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Original article

Molecular identification of *Babesia* spp isolated from Polish cattle with asymptomatic protozoa infections

M. Staniec, Ł. Adaszek, K. Buczek, S. Winiarczyk

Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences Lublin, Głęboka 30, 20-612 Lublin, Poland

Abstract

The aim of the paper was to study the epizootic situation of babesiosis in the cattle population in eastern Poland and possibly to determine what species of protozoa infects Polish cattle. Blood samples for molecular analysis (real time PCR) were collected from 192 dairy cows from various farms located in eastern Poland. The infection was detected in 10.4% of the samples. All animals were infected with *Babesia occultans* which sequence of the 18S RNA gene fragment showed a 93.1%, homology with the sequence of *B. occultans* EU 376017. This is the first report about the detection of *B. occultans* DNA in asymptomatic cattle in eastern Poland.

Key words: babesiosis, cattle, sequencing

Introduction

Bovine babesiosis is a tick-borne disease. Its etiologic agents are intraerythrocytary protozoa belonging to the genus *Babesia*, family *Babesidae*, order *Piroplasmida*, phylum *Apicomplexa* (Vial and Gorenflot 2006). In the body of the animals, the protozoa proliferate in the host's red blood cells, causing their destruction. If untreated, the disease often leads to death of the infected animal. Two groups of *Babesia* parasites capable of infecting cattle can be distinguished: large *Babesia* (*B. bigemina, B. major, B. occultans, B. ovata, B. jakimovi*), whose merozoites are longer than cattle erythrocyte radius, and small *Babesia* (*B. bovis, B. divergens*), with merozoites are smaller than the erythrocyte radius (Chauvin et al. 2009, Cao et al. 2012). The greatest pathogenicity in cattle is observed for three species: *B. bovis, B. bigemina* and *B. divergens*. Their range depends on the presence of their vector – ticks belonging to the *Ixodidae* family (Gohil et al. 2013). The protozoan species with lesser pathogenicity include e.g. *B. major* and *B. occultans* (Decaro et al. 2013).

Cattle babesiosis occurs mainly in areas with a tropical or subtropical climate, there are relatively few reports of cases of this disease in Europe (Hornok et al. 2006, Cassini et al. 2012, Ionita et al. 2013). Until now, no cases of babesiosis in this animal species have been reported in Poland. There are also no reports about the presence of the etiologic agent in ticks collected on the territory of Poland.

The study was aimed at evaluating the epizootic situation of babesiosis in the selected bovine herds in eastern Poland and possibly determining what species of protozoa infects Polish cattle.

Correspondence to: Ł. Adaszek, e-mail: ukaszek0@wp.pl, tel.: +48 81 445 61 92, fax: +48 81 445 62 02

Materials and Methods

Animals used in the study and sample collection

The study included 192 dairy cows, the Polish Black-and-White breed, in the age range of 4-9 years, coming from eastern Poland and bred in a pasture management system. The study animals originated from ten farms (in 9 of them, the blood was collected randomly from 20 cows, in 1 from 12 cows) separated from one another by no less than 200 km. The animals had repeated contact with ticks but none of the animals studied had any clinical symptoms of babesiosis.

DNA extraction and amplification

The DNA from the blood of the animals was isolated using a DNA Blood kit (A&A Biotechnology, Gdańsk, Poland) according to the procedure as provided by the manufacturer. Purified DNA was suspended in 100 μl of Tris buffer to be used in further analysis.

The PCR in real time with the SYBR Green 1 dye was carried out in thin-walled test tubes with a capacity of 100 μl using the Corbett apparatus. A DyNAmo HS SYBR Green qPCR Kit (Finnzymes) was used in the method thus allowing a high specificity reaction. PCR was performed according to the method described by Altay et al. (2008) with a small modification with the primers RLBF2 (5-GACACAGGGAGGTAGTGACAAG-3) and RLBR2 (5'-CTAAGAATTTCACCTCTGACAGT-3'), used to amplify the 18S rRNA gene fragment *Babesia*/*Theileria* spp. The primers allow the amplification of the product with a size of 390-430 bp. The reaction mixture with a capacity of 20 μl consisted of the following components: 2 μl of the DNA matrix, 7.2 μl of water, 0.4 μl of each of the RLBF2 and RLBR2 primers (the final concentration of 50 pM), 10 μl of the Master Mix containing a hot start version of the modified polymerase Tbr (Thermus brockianus), buffer for the polymerase Tbr, dNTP, MgCl2 and the intercalating SYBR Green 1 dye.

The optimised real-time PCR included 40 subsequent cycles, each of them consisting of three stages: denaturation at 94°C for 35 s, annealing at 51°C for 35 s and extension at 72°C for 35 s. The measurement of the reaction mixture and the determination of the Ct indicator value (the number of amplification cycles, after which the fluorescence intensity of the formed product is higher than the background fluorescence) were carried out in real time at the stage of elongation of a helix complementary to the DNA matrix. In order to prove the specificity of amplification, the melting temperature of the PCR products was defined (HRM) by a gradual increase in the reaction mixture temperature from 70°C to 95°C while continually measuring the fluorescence intensity. Additionally, after the completion of amplification, all the real-time PCR reaction products were analysed with the electrophoretic method in a 1% agarose gel.

DNA sequencing

The products were purified using QIAquick spin columns (Qiagen, Germany) and eluted in 50 μl of Tris10 mM, pH 7.6. DNA sequencing was performed on both strands using the same primers employed for PCR at the DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. DNA sequences were assembled and edited using SeqMan (DNAStar, Lasergene, USA) and MegAlign (DNAStar, Lasergene, USA), with alignments to the published Babesia/Theileria spp 18S rRNA gene isolated from cattle: *B. occultans* (EU376017), *B. bigemina* (DQ785311), *B. bigemina* (EF458198), *B. bigemina* (FJ 426362), *B. divergens* (AY789076), *B. divergens* (AY572456), *B. divergens* (AJ 439713), *B. major* (EU622907).

Results

PCR

Out of 192 animals studied, a PCR test for the presence of *Babesia*/*Theileria* genetic materials gave positive results in 20 animals, which constitutes 10.4% of the study population. The real-time PCR products were visualised with the electrophoresis method in the agarose gel. Their size compared with the mass standard was 412 bp. The Ct values read from the amplification curve fluctuated around 23 cycles for all the examined samples. The melting temperature (Tm) of 20 products was 80°C.

Sequence analysis

Clear sequences of *Babesia*/*Theileria* DNA amplification products were obtained from all 20 samples. The comparison of obtained nucleotide isolate sequences in own studies with the use of the software DNA Star MegAligne determined the level of their mutual homology to be in the range of 99-100.0%.

The phylogenetic analysis of the *Babesia*/*Theileria* sequences obtained in own research enabled the differentiation of two groups of protozoa, called A and B. Group A consisted of 18 isolates (No. 1-18), with a mutual homology of nucleotide sequences of about 99.5-100% and a consensus sequence of a part of 18S RNA gene:

GACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCTACTGTCTTGTAATT

GGAATGATGGTGACTTAAAACCTCACCAGAGTACCAATTGGAGGGCAAGTCTGG

TGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGT

TAAAAAGCTCGTAGTTGAATTTCAGCGTTTCGCTTGTGCCCTCTTGATCTGTGCAC

ATTCTCTCTCGCTTTTTTTTTCCTATTACTTTGAGAAAATTAGAGTGTTTCAAGCAG

GCTTTTGTCTTGAATACTTGAGCATGGAATAATAGAGTAGGACCTTGGTTCTATTT

TGTTGGTTTTGTAACCTTGGTAATGGTTAATAGGAACGGTTGGGGGCATTCGTATT

TAACTGTCAGAGGTGAAATTCTAG

while Group B consisted of only two isolates: (No 19-20), with an identical sequence:

GACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCTACTGTCTTGTAATT

GGAATGATGGTGACCCAAACCCTCACCAGAGTAGCAATTGGAGGGCAAGTCTGG

TGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGT

TAAAAAGCTCGTAGTTGAATTTCAGCGTTTCGCTTGTGCCCTCTTGATCTGTGCAC

ATTCTCTCTCGCTTTTTTTTTCCTATTACTTTGAGAAAATTAGAGTGTTTCAAGCAG

GCTTTTGTCTTGAATACTTGAGCATGGAATAATAGAGTAGGACCTTGGTTCTATTT

TGTTGGTTTTGTAACCTTGGTAATGGTTAATAGGAACGGTTGGGGGCATTCGTATT

TAACTGTCAGAGGTGAAATTCTAG

The sequences of own isolates were compared with the use of DNA Star MegAligne software with correspondent sequences of *Babesia*/*Theileria* isolates available in the PubMed NCBI database: The analysis of this comparison showed that the sequences of own isolates exhibit 84.2-93.1% homology with the published nucleotide sequences of 18S RNA from protozoa isolated from cattle around the world. The greatest similarity (93.1%) was observed between the sequences of own isolates and the sequence *B. occultans* EU 376017 (Fig. 1). These two sequences were located on the same branch of the phylogenetic tree, constituting one monophyletic group which was clearly separated from the remaining sequences obtained from the gene bank. On this basis, the parasites detected in own studies were considered to be strains of *B. occultans*.

Discussion

In spite of numerous publications about bovine babesiosis in various regions of the world, there are few comprehensive works which would enable the analysis of the epidemiologic situation of this disease in Europe, and especially in Poland.

Bovine and equine babesiosis was present in Poland in the Interwar period. After the Second World War, the disease became gradually less and less frequent. However it may be presumed that it was not eliminated from cattle totally, but only its course gradually changed (Milczak 2003).

Uilenberg (1983) points out that the course of babesiosis may be very variable and it is difficult to predict the direction of the disease development, while infected animals do not always exhibit clinical symptoms. This phenomenon is especially frequent on endemic territories characterised by the enzootic equilibrium between the mammal, the vector and the pathogen.

In our own PCR-based research, the presence of the DNA of *Babesia* sp. was detected in 10.4% cows (20 out of 192 studied animals). On the basis of the analysis of the sequences obtained it was determined that own isolates exhibit mutual homology (99%) but relatively low similarity (84.2-93.1%) to the earlier published nucleotide sequences of 18S RNA gene of protozoa isolated from cattle around the world.

The greatest similarity (93.1%) was observed between the sequences of the own isolates and the EU 376017 *B. occultans* sequence. *B. occultans* is classified to the group of least virulent bovine pathogens and its

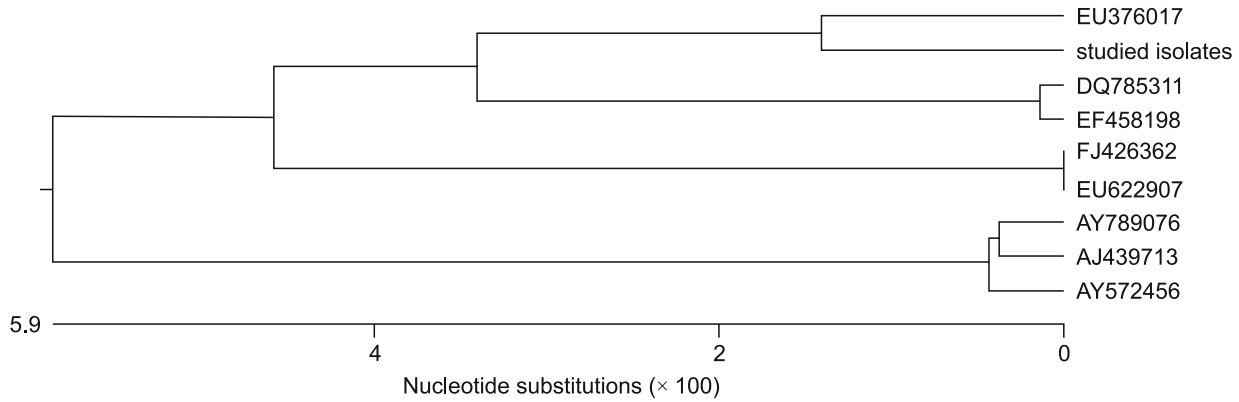

Fig. 1. Phylogenetic relationships between sequences of the isolates in the present study and sequences deposited in GenBank.

presence in Europe has been noted only recently (Ros-García A. et al. 2012, Ionita et al. 2013).

In Europe, the prevalence of *Babesia* spp. in cattle is very variable. In studies conducted in France on 68 cows, the genetic material of *B. major* was found in PCR test in only one specimen (Criado-Fornelio et al. 2009). Also in Italy, a study conducted on a group of asymptomatic animals revealed the presence of the genetic material of *B. divergens* in only 1 out of 394 samples (Cassini et al. 2012). Using the PCR technique, Torina et al. (2007) detected the genetic material of *B. bigemina* in 7.69%, and *B. bovis* in 3.39% of the cattle studied from Sicily.

Similar results were obtained in a study on a group of 133 specimens from the territory of Minorca (Almerìa et al. 2001 B). The genetic material of *B. bigemina* was found in 8 samples studied (6%), while *B. bovis* was detected in only one case (0.75%). In other studies of this author, the invasion rates were 3% (*B. bovis*) and 5.7% (*B. bigemina*) (Almerìa et al. 2001 A, Almerìa et al. 2009).

García-Sanmartín et al. (2006) studied 263 cows from northern Spain. The rate of specimens with detected *Babesia* spp., was 9.1%, out of which *B. bigemina* was detected in 7 samples (2.7%), *B. bovis* in 6 samples (2.2%) while*B. divergens* in 3 samples (1.1%).

Other studies conducted in central and southern Portugal indicated the presence of the genetic material of *B. bigemina* in 34.2% of the animals studied (139/406) and *B. bovis* in 70.7% of them (287/406).

Devos and Geysen (2004) examined 254 blood samples coming from cows from 24 herds in France. Molecular studies resulted in 20% of positive samples, where the genetic material of *B. divergens* was detected.

The aforementioned literature review leads to the conclusion that the main piroplasm species attacking cattle in Europe are: *B. bigemina*, *B. bovis*, and *B. divergens*. Finding the genetic material of *B. occultans* in the blood of Polish cattle sheds a new light on the epidemiology of bovine babesiosis and urges for a revision of the range of these parasites.

The detection of this protozoan in continental Europe suggests that thorough surveillance programs should be undertaken for this tick-borne disease in order to implement effective control measures in cattle populations.

Acknowledgements

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