Molecular surveillance and phylogenetic analysis of *Theileria annulata* in bovine at Baghdad city/ Iraq

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**Summary**

This study is conducted to investigate Theileria spp. by traditional and molecular methods. A total of 150 blood and 50 lymph samples were collected from local breed symptomatically and asymptotically cattle of both sexes with age ranging from less than 6 months to more than 1 year during the four seasons of 2018, in different parts at Baghdad city / Iraq. Microscopic examination of Giemsa stained blood smears revealed 39.33 % (59/150) rate of infection with bovine theileriosis and 34 % (17/50) positive lymph smears. Statistically no significant difference recorded between female and male: 42.04 % (37/88) and 35.48 % (22/62) respectively. Higher rate of infection 57.97 % (40/69) were recorded in more than 1 year age and 0 % in less than 6 months. 48.93 % (23/47) rate of bovine theileriosis was recorded during summer and 39.53 % (17/43); 37.5 % (15/40) rates were recorded during spring and autumn respectively, while the lower rate recorded in winter 20 % (4/20). DNA extraction and polymerase chain reaction (conventional PCR) were done on all cattle blood samples the result recorded that 22 out of 25 samples were positive for *Theileria spp* and *Theileria annulata* with percentage of 88 %. Also DNA sequencing analysis and genetic relationship were conducted by phylogenetic analysis.

**Key words:** PCR, Theileria, Molecular.

**Introduction**

Tick borne diseases are related to livestock production in many developing countries including Iraq, and are responsible for high morbidity and mortality which results in decrease production of milk and meat (1). Theileriosis is parasitic tick borne diseases caused by parasite belong to genus Theileria, which infect cattle in tropical and subtropical areas (2 and 3). Theileria spp. is intracellular parasite cause active latent bovine theileriosis, primary diagnosis of disease based on microscopy as a traditional method, and conventional PCR in case of lower parasitemia and absence of clear symptoms of disease (4).

Bovine theileriosis is caused by most pathogenic *Theileria annulata*, is prevalent and economically disease of cattle and water buffaloes, known as Mediterranean coast fever (5 and 6). Tropical theileriosis or *Theileria annulata* infection is characterized by high fever, weakness, loss of appetite, weight loss, enlarged lymph nodes, anemia, conjunctival petechial paleness, diarrhea in advance stages and recumbency (7 and 8).

Diagnosis of theileriosis is based on clinical signs and is confirmed by examination of Giemsa stained blood smears for detection of piroplasms in erythrocytes and schizonts in lymphocytes (9). Molecular diagnosis is used as rapid identification of Theilerial infection in animals give a negative result in serological tests and still infected with ticks (10 and 11).

Al-Khaledi (12) recorded 32.93 % rate bovine theileriosis and recorded mixed infection with Theileria and Babesia 16.78 % in Al-Qadisiya / Iraq, while (13) recorded 13.23 %rate of infection with theileriosis in cattle by microscopic examination and recorded 33 positive cases of 100 blood samples by PCR analysis in Al-Muthanna province, Iraq.

This study was conducted for detection and investigation Theileria spp. in local breed Iraqi cattle, in different areas of Baghdad city using microscopic examination of stained blood smears, molecular technique, DNA sequence and phylogenetic tree analysis.

**Materials and Methods**

A total of 150 blood samples were collected from Jugular vein of each local breed both sexes Iraqi cattle suffered from weakness, anemia, high fever and high infestation of ticks and other asymptomatic with age ranging from
less than 6 months to more than one year along four seasons of 2018 also 50 lymph samples were aspirated from pre-scapular lymph nodes of same animals blood samples were collected in anticoagulant tubes (EDTA) for microscopic and molecular detection of Theileria parasite after transferred in cooling box to parasitology laboratory at Veterinary Medicine College, University of Baghdad/ Iraq.

Thin blood and lymph smears were prepared and stained by Giemsa stain (10%) and examined under oil immersion (X 100) according to (14).

Genomic DNA from cattle blood samples were extracted by using (Quantipher Genomic DNA Extraction spin kit - Turkey) and done according to company recommendations. The blood Genomic DNA checked by using Nanodrop spectro photometer that measured DNA concentration and purity at (260/280 nm) absorbance.

Conventional PCR by specific primers was used for detection Theileria spp. and Theileria annulata (15) provided by IDT Canada. (Table, 1). The preparation of PCR master mix was : 10 µl PCR premix, 3 µl from forword and reverse primer, 5 µl of DNA template and 2 µl of PCR water for final volume of 20 µl. The PCR products were analyzed by agaros gel electrophoresis and visualized using ultraviolet Trans illuminator.

DNA Sequencing was performed to confirm the detection of local Theileria spp. and Theileria annulata isolates in cattle, and for phylogenetic relationship analysis of 18 ribosomal RNA and cytochrome b genes respectively with NCBI–Gene Bank Global. The PCR sequence result were edited and analyzed by (Mega x) program and using nucleotide collection (nt/nr) database, also to specify the parasite blasted sequences (Neighbor-joining method) were used for aligned sequences. phylogenetic analysis were carried out to find the relationship of sequences determined in the study obtained along with relevant sequences deposited in Genbank DNA Sequencing carried out by (Suol–Korea).

Data were analyzed by using SAS program (2014) the proportions were compared using Chi-square test, P ≤0.05 is considered as significant.

Table 1: Primers and their sequence that used in study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5′ – 3′)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
</table>
| Theileria spp. 18s ribosomal RNA Gene | F: 5′ – AGTTTCTGACCTATCAG – 3′  
R: 5′ – TTGCGTTAAAACCTCTTG – 3′ | 1100 |
| Theileria annulata Cytochrome b | F: 5′ – ACTTTGCGTAATGTAAC – 3′  
R: 5′ – CTCTGGACCAACTGTTGG – 3′ | 312 |

Table 2: PCR Thermocycler system (Amplification program).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Initial Denaturation</th>
<th>Cycle Conditions</th>
<th>Final Extention</th>
<th>Cycle No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
<td>Annealing</td>
<td>Extention</td>
</tr>
<tr>
<td>Theileria spp.</td>
<td>94 °C/3min</td>
<td>94 °C/30 sec</td>
<td>52 °C/30 sec</td>
<td>72 °C/30 sec</td>
</tr>
<tr>
<td>Theileria annulata</td>
<td>94 °C/3 min</td>
<td>94 °C/30 sec</td>
<td>58 °C/30 sec</td>
<td>72 °C/30 sec</td>
</tr>
</tbody>
</table>
Results and Discussion

The overall rate of infection with bovine theileriosis after examining 150 blood smears was 39.33 % (59/150). The piroplasm forms were detected in the positive animals as in (Fig. 1), also 17 animals showed positive lymph smears for schizont stage with rate of 34 % (17/50), (Fig. 2).

Figure, 1: Erythrocytic stage, Piroplasm of Theileria spp. In cattle blood smear (x 100).

Figure, 2: Lymphocytic stage, Schizont of Theileria spp in cattle lymph smear (x 100).

Female and male recorded 42.04 % (37/88) and 35.48 % (22/62) infection rates respectively without significant difference P= 0.50 (Table 3).

Table, 3: Infection rate of bovine theileriosis by microscopic examination according to sex.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of animals examined</th>
<th>No. of positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>88</td>
<td>37</td>
<td>42.04</td>
</tr>
<tr>
<td>Male</td>
<td>62</td>
<td>22</td>
<td>35.48</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>59</td>
<td>39.33</td>
</tr>
</tbody>
</table>

X2 : chi square : 0.45P=0.50.

Infection rates of bovine theileriosis in this study didn't affected by sex of animals, and this coincided with studies of (12, 18 and 19). Higher rate of infection with bovine theileriosis was recorded in animals with more than 1 year age group 57.97 % (40/69), while 0 % (0/30) recorded in less than 6 months age group with significant differences P>0.0001 (Table 4).

Table, 4: Infection rate of bovine theileriosis by microscopic examination according to age groups.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>No. of animals examined</th>
<th>No. of positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 6 Months</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 Months – 1 Year</td>
<td>51</td>
<td>19</td>
<td>37.25</td>
</tr>
<tr>
<td>&gt; 1 Year</td>
<td>69</td>
<td>40</td>
<td>57.97</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>59</td>
<td>39.33</td>
</tr>
</tbody>
</table>

X2 : chi square : 35.86P>0.0001

Our results recorded higher rate of infection in 1 year age group in a coordinate with study of (20) who showed that the infection with Theileria parasite increase with the age. This is might be due to subsequent exposures to infested ticks that represent the important
source of infection (21). Summer showed highest rate of infection with Theileria spp 48.93 % (23/47), spring and autumn recorded 39.53 % (17/43) and 37.5 % (15/40) respectively, the lower rate recorded in winter 20 % (4/20) (Table, 5).

Table, 5: Infection rate of bovine theileriosis by microscopic examination according to seasons of study.

<table>
<thead>
<tr>
<th>Season</th>
<th>No .of animals examined</th>
<th>No. of positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td>43</td>
<td>17</td>
<td>39.53</td>
</tr>
<tr>
<td>Summer</td>
<td>47</td>
<td>23</td>
<td>48.93</td>
</tr>
<tr>
<td>Autumn</td>
<td>40</td>
<td>15</td>
<td>37.5</td>
</tr>
<tr>
<td>Winter</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>59</td>
<td>39.33</td>
</tr>
</tbody>
</table>

X2 : chi square : 5.006P=0. 17.

According to seasons, bovine theileriosis was more prevalent in summer 48.93 %, spring and autumn recorded nearest rates 39.53 % and 37.5 respectively, these result agreed with (12) in Al-Qadisiya province/Iraq who recorded 74.77 % and 71.77 % during summer and spring respectively prevalence increase with warm moist climate that is favorable environment for Theileria sporozoites develop as a result of (22), tick's activity increase in summer, and as a result the salivary gland of single tick contain large amount of sporozoites which can be fatal (7).

Out of 25 blood samples examined by conventional PCR technique 22 was positive for Theileria SPP., that revealed PCR product of 1100 bp in length , and 22 was positive for Theileria annulata that revealed PCR product of 312 bp in length (Figure 3 and 4).

![Figure 3: Agarose gel (1.5 %) electrophoresis of amplified DNA from Theileria spp. lane M: 15, DNA Ladder (100-3000 bp) some positive product showed clear band in 1100 bp.](image)

DNA sequencing result:Twenty samples purified by PCR technique analyzed by sequencing to get nucleotide sets of (18s ribosomal RNA) gene (1100 bp) for Theileria spp. And ( cytochrom b) gene (312 bp) for Theileria annulata isolated from cattle in different parts of Baghdad city /Iraq and recorded in gene bank with accession numbers: MK 182862 , MK 182863 , MK 182864 , MK 182865 , MK 182866 ,MK 182867 , MK 182868 , MK 182869 , MK 182870 , MK 182871.

The analysis of phylogenetic tree based on sequences of(1100 bp) 18 s ribosomal RNA gene, and (312 bp )cytochrom b gene for Theileria spp. and Theileria annulata respectively, phylogenetic tree using constructed neighbor joining bootstrab1000 radiation tree. Analysis result showed that homology of nucleotides sequences between local isolate of Iraqi Theileria spp. was nearly closed to India , Spain , Turkey , Tajikistan , China , Iran , and Pakistan isolates (MF 287926 , FJ426369 , MG569892 , KM288519 ,KF 559356 , KF429794 and JQ 743634 ) respectively with homology sequence identity 99% (Figure, 5 and 6).

Theileria species can be differentiate by Molecular tools, this technique have proved to be highly sensitive and specific in detecting blood parasite DNA (23 ). Piroplasm in carrier animals determine by using diagnostic Molecular species–specific assay in veterinary parasitology (24 and 25). PCR have been used
to detect and differentiate Theileria spp. In carrier and clinically infected animals, even with very low amount of parasite DNA.

PCR technique showed high rate of infection with Theileria spp. in different parts of Baghdad city, using specific primers: 18s ribosomal RNA gene (1100 bp) and cytochrom b gene (312 bp), that was accurate and species–specific (GenBank, MG 787986.1 and KP 731977.1). The results were higher than that recorded by (26) 68.9% and (19) 88.23% in Kurdistan and Basrah province/Iraq respectively and higher than (21) in Algeria 30.16% and (27) in Egypt 24.05%.

The conventional PCR result using 18s rRNA gene and confirmed our result that detected Theileria spp. by examining Giemsa Stained blood smears, also PCR is a successful and sensitive method in detection Theileria annulata by using cytochrom b gene. The highest percentage rate recorded by PCR was good indication for highest prevalence of bovine theileriosis in Baghdad.

Our result recorder 88% rate by PCR and 39.33% microscopically, these agreed with many researchers, (28) in Turkey and (29) in India, the differences between microscopic and molecular methods attributed to several reasons as chronic and carrier cases with low parasitemia and parasite misdiagnosis with staining artifacts (30).

Phylogenetic analysis confirmed low differences between Iraqi strains of Theileria annulata and other countries. The genetic variation that recorded may be due to variation on area size of reference sequence and differences in geographical areas where isolates collected. Using different methods like PCR based on gene sequencing of partial or complete genes, Phylogenetic have been used for genetic analysis. When environment of parasite change, genetic diversity plays important role in survival and makes accurate analysis of variation applicable for studies on taxonomy, biology, epidemiology and pathogenesis of parasites. Result of this study agreed with some studies that reported the existence of genetic variation and phylogenetic relationships based on mitochondrial and nuclear gene sequences among populations of parasite that analyze genetic variations of Theilerial protozoa in the world.

Figure 5: Analysis of phylogenetic tree based on the partial sequence small subunit of 18s rRNA of Theileria spp., local and global sequence using neighbor joining bootstrap 1000 radiation tree figure evolutionary relationships.
Results of study explained that Theileria spp. distributed in local breed cattle in different areas at Baghdad city/Iraq according to microscopic examination of Giemsa stained blood smears and PCR technique. The molecular and phylogenetic analysis are very sensitive and accurate in determining parasite species. The scientific diagnostic molecular methods easily task production of vaccines and proper use of different treatments to prevent spreading of disease.

References

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التهذيب الجيني وتحليل الشجرة الوراثية لطفيلي Theileria annulata في الابقار في مناطق مختلفة من مدينة بغداد/ العراق

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الخلاصة

اجريت الدراسة للتحري عن طفيلي Theileria spp. باستخدام الطرق التقليدية والجينية. جمعت 150 عينة دم و 50 عينة لعصف من ابقار عرفوا بعطف IoT من كلا الجنسين وذات فئات عمرية تراوحت أقل من 6 أشهر إلى أكثر من سنة خلال الفصول الأربعة للعام 2018 ومن مناطق مختلفة في العراق. أظهر الفحص المجهري للمسحات الدموية المصبوغة بالكمزا نسبة اصابة كلية بايثيلريبا الابقار (59/150) 39.33%، وسجلت المسحات المفيدة اصابة بنسبة (50/17) 34.65%. احصائياماً لم تسجل فروق معنوية بين الاناث (36/85 40.90%) والذكور (22/62) 35.48%. أعلى نسبة اصابة (40.69)%. سجلت في اعمار أكثر من سنة ونسبة 0% كانت في أقل من 6 أشهر. وكانت نسبة الاصابة بايثيلريبا الابقار أعلى في فصل الصيف (34/23) 47.83% ونسبة (17/41) 39.53% في الربيع و نسب (37.5) في الخريف و اقل نسبة كانت في فصل الشتاء (4/20) 20%. اظهرت نتائج اختلاف الحمض النووي DNA في فحص سلسلة البلمرة بناجا 22 عينة من اصل 25 عينة موجبة لكل من جنس طفيلي Theileria ونوع Theileria annulata ونسبة 88%. كما اجري التسلسل والعلاقة الجينية للعينات عن طريق تحليل الشجرة الوراثية.

الكلمات المفتاحية:تفاعل سلسلة البلمرة،الثيليريا،التهذيب الجيني.