MOLECULAR DIAGNOSIS OF THEILERIA ANNULATA IN CATTLE IN SALAH EL-DIN PROVINCE AND ESTIMATE SOME HEMATOLOGICAL CRITERIA

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ABSTRACT

Theileria annulata has been identified as a major cause of bovine theileriosis in Iraq. The research was designed to reveal the prevalence of Theileriosis in cattle in Salah El-din province, Iraq from the first of September to the end of December 2020. Sixty blood samples were collected from suspected animals and microscopic and PCR techniques were used. The results of microscopic examination and PCR assay showed that 37% and 88% of cattle were infected respectively. Examination of positive blood smears by using Giemsa-stained revealed the typical morphological structure of piroplasm. According to the phylogenetic analysis of the partial 18S ribosomal RNA gene, Theileria sp. isolates were shown to be related to T. annulata strains, including those from Italy, Turkey, and Pakistan. According to hematological tests, a significant decrease was observed in total erythrocyte count (TEC), hemoglobin concentration (Hb), packed cell volume (PCV), and lymphocyte proportion, while significant increase recorded in total leukocyte count (TLC) (P<0.01). The infected animals complained macrocytic normochromic anemia with abnormal morphology of erythrocyte as poikilocytosis. This study is the first report in Salah El-din province concludes that T. annulata is the causative agent of theileriosis of cattle and suggests that PCR technique is excellent diagnostic tools.

I. INTRODUCTION

Hemoparasitic infections are the main problem in livestock because of the heavy economic losses that lead to reduced livestock production, increased susceptibility to other secondary bacterial infections and higher mortality rates[1]. Theileriosis is a parasitic ticks’ borne disease caused by the genus Theileria spp. which generally infect ruminants, especially wild and domestic cattle in tropical and subtropical areas[2]. Theileria are obligate intracellular protozoan parasites transmitted by ticks (ixodid ticks), and have complex life cycles in both vertebrate and invertebrate host. The most pathogenic and economically important species are T. annulata, which transmitted by ticksgenus Hyalomma and causes Tropical Theileriosis (TT) or Mediterranean theileriosis, T. parva, which causes East Coast fever (ECF)[3].

Tropical theileriosis or T.annulata infection prevalent in southern Europe, North Africa, the Middle East and Asia. characterized by high fever, loss of appetite, weakness, weight loss, enlarged lymph nodes, Suppressed cough, anemia, conjunctival petechial paleness, recumbency and diarrhea in advance stages[4,5]. The classical methods for the diagnosis of theileriosis depend on using microscopy involving lymph and blood smears stained by Giemsa stain for checking the presence of Koch’s bodies and the piroplasm[6]. Many problems were shown due to the use of the smear methods that reflectlow sensitivity for the diagnosis of carrier animals[7]. For a quick and accurate diagnosis, and detect of different Theileria species in infected cattle molecular technique such as PCR was developed which have a high degree of sensitivity and specificity[8,9]. Iraq is one of many countries complaining from Tropical Theileriosis and this disease is represented of the really challenge[10]. current study aimed to diagnosis of T.annulata using conventional microscopy and PCR technique and study some blood criteria.

II. MATERIAL AND METHODS

2. 1. Samples Collection

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The study was undertaken in Salah El-din province Iraq from the first of September to the end of December 2020. A total number of 60 (male and female) with clinical manifestations of theileriosis were noted on animals during sampling such as high temperature, weakness, lymph nodes swelling in some cases, increased respiration, and nasal discharge. In addition, tick infestations were seen on some examined animals. Approximately 6ml of blood samples from Jugular vein of cattle were collected, 2ml of the blood were put in tube containing EDTA. Giemsa’s stain was used for the blood samples of EDTA tubes to determine of piroplasms microscopically. Then the blood stored at -20°C until performed PCR test.

2.2. Microscopic Examination

After preparation of peripheral blood smears on glass slides, the slides were dried and fixed with methanol for about 5 minutes, then stained by 10% Giemsa stain for 30 minutes and examined under oil immersion lens (100 x magnifications).

2.3. DNA extraction

The DNA was extracted used (200 μl) of the whole blood samples with DNA extraction Kit (ReliaPrep™ Blood gDNA Minipre System, Promega - USA) according to the manufacturer’s instructions. The DNAs were eluted into 100 μl Elution buffer at room temperature, samples stored at - 20 °C for PCR test. Ethical standards were considered during sampling. The blood Genomic DNA checked by using Nanodrop spectrophotometer that measured DNA concentration and purity at (260/280 nm) absorbance.

2.4. PCR amplification and sequencing

Conventional PCR reactions were performed using specific primer to amplify a 860 bp fragment of highly conserved regions of 18s ribosomal RNA gene in all Theileria spp. provided by (Macrogen, Korea). The 18s ribosomal RNA gene was amplified using PCR Premix (2X). The preparation of PCR master mix was: 10 μl PCR premix, 1 μm from forward and 1 μm from reverse primer, 8 μl of DNA template, for final volume of 20 μl. The reactions were performed in an automatic DNA thermocycler (Thermo Fisher Scientific, USA) was programmed for 40 cycles as followed: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s; annealing at 59°C for 30s, and extension at 72 °C for 60s and a final extension at 72 °C for 7 - min. After electrophoresis on 1.5% agarose gels stained with Ethidium Bromide (Promega, USA) at 100 volt for 90 minutes, the obtained PCR products were analyzed by agaros gel electrophoresis and visualized using ultraviolet Trans illuminator. The PCR product for Theileria sp. was estimated to be 860 bp. Sequencing was performed to confirm the detection of T. annulata in cattle, and for phylogenetic relationship analysis of 18s ribosomal RNA with NCBI–Gene Bank Global. All positive samples of PCR products were sent for Sanger sequencing using ABI3730XL automated DNA sequencer, by (Macrogen, Korea). The results were received by email then analyzed using Geneious software.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequencing</th>
<th>Table (1)</th>
<th>Primers and their sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theileria spp. 18s ribosomal RNA Gene</td>
<td>F: 5<code>-GCATTCGTATTTAACTGTCAGAGG-3</code>&lt;br&gt;5`-TAAGGTTCACAAAACTTCCCTAG-</td>
<td>5<code>-GCATTCGTATTTAACTGTCAGAGG-3</code>&lt;br&gt;5`-TAAGGTTCACAAAACTTCCCTAG-</td>
<td>860</td>
</tr>
</tbody>
</table>

2.5. Hematological parameters

Blood mixed with EDTA (2.5 mL) collected from cattle infected with Theileria spp. (n=22) served as the infected group while non-infected cattle (n=10) served as control, used to determine erythrocyte count (ER), haemoglobin (HB), packed cell volume (PCV), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin concentration (MCHC), total leukocyte counts (TLC) on an automatic hematology analyzer (BC -30s Mindray, Germany).

2.6. Statistical analysis

The data succumbed to T- Independent test was used for comparison between two groups by using SPSS program.

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III. RESULTS

3.1. Microscopic examination

Out of 60 samples, 22 (37%) were found positive for tropical theileriosis by conventional microscopic technique using Giemsa staining method. The results showed the erythrocytic forms (piroplasm) have piroplasms cocci, rod, comma, signet-ring, and pear-shaped forms with poikilocytosis. (Fig. 1)

![Image](https://example.com/image1.png)

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

3.2. Molecular detection

Twenty four out of 27 (88%) samples were detected positive by PCR assay, that revealed PCR product of 861 bp in length (Fig. 2).

3.3. Sequencing results

The local *Theileria annulata* isolates show closely related to NCBI-BLAST *Theileria annulata* isolates (MT341858.1) with Nucleotide sequence identity 99%. Analysis result showed that homology of nucleotides sequences between local isolate of Iraqi *Theileria* spp. was nearly closed to Bangladesh, turkey, India, and Pakistan isolates.

![Image](https://example.com/image2.png)

**Fig. 2**: Agarose gel electrophoresis image shows the PCR product analysis of 18s ribosomal RNA gene of *Theileria spp*. in blood samples of cattle. Lanes 1, 3, 5, 7, 9, 11, 12, and 13 were positive samples (861bp). Lanes 1, 2, 3, 4, 5, 6, 7, 8, 11, 12 and 9, 10 were negative samples, 100-bp ladder.

3.4. Hematological parameters
The comparison of some hematological parameters between infected and non-infected cattle showed in (Table 2). The hematological analysis showed statistically a significant decreases in total erythrocyte count (TEC), hemoglobin concentration (Hb), packet cell volume (PCV), and lymphocyte. (P<0.01). There was a significant increase in mean corpuscular volume (MCV), leukocytosis, (P<0.01) while not change recorded in mean corpuscular hemoglobin concentration (MCHC). Based on the means of corpuscular volume (MCV) and corpuscular hemoglobin concentration (MCHC), infected animals complained anemia which classified as macrocytic normochromic.

Table (2). Mean ± SEM of blood parameters in uninfected cattle and cattle infected with T. annulata.

<table>
<thead>
<tr>
<th>Blood parameter</th>
<th>Unit</th>
<th>Control (n=10)</th>
<th>Infected animals n=22</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>10¹²/L</td>
<td>7.05 ± 0.48</td>
<td>5.13 ± 0.43**</td>
</tr>
<tr>
<td>Hb</td>
<td>g/dl</td>
<td>10.14 ± 0.45</td>
<td>8.80 ± 0.61**</td>
</tr>
<tr>
<td>PCV</td>
<td>%</td>
<td>32.58 ± 1.5</td>
<td>28.04 ± 1.8**</td>
</tr>
<tr>
<td>Plt1</td>
<td>0⁹/L</td>
<td>284.4 ± 88</td>
<td>283.9 ± 39</td>
</tr>
<tr>
<td>MCV</td>
<td>fl</td>
<td>46.78 ± 2.7</td>
<td>57.30 ± 4.4**</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>14.54 ± 0.77</td>
<td>18.01 ± 1.3**</td>
</tr>
<tr>
<td>MCHC</td>
<td>10¹²/L</td>
<td>31.160 ± 0.30</td>
<td>31.46 ± 0.58</td>
</tr>
<tr>
<td>WBC</td>
<td>10⁹/L</td>
<td>7.92 ± 0.89</td>
<td>8.56 ± 0.96</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>%</td>
<td>50.820 ± 3.0</td>
<td>47.625 ± 3.0</td>
</tr>
<tr>
<td>Monocyte</td>
<td>%</td>
<td>10.44 ± 2.0</td>
<td>11.48 ± 1.8**</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>%</td>
<td>38.74 ± 4.0</td>
<td>40.90 ± 3.1**</td>
</tr>
</tbody>
</table>

IV. DISCUSSION

Theileriosis is a significant clinical illness of cattle in many countries. It is also causes major economic losses as well as reduced production [12]. The current study showed that, the infection rate with Theileriosis in cattle was 37% (22/60) by using microscopic examination. This result was higher than the results conducted by Al-Samarai (2019) [13] and Ahmed et al. (2021) [14] 33.33%, 27.5% in Baghdad and Sulaimani respectively, and approximately near to the result conducted by [16] 38.65% in Egypt, but less than Al-Emarah et al. (2012) [4] 69.43% in Basra province.

Sometimes parasite is difficult to identify in carrier animals by using traditional method. Thus, alternative techniques (PCR) was used as an accurate technique to identify of persistently infected cattle with Theileria spp. [3,16]. The Polymerase Chain Reaction (PCR) technique has proved to be highly sensitive and specific for detecting parasite DNA in blood [12]. PCR is broadly utilized as species-specific molecular diagnostic assay in veterinary parasitology to determine piroplasm-carrier animals [16,18].


The prevalence of T. annulata in east of Turkey was established by PCR 39% [22]. Dehkordi and et al., recorded that the prevalence of T. annulata in southwest Iran was 28.11% (338/1202) [23]. Beside, in Asia 30.8% (380/1,235) were infected with T. annulata in ruminants from nine provinces of China [24].
Despite the fact that anemia is one of the main symptoms of tropical theileriosis, the precise mechanism is still unknown. It has been claimed to be a result of removal of erythrocytes by phagocytosis rather than parasite-induced Lysis[13]. Generally, cattle infected with Theileria spp had significantly lower values of total RBC, Hb and PCV when compared with the control. This which agreed with[26]. The number of RBC decreased due to its destruction caused by parasites which lead to anemia and the Hb decreased due to destruction of RBC [25]. This decline in levels of Hb, PCV and RBC count is attributed to Lysis of erythrocytes by piroplasmwhich infects and replicate in it and erythro-phagocytosis[4]. In case of theileriosis, the low RBC count is attributed to removal of infected erythrocytes by spleen and liver and not due to the destruction of erythrocytes by the parasite[13]. On the other hand the animals infected by Theileria annulata in current study are complained from macrocyt精彩normochromic anemia which agreed with Haron et al.,(2015)[27]and Agina(2017)[32], but disagreed with Yasini et al.,(2014)[28], minnat et al.,(2016)[26] and Ismael and Al-Samarai(2019)[13], who recorded Macrocytic hyperchromic anemia. In addition to omar et al.,(2002)[25] and Nazifi et al.,(2009)[29] who recorded macrocyt精彩 hyperchromic anemia. White blood cell (WBC) counts were increased significantly (P<0.01) in cows as compared to the non-infected. this result agreed with Mahmoudet et al.,(2016)[31], Ismael and Al-Samarai(2019)[13] and Agina(2019)[32], but disagreed with omar et al.,(2002)[25], Nazifi et al.,(2009)[29] Quiyyummet et al.,(2010)[32] and Salem and El-Sherif(2015)[34]. Such change in leucogram might be attributed to the persistent harmful effects of toxic metabolites of Theileria in haemopiotic organs especially bone marrow and their interference with process of leucogenesis [4]. The contradictions in the results among studies may be due to differences in the status of disease: clinical, sub clinical, acute and chronic[27].

V. CONCLUSIONS

The findings of the current study illustrated that traditional method could be a suitable technique to determine tropical theileriosis. Proper formulation of control strategies and reduction of economic loss caused by theileriosis in cattle can be performed via further identification trial of T. annulata based on 18S rRNA gene sequences. In addition, the study showed that T. annulata infection was dominant Theileria species in cattle of the studied area. PCR technique accepted as an accurate tool to determine Theileria even in asymptomatic carrier animals which is quite necessary for carrying out of control programs successfully. Therefore, further field studies to verify these results are suggested.

REFERENCE


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