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Original Research Paper

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM AVIUM* SUBSP. *AVIUM* FROM ANIMALS IN CROATIA USING IS901 RFLP AND MIRU-VNTR TYPING

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Summary: *Mycobacterium (M.) avium* subsp. *avium*, the causative agent of avian tuberculosis, primarily affects the birds but may often be isolated from granulomatous lesions in pigs and occasionally from cattle and other animals. In this study, a total of nine *M. avium* subsp. *avium* isolates collected between 2001 and 2006 from poultry (n=4), wild boars (n=2), pigs (n=2) and cattle (n=1) were investigated by IS*901* restriction fragment length polymorphism (RFLP) analysis using two restriction endonucleases (*Pvu*II and *Pst*I) and by mycobacterial interspersed repetitive units – variable-number tandem repeat (MIRU-VNTR) typing. Digestion with the restriction endonuclease *Pvu*II resulted in three RFLP types F, Q and M. Digestion with *Pst*I was successfully accomplished in eight isolates demonstrating four RFLP types A29, A31, A32 and A33, of which the last three have not been described before. Combination of *Pvu*II and *Pst*I restriction patterns revealed four RFLP types F-A29, F-A31, F-A32 and M-A33, respectively. No epizootiological connection was found among the isolates expressing the predominant RFLP type F-A29, which was discovered in pig, wild boar and poultry. MIRU-VNTR typing resulted in four MIRU-VNTR types; among them, two were regarded as new. The most frequent type 34131127 was detected in four isolates from wild boars, pig and poultry. The combination of both typing methods revealed seven distinct RFLP/MIRU-VNTR genotypes; among them, six were unique.
This work represents the first genotyping research of *M. avium* subsp. *avium* strains isolated from different animal species in Croatia. Notwithstanding the small number of investigated isolates, the results indicate a relatively high genetic diversity of *M. avium* subsp. *avium* in animals and suggest a combination of RFLP and MIRU-VNTR typing as a suitable approach to genotyping of *M. avium* subsp. *avium* isolates.

Key words: IS*901* RFLP; MIRU-VNTR typing; avian tuberculosis; pigs; poultry; cattle; wild boars

Introduction

Mycobacterium (*M.) avium*, comprising organisms that range from ubiquitous mycobacteria causing opportunistic infections in a variety of hosts to obligate pathogens of birds and ruminants, is currently divided into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *hominissuis* (1-3).

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M. avium subsp. *avium* is the causative agent of avian tuberculosis; it may infect many animal species but birds are particularly susceptible to infection which often leads to fatal outcome. In farm animals, particularly in pigs and cattle, it causes mycobacteriosis with tuberculous lesions mostly localized in the lymph nodes of the head and intestine (4). *M. avium* subsp. *avium* genome contains mobile elements, e.g. insertion sequences IS*901* and IS*1245*, which are used as markers for identification and typing.

Molecular techniques with a high discriminatory power, e.g. restriction fragment length polymor-

phism (RFLP) and pulsed-field gel electrophoresis (PFGE) are considered as a useful tool for the epidemiological studies of *M. avium* infections. IS*901* RFLP typing is used for the differentiation of *M. avium* subsp. *avium* isolates despite its rather limited polymorphism (5, 6). Lately, other typing methods that target different structures in the genome have been developed with the aim to facilitate and accelerate strain typing. Recent studies identified loci containing variable-number tandem repeats (VNTRs) of specific mycobacterial interspersed repetitive units (MIRUs) in *M. avium* isolates. This PCR-based typing method has been investigated as an alternative and rapid tool for genotyping of *M. avium* isolates in the past few years (7-10).

The aim of this study was to characterize *M. avium* subsp. *avium* isolates from poultry, pigs, cattle and wild boars by using IS*901* RFLP analysis and MIRU-VNTR typing based on some of the recently described markers (9).

Materials and methods

Mycobacterial isolates

A total of nine *M. avium* subsp. *avium* isolates, obtained between 2001 and 2006 from six regions in Croatia, were studied. The selection of isolates was based on their animal origin, namely they were isolated from different animal species with distinct biological and ecological traits. One isolate originated from cattle and two from pigs from two farms located in distinct regions; these animals showed positive reaction to avian tuberculin and were slaughtered. Two wild boar isolates from different regions were obtained from the laboratory strain collection. A total of four poultry isolates originated from animals that died of avian tuberculosis on small farms in two different regions (Table 1).

Identification of the isolates

Isolates were identified as *M. avium* with molecular identification kit GenoType Mycobacterium CM (Hain Lifescience, Germany) and as *M. avium* subsp. *avium* by IS*901* PCR using primers described previously (11). Amplification products were run on 2% agarose gels and stained with ethidium bromide.

RFLP analysis

RFLP typing was performed according to previously published instructions (12, 13) with slight modifications described by Pate et al. (14). RFLP types were analysed with BioNumerics software (v. 4.0, Applied Maths, Belgium), using *M. avium* subsp. *avium* strain R13 as a reference for band normalization and UPGMA (Dice coefficient) algorithm to

Table 1: Animal isolates of *M. avium* subsp. *avium* investigated in this study: origin, IS*901* RFLP types and MIRU-VNTR types

Isolate code	Region	Host	Sample	Year of isolation	*Pvu*II *Pst*I IS*901* RFLP type[a]	MIRU-VNTR type[b]
S44	VP	pig	SLN	2004	F-A29	34131127
S49	KK	pig	SLN	2002	F-A31	34131137
DS126	VV	wild boar	MesLN	2004	F-A29	34131127
DS125	SM	wild boar	MesLN	2003	F-A32	34131127
P127	Z	poultry	L	2001	F-A29	22131127
P128	S	poultry	L, I, S	2004	Q-ns	35131127
P129	Z	poultry	L	2005	F-A29	34131127
P130	Z	poultry	L	2006	F-A29	ns
G83	Z	cattle	MedLN	2004	M-A33	35131127

Legend: SLN – submandibular lymph node, MesLN – mesenteric lymph node, L – liver, I – intestine, S – spleen, MedLN – mediastinal lymph node, ns – typing not successful

[a] RFLP types are designated according to Dvorska et al. (2003) – the nomenclature established and used at Veterinary research Institute, Brno, Czech Republic

[b] MIRU-VNTR types are designated by the number of tandem repeats in the following sequence: TR 292-X3-25-47-3-7-10-32 (Thibault et al., 2007)

generate dendrograms with 1.2% position tolerance. The nomenclature of RFLP types described herein is in concordance with the nomenclature established and employed at the OIE Reference Laboratory for Avian Tuberculosis in Brno, Czech Republic (13).

MIRU-VNTR typing

PCR amplification of the eight loci described by Thibault et al. (9) was applied with slight modifications, as described by Pate et al. (15). PCR products were analysed by agarose gel electrophoresis and detected by ethidium bromide staining. Reference strain *M. avium* subsp. *avium* R13 was used as positive control. MIRU-VNTR types described herein were designated by the number of tandem repeats in the following sequence: TR 292-X3-25-47-3-7-10-32.

Results

RFLP analysis

Digestion with *Pvu*II resulted in three RFLP types F, Q and M (Figure 1) of an average similarity of 93.5% (data not shown). The majority of isolates (7/9) were of RFLP type F, which was found in pigs, wild boars and poultry. One poultry isolate showed RFLP type Q, while a single isolate from cattle demonstrated RFLP type M (Table 1).

In eight isolates, four RFLP types were detected by *Pst*I digestion: A29, A31, A32 and A33 (Figure 1), exhibiting an average similarity of 93% (data not shown); digestion failed in one poultry isolate. RFLP type A29 was observed in five isolates from poultry, pig and wild boar. Unique RFLP types A31, A32 and A33 were detected in pig, wild boar and cattle, respectively (Table 1).

Parallel digestion with both restriction endonucleases resulted in four combined *Pvu*II *Pst*I RFLP types F-A29, F-A31, F-A32 and M-A33. The predominant RFLP type F-A29 was detected in different time periods and regions in five isolates from poultry, pig and wild boar. Unique RFLP types F-A31, F-A32 and M-A33 were found in pig, wild boar and cattle, respectively (Table 1).

MIRU-VNTR typing

Tested isolates demonstrated four MIRU-VNTR types, including one type which could not be fully determined due to repeated absence of locus TR32 amplification product. The types differed either in

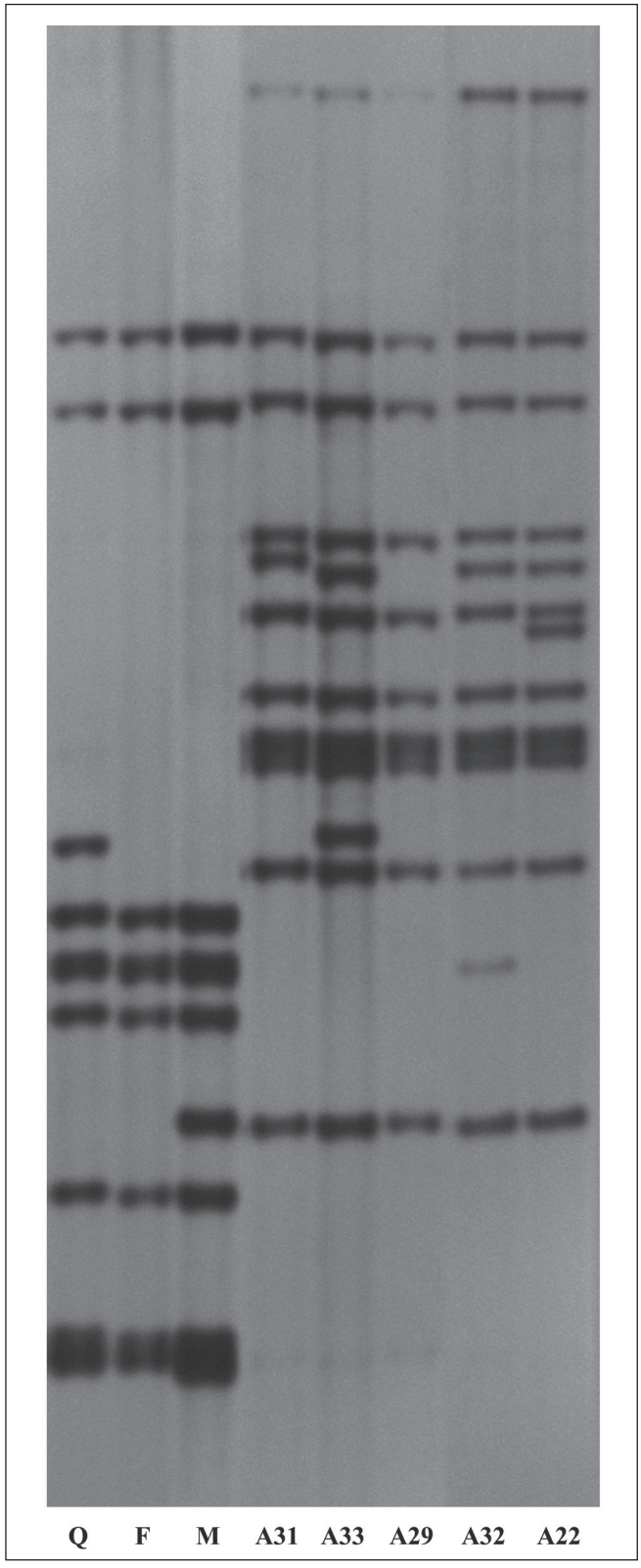
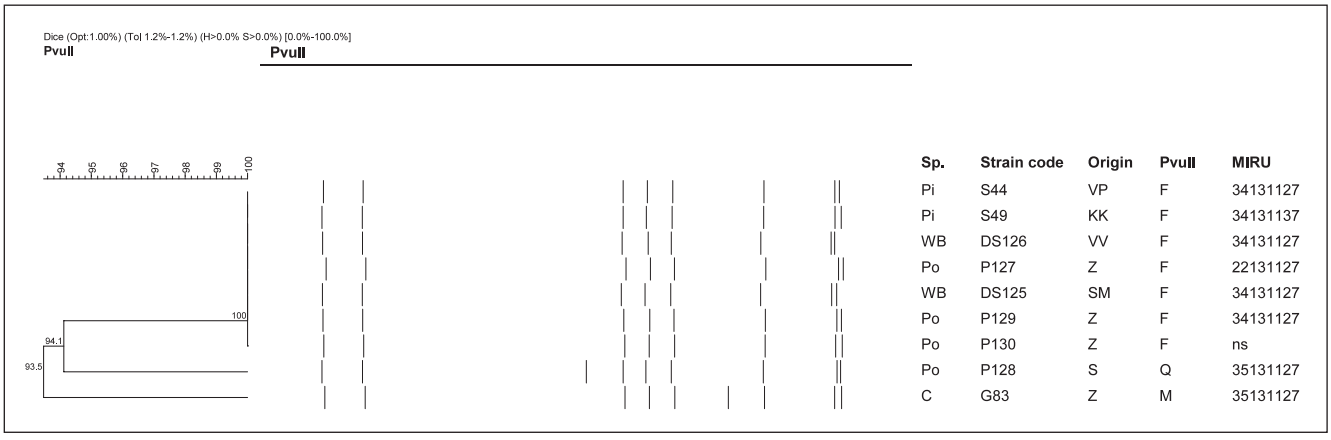


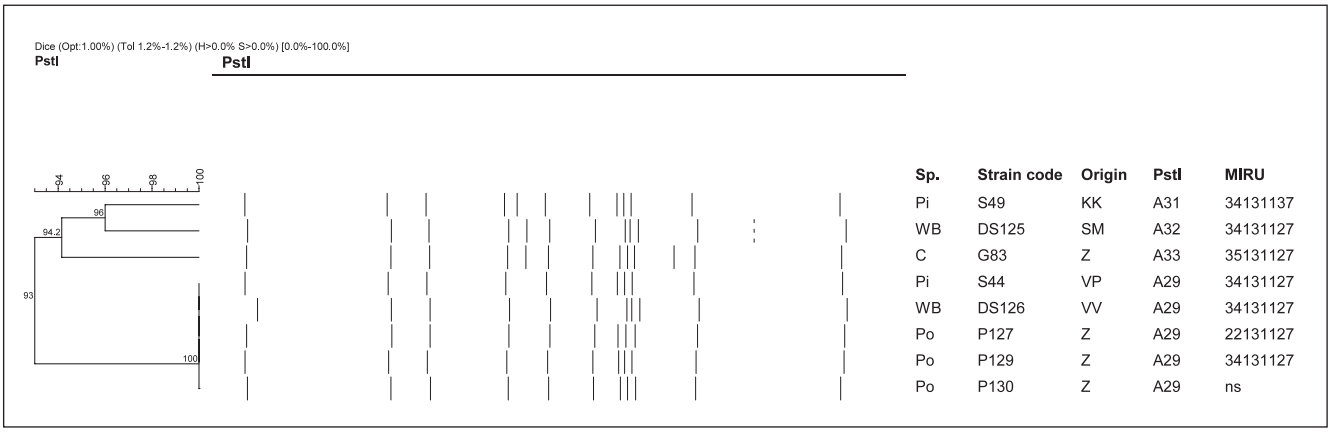
Figure 1: IS*901* RFLP types discovered in nine *Mycobacterium avium* subsp. *avium* isolates in this study: *Pvu*II RFLP types Q to M and *Pst*I RFLP types A31 to A22. RFLP types are designated according to Dvorska et al. (2003). Reference *Mycobacterium avium* subsp. *avium* strain R13 showed the *Pvu*II *Pst*I RFLP type F-A22



Sp.	Strain code	Origin	PvuII	MIRU
Pi	S44	VP	F	34131127
Pi	S49	KK	F	34131137
WB	DS126	VV	F	34131127
Po	P127	Z	F	22131127
WB	DS125	SM	F	34131127
Po	P129	Z	F	34131127
Po	P130	Z	F	ns
Po	P128	S	Q	35131127
C	G83	Z	M	35131127

Figure 2: Comparison of *Pvu*II IS901 RFLP types and MIRU-VNTR types of nine *Mycobacterium avium* subsp. *avium* isolates from animals (UPGMA dendrogram, Dice coefficient, 1.2% position tolerance)

Legend:
Sp. – host species; Pi – pig; WB – wild boar; Po – poultry; VP, KK, VV, Z, SM, S – codes of regions; *Pvu*II – *Pvu*II RFLP type; MIRU – MIRU-VNTR type; ns – typing not successful



Sp.	Strain code	Origin	PstI	MIRU
Pi	S49	KK	A31	34131137
WB	DS125	SM	A32	34131127
C	G83	Z	A33	35131127
Pi	S44	VP	A29	34131127
WB	DS126	VV	A29	34131127
Po	P127	Z	A29	22131127
Po	P129	Z	A29	34131127
Po	P130	Z	A29	ns

Figure 3: Comparison of *Pst*I IS*901* RFLP types and MIRU-VNTR types of nine *Mycobacterium avium* subsp. *avium* isolates from animals (UPGMA dendrogram, Dice coefficient, 1.2% position tolerance)

Legend: See Figure 2

single locus (X3 or TR10) or in two loci (TR292 and X3). The most frequent was type 34131127, detected in four isolates: two from wild boars, one from pig and one from poultry. These isolates were obtained from different regions in different time periods. Three other isolates, originating from the same time period but from different animal species, shared a common type (35131127). The remaining two types were unique. The results are summarized in Table 1.

RFLP and MIRU-VNTR typing

The combination of both typing methods revealed seven distinct RFLP/MIRU-VNTR genotypes, i.e. R1-M1, R2-M2, R3-M1, R1-M3, R4-M4, R1-M4 and R5-M4 (Table 1). Among these, only genotype R1-M1 was found in several isolates, namely one from pig, one form wild boar and one from poultry, originating from different regions. The remaining genotypes were unique.

Discussion

The prevalence of *M. avium* subsp. *avium* in pigs varies and depends on the presence of this subspecies in the environment. Some early studies failed to isolate *M. avium* subsp. *avium* from pigs (16, 17), while other reports described different proportions

of *M. avium* subsp. *avium* in pigs, rising up to more than 50% (18-22). A recent study in Croatia reported 21.1% of *M. avium* subsp. *avium* in pigs (23), which was about 45% less compared to the data from one of the past reports (24).

Development of genotyping methods enabled differentiation within *M. avium* subsp. *avium* isolates. In spite of rather limited polymorphism reported in the first IS*901* RFLP studies (5, 6), the method has been used for typing of *M. avium* subsp. *avium* isolates in several other studies (13, 14, 25-27). Its discriminatory power was improved by using a combination of different restriction endonucleases in parallel to increase the number of discernable RFLP types. The first extensive IS*901* RFLP study (13) revealed 25 *Pvu*II RFLP types and 25 *Pst*I RFLP types which gave a total of 52 combined *Pvu*II *Pst*I RFLP types. Several other RFLP types have been identified (14, 25, 26). In order to compare RFLP types detected in our study with the previously identified, they were submitted to the database of IS*901* RFLP types reported by several central European countries, managed by the OIE Reference Laboratory for Avian Tuberculosis in Brno, Czech Republic.

Using the restriction endonuclease *Pvu*II, three RFLP types were detected. The predominant type F was found in isolates from different animal species from different regions, which was in congruence with the results of Dvorska et al. (13, 25, 27), Moravkova et al. (26) and Pate et al. (14). RFLP type M that was detected in cattle in our study, was found also in a pig in Slovenia (26). RFLP type Q has also been detected previously (13, 14, 26).

Digestion with restriction endonuclease *Pst*I revealed four RFLP types. Among them, types A31, A32 and A33 have not been found in the database and were regarded as new. The most prevalent type A29, found in poultry, wild boar and pig, was detected also in poultry in Slovenia (14). In our study, digestion with *Pst*I failed in one isolate, however similar cases were observed before (14).

Parallel digestion with both restriction endonucleases resulted in four *Pvu*II *Pst*I RFLP types. The predominant type F-A29 was detected in five isolates from poultry, wild boar and pig. This RFLP type was first described by Pate et al. (14) in one isolate from poultry. The remaining unique *Pvu*II *Pst*I RFLP types described herein (F-A31 from pig, F-A32 from wild boar and M-A33 from cattle) were detected for the first time.

In the reports published up to date, MIRU-VNTR typing of *M. avium* was used for differentiation of *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* strains (7-10), but not for differentiation within *M. avium* subsp. *avium* which was aimed for in the present study. Our results show that the method provided less discrimination among *M. avium* subsp. *avium* isolates compared to RFLP. Among nine isolates of different origin, four isolates exhibiting two different RFLP types shared a common MIRU-VNTR type. The same applied for three other isolates of different origin that exerted different RFLP types but shared the MIRU-VNTR type. These results are in congruence with the commonly reported lower discriminatory power of MIRU-VNTR compared to RFLP genotyping of *M. avium* (9, 10, 15, 28). Nevertheless, discrimination of RFLP typing could be improved by MIRU-VNTR analysis, since two of the five isolates from our study exhibiting F-A29 RFLP type demonstrated a different MIRU-VNTR type. However, one of these two types could not be fully determined due to the absence of TR32 amplification product, but was deducted from the calculations of the allelic diversity (*h*) for this locus (*h*=0.00) performed in a study by Pate et al. (15) on 41 *M. avium* subsp. *avium* isolates; this type most probably represented MIRU-VNTR type 35131127 and was regarded as such. In the case of one isolate, both RFLP and MIRU-VNTR typing generated unique profiles. In general, the combination of both methods in our study subdivided the nine isolates into seven RFLP/MIRU-VNTR types, which was more discriminative than applying RFLP or MIRU-VNTR typing alone (obtaining five or four types, respectively). The complementarity of both typing methods was published before when the increased number of discernable types obtained from the combined approach was reported (9, 10, 15, 28).

The loci tested in this study for MIRU-VNTR typing exhibited a relatively low allelic diversity, namely a limited polymorphism was documented only for loci TR292, X3 and TR10. The reason for the observed phenomenon might lie in the selection of the markers, which was done on the basis of complete genome sequences of *M. avium* subsp. *hominissuis* strain 104 and of *M. avium* subsp. *paratuberculosis* strain K10, respectively, since it has been reported(29) that the IS*901*-positive strains contain certain genomic regions that vary between *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*. Nevertheless, the MIRU-VNTR diversity of *M. avium* subsp. *avium* isolates observed in this study is considerably higher compared to diversity established among Slovenian *M. avium* sub-

sp. *avium* isolates (15): herein, four types were detected among nine isolates while in Slovenia, a total of five MIRU-VNTR types were identified among 41 isolates. Types 34131127 and 35131127 were found in both countries with the former being the most prevalent one, which was detected in poultry, pig, wild boar and cattle. The remaining types described in this study seem to be unique among the types discovered in previous publications, although the comparison with the previously described types is hampered due to diverse typing schemes used. However, the main reason for the incongruence among the types most probably lies in the fact that previous studies (9, 28) regarded *M. avium* subsp. *hominissuis* isolates instead of *M. avium* subsp. *avium* isolates. This indicates that *M. avium* subsp. *avium* harbors unique genomic elements not found in other *M. avium* subspecies.

This is the first genotyping study of *M. avium* subsp. *avium* isolates from different animal species in Croatia. Considering the small number of investigated isolates, a relatively high genetic diversity of *M. avium* subsp. *avium* was observed. The combination of RFLP and MIRU-VNTR typing seems to be a suitable approach to genotyping of *M. avium* subsp. *avium* isolates. However, it should be remarked that MIRU-VNTR typing needs improvement in terms of identifying suitable markers for this subspecies. In order to get a better perspective on the genetic diversity of *M. avium* subsp. *avium* strains in Croatia, the research should undoubtedly be expanded by testing a larger collection of strains.

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MOLEKULARNA OPREDELITEV MIKOBAKTERIJ PODVRSTE *MYCOBACTERIUM AVIUM* SUBSP. *AVIUM* PRI ŽIVALIH NA HRVAŠKEM Z METODAMA IS*901* RFLP IN MIRU-VNTR

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Povzetek: Mikobakterije podvrste *Mycobacterium (M.) avium* subsp. *avium*, povzročiteljice aviarne tuberkuloze, prizadenejo predvsem ptice. Pogosto jih lahko izoliramo tudi iz granulomatoznih sprememb pri prašičih, redkeje pa pri govedu in drugih živalih. V okviru te raziskave smo z analizo polimorfizmov dolžin restrikcijskih fragmentov (RFLP) na podlagi cepitve DNK z restrikcijskima endonukleazama *Pvu*II in *Pst*I in s tipizacijo na podlagi razpršenih ponavljajočih se enot v genomu mikobakterij (spremenljivega števila tandemskih ponovitev MIRU-VNTR) opredelili devet sevov podvrste *M. avium* subsp. *avium*, izoliranih v obdobju 2001–2006 pri perutnini (n=4), divjih prašičih (n=2), domačih prašičih (n=2) in govedu (n=1). Po cepitvi DNK z restrikcijsko endonukleazo *Pvu*II smo ugotovili tri tipe RFLP (F, Q in M), medtem ko je bila cepitev s *Pst*I uspešna le pri osmih izolatih, pri katerih smo ugotovili štiri tipe RFLP: A29, A31, A32 in A33. Zadnji trije tipi v literaturi še niso bili opisani. S kombinacijo rezultatov obeh cepitev za posamezni izolat smo določili štiri kombinirane tipe RFLP: F-A29, F-A31, F-A32 in M-A33. Med izolati prevladujočega tipa F-A29, ki smo ga odkrili pri domačem prašiču, divjem prašiču in perutnini, nismo ugotovili nobene epizootiološke povezave.

S tipizacijo smo ugotovili štiri tipe MIRU-VNTR, med njimi dva nova. Najpogostejši tip 34131127 smo odkrili pri štirih izolatih iz divjih prašičev, domačega prašiča in perutnine. Kombinacija obeh tipizacijskih metod je razkrila sedem različnih genotipov RFLP/MIRU-VNTR, šest izmed njih je bilo unikatnih.

To je prva raziskava na področju genotipizacije mikobakterij podvrste *M. avium* subsp. *avium* pri različnih živalskih vrstah na Hrvaškem. Rezultati kljub majhnemu številu v raziskavo zajetih izolatov nakazujejo precejšnjo genetsko pestrost mikobakterij te podvrste, kombinacijo metod RFLP in MIRU-VNTR pa kot uporaben pristop h genotipizaciji izolatov mikobakterij podvrste *M. avium* subsp. *avium*.

Ključne besede: IS*901* RFLP; tipizacija MIRU-VNTR; aviarna tuberkuloza; domači prašiči; perutnina; govedo; divji prašiči