



Clinical and Molecular detection of *Theileria annulata* isolated from calves and ticks in Iraq

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Abstract

Bovine theileriosis is an important tick-borne disease caused by intraerythrocytic parasites from genus *Theileria*. *Theileria annulata* is the causative agent of tropical theileriosis or Mediterranean theileriosis. This study directed to diagnose theileriosis among calves and ticks in Al-Qasim city, Iraq. Seventy blood samples from seventy calves and seventy ticks were collected in April-August of 2022. These animals were a local breed with clinical signs of theileriosis; lymph node enlargement, fever (40c-41c), paley mucus membrane, loss of appetite; living in different areas of Al-Qasim city. The blood smear was prepared and stained with Giemsa for microscopic examination of *T.annulata*. Polymerase chain reaction (PCR) assay was performed to detect *T.annulata* using primer pairs targeted to 18S ribosomal RNA gene. The molecular assays revealed that seventy calves were infected as well as the samples of ticks.

Keywords: Blood Parasite; Tick, 18S rRNA, Molecular, Theileria.

Introduction

Theileriosis is a devastating protozoan disease that has impacted cattle and buffalo all over the world. Theileriosis is known to be caused by *Theileria* species (spp.). Nine *Theileria* species have so far been identified in dairy cattle (1). Four types of *Theileria* are geographically distributed in Sub-Saharan Africa (*Theileria parva*, *Theileria mutans*, *Theileria taurotragi*, and *Theileria velifera*), and the other three of which (*Theileria annulata*, *Theileria orientalis*, and *Theileria buffeli*), are found in Australia, Asia, North America, and Northern Africa. While other *Theileria* species are either non-pathogenic or slightly harmful to cattle, *T. parva* and *T. annulata* are considered to be extremely pathogenic (2).

Many of the parasites in this group were historically categorized according to their morphology, the presence of schizogony in the host cells, the presence of piroplasms in the red blood cells that are linked to disease manifestation, and host-vector specialization (3). *Theileria parva*, *Theileria annulata*, *Theileria mutans*, and *Theileria velifera* have all been identified as the causal organisms of bovine theileriosis (4).

One of the most important livestock diseases in Asia and North Africa is tropical theileriosis, which caused by *Theileria annulata* and transmitted by Hyalomma ticks. The sickness affects mammalian hosts' mononuclear cells and is very pathogenic (5).

Theileria parva and *Theileria annulata* are the pathogen species mainly responsible for theileriosis disease, which is categorized as lympho-proliferative and has a high morbidity and mortality rate, among the *Theileria* species, according to (7). *Theileria annulata* is the tropical theileriosis causing agent, widespread in tropical and subtropical regions (8).

The tick's saliva was used to inject the sporozoites that were created throughout the cyclical development of the tick into the mammalian host (9). They first transform into schizonts in white blood cells before becoming piroplasms (merozoites) in red blood cells. *Theileria annulata*, an intracellular protozoan parasite causes bovine theileriosis (10).

Hyalomma; tick species responsible of spreading *Theileria annulata*; a disease that is widespread in North Africa, India, the Middle East, and Central Asia (11). The genera



Rhipicephalus, Hyalomma, Amblyomma, and Haemaphysalis of Ixodid ticks are known to transmit *Theileria* spp. in tropical and subtropical areas of the world, tick-borne diseases significantly affect domestic cattle management and health (12). *Theileria*, an *Apicomplexan* protozoan parasite that causes the disease, affects millions of cattle throughout Africa (1).

Frequently observed clinical symptoms of tropical theileriosis are high temperature, weight loss, weakness, and loss of appetite, along with enlarged lymph nodes, icterus, conjunctiva petechial, and anemia. Particularly in the advanced stages of the illness, some animals manifested diarrhea and dysentery (13). The turning sickness, which causes neurologic symptoms by blocking capillaries in the central nervous system caused by infected cells, may also have been present in bovine calves (14).

Thin blood smears were taken from the ear marginal vein to see piroplasm forms, and lymph node smears were taken to observe macroschizont (also known as Koch's blue body) stage. Both samples were stained with 5% Giemsa in buffers at (pH 7.2) for 40 min, and the results were then studied under a 1000x microscope (15). Giemsa-stained lymph node biopsy smears are typically used as the basis for diagnosis of clinical *Theileria annulata* infection in cattle. Low numbers of erythrocytes remain infected with *Theileria* piroplasms in a long-lasting carrier state upon recovery (16). Due to its better sensitivity and specificity compared to other procedures.

Polymerase Chain Reaction (PCR) has been described as best diagnostic assay for the identification of *T. annulata* (17). The most accurate test for identifying subclinical carriers is PCR. In comparison to microscopic examination and serological tests, it is the most sensitive and specific test for theileriosis diagnosis (18). For the detection and quantification of numerous infectious pathogens, molecular tools have been created, and they have proven to be extremely precise and sensitive. Due to its importance in the fields in Iraq as shown by (19); this study has

been designed for the detection of theileriosis in calves less than six years old with clinical examination, blood smear and molecular diagnosis. Suspected calves in Al-Qasim city were the target of this work.

Material and Methods

Ethical Standards statements:

University of Al-Qadisiyah /Iraq/ certifies the ethical approval (IRAS0822019).

Sample

The study was planned to investigate the presence of *Theileria* in calves and the ticks picked up on them in order to find out its epidemiology, molecular features, resistance to drugs and open doors for other projects.

Clinical examination

Clinically calves examined by tacking case history and clinical signs which showed on infected calves include lymph node enlargement, fever(40c-41c), pale mucus membrane, loss of appetite, urine is yellow in color and data including the age of animal are recorded and calves, selected in different age from two to six months and breed. In regard to gender, fifty five males and fifteen females all these were infected.

Microscopic detection

After preparation of thin blood smears on glass slides the slides were dried and fixed by methanol for about five minutes, after that stained by 10% Giemsa stain for 30 minutes and examined under Oil immersion lens (100 x magnifications). (21)

Blood Samples

A total of seventy EDTA whole blood samples and ticks suspected to piroplasmosis were obtained from calves in Iraq, Babylon AL- Qasim city, during April-August 2022. All the blood samples obtained from the jugular vein and transported to the laboratory to use later in the study.

Tick samples

Tick also collected from the same infected calves, which directly sprayed with ethanol and by tongs put in beaker and transported to the laboratory to make DNA extraction for PCR. The DNA stored at -20°C until they are used in the study (19). The entire sample collected during April-August in 2022



from variably areas in Babylon, AL-Qasim city.

Molecular method

DNA extraction:

The DNA was extracted from EDTA blood tubes by DNA isolation kit (Genomic DNA mini kit, blood /cultured, Korea). After that, extracted DNAs were kept at -20c till the use in PCR. (19).

Primer Design

The researchers designed two sets of primers and utilized them in the PCR, for specific detection of *T.annulata*; a common 18S rRNA primer pair was also designed to diagnose *Theileria annulata*; F/TAATTTGACTCAACACG and R/ATCACAGACCTGTTATTGCC. 18S rRNA primers produce 256-260bp fragment lengths on *Theileria* and *Babesia* positive samples (22). All the primers were designed using Gene Runner and Oligo 7 software and checked for complementarity and primer dimers. The specificity of primers was checked by the Basic Local Alignment Search Toll (BLAST) of the national center for biotechnology information (NCBI) before primer synthesis.

Polymerase Chain Reactions (PCR)

PCR was set out as follow: 12.5µl of master mix ,8.5µl of DEPC water, 1µl of each primer, 2µl of DNA template then nuclease free water were add until the reaction reach to 25 µl . The PCR conditions were performed as in the table (1).

Table 1: PCR conditions.

| PCR step | °C | Time | Repeat |
|-----------------------------|----|--------|--------|
| Initial denaturation | 95 | 3 min | 1 |
| Denaturation | 95 | 35 sec | 39 |
| Annealing | 51 | 35 sec | |
| Extension | 72 | 35 sec | |

| | | | |
|------------------------|----|-------|---|
| Final extension | 72 | 5 min | 1 |
|------------------------|----|-------|---|

Results

Sampling

More than 100 calves less than six months old suspected to be infected with ECF were referred to the clinic. Seventy blood samples and seventy ticks randomly selected from different part of heat area; Babylon, AL-Qasim city during the seasonal tick activity from April to August months in 2022. All the animals selected in this study were positive according to the clinical examination confirmed by the blood smear and the molecular detection.

Clinical examination

All the infected animals suffered from fever (40c-41c), enlargement of superficial lymph nodes, anorexia, tachycardia, dyspnea and icterus pale mucus membrane, loss of weight and wasting. Also; presence of ticks on the infected animals was significant because it is the transmitting host.

Microscopic Examination of Blood Films

Under light microscope, the found of piroplasms was appraisal by the observation of seventy blood samples from calves in microscopic fields which confirm the infection with *theileria*.

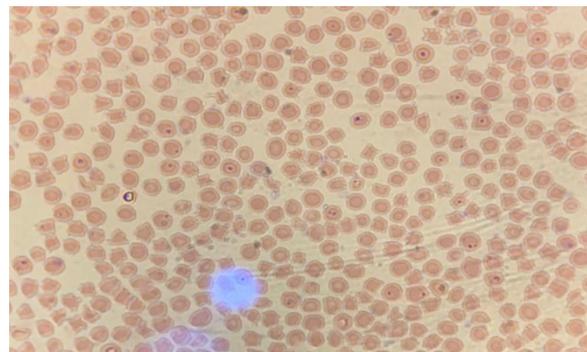


Figure 1: Giemsa stain usage. Piroplasm of *Theileria annulata* in red blood cells (RBC), under the oil emanation 100X microscope

PCR detection

Seventy blood samples which were positive in microscopic examination were

positive in polymerase chain reaction (PCR). The samples used in molecular diagnosis, showed the positive results; as in figure (2); (C) was control negative, in which water

(H₂O) was added instead of DNA. Lane (M): ladder 100pb. Lane (1-17) showed the positive results of infected calves.



Figure 2. Gelelectrophoresis image (1.7 %); showed positive (+ve) samples (lane 1- 17) of *Theileria annulata* in cattle host (PCR amplicon size; 250bp). C was control negative (-ve), DNA was replaced with water (H₂O). The molecular marker is M. (Favor prep, KOREA).

Detection of *T.annulata* by 18s rRNA:

Using 18s rRNA gene with 250bp; sixty three samples were positive and the other were negative. Fragment to detection of *Theileria annulata* in tick samples by PCR test showed positive samples (lane 1-17 except 5,

9 and 16 which were negative) of *Theileria annulata* in tick vector with the same size 250 bp. © was control negative (-ve) in which water (H₂O) was added instead of DNA as shown in the Figure (3).



Figure 3: Gel electrophoresis image (1.7 %) showed positive samples (lane 1-17 except 5 which was negative) of *Theileria annulata* in tick vector (PCR amplicon size = 250 bp). C was control negative in which H₂O was added instead of DNA. M is molecular marker (Favor prep, Korea).

Discussion

The clinical signs of acute tropical theileriosis were evident in calves in this



investigation. The study was the first in AL-Qasim city in Babylon government of Iraq. (23) had found the same clinical signs. Tropical theileriosis is an endemic disease in Iraq with typical form and easily diagnosed from clinical signs simultaneously with blood smear.

Theileria infection can be diagnosed through microscopic examination of a thin blood smear, as shown in figure (4-1). According to (18) and (24), the stained blood smears contained intra-erythrocytic stage of *Theileria annulata*. However, the physical resemblance of piroplasms, false-negative diagnoses as a result of the parasite's scarcity (particularly in carrier animals), and complications in detecting mixed infections have some difficulty with microscopic identification (25). In the current study seventy blood samples were used for examination which showed positive result.

Use of the 18srRNA gene in the diagnosis of *Theileria annulata* in both calves and ticks by PCR showed in figure (4-2) calves and figure (4-3) ticks was although recommended by (26), it is highly concerned for the majority of their phylogenetic analysis on *Theileria annulata* studies.

Current study showed that using the positive result from the 18srRNA gene of *Theileria annulata* and cytochrome b gene to diagnose the resistant gene of *Theileria annulata* in calves and ticks was compared with results in Tunisia by (11), who found sensitive and resistant strains as indicated in figure (4-7) in calves and figure (4-8) in ticks.

Conclusion

Based on the clinical symptoms; diagnosis of *T.annulata* in different area of AL-Qasim city was easy followed by microscopic detection on blood film. Detection of *Theileria* in both calves and ticks using the 18s rRNA gene is major and recommended for more studies. *Theileria* is the most important blood parasite affecting calves with horrible losses.

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Conflict of interest

The authors declare no conflict of interest

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