Research Article

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Microscopically and Molecular Detection of *Theileria* Species in Sheep in Baghdad Province, Iraq

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Abstract

The study was conducted to estimate the prevalence of Theileriosis in sheep in Baghdad city, as well as study the effect of age, sex, and months in the infection rate by examination of 180 of blood samples collected during the period from the beginning of October 2020 to the end of March 2021. All samples were examined by microscopic examination, and 100 blood samples collected randomly from total 180 were molecular detection by using conventional PCR. The total infection rate of the microscopic infection was 33.33%(60/180). The result showed a significant difference (P<0.05) between two different ages of animals that infected with *Theileria* spp and the adult sheep(>1y), which recorded the higher 50% (50/100), while the lower infection rate 12.5% (10/80) was among the young sheep(6mns-1y). concerning sex and months, there was no significant difference between female and male ,also among study months. The conventional PCR technique was used in the molecular study; 70(70%) out of 100 blood samples were selected randomly from 180 sheep. PCR products of teen were selected randomly for DNA analysis to obtain the partial nucleotides sequence of 18S ribosomal RNA gene . The PCR product was processed as a wave-like shape at (1104bp) after that sequences were recorded in NCBI with No.1 (MW735685.1), No.2(MW735686.1), No.3 (MW735687.1), **No.5** (MW735689.1), No.7 (MW735691.1),No.8 (MW735692.1), No.10 (MW735694.1) for *Theileria ovis* and No.1 **No.4** (MW735688.1), **No.6** (MW735690.1), No.9 (MW735693.1) for *Theileria lestoquardi.*

Keywards: *Theileria ovis* , *Theileria lestoquardi*, Sheep, Iraq.

التحري المجهري والجزيئي لطفيلي *Theileria* في الاغنام في محافظة بغداد ،العراق

الخلاصة

اجريت الدراسة لمعرفة انتشار الثاليريا في الاغنام في مدينة بغداد ودراسة تاثير العمر والجنس واشهر الدراسة على نسبة الاصابة في 180 عينة دم للمدة من اول شهر ايلول 2020 ولغاية نهايةشهر اذار 2021. فحصت جميع العينات مجهريا ومن خلال تقنية تفاعل البلمرة المتسلسل التقليدي 100 عينة تم اختيارها عشوائيا من 180 عينة. بلغ معدل الاصابة الكلية في الاغنام 33.3% (60/180) بالفحص المجهري ، اظهرت النتائج فرقا معنويا في الاصابة بالثاليريا في الاعمار عند مستوى (P<0.05) حيث بلغت في الاعمار الكبيرية اكبر من سنة 50%(50/100) وبلغت بمعدل12.5 %(10/80) في الاعمار الصغيرة من ستة اشهر الى سنة،وفقا للاجناس والاشهر عدم وجود فروق معنوية بين الذكور والاناث وايضا بين اشهر الدراسة.في هذة الدراسة تم استخدام تقنية تفاعل البلمرة المتسلسل التقليدي، وكان معدل الاصابةالكلي 70% (70/100) وتم اختيار عشرعينات موجبةمن PCRلتحليل الحامض النووي للحصول على مجموعات النيوكلوتيدات *18SrRNA*جين تمت ظهور النتائج بشكل شبة موجةفي 1104لمنتجPCR وبعد ذلك تم تسجيل الجينات في قاعدةاظهرت سبع عزلات بيانات بنك **الجينات** العالمي تنتمي الى ***Theileria ovis*** سجلت بالارقام, No2(MW735686.1), No1(MW735685.1) No.3 (MW735687.1), No.5 (MW735689.1), No.7 (MW735691.1),No.8 (MW735692.1), No.10 (MW735694.1). وثلاث عزلات تنتمي الى *Theileria lestoquardi* سجلت بالارقام ، No.4 (MW735688.1), No.6 (MW735690.1), No.9 (MW735693.1) .

Introduction

Theileriosis is a tick-borne protozoan illness caused by *Theileria spp*. (*Piroplasmida, Theileridae*) that affects both domestic and wild ruminants. Theileria is a member of the Apicomplexa phylum, which also includes *Babesia, Toxoplasma, Neospora, Plasmodium*, and other parasites **(1)**. Tick-borne theileriosis caused high significant morbidity and mortality in afflicted animals, resulting in higher financial losses in tropical and subtropical areas across the world. **(2).** *Thieleria hirci* (synonym *T.lestoquardi*) was the most virulent of the six *Theileria* species that caused ovine and caprine theileriosis, whereas *Thieleria ovis* produced subclinical illness in sheep and goats. **(3).** Acute and chronic *Theileria* infections are also possible. *T. lestoquardi* causes ovine malignant theileriosis, which has a high fatality rate **(4).** Fever, emaciation, lymphadenopathy, wasting, malaise anorexia, fast heartbeat, dyspnea, listlessness, anaemia, icterus, jaundice, pyrexia, intermittent diarrhoea or constipation, weakness, and the cessation of rumination are all symptoms of *Theileria lestoquardi.* **(5).** During theileriosis, *Theileria* schizonts are frequently observed in liver, spleen, lungs, kidneys and lymph **(6).** Electrolyte imbalance, calcium overdose, digoxin overdosing, and cardiomyopathy are all possible indications of theileriosis **(7).** Each haemoparasite detection technique has been designed specifically for use in each species. The microscopic inspection approach is typically sufficient for detecting acute infection, but not for detecting carrier animals with low parasiteemia. **(8)** For the detection and identification of *Theileria* species, a polymerase chain reaction (PCR) approach has been devised. **(9 and 10).** The objective of this research was to use microscopic and molecular approaches to identify the incidence of *Theileriosis* in sheep in Baghdad city,Iraq.

Materials and Methods

Specimens Collection:

This study is conducted during the period from October 2020 to 30th March2021 in Baghdad city. A total of 180 blood samples were collected from sheep of different ages and of sexes. five milliliters of blood sample were collected during the slaughtering of animal and kept in tubes with anticoagulant ethylene diamine -tetra acetic acid (EDTA). All samples were transferred in cooling conditions to the laboratory of College of Veterinary Medicine / University of Baghdad to conduct the necessary tests to determine the infection with *Theileria* spp. according to the experimental design.

Microscopic examination:

Blood smears were prepared according to **(11)**, stained with Giemsa-stain then examined for detection of *Theileria* parasite under light microscopy by X100 oil-immersion described according to **(12).**

Molecular Diagnosis of *Theileria* by Conventional PCR:

DNA Extraction:

Genomic DNA of *Theileria* isolate was extracted from 100 blood sample according to the protocol ReliaPrep™ Blood gDNA Miniprep System, Promega

Primers:

For detection *Theileria* one primer were used in this study were obtained from IDT company (*18S RNA gene*). This primer were prepared according to the information of the company (Table 1)

Table 1: The primers with their sequences and product size.

Primer Name	Primer sequence (5′ to 3′)	Product size (bp)	References
Theileria spp-F *Theileria* spp-R	5`-AGTTTCTGACCTATCAG-3` 5`-TTGCCTTAAACTTCCTTG-3`	1104	**(13)**

PCR master mix preparation

PCR master mix was implemented by (AccuPower PCR PreMix Kit) ,according to manufacturer's instructions as in (Table2).

Table2: Protocol of PCR reaction mixture volume

PCR Master mix	Volume
DNA template 5-5ng/µl	**5µl**
Forward primer (10pmol)	**1µl**
Reveres primer (10pmol)	**1µl**
PCR water	**13µl**
Total volume	**20µl**

After then, These PCR master mix constituents transferred into thermocycler (T100 Thermal cycler BioRad. USA).

PCR Thermocycler Conditions:

PCR thermocycler conditions was implemented by utilizing conventional PCR thermocycler system as in (Table3).

Table3: The optimum condition of detection *Theileria spp.*

PCR step	Temp.	Time	repeat
Initial Denaturation	**95ºC**	**5min**	**1**
Denaturation	**95 ºC**	**30sec.**	**35 cycle**
Annealing	**59 ºC**	**30sec**	
Extension	**72 ºC**	**1 min**	
Final extension	**72 ºC**	**5min**	**1**
Hold	**4 ºC**	**Forever**	**-**

Preparation of agarose

One hundred milliliters of 1X TAE were poured into a flask and 1.5 gm (for 1.5%) agarose ware added to the buffer and the solution ware heated to boiling (using microwave) until all the gel particles were dissolved. After that, 1µl of Ethidium Bromide (10mg/ml) was added to the agarose. The agarose stirred in order to get mixed and to avoid bubbles then the solution was left to cool down at 50-60C°.

DNA sequencing method:

Ten samples that were positive from sheep by PCR technique were subjected to sequencing for detection *Theileria* spp. These PCR ribosomal RNA genes positive products were sent by ice bag by DHL to Macrogen Company in Korea for performed the DNA sequencing by AB DNA sequencing system. The genetic analysis done by phylogenetic tree analysis between local *Theileria* species isolates and NCBI-Blast submission *Theileria* species . Then the identification species isolates were submitted into of NCBI-GenBank .The DNA sequencing analysis by utilizing Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis of the partial small subunit ribosomal rRNA gene depend on analysis of ClustalW alignment analysis and The development distances were computed by the Maximum Composite Likelihood method by phylogenetic tree UPGMA method.

Statistical Analysis

All data were statistically analyzed by Chi-square tests for significance using Statistical Analysis System- SAS**. 2012**version**(14).**

Results and Discussion

Microscopically Examination

The results with *Theileria* spp. of infection was showed that among (180) samples were examined microscopically 33.33%(60/180), were given positive result table(4) figure(1).

Table(4): Total of infection rate *Theileria spp.* in sheep by using microscopic examination.

Number of blood samples	Positive	%
180	60	33.33

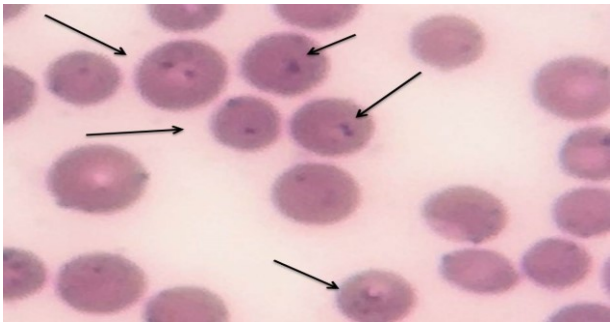

Figure (1): Blood smear stained with Giemsa stain, show *Theileria* spp. inside red blood cells of sheep(black arrow) (X 100).

The result showed a significant difference ($P<0.05$) between two different ages of animals that infected with *Theileria* spp and the adult sheep (>1y), which recorded the higher 50% (50/100), while the lower infection rate 12.5% (10/80) was among the young sheep (6mns-1y) (Table 5) .

Table (5): Prevalence of *Theileria spp.* in sheep according to age.

Age	Total number examined	Positive	Percentage (%)
Young's(6mns-1y)	80	10	12.50
Adults(>1y)	100	50	50.00
Total	180	60	33.33
Chi-Square (χ^2-)	---	---	26.666 **
** (P≤0.01)-Highly Sig.			

Concerning sex susceptibility to infection present work showed no statistical significance between sex and *Theileria* infection. The prevalence of *Theileria* in males was 33.63% (37/110), while in females was 32.85%(15/80) (Table 6).

Table(6): Prevalence of *Theileria spp.* in sheep according to sex.

sex	Total number examined	Positive	Percentage (%)
Male	110	37	33.63
Female	70	23	32.85
Total	180	60	33.33
Chi-Square (χ^2)	--	--	3.266 NS
NS: Non-Significant.			

The present results showed that no significance were found between the study of months on the rate of infection (P>0.05) (Table 7)

Table(7): Prevalence of *Theileria spp.* in sheep according to months .

Months	Total number examined	Positive	Percentage (%)
October (2020)	30	11	36.66
November	30	10	33.33
December	30	9	30.00
January(2021)	30	10	33.33
February	30	9	30.00
March	30	11	36.66
Total	180	60	33.33
Chi-Square (χ^2)	---	---	2.094 NS
NS: Non-Significant.			

The current study recorded that infection rate by *Theileria* spp. examined under a microscope using Giemsa dye was 33.33 percent. This study's infection rate was remarkably identical to some experiments conducted in Iraq. Alfetly **(15)** and Dhaim and A'aiz **(16)** when they recorded a rate 26.51 and22.8 % respectively. Different results were found in nearby countries, including 8.6% and 11.9 percent , respectively in Iran. **(17),** 15.5 % in Turkey **(18)** and 5-24 % in Saudi Arabia **(19).** Changes in the above results might be due to differences in environmental and climatic circumstances, the quantity of samples studied, the methodologies employed in their diagnosis, the density of vectors, and the health of the animals. There were no significant differences

between the sexes in this investigation. This may because of exposure to same environmental condition and vector . These results are in agreement with Naz *et al.* **(4)** when reported that percentage of infection in male was 14.1 % and in female 13.6 % .With related to the effect of age on theileriosis, the results showed that the highest infection rate in adult sheep was 50%, while the lowest rate in young sheep less than was 12.5%. This result correspond with *Naz et al.* **(4)** reported low prevalence parasite in lambs below six months of age. Zangana and Naqid **(20),** also said that the high rate of infection occur in age above three years . The low rate of infection in young sheep may be due to received immunity from their dams through placenta or colostrum (**21 and 22).**

The present results showed that no significance were found between the study of months on the rate of infection (P>0.05). These findings supported with **(23)** and **(2)** As they discovered, the incidence of *Theileria* infection did not fluctuate much over time. The lack of a significant influence of months might be ascribed to continued transmission of *Theileria* spp. throughout the year in Iraq, which supports the fact that the vector was found to be active during most of the year in the research region, albeit in modest numbers.

PCR Examination.

The result of present study showed high rate infection, amplification conditions were optimized for the PCR assay, using specific primers sequences of *18S rRNA* (1104bp), (Table 8), (Figure 2), out of the samples , (70 %) (70/100) were positive.

Table (8). Showed the positive of *Theileria* by using PCR examination.

Diagnostic methods	Total number	positive	Percentage
PCR	100	70	70

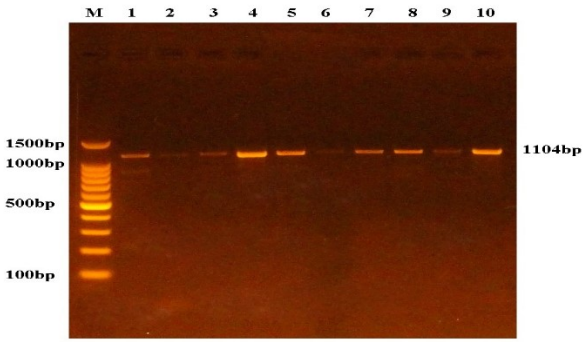

Figure 2: 1.5 % agarose gel electrophoresis ,showed the PCR product analysis of *18SrRNA* gene of *Theileria* spp from sheep blood samples. Lanes (1,2,3,4,5,6,7,8,9,10) are positive results at 1104bp products M:marker (100-1500).

Submission of local Iraq isolate in NCBI

Ten samples of PCR products were taken from seventy PCR samples chosen randomly positive sequencing by forward and reverse primers. The sequences were employed in the NCBI gene bank database No.1 (MW735685.1), No.2(MW735686.1), No.3 (MW735687.1), **No.4 (**MW735688.1), **No.5 (**MW735689.1), **No.6 (**MW735690.1), No.7 (MW735691.1),No.8 (MW735692.1),No.9 **(**MW735693.1),No.10 (MW735694.1) . These sequences were analyzed by BLAST- NCBI program to determine the converging sequences recorded in the gene bank.

Phylogenetic Analysis

The present study ,the sequences have been registered in NCBI under accession numbers No.1 (MW735685.1), No.2(MW735686.1), No.3 (MW735687.1), **No.5 (**MW735689.1), No.7 (MW735691.1),No.8 (MW735692.1), No.10 (MW735694.1),belong to *Theileria ovis* for analysis and were compared with the NCBI-genbank *Theileria* strain isolates. shown in (Table 9) (Figure 4). the sequences have been registered in NCBI under accession numbers No.4 **(**MW735688.1), **No.6 (**MW735690.1), No.9**(**MW735693.1), belong to *Theileria lestoquardi* for analysis and were compared with the NCBI- genbank *Theileria* strain isolates. shown in(Table 10) (Figure 4).

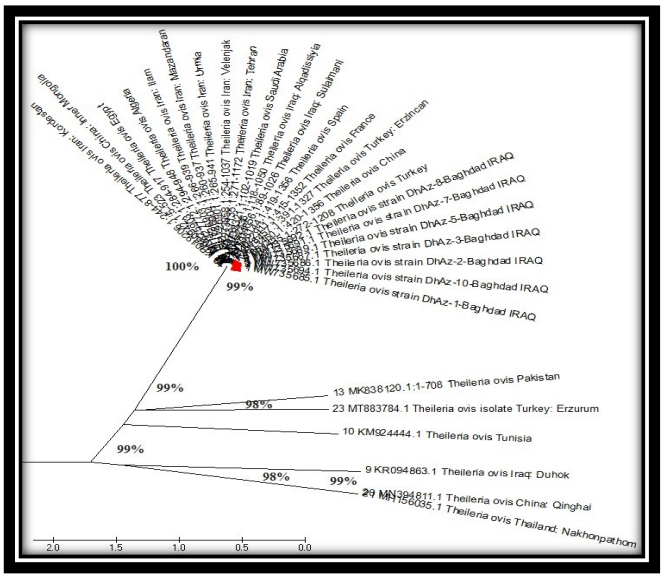

Figure (3):Neighbor-joining tree *Theileria ovis* of *18S ribosomal RNA* gene.

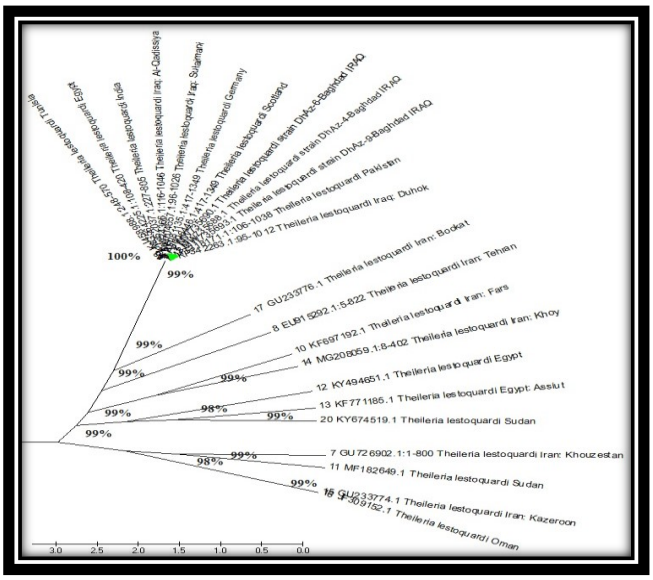

Figure (4):Neighbor-joining tree *Theileria lestoquardi* of *18S ribosomal RNA gene.*

The present study recorded the total infection rate with *Theileria* spp in sheep that examined by Molecular assay was 70%, which was in agreement with Yaghfoori *et al*. **(24)** in Iran, they recorded the rate of infection in sheep was (76 %).In Turkey by **(18)** who recorded 41.2%. %. In Pakistan**(25),** was recorded 35% prevalence infection in sheep .

Its role in antigenic diversity of the surface receptor for avoiding host immune responses might explain the greater infection rate in our research, this might have a part in the chronic nature of the disease in sheep, as well as dealing with parasite virulence and capacity to infect a wide variety of host species**(26).**

The PCR approach employed in this investigation was consistently the most sensitive tool for detecting chronic infection and low parasitaemia. In comparison to Classical method, PCR provides a number of significant benefits.

Sample processing does not need to be completed within a certain amount of time after collection, but it must be done within 180 days after preservation at 20°C. The PCR approach was tested on blood samples from infected sheep, indicating that it had greater sensitivity and specificity than parasitological procedures. This was in line with previous research **(27 and 28).**

The PCR approach solves the problem of nonspecific reactions in serological testing, allowing it to identify low parasite sheep in chronic cases. The efficiency of PCR was demonstrated in the diagnosis of infection in a parasitaemic sheep with clinical indications of sickness who had previously tested negative for parasites using parasitological testing. The disease's persistent infection and the parasitological tests' limited sensitivity. Furthermore, the use of PCR as a precise and specific diagnostic tool was agreed with **(29).**

Conclusion

The results of this study recorded high prevalence of theileriosis and the disease was endemic in Baghdad city.PCR is important technique in the epidemiological studies for diagnosis at both carrier and infected animal. According to the molecular study, *Theileria ovis and Theileria lestoquardi* are considered the main species that infect sheep in Baghdad city.

Acknowledgment

Authors appreciate all the laboratory assistance technical support from the department of parasitology in the college of veterinary medicine university of Baghdad.

Conflicts of interest

All author declared there are no conflicts of interests.

Table 9: The NCBI-BLAST Homology Sequence identity (%) between local *Theileria ovis* sheep isolates and NCBI-BLAST submitted *Theileria* spp. strain

	Accession	Country	Host	Source	Compatibility
1.	ID: KT851435.1	Turkey	sheep	Theileria ovis	99%
2.	ID: FJ603460.1	China	sheep	Theileria ovis	99%
3.	ID: AY508457.1	Turkey: Erzincan	sheep	Theileria ovis	99%
4.	ID: MN493111.1	France	sheep	Theileria ovis	99%
5.	ID: AY533144.1	Spain	sheep	Theileria ovis	99%
6.	ID: MN704656.1	Iraq: Sulaimani	sheep	Theileria ovis	99%
7.	ID: KJ452336.1	Iraq: Alqadissiyia	sheep	Theileria ovis	99%
8.	ID: MG738321.1	Saudi Arabia	sheep	Theileria ovis	99%
9.	ID: KR094863.1	Iraq: Duhok	sheep	Theileria ovis	99%
10.	ID: KM924444.1	Tunisia	sheep	Theileria ovis	99%
11.	ID: JQ737135.1	Iran: Tehran	sheep	Theileria ovis	99%
12.	ID: KX273858.1	Iran: Velenjak	sheep	Theileria ovis	99%
13.	ID: MK838120.1	Pakistan	sheep	Theileria ovis	99%
14.	ID: GU726901.1	Iran: Urmia	sheep	Theileria ovis	99%
15.	ID: GU726904.1	Iran: Mazandaran	sheep	Theileria ovis	99%
16.	ID: GU726903.1	Iran: Ilam	sheep	Theileria ovis	99%
17.	ID: MH327772.1	Algeria	sheep	Theileria ovis	99%
18.	ID: MN625903.1	Egypt	sheep	Theileria ovis	99%
19.	ID: KP019206.1	Iran: Kordestan	sheep	Theileria ovis	99%
20.	ID: MN394811.1	China: Qinghai	sheep	Theileria ovis	99%
21.	ID: MH156035.1	Thailand: Nakhonpathom	sheep	Theileria ovis	99%
22.	ID: KJ850942.1	China: Inner Mongolia	sheep	Theileria ovis	99%
23.	ID: MT883784.1	Turkey: Erzurum	sheep	Theileria ovis	99%

Table 10: The NCBI-BLAST Homology Sequence identity (%) between local *Theileria lestoquardi* sheep isolates and NCBI-BLAST submitted *Theileria* spp. strain.

	Accession	Country	Host	Source	Compatibility
24.	ID: MT318171.1	Pakistan	sheep	*Theileria lestoquardi*	99%
25.	ID: AF081135.1	Germany	sheep	*Theileria lestoquardi*	99%
26.	ID: AJ006446.1	Scotland	sheep	*Theileria lestoquardi*	99%
27.	ID: MN704657.1	Iraq: Sulaimani	sheep	*Theileria lestoquardi*	99%
28.	ID: KJ024366.1	Iraq: Al-Qadissiya	sheep	*Theileria lestoquardi*	99%
29.	ID: KP342263.1	Iraq: Duhok	sheep	*Theileria lestoquardi*	98%
30.	ID: GU726902.1	Iran: Khouzestan	sheep	*Theileria lestoquardi*	99%
31.	ID: EU915292.1	Iran: Tehran	goat	*Theileria lestoquardi*	98%
32.	ID: KY352037.1	India	sheep	*Theileria lestoquardi*	99%
33.	ID: KF697192.1	Iran: Fars	sheep	*Theileria lestoquardi*	99%
34.	ID: MF182649.1	Sudan	sheep	*Theileria lestoquardi*	99%
35.	ID: KY494651.1	Egypt	sheep	*Theileria lestoquardi*	99%
36.	ID: KF771185.1	Egypt: Assiut	buffalo	*Theileria lestoquardi*	99%
37.	ID: MG208059.1	Iran: Khoy	goat	*Theileria lestoquardi*	98%
38.	ID: GU233774.1	Iran: Kazeroon	bovine	*Theileria lestoquardi*	99%
39.	ID: JF309152.1	Oman	sheep	*Theileria lestoquardi*	99%
40.	ID: GU233776.1	Iran: Bookat	sheep	*Theileria lestoquardi*	99%
41.	ID: KJ458988.1	Tunisia	Sheep	*Theileria lestoquardi*	99%
42.	ID: MG564225.1	Egypt	Sheep	*Theileria lestoquardi*	99%
43.	ID: KY674519.1	Sudan	sheep	*Theileria lestoquardi*	99%

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