Ticks and Tick-borne Diseases 10 (2019) 251–257

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Molecular survey and genetic characterization of *Anaplasma marginale* isolates in cattle from two regions of Russia

Ekaterina A. Fedorina*, Anna L. Arkhipova, Gleb Y. Kosovskiy, Svetlana N. Kovalchuk

Department of Molecular Biotechnology, Center of Experimental Embryology and Reproductive Biotechnology, Moscow, Russian Federation

ARTICLE INFO

Keywords:
Anaplasma marginale
Genetic diversity
msp4
msp1α
Russia

ABSTRACT

Anaplasma marginale is an intraerythrocytic tick-borne rickettsial pathogen that causes bovine anaplasmosis, an economically important disease of cattle worldwide. Major surface protein MSP1α has been used as a stable marker in identifying geographical strains of *A. marginale*. The genetic diversity of *A. marginale* based on MSP1α has been reported in several countries all over the world. Only a few molecular surveys of *A. marginale* strains have been conducted in Russia. The aim of this study was molecular detection and characterization of *A. marginale* isolates in cattle from two regions of Russia. Blood samples from 62 cattle were collected and screened for the presence of *A. marginale* by real-time PCR targeting the *msp4* gene. *Anaplasma marginale* DNA was detected in 26 cattle (42%). The partial *msp1α* gene containing tandem repeat sequences and *msp4* gene were amplified from *msp4*-positive samples, cloned and sequenced. Sequence analysis revealed that two *msp4* genotypes were found. The genetic diversity of *A. marginale* strains was analyzed based on the MSP1α tandem repeats structure and 5′-UTR microsatellite. Sixteen new genotypes of *A. marginale* were found in 17 animals. Seven animals (41%) were infected by more than one genotype. Eight new tandem repeats are described for the first time. The number of repeats differed between 1 and 6 across the isolates. The *msp1α* microsatellite analysis revealed that six genotypes were identified; one of them was not previously described. Phylogenetic analysis revealed that Russian isolates formed four separate clades. The tandem repeat and microsatellite analyses of the *msp1α* gene showed a high genetic diversity among the isolates. The present study provided the first evidence of genetic diversity of *A. marginale* in cattle in Russia.

1. Introduction

Anaplasma marginale (Rickettsiales: Anaplasmataceae) is an obligate intracellular pathogen that causes bovine anaplasmosis throughout the world (Kocan et al., 2003). The disease is characterized by fever, anemia, weight loss, decreased milk production, abortion, lethargy, icterus and death. Animals that recover from acute infection become persistently infected and serve as reservoirs for pathogen transmission (Aubry and Geale, 2011). Outbreaks of anaplasmosis cause economic losses to the cattle industry over the world. Clinical anaplasmosis is most notable in cattle, but other ruminants can also become infected (Battilani et al., 2017; Kocan et al., 2003). *Anaplasma marginale* is transmitted biologically by ticks, mechanically by biting insects or blood-contaminated fomites and transplacentally (Aubry and Geale, 2011; Kocan et al., 2010; Silvestre et al., 2016).

In Russia, bovine anaplasmosis is distributed in many regions, including the southern areas of Russia, Bryansk, Kaluga, Ryazan, Kaliningrad, Saratov, Tver, Tyumen, Vladimir, Nizhny Novgorod, Novosibirsk, Ulyanovsk regions and Altay (Georgiou and Belimenko, 2015; Gulyukin et al., 2013; Kazakov and Idina, 2009). *Anaplasma marginale* has been detected by peripheral blood smear microscopic examinations and serological methods. Molecular methods for the pathogen identification were used only in several studies (Krasikov et al., 2007; Rar et al., 2015; Vasilevich et al., 2017).

Knowledge of the genetic diversity of *A. marginale* strains is important for epidemiological and virulence studies and control strategies (Lis et al., 2015). Many geographical strains of *A. marginale* have been identified, which differ in genotype, antigenic characteristic and transmissibility by ticks (de la Fuente et al., 2001b, 2007; Smith et al., 1986). The genetic diversity of *A. marginale* has been classified by using major surface proteins (MSP) such as MSP1α, *MSP4* and MSP5, which are encoded by single genes (Aubry and Geale, 2011). MSP1α is involved in the adhesion and transmission of *A. marginale* by ticks and varies among strains in the number and sequence of amino-terminal

---

* Corresponding author at: Department of Molecular Biotechnology, Center of Experimental Embryology and Reproductive Biotechnology, ul. Kostyakova 12/4, Moscow 127422, Russian Federation.

E-mail address: efedorina@inbox.ru (E.A. Fedorina).

https://doi.org/10.1016/j.ttbdis.2018.10.011
Received 9 July 2018; Received in revised form 24 September 2018; Accepted 24 October 2018

tandem repeats (de la Fuente et al., 2001a; McGarey et al., 1994). Despite the high genetic diversity *msp1α* gene is considered a stable genetic marker conserved during infections in cattle and ticks (Bowie et al., 2002; de la Fuente et al., 2003b; Palmer et al., 2001). In addition, MSP1α contains T- and B - cell epitopes required for development of a protective immune response (Brown et al., 2002; de la Fuente et al., 2003c; Palmer et al., 1987).

The genetic diversity of *A. marginale* based on MSP1α has been reported in several countries worldwide (Cabezas-Cruz et al., 2013; Castaneda-Ortiz et al., 2015; de la Fuente et al., 2005, 2007; Mutshembele et al., 2014; Pothmann et al., 2016; Silva et al., 2015; Yang et al., 2017; Ybañez et al., 2014).

There is no information about genetic diversity of *A. marginale* in Russia. The aim of this study was detection, molecular characterization and assessment of the genetic diversity of *A. marginale* isolates in cattle from Moscow region and Saratov region of the European part of Russia.

2. Materials and methods

2.1. Samples collection and DNA extraction

This study was conducted in 2015–2016 on two different dairy herds from Moscow (55 °N, 37 °E) and Saratov (51 °N, 47 °E) regions of Russia. Blood samples were collected from 42 cattle (Moscow region) and 20 cattle (Saratov region). The sampled animals were adult cows of Holsteinized black and white breed. All of them were clinically healthy. Blood was collected aseptically from the jugular vein of cattle by a veterinarian after obtaining farm owner's permission. In Russia, blood samples can be taken from animals without approval from ethics committee.

Genomic DNA was extracted from 100 μL of EDTA-treated blood using the Sorb-M kit (Sintol, Russia) according to manufacturer's recommendations.

2.2. Real-time PCR for detection of A. marginale

The amplification of a 177 bp fragment of the *msp4* gene was performed in real-time PCR in a mixture of 10 μl LightCycler® 480 Probes Master PCR reagent (Roche, Switzerland); *MSP4*-F 5′-CATGAGTCACGAAGTGGCT-3′ primer (0.5 μM), *MSP4*-R 5′-GGCACACTCACATCAATC-3′ primer (0.5 μM); *MSP4*-probe 5′-(Cy5)-AAGGGGGAGTAATGGGAGGTAGCT-3′ fluorescently labeled probe (0.1 μM); 3 μl of DNA; total reaction volume was 20 μl. The DNA extracted from blood of cow naturally infected with *A. marginale,* previously detected by rtPCR and sequence analysis (Kovalchuk et al., 2015) was used as positive control. Sterile deionized nuclease-free water for PCR (Eurogen, Russia) was used as negative control. PCR was performed using a LightCycler 96 instrument (Roche, Switzerland) under the following conditions: initial denaturation for 2 min at 95 °C; 45 cycles of 15 s at 95 °C, 15 s at 58 °C, 15 s at 72 °C (Kovalchuk et al., 2015).

2.3. Msp4-PCR

A genotyping method based on the *msp4* gene (de la Fuente et al., 2001b) with modifications of the primer structure was used to obtain 849 bp fragment. Amplifications were carried out in reaction mixture containing FastStartHiFi PCR System (Roche, Switzerland), MSP4phyl-D 5′-ATGAATTACAGAGAATTGTTTAC-3′ primer (0.5 μM), MSP4phyl-R 5′-TTAGCTGAACAGGAATCTTGC-3′ primer (0.5 μM), 3 μl of DNA. Sterilized deionized water was used as negative control. Cycling conditions were initial denaturation for 2 min at 95 °C, 45 cycles of 15 s at 95 °C, 15 s at 62 °C, 40 s at 72 °C.

2.4. Msp1α -semi-nested PCR

The partial *msp1α* gene containing the tandem repeats was further amplified from *msp4*-positive samples by semi-nested PCR (Castaneda-Ortiz et al., 2015). The primers (forward 5′-GTGCTTATGGCAGACATTTCC-3′ and reverse 5′-CTCAACACTCGCAACCTTGG-3′) were used with PCR conditions of 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and a final extension of 72 °C for 5 min. An internal primer msp1α_phylD2 (5′-CGCATTACACGTTCCGTATG-3′) was used with the reverse primer from the first reaction. For the second PCR reaction, the conditions were: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 65 °C for 20 s, 72 °C for 40 s and a final extension of 72 °C for 7 min. Sterilized deionized water was used as negative control.

2.5. Cloning and sequencing of PCR products

The PCR products were analyzed by 1% agarose gel electrophoresis and visualized under UV light, excised from the gel, purified Cleanup Mini Kit (Eurogen, Russia) and cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA) in *E.coli* DH5alpha. All *msp1α* amplicons obtained in a given sample were cloned to study the genotype diversity. Recombinant plasmids were screened by colony PCR using vector-specific primers pJET1.2 F and pJET1.2R. Plasmid DNA was extracted from recombinants using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). Plasmids containing the correct insert were sequenced in both directions. Sequencing was performed by Evrogen (Moscow, Russia). Each construct was sequenced at least three times.

2.6. Sequence analysis

Sequence analysis was performed using the BLAST search (http://blast.ncbi.nlm.nih.gov/). Sequences were aligned, edited, and analyzed using MEGA 6.0 (Tamura et al., 2013). Tandem repeat analysis was performed according to the nomenclature proposed by de la Fuente et al. (2007) and Cabezas-Cruz et al. (2013). A microsatellite was located at the 5′- untranslated region between the putative Shine-Dalgarno sequence (GTAGG) and the translation initiation codon (ATG). Its structure is (G/A TTT)m (GT)n T ATG (Cabezas-Cruz et al., 2013; Estrada-Peña et al., 2009). The SD-ATG distance was calculated in nucleotides as (4 × m) + (2 × n) + 1.

2.7. Phylogenetic analysis

The phylogenetic analysis was performed using partial MSP1α amino acid sequences aligned with MUSCLE from MEGA 6.0 package (Tamura et al., 2013). The phylogenetic trees were reconstructed by maximum likelihood (ML) based on the Jones-Taylor-Thornton model (JTT + F + G) and neighbor joining (NJ) based on the Jones-Taylor-Thornton (JTT + G) methods in MEGA 6.0 software. The Akaike information criterion was used to identify the most appropriate model of nucleotide substitution. Internal branches of ML and NJ trees were assessed using the bootstrapping method (500 bootstrap replicates). MSP1α sequences of 30 *A. marginale* isolates worldwide available in GenBank were included. The graphical representation and editing of the phylogenetic trees were done using TreeDyn (v 198.3) (Chevenet et al., 2006).

3. Results

3.1. Detection of A. marginale infections

Anaplasma marginale DNA was detected in 26 of 62 cattle, with an overall infection rate of 42%. The infection rate in Moscow region was 57% (24 positive/42 sampled), while that Saratov region was 10% (2/20).

3.2. Analysis of A. marginale msp4 sequences

The PCR results were validated by sequencing 849 bp of the *msp4* gene from positive samples. In this study two *msp4* sequence variants were obtained with 100% identity to sequences already registered in GenBank. The *msp4* sequence variant from the RM8 isolate (GenBank MH191397) was identical to the *A. marginale* strain 50(G16) (GenBank EU315782) from Hungary (Hornok et al., 2008). The sequence variant from the isolate RM4 (GenBank MH191396) has 100% identity to strains Israeli non-tailed (GenBank AY786993) and 1.6 (GenBank AY666006) from Israel and Zimbabwe, respectively.

3.3. Analysis of A. marginale msp1α sequences

Anaplasma marginale msp4-positive blood samples were further analyzed by semi-nested PCR targeting the *msp1α* gene. The tandem repeat region was successfully amplified, cloned and completely sequenced in seventeen samples only. The analysis of the MSP1α repeat region structure revealed eleven novel genotypes in cattle from Moscow region and five in samples from Saratov region. The predominant genotype was RM8, occurred in six samples (35%). In this study eight new tandem repeats were identified and designated ru1 - ru8 (Fig. 1). All of them were 28 amino acids in length. The number of MSP1α repeats in different isolates of *A. marginale* varied from one to six. Most of these tandem repeats were shared between different genotypes and isolates of *A. marginale*, while ru1 was found in a single genotype and isolate. The most common was ru6, occurred in 9 genotypes and 12 isolates (Tables 1 and 2).

Analysis of MSP1α repeats revealed the presence of the sequences EASTSS the neutralisation-sensitive epitope (positions 20–26) (Allred et al., 1990) and of glutamic acid (E) at position 20. The novel repeats contained 4 variations of the previously reported immunodominant B-cell epitope (positions 4–14) (Garcia-Garcia et al., 2004) (Fig. 1).

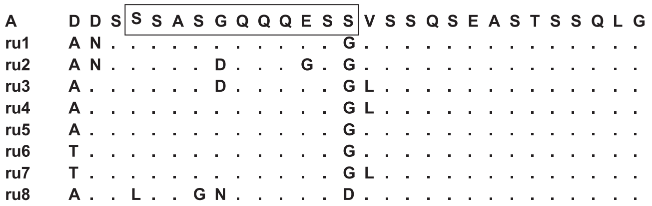
The *msp1α* microsatellite analysis resulted in six distinct genotypes (Table 1). The microsatellite sequences produced SD ATG distances between 19 and 25 nucleotides, and the predominant distance was 23. The new microsatellite structure designated as L (m = 1, n = 10, SD-ATG distance = 25) was described. According to the structure of the *msp1α* microsatellite of *A. marginale*, the genotypes J, G and E were observed in the samples from Moscow region and the genotypes A, B and L were found in cattle from Saratov. The predominant *msp1α* microsatellite was G (occurred in 4 genotypes and 8 samples), and another was J (4 genotypes and 6 samples) (Tables 1 and 2).

The new *msp1α* sequences have been deposited in GenBank (accession numbers MG570149 – MG570164).

Seven animals (41%) were infected by more than one genotype of *A. marginale* (Table 2).

3.4. Phylogenetic analysis

Phylogenetic analysis based on the alignment of the 16 MSP1α genotypes identified in this study with 30 MSP1α sequences of *A. marginale* isolates worldwide represented in ML tree and demonstrated that Russian *A. marginale* isolates formed four separate lineages (Fig. 2).



```
A     D D S S S A S G Q Q Q E S S V S S Q S E A S T S S Q L G
ru1   A N . . . . . . . . . . . G . . . . . . . . . . . . . .
ru2   A N . . . . . D . . . G . G . . . . . . . . . . . . . .
ru3   A . . . . . . D . . . . . G L . . . . . . . . . . . . .
ru4   A . . . . . . . . . . . . G L . . . . . . . . . . . . .
ru5   A . . . . . . . . . . . . G . . . . . . . . . . . . . .
ru6   T . . . . . . . . . . . . G . . . . . . . . . . . . . .
ru7   T . . . . . . . . . . . . G L . . . . . . . . . . . . .
ru8   A . . L . . G N . . . . . D . . . . . . . . . . . . . .
```

Fig. 1. New tandem repeat sequences of *A. marginale* MSP1α identified in the Russian isolates. The one letter amino acid code was used to depict the differences found in MSP1α repeats. Dots indicate identical amino acids the sequences. Tandem repeat A was used as a model to compare (de la Fuente et al., 2007). The immunodominant linear B-cell epitope (positions 4–14) (Garcia-Garcia et al., 2004) is framed.

4. Discussion

Although bovine anaplasmosis is increasingly registered in many regions of Russia, information available for *A. marginale* in Russia is limited. Identification of the pathogen is based in most cases on the analysis of blood smears. Molecular detection methods are not routinely used. In 2008 *A. marginale* infection has been reported in cattle in 13 areas of Tver region, blood samples from cattle were screened by microscopy (Kazakov and Idina, 2009). Microscopic examination has been conducted in 2005–2009 in Kirov region. Examination of blood smears showed the *A. marginale* infection rate 78–100% in some areas of the region (Skornyakova, 2014). In a previous study by Liberman and Khlyzova (2015), reported large-scale serological and microscopic studies of anaplasmosis in cattle had been conducted in different farms in the south of the Tyumen region (Western Siberia) in 2010-2014. The *A. marginale* infection rate ranged from 21.4% to 56%.

A molecular survey of *Anaplasma* spp. has previously been conducted during anaplasmosis outbreak in cattle in the Omsk region (Western Siberia), *Anaplasma* DNA was determined by *16SrRNA* gene sequencing. The causative agent of this infection was designated as *Anaplasma* sp. *Omsk* (GenBank AY649325) (Krasikov et al., 2007). In 2012–2013 blood samples of livestock collected in different regions of Altai Republic, Altai region, Novosibirsk and Irkutsk regions (Western and Eastern Siberia) were examined on the presence of *Anaplasma* DNA using nested PCR by genus-specific primers. *Anaplasma* DNA was found in 49.3% samples of cattle, the infection rate ranged from 21.2% to 84% in different farms. Sequence analysis of *16SrRNA* gene revealed the presence of the *Anaplasma* sp. *Omsk* (GenBank AY649325) and a new genetic variant designated as *Anaplasma* sp. *Sib122* (GenBank KT734729.) (Rar et al., 2015). In present study *A. marginale* was detected by species-specific rtPCR in cattle from two regions of the European part of Russia with an overall infection rate of 42%.

There are six major surface-exposed proteins that have been well characterized in *A. marginale*, and were considered to be involved in the interactions of pathogen with both ticks and hosts (Brayton et al., 2005; Kocan et al., 2004, 2010). The genetic variability of *A. marginale* was frequently characterized on the basis of the *msp4* and *msp1α* genes (Aktas and Özübek, 2017; Belkahia et al., 2015; de la Fuente et al., 2002b; Jaimes-Dueñez et al., 2018). However, the *msp4* gene is highly conserved and stable among different strains of *A. marginale* (de la Fuente et al., 2005). In present study the *msp4* sequences of *A. marginale* isolates found in cattle had 100% sequence identity to previously been reported (Hornok et al., 2008). This finding was consistent with previously report by Vasilevich et al. (2017). Similar results had been obtained in China (Yang et al., 2017).

Anaplasma marginale geographical strains differing in their biological properties have been genetically characterized, over 250 MSP1α repeats have been described (Catanese et al., 2016; Cabezas-Cruz et al., 2013; de la Fuente et al., 2005, 2007). *Anaplasma marginale* strains vary in the copy number and sequence of tandem 23–31 amino acid repeats in MSP1α. The majority of *A. marginale* strains had more than one MSP1α tandem repeat and the maximum number of repeats was 10. The majority of strains were seen in only a given region, although several strains were isolated from multiple countries. Most of the MSP1α tandem repeats were shared between different strains, while some tandem repeats were unique (Cabezas-Cruz et al., 2013). In this study, sixteen novel genotypes of *A. marginale* have been identified in 17 sequenced samples from cattle. Eight new 28 amino acid tandem repeats are described; its number differed between 1 and 6 across the isolates. Interestingly, all repeats and genotypes found in this study were unique to Russian samples. These evidences may suggest that novel repeats originate independently in different geographical regions,

Table 1
Structure of the *msp1a* microsatellite sequences and MSP1a tandem repeat region of 16 *A. marginale* isolates from cattle from Russia. SD, Shine-Dalgarno sequences (GTAGG); ATG, translation initiation codon. The SD-ATG distance was calculated in nucleotides as (4 × m) + (2 × n) + 1.

Isolate	GenBank accession number	Origin	Microsatellite sequence				Number of repeats	Structure of *msp1a* tandem repeats					
			m*	n**	Genotype	SD-ATG distance, nt							
RM1	MG570149	Moscow region	1	8	J	21	1	ru4					
RM2	MG570150	Moscow region	1	8	J	21	2	ru4	ru7				
RM3	MG570151	Moscow region	1	8	J	21	2	ru3	ru7				
RM4	MG570152	Moscow region	1	8	J	21	6	ru3	ru7	ru7	ru7	ru7	ru7
RM5	MG570153	Moscow region	3	5	G	23	3	ru1	ru6	ru6			
RM6	MG570154	Moscow region	3	5	G	23	2	ru2	ru6				
RM7	MG570155	Moscow region	3	5	G	23	3	ru2	ru6	ru6			
RM8	MG570156	Moscow region	3	5	G	23	4	ru2	ru6	ru6	ru6		
RM9	MG570157	Moscow region	2	7	E	23	2	ru5	ru8				
RM10	MG570158	Moscow region	2	7	E	23	6	ru5	ru8	ru5	ru8	ru5	ru8
RM11	MG570159	Moscow region	2	7	E	23	4	ru8	ru8	ru5	ru8		
RS1	MG570160	Saratov region	1	9	B	23	4	ru5	ru6	ru6	ru6		
RS2	MG570161	Saratov region	1	9	B	23	5	ru5	ru6	ru6	ru6	ru6	
RS3	MG570162	Saratov region	1	10	L	25	5	ru5	ru6	ru6	ru6	ru6	
RS4	MG570163	Saratov region	1	7	A	19	5	ru5	ru6	ru6	ru6	ru6	
RS5	MG570164	Saratov region	1	7	A	19	6	ru5	ru6	ru6	ru6	ru6	ru6

*m is the number of repetitions of the nucleotide sequence G/ATTT.
**n is the number of repetitions of the nucleotide sequence GT.

resulting in the emergence of new variants of genotypes. These data were consistent with a recent study by Hove et al. (2018), which reported a comparison of the *msp1α* genotypes and MSP1a repeats occurring in the five selected countries; the highest proportions of unique genotypes were found in USA (100%) and South Africa (99.0%), the highest percentage of unique repeats (71.7%) was found in South Africa.

According to de la Fuente et al. (2003b) *A. marginale* strains with negatively charged amino acids such as aspartic acid (D) and glutamic acid (E) at position 20 of the MSP1α tandem repeat region bind to the tick cell extract (TCEs). In present study all MSP1α tandem repeats have the glutamic acid (E) at position 20, these data suggest the high binding capacity of these isolates to bind to tick TCEs and its likely infection and transmission by ticks (Cabezas-Cruz et al., 2013).

In total, six different genotypes of the *msp1α* microsatellite were identified, three (J, G and E) were observed in the samples from Moscow region and other three (A, B and L) were found in cattle from Saratov region. The predominant microsatellite genotypes were G and J. The findings were consistent with the previous report (Machado et al., 2015), in which the genotypes E and H were observed in the samples from Lins and the genotypes C, D and E were found in cattle from Mambaí (Brazil). Similar results reported by Pohl et al. (2013) in which five distinct microsatellite genotypes (B, C, D, E, and G) have been identified in 13 sequenced samples from cattle in Brazil, and Aktas and Özübek (2017), in which three microsatellite genotypes (C, E, and G) had been observed in eight samples from cattle in Turkey. Genotype G is the most frequently found genotype around the world and has been seen to be the most prevalent type in the ecoregions of South Africa and parts of the USA and Mexico (Estrada-Peña et al., 2009). The length of the *msp1α* microsatellite is important for the expression of *msp1α* gene, affecting the infection and transmission of *A. marginale*. SD-ATG distances of 23 and 29 nucleotides have been found a higher expression levels than 19 nucleotides (Estrada-Peña et al., 2009). In this study, 63% (10/16) of the isolates had genotypes with SD-ATG distances 23 or 25 nucleotides long, suggesting that a high capacity for infection and transmission of the *A. marginale* strains detected.

Although MSP1α repeat sequences did not group in clusters geographically related or offered phylogenetic relationships, they did

Table 2
A. marginale msp1a genotypes present in singly and superinfected cattle in Moscow (1–15) and Saratov (16, 17) regions.

Animal	Number of unique *msp1α* genotypes detected and sequenced		
	1	2	3
1	G - ru1, ru6, ru6		
2	G - ru2, ru6, ru6, ru6		
3	G - ru2, ru6, ru6, ru6		
4	J - ru3, ru7		
5	G - ru2, ru6, ru6, ru6		
6	J - ru3, ru7	J - ru4	G - ru2, ru6
7	G - ru2, ru6, ru6	G - ru2, ru6, ru6, ru6	
8	G - ru2, ru6, ru6, ru6		
9	J - ru4, ru7		
10	J - ru3, ru7, ru7, ru7, ru7, ru7		
11	J - ru3, ru7, ru7, ru7, ru7, ru7	E - ru5, ru8, ru5, ru8, ru5, ru8	
12	E - ru5, ru8		
13	E - ru5, ru8, ru5, ru8, ru5, ru8		
14	E - ru8, ru8, ru5, ru8	E - ru5, ru8	
15	G - ru2, ru6, ru6, ru6	G - ru2, ru6	J - ru3, ru7, ru7, ru7, ru7, ru7
16	A – ru5, ru6, ru6, ru6, ru6, ru6	A – ru5, ru6, ru6, ru6, ru6	
17	B – ru5, ru6, ru6, ru6	B – ru5, ru6, ru6, ru6, ru6	L – ru5, ru6, ru6, ru6, ru6

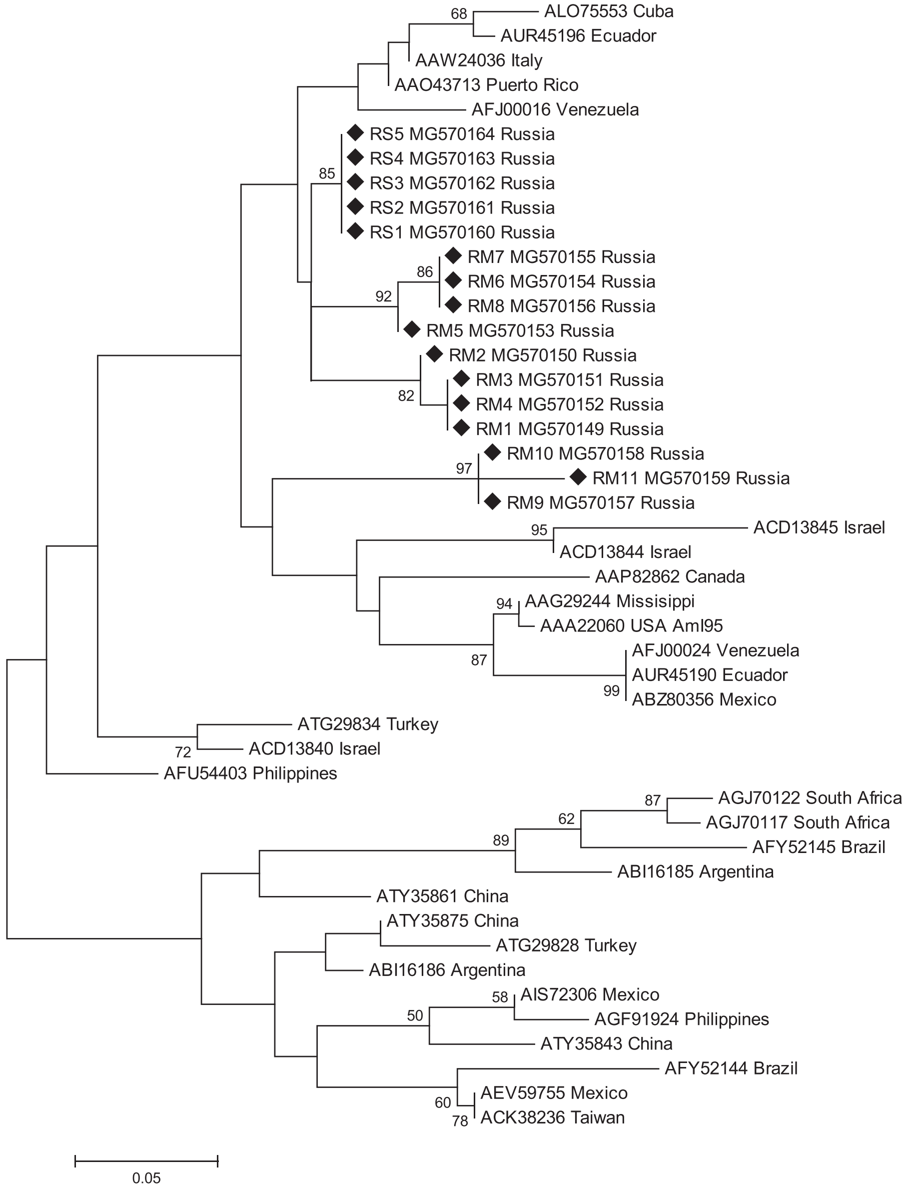


Fig. 2. Phylogenetic tree based on *msp1a* gene of *Anaplasma marginale* Russian isolates. The tree was reconstructed using the Maximum Likelihood method based on the partial MSP1α amino acid sequences. The percentage of replicate in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches (only percentages greater than 50% were represented). The GenBank accession numbers of the respective sequences used for the phylogenetic analysis are shown. Legend: ◆ Russian isolates of *A. marginale*.

provide phylogeographic information, as 78% of the repeat sequences were present in strains from a single geographic region. Some MSP1α repeats clustered and were unique to certain regions such as Italy, Spain, China, Argentina, and South America (Quiroz-Castaneda et al., 2016). The phylogenetic analysis of Russian *A. marginale* isolates identified divergence between the samples studied.

Although infection exclusion was thought to result in only one *A. marginale* genotype in individual cattle and ticks (de la Fuente et al., 2002a, 2003a), more recently, infections with multiple different *msp1α* genotypes have been identified in herds in areas with high levels of infection prevalence and genetic diversity (Castaneda-Ortiz et al., 2015; Hove et al., 2018; Ueti et al., 2012; Vallejo Esquerra et al., 2014). In this study, 41% animals (7/17) were infected by multiple *A. marginale* genotypes. This result was consistent with the previous studies (Hove et al., 2018; Palmer et al., 2004; Ybañez et al., 2014).

It has been demonstrated that *A. marginale* is widespread in the tropics and subtropics, and in temperate climates has low variability and prevalence (Castaneda-Ortiz et al., 2015; Palmer et al., 2004; Ueti et al., 2012; Vallejo Esquerra et al., 2014). The present study shows a significant infection rate (up to 57% in Moscow region), the high variability and superinfection of animals with different genotypes of *A. marginale*.

The heterogeneity and variability of the *A. marginale* population observed in the studied farms could be explained both a movement of animals from different geographic regions within the country and the expansion of the habitat of ticks in conditions of the changing climate (Yasyukevich et al., 2009). In addition, the high genetic diversity and the evolution of new sequences unique to the region may be explained by other factors, such as selective pressures exerted by host immune system and host-parasite interactions. The obtained results can help to improve the design of epidemiological studies and control strategies for *A. marginale* in Russia.

5. Conclusions

Anaplasma marginale was detected by rtPCR in cattle from two regions of Russia with an overall infection rate of 42%. The molecular characterization of *A. marginale* isolates based on the *msp1α* showed the high genetic diversity. Sixteen novel genotypes of *A. marginale* were found in 17 animals. Eight new tandem repeats are described, six microsatellite genotypes were identified, and one of them described for the first time. Seven animals (41%) were infected by more than one genotype. The present study is the first report of the genetic diversity of *A. marginale* in cattle in Russia.

Conflict of interests

The authors declare that they have no conflict of interests.

References

Aktas, M., Özübek, S., 2017. Outbreak of anaplasmosis associated with novel genetic variants of *Anaplasma marginale* in a dairy cattle. Comp. Immunol. Microbiol. Infect. Dis. 54, 20–26.

Allred, D.R., McGuire, T.C., Palmer, G.H., Leib, S.R., Harkins, T.M., McElwain, T.F., Barbet, A.F., 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in Anaplasma marginale. Proc. Natl. Acad. Sci. U.S.A. 87, 3220–3224.

Aubry, P., Geale, D.W., 2011. A review of Bovine anaplasmosis. Transbound. Emerg. Dis. 58, 1–30.

Battilani, M., De Arcangeli, S., Balboni, A., Dondi, F., 2017. Genetic diversity and molecular epidemiology of Anaplasma. Infect. Genet. Evol. 49, 195–211.

Belkahia, H., Said, M.B., Alberti, A., Abdi, K., Issaoui, Z., Hattab, D., Gharbi, M., Messadi, L., 2015. First molecular survey and novel genetic variants' identification of *Anaplasma marginale*, *A. centrale* and *A. bovis* in cattle from Tunisia. Infect. Genet. Evol. 34, 361–371.

Bowie, M.V., de la Fuente, J., Kocan, K.M., Blouin, E.F., Barbet, A.F., 2002. Conservation of major surface protein 1 genes of *Anaplasma marginale* during cyclic transmission between ticks and cattle. Gene 282, 95–102.

Brayton, K.A., Kappmeyer, L.S., Herndon, D.R., Dark, M.J., Tibbals, D.L., Palmer, G.H., McGuire, T.C., Knowles, D.P.Jr., 2005. Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins. Proc. Natl. Acad. Sci. U.S.A. 102, 844–849.

Brown, W.C., McGuire, T.C., Mwangi, W., Kegerreis, K.A., Macmillan, H., Lewin, H.A., Palmer, G.H., 2002. Major histocompatibility complex class II DR-restricted memory CD4+ T lymphocytes recognize conserved immunodominant epitopes of *Anaplasma marginale* major surface protein 1a. Infect. Immun. 70, 5521–5532.

Cabezas-Cruz, A., Passos, L.M.F., Lis, K., Kenneil, R., Valdés, J.J., Ferrolho, J., Tonk, M., Pohl, A.E., Grubhoffer, L., Zweygarth, E., Shkap, V., Ribeiro, M.F.B., Estrada- Peña, A., Kocan, K.M., de la Fuente, J., 2013. Functional and immunological relevance of *Anaplasma marginale* major surface protein 1a sequence and structural analysis. PLoS One 8, 1–13.

Castaneda-Ortiz, E.J., Ueti, M.W., Camacho-Nuez, M., Mosqueda, J.J., Mousel, M.R., Johnson, W.C., Palmer, G.H., 2015. Association of *Anaplasma marginale* strain superinfection with infection prevalence within tropical regions. PLoS One 10, e0120748.

Catanese, H.N., Brayton, K.A., Gebremedhin, A.H., 2016. RepeatAnalyzer: a tool for analysing and managing short-sequence repeat data. BMC Genomics 17, 422.

Chevenet, F., Brun, C., Bañuls, A.L., Jacq, B., Chisten, R., 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinf 10, 439.

de la Fuente, J., Blouin, E.F., Kocan, K.M., 2003a. Infection exclusion of the rickettsial pathogen, *Anaplasma marginale* in the tick vector, *Dermacentor variabilis*. Clin. Diagn. Lab. Immunol. 10, 182–184.

de la Fuente, J., Garcıa-Garcıa, J.C., Blouin, E.F., Saliki, J.T., Kocan, K.M., 2002a. Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. Clin. Diagn. Lab. Immunol. 9, 658–668.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2003b. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet. Microbiol. 91, 265–283.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2001a. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int. J. Parasitol. 31, 145–153.

de la Fuente, J., Lew, A., Lutz, H., Meli, M.L., Hofmann-Lehmann, R., Shkap, V., Molad, T., Mangold, A.J., Almazán, C., Naranjo, V., Gortázar, C., Torina, A., Caracappa, S., García-Pérez, A.L., Barral, M., Oporto, B., Ceci, L., Carelli, G., Blouin, E.F., Kocan, K.M., 2005. Genetic diversity of anaplasma species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. Anim. Health Res. Rev. 6, 75–89.

de la Fuente, J., Ruybal, P., Mtshali, M.S., Naranjo, V., Shuqing, L., Mangold, A.J., Rodríguez, S.D., Jiménez, R., Vicente, J., Moretta, R., Torina, A., Almazán, C., Mbati, P.M., de Echaide, S.T., Farber, M., Rosario-Cruz, R., Gortazar, C., Kocan, K.M., 2007. Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences. Vet. Microbiol. 119, 382–390.

de la Fuente, J., Van Den Bussche, R., Prado, T., Kocan, K., 2003c. *Anaplasma marginale msp1α* genotypes evolved under positive selection pressure but are not markers for geographic isolates. J. Clin. Microbiol. 41, 1609–1616.

de la Fuente, J., Van Den Bussche, R.A., Garcia-Garcia, J.C., Rodriguez, S.D., Garcia, M.A., Guglielmone, A.A., Mangold, A.J., Friche Passos, L.M., Barbosa Ribeiro, M.F., Blouin, E.F., Kocan, K.M., 2002b. Phylogeography of new world isolates of *Anaplasma marginale* based on major surface protein sequences. Vet. Microbiol. 88, 275–285.

de la Fuente, J., Van Den Bussche, R.A., Kocan, K.M., 2001b. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). Vet. Parasitol. 97, 65–76.

Estrada-Peña, A., Naranjo, V., Acevedo-Whitehouse, K., Mangold, A.J., Kocan, K.M., de la Fuente, J., 2009. Phylogeographic analysis reveals association of tick-borne pathogen, *Anaplasma marginale*, MSP1a sequences with ecological traits affecting tick vector performance. BMC Biol. 7, 57.

Garcia-Garcia, J.C., de la Fuente, J., Kocan, K.M., Blouin, E.F., Albur, T., Onet, V.C., Saliki, J.T., 2004. Mapping of B-cell epitopes in the N-terminal repeated peptides of *Anaplasma marginale* major surface protein 1a and characterization of the humoral immune response of cattle immunized with recombinant and whole organism antigens. Vet. Immunol. Immunopathol. 98, 137–151.

Georgiou, Ch., Belimenko, V.V., 2015. Bovine Anaplasmosis. Russ. Vet. J. 1, 5–7 (In Russ.).

Gulyukin, M.I., Zablotskiy, V.T., Belimenko, W.V., 2013. Monitoring of the protozoan blood parasitic diseases epizootic situation in Russia (2007–2012). Russ. Vet. J. Farm Anim. 2, 36–40 (In Russ.).

Hornok, S., Foldvari, G., Elek, V., Naranjo, V., Farkas, R., de la Fuente, J., 2008. Molecular identification of *Anaplasma marginale* and rickettsial endosymbionts in blood-sucking flies (Diptera: Tabanidae, Muscidae) and hard ticks (Acari: Ixodidae). Vet. Parasitol. 154, 354–359.

Hove, P., Chaisi, M.E., Brayton, K.A., Ganesan, H., Catanese, H.N., Mtshali, M.S., Mutshembele, A.M., Oosthuizen, M.C., Collins, N.E., 2018. Co-infections with multiple genotypes of *Anaplasma marginale* in cattle indicate pathogen diversity. Parasit. Vectors 11, 5.

Jaimes-Dueñez, J., Triana-Chávez, O., Mejía-Jaramillo, A.M., 2018. Genetic, host and environmental factors associated with a high prevalence of *Anaplasma marginale*. Ticks Tick Borne Dis. 5, 1286–1295.

Kazakov, N.A., Idina, M.F., 2009. Anaplasmosis of cattle in the Tver region. Vet. Pathol. 2, 72–79 (In Russ.).

Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The natural history of Anaplasma marginale. Vet. Parasitol. 167, 95–107.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Garcia-Garcia, J.C., 2004. *Anaplasma marginale* (Rickettsiales: anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. Parasitology 129 (Suppl), S285–S300.

Kocan, K.M., de la Fuente, J., Guglielmone, A.A., Meléndez, R.D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin. Microbiol. Rev. 16, 698–712.

Kovalchuk, S.N., Kosovskii, G.Yu., Arkhipov, A.V., Glazko, T.T., Glazko, V.I., 2015. Development of real-time PCR assay for detection of *Anaplasma marginale*. Agric. Biol. 50, 825–831.

Krasikov, A.P., Rudakov, N.V., Bejsembayev, K.K., Kupman, L.V., 2007. The main biological properties of causative agent of anaplasmosis, isolated from pathological material from cattle. Vet. Pathol. 3, 89–95 (In Russ.).

Liberman, E., Khlyzova, T., 2015. Dependence infestation of cattle anaplasmosis on the number of blood-sucking Diptera. Int. Sci. Inst. Educ. 3, 46–50 (In Russ.).

Lis, K., Fernández de Mera, I.G., Popara, M., Cabezas-Cruz, A., Ayllón, N., Zweygarth, E., Passos, L.M., Broniszewska, M., Villar, M., Kocan, K.M., Ribeiro, M.F., Pfister, K., de la Fuente, J., 2015. Molecular and immunological characterization of three strains of *Anaplasma marginale* grown in cultured tick cells. Ticks Tick Borne Dis. 6, 522–529.

Machado, R.Z., da Silva, J.B., Andre, M.R., Goncalves, L.R., Matos, C.A., Obregron, D., 2015. Outbreak of anaplasmosis associated with the presence of different *Anaplasma marginale* strains in dairy cattle in the states of Sao Paulo and Goias, Brazil. Braz. J. Vet. Parasitol. 24, 438–446.

McGarey, D.J., Barbet, A.F., Palmer, G.H., McGuire, T.C., Allred, D.R., 1994. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect. Immun. 62, 4594–4601.

Mutshembele, A.M., Cabezas-Cruz, A., Mtshali, M.S., Thekisoe, O.M.M., Galindo, R.C., de la Fuente, J., 2014. Epidemiology and evolution of the genetic variability of *Anaplasma marginale* in South Africa. Ticks Tick Borne Dis. 5, 624–631.

Palmer, G.H., Knowles, D.P., Rodriguez, J.L., Gnad, D.P., Hollis, L.C., Marston, T., Brayton, K.A., 2004. Stochastic transmission of multiple genotypically distinct *Anaplasma marginale* strains in a herd with high prevalence of Anaplasma infection. J. Clin. Microbiol. 42, 5381–5384.

Palmer, G.H., Rurangirwa, F.R., McElwain, T.F., 2001. Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. J. Clin. Microbiol. 39, 631–635.

Palmer, G.H., Waghela, S.D., Barbet, A.F., Davis, W.C., McGuire, T.C., 1987. Characterization of a neutralization-sensitive epitope on the Am 105 surface protein of *Anaplasma marginale*. Int. J. Parasitol. 17, 1279–1285.

Pohl, A.E., Cabezas-Cruz, A., Ribeiro, M.F., Silveira, J.A., Silaghi, C., Pfister, K., Passos, L.M., 2013. Detection of genetic diversity of *Anaplasma marginale* isolates in Minas Gerais, Brazil. Rev. Bras. Parasitol. Vet. 22, 129–135.

Pothmann, D., Poppert, S., Rakotozandrindrainy, R., Hogan, B., Mastropaolo, M., Thiel, C., Silaghi, C., 2016. Prevalence and genetic characterization of *Anaplasma marginale* in zebu cattle (*Bos indicus*) and their ticks (*Amblyomma variegatum*, *Rhipicephalus microplus*) from Madagascar. Ticks Tick Borne Dis. 7, 1116–1123.

Quiroz-Castaneda, R.E., Amaro-Estrada, I., Rodriguez-Camarillo, S.D., 2016. *Anaplasma marginale*: diversity, virulence, and vaccine landscape through a genomics approach. Biomed. Res. Int 9032085.

Rar, V.A., Yepikhina, T.I., Yefremova, E.A., Marchenko, V.A., Suntsova, O.V., Lisak, O.V., Doroshchenko, E.K., Zubareva, I.M., Tikunov, A.Yu., Tikunova, N.V., 2015. Molecular genetic analysis of infection agents of farm animals anaplasmosis on the territory of Western and Eastern Siberia. Bjull. VSNC SO RAMN. 5, 83–87 (In Russ.).

Silva, J.B., Gonçalves, L.R., Varani, A.M., Andre, M.R., Machado, R.Z., 2015. Genetic diversity and molecular phylogeny of *Anaplasma marginale* studied longitudinally under natural transmission conditions in Rio de Janeiro, Brazil. Ticks Tick Borne Dis. 6, 499–507.

Silvestre, B.T., Silveira, J.A.G., Meneses, R.M., Facury-Filho, E.J., Carvalho, A.U., Ribeiro, M.F.B., 2016. Identification of a vertically transmitted strain from Anaplasma marginale (UFMG3): molecular and phylogenetic characterization, and evaluation of virulence. Ticks Tick Borne Dis. 1, 80–84.

Skornyakova, O.O., 2014. Epizootological monitoring and dynamics of seasonal sensitivity of cattle against babesiosis and anaplasmosis. Russ. J. Parasitol. 4, 34–39 (In Russ.).

Smith, R.D., Levy, M.G., Kuhlenschmidt, M.S., Adams, J.H., Rzechula, D.L., 1986. Isolate of *Anaplasma marginale* not transmitted by ticks. Am. J. Vet. Res. 47, 127–129.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Ueti, M.W., Tan, Y., Broschat, S.L., Castañeda Ortiz, E.J., Camacho-Nuez, M., Mosqueda, J.J., Scoles, G.A., Grimes, M., Brayton, K.A., Palmer, G.H., 2012. Expansion of variant diversity associated with a high prevalence of pathogen strain superinfection under conditions of natural transmission. Infect. Immun. 80, 2354–2360.

Vallejo Esquerra, E., Herndon, D.R., Alpirez Mendoza, F., Mosqueda, J., Palmer, G.H., 2014. *Anaplasma marginale* superinfection attributable to pathogen strains with distinct genomic backgrounds. Infect. Immun. 82, 5286–5292.

Vasilevich, F.I., Kovalchuk, S.N., Babiy, A.V., Arkhipov, A.V., Arkhipova, A.L., Glazko, T.T., Kosovskiy, G.Yu., 2017. Molecular identification of isolates *Anaplasma marginale* found in blood of cattle on the territory of Moscow region. Russ. J. Parasitol. 2, 179–182 (In Russ.).

Yang, J., Han, R., Liu, Z., Niu, Q., Guan, G., Liu, G., Luo, J., Yin, H., 2017. Insight into the genetic diversity of *Anaplasma marginale* in cattle from ten provinces of China. Parasit. Vectors 10, 565.

Yasyukevich, V.V., Kazakova, E.V., Popov, I.O., Semenov, S.M., 2009. Distribution of *Ixodes ricinus L.*, 1758 and *Ixodes persulcatus Shulze*, 1930 (Parasitoformes, Ixodidae) in Russia and adjacent countries in view of observable climate changes. Dokl. Earth Sci. 427, 1030–1034.

Ybañez, A.P., Ybañez, R.H.D., Claveria, F.G., Cruz-Flores, M.J., Xuenan, X., Yokoyama, N., Inokuma, H., 2014. High genetic diversity of *Anaplasma marginale* detected from Philippine cattle. J. Vet. Med. Sci. 76, 1009–1014.