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Detection of the cytochrome B (cytb) insecticide resistance gene in Theileria annulata isolated from cattle in Hilla city, Babylon governorate, Iraq

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Abstract

Clinical features, lesions, and light microscopy of thin blood smears can be used to identify Theileria infection, but the structural similarity to piroplasm poses a challenge in identification under the microscope, so the use of PCR gives accuracy to the diagnosis. The current study was conducted to identify the presence of the cytochrome B (*cytb*), an insecticide resistance gene, in *Theileria spp* isolated from infected cattle in Al-Hilla City, Babel, Iraq. Based on a previous microscopy identification, 63 infected blood samples with *Theileria* spp were subjected to an *18S rRNA* and *cytb-based* polymerase chain reaction (PCR) technique to identify species and the presence of the resistance gene, respectively. The results of the *18S rRNA*-PCR revealed that 45 (71.4%) of the samples were infected with *Theileria annulata*. The finding of the *cytb*-PCR demonstrated that 37 (82.2%) of the *T. annulata* infected samples carried the *cytb* resistance gene in their genomes, according to the amplified 1092bp-piece. The fragment of 10 *cytb*-PCR products was DNA-sequenced, showing close identity to NCBI-sequences isolated from different parts of the world, such as Tunisia. The current findings recorded a high presence rate of the cytochrome B (*cytb*) insecticide resistance gene in *Theileria annulata* species isolated from cattle in Al-Hilla City, Babel, Iraq.

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Introduction

Regarding Iraqi ruminants, both large and small, *T. annulata* is the most common pathogenic *Theileria* species. The evaluation of illness with *Theileria* protozoa can be made through the use of case history, clinical manifestations, lesions, and light microscope inspection of thin blood smears; however, there are some challenges linked to microscopic identification, such as the structural similarity of piroplasms, unrealistic results due to the few numbers of the protozoa (especially in carrier cattle), and complexity in the identification of mixed infections (1). Thus, sensitivity and specificity in diagnostic techniques, such as polymerase chain reaction (PCR), might be used to diagnose and differentiate between several species of piroplasms (2,3). Livestock farmers suffer significant financial losses due to tick-borne illnesses. Recent research suggests that climatic and environmental changes are increasing the prevalence of ticks and tick-borne diseases, impacting domesticated ruminants and people, resulting in yearly losses of USD 13.9 to 18.7 billion (4). Cattle in tropical and subtropical regions are particularly vulnerable to theileriosis, a disease transmitted by ticks and characterized by several different types of the microorganism *Theileria*. Disease epidemics, high levels of fatality and morbidity, lower productivity, and consequentially significant economic losses are caused by certain of these species (5-7). The expenses of diminished milk supply, weight loss, chemical pesticide treatments, and vaccination were all included. Tropical theileriosis (caused by *Theileria annulata*) is thought to threaten over 250 million cattle globally. Funding in studies into effective control measures for theileriosis is essential to lowering cattle production losses and ensuring food and nutritional security throughout the globe (8-10). *Theileria* protozoa

(Piroplasmida, Theileridae family) infect a wide variety of domesticated and non-domesticated animal species and are delivered by the Ixodidae tick genera, such as *Haemaphysalis* and *Hyalomma* (11-13). The disorders induced by *Theileria* spp. have gained more and more attention in recent years, partly because diagnostic techniques have become more reliable. *T. annulata*, the causative agent of tropical theileriosis in cattle, induces a proliferative response of the immune system to the invading pathogen, accompanied by fever, lymphadenitis, and anemia. Without treatment, infected animals often die within a month. This condition also causes a considerable decline in animal reproduction (14-16). Theileriosis is one of the most frequently seen tick-borne illnesses in Babel province, with yearly epidemics being reported on several occasions. The present management approaches include the employment of acaricides for vector reduction, treatment with buparvaquone, and immunization. The effectiveness of the therapeutic interventions relies on the timeliness of administering the pharmacological intervention. Because molecular technologies, including PCR, enable immediate detection of the pathogen and swiftly commencing control efforts, molecular biology provides a very effective diagnostic tool to distinguish the illness (17-19). Also, developing methods for analyzing sequencing data has facilitated better pathogen detection and characterization. According to the consistent activity and architecture, the *18S rRNA* component is the most common genetic marker for detecting species levels and phylogenetic study in eukaryotic species based on the presence of hypervariable regions in this gene. It is commonly used to detect evolutionary trends and similarities across *T. annulata* species (15,20-23). This illness is common in many parts of the world, including North Africa, Southern Europe, the Middle East, and Asia. Exotic cattle species have a 40-60% mortality rate due to illness, but domestic cattle only suffer a lower mortality rate (24). There are now three primary approaches used in the fight against tropical theileriosis. The first is the pricey and controversial topic of preventing the spread of vector ticks, which poses risks to both human health and the environment. The other involves immunizing cattle with infected cell lines that have been proven less infectious by prolonged in vitro cultivation. Long-term vaccine efficacy in endemic places is controversial, and the impact of immunization on parasite diversity in the field is unclear (25). The third and most common strategy involves administering the hydroxynaphthoquinone antiprotozoal medication buparvaquone to animals suffering from acute infections. The condition has been treated with buparvaquone since the 1980s, and it is the only medicine shown to be particularly advantageous against *Theileria* spp. Nevertheless, other countries, particularly Tunisia, Iran, Sudan, and Egypt, have documented a rise in the percentage of buparvaquone therapy failure. The process of buparvaquone resistance and its action method remain uncertain (26-30). Evidence for the 1,4-naphthoquinone family of hydroxynaphthoquinones and the similar medication atovaquone supported the hypothesis that buparvaquone interrupts the mitochondrial electron transport chain at the cytochrome bc1 complex. Mutations in the Cyto b gene provide resistance to the medication, as shown in investigations of *Plasmodium falciparum*, *Toxoplasma gondii*, and *Pneumocystis carinii* (24).

The current study identified the presence of cytochrome B (*cytb*), an insecticide resistance gene, from *Theileria spp* isolated from infected cattle in Al-Hilla City, Babel, Iraq.

Materials and methods

Ethical approve

All the authors of the present work ensure that all procedures of our experiment were performed under the Ethical Norms approved by the scientific board of the College of Veterinary Medicine, University of Al-Qadisiyah (committee approval number 1314 on 18/10/2022).

Samples

The current study identified the presence of cytochrome B (*cytb*), an insecticide resistance gene, from *Theileria spp* isolated from infected cattle in Al-Hilla City, Babel, Iraq. The jugular vein of cattle was used to collect the blood samples (2ml/each), utilizing clean, sterile EDTA-containing tubes, which were immediately ice-packed and transported to the College of Veterinary Medicine/University of Al-Qadisiyah laboratory. The study lasted from September 2021 to February 2022. Based on a previous microscopy identification, 63 infected blood samples with *Theileria* spp were subjected to an *18S rRNA* and *cytb-based* PCR technique to identify species and the presence of the resistance gene, respectively.

Extraction of DNA

The DNA was extracted from the cattle blood samples by employing the DNA extraction kit (Geneaid, Korea) and following the kit instructions. After the extraction, the DNA was evaluated as in the cases of its purity and concentration using a NanoDrop. The DNA was -20ºC-stored for later analyses.

18S rRNA and *cytb-based* polymerase chain reaction

The total reaction volume for the detection of the target genetic pieces was 20µl, which included 10µl green master mix 2X Kit (Promega, USA), 1µl from each of the upstream primer or downstream primer (F: GAGACAAGGAATATTCTGAGTCC and R: TTA AGT GGC ATA TAA TGA CTT AAGC) (Promega, USA) (21) of the *18S rRNA* gene (F: GAG ACA AGG AAT ATT CTG AGT CC and R: TTA AGT GGC ATA TAA TGA CTT AAGC) (Promega, USA) (31) and the *cytb* gene (F: CAG GGC TTT AAC CTA CAA ATT AAC and R: CCCCTCCACTAAGCGTCTTTCGACAC) (32), 2µl of

extracted DNA, 5.5µl for-molecular-use-water, and 0.5µl $MgCl_2$. The thermocycler conditions were one cycle of 95ºC at 5mins, 39 cycles of (95ºC at 35s, 57ºC at 35s, and 72ºC at 40s), and one cycle of 72ºC at 5mins for the initial denaturation (denaturation, annealing, and extension), and final extension, respectively. A 1.5% agarose gel electrophoresis at 100 volts and 80A for one hour was performed to run the PCR products, which were UV-visualized later.

Partial *cytb* gene sequencing

Ten purified PCR products of the *cytb* gene of the *Theileria annulata* isolates were sent out for sequencing at Macrogen Company in Korea. The Phylogenetic tree was established using multiple tools, including the NCBI-nucleotide-based websites and the Mega X version (33,34).

Results

The results of the *18S rRNA*-PCR revealed that 45 (71.4%) of the samples were infected with *T. annulata*. The finding of the *cytb*-PCR demonstrated that 37 (82.2%) of the *T. annulata* infected samples carried the *cytb* resistance gene in their genomes, according to the amplified 1092bp-piece (Figures 1 and 2). The fragment of 10 *cytb*-PCR products was DNA-sequenced, showing close identity to NCBI-sequences isolated from different parts of the world, such as Tunisia (Figure 3).

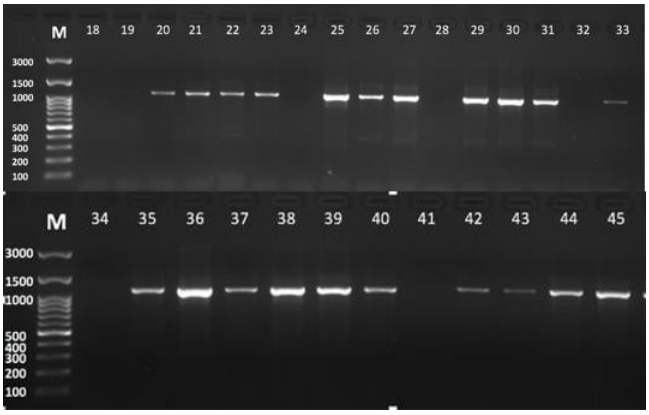

Figure 1: PCR products of the *Theileria annulata cytb* gene from blood samples of cattle. M: ladder, PCR products at 1092bp.

Discussion

During the last few years, researchers have employed a range of genetic biomarkers, including the *18S rRNA* and *cytb* genes, to establish the evolutionary connection between the various piroplasms. Using the cytb gene in phylogenetic studies at the family and genus levels is normal practice. The current research employed *cytb* gene sequencing to generate a phylogenetic tree connecting several pathogens isolates of *Theileria* from around the world (35,36).

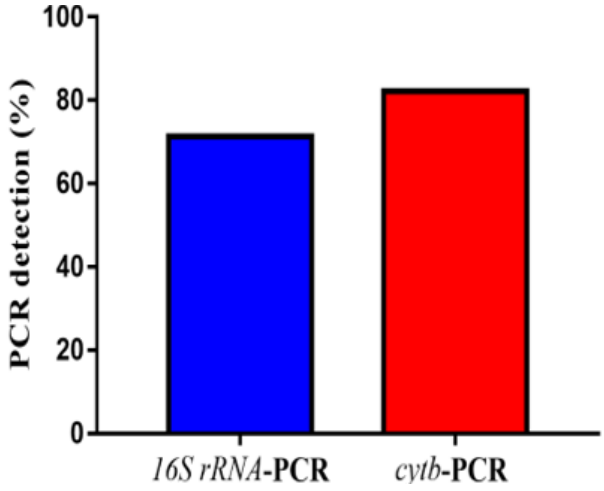

Figure 2: The rate of detection of both PCR methods of the *Theileria annulata 16S rRNA* gene and *cytb* gene from blood samples of cattle.

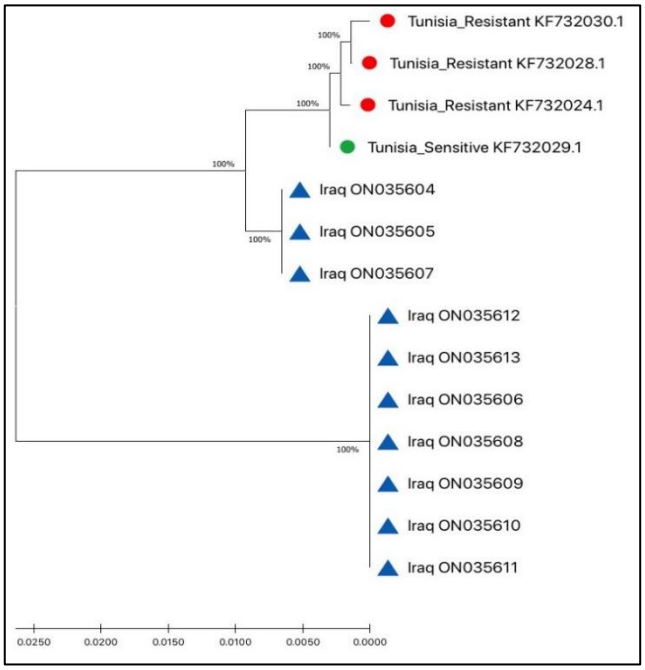

Figure 3: Phylogenetic tree of the *Theileria annulata cytb* gene from blood samples of cattle (Blue triangle).

The current study findings reflect that the local isolates of *T. annulata* were in genetic matching with those from the world, such as from Tunisia. Some studies from neighboring countries, such as Iran, revealed genetic identities closely related to those isolated from Turkey, India, Sudan, and Africa (1).

Our findings demonstrated that the *cytb* sequencing could efficiently recognize the difference between *Thieleria* spp. in various lineages, even though most phylogenies of *Theileria* have relied on the 18S rRNA gene, which contains a large region of conserved fragments that does not very often make a distinction among tightly connected organisms (37).

Modifications in the *ctyb* gene of *T. annulata* populations obtained from clinical incidences of therapeutic failures in countries such as Iran, Tunisia, and Sudan have previously been demonstrated employing low throughput sequencing techniques. However, some studies used high-throughput sequencing to find 14 mutations in the gene of *T. annulata* isolated from isolates with treatment-resistant infections in the endemic area of Tunisia. Similar alterations were discovered in the binding sites, which provide the genetic connection between polymorphisms and the buparvaquone drug in *T. annulata* and the high throughput sequencing investigation of *cytb* loci implicated in the evolution of buparvaquone resistance (38).

The antiprotozoal buparvaquone is effective against theileriosis but is not permitted to treat cattle in many nations. This includes several European countries. Treatment with oxytetracycline, imidocarb, halofunginone, or erythromycin for theileriosis has shown little success. Additional medications may be prescribed based on the variety of clinical symptoms. For instance, Meloxicam or Paracetamol could be prescribed for overheating animals. Sodium acid phosphate helps lame animals get up and move around. Blood transfusions may help severely anemic animals by relieving their symptoms (39-47).

Conclusion

The current findings recorded a high presence rate of the cytochrome B (*cytb*) insecticide resistance gene in *Theileria annulata* species isolated from cattle in Al-Hilla City, Babel, Iraq.

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Conflict of interests

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

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الكشف عن الجين المقاوم لمبيدات السيتوكروم ب في الثيليرية الحلقية المعزولة من الأبقار في مدينة الحلة، محافظة بابل، العراق

آيات قاسم جواد و منير عبد الأمير الفتلاوي

فرع الأحياء المجهرية البيطرية، كلية الطب البيطري، جامعة القادسية، الديوانية، العراق

الخلاصة

يمكن استخدام المظاهر السريرية والآفات والمجهر الضوئي للطاخات الدموية الرقيقة لتحديد عدوى الثيليريا، لكن التشابه البنيوي مع البيروبلازما يشكل تحديًا في تحديد الهوية تحت المجهر، لذا فإن استخدام تفاعل البوليميراز المتسلسل يعطي دقة في التشخيص. أجريت الدراسة الحالية للتعرف على وجود جين السايتوكروم ب، وهو جين مقاوم للمبيدات الحشرية، في الثلاريا الحلقية والمعزولة من الماشية المصابة في مدينة الحلة، محافظة بابل، العراق. في هذه الدراسة، واعتمادا على فحص مجهري سابق، فحصت ٦٣ عينة دم مصابة بال الثلاريا الحلقية بتقنية تفاعل أنزيم البلمرة المتسلسل المستهدف لكل من جيني الثماني عشرة الرايبوسومي والسايتوكروم لتحديد الأنواع ووجود جين المقاومة، على التوالي. أظهرت نتائج التفاعل الإنزيمي للبلمرة المتسلسل لجين الثماني عشرة الريبوسومي أن ٤٥ (٧١,٤٪) من العينات كانت مصابة بالثيلاريا الحلقية. أظهرت نتائج التفاعل الانزيمي للبلمرة المتسلسل لجين السايتوكروم بي أن ٣٧ (٨٢,٢٪) من العينات المصابة بالثيلاريا الحلقية أنها تحمل الجين المقاوم السايتوكرومي، وفقًا للقطعة ١٠٩٢ قاعدة جينية المضخمة من الجين. تم دراسة تعاقب القواعد النيتروجينية لهذه القطعة من ١٠ من منتجات التفاعل الإنزيمي للبلمرة المتسلسل لجين السايتوكروم والذي أظهر هوية للعزلات المحلية قريبة لتسلسلات مخزنة في بنك الجينات الأمريكي والمعزولة من أجزاء مختلفة من العالم، مثل تونس. سجلت النتائج الحالية نسبة عالية من تواجد الجين المقاوم للمبيدات الحشرية السايتوكروم بي في أنواع الثيلاريا الحلقية المعزولة من الماشية في مدينة الحلة، محافظة بابل، العراق.