

**University of Veterinary Medicine  
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**Genetic diversity and antibiotic resistance  
of *Mycoplasma bovis***

Ph.D. thesis

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## Introduction

*Mycoplasma bovis* is a worldwide pathogen, the causative agent of respiratory disease, mastitis and arthritis of intensively farmed cattle, for which it had emerged as an economically significant infectious agent in North America and Europe. In Hungary, *M. bovis* infection was first reported in 1975 (Hale et al. 1962). By the beginning of this century, the proportion of seropositive cattle herds in Hungary was almost two thirds (Tenk et al. 2004), which value increased to 100% by 2008 (Fodor et al. 2017).

This agent is primarily transmitted by direct contact and spread through animal movements to numerous countries of the world (Nicholas és Ayling 2003). Recently, several multi-locus sequence typing (MLST) systems (Manoso-Silvan et al. 2012, Register et al. 2015, Becker et al. 2015, Rosales et al. 2015) and a multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) (Pinho et al. 2012) have been developed to reveal new insights into the epidemiology of *M. bovis*. These techniques have several advantages, such as the good reproducibility and the lower equipment and time requirements compared to methods applied earlier.

Since no effective vaccine is available against *M. bovis*, adequate housing and appropriate antibiotic treatment are promoted in the control of the diseases caused by this pathogen. Antibiotic therapy of mastitis often fails, but antimicrobial treatment of pneumonia has shown success in certain cases and it may help reduce economic losses. The number of antimicrobial classes active against *M. bovis* is limited, as mycoplasmas are naturally resistant to  $\beta$ -lactams, polymyxins and sulfonamides (Lysnyansky és Ayling 2016). Among the few antibiotics licenced for treatment of *M. bovis* (generally act on DNA or protein synthesis), there is an increasing evidence for resistance (Ayling et al. 2014, Gautier-Bouchardon et al. 2014).

Traditionally, antibiotic susceptibility examination of mycoplasmas is technically demanding, time-consuming and rarely performed in diagnostic laboratories, thereby, empirical treatment is often introduced leading to therapeutic failure and development of resistance to critically important antimicrobials (Hannan 2000). The application of rapid genetic-based diagnostic assays allows the selection of appropriate antibiotic therapy and also the prevention of the spread of resistance to antibiotics. For this, information about the genetic background of antibiotic resistance is essential.

The main mechanisms of antimicrobial resistance in *M. bovis* are based on genetic point mutations resulting in target modification. The DNA synthesis inhibitory fluoroquinolones acquire resistance by alterations within the subunit A of DNA gyrase (GyrA) or topoisomerase IV (ParC). Decreased susceptibility to antibiotics acting on the 30S ribosomal subunit (eg. tetracyclines, spectinomycin) was associated with mutations present on genes encoding the 16S rRNA. Acquired resistance to antimicrobials binding to the 50S ribosomal subunit (eg. macrolides, lincosamides) is caused by point mutations of the 23S rRNA.

## Aims of the study

The aims of the study were:

**Ad 1.** to genetically characterize the Hungarian *M. bovis* population with the MLST and MLVA methods in order to evaluate and compare these two typing systems and to better understand the epidemiology of *M. bovis* in Hungary.

**Ad 2.** to determine the *in vitro* susceptibility profile of the Hungarian *M. bovis* isolates to fifteen antibiotics of eight antimicrobial groups that could potentially be used in the therapy.

**Ad 3.** to investigate the molecular mechanisms involved in the resistance of *M. bovis* and to identify mutations responsible for the high MICs (minimum inhibitory concentration) to seven different antimicrobial families (fluoroquinolones, tetracyclines, aminocyclitol, macrolides, lincosamide, phenicol and pleuromutilins) by using whole-genome sequencing of field isolates and laboratory-derived mutants.

**Ad 4.** to develop and to characterize rapid and cost-effective real-time PCR based assays for the simultaneous determination of antibiotic susceptibility profile of *M. bovis* isolates in the case of seven antimicrobial groups.

## Materials and methods

### ***Mycoplasma bovis* isolates**

*M. bovis* isolates originating from nasal swabs, lung samples and a single lymph node collected from different parts of Hungary and the *M. bovis* type strain (NCTC10131; PG45) were included in the present study. The samples were cultured in Mycoplasma broth medium at 37°C in a 5% CO<sub>2</sub> atmosphere. All isolates were identified by PCR amplifying the *uvrC* gene of *M. bovis* (Subramaniam et al. 1998). The purity of the cultures was confirmed by an universal mycoplasma PCR system targeting the 16S/23S rRNA intergenic spacer region in Mollicutes, and sequencing (Lauerman et al. 1995). Thirty-five isolates were collected between 2010 and 2013, while another twenty isolates were obtained between 2013 and 2016.

### **Genotyping of the Hungarian *Mycoplasma bovis* isolates**

The typing of 31 *M. bovis* isolates from Hungary was performed by using a previously published MLST assay based on four housekeeping genes (*fusA*, *gyrB*, *lepA*, *rpoB*) (Manso-Silván et al. 2012). In order to determine the level of genetic diversity within each subclade identified by MLST Hungarian isolates were screened with an MLVA system based on nine tandem repeats described by Pinho et al. (2012).

Phylogenetic analysis was performed with a neighbour-joining method. The discriminatory power of the different typing schemes was calculated using Simpson's index of diversity (Hunter és Gaston 1988). The quantitative level of congruence between the two typing methods was calculated using the adjusted Rand and Wallace coefficients (Carriço et al. 2006).

### **Antibiotic susceptibility testing**

The susceptibility of 35 *M. bovis* isolates from Hungary and the PG45 was determined by microbroth dilution method to 15 antimicrobial agents of 8 antimicrobial groups (Hannan 2000).

The MIC value was defined after one week incubation period at 37°C to the following antibiotics: fluoroquinolones (danofloxacin, enrofloxacin, marbofloxacin); tetracyclines (tetracycline, oxytetracycline); aminocyclitols (spectinomycin); aminoglycosides (gentamycin); macrolides (tylosin, tilmicosin, gamithromycin, tulathromycin); lincosamide (lincomycin); phenicol (florfenicol) and pleuromutilines (tiamulin, valnemulin).

## **Identification of mutations responsible for the high MICs to seven antimicrobial families**

The molecular mechanisms of resistance to 12 antibiotics of 7 antimicrobial families (danofloxacin, enrofloxacin, marbofloxacin, tetracycline, oxytetracycline, spectinomycin, tilimicosin, tylosin, lincomycin, florfenicol, tiamulin and valnemulin) were investigated. Antibiotic-resistant mutants were selected by serial passages of three isolates with low MIC values to the tested antibiotics. The serial passages were performed in Mycoplasma broth medium containing subinhibitory concentrations of each of the examined antibiotics. Resistant mutants were subcultivated in antibiotic-free medium at least five times and MICs of all drugs were determined again by the end of the passages to check whether the phenotype was stable without selection pressure (Pereyre et al. 2004). Cross-resistance was also examined with microbroth dilution tests among the 12 examined drugs.

Next-generation sequencing of the 35 Hungarian *M. bovis* isolates, the reference strain (PG45, NCTC 10131) and the 36 *in vitro* selected mutants was performed on Ion Torrent platform as previously described (Rónai et al. 2015). Mutations associated with high MIC were identified.

## **Development of molecular assays for the rapid detection of antibiotic susceptibility of *Mycoplasma bovis***

For the simultaneous detection of multiple mutations responsible for high MICs to fluoroquinolones, tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis*, nine mismatch amplification mutation assays (MAMA) and seven high resolution melt (HRM) tests were designed.

Initially, evaluation of the MAMA and HRM assays was performed on DNAs extracted from purified culture of *M. bovis* field isolates (n=35) and laboratory derived mutants (n=36) with known sequence and MIC values. In order to test the sensitivity of the assays, tenfold dilutions of each genotype were used in the range of  $10^6$ - $10^0$  copy number/ $\mu$ l. The specificity of the assays was tested by bovine *Mycoplasma* species and by the genetically closely related species, *M. agalactiae*. For further evaluation of the assays, HRM and melt-MAMA tests were challenged with the DNA of clinical samples (n=30).

# Results

## **Genetic diversity of the Hungarian *Mycoplasma bovis* isolates**

Six STs were discriminated by MLST and twenty different MLVA profiles were identified among the 31 Hungarian *M. bovis* isolates and PG45. The Simpson's index of diversity was 0.776 in MLST and 0.970 in MLVA indicating high genetic variation. The analysed isolates were grouped into two clades by both methods with little congruence between the results of the two typing systems (Adjusted Rand coefficient 0.178, Adjusted Wallance coefficient MLVA → MLST 0.099; Adjusted Wallance coefficient MLST → MLVA 0.914). Isolates originating from the same herds had the same sequence types except for one case (MYC65-MYC68). Correlation was not found between the genotype and source (lung, nasal swab or lymph node) of the *M. bovis* isolates.

## **Antibiotic susceptibility of the Hungarian *Mycoplasma bovis* isolates**

Ninety percent of the Hungarian *M. bovis* isolates were inhibited with high MIC values of macrolides (tylosin  $\geq 128$   $\mu\text{g/ml}$ , tilmicosin  $\geq 128$   $\mu\text{g/ml}$ , gamithromycin  $\geq 128$   $\mu\text{g/ml}$ , tulathromycin  $\geq 128$   $\mu\text{g/ml}$ ), aminoglycosides (gentamycin 8  $\mu\text{g/ml}$ ), aminocyclitols (spectinomycin  $\geq 256$   $\mu\text{g/ml}$ ), tetracyclines (tetracycline 16  $\mu\text{g/ml}$ , oxytetracycline  $\geq 64$   $\mu\text{g/ml}$ ), lincosamides (lincomycin  $\geq 64$   $\mu\text{g/ml}$ ) and phenicols (florfenicol 8  $\mu\text{g/ml}$ ). Fluoroquinolones (MIC<sub>90</sub> values: 0.312  $\mu\text{g/ml}$  to danofloxacin, 0.312  $\mu\text{g/ml}$  to enrofloxacin, 0.625  $\mu\text{g/ml}$  to marbofloxacin) and pleuromutilins (MIC<sub>90</sub> values: 0.312  $\mu\text{g/ml}$  to tiamulin,  $\leq 0.039$   $\mu\text{g/ml}$  to valnemulin) were found to be the most active compounds *in vitro* for the therapy of *M. bovis* infections in Hungary. Isolates originating from the same herd showed similar antibiotic susceptibility profiles.

## **Mutations responsible for the high MICs to seven antimicrobial groups**

Mutants with high MICs to fluoroquinolones ( $\geq 10$   $\mu\text{g/ml}$ ) were selected *in vitro* by 4-10 passages of the parental strains. All *M. bovis* field isolates and laboratory-derived mutants with MIC  $\geq 10$   $\mu\text{g/ml}$  to fluoroquinolones harboured mutations within hot spot regions of *gyrA* and *parC* genes (244-260 and 232-250, according to *Escherichia coli* nucleotide numbering). In each case, cross-resistance ( $\geq 10$   $\mu\text{g/ml}$ ) developed in the selected mutants to the other fluoroquinolones as well.

In contrast with the observations of the development of resistance to tetracyclines (9-18 passages), resistance to spectinomycin (MIC  $\geq 256$   $\mu\text{g/ml}$ ) evolved rapidly (2-3 passages). This finding is in harmony with the detected spectinomycin susceptibility profile of the 35



Hungarian isolates. Mutations associated with elevated MICs to tetracyclines ( $\geq 16$   $\mu\text{g/ml}$  and  $\geq 4$   $\mu\text{g/ml}$  to oxytetracycline and tetracycline) were identified at positions 962-967, 1058, 1195, 1196 in genes encoding the 16S rRNA. All isolates with high MICs to spectinomycin ( $\geq 256$   $\mu\text{g/ml}$ ) harboured a single mutation at position 1192 of *rrs1* gene. Cross-resistance was observed between tetracycline and oxytetracycline resistant mutants and only slightly elevated MICs to spectinomycin (fourfold) were detected.

Evolution of resistance to 50S inhibitory antibiotics (tylosin, tilmicosin, lincomycin, florfenicol, tiamulin, valnemulin) strongly differed: two out of three valnemulin resistant mutants were obtained after 10-14 passages, while the rest of the resistant mutants developed more rapidly (2-6 passages). In field isolates with MICs  $\geq 128$   $\mu\text{g/ml}$  to tilmicosin substitution G748A of genes encoding the 23S rRNA was detected. In the Hungarian isolates with high MICs to tylosin ( $\geq 128$   $\mu\text{g/ml}$ ) and lincomycin ( $\geq 64$   $\mu\text{g/ml}$ ) the additional substitution A2059G in both genes were observed. The following nucleotide regions were taking part in the evolution of resistance to 50S inhibitory antibiotics: 748-752, 2059-2067, 2500-2506 and 2611-2612. Cross-resistance between macrolides and lincomycin were often observed, moreover one valnemulin resistant mutant had elevated MICs to all of the tested 50S inhibitors.

### **Rapid detection of antibiotic susceptibility of *Mycoplasma bovis***

For the detection of point mutations responsible for high MICs to certain antibiotics in *M. bovis*, nine MAMA (3 to fluoroquinolones; 3 to tetracyclines; 1 to spectinomycin; 2 to macrolides including 1 to lincomycin), while for the detection of hot-spot regions seven HRM (1 to fluoroquinolones, 2 to 30S inhibitors and 4 to 50S inhibitors) tests were designed.

In the current study all systems (MAMAs and HRMs) were designed with the same thermal profile allowing simultaneous application of the assays. The sensitivity of real-time PCR based methods (melt-MAMA and HRM) was  $10^2$ - $10^5$  copy numbers/reaction, while agarose-MAMAs showed a sensitivity of  $10^3$ - $10^5$  copies/reaction.

The reliability of the reported methods was 100% tested on the DNA of *M. bovis* pure cultures. However, the reliability varied notably in the case of DNA extracted directly from clinical samples, especially in assays targeting universal regions (e.g. 16S rRNA), because of their lower specificity. Nevertheless, the tests of clinical samples containing high amount of *M. bovis* DNA were congruent even in the presence of other *Mycoplasma* spp.

## Discussion

### **Genetic diversity of the Hungarian *Mycoplasma bovis* isolates**

Information on the genetic heterogeneity within the Hungarian *M. bovis* population is restricted. The thirty-one examined Hungarian *M. bovis* isolates showed high genetic variation by both MLST and MLVA with little congruence between the results of the two typing systems. The high genetic variation detected among the Hungarian isolates is similar to the results of more recent examinations worldwide (Register et al. 2015, Rosales et al. 2015). At least partially, the high genetic diversity of *M. bovis* is possibly due to the intensive cattle trade. The loss of diversity of French isolates has been reported as an exception from the generally described genetic heterogeneity of *M. bovis* due to the spread of a multiresistant clone (Becker et al. 2015).

Based on our data, the combined use of the two molecular epidemiological typing techniques are recommended for analysis of *M. bovis* isolates, as MLST provides information about mid-term evolutionary events while the short-term epidemiological relationships can be revealed by MLVA.

### **Antibiotic susceptibility of the Hungarian *Mycoplasma bovis* isolates**

Since no effective vaccine is available against *M. bovis*, appropriate antibiotic treatment is promoted in the control of the diseases caused by this pathogen. In routine practice, the potential effectiveness of antimicrobials *in vivo* can be estimated by *in vitro* susceptibility testing with the determination of the MIC values. However, interpretation of the results of the *in vitro* examinations should be handled with caution as *in vivo* factors can also influence the efficiency of the treatment (Lysnyansky és Ayling 2016). Comprehensive study about the antibiotic susceptibility profiles of *M. bovis* strains isolated in Hungary has not been performed previously.

Based on our *in vitro* examinations on 15 antibiotics of 8 antimicrobial families, fluoroquinolones are supposed to be the most effective agents in the therapy of *M. bovis* infections in Hungary. However, current antimicrobial usage policies have to be taken into account to avoid further antibiotic resistance development and to reserve fluoroquinolones for the treatment of severe infections which have responded poorly to other classes of antimicrobials. Our results confirmed the increasing resistance to antibiotics commonly used for the treatment of mycoplasma infections, primarily to tetracyclines and macrolides emphasizing the necessity of periodic testing of antibiotic susceptibility within a geographic region. In accordance with previous *in vivo* examinations (Stipkovits et al. 2001, Stipkovits et al. 2005), pleuromutilins effectively inhibited the growth of this bacteria *in vitro* suggesting the potential of this antibiotic group in the therapy of *M. bovis* infections.

### **Mutations responsible for the high MICs to seven antimicrobial families**

Antibiotics are among the most important therapeutic tools in the veterinary and human medicine, but their use is limited as resistance tends to evolve in pathogenic bacteria (Perron et al. 2015).

All *M. bovis* isolates of the present study with high MICs ( $\geq 10$   $\mu\text{g/ml}$ ) to fluoroquinolones contained at least one substitution both in GyrA and ParC. Our results confirm that for the increase of MICs to fluoroquinolones substitutions in GyrA are sufficient and resistance to fluoroquinolones is achieved by an additional change in ParC (Lysnyansky et al. 2009, Sato et al. 2013).

In accordance with previous studies, mutations in the region of primary tetracycline binding site (Tet-1) were responsible for the increase of MICs to tetracycline in *M. bovis* isolates. In the present study, all of the previously determined mutations in Tet-1 site formed by 16S rRNA were identified with one exception (Amram et al. 2015). The current study confirmed the role of 1192 position of 16S rRNA in the antibiotic susceptibility of *M. bovis* as substitution C to A or T occurred in isolates with MICs of  $\geq 256$   $\mu\text{g/ml}$  (Schnee et al. 2014).

Several mutations were identified in genes encoding the 23S rRNA, which were responsible for high MICs to macrolides, lincomycin, florfenicol and pleuromutilins. Some of these mutations led to cross-resistance to other classes of antibiotics with a similar mechanism of action. Macrolide and lincomycin related mutations identified in the Hungarian *M. bovis* population were all described in other countries; however the frequency of certain mutations differed according to the countries (Lerner et al. 2014).

### **Rapid detection of antibiotic susceptibility of *Mycoplasma bovis***

In the current study, 9 MAMA and 7 HRM tests were designed for the simultaneous detection of markers responsible for high MICs to fluoroquinolones, tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis*.

The mutations of the field isolates have higher diagnostic importance compared to the mutations in laboratory selected mutants (Sundsford et al. 2004), which was considered in the development of the diagnostic assays. The reported method reliably worked with the DNA of purified *M. bovis* cultures and clinical samples containing high amount of *M. bovis* DNA even in the presence of other *Mycoplasma* spp., assuming the usefulness of the assays during the therapy of clinical *M. bovis* infections. For the most reliable antibiotic resistance determination, especially in the case of clinical samples, the regions examined by the MAMA and HRM tests are overlapping. The presented method is rapid, highly cost-effective and can provide the antibiogram of *M. bovis* to 12 antibiotics of 7 antimicrobial groups, thus it may represent a suitable alternative for the conventional antibiotic susceptibility tests and it can promote the effectiveness of the therapy.

## Overview of the new scientific results

**Ad 1.** High genetic heterogeneity was described of the *M. bovis* isolates originating from Hungary with both MLVA and MLST methods. The combined use of the two molecular epidemiological typing techniques is recommended in the case of *M. bovis* isolates. Strains have to be characterized first by MLST, as an intermediate scale typing, and it should be followed by the fine scale typing of identical sequence types with MLVA.

**Ad 2.** Based on our *in vitro* examinations of fifteen antibiotics of eight antimicrobial families, fluoroquinolones proved to be the most effective drugs for the therapy of *M. bovis* infections in Hungary. Increasing resistance to antibiotics commonly used in the therapy of mycoplasma infections, primarily to tetracyclines and macrolides was described in Hungary. The results emphasize the necessity of periodic testing for antibiotic susceptibility in *M. bovis*. The effectiveness of pleuromutilins in the *in vitro* examinations suggests the potential of this antibiotic group in the therapy of *M. bovis* infections.

**Ad 3.** The whole genomes of 35 Hungarian *M. bovis* isolates were sequenced for the first time. Molecular markers responsible for high MICs to 3<sup>rd</sup> generation fluoroquinolones, tetracyclines, spectinomycin, 16-membered macrolides and to lincomycin were identified within the Hungarian *M. bovis* population.

*In vitro* development of resistance to tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis* was described for the first time. In laboratory derived *M. bovis* mutants novel molecular markers of high MICs to fluoroquinolones, tetracyclines, spectinomycin and macrolides were described. Molecular mechanism of acquired resistance to florfenicol and pleuromutilins in *M. bovis* were investigated for the first time identifying mutations in the 23S rRNA genes.

**Ad 4.** Nine MAMA and seven HRM assays were designed and evaluated for the simultaneous detection of multiple mutations responsible for high MICs to fluoroquinolones, tetracyclines, spectinomycin, macrolides, lincomycin, florfenicol and pleuromutilins in *M. bovis*. The presented method is highly cost-effective and can provide an antibiogram to 12 antibiotics of 7 antimicrobial groups within a day performing directly on the clinical samples, while approximately 3-4 days are needed when previous isolation of *M. bovis* is applied. For the most reliable antibiotic resistance determination, especially in the case of clinical samples, the combined use of MAMA and HRM is recommended.

## Scientific publications

### In peer-reviewed journals

Sulyok, K.M., Bekő, K., Kreizinger, Z., Wehmann, E., Jerzsele, Á., Rónai, Z., Turcsányi, I., Makrai, L., Szeredi, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M.: **Development of molecular methods for the rapid detection of antibiotic susceptibility of *Mycoplasma bovis***, Vet. Microbiol., [submitted].

Sulyok, K.M., Kreizinger, Z., Wehmann, E., Lysnyansky, I., Bányai, K., Marton, S., Jerzsele, Á., Rónai, Z., Turcsányi, I., Makrai, L., Jánosi, S., Nagy, S.Á., Gyuranecz, M.: **Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains**, Antimicrob. Agents Chemother., 61. e01983-16, 2017.

Sulyok, K.M., Kreizinger, Z., Fekete, L., Hrivnák, V., Magyar, T., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., Gyuranecz, M.: **Hazai *Mycoplasma bovis* törzsek antibiotikum-érzékenységének vizsgálata** (bővített másodközlés), Magy. Állatorvosok, 139. 169-179, 2017.

Sulyok, K.M., Kreizinger, Z., Fekete, L., Hrivnák, V., Magyar, T., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., Gyuranecz, M.: **Antibiotic susceptibility profiles of *Mycoplasma bovis* strains isolated from cattle in Hungary, Central Europe**, BMC Vet. Res., 10. 256, 2014.

Sulyok, K.M., Kreizinger, Z., Fekete, L., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., Gyuranecz, M.: **Phylogeny of *Mycoplasma bovis* isolates from Hungary based on multi locus sequence typing and multiple-locus variable-number tandem repeat analysis**, BMC Vet. Res., 10. 108, 2014.

## Other publications in peer-reviewed journals

- Sulyok, K.M.\*, Gyuranecz, M.\*, Balla, E., Mag, T., Balázs, A., Simor, Z., Dénes, B., Hornok, S., Bajnóczi, P., Hornstra, H.M., Pearson, T., Keim, P., Dán, Á.: **Q fever epidemic in Hungary, April to July 2013**, Euro Surveill., 19. 20863, 2014. \*These authors contributed equally to this article
- Sulyok, K.M., Kreizinger, Z., Hornstra, H.M., Pearson, T., Szigeti, A., Dán, Á., Balla, E., Keim, P.S., Gyuranecz, M.: **Genotyping of *Coxiella burnetii* from domestic ruminants and human in Hungary: indication of various genotypes**, BMC Vet. Res., 10. 107, 2014.
- Sulyok, K.M., Hornok, S., Abichu, G., Erdélyi, K., Gyuranecz, M.: **Identification of novel *Coxiella burnetii* genotypes from Ethiopian ticks**, Plos ONE 9. e113213, 2014.
- Flaisz, B., Sulyok, K.M., Kováts, D., Kontschán, J., Csörgő, T., Csapak, Á., Gyuranecz, M., Hornok, S.: **Babesia genotypes in *Haemaphysalis concinna* collected from birds in Hungary reflect phylogeographic connections with Siberia and the Far East**, Ticks Tick Borne Dis. [in press].
- Hornok, S., Mulvihill, M., Szőke, K., Gönczi, E., Sulyok, K.M., Gyuranecz, M., Hofman-Lehman, R.: **Impact of a freeway on the dispersal of ticks and *Ixodes ricinus*-borne pathogens: forested resting areas may become Lyme disease hotspots**, Acta Vet. Hung., 65. 242-252, 2017.
- Kreizinger, Z., Sulyok, K.M., Bekő, K., Szabó, Z., Gyuranecz, M.: **Development of mismatch amplification mutation assays for the differentiation of MS1 vaccine strain from wild-type *Mycoplasma synoviae* and MS-H vaccine strains**, Plos ONE, 12. e0175969, 2017.
- Kreizinger, Z., Erdélyi, K., Felde, O., Fabbi, M., Sulyok, K.M., Magyar, T., Gyuranecz, M.: **Comparison of virulence of *Francisella tularensis* ssp. *holarctica* genotypes B.12 and B.FTNF002-00**, BMC Vet. Res., 13. 46, 2017.
- Gróznér, D., Kreizinger, Z., Sulyok, K.M., Rónai, Z., Hrivnák, V., Turcsányi, I., Jánosi, S., Gyuranecz, M.: **Antibiotic susceptibility profiles of *Mycoplasma* sp. 1220 strains isolated from geese in Hungary**, BMC Vet. Res., 12. 170, 2016.
- Kreizinger, Z., Sulyok, K.M., Makrai, L., Rónai, Z., Fodor, L., Jánosi, S., Gyuranecz, M., **Antimicrobial susceptibility of *Bacillus anthracis* strains from Hungary**, Acta Vet. Hung., 64. 141-147, 2016.

- Kreizinger, Z., Szeredi, L., Bacsadi, Á., Nemes, C., Sugár, L., Varga, T., Sulyok, K.M., Szigeti, A., Ács, K., Tóbiás, E., Borel, N., Gyuranecz, M.: **Occurrence and significance of *Coxiella burnetii* and Chlamydiales in abortions of domestic ruminants and in wild ruminants in Hungary, Central Europe**, J. Vet. Diagn. Invest., 27. 206-210, 2015.
- Kreizinger, Z., Sulyok, K.M., Pásztor, A., Erdélyi, K., Felde, O., Povaszán, J., Kőrösi, L., Gyuranecz, M.: **Rapid, simple and cost-effective molecular method to differentiate the temperature sensitive (ts+) MS-H vaccine strain and wild-type *Mycoplasma synoviae* isolates**, Plos ONE, 10. e0133554, 2015.
- Kreizinger, Z., Foster, J.T., Rónai, Z., Sulyok, K.M., Wehmann, E., Jánosi, S., Gyuranecz, M.: **Genetic relatedness of *Brucella suis* biovar 2 isolates from hares, wild boars and domestic pigs**, Vet. Microbiol., 172. 492, 2014.
- Hornok, S., Abichu, G., Meli, M.L., Tánczos, B., Sulyok, K.M., Gyuranecz, M., Gönczi, E., Farkas, R., Hofmann-Lehmann, R.: **Influence of the biotope on the tick infestation of cattle and on the tick-borne pathogen repertoire of cattle ticks in Ethiopia**, Plos ONE, 9. e106452, 2014.

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