراسة للتحري عن بعض طفيليات الدم والتي شملت *Trypanosoma spp* التريبانوسوما , *Babesia spp* البابيزيا *Theileria spp*, الثليريا و*Anaplasma spp* الانابلازما بالطرق الجزيئية .

أجريت هذه الدراسة خلال الفترة الممتدة من شهر أيلول 2017 ولغاية شهر اذار 2018 تم جمع 200 عينة دم عشوائية من الابل (125) اناث و(75) ذكور وبمجموعتين من الاعمار(150)عينه اكبر من سنه و(50) عينه اصغر من سنه التي ظهرت عليها علامات سريرية ومنها لم تظهر عليها اي اعراض من مجزرة محافظة الديوانية .

صممت هذه الدراسة لتشخيص هذه الطفيليات اولا باستخدام صبغة كمزة(Geimesa stain) وتم الفحص في مختبر الطفيليات في كلية الطب البيطري/جامعة القادسية وكانت النتائج 167 عينة موجبه من مجموع 200 عينه وبنسبة (83.5%).وتم تشخيص 76(38%) تريبانوسوما ,106(53%) بابيزيا, 93(46.5%) ثيليريا و 132(66%) انابلازما .

وبينت الدراسة بأن أعلى نسب للإصابة *Trypanosoma spp* ظهرت في الاناث(57/125) (45.6%) اما الذكور(75/ 19) (25.3 %) وبمختلف الاعمار.

وسجلت اعلى نسبة اصابة *Babesia spp* في الاناث عما عنه في الذكور (125/ 76)((60.8%), (30/75)(40%) وبمختلف الاعمار .

Theileria spp الثيليريا ايضا سجلت اعلى نسبة اصابة في الاناث عما عنه في الذكور (125 69) (55.2%) (75 24)(32%) وبمختلف الاعمار .

وكذلك *Anaplasma spp* الانابلازما سجلت اعلى نسبة اصابة في الاناث عما عنه في الذكور (75 37)(49.33%) و (125 95)(76%) وبمختلف الاعمار .

سجلت اعلى نسبة اصابه في الاشهر (ايلول ,تشرين الاول واذار) (88.3% , 96.2% و75%) على التوالي حسب وقت الدراسة .

وتبين لنا من خلال النتائج انه من الممكن اصابة الحيوان بأكثر من طفيلي حيث سجلت 80 حالة اصابة مشتركة من 167عينه موجبة مثل (التريبانوسوما و الانابلازما) (البابيزيا والثليريا), (التريبانوسوما ,الثيليريا

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
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كلية الطب البيطري

دراسة جزيئية لبعض طفيليات الدم في الجمال في محافظة الديوانية

رسـالة مقدمة إلى

مجلس كلية الطب البيطري في جامعة القادسية وهي جزء من متطلبات نيل درجة الماجستير في علوم الطب البيطري/ الطفيليات

من قبل

زينب حسين جياد النائلي

بكالوريوس طب وجراحة بيطرية عامة 2008
دبلوم عالي طب باطني ووقائي 2014

بإشراف

أ. د. غيداء عباس جاسم

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