

Clinical and molecular identification of ruling *Theileria annulata* strains in cattle calves in Al-Diwaniyah province, Iraq

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Abstract

This study aimed to investigate the evolutionary status of *T. annulata* in Al-Diwaniyah province, Iraq. In this study, the clinical examination of 50 infected animals was performed with blood sample collection (2.5ml per animal), and drug targets cytochrome b, a vital component of the electron transfer chain in the mitochondria of the protozoan, *cytb* gene was targeted using a polymerase chain reaction (PCR) procedure. Also, *18S rRNA* gene as a molecular target for the PCR and a partial gene sequencing (PGS) were included. The PCR that involved using the *18S rRNA* and *cytb* genes as genetic targets revealed amplification of the targeted pieces at 620bp and 1092bp, respectively, in all tested samples. The 18S rRNA gene sequence of local *T. annulata* isolates were aligned with global reference strains for *T. annulata* recorded in the GenBank. The local strains were close, 100%, in their identity to isolates from Iran, Turkey, and Pakistan; however, they were 99% similar to a nucleotide sequences from India and Bangladesh. Diseased calves showed clinical signs such as high fever (40.3-41.5°C), decreased appetite or inappetence, asymmetrical enlargement of superficial lymph nodes particularly the pre-scapular ones, some cases with diarrhea, pale or icteric mucus membrane of eyes, bulging eyes, lacrimation, ecchymotic hemorrhages on the sclera, incoordination, nervous signs (Dullness, depression, lethargy), salivation, and bloated young calves. The data observed from the present inspecting work may reveal genetic evolution in the local strains with others recorded in the GeneBank. This means that our local strains might have close relationships with some global strains.

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Introduction

Theileria annulata is an important parasitic species responsible for economic crises leading to high global morbidity and mortality in animals (1,2). The genus *Hyalomma* is well-known for transmitting these protozoa leading to the induction of bovine tropical theileriosis (BTT) in the Middle East countries including Iraq (3,4). Molecular study of the tick *Hyalomma anatolicum* in Iraq have been recorded this spp. in Najaf city (5). The severity of clinical signs of tropical theileriosis depends on the type of animal breeds and on the age of animals; however, and

the most common signs are anorexia, high body temperature, superficial lymph node enlargement (especially the pre-scapular, submandibular, and pre-femoral lymph nodes), pale of mucus membranes at early stages followed by an icteration at the late stages, uncommon blood-stained diarrhea, dehydration, salivation, lacrimation, nasal discharge, nervous signs, and dyspnea (6,7).

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 (8). Many problems were shown due to the use of the smear methods that reflect

low sensitivity for the diagnosis of carrier animals (9). For a quick and accurate diagnosis, molecular methods such as PCR techniques have been employed for the identification of *Theileria* spp (10-12).

For treatment, buparvaquone, the most effective drug against bovine theileriosis (13,14), at 2.5mg/kg.bw intramuscular (IM) as a single dose (15). However, Tunisian and Sudan veterinarians have reported failures of this drug at the recommended dose (2) probably due to developed resistance in those protozoa against the drug (16). Previous studies showed that hydroxynaphthoquinone works via its binding to one of the electron transport chain components, cytochrome b (*cytb* gene), resulting in suppressing this vital biological process in the target parasite. Resistance to antiprotozoal drugs can be resulted from certain types of primary or secondary mutations or due to a complete gene deletion (17,18). The present work was aimed to investigate the evolutionary status of *T. annulata* in Al-Diwaniyah province, Iraq.

Materials and methods

Sample collection

Fifty crossbred calves (age at 15 days to 6 months old) were brought to the Veterinary Teaching Hospital, Al-Diwaniyah City, Al-Diwaniyah Province, Iraq. After clinical examination of the calves, signs of high rectal temperature, enlarged superficial lymph nodes, pale mucous membranes, and tick infestation were recorded. Then, 2.5ml of the jugular vein blood was collected from each calf clinically diagnosed with BTT using high aseptic conditions; EDTA-preloaded blood collecting tubes were utilized. The blood samples were employed in the direct blood smears and molecular analyses.

Microscopic examination

The blood films were Giemsa's-stained according to a method described by Al-Hosary and Nordengrahen (19). Briefly, the fixed smears were stained with 5% Giemsa diluted in phosphate buffer (pH 7.2). The analysis was performed by microscopic examination.

Genomic DNA extraction

The DNA was extracted as a genomic type from the 50 calf blood sample utilizing a blood DNA extraction Kit (Bioneer, Korea) with relying on the steps included in the kit package. The process was initiated with a Proteinase-K-based lysing step. DNA was Nano Drop (THERMO/USA) evaluated for its amount and purity and stored at -20°C until for next test accomplishment.

Polymerase Chain Reaction

The *cytb* (1092bp) gene primers for detecting the *cytb* gene were F: CAGGGCTTTAACCTACAAATTAAC while, R: CCCCTCC ACTAAG CGTCTTT CGACAC

ordered through Bioneer (Korea). The *18S rRNA*-(620bp) gene targeted designed set of primers (10pmol/each direction) for *T. annulata* (F: ATTGCTTGTGTCCCTCTGGG and R: TCCACCAACTAAGAACGGCC) were employed for the PCR technique. Applying the instructions of the AccuPower® PCR PreMix kit (Bioneer, Korea), the 20µl-PCR total volume mix was prepared by adding the primers (1.5ul of 10pmole) and 5µl of DNA into the kit tube, that contained components such as 1U DNA polymerase, 250µM of dNTPs, Tris-HCl (pH 9.0) 10mM, 30mM of KCl, 1.5mM of MgCl₂, which was completed by adding deionizer PCR water to reach the total volume. The reaction conditions in the thermocycler were the launching step of denaturation programmed at 95°C for 5mins for one cycle. Later, the 30 cycles of the reaction were set up for (the main denaturing process at 95°C for 30s, followed by an annealing step at 58°C for 30s, and the later step of the core extension at 72°C for 1min), and accomplished by applying the final extension process at 72°C for 5mins for one cycle. Electrophoresis was lasted for one hour at 80 volts and 100 am. The visualizing process of the PCR products was performed by placing an electrophoresed 1%-agarose gel treated with ethidium bromide, under a UV transilluminator.

DNA sequencing method

Four purified positive PCR products of the *18S rRNA* gene were F-primer-based sequenced by the AB DNA sequencing system (Bioneer Company, Korea) for doing the phylogenetic analysis represented by drawing the phylogenetic tree. The alignment processes and constructing the tree were conducted using the NCBI-Blast alignment and Neighbor Distance (MEGA v6), respectively.

Results

Results of clinical examinations

Diseased calves appear high fever 40.3-41.5°C, decreased appetite or in appetence, asymmetrical enlargement of superficial lymph nodes particularly the pre-scapular ones, some cases with diarrhea, pale and/or icteric mucus membrane of eyes, bulging eyes, lacrimation, ecchymosis hemorrhages on the sclera, incoordination, nervous signs as dullness, depression, lethargy, depression, lethargy in addition to salivation, presence of ticks, and bloated young calves (Table 1).

Microscopic examination

The microscopic examination of blood film stained with 5% Giemsa, showed *T. annulata* in the infected calves (Figure 1).

Table 1: Number and percentage of infected calves with *T. annulata* according to clinical signs

Clinical signs	Number with signs	%
Fever, decreased appetite or in appetence, asymmetrical enlargement of superficial lymph nodes, nervous signs and presence of ticks.	28/50	56
Fever, diarrhea, presence of ticks and bloated young calves.	13/50	26
Fever, bulging eyes, pale or icteric mucus membrane of eyes, lacrimation and presence of ticks.	7/50	14
Fever, incoordination, nervous signs, salivation and presence of ticks.	2/50	4

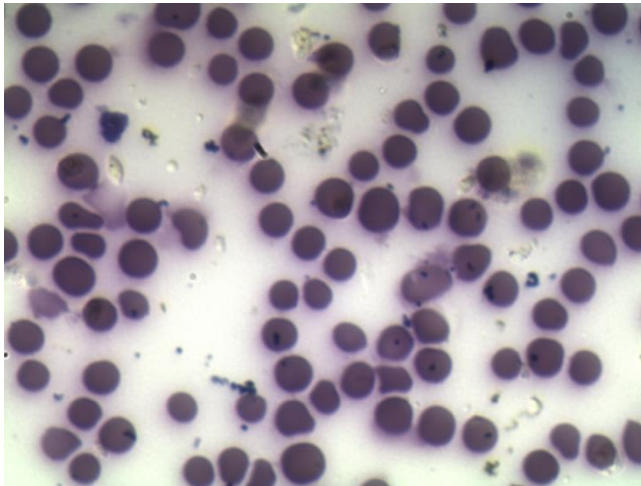


Figure 1: Microscopical image of blood stained film with giemsa showed *T. annulata cytb* in the RBC blood samples of calves, oil immersion (X1000).

PCR findings

For the *cytb* gene, the PCR results unveiled amplification of the genetic piece at 1092bp in all tested samples (Figure 2).

The PCR that involved using the *18S rRNA* gene as a genetic target revealed amplification of the targeted piece at 620bp in all tested blood samples (Figure 3).

Sequencing and phylogenetic tree construction of 18S rRNA gene

The *18S rRNA* gene of local *T. annulata* isolates were aligned with global reference strains for *T. annulata* recorded in the GenBank. The local strains were close, 100%, in their identity to isolates from Iran, Turkey, and Pakistan; however, they were 99% similar to a nucleotide sequences from India and Bangladesh, figure 4. The detected isolates of *T. annulata* were deposited in the GeneBank with the following accession numbers;

MN420930.1, MN420931.1, MN420932.1, and MN420933.1, table 2.

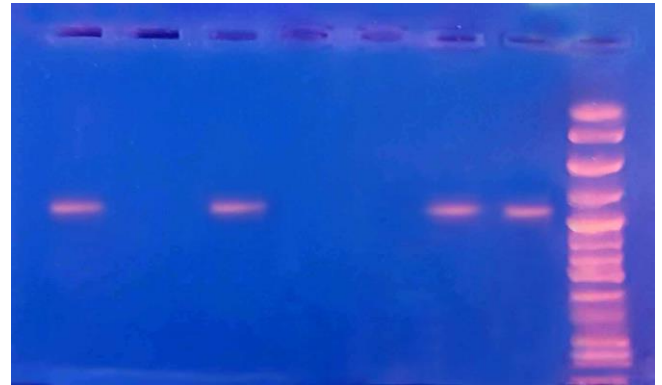


Figure 2: Electrophoresed image of 1% agarose gel with the PCR products of *T. annulata cytb* gene from blood samples of calves. M lane is the ladder (1500-100bp), Lanes (1 to 12) are positive samples at 1092bp.

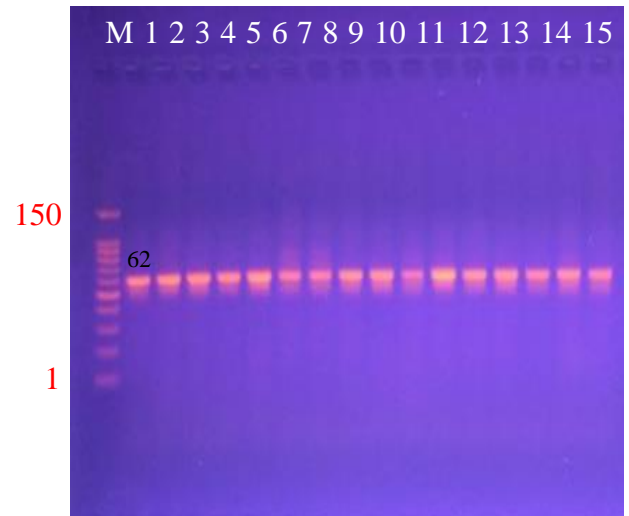


Figure 3: Electrophoresed Image of 1% agarose gel with the PCR products of *T. annulata 18S rRNA* gene from DNA samples of calves. M lane is the ladder (1500-100bp), Lanes (1 to 16) are positive samples at 620bp.

Table 2: Local *T. annulata* isolates with their NCBI-Genbank accession numbers

<i>T. annulata</i> isolates	Accession number
<i>T. annulata</i> IQ.1	MN420930.1
<i>T. annulata</i> IQ.2	MN420931.1
<i>T. annulata</i> IQ.3	MN420932.1
<i>T. annulata</i> IQ.4	MN420933.1

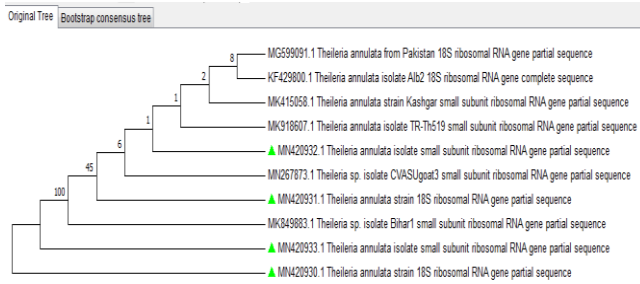


Figure 4: Phylogenetic tree analysis based on 18S rRNA gene in local *T. annulata* isolates (green triangles). The UPGMA method in MEGA v6.0 was used to calculate the evolutionary distances.

Discussion

The present investigation study findings showed the presence of the *T. annulata* in the tested blood samples of the calves. Moreover, four different isolates were detected via the use of the partial gene sequencing analysis. There are increasing incidences when the drug treatment failure is reported when used against *T. annulata*, in which a study by Mhadhbi *et al.* (16) who unveiled that the occurrence of mutations at the drug-binding site including changing some of the amino acids residues with different ones may introduce incomplete binding of the drug to the CYTB components leading to decrease the activity or complete failure in the activity of the drug in deactivating the electron transfer chain of the parasite mitochondria. In addition to those mutations at the binding site, drug inefficiency was also discovered to be due to the occurrence of mutations at the binding-site-neighboring regions of the CYTB protein which can also enhance the failure of the drug actions in defeating the parasite (16,20).

It has been suggested that increasing the occurrence of the BTT due to *T. annulata* may increase the percentage of developing mutations in the above mentioned sites or any other structural regions of the protein leading to the failure of the drug in treating the disease, and this supports the idea that our strains, since Iraq is an endemic area with the disease, might have evolved resistance against the drug due to the mass use of the drug in the field. This is supported by a study performed in Sudan by Chatanga *et al.* (21) who showed that multiple-point mutations in the *cytb* gene may have been responsible of appearing resistance of the parasite against buparvaquone.

The data observed from the present inspecting work may reveal genetic evolution of the local strains with more confidence that our local strains might have been sisters or cousins to global strains that carry parasitic resistance against buparvaquone.

Conclusion

To investigate the evolutionary status of *T. annulata* in Al-Diwaniyah province, Iraq. We collect 50 blood samples for Molecular identification of *T. annulata* between cattle calves. The results reveal genetic evolution in the local strains with others recorded in the GeneBank. This means that our local strains have close relationships with some global strains.

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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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التعريف السريري والجزئي لسلاسل ثايليريا الحلقيّة في عجول الماشية في محافظة الديوانية، العراق

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الخلاصة

هدفت هذه الدراسة إلى دراسة الحالة التطورية للثايليريا الحلقيّة في محافظة الديوانية، العراق. في هذه الدراسة، تم إجراء الفحص السريري لخمسون عجلاً مصاباً عن طريق جمع عينة دم (2,5 مل لكل حيوان). أيضاً، كما تضمن البحث فحص تفاعل السلسلة المتبلّمة لجينات الرنا الريباسومي 18 كهدف وتسلسل الجينات الجزئي. كشف فحص تفاعل السلسلة المتبلّمة الذي تضمن استخدام جينات الرنا الريباسومي 18 و cytb كأهداف وراثية تضخيم القطع المستهدفة عند 620 نقطة أساس و 1092 نقطة أساس، على التوالي، في جميع العينات التي تم اختبارها. تم الاستقصاء عن تسلسل جين الرنا الريباسومي 18 لعزل *T. annulata* المحلي مع السلالات المرجعية العالمية إلى *T. annulata* المسجلة في بنك الجينات. كانت السلالات المحلية متقاربة بنسبة 100% في هويتها لعزلة من إيران وتركيا وباكستان. ومع ذلك، كانت 99% مماثلة لسلسلة النيوكليدات من الهند وبنغلاديش. تظهر العجول المصابة علامات سريرية مثل ارتفاع في درجة الحرارة 3,40-5,41 درجة مئوية، انخفاض الشهية أو انعدامها، تضخم غير متمائل في الغدد الليمفاوية السطحية وخاصة تلك التي تسبق الكتف، بعض الحالات تصاحبها إسهال أو غشاء مخاطي شاحب أو مصفر للعيون، عيون منتفخة، تسمم، بقع نزيف في العين وخلل في الحركة وأعراض عصبية كالتحسس، والاكنتاب، والخمول، وزيادة إفراز اللعاب، والعجول تعاني من الانتفاخ. كشفت البيانات التي تم رصدها من أعمال الفحص الحالية عن التطور الوراثي للسلالات المحلية مقارنة مع سلالات أخرى مسجلة في بنك الجينات. هذا يعني أن سلالاتنا المحلية قد تكون لها علاقات وثيقة مع بعض السلالات العالمية.