

Phylogenetic study of *Theileria lestoquardi* based on 18SrRNA gene Isolated from sheep in the middle region of Iraq

M.J.A. Alkhaled*, N.N. A'aiz and H.H. Naser

Department of Microbiology and Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Qadisiyah, Iraq *(mansoor.ali@qu.edu.iq)

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Abstract

Theileriosis is parasitic infection causes by obligate intracellular protozoa of the genus *Theileria*. *T. lestoquardi* is the most virulent species in sheep and goats which causes a severe disease with a high morbidity and mortality rate. In this study the phylogenetic relationships between two local isolate of *T. lestoquardi* and nine *T. lestoquardi* global isolates as well as *Babesia ovis* out-group isolate were analyzed using the 18S rRNA gene sequence. The multiple sequence alignment analysis and neighbor joining phylogenetic tree analysis were performed by using ClustalW multiple sequence alignment online based analysis of 1098bp 18S rRNA gene was amplified by polymerase chain reaction. Phylogenetic analysis results of these gene sequences revealed that *T. lestoquardi* local isolates were closely related to *T. lestoquardi* Iran isolate (JQ917458.1) and two Iraq Kurdistan isolates (KC778786.1 and KC778785.1) more than other countries. This study represents the first report on the use of molecular phylogeny to classify *T. lestoquardi* obtained in Middle Region of Iraq.

Keywords: *Theileria*, Phylogenetic Analysis, Ovine, PCR, 18S rRNA gene, Iraq

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دراسة الشجرة الوراثية لطفيلي *Theileria lestoquardi* للجين 18S rRNA المعزول من الاغنام في المنطقة الوسطى من العراق

منصور جدعان على الخالد، نعمان ناجي عايز و حسن حاجم ناصر

فرع الاحياء المجهرية والطفيليات، كلية الطب البيطري، جامعة القادسية، القادسية، العراق

الخلاصة

يعد داء الثايليريا من الامراض المتسبب عن طفيلي من جنس *Theileria*، وهي طفيليات من الاوالي داخل خلوي اجبارية المعيشة ويعتبر النوع *T. lestoquardi* والذي يصيب الاغنام والماعز من الانواع الاكثر ضراوة كما ينتج عنه مرضا شديدا مع ارتفاع في نسبة الاصابة والهلاكات. تناولت هذه الدراسة تحليل علاقات النشوء والتطور بين اثنين من العزلات المحلية من *T. lestoquardi* وتسعة من العزلات العالمية *T. lestoquardi* وكذلك *Babesia ovis* خارج المجموعة باستخدام تتابع الجين 18S rRNA. استخدم تحليل الترتيب الجيني المتعدد وشجرة العلاقة الوراثية الجينية بواسطة برنامج ClustalW عبر الانترنت اعتمادا على الناتج 1098 زوج قاعدي للجين 18S rRNA نتيجة تفاعل السلسلة المتبلعمة التقليدي. بينت نتائج دراسة شجرة العلاقة الوراثية الجينية للجين 18S rRNA ان العزلات المحلية لطفيلي *T. lestoquard* كانت متطابقة تماما مع العزلة الايرانية لطفيلي *T. lestoquard* والتي تحمل الرقم التسلسلي (JQ917458.1) وكذلك كانت متطابقة مع العزلتين من اقليم كردستان العراق ذات الارقام التسلسلية (KC778786.1 and KC778785.1) اكثر من بقية عزلات بعض الدول التي استخدمت للمقارنة في التحليل. وتعد هذه الدراسة هي الاولى من نوعها في استخدام الشجرة التطورية الجينية الوراثية لتصنيف *T. lestoquardi* المعزول من المنطقة الوسطى في العراق.

Introduction

Tick-borne protozoan parasites of the genus *Theileria* infect wild and domestic ruminants in the tropical and subtropical regions of the world. Although *Theileria* infection in cattle has been extensively studied little is known about theileriosis in sheep (1). Recently, interest has arisen in sheep-infecting *Theileria* parasites. Among known *Theileria* parasites of sheep, *T. lestoquardi* and *Theileria spp.* from North China are considered highly pathogenic. The other species, *Theileria ovis*, *Theileria separata* and *Theileria recondita* cause subclinical infection in small ruminants (2). In order to prioritize future research on the development of improved control measures against tick-borne diseases, it is essential to define the prevalence of tick borne pathogens in target populations (3). The precise identification of these organisms is essential to understand their epidemiology and classification. The methods traditionally used to detect and identify these hemoparasites consist of microscopic examinations of thin blood smears and serological tests. In contrast to these conventional methods, the application of molecular techniques would allow direct, specific and sensitive detection of parasites, and rapid, simultaneous detection and differentiation of different *Theileria* infecting a given animal (4). The tools of molecular biology are increasingly relevant to veterinary parasitology. The techniques used with eukaryotic cells are generally applicable to the study of parasites and their hosts. The sequencing of the complete genomes of helminthes and protozoa is allowing great advances in studying the biology, and improving diagnosis and control of parasites, especially sequence data analysis allowed the researchers to identify and characterize the hemoparasites species in particular *Theileria* group (5-6). The comparison of srRNA has been introduced for deducing phylogenetic relationships of the Piroplasmidia and other Apicomplexa (7-8). These studies on srRNA confirmed that the pathogenic and benign *Theileria* parasites existed in many areas of the world and defined their phylogenetic relationship. Nevertheless, the status of *Theileria spp.* of the Iraq remained unclear.

Aim of study: In this study the small subunit ribosomal RNA genes were examined and analyzed for construction of phylogenetic trees of Iraqi *Theileria* parasite in comparison to those of other *Theileria spp.*

Materials and methods

Samples collections

43 blood samples collected from sheep clinically infected by theileriosis in Wasit province and placed in anticoagulant tubes, then transported to laboratory and stored in refrigerator until genomic DNA extraction step.

Genomic DNA Extraction

Genomic DNA was extracted from frozen blood by using (Genomic DNA Mini Kit, Geneaid. USA). The extraction was done according to company instructions by using frozen blood extraction Protocol method with Proteinase K. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, and then stored at -20°C at refrigerator until used in PCR amplification.

Polymerase Chain Reaction (PCR)

PCR assay was carried out by using specific primer which was designed by (7) to amplify a 1098bp fragment of highly conserved regions of 18S ribosomal RNA gene in all *Theileria spp.* 18SrRNA forward primer (AGTTTCTGACCTATCAG) and 18SrRNA Reverse primer (TTGCCTTAAACTTCCTTG) were provided by (Bioneer company. Korea). Then PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250 µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, MgCl₂ 1.5 mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20 µl total volume by added 5 µl of purified genomic DNA and 1.5 µl of 10 pmole of forward primer and 1.5 µl of 10 pmole of reverse primer, then complete the PCR premix tube by deionized PCR water in to 20 µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Techne TC-3000. USA) by set up the following thermocycler conditions; initial denaturation temperature of 95°C for 5 min; followed by 30 cycles at denaturation 95°C for 30 s, annealing 55°C for 30 s, and extension 72°C for 30 s and then final extension at 72°C for 7 min. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

DNA sequencing method

DNA sequencing method was performed for confirmative detection and Phylogenetic relationship of *T. lestoquardi* based on 18SrRNA gene by Phylogenetic tree analysis using ClustalW multiple sequence alignment program. 1098bp PCR product was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). The purified 18S rRNA gene PCR product samples were sent to Bioneer Company in Korea for performed the DNA sequencing using 18SrRNA forward primer by (AB DNA sequencing system).

Results

Out of 43 blood samples of clinical suspected theileriosis were tested by conventional PCR assay, only 10 samples which appeared positive for *Theileria spp.* at

1098bp PCR product of 18S rRNA gene on agarose gel electrophoresis (Fig. 1). Sequence analysis of two samples positive for *T. lestoquardi* was performed to confirm the PCR results. These sequences for the 18S ribosomal RNA genes of the pathogens can be found under the accession numbers KJ024366 and KJ024367 at NCBI –GenBank submissions. The DNA sequencing analysis of 18S rRNA gene 1098bp PCR product by multiple sequence alignment (ClustalW2) showed specific detection of *T. lestoquardi* Where, the results of Phylogenetic sequence alignment of

(*Theileria lestoquardi* local isolate) was 99% identity to *Theileria lestoquardi* strain (KF429800.1) more than other *Theileria spp.* as following (Fig. 2). The phylogenetic tree was constructed based on the two *T. lestoquardi* Iraq isolate, seven *T. spp* and out group sequences including *Babesia ovis* (Fig. 3) The result of current study was refer to that the Iraq isolate more closely related to Iran_1 (JQ917458.1) more than other of countries included in phylogenetic tree (Fig. 4).

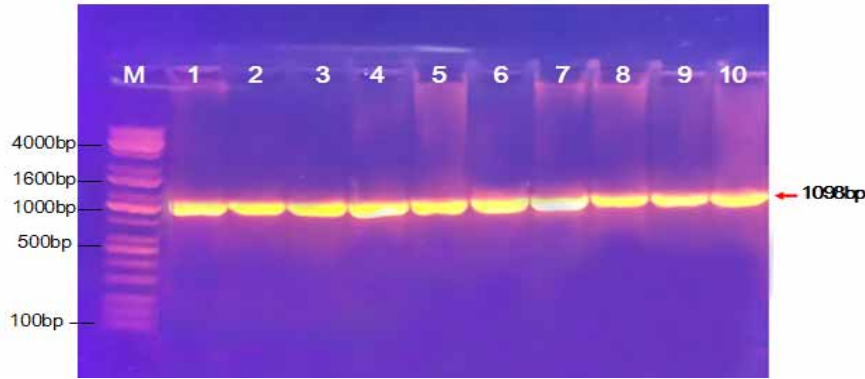


Figure 1: Agarose gel electrophoresis image, which show the PCR product results for *Theileria spp* of 18S rRNA gene at 1098bp PCR product size, where M: Marker 100bp, Lane (1-10) are positive samples.

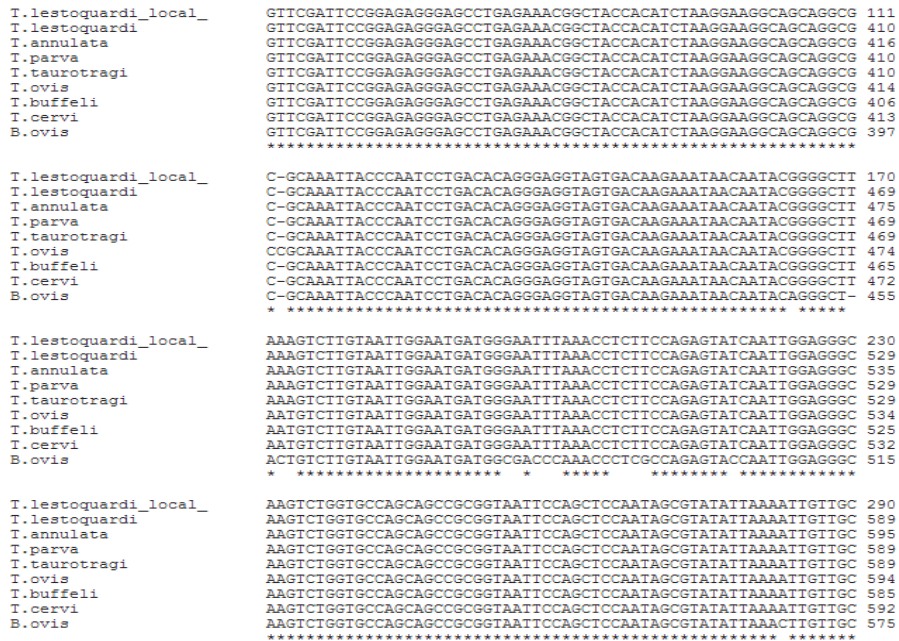


Figure 2: Multiple sequence alignment analysis of 18S rRNA gene 1098bp PCR product (A8) isolate with standard *Theileria spp.* strains *T. lestoquardi* (AF081135.1), *T. annulata* (KF429800.1), *T. parva* (AF013418.1), *T. taurotragi* (L19082.1), *T. buffeli* (FJ426360.1), *T. ovis* (AY260172.1), *T. cervi* (AY735129.1) and *B. ovis* (AY533146.1) by CLUSTAL 2.1 multiple sequence alignment.

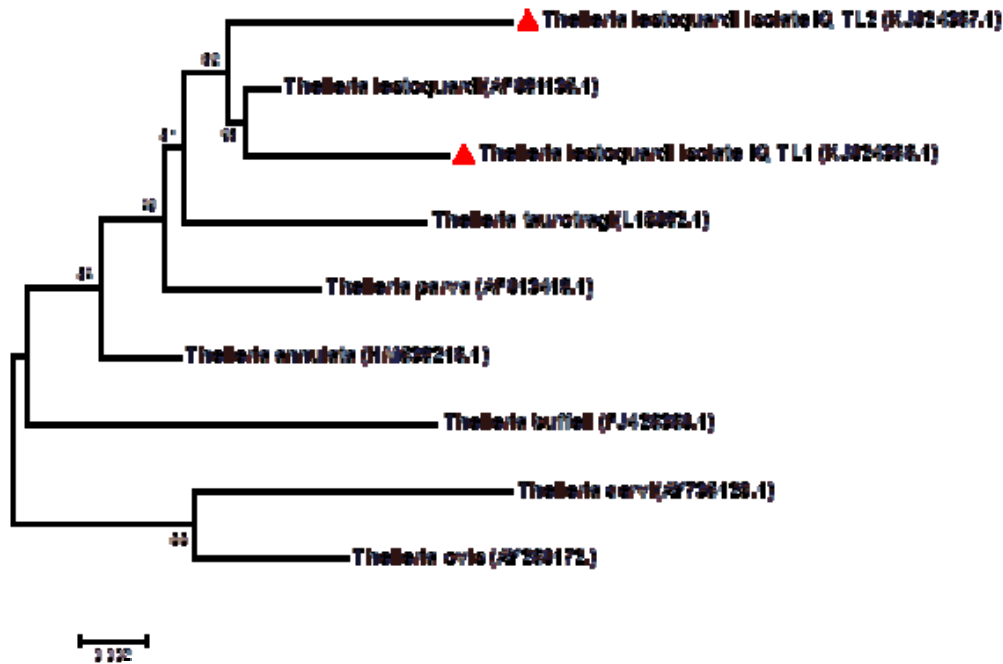


Figure 3: Phylogenetic analysis of 18S rRNA gene of two *T. lestoquardi* Iraq isolate along with other *Theileria spp.* such as *T. lestoquardi* (AF081135.1), *T. annulata* (KF429800.1), *T. parva* (AF013418.1), *T. taurotragi* (L19082.1), *T. buffeli* (FJ426360.1), *T. ovis* (AY260172.1), *T. cervi* (AY735129.1) and *B. ovis* (AY533146.1) by CLUSTAL 2.1 multiple sequence alignment. We show the two *T. lestoquardi* Iraq isolate is closely related *T. lestoquardi* (AF081135.1).

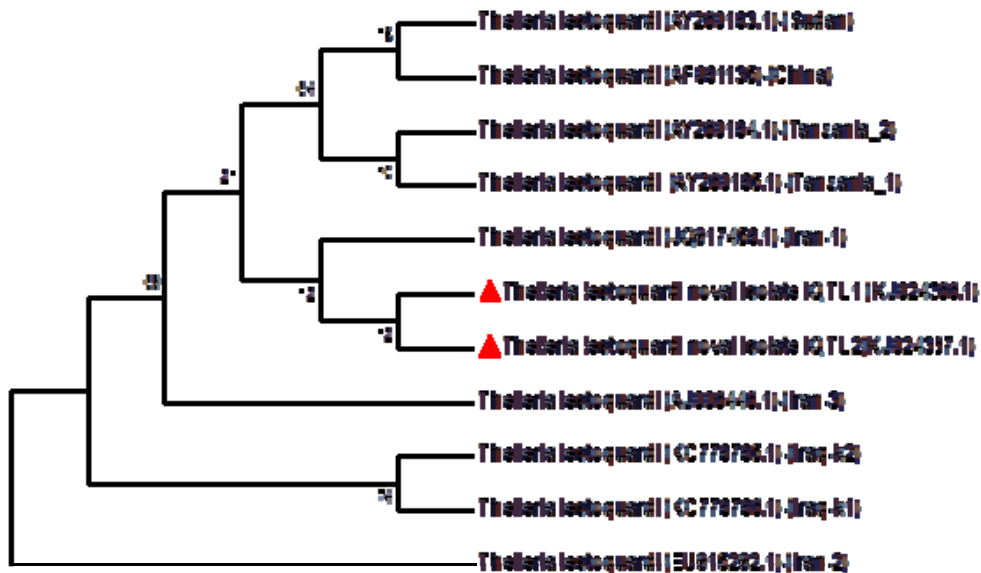


Figure 4: The phylogenetic tree of selected seven *T. lestoquardi* strain and isolate from some countries, two Iraq Kurdistan isolate with two Iraq isolate based upon 18S ribosomal RNA genes using neighbor joining phylogenetic tree analysis by CLUSTAL 2.1 multiple sequence alignment.

The identity percent of two *T. lestoquardi* Iraq isolate and other *T. lestoquardi* of selected countries were 98% to 99%, furthermore the score were high in china and iran3,

moderate in iran2, iran1, Iraq K1 and Iraq K2 comparable to low score in Tanzania1, tanzania2 and Sudan (Table 1).

Table 1: Homology sequence identity for *T. lestoquardi* Iraq isolate

No.	Accession Number	Name of Sequences	Country	IQ TL1		IQ TL2	
				Max score	Identity	Max score	Identity
1	AF081135.1	<i>Theileria lestoquardi</i> small subunit ribosomal RNA gene, complete sequence	China	1895	99%	1906	99%
2	AJ006446.1	<i>Theileria lestoquardi</i> 5.8S rRNA gene, partial	IRAN3	1884	99%	1895	99%
3	EU915292.1	<i>Theileria lestoquardi</i> small subunit ribosomal RNA gene, partial sequence	IRAN2	1426	99%	1435	99%
4	JQ917458.1	<i>Theileria lestoquardi</i> 18S ribosomal RNA gene, partial sequence	IRAN1	1395	98%	1397	98%
5	KC778786.1	<i>Theileria lestoquardi</i> isolate TlestoIraq21.3 18S ribosomal RNA gene, partial sequence	Iraq K1	1262	99%	1264	99%
6	KC778785.1	<i>Theileria lestoquardi</i> isolate TlestoIraq42.8 18S ribosomal RNA gene, partial sequence	Iraq K2	1262	99%	1264	99%
7	AY260183.1	<i>Theileria cf. lestoquardi</i> G4 18S ribosomal RNA gene, complete sequence	Tanzania 1	924	99%	924	99%
8	AY260184.1	<i>Theileria cf. lestoquardi</i> G6 18S ribosomal RNA gene, complete sequence	Tanzania 2	924	99%	924	99%
9	AY260185.1	<i>Theileria cf. lestoquardi</i> (Atbara) 18S ribosomal RNA gene, complete sequence	Sudan	924	99%	924	99%

Discussion

Ribosomal RNA is the most abundant constituent of nucleic acids in any non-viral organism with the eukaryotic RNA transcription unit. The rRNA gene has been sequenced from a variety of different organisms, resulting in a large database for sequence comparisons (9-10). Moreover, the 18S rRNA gene is valuable for phylogenetic analysis due to its high levels of conservation (11). However, the molecule also possesses phylogenetically informative variable regions that are useful for determining relationships among species (12). Results from the present study illustrate relationships among *Theileria* parasites where their previous taxonomic classification was not clear. Molecular techniques such as sequencing of S r RNA genes described in this study are promising tools for classification of these parasites. However, basic information based on life cycle differences, vectors, modes of transmission, virulence and genetic compatibility is essential for clearer taxonomic definition of the *Theileria* parasites. A phylogenetic tree

was inferred based on the 18S rRNA gene sequence of the Iraq isolates, and other species of *Theileria* available in GenBank. In the constructed tree, *Theileria lestoquardi* (Iraq isolates) was closely related to *T. lestoquardi* (AF081135.1). Based on phylogenetic analysis study of *T. lestoquardi* Iraq isolates which isolated from clinically infected sheep in middle of Iraq, by using 18S rRNA gene sequence. In addition, *Theileria* species are host and vector specific (13-14) but in Iraq more than one species can infect sheep (15) which causes a problem in diagnosis and epidemiology. In this study, the fragment of 18S rRNA gene sequences of *T. spp*1098bp were amplified for examined samples. Nucleotide sequence identity data demonstrated that the *T. lestoquardi* Iraq isolate has nucleotide identity percent of 99% with *T. lestoquardi* (AF081135.1) and with *T. annulata*(KF429800.1) were 99%. The results of the current study was nearly identical with (16) when he show the similarity identity percent

between *T. annulata* Iran strain and *T. lestoquardi* was 99.5%. In spite of The identity percent of two *T. lestoquardi* Iraq isolate and other *T. lestoquardi* of selected countries were 98% to 99% but the score of identity was higher in china and iran3, moderate in iran2, iran1, Iraq K1 and Iraq K2 From this perspective, the Iraq isolate has a great homology with other *T. lestoquardi* isolates from above countries which most of them in neighborhood.

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