



Original article

Co-infection with tick-borne disease agents in cattle in Russia

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ABSTRACT

Tick-borne diseases cause significant livestock losses worldwide. In Russia, information concerning single or mixed infections with different *Anaplasma*, *Theileria* and *Babesia* species in cattle is very limited. This study was conducted to determine the level of co-infection with protozoan pathogens (*Theileria* spp. and *Babesia* spp.) and rickettsial pathogens (*A. marginale* and *A. phagocytophilum*) in cattle in central Russia. Blood samples were examined with real time polymerase chain reaction (RT-PCR) for *A. marginale* and *A. phagocytophilum*, and by amplifying the V4 hypervariable region of the 18S rRNA gene, followed by cloning, DNA sequencing, and phylogenetic analyses, for *Babesia* and *Theileria* species. In total 67% of examined blood samples were positive for *Theileria* spp. or *A. marginale*, and 19% of the animals were co-infected with *Theileria* spp. and *A. marginale*. Seasonal variation in prevalence was found for *Theileria* spp. Phylogenetic analysis based on 18S rRNA gene sequences revealed the presence of five *Theileria* species: *T. annulata*, *T. orientalis*, *T. buffeli*, *T. sergenti*, and *T. sinensis*. No samples were positive for *Babesia* spp. or *A. phagocytophilum*. The data obtained for prevalence of bovine theileriosis and anaplasmosis in the central part of Russia underscore the need for improved surveillance and control programs to reduce tick-borne diseases in cattle.

1. Introduction

Ticks are the most important vectors of pathogens affecting cattle worldwide (Guglielmo, 1995; Manzano-Román et al., 2012; Sivakumar et al., 2016; Zhou et al., 2016; Lagrée et al., 2018; Rashid et al., 2018; Wikel, 2018). Tick-borne diseases (bovine anaplasmosis, theileriosis, and babesiosis) cause significant economic losses in many countries (Kocan et al., 2003; Kivaria, 2006; Rar and Golovljova, 2011).

Bovine anaplasmosis is considered one of the top ten economically important infectious non-contagious rickettsial diseases affecting ruminants. The epizootiology of anaplasmosis is complicated by the life-long carrier state of animals that have recovered from clinical disease (Amorim et al., 2013). In cattle, anaplasmosis is caused by *A. marginale*, *A. phagocytophilum*, *A. centrale*, and *A. bovis*. *Anaplasma marginale*, the most common etiologic agent of bovine anaplasmosis, is widespread throughout the world (Kocan et al., 2003). It causes mild to severe febrile hemolytic anemia, and animals that survive an acute infection develop persistent infections and serve as reservoirs of *A. marginale* (Kocan et al., 2003). *Anaplasma phagocytophilum* is an obligate intracellular multi-host parasite that infects granulocytes and causes tick-borne fever (TBF) in ruminants (Woldehiwet, 2008; Atif, 2015). The prevalence of bovine anaplasmosis and TBF varies according to locality, bioclimatic area, tick species composition, animal breed, and type of

breeding (Belkahia et al., 2015; M'ghirbi et al., 2016).

Theileriosis and babesiosis are other widespread hemoparasitic transmissible diseases of cattle caused by intracellular protozoan parasites of the genera *Theileria* and *Babesia*. Economic damage is determined not only by the animals' illness, but also by a sharp decline in productivity. In most cases, these protozoan diseases manifest themselves as a mixed and often latent invasion, which complicates its diagnosis, treatment, and measures for its elimination.

Taking into account that tick-borne hemoparasitic diseases present a serious threat to the livestock populations, reliable methods for identification of their causative agents is important for their control (Reetha et al., 2012; Maharana et al., 2016; Mohamed et al., 2018). Molecular genetic methods based on polymerase chain reaction (PCR) possess more sensitivity and specificity in pathogen detection compared with microscopic and serological techniques. They are independent of the immunocompetence of animals, can differentiate between morphologically similar parasites and facilitate detection of infection in the latent phase of the disease when the level of parasitemia is often below the detection limit of microscopic and serological methods. In addition, PCR-based methods allow dealing with a large number of samples and are flexible to be automated (Gasser, 2006; Michelet et al., 2014).

PCR is the most sensitive and specific tool for diagnosis of anaplasmosis, theileriosis and babesiosis during the carrier state in the

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absence of clinical symptoms (Kocan et al., 2010; Mans et al., 2015). Several gel-based and real-time PCR assays have been developed and used for diagnosis of *A. marginale* (Torioni de Echaide et al., 1998; Carelli et al., 2007; Torina et al., 2012; Kovalchuk et al., 2015) and *A. phagocytophilum* (Courtney et al., 2004; de la Fuente et al., 2005; Henningson et al., 2015; Arkhipova et al., 2018). RT-PCR assays (Kovalchuk et al., 2015; Arkhipova et al., 2018) allows detecting 10^2 copies of *msp4* gene in the analyzed sample. Specific amplification and sequencing of the V4 hypervariable region of the 18S rRNA gene encoding rRNA of the small ribosomal subunit (SSU rRNA) is widely used to detect *Babesia* and *Theileria* species (Gubbels et al., 1999; Sanmartin et al., 2006; Lempereur et al., 2017).

Until now, data on prevalence of bovine anaplasmosis, theileriosis and babesiosis in Russia is scarce. Bovine babesiosis was reported to affect cattle in the southern areas of Russia (Belimenko et al., 2012; Gulyukin et al., 2013). Bovine anaplasmosis is distributed in the southern and central areas of Russia, Western Siberia (Tyumen, Novosibirsk and Altay), Kaliningrad and Ulyanovsk regions (Kazakov and Idina, 2009; Gulyukin et al., 2013; Georgiou and Belimenko, 2015; Liberman and Khlyzova, 2015). Theileriosis was detected in the republic of Dagestan (Darbisheva et al., 2014). A distinctive characteristic of these diseases in the central part of Russia is their seasonal natural focality associated with vectors (ixodid ticks) and with prolonged, sometimes lifelong presence of parasites in the ill animals, during which relapses are possible due to a decrease in the resistance of the organism (Skornyakova, 2014).

A better understanding of the epidemiology of single and mixed infections is essential for evaluating the impact of tick-borne diseases and their potential for spread. Co-infections can alter host susceptibility, infection duration, transmission risks, and clinical symptoms (Vaumourin et al., 2015) and might play a role in incidence, distribution, and possible control of diseases (Anderssons et al., 2017). The objective of this study was to determine the prevalence of co-infections of the cattle with tick-borne diseases agents in the Moscow Region of Russia.

2. Materials and methods

2.1. Samples collection from cattle and DNA extraction

Blood samples were collected in 2015–2016 at the end of March and in the first days of August from the jugular vein of black-and-white Holsteinized adult clinically healthy cows ($n = 113$) of one dairy herd from the Moscow region of Russia (55.3°N, 36.1°E). A veterinarian sampled the animals after obtaining farm owner's permission without approval from ethics committee (allowed in Russia). DNA was isolated from 100 μ l EDTA-preserved whole blood samples using M-Sorb Kit (Synthol, Moscow, Russia) according to the manufacturer's instructions. DNA samples were analyzed spectrophotometrically (BioPhotometer Plus, Eppendorf, Germany) and stored at -20°C until used as a template for PCR.

2.2. Molecular detection of tick-borne pathogens and sequencing

Testing for presence of *Theileria* spp. and *Babesia* spp. was performed by amplifying, cloning and sequencing of ~ 420 bp fragments of V4 hypervariable region of rRNA gene as described earlier (Mans et al., 2011a, b). This approach is widely used for study of *Theileria* and *Babesia* spp. diversity in different parts of the world (Criado-Fornelio et al., 2009; Liu et al., 2010; Mans et al., 2011a; Gebrekidan et al., 2014). Amplification of a 390–430 bp fragment of the 18S SSU ribosomal ribonucleic acid (rRNA) gene spanning the V4 region of *Babesia* and *Theileria* species was carried out with forward primer, FThBa (5'-GAGGTAGTGACAAGAAATAACAATA-3'), and reverse primer RThBa (5'-CTAAGAATTCACCTCTGACAGT-3') (Gubbels et al., 1999) hybridized with regions conserved for *Theileria* and *Babesia*. Primers were

synthesized by Synthol (Moscow, Russia). The PCR reactions were performed in a 20 μ l reaction mixture consisting 0.2 mM of each dNTPs (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 μ M of each primer, 1 \times Phusion HF buffer and 0.02 U/ μ l Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, USA) and 3 μ l of purified DNA sample as a template. The reactions were performed with an automated DNA thermal cycler (Nyx Technik, San Diego, CA, USA) with a 5 min denaturation step at 95°C followed by 40 cycles. Each cycle consisted of a denaturing step of 45 s at 95°C , an annealing step of 45 s at 50°C , and an extension step of 1 min at 72°C and a final extension at 72°C for 10 min.

Amplification products from positive samples were analyzed by agarose gel electrophoresis and expected bands were excised and purified by the Cleanup Mini Gel DNA Recovery Kit (Evrogen, Moscow, Russia) according to the manufacturer's instructions. DNA cloned with plasmid pJET1.2/blunt Vector CloneJET PCR Cloning kit (Thermo Fisher Scientific) and recombinant plasmid vectors were used for competent cells *E. coli* DH5 α transformation according to the manufacturer recommendations. After plasmid purification by Plasmid Miniprep (Evrogen), three positive clones from each sample were sequenced bidirectionally by the Sanger method at Evrogen JSC (Moscow, Russia). The obtained sequences were analyzed by BLASTn search in GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Multiple alignments of ~ 430 bp fragments of 18S rRNA gene were carry out by Muscle algorithm (<http://www.drive5.com/muscle/downloads.htm>) of Molecular Evolutionary Genetics Analysis (MEGA) program (www.megasoftware.net). Obtained sequences were submitted to GenBank (<https://submit.ncbi.nlm.nih.gov/>). Phylogenetic analyses were conducted with MEGA 10.0 X program (Kumar et al., 2018), using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993). The tree involved 49 nucleotide sequences. The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar, 2002).

The real-time PCR assay for detection of *A. marginale* was performed in 10 μ l of mixture, containing 5 μ l of the LightCycler[®] 480 Probes Master reagent (Roche, Basel, Switzerland) (Kovalchuk et al., 2015). The forward and reverse primers were are MSP4-D1 5'-CATGAGTCAC GAAGTGGCT-3' and MSP4-R2 5'-GGCACACTCACATCAATC-3' (0.2 μ M of each), fluorescently labeled probe MSP4-probe5'-Cy5) AAGGGG AGTAATGGAGAGTAGCT-3' (0.1 μ M), 30 ng of DNA, as described earlier (Kovalchuk et al., 2015). The PCRs were performed in a 96-well plate using a LightCycler 96 instrument (Roche) under the following conditions: a hot-start DNA polymerase activation at 95°C for 2 min at 95°C followed by 45 amplification cycles: denaturation at 95°C , for 15 s, primer annealing at 58°C , during 15 s, elongation at 72°C , for 15 s; final annealing for 2 min at 72°C . The fluorescence signal was recorded on the Cy5 channel. The results were analyzed using software for the LightCycler 96 instrument (Roche) version SW1.1.

Amplification of 207 bp fragment of *A. phagocytophilum msp4* gene was carried out in 10 μ l of mixture containing 2 μ l of 5x qPCRmix-HS SYBR master mix (Evrogen), primers A_phag_msp4RT-D 5'-TTTAATTA GTGGATCCTCATTTC-3' and A_phag_msp4RT-R 5'-GGATCTGAAAYA-TTCAACGTGC-3' (0.2 μ M of each), 30 ng of DNA. PCR conditions were as follows: activation of DNA polymerase at 95°C for 2 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 15 s and extension at 72°C for 15 s (Arkhipova et al., 2018).

3. Results and discussion

Blood samples from black-and-white Holsteinized cattle were collected in spring ($n_1 = 67$) and summer ($n_2 = 46$) in one herd from the Moscow region of Russia and tested for presence of *Anaplasma* (*A. marginale*, *A. phagocytophilum*), *Theileria* spp., and *Babesia* spp. by molecular genetic methods.

Table 1
Infection and co-infection of cattle with tick-borne pathogens.

	[*] n ₁ = 67		^{**} n ₂ = 46		^{***} (n ₁ + n ₂) = 113	
	No of cattle	%	No of cattle	%	No of cattle	%
Not infected	22	33	15	33	37	33
<i>A. marginale</i>	39	58	26	57	65	58
<i>Theileria</i> spp.	13	19	20	43	33	29
Co-infected with <i>A. marginale</i> + <i>Theileria</i> spp.	7	10	15	33	22	19

^{*} n₁ – the number of cattle examined in spring.

^{**} n₂ – the number of cattle examined in summer.

^{***} (n₁ + n₂) - total number of cattle examined.

According to the PCR results, 29% of tested cows were infected with *Theileria* spp. and/or *Babesia* spp. (Table 1). Because this PCR assay did not allow discriminating *Theileria* and *Babesia* species, obtained PCR products were cloned and sequenced. Phylogenetic analysis of sequences of ~420 bp fragments 18S rRNA gene obtained by us or retrieved from GenBank (Fig. 1) revealed that all sequences have the highest identity to 18S rRNA sequences of pathogenic *T. annulata* and mildly pathogenic *T. orientalis*, *T. sergenti*, *T. sinensis* and *T. buffeli*. Most 18S rRNA gene sequences (n = 30) from this study formed one clade with sequences from *T. annulata*, but two sequences formed one clade with *T. sinensis*. Despite the fact that *Theileria* specie affiliation for one sequence was not clearly defined, no sequences obtained by us belonged to *Babesia* species. Thus, in total 29% of cows were infected with *Theileria* spp. and no cow were *Babesia*-positive.

Specificity of the assays makes it possible to reliably differentiate *A. marginale* from *A. phagocytophilum* as well as from other *Anaplasma* species due to targeting of species-specific fragments of the *A. marginale* and *A. phagocytophilum* *msp4* gene. RT-PCR results showed that 65 of 113 cows (58%) were infected with *A. marginale* and no cows were positive for *A. phagocytophilum* (Table 1).

The absence of *Babesia* spp. and *A. phagocytophilum* may be explained by seasonality of infection, a low level of parasites in the peripheral blood that was below the threshold of PCR detection (Suarez et al., 2012) or a phenomenon of PCR competition among *Theileria* and *Babesia* with insufficiency of the clones number sequenced (Pienaar et al., 2011).

In total, 67% of examined blood samples were positive for *Theileria* spp. or *A. marginale*. Co-infection with *Theileria* spp. and *A. marginale* were found for 22 of 113 (19%) examined blood samples (Table 1). The high frequency of co-infections suggests that clinical manifestations might be complex. The impact of ticks and tick-borne diseases on health and productivity will continue to increase in many parts of the world and in Russia. In some tropical countries, and perhaps also elsewhere, co-infections in cattle may involve six or more different tick-transmitted pathogens (Hailemariam et al., 2017; Ringo et al., 2018). Co-infections ranging from double to sextuple were observed for cattle in South-western Ethiopia (Hailemariam et al., 2017) and Tanzania (Ringo et al., 2018). The majority of the mixed infections occurred as double infections (42.7%) (Hailemariam et al., 2017).

Characterization of microbial interactions within the tick host in multiple infections offers a big scientific challenge for understanding the epidemiology of tick-borne infectious diseases (Karim et al., 2017). The tick vectors are co-infected with a variety of diverse pathogens and non-pathogenic symbionts that may play important roles in tick biology, and could interfere with or aid in pathogen maintenance and transmission (Moutailler et al., 2016; Lempereur et al., 2017). Co-transmission of multiple pathogens may result in a co-infection which enhances disease severity or which might evolve with atypical symptoms, resulting in diagnostic difficulties (Moutailler et al., 2016; Karim

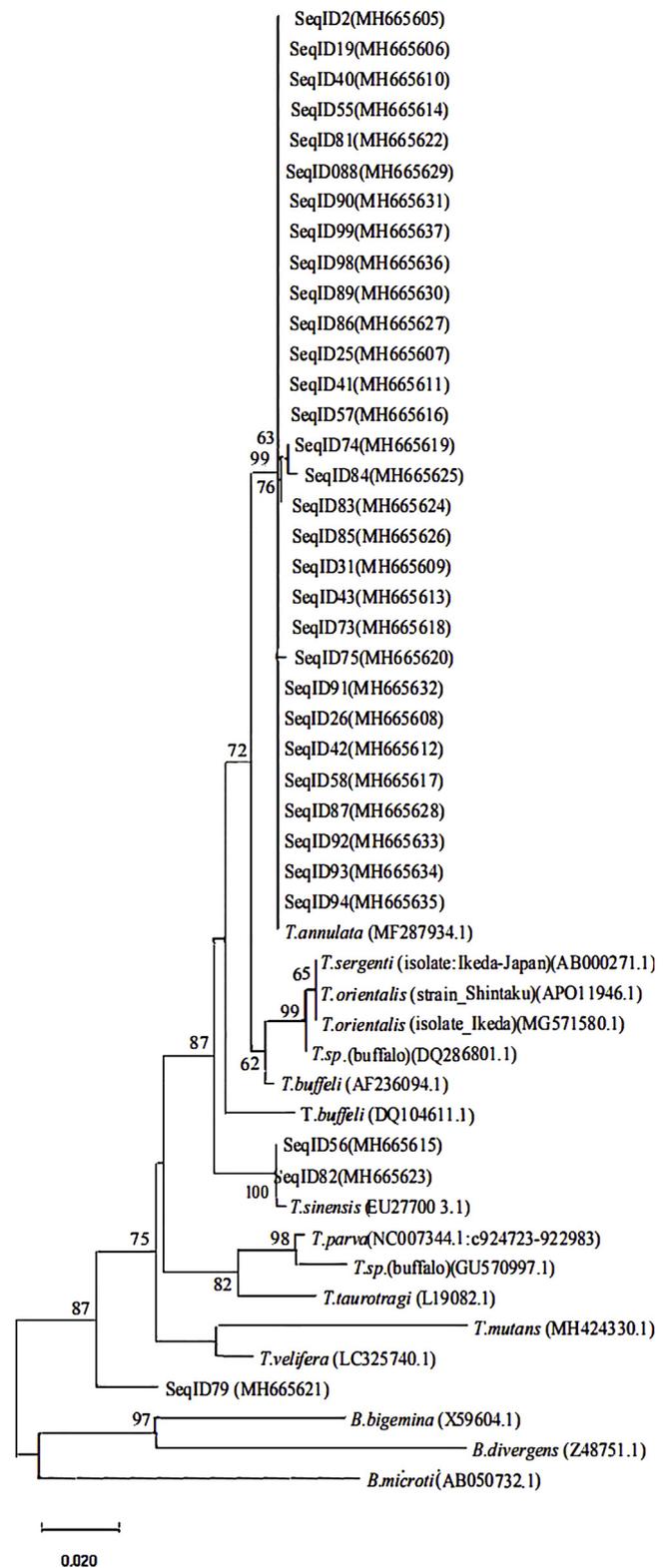


Fig. 1. Phylogenetic tree of *Theileria* spp. using 18S rRNA gene sequences constructed by neighbor-joining method, using the Tamura-Nei method as evolution model. The GenBank accession numbers of sequences are presented after each sequence ID number. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was outgroup rooted using *B. bigemina*, *B. divergens* and *B. microti*.

et al., 2017). In our study, 10–33% of cattle were found to be simultaneously co-infected with two pathogens - *Theileria* spp. and *A. marginale*. These data pose question about possible co-transmission of these agents to animals.

Seasonality was demonstrated for various blood parasitic diseases, mostly in tropic countries (Dharanesha et al., 2017; Kimaro et al., 2017). Data on seasonal prevalence of tick-borne diseases in cattle in Russia are very limited but include information for babesiosis and anaplasmosis (Skorniyakova, 2014). Babesiosis was documented in the Kirov region of Russia from June to the beginning of September, with peaks in infection prevalence (58–69%) during June–July. Anaplasmosis occurred in subclinical form and is characterized by three peaks in affected areas: the peak of infection was recorded in June (97%), with some decrease in October (78%) and an increase in February (86%) (Skorniyakova, 2014). In our study, the percentage of *Theileria*-positive cows examined in spring (19%) and in summer (43%) differed significantly (Table 1). Moreover, the prevalence co-infected animals increased from 10% in spring to 33% in summer (Table 1). A similar change of *A. marginale* infection prevalence was not observed.

This study provides data on prevalence of bovine anaplasmosis and theileriosis, as well as co-occurrences of *Theileria* spp. and *A. marginale* in cattle from Moscow region of central Russia. Additional investigations are needed to determine which tick species serve as vectors of *Theileria* spp. and *A. marginale* to cattle in central Russia, and the degree of co-infections in the ticks. New knowledge in vector and pathogens biology of interactions among different tick-borne pathogens within tick vectors and mammalian hosts should open new possibilities in illness control and prevention. Obtained data can contribute to estimation of economic loss for Russia associated with tick-borne diseases.

4. Conclusion

We found a high prevalence of hemoprotozoan parasite infections in cattle of the central part of Russia. The overall rate of hemoparasite infection in cattle was 67%; and from 10% to 33% of the animals were co-infected with *A. marginale* and *Theileria* spp. These data on prevalence of bovine theileriosis and anaplasmosis in Moscow region of Russia underscore the need for improved surveillance and control programs to reduce tick-borne diseases in cattle. Obtained data should draw attention to research programs in the fields of epidemiology, rapid pathogen identification, and efficient prophylactics for tick-borne diseases. Better understanding the epidemiological distribution of pathogenic and non-pathogenic parasites in cattle herds should contribute to the development of improved strategies to control theileriosis and anaplasmosis in Russia.

Conflict of interests

The authors declare that they have no conflict of interests.

Ethical approval

None

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