ISSN: 1220-3173; E-ISSN: 2457-7618

MOLECULAR IDENTIFICATION OF *BABESIA CABALLI* IN VECTOR TICKS IN SOUTHWESTERN AND WESTERN ROMANIA

IDENTIFICAREA MOLECULARĂ A LUI *BABESIA CABALLI* ÎN CĂPUȘELE VECTOR ÎN SUD-VESTUL ȘI VESTUL ROMÂNIEI

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ABSTRACT

An increasing number of pathogens are transmitted via arthropod vectors. The detection of *Babesia caballi* in its invertebrate host is important for understanding parasite biology to gain more knowledge about host-parasite-vector interactions. The aim of this study was to determine the prevalence of *B. caballi* in tick vectors in three distinct counties in Romania, namely, Gorj, Caras-Severin, and Timiș. A total of 356 ticks were collected for the present study from the southwestern and western areas of Romania, i.e., the counties of Gorj, Caras-Severin, and Timis. The ticks were identified to the genus level, and DNA was subsequently extracted, quantified, and amplified with the Bc_18SF402 and Bc_18SR496 primer pairs and a TaqMan MGB probe. There were 236 ticks in the genus *Dermacentor,* 30 in the genus *Ixodes* and 90 in the genus Rhipicephalus. The total prevalence of *B. caballi* was 13.79%, and positive samples were identified in only one county, namely, Gorj County, with a positivity percentage of 30.30%. The identification of *Babesia caballi* parasitism represents an important threat to the health of horses in Romania, and vector ticks act as reservoirs of infection.

Keywords: ticks; *Babesia caballi;* real-time PCR

REZUMAT

Din ce în ce mai mulți patogeni sunt transmiși prin intermediul unor artropode vectori. Detectarea *Babesia caballi* în gazda sa nevertebrată este un punct important în înțelegerea biologiei parazitului pentru a dobândi mai multe cunoștințe despre interacțiunile gazdă-parazit-vector. Scopul acestui studiu a fost de a identifica prevalența *B. caballi* în căpușele vector în trei județe distincte din România, respectiv Gorj, Caraș-Severin și Timiș. Un număr total de 356 de căpușe au fost colectate pentru prezentul studiu, din zonele sud-vest și vest ale României, respectiv din județele Gorj, Caraș-Severin și Timiș. Căpușele au fost identificate la nivel de gen, ulterior fiind extras ADN-ul, cuantificat și amplificat cu o pereche de primeri Bc_18SF402, Bc_18SR496 și o sondă TaqMan MGB. Din numărul total de căpușe genul *Dermacentor* au fost 236, genul *Ixodes* 30 și *Rhipicephalus* 90. Prevalența totală a *B. caballi* a fost de 13,79%, probele pozitive fiind identificate într-un singur județ și anume județul Gorj, cu un procent de pozitivitate de 30,30%. Identificarea parazitismului cu *Babesia caballi* reprezintă o amenințare importantă pentru sănătatea cailor din România, iar căpușele vector acționează ca un rezervor de infecție.

Cuvinte cheie: căpușe; *Babesia caballi*; Real Time PCR

Ticks (*Acari: Ixodida*) are ubiquitous ectoparasitic arthropods and vectors of many pathogenic microorganisms, such as *Babesia* spp., *Borrelia* spp., *Rickettsia* spp., and *Ehrlichia* spp., all of which represent a significant medical threat to humans and animals (7). Active tick vector surveillance can provide information on the frequency and distribution of important pathogens in the environment. The detection of *Babesia caballi* in its invertebrate host is important for understanding parasite biology to gain more knowledge about host-parasite-vector interactions. In addition, parasite detection is necessary to determine the prevalence of these protozoa in different tick genera. An increasing number of pathogens are transmitted via arthropod vectors. Undoubtedly, the first arthropods to be recognised as vectors were ticks. Ticks now rank second in the transmission of pathogens to humans and first in the veterinary field (12). The main mode of transmission of piroplasmas is by competent ticks. With approximately 850 tick species worldwide, the potential for transmission is high (22). The tick species confirmed or suspected to be vectors of the causative pathogens of equine piroplasmosis are *Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp., *Hyalomma* spp., *Ixodes* spp., and *Rhipicephalus* spp. (21). Five genera have been identified in Europe, namely, *Rhipicephalus, Dermacentor, Hyalomma, Haemaphysalis* and *Ixodes*, and their vector competence has been demonstrated based on the detection of parasite DNA in tick species. Only one species of *Amblyomma*, namely, *Amblyomma cajennense*, has been implicated in the transmission of piroplasmas in the USA (21).

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MATERIALS AND METHODS

A total of 356 ticks were collected for the present study. Samples were collected between September 2023 and June 2024 from the southwestern and wes-

tern areas of Romania, respectively, in Gorj, Caras-Severin and Timis counties. For the number of ticks collected in each county, there were 265 in Gorj County, 39 in Caras-Severin County and 52 in Timis County.

All tick samples collected were from infested animals and were identified to the genus level based on standard keys (Estrada-Pena, 2004) using a stereomicroscope (Olympus®).

Of the 356 ticks collected and identified, 145 ticks were randomly selected for parasite DNA extraction and amplification. For better homogenisation and release of the contents, ticks were sectioned into two or four parts. Prior to the extraction process, mechanical lysis was performed to break the chitinous coating and release the contents. Specifically, the ticks were homogenised in 2 ml tubes with 1.4 mm diameter ceramic beads together with 500 µl of PBS. The samples were homogenised in a tissue homogeniser at 4000 m/s for 10 min, followed by centrifugation at 13000 rpm for 3 min. A MAG MAX Core Nucleic Acid Purification Kit (Applied Biosystems, Thermo Fisher Scientific), and a King Fisher Flex automated extractor were used for DNA/RNA extraction, and a AgPath-ID™ One -Step RT–PCR Reagent Kit together with a TaqMan MGB (minor groove binder) probe and a pair of primers (Bc_18SF402 and Bc_18SR496) were used for amplification (17) (Table 1).

Table 1

Real-time PCR amplification parameters of the study samples

No. cycle	**Temperature**	**Time**
1	45°C	10 minutes
1	95°C	10 minutes
40	95°C	15 seconds
	60°C	45 seconds

For sequencing, 3 randomly chosen positive samples were tested by classical PCR, amplifying a 95 bp segment and using a total reaction volume of 25 µl. The protocol comprised 5 µl of DNA, 12.5 µl of MyTaq TM Red Mix (BIOLINE® UK Ltd., London, UK), 1 µl of primer Bc_18SF402, 1 µl of primer Bc_18SR496, and 5.5 µl of ultrapure water. Amplification was performed with a My Cycler® thermocycler (Bio-Rad®, Berkeley, CA, USA) using a 32-cycle amplification program, i.e., initial DNA denaturation at 95°C for 1 min, followed by denaturation at 95°C for 30 s, alignment at 58°C for 30 s and extension at 72°C for 30 s, followed by incubation at 4°C to allow full extension of the PCR products. Amplicon analysis was performed by horizontal electrophoresis in a 1.5% agarose gel immersion system with the addition of the fluorescent dye Midori Green (Nippon Genetics® Europe). RedSafe™ (iNtRON Biotechnology,Inc., Gyeonggi-do, Korea) was used at 120 V and 90 mA for 60 min. A 100 bp DNA ladder marker (BIOLINE® UK Ltd., London, UK) was used as the molecular marker. Gel images of migrated DNA fragments were captured using a UV photodocumentation system (UVP®). To confirm the results, the PCR products of the isolates were bidirectionally sequenced by Macrogen Europe B.V., Amsterdam. The results were compared with those available in the Gen Bank database using BLAST analysis (24).

RESULTS AND DISCUSSION

Differentiation by morphological keys (4) resulted in the identification of ticks belonging to three genera (Fig. 1), namely, *Ixodes, Dermacentor* and *Rhipicephalus* (Figs. 2-4).

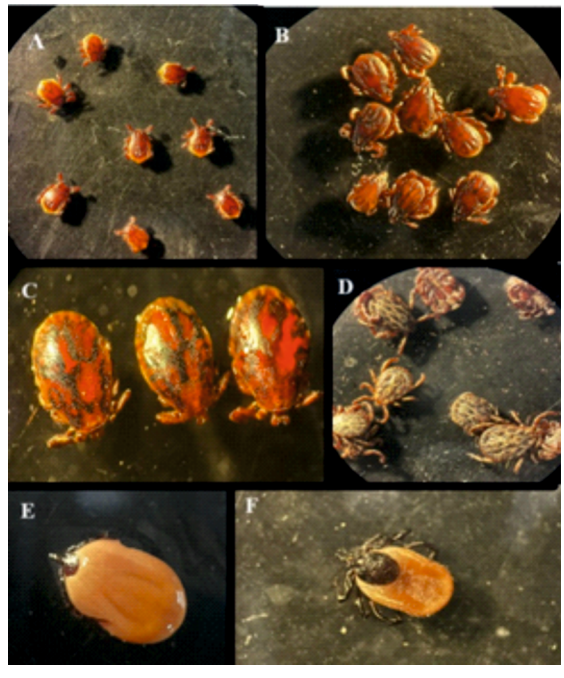

Fig. 1. A-C. *Rhipicephalus* spp., D. *Dermacentor* spp., E-F. *Ixodes* spp.

Fig. 2. *Dermacentor* spp., male, dorsal (left) and ventral (right) views 1. Anterior mouth parts; 2. Large palp; 3. Legs without pale rings; 4. Conscutum with ornamentation, forming a white pattern; 5. Presence of festoons; 6. Coxae, one with large and equal spurs; 7. Coxae four, very large; 8. Presence of festoons; 9. Posterior anal groove of the anus; 10. The spiracular plates were large and positioned posteriorly to the legs.

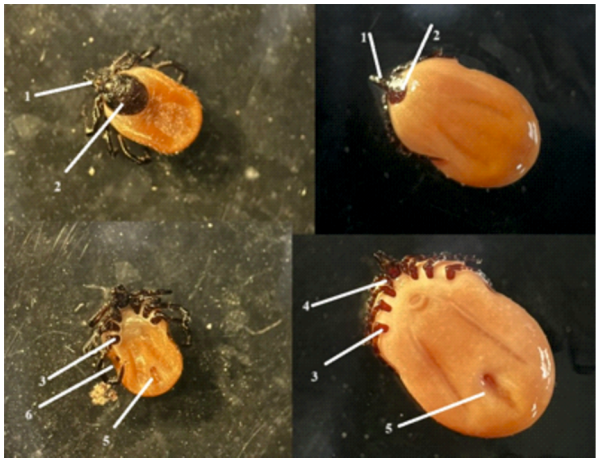

Fig. 3. *Ixodes* spp., female, dorsal (top) and ventral (bottom) views 1. Palp longer than in other genera; 2. Scutum present, ornamentation absent; 3. Coxae four of normal size; 4. Coxae one with unequal paired spurs; 5. The anal groove forms a loop anterior to the anus; 6. The spiracular plates were large and positioned posterior to the legs.

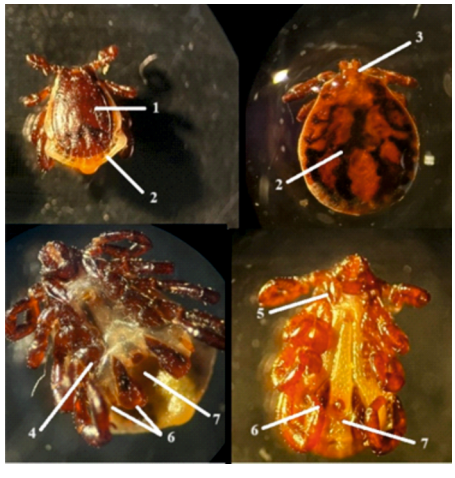

Fig. 4. *Rhipicephalus* spp., male, dorsal (top) and ventral (bottom) views 1. Conscutum; 2. Festoons present; 3. The base capituli were hexagonal in shape; 4. Coxae four of normal size; 5. Coxae, one with large equal spurs; 6. Ventral plates only in males, usually in pairs; 7. Anal groove posterior to the anus.

Of the total number of ticks, 217 were female, and 139 were male; these ticks belonged to the three identified genera (Fig.5).Thus, for the genus *Dermacentor, there* were 236 females and 100 males; for the genus *Ixodes, there were* 30 females; and for *Rhipicephalus* 90, there were 51 females and 39 males (Fig. 6).

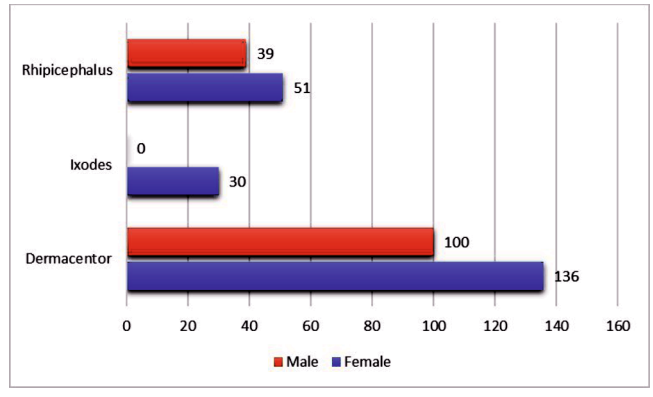

Fig. 5. Graphical representation of ticks by sex

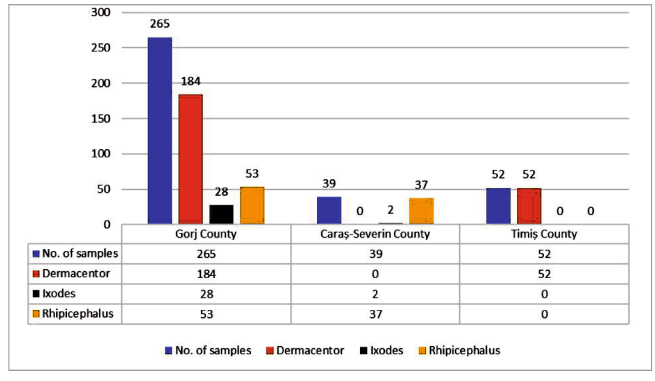

Fig. 6. Graphical representation of the number of ticks/gender/county

A total of 66 ticks were selected, from Gorj County, 40 from Timiș County and 39 from Caraș-Severin County, for DNA extraction and amplification to detect the parasitic genome of B*. caballi*; these ticks belong to the three previously identified genera, *Ixodes, Dermacentor* and *Rhipicephalus* (Table 2).

Examination of the DNA tick samples by PCR revealed that 20 were positive for *B. caballi* (Fig. 7).

A total of three from 20, positive samples, randomly selected, were tested by classical PCR, and the results are shown in Fig. 8.

For confirmation of the real-time PCR and classical PCR results, the same three samples,one for each genus of tick, were bidirectionally sequenced and compared with those available in the GenBank database via BLAST analysis, with similarity ratios of 97.96% with Or794983. 1, OR794984.1, OR794989.1, and OR794991.1 and 91% with MT355491.1 and MN 907451.1. The overall prevalence of *B caballi* in ticks was 13.79%, and positive samples were identified in only one county, namely, Gorj County, with a positivity of 30.30%.

Table 2

Number of ticks by sex in each county

County	No. of ticks	*Ixodes*	*Dermacentor*	*Rhipicephalus*
Gorj	66	28	29	9
Caraș-Severin	39	2	-	37
Timiș	40	-	40	-

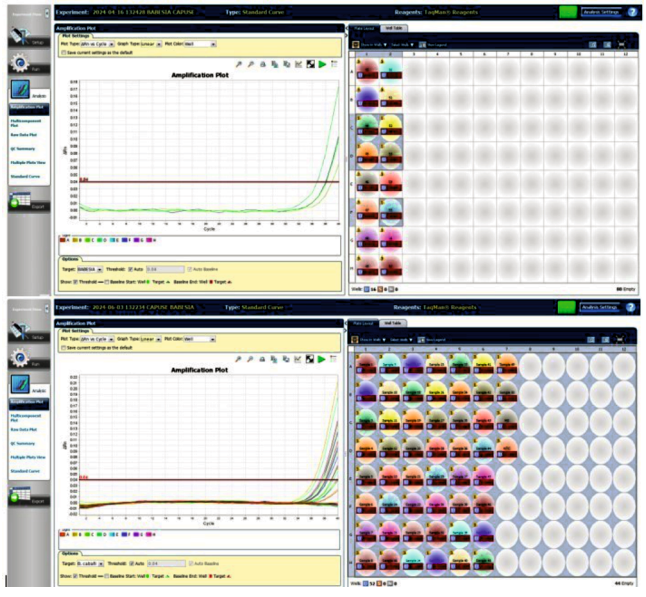

Fig. 7. Positive sample amplification curves – linear

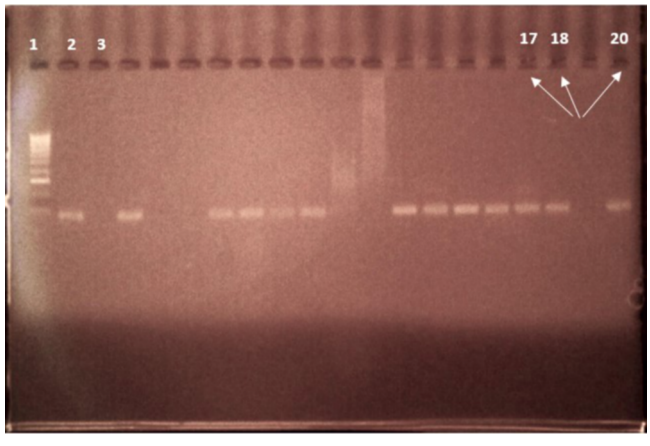

Fig. 8. Gel electrophoresis of PCR products of the 18S rRNA gene of *B. caballi* isolates. Lane 1-100 bp DNA ladder (BIOLINE® UK Ltd., London, UK), Lane 2-Positive control *B. caballi*, Line 3 negative control, Lines 17,18, 20 positive samples *(arrows).*

Of the total positive samples, 6 were from the genus *Ixodes* (4.13%), 12 from *Dermacentor* (8.27%) and 2 from the genus *Rhipicephalus* (1.37%) (Fig. 9).

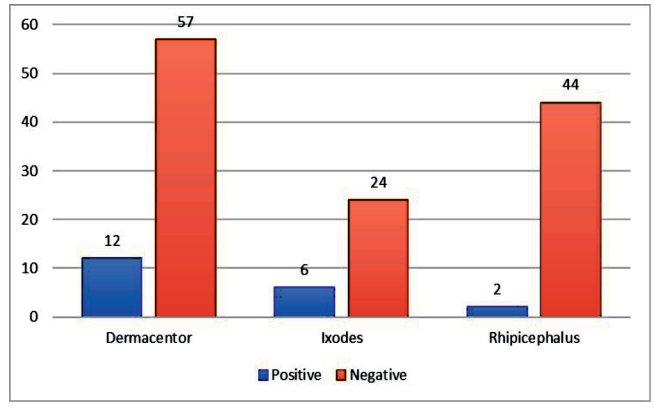

Fig. 9. *Number of positive samples per genus*

Studies carried out in Europe have identified eight tick species as carriers of one or both of the pathogens responsible for equine piroplasmosis (EP): *D. reticulatus, D. marginatus, R. bursa, R. sanguineus, R. annulatus, H. marginatum, H. punctata,* and *I. ricinus* (15).

In Romania, according to the specialised literature, 25 tick species belonging to the *Ixodidae* family have been identified thus far, thus also identifying the species involved in the transmission of piroplasmas (5, 13, 14). The tick species confirmed or suspected to be vectors of pathogens causing equine piroplasmosis are *Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp., *Hyalomma* spp., *Ixodes* spp., and *Rhipicephalus* spp. (21). Five genera have been identified in Europe, namely, *Rhipicephalus, Dermacentor, Hyalomma, Haemaphysalis* and *Ixodes*, and their vector competence has been demonstrated based on the detection of parasite DNA in tick species (15). Only one species of *Amblyomma* has been implicated in pyroplasma transmission in the USA (21).

Our results indicate that there are competent vectors infected with *Babesia caballi in* the southwestern region of Romania that act as a reservoir of infection. Positive samples belonged to all three identified gene-

Table 3

Distribution of positive ticks according to risk factors

Factor	No. of ticks tested	N* (%)*	*B. caballi* OR* *(95%CI*)*
Gender			
Female	217	12 (5.52%)	0.0597 (0.0345-0.1014)
Male	139	6 (4.31%)	0.0432 (0.0199-0.0910)
Genera			
Dermacentor	236	12 (8.27%)	0.0508 (0.0293-0.0868)
Ixodes	30	6 (4.13%)	0.0254 (0.0117-0.054)
Rhipicephalus	90	2 (1.37%)	0.0222 (0.0061-0.0774)

* N-number of positive samples; *% prevalence;
* OR odds ratio; * CI confidence interval; p *p value

ra. The involvement of *Ixodes* has been intensively debated, with studies claiming that this tick cannot transmit piroplasmas and others claiming that the pathogen can be transmitted transstadially because ticks collected from PCR-negative animals were found to be positive (21).

In Europe, 16 studies refer to potential vectors, and their competence has been demonstrated by the detection of parasite DNA in tick species (15). In a study carried out in France by Rocafort-Ferrer et al., out of a total of 585 ticks tested, a prevalence of 0.85% for *B. caballi was found* (18). In a study conducted in Italy of a total of 11 ixodid tick species identified, 5 were found to be positive for piroplasmas, with prevalences ranging from 1.2% for *Rhipicephalus* to 5% for *Dermacentor* (9). In China, Zhang et al. reported a prevalence of 6.7% for *Babesia caballi* out of a total of 119 ticks analysed (23). Another study in Italy showed a prevalence of 14.55% for *B. caballi* in 110 ticks belonging to the genus *Rhipicephalus* (19). In a study conducted in the UK, DNA was extracted from 879 ticks, seven species of *Babesia* were detected in 7.5%, and *B. caballi* had a prevalence of 0.68% (20). Other studies conducted in Romania have identified ticks and tick-borne pathogens (*Anaplasma phagocytophilum, Borrelia burgdoferi* and *Ehrlichia canis*) in other animal species as well (1, 2, 3, 8, 10). Other studies have documented *Ixodes ricinus, Ixodes frontalis, Dermacentor marginatus* and *Dermacentor reticulatus* as the most commonly identified species (6, 11, 14, 16).

CONCLUSIONS

The resulting prevalence of babesiosis determined by molecular methods was 13.79% in ticks from three Romanian counties. Positive samples were found only in Gorj County, with a positivity percentage of 30.30%. All three identified genera represent competent vectors for *Babesia caballi*, and their ability to detect parasitic DNA in tick species has been demonstrated. The implementation of an epidemiological surveillance program for both vectors and animals could contribute to the control of this disease in our country.

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