Detection of *Theileria annulata* in the parasitic ixodids of the spanish fightingbull by the nested-PCR technique.

García Fernández, P.; Caballero González, V. and Víseras Alarcón, J.
Área de la Producción Agraria. Centro IFAPA Camino de Purchil.
IFAPA. Consejería de Innovación, Ciencia y Empresa. Junta de Andalucía. Granada (Spain).

Received: 05.07.2005

In Memoriam: "La vida es corta; pero a juzgar por la obra de los que han sabido trabajar bien, es larga"

(Lucio Anneo Séneca).

Accepted: 20.09.05

Abstract: We report on the detection of *Theileria annulata* in ixodid ticks by the technique of nested-PCR using primers derived from the gene 18S rRNA. 135 specimens of adult ticks gathered in 51 fightingbull coming from 10 located different cattle ranches in five Spanish provinces have been processed (Cadiz, Sevilla, Cordoba, Albacete, Salamanca). The results obtained in this sampling, indicate to us that the PCR technique is a good tool to detect to *T.annulata* in the ticks, although the heads of cattle were carrying healthy with rates of parasitación in blood very low (TP media=1,82; min. 0.1 to max.10 by thousand) and titles of antibodies by the IFI test that oscillated between 0-1280 (average 160). The technique of nested PCR has been a good tool to detect *T. annulata* in the ticks with a 50.37% of positive specimens. The positive results by species have been: *Hyalomma lusitanicum* 20 of 25 samples (83.33%); *Hyalomma m. marginatum* 28 of 56 (50%); *Rhipicephalus bursa* 20 of 54(37,04% and *Hyalomma a. excavatum* 0 of 1 (0%); The importance of *H. lusitanicum* is ratified how main vector of the Mediterranean theileriosis in Spain.

Key words: Mediterranean theileriosis, Nested-PCR, *Theileria annulata*, Ixodid Ticks, *Hyalomma lusitanicum*, fightingbull.

Resumen: Se ha realizado un estudio sobre la detección de *Theileria annulata* en garrapatas utilizando la técnica de nested-PCR usando iniciadores derivados del gen 18S rRNA. Se han procesado 135 ejemplares de garrapatas adultas recogidas en 51 toros de lidia procedentes de 10 ganaderías distintas localizadas en diferentes provincias españolas (Cádiz, Sevilla, Córdoba, Albacete, Salamanca). Los resultados obtenidos en este muestreo, nos indican que la técnica de PCR es una buena herramienta para detectar a *T. annulata* en la garrapata, a pesar de que las reses eran portadoras sanas con tasas de parasitación en sangre muy bajas (TP media=1,82; min. 0,1 a máx.10 por mil) y titulos de anticuerpos por el test de IFI que oscilaban entre 0-1280 (media 160). La técnica de nested PCR ha sido una buena herramienta para detectar *T. annulata* en las garrapatas con un 50.37% de garrapatas positivas. Los resultados de positividad por especies han sido: *Hyalomma lusitanicum* 20 de 25 ejemplares (83.33%); *Hyalomma m. marginatum* 28 de 56 (50%); *Rhipicephalus bursa* 20 de 54 (37,04%) y *Hyalomma a. excavatum* 0 de 1 (0%); Se ratifica la importancia de *H. lusitanicum* como vector de la theileriosis mediterránea en España.

Palabras clave: Theileriosis mediterránea, Nested-PCR, *Theileria annulata*, garrapatas ixodidos, *Hyalomma lusitanicum*, toros de lídia.

1. Introduction.

The Mediterranean Theileriosis is a parasitic disease of the bovine cattle produced by the protozoan *Theileria annulata* and transmitted by ticks of the Ixodidae family. Between the recognized vectors it mentions the species of the genre Hyalomma, such as *H. detritum* (Samish and Pipano, 1978), *H. anatolicum excavatum* (Schein, 1975) and *H.anatolicum anatolicum* (Samish, 1977).

In Spain, *Hyalomma lusitanicum* is recognized like the most important vector of Mediterranean Theileriosis (Viseras *et al.*, 1999), mentioning to other species like potential vectors, such as *Hyalomma marginatum marginatum* and *Demacentor marginatus* (Habela *et al.*, 1993 a and b).

Corresponding author:
Dr. Pedro García Fernández
Área de la Producción Agraria. Centro IFAPA Camino de Purchil.
IFAPA. Consejería de Innovación, Ciencia y Empresa.
Junta de Andalucia,
Camino de Purchil sn. 18004 Granada (Spain).
Teléfono: +34 958 895311
Fax: +34 958 895203
Email: pedro.garcia@juntadeandalucia.es

The techniques used in the demonstration of the vectorial paper of ixodids, such as the xenodiagnostic, with the feeding of noninfected specimenes on ill experimental animals and their later healthy animals feeding, are very expensive and difficult to carry out. Other traditional techniques, such as the histological, need a long time and a highly qualified staff, to identify the protozoan within the tissue of the tick. The recent use of the techniques of molecular biology in the diagnosis of the diseases, such as the Polymerase Chain Reaction (PCR), has increased the specificity and sensitivity of the obtained results (Kok *et al.*, 1993 and Martín Sánchez et cols., 1999). With this technique we demonstrated the presence of *T. annulata* in the tick and after analyzing the different epidemological factors such as the rate of parasitation (RP) of the cattle, degree of immunization (IFI test), degree of feeding of the tick specimenes, etc., we can obtain trustworthy results that ratify if the tick is a carrier of the parasite or if it is the vector of the disease.

2. Material and Methods

The sampling has been taken in the month of June of 1999 on the 51 fighting bulls in the bullring of Granada, belonging to ten livestocks of fight race, located in the following provinces: Cadiz, Sevilla, Cordoba, Albacete and Salamanca (Table 1).

Tabla 1.- Results of sampling in fightingbull for the detection of the Mediterranean theileriosis

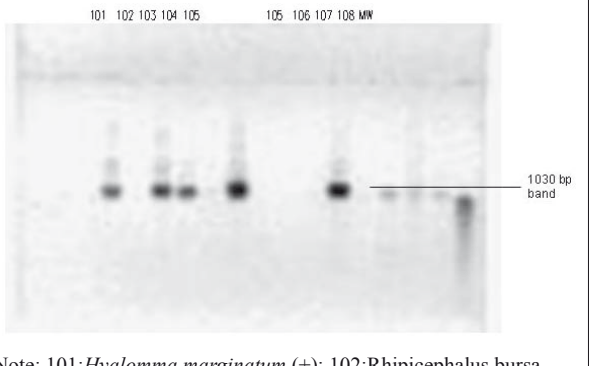
Tick species	Number Samples Ticks/ Fr. presence	Cattle Procedence	Parasited Heads Cattle /Total Heads	Titles IFI Min-max (Mean)	RPM *T. annulata* Heads
H. lusitanicum	51/27.77	Ab, Se, Ca, Sa	20/51	160-640(320)	3.30
H. m. marginatum	95/34.72	Ab, Se, Co, Sa	25/51	40-640 (160)	2.26
R. bursa	127/36.11	Ab, Se, Sa	26/51	0-1280 (80)	1,44
H. a. excavatum	1/1.38	Se	1/51	320	6.6

RPM= Rate Parasization Mean;
Ab= Albacete; Se= Sevilla; Ca= Cádiz; Sa= Salamanca; Co= Córdoba

In this study we have designed a protocol of the technique of nested PCR to detect the presence of *T. annulata* in the ixodid tick in parasitic phase and it has also been identified the rate of parasitation and the degree of immunization of the heads of cattle with the purpose of correlating its presence in the ixodid ticks with the possible vectorial paper of each tick. Two oligonucleotide PCR primers specific of the genres Theileria and Babesia have been used, belonging to the sequence of the gene 18S rRNA of *Theileria annulata*. 135 samples of adult ticks have been taken in different states of feeding have been processed.

To these head of cattle, blood has been also taken with EDTA-triK for the hematological diagnosis and blood without additives for the obtaining of serum and its later use in the serological diagnosis by means of the IFI technique. The samples of ticks were preserved in ethanol of 70º until their identification in the laboratory and its later processing. Later the ticks were sectioned, under conditions of sterility, by means of a longitudinal section obtaining two parts, one of which was used for the molecular proofs and the other was conserved with the purpose of detecting the parasite in the salivary glands by means of histological techniques (This results are not included in this work). With the halves of each sample one came to the extraction and purification of the total DNA from the ixodid tick and the protozoan using the Quiagen Ltd kit ©. For the detection of the specific sequence of *T. annulata* two consecutive amplifications with the PCR (Nested-PCR) technique were made.

In the first reaction we used a set of specific primers of the genres *Theileria* and *Babesia* designed in our laboratory (Direct Oligo Thefor1: TACGACTTCTCCTTCCTTT and reverse Therev1: ATTACCCAATCCTGACACAG), amplifying a region of 1100 pairs of bases (pb). In the second, two specific primers for *Theileria annulata* (direct oligo Thefor2: CCAATGACACAGGGAGGTAG and reverse oligo Therev2: TCCCTAGACGTTCCCTGACGG, obtaining one second region of 1030 pb. (Fig. 1).



Note: 101:*Hyalomma marginatum* (+); 102:Rhipicephalus bursa (++); 103: *R.bursa* (+);104 *R.bursa* (-); 105 *H. lusitanicum* (+++); 106, 107, 108 *H. lusitanicum* (-); **MW**: Moleculars Weight

Fig 1.- Results of the Nested-PCR to *Theileria annulata* (Band 1030 bp) of various species of ticks (numbers: 101-108) in 1.2% agarose gel.

In the first PCR it was started from a final reaction volume of 50 µl and the concentrations that were used for the preparation of the reaction mixture were: 5 µl 10X PCR buffer, 50mM $MgCl_2$, 12.5mM dNTP, 10µM Thefor1, 10 µM Therev1 and 2.5 u/µl Taq polimerase (Promega, Madison, WI, USA). The program used for the amplification with a Thechne Progene thermal cycler (Cambridge, UK), uncluded 26 cycles. An initial denaturation step at 94ºC for 12 min was followed by 1 cycle 94 ºC/1,5min and 25 cycles at 94 ºC for 30 s, annealing at 55,5ºC for 30 s and extension is obtained at 72ºC for 60 s.

The concentrations of the reagents of the second PCR were the following ones: 5 µl 10X PCR buffer, 50mM $MgCl_2$, 12.5mM dNTP, 10 µM Thefor2, 5 µl DNA and 2.5 u/µl Taq polymerase (Promega, Madison, WI, USA). Finally, the program to which the cocktail of 28 cycles was subjected has the following conditions: An initial denaturation step at 94ºC for 30 s, annealing at 58ºC for 30 s and the extension was obtained at 72ºC during 45 s. Final extension was done at 72 ºC for 7 min followed a hold step at 4ºC. Amplified DNA was subjected to electrophoresis in a 1,2% agarose gel (100V, 40min), pre-stained with ethidium-bromide and viewed under ultra-violet ligth.

3. Results and Discussion

The global results of the analyses by nested-PCR are expressed in Table 2, that indicate to us that 50.37% of all the samples of analyzed tick are carrier of *T. annulata*.

Taking into account the percentage of positivity in the different species, it is ratified that *Hyalomma lusitanicum* with a 83.33% (n=24) is the tick with greater presence of piroplasm, followed by *Hyalomma m. marginatum* with a 50.0% (n=56) and already with a lower value *Rhipicephalus bursa* (37.04%; n=54).

Table 2. Results obstained in analisis of the ixodid tick parasites of cattle by the Nested PCR to *Theileria annulata*

Results Nested-PCR / Tick	**Tick species**								
	H. lusitanicum		*H. marginatum*		*Rhipicephalus bursa*		*Hyalomma a. excavatum*		Totales
	Males	Females	Males	Females	Males	Females	Males	Females	
Positives	15	5	25	3	9	11	0	0	68
Negatives	1	3	25	3	20	14	1	0	67
Sums	16	8	50	6	29	25	1	0	135
Total	24		56		54		1		135
% PCR Positives	83,33		50,00		37,04		0,00		50,37

These results indicate the presence of piroplasm in the tissue of the tick, located mainly in its intestine when it is in feeding state (sexual phase of the cycle), or in the salivary glands in the esporozoite stage (asexual phase). In order to discern where is located, it will be necessary to dissect the salivary glands and to make the PCR just by this organ. If we consider the results of the head of cattle on which the ticks were fed we observed that *H. lusitanicum* has located preferably in the southern west of the Iberian peninsula (Cádiz and Sevilla), where the 100% of the analyzed head of cattle were positive to *T. annulata*, with a rate parasitation (R.P.) mean = 3.3 by thousands/head of cattle and a positives titles between 160 to 640. This species play an important roll in the spread of Mediterranean Theileriosis.

Hyalomma m. marginatum is the most cosmopolitan tick of cattle in Spain, being located in Andalusia (Córdoba and Sevilla), Castilla-La Mancha (Albacete) and Castilla León (Salamanca), with a lower R.P.mean to *Theileria annulata* (2.26 thousands/head), as well as lower mean titles (160), which is reflected in the smaller infectation of the ticks (50.0%).

On the other hand *Rhipicephalus bursa* presents a geographic distribution similar to the previous species, this tick is not a recognized vector of the Mediterranean Theileriosis; nevertheless, it presents a 37.04% of positives in our results of the PCR, what it indicates to us that is carrier of the piroplasm, having to discern if it is present in the salivary glands in order to assert that it can be a possible vector. The RP of the head cattle what is parasitized with this tick is still more bass (1.44 by thousands) and the titles of IFI oscillated between 0- 1280, mean 80.

Hyalomma a. excavatum is a very rare species of tick in spanish cattle, nevertheless we has find a single male specimen in Aznalcollar (Seville) wich resulted negative to PCR.

These findings confirm presumably that *H. lusitanicum* is the main vector of *T. annulata* in Spain and that the PCR is a useful method of determining the infection rates in ticks collected from cattle carrying low levels of this piroplasm.

4. Acknowledgements.

To Dr. F.J. Márquez of the Department of Animal Biology of the University of Jaén by his help in the design of the Nested-PCR technique during a stay in our Centre. This study has been subsidized by the INIA project 98-102.

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