# Prevalence and risk factors of tropical theileriosis, and sequencing of *Theileria annulata*, the causative pathogen, in Setif region (Algeria) before and after tick season

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***Keywords***

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***Summary***

To determine the prevalence of *Theileria annulata* infection and the influence of some risk factors, a molecular survey was carried out in the Setif region (Algeria). A total of 134 cattle blood samples from 21 farms were collected twice, in April and November 2015, before and after the tick vector season. *Theileria annulata* molecular prevalence was 25.4% in April and and 50% in November, indicating a significant increase ($p < 0.001$) in the number of asymptomatic carrier animals. The molecular prevalence was significantly higher than the prevalence in Giemsa-stained blood smears for the two periods. In April, the Fleckvieh breed had the lowest molecular prevalence, but this prevalence increased significantly in November ($p < 0.001$). The breeding type and sex had no influence on *T. annulata* prevalence. However, the infection rate increased significantly during the tick infestation period as the walls were not roughcast and had cracks ($p < 0.001$). The Algerian Tams1 gene sequence was very similar to the already known Mauritanian, Tunisian and Egyptian sequences.

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## ■ INTRODUCTION

Bovine tropical theileriosis (*Theileria annulata* infection) is a tick-borne disease whose causative pathogen (*Theileria annulata*) is transmitted by *Hyalomma* ticks (Neitz, 1959; Barnett, 1968; Robinson, 1982; El Hussein et al., 2012). This disease occurs in Southern Europe, North Africa and Asia, from the Middle East to China (Uilenberg, 1981). Local breeds are resistant to *T. annulata* infection and can remain carriers for 11 years (Sergent et al., 1945). Exotic cattle are, on the other hand, highly susceptible to this infection which can cause significant economic losses (Robinson, 1982; Ait Hamou et al., 2012). After recovery, sick animals become carriers and reservoirs of the parasite (Brown, 1990; Bilgic et al., 2010).

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*Theileria annulata* is the most prevalent piroplasm affecting cattle in Tunisia (Darghouth, 2004) and Morocco (El Haj et al., 2002). In Algeria, Ziam and Benaouf (2004) estimated the prevalence of *T. annulata* by examination of Giemsa-stained blood smears to be 53.7%. In 2015, molecular prevalence of *T. annulata* was estimated by Ziam et al. (2015) at 36.8% in randomly chosen healthy cattle.

In Algeria, tropical theileriosis is enzootic in humid, subhumid and semiarid regions, favorable to the development of tick vectors (Sergent et al., 1945; Ziam and Benaouf, 2004; Ziam et al., 2015). The tick *Hyalomma scupense* is thus abundant in Mila (39.2% of the collected ticks; Benchikh Elfegoun et al., 2013) and in Tiaret (28% of the ticks; Boulkaboul, 2003) areas, where the climate is semiarid, whereas lower frequencies are present in more humid regions such as Jijel (2.5% of the ticks; Benchikh Elfegoun et al., 2007) and El Tarf (1.8%; Benchikh Elfegoun et al., 2013). The highest prevalence of the other vector – *H. lusitanicum* – was found in Tiaret (20% of the ticks; Boulkaboul, 2003) and Oran regions (15%; Yousfi-Monod and Aeschlimann, 1986). This prevalence was lower in Jijel (5.5%; Benchikh Elfegoun et al., 2007), Mila (1.1%) and El Tarf (0.02%) regions (Benchikh Elfegoun et al., 2013).

Several techniques are used to detect *T. annulata* in cattle (Dumanli et al., 2005; Durrani et al., 2010). The Giemsa-stained blood smear is a fast and low cost tool, but it is not suitable for epidemiological

studies because of its low sensitivity (Darghouth et al., 1996; Uilenberg, 2004). On the other hand, the polymerase chain reaction (PCR) method allows the detection of a single piroplasm in 4 µL of blood sample (Ilhan et al., 1998).

Recently, Gharbi and Darghouth (2015) wrote a review on the disease and on the measures to control it in North Africa. The aim of the present study was to estimate the prevalence of *T. annulata* infection in carrier cattle by microscopic and molecular methods, before and after the vector tick season.

## ■ MATERIALS AND METHODS

### *Study area*

The present study was carried out in El Eulma region, *wilaya* of Setif (North-East Algeria, Figure 1). The altitude of this locality varies between 800 and 1300 meters. The climate is semiarid with a mean annual rainfall of 482 mm, hot summers and cold winters with mean temperatures of 23.7 °C in summer and 5.1 °C in winter.

The cattle population, estimated at 30,000 animals, is composed of local and exotic breeds (Montbeliard, Holstein Friesian, Red Holstein and Fleckvieh). Generally, dairy cattle farms are conducted semi-intensively, whereas beef cattle are intensively bred (data from the Algerian Agricultural Services Branch, 2014).

### *Animal sampling*

Twenty-one cattle farms, randomly chosen among those whose owner agreed to collaborate and were available, were included in the study. EDTA blood samples from 180 healthy cattle, aged three months to seven years, were randomly collected in late April 2015. Some of these cattle had been introduced in the farms from other Algerian regions or even from other countries (particularly the Fleckvieh cattle), after the previous tick infestation period, but no precise information regarding animals' movements was available.

It had been planned to sample again the same animals after the *H. scupense* vector tick season, i.e. in November 2015, but only 134 of them were present at that time. Of the 46 missing cattle, an unknown number died of theileriosis and others were sold. They were thus removed from the study, and the analysis concerned only the 134 cattle sampled twice.

### Theileria annulata *infection study*

Giemsa-stained blood smears were examined under microscope at x1000 magnification using immersion oil. DNA was extracted from 300 µL blood with the BioBasic DNA (extraction) Kit (Markham, Canada) following the manufacturer's instructions. *Theileria annulata* PCR amplifying a 721 bp DNA fragment was performed with a set of specific primers of the gene encoding *T. annulata* Tams1 antigen. The forward primer was N516 (5'-GTAACCTTTAAAAACGT-3') and the reverse primer was N517 (5'-GTTACGAACATGGGTTT-3') (d'Oliveira et al., 1995). PCR was carried out in 25 µL volume for each reaction consisting of 2.5 µL of 10x PCR buffer (20 mmol Tris-HCl; pH 8.5; 50 mmol KCl), 0.4 mmol of each dNTP, 0.5 µmol of each primer, 3 mmol $MgCl_2$, 0.05 U/µL of Taq DNA Polymerase (Vivantis, Chino, California) and 2 µL of the extracted DNA as template.

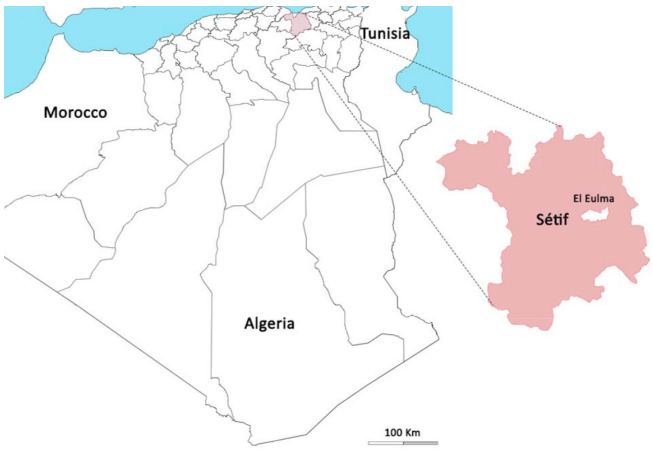


***Figure 1:*** *Geographical location of the region of El Eulma, Algeria.*

Samples were amplified in an automated DNA thermocycler (ESCO Swift MaxPro, Kintex, Korea) using the following program: initial denaturation at 94 °C for 5 min followed by 30 cycles (94 °C, 55 °C and 72 °C during 1 min each) and a final extension at 72 °C for 10 min. Amplified DNA was electophoresed, then examined under an ultraviolet transilluminator.

### *DNA sequencing and phylogenetic analyses*

Four PCR randomly chosen products obtained with N516/N517 primers were purified with Wizard SV gel and PCR clean-up (Promega, Madison, WI, USA). The PCR products were sequenced in both directions, using the same primers as for PCR. Sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) with the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. MEGA 6.1 software was used to perform multiple sequence alignments using MUSCLE (Tamura et al., 2013). Obtained DNA sequences were compared for similarity to sequences deposited in GenBank using the BLAST program.

The Tams1 partial sequences of *T. annulata* (Tabv A1) variant have been deposited in GenBank (accession number: KX196177). A phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were computed with the Tajima-Nei method (Tajima and Nei, 1984) and expressed in a number of base substitutions per site between sequences.

### *Statistical analysis*

Data were analyzed with SPSS 21 (IBM). Chi square tests were performed and differences were considered significant at $p \leq 0.05$.

## ■ RESULTS

The molecular study showed that 25.4% and 50% of the samples were positive for *T. annulata* in April and November, respectively ($p < 0.001$). The percentages of positive Giemsa-stained blood smears, before and after the tick season, were 10.4% and 16.4%, respectively. They were significantly lower than those obtained by PCR ($p \leq 0.001$) (Table I).

In April, the Fleckvieh cattle had the lowest molecular prevalence (14.6%; $p = 0.047$). This prevalence increased significantly in November ($p < 0.001$). At that time, there was no difference according to the breed ($p > 0.05$). Moreover, there was no difference according to the sex regardless of the period. However, between April and November, the infection rate increased significantly for both males ($p = 0.004$) and females ($p = 0.002$) (Table II). The infection rate increased significantly in farms without roughcast walls and with cracks in the walls ($p < 0.001$), and in the older cattle (> 48 months). No significant difference between dairy semi-extensive and intensive herds (milk or meat) was detected (Table II).

**Table I**

Prevalence comparison between blood smears and PCR before and after the tropical theileriosis season

| | Positive/examined (prevalence ± CI) | | |
|---|---|---|---|
| **Period** | **Blood smears** | **PCR** | **P value** |
| Before theileriosis season | 14/134 (10.4 ± 0.05) | 34/134 (25.4 ± 0.07) | 0.001* |
| After theileriosis season | 22/134 (16.4 ± 0.06) | 67/134 (50.0 ± 0.08) | < 0.001* |
| P value | 0.152 | < 0.001* | |

CI: Confidence interval; * Statistically significant

**Table II**

*Theileria annulata* risk factors in carriers before (April) and after (November) the tropical theileriosis season

| | Positive/examined (prevalence ± CI) | | |
|---|---|---|---|
| **Parameter** | **Before theileriosis season** | **After theileriosis season** | **P value** |
| Sex | | | |
| Male | 7/41 (17.1 ± 0.12) | 19/41 (46.3 ± 0.15) | 0.004* |
| Female | 27/93 (29.0 ± 0.09) | 48/93 (51.6 ± 0.10) | 0.002* |
| P value | 0.143 | 0.574 | |
| Breed | | | |
| Local | 9/42 (21.4 ± 0.12) | 16/42 (38.1 ± 0.15) | 0.095 |
| Fleckvieh | 6/41 (14.6 ± 0.11) | 21/41 (51.2 ± 0.15) | < 0.001* |
| Holstein | 11/34 (32.4 ± 0.16) | 19/34 (55.9 ± 0.17) | 0.051 |
| Montbeliard | 8/17 (47.1 ± 0.24) | 11/17 (64.7 ± 0.23) | 0.303 |
| P value | 0.047* | 0.226 | |
| Age (months) | | | |
| 0–6 | 1/23 (4.3 ± 0.08) | 0/0 (0.0) | – |
| 7–12 | 8/28 (28.6 ± 0.17) | 9/23 (39.1 ± 0.20) | 0.426 |
| 13–24 | 3/9 (33.3 ± 0.31) | 17/38 (44.7 ± 0.16) | 0.534 |
| 25–48 | 12/43 (27.9 ± 0.13) | 8/16 (50.0 ± 0.25) | 0.111 |
| > 48 | 10/31 (32.3 ± 0.16) | 33/57 (57.9 ± 0.13) | 0.022* |
| P value | 0.150 | 0.403 | |
| Type of livestock | | | |
| Dairy, intensive farming | 1/8 (12.5 ± 0.23) | 3/8 (37.5 ± 0.34) | 0.248 |
| Dairy, semi-intensive farming | 20/58 (34.5 ± 0.12) | 34/58 (58.6 ± 0.13) | 0.009* |
| Beef, intensive farming | 13/68 (19.1 ± 0.09) | 30/68 (44.1 ± 0.12) | 0.002* |
| P value | 0.098 | 0.205 | |
| Presence of cracks | | | |
| Yes | 27/117 (23.1 ± 0.08) | 59/117 (50.4 ± 0.09) | < 0.001* |
| No | 7/17 (41.2 ± 0.23) | 8/17 (47.1 ± 0.24) | 0.730 |
| P value | 0.109 | 0.795 | |
| Whitewashed walls | | | |
| Yes | 15/73 (20.5 ± 0.09) | 34/73 (46.6 ± 0.11) | < 0.001* |
| No | 19/61 (31.1 ± 0.12) | 33/61 (54.1 ± 0.13) | 0.010* |
| P value | 0.160 | 0.386 | |
| Roughcast walls | | | |
| Yes | 11/37 (29.7 ± 0.15) | 17/37 (45.9 ± 0.16) | 0.150 |
| No | 23/97 (23.7 ± 0.08) | 50/97 (51.5 ± 0.10) | < 0.001* |
| P value | 0.474 | 0.562 | |
| **Total** | 34/134 (25.4 ± 0.07) | 67/134 (50.0 ± 0.08) | 0.001* |

CI: Confidence interval; * Statistically significant

The four *T. annulata* sequenced amplicons were 100% homologous (GenBank Accession Number: KX196177). This Algerian *T. annulata* strain shared high homology with Egyptian (96.9%; AB917290), Tunisian (98.6%; AF214881) and Mauritanian isolates (98.9%; AF214819): all of them were clustered in a single clade. Our isolate also had 96.1% identity with the Bahraini isolate (AF214795). It was, on the other hand, different from the isolates from Spain, Italy, Iran, Sudan and India (Figure 2). However, the tree was not supported by high bootstrap values.

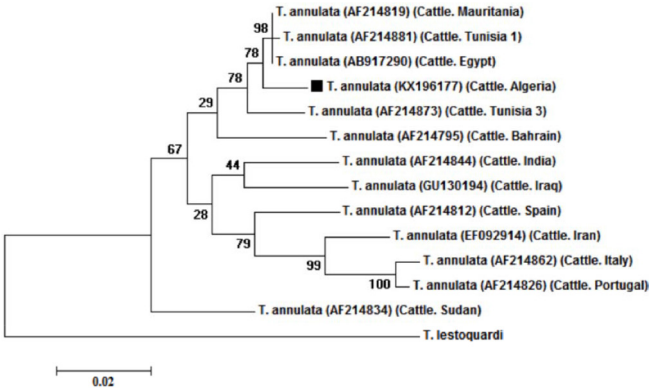


***Figure 2:*** *Partial sequence Tams1 gene phylogenetic tree of* Theileria annulata *identified in cattle in the present survey and those deposited in GenBank.*
*The tree was constructed with the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1100 replicates) is shown next to the branches. The evolutionary distances were computed with the Tajima-Nei method (Tajima and Nei., 1984) and expressed in a number of base substitutions per site between sequences. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). GenBank accession numbers are shown in parentheses. The isolates described in this study are preceded by a black square.*

## ■ DISCUSSION

Tropical theileriosis is caused by the protozoan *T. annulata* and transmitted by *Hyalomma* ticks (Robinson, 1982). In North Africa, *H. scupense* is the main vector of *T. annulata* (Gharbi et al., 2013). The higher infection rate observed in our results in November indicates that *T. annulata* was actually transmitted during the adult tick infestation season.

Two techniques were used in this study to identify *T. annulata* carrier animals, namely the microscopic examination of Giemsa-stained blood smears, and PCR. Several studies reported that PCR is more specific and sensitive than blood microscopic examination for detecting carriers (Dumanli et al., 2005; Aktas et al., 2006; Azizi et al., 2008; Safarpoor Dehkordi et al., 2012), which was also observed in this study. The number of infected red blood cells is high during early infection, allowing a better detection by Giemsa-stained blood smears at that time. However, after the establishment of an immune response, the number of infected red blood cells decreases drastically. Similarly, during the tick season, the cattle immune system is boosted by new infections, inducing a better control of the parasites (Darghouth et al., 1996) and leading to a consequent decrease in sensitivity of this microscopic method.

Cattle under six months of age had the lowest prevalence in April, because they were born after the previous season of theileriosis. A similar trend was reported by other authors (Ait Hamou et al., 2012; Khattak et al., 2012; Ziam et al., 2015). Flach et al. (1995) argue that this difference is caused by higher tick burdens in adult cattle. The infection rate evolution in our study showed that the older cattle were more infected than younger animals, which may be caused by the accumulation of infection during the successive tick seasons. However, as animals of different ages and origins were regularly introduced or removed from the farms before the tick season, it was not possible to compare further the evolution of infection rates between age groups.

During both visits, there was no difference in *T. annulata* molecular prevalence between males and females, which was also reported in other surveys (Ait Hamou et al., 2012; Khattak et al., 2012). On the other hand, Saleem et al. (2014) report that females are more infected than males (89.3% vs 10.7%), suggesting that females are less resistant to infection than males because of a regular decrease in their immune system (pregnancy and lactation). This was not observed in our study, although the infection rate of females was slightly higher than that of males during the first visit.

Cattle of local breeds are generally more resistant than exotic animals (Gharbi et al., 2014; Saleem et al., 2014). Glass et al. (2005) and Jensen et al. (2008) attribute the difference in sensitivity to tropical theileriosis between Holstein and Sahiwal breeds to a genetic difference leading to a high production level of pro-inflammatory cytokines in Holstein cattle. Moreover, the immune resistance of the local breed can block the multiplication of the parasite, preventing its release into the blood stream, which can give false negative results. A similar trend was observed in our study: the infection rate did not increase significantly in the local breed indicating that these cattle were resistant to infection.

The absence of a significant prevalence increase in Montbeliard and Holstein cattle may be due to the fact that the majority of these cows received a special care, including tick control, because of their high market value. In Fleckvieh cattle, a high increase of infection prevalence was observed which can be explained by the recent introduction of naïve Fleckvieh animals in the studied farms.

Cracks in cowshed walls are favorable to the overwintering and egg-laying endophilic tick vector *H. scupense*, whereas roughcasting and smoothing walls significantly reduce cracks and thus prevent nymphs and young adults of *H. scupense* to hide for overwintering (Gharbi et al., 2014). This should decrease the tick infestation of cattle and consequently the prevalence of theileriosis. In our study, the infection rate increased significantly during the tick season when the walls were not roughcast and had cracks. On the contrary, whitewashing walls had no influence on the cattle infection rate. The proportion of *H. lusitanicum* among the ticks infesting cattle is high in Western Algeria, in Oran (Yousfi-Monod and Aeschlimann, 1986) or Tiaret (Boulkaboul, 2003), but lower in Eastern parts of the country, in Jijel (Benchikh Elfegoun et al., 2007), El Tarf and Mila regions (Benchikh Elfegoun et al., 2013). Viseras et al. (1999) showed that *H. lusitanicum*, an exophilic tick, is a vector of *T. annulata* in Spain. This species could also be a vector of tropical theileriosis in Algeria. Cattle infestation by ticks was not studied during this survey. But, because Setif *wilaya* is close to Jilel and Mila areas, *H. lusitanicum* is probably not predominant in the region. However, its presence can make the control of tropical theileriosis more complicated and explain the occurrence of tropical theileriosis in farms with walls unfavorable to *H. scupense* overwintering. The importance of this vector should be assessed.

The partial sequencing of Tams-1 gene revealed that the Algerian amplicon is closely related to the other isolates from North Africa (from Egypt to Mauritania), but not to isolates from Europe or Asia. However, as the bootstrap values were low, this observation, favorable to the development of a single vaccine for the whole of Maghreb, should still be confirmed.

## ■ CONCLUSION

The PCR study of *T. annulata* infection in apparently healthy animals during the adult tick infestation period provided information on the prevalence in the region during this period, whereas the study of the infection rate increase between pre- and post-infection periods provided accurate information about risk factors. It appeared that only acaricide-treated and non-infected animals should be considered for trading in the country in order to decrease the dissemination of both tick vectors and *T. annulata* in farms and regions free of ticks and theileriosis. The elimination of cracks, plastering and smoothing the inner and outer surfaces of shed walls should drastically reduce the incidence of tropical theileriosis, when the main vector is *H. scupense*, because the cracks represent a favorable place for overwintering ticks. But in Algeria the control of this disease should also include *H. lusitanicum*, the exophilic tick species.

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## *Résumé*

**Ayadi O., Rjeibi M.R., Benchikh Elfegoun M.C., Gharbi M.** Prévalence et facteurs de risque de la theiléríose tropicale, et séquençage de l'agent pathogène causal *Theileria annulata*, dans la région de Sétif (Algérie), avant et après la période d'infestation par les tiques

Pour déterminer la prévalence de l'infection par *Theileria annulata* et l'influence de certains facteurs de risque, une étude moléculaire a été menée dans la région de Sétif (Algérie). Au total, 134 échantillons sanguins de bovins provenant de 21 fermes ont été collectés deux fois, en avril et novembre 2015, avant et après la saison des vecteurs de tiques. La prévalence moléculaire de *T. annulata* a été de 25,4 % en avril et de 50 % en novembre, indiquant une augmentation significative ($p < 0,001$) du nombre d'animaux porteurs asymptomatiques. La prévalence moléculaire a été significativement plus élevée que la prévalence sur frottis sanguins colorés au Giemsa pour les deux périodes. En avril, la race Fleckvieh a eu la prévalence moléculaire la plus faible mais cette prévalence a considérablement augmenté en novembre ($p < 0,001$). Le type d'élevage et le sexe n'ont eu aucune influence sur la prévalence de *T. annulata*. Toutefois, le taux d'infection a augmenté de manière significative pendant la période d'infestation par les tiques, car les murs n'étaient pas crépis et présentaient des fissures ($p < 0,001$). La séquence algérienne du gène Tams1 a été très similaire aux séquences mauritaniennes, tunisiennes et égyptiennes déjà connues.

***Mots-clés :*** bovin, *Theileria annulata*, PCR, séquence d'ADN, Algérie

## *Resumen*

**Ayadi O., Rjeibi M.R., Benchikh Elfegoun M.C., Gharbi M.** Prevalencia y factores de riesgo de la theilerosis tropical y secuenciación de *Theileria annulata*, agente patógeno causal, en la región de Setif (Argelia) antes y después de la estación de garrapatas

Con el fin de determinar la prevalencia de infección de *Theileria annulata* y la influencia de algunos factores de riesgo, se llevó a cabo un estudio molecular en la región de Setif (Argelia). Se colectaron un total de 134 muestras de sangre de ganado en 21 fincas, dos veces, en abril y noviembre 2015, antes y después de la estación de las garrapatas vectores. La prevalencia molecular de *Theileria annulata* fue de 25,4% en abril y de 50% en noviembre, indicando un aumento significativo ($p < 0,001$) en el número de animales portadores asintomáticos. La prevalencia molecular fue significativamente más elevada que la prevalencia en los frotis con tinción Giemsa en ambos periodos. En abril, la raza Fleckvieh presentó la menor prevalencia molecular, pero esta prevalencia aumentó significativamente en noviembre ($p < 0,001$). El tipo de raza y sexo no tuvo influencia en la prevalencia de *T. annulata*. Sin embargo, la tasa de infección aumentó significativamente durante el periodo de infestación de garrapatas, ya que las paredes no estaban revocadas y presentaban grietas ($p < 0,001$). La secuenciación del gen Algerian Tams1 fue muy similar a la ya conocida secuenciación mauritana, tunicina y egipcia.

***Palabras clave:*** ganado bovino, *Theileria annulata*, PCR, secuencia de ADN, Argelia