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Epidemiological and molecular study of Theileriosis in cattle in Al- Muthanna province- Iraq

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Abstract
The present study was carried out to investigate the prevalence of Theileriosis in cattle in AL-Muthanna province - Iraq during the period from 1st November 2016 to 30th April 2017. The study was accomplished on 204 blood samples collected from 133 females and 71 males, from different regions in the province. A blood smears were perpetrated and stained utilizing Giemsa stain, and examined by microscopically. Then polymerase chain reaction (PCR) technique was conducted to accurate detection and determination species of *Theileria*. The results of microscopic examination revealed that 13.23% of blood samples were infected with *Theileria* spp. The most sensitive and accurate method utilized for the diagnosis of Theileriosis was PCR technique, DNA extraction was performed only on 100 blood samples, the extracted DNA from blood samples were analyzed using primers derived from TAMS1 gene. The result of PCR technique revealed that (33/100) blood samples were positive for *Theileria annulata*. Also phylogenetic analysis of *Theileria annulata* local isolates based on Tams1 gene, two local isolates (MIQ1, MIQ1) sent to Korea (Bioneer company) to analysis and compared with the NCBI-Genbank *Theileria annulata* isolates.

Keywords: Microscopic examination, Molecular diagnosis, *Theileria annulata*, cattle

1. Introduction
Cattle are raised as livestock for beef and veal meat, and dairy animals for milk and other dairy products, Theileriosis is a tick borne parasitic disease that caused by hemoprotozoan parasites belong the genus *Theileria* (T.), which generally infect ruminants especially cattle (both wild and local Bovidae throughout many of the world countries) in tropical and subtropical regions [1, 2]. *Theileria* is oblige intracellular protozoan parasites transmitted by ticks and have complex life cycles in vertebrate and invertebrate hosts together [3, 4]. The diagnosis of tropical theileriosis is mainly base on clinical signs and confirmed by microscopic examination of Giemsa stained blood or lymph node smears for detect of piroplasms in erythrocyte and macroschizonts in lymphocytes [5].In addition, serological tests can be utilized to detect circulating antibodies of *Theileria annulata* by using either piroplasms or schizonts as the antigen [6]. Molecular methods have been developed to accurate and rapid identification of Theileria spp. in animals having negative serological test that can still infect ticks [7, 8]. Iraq is one of many countries complaining from Tropical Theileriosis and this disease is represented of the really challenge, many epidemiological studies was performed and indicated that the disease is endemic in many provinces such as, Kurdistan, Mosul, Diyala, Baghdad, Hilla, Qadsiya and Basra [9-15]. And because of unavailable of any epidemiological data about bovine theileriosis in southern part of Iraq especially in Al-Muthanna province. The present study was aimed to investigate the prevalence of theileriosis infection in cattle in Al- Muthanna Province- Iraq utilizing conventional and molecular methods, investigate effect of age and sex on infection rate, phylogenetic analysis of *Theileria annulata* local isolates.

2. Materials and Methods
A total number of 204 local and cross breed cattle from both sex were selected randomly and examined for theileriosis, these animals include fourth age groups: the first one, from 10 day to 6 months, the second above 6 months to 1 year, the third above 1 year to 2 years, and the fourth above 2 years. Investigate the prevalence of Theileriosis in cattle in AL-Muthanna province - Iraq during the period from 1st November 2016 to 30th April 2017.

2.1. Samples collection
Blood samples were collected from each animal examined for infection with *Thieleria* spp. One from ear sublime vein by puncturing after hair trimming and shaving and disinfecting the ear with 70% alcohol for making blood smear [16]. blood samples (2.5) ml were taken from the jugular vein from 100 cattle after sterilizing the area with 70% alcohol using disposable syringes size (5) ml, the blood was collected with plastic EDTA tubes and stirred to prevent blood clotting then transferred in cooler box to the laboratory of parasitology in Veterinary Medicine College in Al-Qadsiya university and freezing at -20 °C until used for DNA Extraction [17, 18].

2.2. Smear preparation
Primarily, a thin layer smear was prepared from ear sublime vein blood from 204 native cattle randomly selected and was fixed with methanol and stained with Giemsa dye [19]. Giemsa-stained blood smears were examined for the presence of parasites; at least 50 microscopical areas were carefully examined for *Theileria* piroplasms under the oil immersion lens. The presence of even a single piroplasm was considered positive.

2.3. DNA isolation, PCR amplification, and sequencing
Theileria annulata piroplasm DNA was purified from bovine blood with less than 0.5%. parasitemia. Genomic DNA extracted with a gSYNC™ genomic DNA extraction kit (GENEAID, USA) and (BIONEER, Korea) AccuPower™ kit for PCR Amplification. Aliquots of extracted DNA were kept at -20 °C. PCR was performed using one set of primers derived from Tams1 gene (5'-AACGTCGACCCTAACACCAC-3' *T. annulata* specific forward primer, and 5'-CTGCCTGTGACATTTGCACC-3',*T. annulata* specific reverse primer and amplicon 559 bp) in a final reaction volume 20 µl contain 10 µl of (KCl, Tris–HCl pH 9.0, $MgCl_2$, Stabilizer and Tracking dye, deoxyNucleoside TriPhosphates dNTPs, Taq polymerase) and 2µl of DNA template and 1.5µl of Forward primer (10pmol) and 1.5µl of Reveres primer (10pmol) and complete the final volume with PCR water [20]. The reactions were performed in an automatic DNA thermocycler (THECHNE, USA) for 30 cycles. Each cycle consisted of a denaturing step of 30sec. at 95 °C, and annealing step of 30sec. at 58 °C and 1 min of extension step at 72 °C, according to AccuPower® kit, Bioneer company korea.

2.4. Statistical analysis
The data succumbed to Statistical Analysis System (SAS) [21]. The program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentages in this study.

3. Results
Out of 204 smears examined microscopically, 27 (13.23%) were positive for piroplasmic forms of *Theileria annulata*, whereas 33 of 100 (33%) cattle were positive by PCR method. The 559 bp fragment was generated in all samples that were positive by blood smears (Fig. 1). The study showed that all age groups were infected with *Theileria parasite* with variable rates. A significant differences $P<0.01$ was recorded between old and young age groups Table 1.The present study demonstrated that the infection rate in females was 15.03% (20/133) more than in males 9.80% (7/71) without significant differences ($P>0.01$) (Table 2).

Table 1: Infection rate with *Theileria spp.* in cattle according to age.'

Age	Number of samples examined	Positive	Infection rate (%)
10 D – 6 M	46	1	2.17
> 6 M –1 Y	52	4	7.69
> 1 – 2 Y	48	10	20.83
> 2 Y	58	12	20.68
Total	204	27	13.23
x^2	---	---	6.192 **

D: Day, M: Month, Y: Year, x^2: Chi-Square, ** (P<0.01).

Table 2: Infection rate with *Theileria spp.* in cattle according to sex.

Sex	Number of samples examined	Positive	Infection rate (%)
Males	71	7	9.8
Females	133	20	15.03
Total	204	27	13.23
X^2	---	---	2.983 NS

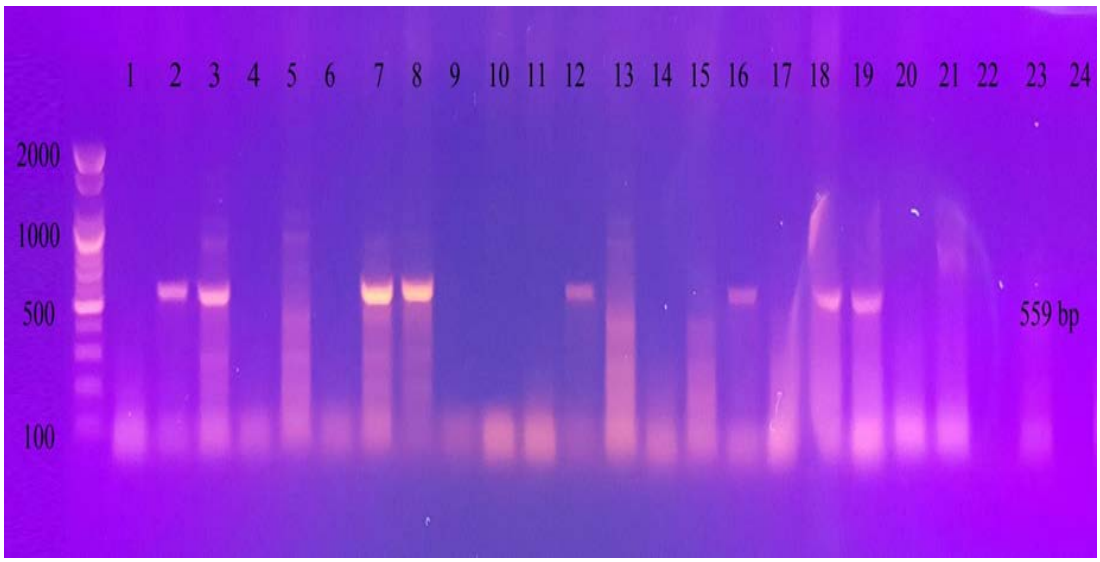

Fig 1: Agarose gel electrophoresis image shows the PCR product analysis of TAM1 gene of *Theileria annulata* in blood samples of cattle.Lanes 2,3,7,8, 12, 16, 18, and19 were positive samples (559bp). Lanes 1,4,5,6,9,10,11,13,14, 15, 17, 20,21,22,23, and 24 were negative samples, 100-bp ladder.

3.1. Phylogenetic analysis

Two *Theileria annulata* local isolates (MIQ1, MIQ1) were taken and sent to Bioneer Company South Korea for analysis and compared with the NCBI-Genbank *Theileria annulata* isolates.

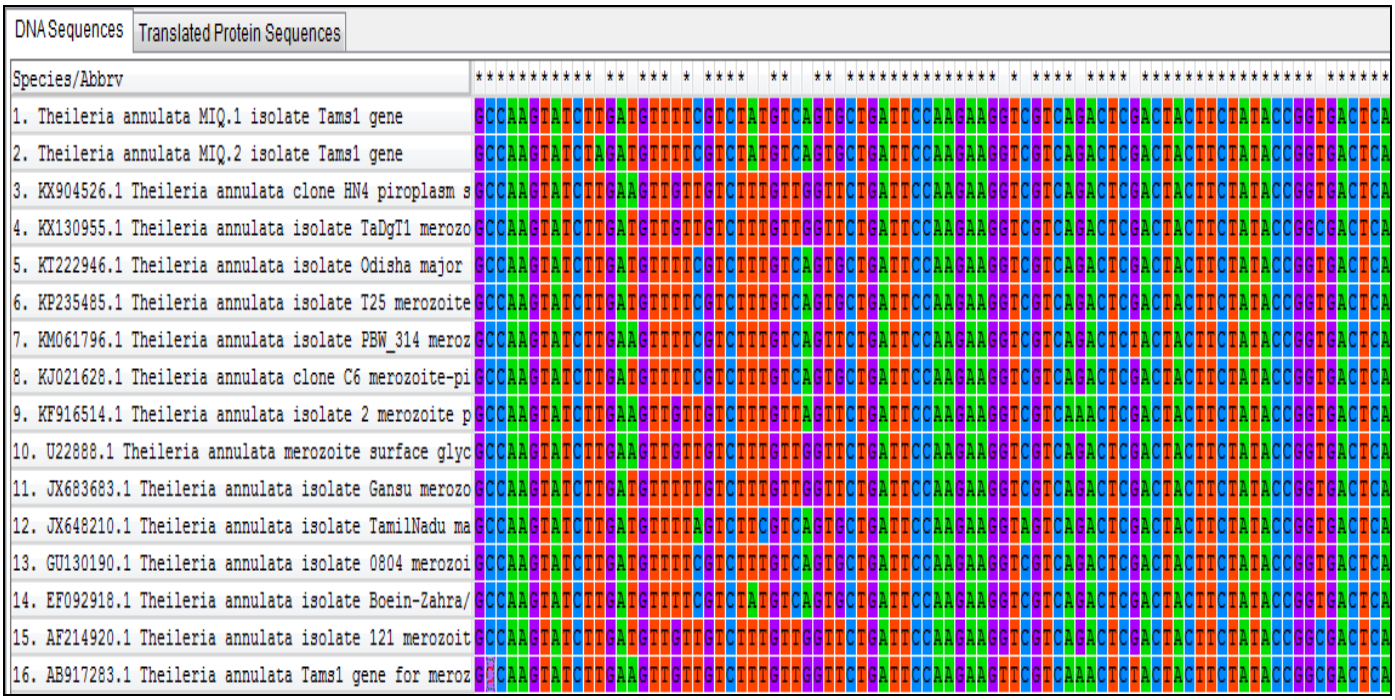

Fig 2: Multiple sequence alignment analysis of the partial merozoite-piroplasm surface antigen Tams1 gene sequence in local *Theileria annulata* MIQ.1 and MIQ.2 isolates and NCBI-Genbank *Theileria annulata* isolates based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis were show similarity (*) and differences based nucleotides substitution mutation in merozoite-piroplasm surface antigen Tams1 gene sequences.

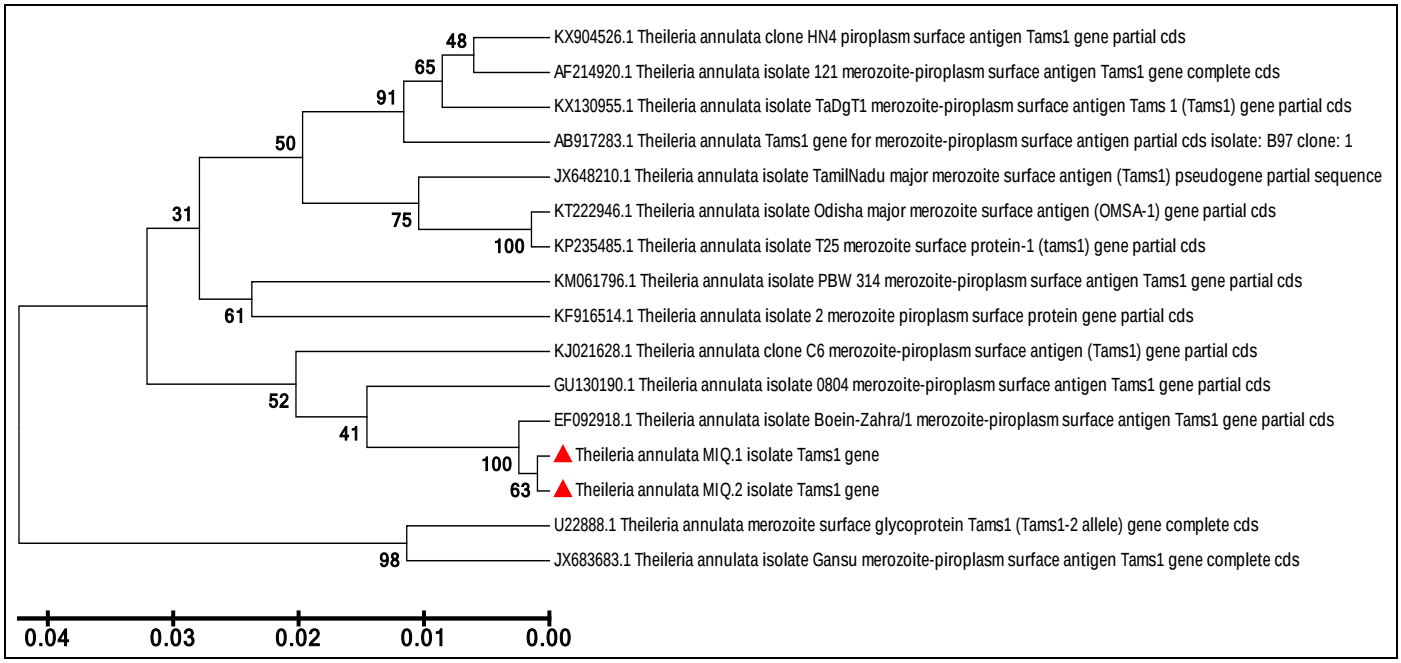

Fig 3: Phylogenetic tree analysis based on the partial sequence of Tams1 gene in *Theileria annulata* local isolates MIQ.1 and MIQ.2 that used for phylogenetic relationship analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method (MEGA 6.0 version). The *Theileria annulata* local MIQ.1 and MIQ.2 isolates were show closed related to NCBI-Blast *Theileria annulata* Iran isolate (EF092918.1) and less related to Kurdistan Region, Iraq *Theileria annulata* isolate (GU130190.1), whereas, other the NCBI-Blast *Theileria annulata* isolate were show more different to *Theileria annulata* local MIQ.1 and MIQ.2 isolates at genetic change (0.01-0.04).

4. Discussion

The present study showed that, the infection rate with Theileriosis in cattle was relatively low 13.23% (27/204) by microscopic examination (blood staining). This results approximately near the result 15.78% (30/220) which recorded by Al-Abadi and Al-Badrani, 2012 in Mosul city in north Iraq [22]. This contributed to climate, which not favorite for tick infestation during the month study which extend from November 2016 to April 2017. The results of this study showed that the native and cross breed of livestock are high

resistant to theileriosis and are affected by subclinical form of the theileriosis. Similar results in microscopic examination in Iran, Algeria and Saudi Arabia which was recorded 10.66% (16/150), 11.69% (42/359) and 15.4% (62/403) respectively [5, 7, 23]. Nourollahi-Fard *et al.* (2013) explained that the diagnosis of piroplasm infections base on clinical signs of disease and microscopic examination of Giemsa-stained blood smears, was low sensitive and unsatisfied in detection of parasite in epidemiological study. While, the low rate of infection may attributed to decrease in tick infestation, which influenced by the sub humid bioclimatic slope and differences in agricultural land use [7]. In consideration of age, the results revealed that the highest rate of infection in cattle was at age over one year and over two year (20.83%), (20.68%), which agreement with [10]. While the lowest rates in the age 10 day to 6 months and in the age over 6 months one year were 2.17%, 7.69%, which disagreed with [9] who recorded 34.35% and 36.84% respectively, the age of the animal in the present study assumes great role in occurrence of the infection, the older animals which previously infected were more like transformed to carrier status and this cases were maintenance the infection in flock.

It has been reported that infection of cattle with *Theileria* increases with age [24]. The older age animals (>15 months) were more likely to infect with *Theileria* due to subsequent exposures to numbers of infected ticks, these chronic carriers represent the most important source of infection for tick [7]. In regarding to the sex, the results showed no any effect on the sex of animals on percentage of infection with bovine theileriosis. The infection can ouccor in both sex at the same level and this coincided with studies of [9, 10, 15, 25].

4.1. Molecular study

The Polymerase Chain Reaction (PCR) technique has proved to be highly sensitive and specific for detecting parasite DNA in blood [24]. PCR is broadly utilized as specie-specific molecular diagnostic assay in veterinary parasitology to determine piroplasm-carrier animals [27, 28].

In the current study, the whole blood PCR examination showed high rate 33.00% (33/100) of animal were positive with *Theileria annulata* in Al-Muthana province/south of Iraq by using specific primers for *Theileria annulata* derived from Tams 1 gene, this was more accurate, and species-specific (Gen Bank,AF214920.1) [18, 29].

However, the results were lower than that recorded in the Kurdistan Region in north Iraq by PCR, the infection rate was 68.9% [11]. In addition the results of Al-Emarah *et al.* (2012) was 88.23% in Basrah province in south Iraq. While, the current results agreed with Ziam *et al.* (2015) in Algeria which was 30.16%, and Nayel *et al.* (2012) in Egypt also was 24.05% [30]. The prevalence of *T. annulata* in east of Turkey was established by PCR was 39% [18]. Dehkordi *et al.* (2012) recorded that the prevalence of *T. annulata* in southwest Iran was 28.11% (338/1202) [31]. Beside, in Asia 30.8% (380/1,235) were infected with *T. annulata* in ruminants from nine provinces of China [32].

4.2. Phylogenetic tree analysis

T. annulata has a haploid nuclear genome and the genome is arranged in 4 chromosomes [33]. The rRNA is the most abundant constituent of nucleic acids in the eukaryotic RNA transcription unit. The rRNA gene has been sequenced from a variety of different organisms, resulting in a large database for sequence comparisons [34, 35]. Several studies found that the Tams1 gene is highly polymorphic, raising questions concerning the suitability of Tams1 gene-targeted primers to detect all *T. annulata* isolates [36]. However, the molecule also possesses phylogenetically informative variable regions that are useful for determining relationships among species [37]. Based on available information, this is the first phylogenetic analysis study of *T. annulata* isolates in southern part of Iraq which isolated from infected cattle, a phylogenetic tree based on the TAMs1 gene sequence of the Al-Muthanna province isolates illustrated that there were relationships between *Theileria annulata* local isolates MIQ.1 and MIQ.2 and other global isolates obtainable from GenBank. In the constructed tree, *Theileria annulata* (MIQ.1 and MIQ.2 local isolates) were closely related to *T. annulata* Iran isolate (EF092918) with Nucleotide sequence identity 99%. In addition, our isolates nearly closed to Kurdistan Region, Iraq *Theileria annulata* isolate (GU130190) with homology sequence identity 97%. However, there were far distant between our isolates and *T. annulata Egypt* isolate (KJ021628**)** with identity as 95% and 96% to both local isolates respectively. While, other the NCBI-Blast *Theileria annulata* isolates were show more different to *Theileria annulata* local MIQ.1 and MIQ.2. In spite of the differences of phylogenetic tree with our local isolates, the identity score was ranged from 95% in India isolate (KM061796) to 90% in China (JX683683**)** and Turkey (U22888**)** isolates.

5. Conclusion

The present study revealed that the prevalence rate of theileriosis in cattle in Al-Muthanna province was low and approximately similar to that in other countries around. The study revealed that the highest rate of infection in cattle aged over than one years, and there is no significant differences regarding sex. The PCR is consider as more sensitive and accurate technique in the studies for diagnosis of both carrier and infected animals and great number of positive cases detected by PCR technique was carrier. Theileriosis was spread in cattle in all over the areas of Al-Muthanna province which includes in the study.

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