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Molecular study and DNA sequence analysis of *Theileria annulata* in cattle in Al-Hilla, Iraq

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Abstract

The current work was conducted to unveil the current situation for the infection by *Theileria annulata* in cattle in Al-Hilla City, Iraq. A total of 225 blood samples (200 from suspected infected animals and 25 from clinically healthy animals as a control group) were collected. These samples were subjected to a direct slide-smearing for detection using a microscope and DNA sequencing, targeting the cytochrome b (Cyt b) gene of 10 polymerase chain reaction (PCR) products. The thin smear findings of the 200 suspected cases revealed that 63 (31.5%) were infected with *Theileria* spp., while 115 (57.5%) cases had no *Theileria* but other blood parasites; however, only 22 (11%) suspected cases showed no presence of any parasites. Unsurprisingly, the 25 blood samples from the control group demonstrated no presence of any blood parasite. Moreover, the DNA sequencing demonstrated that the *Theileria* spp. belonged to *T. annulata* species, and these sequences were nucleotide-based similar to Gene-Bank isolates from Tunisia (ON035604, ON035605, ON035606, ON035607, ON035608, ON035609, ON035610, ON035611, ON035612, and ON035613). The present study outcomes indicate that theileriosis is the dominant parasitic infection in cattle in Al-Hilla City and is highly caused by *Theileria annulata*.

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Introduction

Only a few of the difficulties faced by Iraqi livestock and dairy industries: a widespread shortage of knowledge among livestock farmers about consuming food, tick control techniques, artificial insemination, and financial damages (1). Parasitism is a major cause of health issues in these farm animals, and most of them are infested with ticks that are considered the natural vector for the transmission of blood parasites. Ticks thrive in Iraq, making it an ideal place to grow and reproduce (2-8). Ticks related to the genera *Hyalomma*, *Rhipicephalus*,and *Ixodes* affect various animals from domestic and wild origins, producing different tick-borne illnesses. In addition to harming the health and production of cattle, theileriosis also costs livestock owners a wide range of financial resources (9-12). Cattle theileriosis can occur due to *Theileria annulata*, an intracellular protozoan. Several Ixodid tick genera, including *Rhipicephalus*, *Hyalomma*, and *Amblyomma*, are frequently reported as the main vectors for transmitting *Theileria* spp. (13-16). Host bovines undergo the sporogony and merogony phases, whereas ticks develop zygotes and kinetes. When a tick feeds on a host, the parasite enters the host and quickly invades its leukocytes. Once liberated from the parasitized leukocytes, merozoites invade erythrocytes, where they grow into piroplasms (17-22). Conjunctival petechial hemorrhage, swollen lymph nodes, and anemia are symptoms of theileriosis, in addition to high fever, restricted appetite, loss of body weight, and general weakness (23,24). *Theileria* piroplasms are often seen in animals and acute sings and serve as reservoirs for the parasite community (25). It is thus essential to identify carrier animals in epidemiological investigations to determine the level of disease risk and evaluate control measures (26).

The molecular methods used to identify *Theileria annulata* in current work are the main aim of it in cattle in Al-Hilla, Iraq.

Materials and methods

Ethical approve

The study was approved and carried out at the College of Veterinary Medicine, University of Al-Qadisiyah with approval number (P.G, No. 1890 in 2020) during the period September 2021to February 2022 according to the international guidelines for the care and use of animals.

Blood collection

This study was conducted between September, 2021to February, 2022. A total of 225 jugular-vein blood-samples (200 from suspected infected animals and 25 from clinically healthy animals as a control group) were collected. The animals were of different ages, from six months to 9 years old, and of both sexes. Blood samples (2 ml/each) were inserted in sterile EDTA treated tubes and transported immediately in an icepack to the Parasitology Laboratory, College of Veterinary Medicine, University of Al-Qadisiyah. Thin blood smears were prepared to identify *Theileria* spp., Then the remaining blood was placed in a deep freezer under -20ºC for DNA extraction.

Microscopic examination

Each blood sample was methanol-fixed, Giemsa-stained, and examined using a microscope (100X). The existence of only one piroplasm was reported as positive (27).

DNA Extraction

This extraction of the parasite DNA was made according to the genomic DNA purification Kit supplemented by Geneaid (Korea) and was done depending on the instruction accompanying the kit. As an initial step, 200µl of frozen blood was used as a startup material for the DNA extraction. Ultimately, the final DNA product was Nano Drop - estimated identify its quality and quantity.

PCR

The *Theileria* spp. Was identified using the rRNA gene as a molecular target (primers: F: GAG ACA AGG AAT ATT CTG AGT CC and R: TTA AG TGG CAT ATA ATG ACT TAA GC, (28)). The Cyt b gene was used to identify *Theileria annulata* via sequencing using the primers F: CAG GGC TTT AAC CTA CAA ATT AAC and R: CCC CTC CAC TAA GCG TCT TTC GAC AC, (29), as a molecular target, specifically designed for the current investigation. The 20µl-reaction mixture for the PCR contained 10µl green master mix, 1µl for each upstream primer and downstream primer, 2µl DNA template, 5.5µl for-molecular-use-water, and 0.5 µl $MgCl_2$. The thermocycler conditions were 95ºC for 5mins, (95ºC for 35s, 57ºC for 35s, and 72ºC for the 40s), and 72ºC for 5mins, for the one-cycle for initial denaturation, 39-cycle for (main denaturation, annealing and main extension), and one-cycle for a final extension. For the electrophoresis, 2% agarose gel mixed with 0.5µg/ml ethidium bromide was employed. The bands were then examined utilizing a UV-imager.

Amplicon sequencing analysis

DNA sequencing was conducted for 10 positive-PCR local isolates of *Theileria annulata* from cattle. The PCR products for the Cyt b gene were sent to Macrogen Company in Korea employing the AB DNA sequencing system. The phylogenetic tree analysis was built using MEGA X and the multiple sequence alignment analysis based on Clustal Walignment analysis, and the related evolutionary distances were calculated employing the maximum composite likelihood method via the phylogenetic tree UPGMA method. Comparisons were made using the sequences of the local isolates against isolates from the NCBI-Blast. Finally, the sequences of the local isolates were deposited into the NCBI GenBank get accession numbers.

Results

Microscopic examination

The thin blood smears findings of the 200 suspected cases revealed that 63 (31.5%) were infected with *Theileria* spp., while 115 (57.5%) cases had no *Theileria* but other blood parasites; however, only 22 (11%) suspected cases showed no presence of any parasites. Unsurprisingly, the 25 blood samples from the control group demonstrated no presence of any blood parasite.

DNA sequencing

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Discussion

Dairy sector expansion has been hindered by tick-borne diseases (TBDs) that generate significant economic consequences. According to earlier investigations, *T. annulata* was detected in 33 and 24% of cattle in Pakistan from two districts. Also, in Pakistan, the occurrence of *T. annulata* in cattle in different areas was revealed to be 33, 30, 28, 23.7, 21, 19, and 18.8% (30-35). Additionally, *T. annulata* infection in cattle has been documented in many nations that fall within the tropical or subtropical climate zones. *T. annulata* prevalence in cattle was 23.3, 20, 25.4, 18.2, and 1.9% in India, Egypt, Algeria, Northwest China, and Saudi Arabia, respectively. These data from the

countries mentioned above regarding the infection rates lower than the rate of the current study, probably, due to some failure in the control programs of ticks in the current study areas. Differences in tick eradication strategies, environment compatibility, farm control, husbandry techniques, and abiotic conditions at sampling locations might cause differences and help in the varying infection rates of *T. annulata* from one site to another (30,36,37).

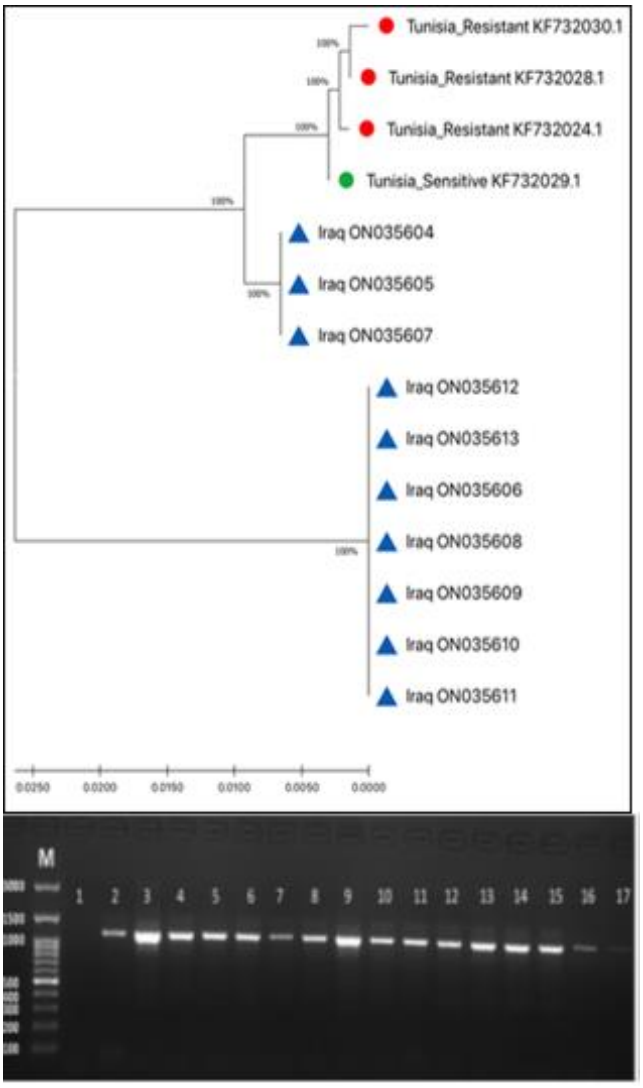

Figure 1: *Theileria annulata* based phylogenetic tree (Cyt b gene) of the study detected sequences (blue triangles + Accession numbers).

The primary ingredient for the evolution of microorganisms is genetic diversity (37), so the genetic variety of *T. annulata* in a host animal enables the parasite to avoid the host's immune system. Chromosomal recombination in tick vectors throughout sexual reproduction is how *T. annulata* acquires its genetic variety (38). Factors like genetic drift and mutation strengthen their genetic variation. Because of this, creating control methods (such as vaccinations and pharmacological treatments) depends on parasite populations acquiring genetic diversity (39). The foundation for genetic differences and evolutionary links between species may be found through phylogenetic analysis. The piroplasm population has recently been studied using molecular markers, including 18S rRNA, ITS1, ITS2, and the Cyt b gene, to identify genetic associations between local and global isolates (40). Marker genes are essential tools for detecting the evolutionary connection between species because of the occurrence of both highly conserved and changeable areas of the genome (41,42). There are many *T. annulata* genetic diversity data from Iraq, especially Al-Hilla City. The current similarity between the present study isolates and the GeneBank isolates could be due to importing cattle infested with ticks from different countries to Iraq, such as India. It could be due to the travel of the tick vectors from different countries to Iraq via some tools, including migrating birds, in which new species of *T. annulata* might be brought in, and new genetic differentiation might occur in Iraq.

Conclusion

The present work demonstrates that cattle from Al-Hilla City, Iraq, were highly infected with *Theileria annulata* compared to those from other countries. The current study shows links between the current identified local and some global isolates of the protozoan.

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Conflict of interests

The authors have not received any funding or benefits from industry, agency of financing, or elsewhere to conduct this study.

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دراسة جزيئية والتحليل الجيني لطفيلي الثلاريا انيولاتا في الأبقار في مدينة الحلة، العراق

آيات قاسم جواد و منير عبد الأمير عبد الفتلاوي

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الخلاصة

تم تنفيذ العمل الحالي لكشف النقاب عن الوضع الحالي لعدوى طفيلي الثلاريا انيولاتا في الماشية في مدينة الحلة، العراق. في هذ الدراسة تم جمع إجمالي ٢٢٥ عينة دم (٢٠٠ من الحيوانات المشتبه بإصابتها و ٢٥ عينة من الحيوانات السليمة سريريا). تم إخضاع هذه العينات إلى الفحص المجهري وفحص تسلسل الحمض النووي، باستهداف جين السيتوكروم ب، لعشرة منتجات من تفاعل أنزيم البلمرة المتسلسل. أظهرت نتائج المسحة الرقيقة للحالات المشتبه بها البالغ عددها ٢٠٠ حالة إصابة ٦٣ (٣١,٥٪) بالثيليريا، بينما كانت ١١٥ حالة (٥٧,٥٪) مصابة بأنواع أخرى من العدوى الطفيلية في الدم. ومع ذلك، لم تظهر سوى ٢٢ حالة (١١٪) مشتبه فيها عدم وجود أي طفيليات. بالإضافة إلى ذلك، لم تظهر أي عدوى طفيلية في ٢٥ عينة دم. علاوة على ذلك، أظهر تسلسل الحمض النووي أن أنواع الثايليريا تنتمي إلى أنواع الانيولاتا، وكانت هذه التسلسلات تعتمد على تشابه القواعد النيتروجينية بين العزلات المحلية للدراسة الحالية وعزلات بنك الجينات من تونس (ON035604, ON035605, ON035606, ON035607, ON035608, ON035609, ON035610, ON035611, ON035612, and ON035613). تشير نتائج الدراسة الحالية إلى أن مرض الثايليريا هي العدوى الطفيلية السائدة في الماشية في مدينة الحلة وتسببها بشكل كبير نوع الثلاريا انيولاتا.

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