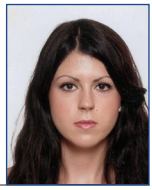
Suspect and positive cases of bovine tuberculosis in the Republic of Croatia from 2017 to 2020



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Abstract

During regular implementation of the bovine tuberculosis-free cattle herd certification programme in the period from 2017 to 2020, the Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases of Croatian Veterinary Institute Zagreb, Croatia tested material from 161 cattle from 27 holdings in 11 counties. The material was submitted following findings of pathoanatomical changes detected in the slaughter line suggesting tuberculosis, or after a positive reaction of cows to the tuberculin comparative methods. Species from the *M. tuberculosis* complex (*M. bovis* and *M. caprae*) were isolated from samples of 58 bovines (36%) from 16 holdings in eight counties. *M. caprae* was confirmed in 55 bovines (34%) originating from 13 holdings in seven counties, and *M. bovis* in three bovines (2%), each from a different holding in a different county. Saprophytic mycobacteria were isolated from four bovine samples (2.5%) from three holdings in two counties, *i.e., M. gordonae* (1), *M. celatum* (1) and two unidentified species (*M. sp.*). Based on the obtained results, we can conclude that the main causative agent of bovine tuberculosis in the Republic of Croatia is *M. caprae,* which confirmed previous findings. Control of bovine tuberculosis in the Republic of Croatia is still needed and, in the future, should be further suppressed using tuberculinisation, controls on slaughter lines, depopulation of infected herds, and etiological determination of the causative agents.

Key words: *Mycobacterium bovis*; *M. caprae*; *tuberculosis; cattle; Republic of Croatia*

Introduction

The genus *Mycobacterium* includes more than 190 species (Parte, 2018) that differ based on their metabolism, growth rate, epidemiology, pathogenicity, geographic distribution and sensitivity to antimicrobials. Within the genus are three main groups: 1. *Mycobacterium tuberculosis* complex - species that

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cause tuberculosis in mammals (*M. tuberculosis*, *M. africanum*, *M. canetti*, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. munghi*, *M. orygis*, *M. suricattae*); 2. *Mycobacterium leprae* – the causative agent of leprosy in humans; 3. Non-tuberculosis mycobacteria (NTM) – includes all other species (more than 160 species) that act as opportunistic pathogens (Falkinham, 1996; Sinha et al., 2016).

Tuberculosis is a chronic infectious disease affecting a wide range of species of wild and domesticated animals and humans. In many countries, bovine tuberculosis is an epidemiological issue causing economic losses. Domesticated and wild animals are considered reservoirs and vectors for bovine tuberculosis, and direct contact, contaminated pastures and contact with wild animals are the dominant means of disease transmission (Delahay et al., 2001; Biet et al., 2005).

In Croatia, the first infections with the species *M. caprae* in cattle, swine and humans were confirmed in 2006. (Cvetnić et al., 2007). A subsequent detailed study gave an incidence of the species *M. caprae* in 85% and *M. bovis* in 15% of 92 isolates obtained belonging to the *M. tuberculosis* complex (Špičić, 2008). As the most common cause of tuberculosis in cattle, *M. caprae* has also been reported in the countries of central and western Europe (Prodinger et al., 2005).

A 2009 decision of the Ministry of Agriculture, Fisheries and Rural Development marked the start of testing cattle for tuberculosis to obtain and maintain the status of tuberculosis-free herds of cattle, in accordance with the procedures and criteria laid down in the Ordinance on veterinary conditions for the trade of cattle and swine (Official Gazette 154/08). With the aim of complete eradication of the disease and obtaining the status of a tuberculosis-free country, the Veterinary Directorate of the Ministry of Agriculture adopted new measures in 2010 to begin the tuberculinisation campaign for the entire cattle population in the Republic of Croatia. This campaign led to the discovery of new hotspots in individual cattle holdings in Croatia. All bovines with a positive reaction to tuberculosis were removed from the holding and sent to slaughter, with the mandatory sampling of organs and tissues for bacteriological confirmation of the causative agent of tuberculosis.

This paper aims to provide an overview of the bacteriological and molecular testing of samples obtained from positive cattle in the period from 2017 to 2020 and to determine the spread and presence of individual species of mycobacteria in the Republic of Croatia.

Materials and methods

In the period from 2017 to 2020, a total of 161 bovine organ and tissue samples collected from 27 holdings in 11 counties were submitted for bacteriological testing to the Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases. These samples were submitted as part of the Bovine Tuberculosis Monitoring and Eradication Programme, prescribed in the Republic of Croatia by the Ministry of Agriculture following results of pathological anatomical changes detected in the slaughter line that are suspected of tuberculosis, or after a positive reaction to the tuberculin comparative method.

Bacteriological testing. Submitted material was homogenised then decontaminated with 5% oxalic acid, with occasional stirring at room temperature. Samples were concentrated by centrifugation, then the supernatant was removed, and the sediment diluted with sterile distilled water. The obtained suspension was inoculated onto selected agar: 2 Löwenstein-Jensen agar with glycerol, 2 Löwenstein-Jensen agar without glycerol and 2 Stonebrink agar

(Kent and Kubica, 1985). Eprouvettes with the inoculated material were incubated for 8 weeks at 37°C. Mycobacterial growth was first controlled after 4–7 days, then at weekly intervals. Colonies were identified based on the growth rate, morphology and Ziehl-Neelsen colouration, and after determination of acid-resistant rods. Further identification and typing were performed using molecular methods. Agar inoculated with bovine material that showed no growth after 8 weeks was considered a negative test and testing was completed (OIE Terrestrial Manual 2018, Section 3.4.6).

Molecular testing. DNA was extracted from the obtained bacterial cultures using a commercial kit DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using the QIACUBE automated system for DNA isolation (Qiagen, Hilden). The obtained DNA was stored at -20°C.

Identification to the genus *Mycobacterium* was proven using the specific primers 16S rRNA F (5′-ACG GTG GGT ACT AGG TGT GGG TTT C-3′) and 16S rRNA R (5′-TCT GCG ATT ACT AGC GAC TCC GAC TTC A-3′) for replication of the region of the 16S rRNA gene (Huard et al., 2003) and primers TB1 (5′-GAG ATC GAG CTG GAG GAT CC-3′) and TB2 (5′-AGC TGC AGC CCA AAG GTG TT-3′) for the gene coding the 65 kDa antigen (Hance et al., 1989) that is common to all mycobacteria. Gene replication was performed with initial denaturation (95°C/15 min), followed by 35 cycles of denaturation (94°C/1 min), primer binding (60°C/1 min) and chain elongation (72°C/1 min) and the final step of chain elongation (72°C/10 min).

Isolates proven by the above method to belong to the genus *Mycobacterium* were further tested using the primers IS 1 (5′-CCT GCG AGC GTA GGC GTC GG-3′) and IS 2 (5′-CTC GTC CAG CGC CGC TTC GG-3′) which prove the insertion sequence IS*6110* characteristic to the species of the *M. tuberculosis* complex (Eisenach et al., 1990). Replication was conducted with an initial denaturation at 95°C for 15 minutes, followed by 35 cycles (94°C/30 sec, 68°/1 min, 72°/1 min) and finally chain elongation at 72°C for 7 minutes.

The PCR reactive mixture in these tests contained 10 μL HotStarTaq Master Mix (Qiagen, Hilden, Germany), 6 μL water (RNase-free Water, Qiagen, Hilden, Germany), 1 μL of each of the listed primers, and 2 μL DNA. Gene replication was performed using the device Veriti 96 Well Thermal Cycler (Applied Biosystems, California, USA). Replication products were analysed using the QIAxcel capillary electrophoresis device (Qiagen, Hilden, Germany).

Determination of species within the *M. tuberculosis* complex was performed using the molecular test GenoType MTBC kit (Hain Lifescience, Nehren, Germany). This kit is based on DNA strip technology and enables the identification of species that belong to the *M. tuberculosis* complex (*M. africanum*, *M. bovis* ssp. BCG, *M. bovis* ssp. *bovis* (*M. bovis*), *M. bovis* ssp. *caprae* (*M. caprae*), *M. microti* and *M. tuberculosis*/*M. canettii*. The species determination procedure using this kit includes PCR replication using biotinylated primers and reverse hybridisation. Hybridisation includes the chemical denaturation of PCR replication products, hybridisation of single-chain biotinylated products on probe membranes, additional of streptavidin-alkaline phosphate conjugates, and interpretation of the obtained sample on the strip.

The final determination of saprophytic and potentially pathogenic species was conducted using the molecular tests GenoType CM based on the same principle as GenoType MTBC Kit (*M. avium* ssp., *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M.*

kansasii, *M. malmoense*, *M. peregrinum*, *M. marinum*/*M. ulcerans*, *M. tuberculosis* kompleks i *M. xenopi*) i GenoType AS kit (Hain Lifescience, Nehren, Germany) (*M. simiae*, *M. mucogenicum*, *M. goodii*, *M. celatum*, *M. smegmatis*, *M. genavense*, *M. lentiflavum*, *M. heckeshornense*, *M. szulgai*/*M.intermedium*, *M. phlei*, *M. haemophilum*, *M. kansasii*, *M. ulcerans*, *M. gastri*, *M. asiaticum* and *M. shimoidei*).

Results

Bacteriological testing and subsequent molecular species identification detected mycobacteria in 62 (38.5%) of the total 161 analysed samples. These positive samples originated from 27 holdings in eleven counties. Molecular methods used to prove the 16S rRNA gene region and the gene coding the 65 kDa antigen confirmed that all 62 samples belonged to the genus *Mycobacterium*. Further detection of the insertion sequence IS*6110* and use of the molecular test kit GenoType MTBC showed that these samples belong to the *M. tuberculosis* complex, with the species *M. bovis* and *M. caprae* found in isolates from 58 bovines (36%) from 16 holdings in eight counties. *M. caprae* was confirmed in 55 animals (34%) from 13 holdings in seven counties, while *M. bovis* was found in three animals (2%), each from a different holding and county. A sample from one holding confirmed dual infection with *M. caprae* and *M. bovis*. Use of the molecular tests GenoType CM and AS proved saprophytic mycobacteria that were isolated from four samples (2.5%) obtained from three holdings in two counties. The following species of mycobacteria were identified: *M. gordonae* (1), *M. celatum* (1) and two unidentified species (*M. sp.*) (Table 1). Mycobacteria were not isolated from 97 samples (60.2%) of cattle.

Discussion

In many European countries, *M. caprae* is listed as a frequent causative agent of bovine tuberculosis, and this disease has also been confirmed in red deer and wild boar. The first evidence of infection in Croatia was proven in cattle, swine and humans in 2006 (Aranaz et al., 2003; Cvetnić et al., 2007). In terms of human infection, it is less common than *M. bovis*, and its global distribution is primarily restricted to European countries (Prodinger et al., 2014). The widespread distribution of *M. bovis* in wild animals presents a serious issue in attempts to eradicate bovine tuberculosis, and in certain countries, wild animals remain a permanent reservoir of infection. In the United Kingdom and Ireland, the European badger (*Meles meles*) is the

Table 1. Overview of the number of cattle and counties in the Republic of Croatia where different species of *Mycobacterium* sp. were isolated

	County	*M. caprae*	*M. bovis*	NTM
1	Bjelovar-Bilogora	2	1	
2	Koprivnica-Križevci	1		
3	Lika-Senj	2	1	
4	Osijek-Baranja	34	1	
5	Sisak-Moslavina	1		1
6	Brod-Posavina	4		
7	Vukovar-Srijem	8		3
8	Zagreb County	3		
	TOTAL	55	3	4

main host of endemic infection with *M. bovis*, while in Spain this is wild boar (*Sus scrofa*). Individual hosts can vary between regions, and can also change over time (Miller and Sweeney, 2013). *M. bovis* is primarily a bovine pathogen, though it can also infect goats, horses, swine, camels, cats and dogs. In many undeveloped countries, this causative agent has high commercial significance.

Špičić (2008) conducted a detailed study in the Republic of Croatia, proving the presence of *M. caprae* in 85%, and *M. bovis* in 15% of a total of 92 samples belonging to the *M. tuberculosis* complex. During 2010, these species were isolated from materials obtained from 117 bovines (90.7%) from 23 holdings (23%) in seven Croatian counties. *M. caprae* was confirmed in 83 bovines (70.9%) from nine holdings in four counties, *M. bovis* in 33 bovines (28.2%) from 14 holdings in five counties, and *M. tuberculosis* in only one animal (0.85%) (Špičić et al., 2011).

In the period 2017-2020, species from the *M. tuberculosis* complex (*M. bovis* and *M. caprae*) were isolated from the samples of 58 bovines (36%) from 16 holdings in 8 counties. *M. caprae* was confirmed in 55 bovines (34%) from 13 holdings in seven counties, and *M. bovis* in three bovines (2%), each from a different holding and a different county. Based on the obtained results, it can be concluded that the main causative agent of bovine tuberculosis in the Republic of Croatia is *M. caprae*, which confirms the results of earlier research. The research conducted in the countries of Central and Western Europe also corroborate this finding (Prodinger et al., 2005).

In comparison with previous studies in the Republic of Croatia, it can be concluded that the number of bovines positive for tuberculosis has declined, but that the disease remains present in nearly the same number of holdings. It is also interesting to note the origin of samples included in the research. Of the 58 tuberculosis-positive bovines, 29 (50%) originated from other European countries, as determined by the ear tags, *i.e.*, 18 animals from Romania, 10 from Hungary and 1 from the Czech Republic.

The prevalence of bovine tuberculosis has decreased over the past decade due to planned eradication efforts, though the disease has still not been fully eradicated. In Croatia, controls of bovine tuberculosis are based on testing of all bovines over the age of six weeks in herds that do not have disease-free status in line with the requirements of EU legislation. Testing is based on a tuberculin skin test. Disease-free status is maintained by testing disease-free herds once every 3 years. For that purpose, Croatian territory is divided in 3 zones which are regularly tested in 3-year cycles. Positive animals are sent for slaughter, while their organs are taken for bacteriological testing for tuberculosis.

It can be concluded that control of bovine tuberculosis in the Republic of Croatia is still necessary to fulfil requirements to be recognised as a country free from bovine tuberculosis and in the future, this disease should be suppressed using tuberculinisation, slaughterhouse line controls, depopulation of infected herds, and etiological determination of causative agents. Special attention should also be focused on controlling migration and controls during animal imports to prevent the introduction and spread of the disease in the Republic of Croatia.

References

1. ARANAZ, A., D. COUSINS, A. MATEOS and L. DOMINGUEZ (2003): Elevation of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz et al. 1999 to species rank as *Mycobacterium caprae* comb. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 53, 1785-1789.
2. BIET, F., M. L. BOSCHIROLI, M. F. THOREL and L. A. GUILLOTEAU (2005): Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). Vet. Res. 36, 411-436.
3. CVETNIĆ, Ž., V. KATALINIĆ-JANKOVIĆ, B. ŠOŠTARIĆ, S. ŠPIČIĆ, M. OBROVAC, S. MARJANOVIĆ, M. BENIĆ, B. K. KIRIN and I. VICKOVIĆ (2007): *Mycobacterium caprae* in cattle and humans in Croatia. Int. J. Tuberc. Lung. Dis. 11, 652-658.

4. DELAHAY, R. J., C. L. CHEESEMAN and R. S. CLIFTON-HADLEY (2001): Wildlife disease reservoirs: the epidemiology of *Mycobacterium bovis* infection. Tuberculosis. (Edinb.) 81, 43-49.
5. EISENACH, K. D., M. D. CAVE, J. H. BATES and J. T. CRAWFORD (1990): Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J. Infect. Dis. 161, 977-981.
6. FALKINHAM, J. O. 3rd (1996): Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. 9, 177-215.
7. HANCE, A. J., B. GRANDCHAMP, V. LÉVY-FRÉBAULT, D. LECOSSIER, J. RAUZIER, D. BOCART and B. GICQUEL (1989): Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol. Microbiol. 3, 843-849.
8. HUARD, R. C., L. C. LAZZARINI, W. R. BUTLER, D. VAN SOOLINGEN and J. L. HO (2003): PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. J. Clin. Microbiol. 41, 1637-1650.
9. KENT, P. T. and G. P. KUBICA (1985): Public health mycobacteriology: a guide for the level III. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta.
10. MILLER, R. S. and S. J. SWEENEY (2013): *Mycobacterium bovis* (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations. Epidemiol. Infect. 141, 1357-1370.
11. PARTE, A. C. (2018): LPSN – list of prokaryotic names with standing in nomenclature (bacterio.net), 20 years on. Int. J. Syst. Evol. Microbiol. 68, 1825-1829.
12. PRODINGER, W. M., A. BRADSTATTER, L. NAUMANN, M. PACCIARINI, T. KUBICA, M. L. BORSCHIROLI, A. ARANAZ, G. NAGY, Ž. CVETNIĆ, M. OCEPEK, A. SKRYPNIK, W. ERLER, S. NIEMAN, I. PAVLIK and I. MOSER (2005): Characterization of *Mycobacterium caprae* isolates from Europe by mycobacterium interspersed repetitive unit genotyping. J. Clin. Microbiol. 43, 4984-4992.
13. PRODINGER, W. M., A. INDRA, O. K. KOKSALAN, Z. KILICASLAN and E. RICHTER (2014): *Mycobacterium caprae* infection in humans. Expert. Rev. Anti. Infect. Ther. 12, 1501-1513.
14. SINHA, P., A. GUPTA, P. PRAKAS, S. AMUPURBA, R. TRIPATHI and G. N. SRIVASTRA (2016): Differentiation of *Mycobacterium tuberculosis* complex from non-tubercular mycobacteria by nested multiplex PCR targeting IS*6110*, MTP40 and 32 kD alpha antigen encording gene fragments. BMC Infect. Dis. 16, 123-132.
15. ŠPIČIĆ, S. (2008): Molekularna epizootiologija vrsta *Mycobacterium tuberculosis* i *Mycobacterium avium* kompleksa izdvojenih iz ljudi, životinja i okoliša. Disertacija. Veterinarski fakultet Sveučilišta u Zagrebu.
16. ŠPIČIĆ, S., I. RAČIĆ, V. KATALINIĆ-JANKOVIĆ, A. LABROVIĆ, T. KIŠ, M. ZDELAR-TUK, S. DUVNJAK, B. HABRUN, G. KOMPES, A. VUJNOVIĆ i Ž. CVETNIĆ (2011): Tuberkuloza goveda u Hrvatskoj s posebnim osvrtom na postupak certifikacije stada slobodnih od tuberkuloze. Vet. stn. 42, 401-406.

Sumnjivi i pozitivni slučajevi tuberkuloze goveda u Republici Hrvatskoj od 2017. do 2020. godine

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U razdoblju od 2017. do 2020. godine tijekom redovitog provođenja programa certifikacije stada goveda slobodnih od tuberkuloze goveda bakteriološki je u Laboratoriju za bakterijske zoonoze i molekularnu dijagnostiku bakterijskih bolesti Hrvatskog veterinarskog instituta u Zagrebu, Hrvatska bio pretražen materijal 161 goveda iz 27 različitih uzgoja u 11 županija. Materijal je dostavljen nakon nalaza patoanatomskih promjena na liniji klanja koje upućuju na tuberkulozu ili komparativnom metodom nakon pozitivne reakcije goveda na tuberkulin. Vrste iz *M. tuberculosis* kompleksa (*M. bovis* i *M. caprae*) su izdvojene iz materijala 58 goveda (36 %) iz 16 uzgoja u osam županija. *M. caprae* je ustvrđen u 55 goveda (34 %) podrijetlom iz 13 uzgoja u sedam županija, a *M. bovis* u tri goveda (2 %) iz dva uzgoja u dvije županije. Saprofitske mikobakterije izdvojene su iz četiri uzorka goveda (2,5 %) dostavljenih iz tri uzgoja (21 %) u dvije županije i to *M. gordonae* (1), *M. celatum* (1) i dvije neidentificirane vrste (*M. sp.*). Na temelju dobivenih rezultata možemo zaključiti da je glavni uzročnik tuberkuloze goveda u Republici Hrvatskoj *M. caprae*, a koji je bio uzročnik i u prijašnjim istraživanjima. Kontrola tuberkuloze goveda u Republici Hrvatskoj i dalje je potrebna te bi se i u budućnosti trebala suzbijati na temelju tuberkulinizacije, kontrolama na liniji klanja te depopulacijom inficiranih stada i etiološkim dokazom vrste uzročnika.

Ključne riječi: *Mycobacterium bovis*, *M. caprae*, *tuberkuloza, goveda, Republika Hrvatska*