MOLECULAR AND PHYLOGENETIC STUDY OF *THEILERIA* SPP ISOLATED FROM TICKS IN AL-DIWANIYAH CITY, IRAQ

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ABSTRACT

The current protozoal study was intended to explore the presence and the evolutionary history of Theileria spp in ticks that infecte 150 out of 200 cows in Al-Diwaniyah City, Iraq. For these purposes, 10 ticks were collected from each cow, identified morphologically, and crushed for extract Theileria-based DNA from the tick tissues. The extracted DNA was examined by polymerase chain reaction (PCR) technique for molecularly identificationusing special primer of 18S rRNAgene. The PCR-targeted products of the 18S rRNA gene were subjected to sequencing for further confirmation of the diagnosis of the Theileria spp. The results revealed 2 distinct sequenced protozoa, SP1 and SP2. After performing database searching and comparing the current 2 isolates to some regional and global species, the results showed that the SP1 is closely related to Theileria lestoquardi, KP342263.1 and the SP2 is closely related to Theileria annulata, MF287951.1. The study reveals important information about the evolutionary history of these protozoa in Al-Diwaniyah City, Iraq.

Keywords: 18S rRNA, cow, PCR, protozoa, tree.
Part of M.Sc. thesis of first author

دراسة جزيئية ونسلية للـ *Theileria* spp المعزولة من قراد في مدينة الديوانية، العراق

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المستخلص

الهدف من دراسة الكائنات الاولية الحالية هو التحري بدقة عن التطور التاريخي لانواع ال *Theileria* في القراد المصيب ل 150 بقرة في مدينة الديوانية، العراق. لهذا الغرض جمعت 10 قرادات من كل بقره، وشخصت مظهريا، وهشمت لاستخلاص ال DNA الخاص ب ال *Theileria* من انسجة القراد. خضع ال DNA المستخلص لتقنية تفاعل البلمرة (PCR) لتحديد وجود هذا الكائن الاولي جزيئيا. عرضت نواتج ال PCR المستهدفة من جين ال 18S rRNA الى دراسة تعاقب القواعد النتروجينية لتأكيد تشخيص انواع ال *Theileria* . اظهرت النتائج تعاقبين لكائنين اوليين مستقلين، SP1 و SP2. بعد اجراء البحث في قاعدة البيانات للعزلتين ومقارنتهما مع بعض الانواع العالمية والاقليمية، بينت النتائج ان ال SP1 قريبة جدا لل Theileria lestoquardi (KP342263.1) وال SP2 قريبة جدا من Theileria annulata (MF287951.1) . اظهرت الدراسة معلومات مهمة عن التاريخ التطوري النوعي لسلالة ال *Theileria* في مدينة الديوانية، العراق.

كلمات مفتاحية: 18S rRNA ، الابقار، السلسلة البلمرة التفاعلية ، الاوالي، الشجرة.

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INTRODUCTION

Protozoan-based infection in animals is considered as a major-global health-problem that subsequently affects the food animal industries (11). *Theileria* spp is well-known for the world-wide spread of its infection that is responsible for a huge-economic loss in the food animal industries (10). The causative agent, *Theileria* spp, is transmitted to animals and causes a disease via blood-feeding ticks (6). The diseases in wild and domestic animals are caused by various species of *Theileria* that are vectored by wide range of ticks such as *Hyalomma* and *Rhipicephalus* (3). This microorganism is divided into two-schizont-based species depending on its transformation occurrence (16). Microscopic appearance and diagnosis of *Theileria* spp have been relied on how long time to diagnose the presence of *Theileria* via Giemsa-based staining of blood samples from suspected-infected animals with this protozoan. However; using such diagnostic technique may face difficulties and challenges of false-negative diagnosis especially in subclinical and carrier animals when low numbers of piroplasms may exist in blood samples of suspected-infected animals. Low-detailed diagnostic-methods such as microscopy may also have some obstacles especially when piroplasms of various species of *Theileria* have similar morphological features (14). Molecular methods such as PCR technique and sequencing of the target region in *Theileria* spp are important in reliable detection of the protozoan especially in low-number presence in blood samples of the expected-affected animals (4). Understanding the reality and correctness of diagnosis of specific-species existence in certain regions or countries needs deeper-detailed-diagnostic methods such as sequencing and phylogenetic analyses (5). For these reasons, this study employed PCR and partial-18S-rRNA-gene sequencing to study the existent *Theileria* species in Al-Diwaniyah City, Iraq.

MATERIALS AND METHODS

Sampling: Ten ticks were collected from infected cows (150 cows) from Al-Diwaniyah city –Iraq. The ticks were identified morphologically according to Shubber *et al*, (17) in the Iraqi Natural History Museum.

DNA extraction

The ticks were crushed to extract *Theileria*-based DNA from the tick tissues of 150 ticks. DNA of the tick tissue was extracted using a tissue-based protocol of gSYAN DNA mini extraction kit (Geneaid, USA). Briefly of pre-extraction preparation of the tissue samples, 200mg of tick tissues were placed in a 1.5-ml tube, and 200 µl of GST solution was added to the tube. Then, the tissues were crushed and homogenized using micropestle. The extracted DNA was tested using a NanoDrop to evaluate its quality and quantity.

Polymerase Chain Reaction and Gel Electrophoresis

Using AccuPower PCR PreMix Kit, the PCR mastermix was generated as follows: DNA template 5 µl, forward primer (10 pmol) 1.5µl: ATTGCTTGTGTCCCTCTGGG and reverse primer (10 pmol) 1.5µl: TCCACCAACTAAGAACGGCC, PCR water (12 µl) to bring the total volume up to 20 µl. The process was performed following the kit information. The primers were designed using NCBI website and primer 3 plus (Macrogen Company, Korea). Amplification aims to detect a PCR product at 620 bp of the 18S rRNA gene. The thermcycler conditions were initial denaturation as 1 cycle at 94℃ for 5min, 35 cycles of (denaturation at 94℃ for 30 sec-annealing at 58℃ for 30 sec-extension at 72℃ for 1min), and final extension as 1 cycle at 72℃ for 5min. Electrophoresis and 1.5% -Agarose gel were used to separate the bands at 100 volt and 80 amp for 1hour. These PCR products were evaluated using a UV imager.

18S rRNA partial sequencing

The PCR products of 5 ticks at 620 bp of the 18S rRNA gene were sent out for sequencing. The results were blasted against NCBI database to compare the resulted species with some global isolates. The phylogenetic tree was generated using MEGA 6.0 and following computing evolutionary distances via Maximum Composite Likelihood Method (15,18).

RESULTS AND DISCUSSION

The PCR results are shown in figure 1, and they revealed the positive product size at 620 bp. The results revealed 2 distinct sequenced protozoa, SP1 and SP2. After performing database searching and comparing the current

2 isolates to some regional and global species, the results showed that the SP1 is closely related to *Theileria lestoquardi* (KP342263.1) and the SP2 is closely related to *Theileria annulata* (MF287951.1), as shown in figure 2. The current study reveals important information about the history of this protozoan in Al-Diwaniyah City, Iraq.

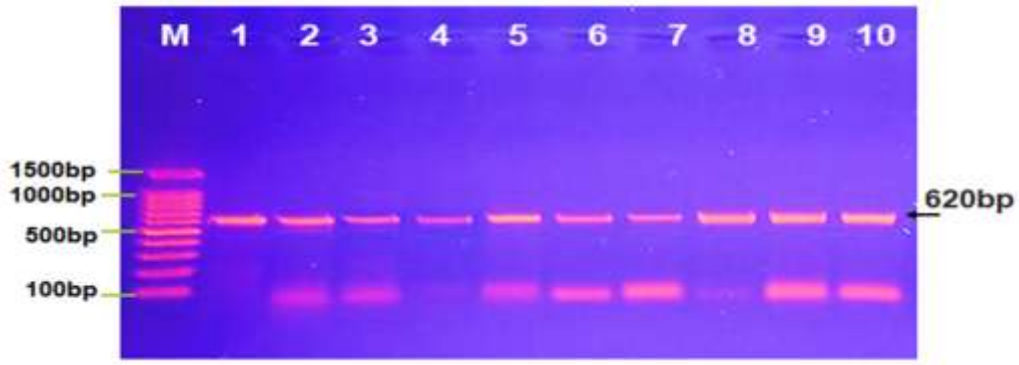

Figure 1. The image of the agarose gel electrophoresis of the 18S rRNA gene of *Theileria* sp. M is the ladder, 1500-100bp, and Lane, 1-10, are some of the positive samples. Product size is at 620bp

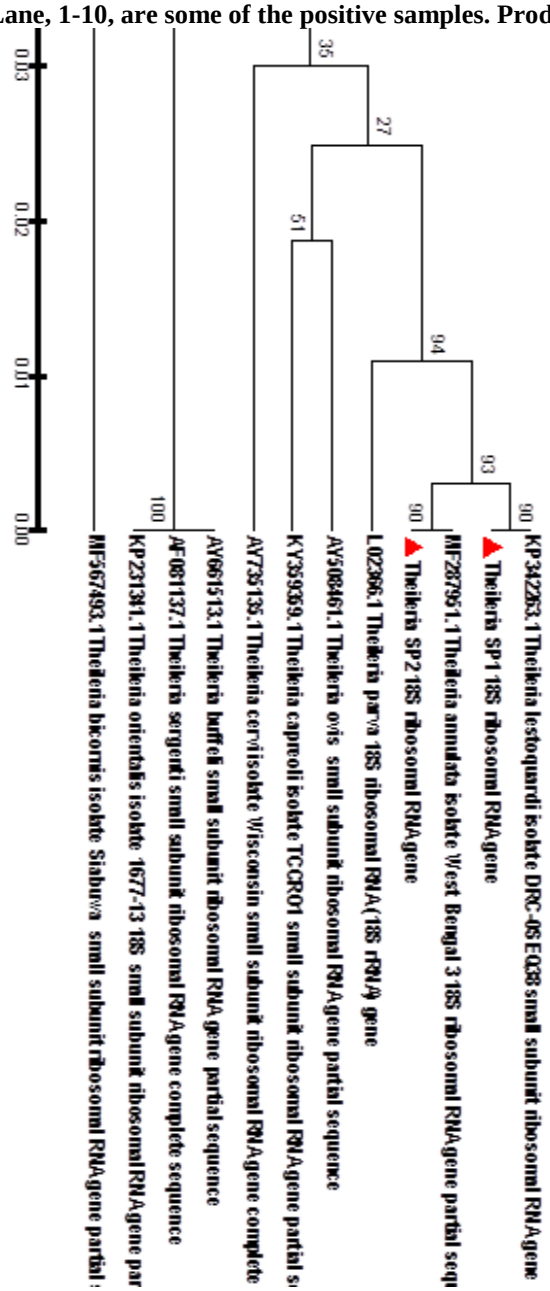

Figure 2. Phylogenetic tree based on the partial sequence of 18S rRNA gene in local *Theileria*-species isolates (SP1 and SP2)

The infections that are related to protozoa and more specifically *Theileria* spp are wide-spread tick-borne diseases. These disease conditions causes economic crisis worldwide (8). For better understanding of the *Theileria* spp in Al-Diwaniyah City, Iraq, partial sequencing of the 18S rRNA gene gave interesting information about the presence of 2 distinct species that branched separately in the phylogenetic tree. The results showed that the SP1 is closely related to *Theileria lestoquardi* (KP342263.1) which is an isolate from Duhok province, Iraq (NCBI base). The SP2 is closely related to *Theileria annulata* (MF287951.1) which is an isolate from India (NCBI base). It seems that our isolates have history of evolution from or related to Iraq and India isolates; however; these strains might have been originated from an isolate specific to certain regions of the world especially when look at the phylogenetic tree. Based on this look, a single-branched isolate (L02366.1) which had been studied in United Kingdom and neighbors our isolates in the phylogenetic tree indicates common identities between these strains. Our results agree with (2,9 and 12) who detected *Theileria* spp using molecular techniques in different various animal species. The results agree with (7) who detected the presence of this protozoan using PCR in ticks in Mauritania. The study also matches the result from (13,1) who recognized these parasites in ticks using PCR techniques. Our study provides accurate information about the evolutionary status of *Theileria* spp in Al-Diwaniyah City, Iraq. The current study also shows the huge role of ticks in transmitting *Theileria* spp between cows. This needs better understanding of the molecular-based life-cycle of this protozoan that could help in generating important, cost effective, and fast methods to control ticks and tick-borne diseases such as theileriosis. These results should be future-followed up to find suitable methods based on the current study findings to control these tick-borne protozoa in Iraq.

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