SHORT-FORMAT PAPER

Clinical bovine piroplasmosis by Babesia occultans, Italy

Nicola Decaro,a # Vittorio Larocca,a Antonio Parisi,b Michele Losurdo,a,b Riccardo Paolo Lia,a Maria Fiorella Greco,a Antonio Miccolis,c Gianpiero Ventrella,a Domenico Otranto,a and Canio Buonavogliaa

a Department of Veterinary Medicine, University of Bari, Valenzano, Italy
b Istituto Zooprofilattico Sperimentale di Puglia e Basilicata, Foggia, Italy
c Veterinary practisioner, Bari, Italy

Running title

Outbreak of Babesia occultans in cattle.

# Corresponding author:

Department of Veterinary Medicine, University of Bari, Strada provinciale per Casamassima km 3, 70010 Valenzano, Bari, Italy

Tel: +390804679832
Fax: +390804679843
E-mail: nicola.decaro@uniba.it

Words in the abstract: 50

Words in the text: 1071
ABSTRACT

A clinical outbreak of bovine piroplasmosis is reported in Italy. The etiological agent was characterized as Babesia occultans, a parasite regarded as apathogenic and never detected before in continental Europe. This report paves the way for further studies to assess the occurrence of this tick-transmitted protozoan in other European regions.

Keywords

Piroplasmosis, Babesia occultans, cattle.
Bovine babesiosis is a tick borne disease of cattle caused by apicomplexan protozoa of the genus *Babesia*, which may induce clinical conditions characterised by haemolytic anemia and fever, with occasional hemoglobinuria and even death of animals. *Babesia* parasites are responsible for severe economic losses in cattle industry, with large social and epidemiological impacts (1). Among the main *Babesia* species infecting cattle, *Babesia bovis* and *Babesia bigemina*, cause a severe and often fatal disease in untreated cattle of tropical and subtropical regions, whereas *Babesia divergens* is associated to bovine and human infection in Europe (2). In addition, *Babesia major* (3) and *Babesia occultans* are less pathogenic. The latter species has been detected, more than 30 years ago in southern Africa, being transmitted by *Hyalomma marginatum* tick (4). *Babesia occultans* has been never associated to clinical signs in animals, even after experimental infection of splenectomised individuals (4). For long time, the distribution of *B. occultans* was believed to be confined to sub-Saharan Africa (4,5), and only recently this protozoan was detected in *H. marginatum* in Tunisia (3) and in cattle in the Balearic Islands (6). However, even in this case clinical disease was not associated to *B. occultans* infection. This study reports on a clinical outbreak of bovine piroplasmosis by *B. occultans* in continental Europe.

In May-June 2012, an outbreak of clinical piroplasmosis occurred in a cattle herd of Apulia region (southern Italy), following a severe tick infestation. At that time, the herd consisted of 48 brown Swiss cows kept in loose housing and fed with forages and purchased feedingstuffs. In early May of each year, all cows were subjected to deltamethrin treatments against ticks, whereas facilities were treated with azamethiphos against flies. However, despite the antiparasitic treatments, tick infestation was repeatedly reported in the farm. Clinical signs were observed in 26 lactating cows (mainly primiparous animals) and consisted of marked pallor of the oral and genital mucosae, fever (up to 40.8°C), and drop of the milk production. Neither gastroenteric nor
respiratory signs were reported. After two days from the onset of fever, the animals received oxytetracycline hydrochloride at the dose of 11 mg/kg, PO, q 12 h for 7 days and two administrations, 36 h apart, of imidocarb dipropionate at the dose of 1.7 mg/kg, IM. Deltamethrin treatments were also repeated in all animals of the farms. The therapy resulted in complete recovery of animals within about ten days, although further clinical cases were sporadically reported on July and a single case occurred on August.

EDTA-blood samples collected from 8 febrile animals were used for haematological investigations, revealing mean haematological parameters generally below the reference ranges (RBC 3.75 ± 1.24 × 10^{12} l^{-1}, lower reference limit 5.0 × 10^{12} l^{-1}; PCV 19.95 ± 4.78%, lower reference limit 24%; Hg 66.1 ± 1.61 g l^{-1}, lower reference limit 80 g l^{-1}). Blood samples were also submitted to a PCR assay for the detection of bovine piroplasms using generic primers RLB-F2 (5′-GACACAGGGAGGTAGTGACAAG-3′) (7) and 18STBR (5′-GATCCTTCTGACAGGCACGTT-3′) as described elsewhere (8). Reactions were performed using LA PCR Kit Ver. 2.1 (TaKaRa Bio Inc., Shiga, Japan) in 25 μl volumes containing 1 μmol l^{-1} of primers, LA PCR Buffer (Mg^{2+} plus) 1x, 4 μl of dNTP mixture (corresponding to 400 μmol l^{-1} of each dNTP), 1.25 units of TaKaRa LA TaqTM and 5 μl of template DNA. The cycling protocol consisted of preheating of 94°C for 3 min following by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final extension of 72°C for 10 min. To rule out other pathogens responsible for anemia and/or fever in cattle, molecular analyses were carried out detecting Anaplasma marginale (9), Anaplasma centrale (10), bovine pestiviruses (11), bovine coronavirus (12), Schmallenberg virus (13), and bluetongue virus (14). Other eubacteria were searched for through amplification of 16S ribosomal DNA (15). All samples tested positive for Babesia spp./Theileria spp. 18SrRNA gene without any evidence of coinfection by other pathogens of cattle. PCR products were purified using Montage® PCR filter units (Millipore, Milan, Italy) and sequenced by BigDye 3.1 Ready reaction mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Sequences were...
imported and assembled with the Bionumerics 5.0 software (Applied Maths, Saint-Martens-Latem, Belgium) and analysed using BioEdit software package (http://www.mbio.ncsu.edu/bioedit/bioedit.html), NCBI’s (http://www.ncbi.nlm.nih.gov) and EMBL’s (http://www.ebi.ac.uk) analysis tools. The 18SrRNA gene sequences obtained from the diseased cows were 100% identical to each other and matched the best nucleotide identity (99.7-100%) to analogous sequences of *B. occultans* retrieved in GenBank database but not those of other protozoa within the genus *Babesia*. Phylogenetic analysis was carried out on a 1050-nucleotide sequence generated from sample 366/12-20 (GenBank accession KC157568), which was considered as the prototype strain (Italy-366/12-20). The neighbor-joining tree obtained with the Mega4.1 software (http://www.megasoftware.net/mega4/mega41.html) over 1000 replicates showed that strain Italy-366/12-20 clustered with the *B. occultans* clade, which also comprises *Babesia orientalis*, *Babesia* sp. Kashi 1 and *Babesia* sp. Kashi 2, but not with other piroplasms (FIG. 1). *Babesia* sp. Sable antelope and *Babesia* sp. Anglona, which was recently isolated from pigs in Italy (16), were also strictly related to the *B. occultans* cluster. This pattern of segregation was confirmed by the maximum-parsimony method in all analysed genomic regions (data not shown).

In this note, we reported a clinical outbreak of bovine piroplasmosis caused by *B. occultans*. Although there was no evidence of coinfections with other pathogens, less common infectious agents of bovine anemia could not be ruled out definitively. *Babesia occultans* is considered as a completely apathogenic or lowly pathogenic parasite of cattle with a geographical distribution restricted to sub-Saharan Africa (4,5). Although this piroplasm has been recently detected in the Balearic Islands (3,6), no reports from continental Europe were available. Nonetheless, *H. marginatum* is diffused in southern Italy, where the infection by *B. occultans* might be more spread than currently acknowledged (17). *Babesia occultans* was molecularly detected in cows that displayed fever, anemia and severe alteration in the haematological parameters. Circumstantial evidence for the condition caused by this protozoa are also represented by the efficacy of imidocarb
dipropionate in animals’ recovery. Phylogeny showed that other babesias, that have not been
classified so far, may likely belong to the species *B. occultans*. Importantly, the detection of this
protozoan in continental Europe suggests that thorough surveillance programs should be undertaken
for this tick borne diseases in order to implement effective control measures in cattle populations
where the proper tick species and the vectored pathogen occur.

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**Figure legend.**

**Fig. 1.** Neighbor-joining tree based on partial 18SrRNA gene sequences of ruminant piroplasms. A statistical support was provided by bootstrapping over 1,000 replicates. The scale bar represents the estimated numbers of nucleotide substitutions per position.
Fig. 1

Babesia motasi-AY533147
Babesia motasi-AY260180
Babesia sp. China-BQ1-AY260182
Babesia ovata-AY081192
B. bigemina-X59605
Babesia bigemina isolate BRC02-FJ426361
Babesia crassa-AY260176
Babesia major-EU622907
Theileria annulata-M64243
Theileria buffeli-AF236094
Babesia caballi strain EB1-AY534883
Babesia sp. EU1-A046575
Babesia sp. DD-2004-A053915
Babesia odocoilei-U16369
Babesia capreoli-AY726009
Babesia divergens-A0789076
Babesia sp. 10-JQ682877
Babesia sp. Xinjiang-2005-DQ159073
Babesia sp. giraffe 544-FJ213577
Babesia sp. sable antelope/2005-EU376016
Babesia sp. Anglona/AA-2011-HQ437690
Babesia orientalis strain DaYe-HQ840969
Babesia orientalis-AY596279
Babesia occultans-EU376017
Babesia sp.-U09834
366/12-20
Babesia sp. Kashi 1-AY726556
B. occultans-58-HQ331478
B. occultans-68-HQ331479
Babesia sp. Kashi 2-AY726557
Babesia ovis-AY533146
Babesia bovis-L19077