DIVERSITY OF NONTUBERCULOUS MYCOBACTERIA ISOLATED FROM ANIMAL SAMPLES

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Abstract

Limited data is available regarding the epidemiology of the nontuberculous mycobacteria (NTM) in Romania, in both human and veterinary medicine. The objective of the present study was the identification and characterization of the Mycobacterium avium complex species isolated from backyard poultry and ruminant samples. The molecular identification and genotyping was performed in ANSES, Laboratory for Animal Health, Maisons-Alfort, France, by real time PCR, with a wide range of targets: hsp65, IS6110, IS1081, IS1245, IS901 and MIRU-VNTR. M. avium avium and M. avium paratuberculosis were the two species identified. Five different profiles were obtained through genotyping, four of which had corresponding INMV (INRA Nouzilly MIRU-VNTR) profiles: INMV 2, INMV 67, INMV 99 and INMV 100. The technique differentiated between the M. avium paratuberculosis isolated from sheep and cattle and expressed a high discriminatory power, proving to be extremely useful for assessing the genetic diversity of the tested animal origin samples and providing comparable information on the general structure and main pathogens belonging to MAC.

***Key words**: NTM, MIRU-VNTR, Mycobacterium*

Introduction

From important pathogens to saprophytic species, the large group of nontuberculous mycobacteria (NTM) comprises more than 150 members (Biet F., Boschiroli M.L., 2014), of which *Mycobacterium avium* Complex (MAC) species are the most frequently isolated (Fawzy et al., 2018; Alvarez et al., 2011) from a variety of animal hosts and also from human beings. Some species may have the ability of surviving and multiplying in various biotopes, resisting water chlorination treatments or extreme temperatures, acid or low oxygenated environments (Biet et al., 2005 A; Coelho et al., 2013).

Little is known on the prevalence of these bacteria and the risk factors for animal populations in Romania. These species gained an increased attention, particularly in countries with well-implemented bovine tuberculosis control and surveillance programs in Europe, North America or Australia. NTM infections present a broad clinical spectrum, in both animals and human, affecting a large number of species, mammals, birds, reptiles that may have asymptomatic or clinical forms, localized or disseminated. Diagnosis of NTM infections is challenging, conventional microbiological methods are not sufficient and molecular methods are currently used in many laboratories. Due to the presence of common antigens, NTM can hamper the routine intradermal test and may induce false positive responses in case of previous exposure or co-infection (Humblet et al, 2011).

MAC is a group of slow growing mycobacteria, comprising *M. avium* (with four subspecies), *M. intracellulare* and other more recently included species: M. chimaera, M. colombiense, M. arosiense, M. vulneris, M. marseillense, M. bouchedurhonense and M. timonense (Cayrou et al., 2010, van Ingen et al., 2009), *M. lepraemurium* (Benjak et al., 2017).

Most often, MAC infection in domestic ruminants is discovered at slaughter and diagnosed by bacteriological procedures. Members of this complex were isolated from a wide range of wildlife hosts, such as rabbits, wild boars, foxes, badgers, mandrills, birds, different ruminant

species, but also from various environmental sources such as potable and river water or soil and from insects and protozoa, suggesting the existence of both environmental and wildlife reservoirs (Biet et al., 2005 A).

M. avium subsp. *paratuberculosis* is the causative agent of paratuberculosis, disease that may affect both domestic and wild ruminants, determining great financial losses. In countries with intensive dairy industry, at herd level, it is estimated that the prevalence is above 50% (Barkema et al., 2010). *M. avium avium* is the most important mycobacterial species in avian pathology and, along with *M. genavense,* is capable of infecting any bird species, causing avian tuberculosis. Due to the high level of genetic homogeneity, *M. avium* subspecies strains are difficult to discriminate without genotyping techniques like IS*900* RFLP, IS*1245* RFLP or MIRU-VNTR.

The objective of the present study was the identification and characterization of the nontuberculous mycobacteria from ruminants and backyard poultry tissue sample, using real time PCR technique and typing by MIRU-VNTR.

Material and methods

Samples

A total number of 29 samples, from animals originating from the North-Eastern region of Romania, consisting of tissue DNA extracts and lysates were analyzed using real time PCR. Liver, spleen and ovary tissues were harvested from eight chickens that presented granulomatous lesions and various samples of mesenteric lymph nodes and intestine from three cows, two sheep and one deer, suspected of infection with *M. avium* subsp. *paratuberculosis,* based on clinical or lesional aspects. Primary isolation was performed onto Löwenstein Jensen (LJ) solid media with and without mycobactin and on liquid Middlebrook 7H9 media, with Middlebrook ADC Growth Supplement (Sigma-Aldrich) and mycobactin, after decontamination with 0.75% HPC.The lysates were obtained from the enrichment cultures on Middlebrook 7H9 liquid media, after a thermic shock inactivation and centrifugation. The DNA extraction was performed using commercial kits (NucleoSpin® Tissue MACHEREY-NAGEL GmbH & Co. KG, High Pure PCR Template Preparation Kit Roche Molecular Systems, Inc.), according to the specifications provided by the producer and the protocol used in the National Reference Laboratory for bovine tuberculosis, Laboratory for Animal Health, ANSES, Maison-Alfort, France.

Molecular identification

The DNA extracts obtained for each sample were further submitted to real time PCR analysis. Initially, the samples were tested for a general mycobacterial target- *hsp65*, and then PCR on insertion sequences were used to in order to differentiate between members of MAC: IS*1245*, IS*901*, IS*900*, but also IS*6110* to distinguish any *M. tuberculosis* Complex members (Table 1 and 2). The PCR reaction was carried out in total final volume of 25 µl, using 2,5 µl of primers and probe and 12,5 µl of TaqMan® Fast Advanced Master Mix, with an initial holding stage (50^{o}C-2 minutes and 95^{o}C, 20 seconds) and 45 cycles (95^{o}C, 3 seconds and 60^{o}C, 30 seconds). Identification of insertion elements was performed, based on Ct (cycle threshold) values. The primers and the used protocol were performed according to the methods used in the National Reference Laboratory for bovine tuberculosis, Laboratory for Animal Health, ANSES, Maison-Alfort, France.

Table 1.

Target sequences used for NTM identification by real time PCR

	Species	*hsp*65	IS*1245*	IS*901*	IS*900*
MAC	*M. avium avium*	++	++	++	—
	M. avium paratuberculosis	++	—	—	++
	M. avium silvaticum	++	++	++	—
	M. avium hominissuis	++	++	—	—
	M. avium intracellulare	++	—	—	—
	Other NTM species	++	—	—	—

MIRU-VNTR typing method

For differentiation and characterization, the MIRU-VNTR technique was used, according to the protocol described by Thibault et al. (Thibault et al., 2007), with adaptations of the reagents, in accordance with the aspects specified by Radomski (Radomski, 2007).

Of all the samples identified at species level by real time PCR, six extracts were selected for genotyping using the MIRU-VNTR technique, included in the *M. avium* Complex and with a Ct value for IS*1245* and IS*900* targets below 30 cycles. Typing the selected strains was performed using the TR loci: MIRU 292, MIRU X3, VNTR 25, 47, 3, 7, 10 and 32, in this order, to obtain numerical profiles. The nucleotide sequences of the primers are described in Table 2.

Table 2

The nucleotide sequence of the MIRU-VNTR primers
(According to Thibault et al., 2007)

Locus	Forward (5'-3')	Reverse (5'-3')
292	CTTGAGCAGCTCGTAAAGCGT	GCTGTATGAGGAAGTCTATTCATGG
X3	AACGAGAGGAAGAACTAAGCCG	TTACGGAGCAGGAAGGCCAGCGGG
25	GTCAAGGGATCGGCGAGG	TGGACTTGAGCACGGTCAT
47	CGTTGCGATTTCTGCGTAGC	GGTGATGGTCGTGGTCATCC
3	CATATCTGGCATGGCTCCAG	ATCGTGTTGACCCCAAAGAAAT
7	GACAACGAAACCTACCTCGTC	GTGAGCTGGCGGCCTAAC
10	GACGAGCAGCTGTCCGAG	GAGAGCGTGGCCATCGAG
32	CCACAGGGTTTTTGGTGAAG	GGAAATCCAACAGCAAGGAC

An aliquot of 2 µl of each DNA samples described previously was used to carry out the amplification. The reactions were performed in a total final volume of 25 µl, using the BIO RAD MyCycler ™ Thermal Cycler System. For each locus a different master mix was used, with the specific primers and the GoTaq® DNA Polymerase kit, according to the previously described protocol (Radomski, 2007). The amplification program comprises one step at 94ºC for 5 minutes, 40 cycles of 94 ºC–58ºC–72ºC, 30 seconds each and a final elongation at 72 ºC for 12 minutes.

Three strains with INMV (INRA Nouzilly MIRU-VNTR) profiles identified in a previous study (Radomski, 2007) were used as positive controls: *M.avium hominissuis*- INMV 86: 05321128, *M. avium avium* INMV 88: 21131117 and *M. avium silvaticum* INMV 99: 24131117.

Analysis of the obtained amplicons was done by electrophoresis using a 3% agarose gel. For this purpose, two types of agarose were used: MetaPhor® Agarose and SeaKem® GTG® Agarose (Lonza, Switzerland) and TAE buffer 0.5X (Tris-acetate-EDTA). Gel visualization was performed with the ChemiDoc XRS (BioRad) system in ultraviolet light and the Quantity One 1-D software was used to process and record the obtained image.

Discriminatory power

The discriminating power of the MIRU VNTR typing method and the discriminatory capacity of each locus was determined by calculating the Discriminatory Index (DI), described by Hunter and Gaston (Hunter, Gaston, 1988). DI was calculated using an on-line software: http://insilico.ehu.es/mini_tools/discriminatory_power/, according to the following formula:

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

N= total number of strains in the typing scheme; ***s***= total number of distinct patterns discriminated by each locus and by the whole typing method; n_j= the number of strains in the j^{th} pattern.

Results

Real time PCR results

Considering the CT values obtained, species identification was possible for almost all the tested samples. As expected, given the host and the type of tissue, *M. avium* subsp. *avium* was identified in all the chicken samples (Table 3). For some of the ruminant samples, the results were inconclusive (sheep 2, cow 3 – Table 4). *M. avium* subsp. *paratuberculosis* was identified in the samples from cow 5 and sheep 1, a MTBC species in the samples from cow 4 (IS*6110*+, further identified as *M. caprae*) and a MAC member for the deer.

Table 3

Real time PCR result obtained for the chicken samples

Species	Tissue sample	Sample type	Target		
			***Hsp*65**	**IS*1245***	**IS*901***
Chicken 1	Liver	Lysate	22,48	23,24	21,33
Chicken 2	Liver	DNA extract	38,59	37,94	36,28
Chicken 2	Liver	Lysate	34,74	indet	38,73
Chicken 3	Liver	Lysate	39,3	indet	38,58
Chicken 3	Liver	DNA extract	31,51	31,68	28,74
Chicken 4	Liver	Lysate	26,24	26,8	25,2
Chicken 5	Liver, Ovary	Lysate	21,7	24,08	22,09
Chicken 5	Liver	DNA extract	34,03	33,24	31,55
Chicken 5	Lung	DNA extract	27,7	27,26	25,51
Chicken 6	Spleen	DNA extract	29,61	28,67	26,13
Chicken 6	Liver	DNA extract	32,66	32,04	30,23
Chicken 7	Spleen	DNA extract	26,36	28,01	26,76
Chicken 7	Intestine	DNA extract	24,36	25,69	23,55
Chicken 8	Liver	DNA extract	19,57	21,14	19,24

*indet= indeterminate

Table 4

Real time PCR results obtained for the ruminant samples

Species	Tissue sample	Sample type	Target				
			Msp2	**IS*1245***	**IS*901***	**IS*900***	**IS*6110***
Cow 2	Intestine	DNA extract	37,92	37,61	38,9	indet	
Cow 2	Mesenteric lymph node	DNA extract	indet	36,84	36,21	38,44	
Cow 3	Lung	DNA extract	36,49	indet	38,78	indet	
Cow 4	Tracheobronchial lymph node	DNA extract	27,07	indet	39,13	indet	*23,06*
Cow 4	Lung	DNA extract	27,13	indet	38,61	indet	*23,74*
Cow 5	Mesenteric lymph node	DNA extract	41,77	indet	-	40,96	
Cow 5	Jejunum	DNA extract	34,31	indet	-	34,48	
Cow 5	Rectus	DNA extract	33,19	indet	-	36,04	
Cow 5	Ileocecal region	DNA extract	30,33	indet	-	27,21	
Sheep 1	Intestine	DNA extract	32	35,54	35,65	27,38	
Sheep 1	Intestine	Lysate	38,49	indet	indet	indet	
Sheep 2	Mesenteric lymph node	DNA extract	37,39	indet	38,3	indet	
Sheep 2	Intestine	DNA extract	37,53	indet	38,8	indet	
Deer 1	Spleen	DNA extract	37,49	36,76	35,94	indet	
Deer 1	Intestine	Lysate	36,82	38,09	indet	Indet	

*indet= indeterminate

MAC strains that can induce generalized tuberculosis in birds are considered of great importance in the field of veterinary epidemiology. The IS*901* positive response is associated with bird virulent strains of *M. avium* serotype 1, 2, 3 (Pavlik et al., 2000). All the poultry tested samples were positive for IS*901*.

MIRU-VNTR results

For all the samples selected for genotyping, complete eight digits MIRU-VNTR profiles were obtained. With the exception of one sample that expressed a double repetition for locus 32, for the other five samples the INMV corresponding profiles could be identified. From the analysis of the eight loci, the following allelic profiles resulted: 23131127, 24131127, 24121117, specific for *M. avium avium* and 32332228, 5834111(5,8) for *M. avium paratuberculosis*.

The discriminatory power of this method is characterized by a DI index of 0.933, with five different profiles being obtained.

Table 5

Discriminatory power of each locus

TR	LOCUS								
	292	**X3**	**25**	**47**	**3**	**7**	**10**	**32**	
-	0	0	0	0	0	0	0	0	**Samples with the same profile**
0	0	0	0	0	0	0	0	0	
1	0	0	4	0	5	5	2	0	
2	4	1	0	0	1	1	4	0	
3	1	1	2	5	0	0	0	0	
4	0	3	0	1	0	0	0	0	
5	1	0	0	0	0	0	0	0	
6	0	0	0	0	0	0	0	0	
7	0	0	0	0	0	0	0	4	
8	0	1	0	0	0	0	0	1	
(5,8)	0	0	0	0	0	0	0	1	
DI	*0.6*	*0.8*	*0.533*	*0.333*	*0.333*	*0.333*	*0.533*	*0.6*	

The discriminatory capacity of the typing method is clearly focused around the three variable markers: X3, 292 and 32. The X3 locus is characterized by much higher discriminatory power (DI = 0.8), allelic diversity being identified for all the used markers (Table 5). Along with X3, high individual DI values (DI = 0.6) were obtained for 292 and 32 loci.

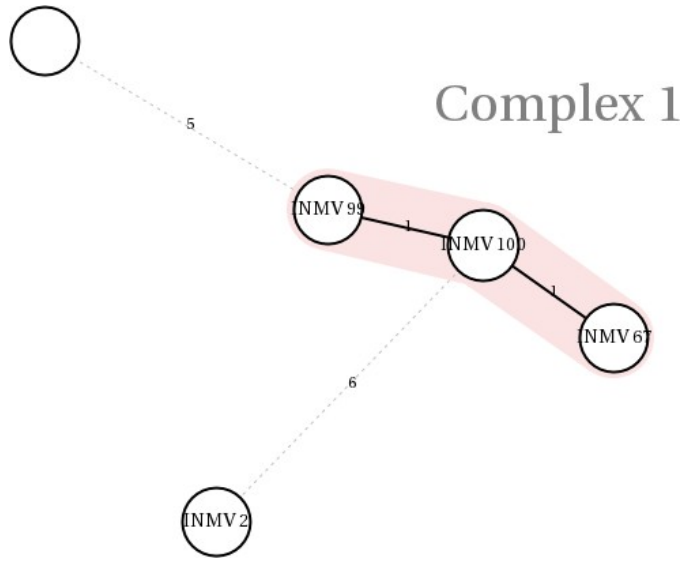

Fig 1. Minimum Spanning Tree based on MIRU—VNTR profiles of the tested samples, using the application in the MIRU—VNTRplus database web

* The sizes of the circles are proportional to the number of isolates with that profile; *the number on the lines joining the different clones is representative for the genetic distance between them; *INMV =INRA Nouzilly MIRU-VNTR

Using the MAC-INMV-PLUS database (http://mac-inmv.tours.inra.fr), the following corresponding INMV profiles were obtained: INMV 67, INMV 100, INMV 99 and INMV 2. The

first three form a clonal complex - *Complex 1*, marked in Figure 1 through a pink halo. The INMV 99 and INMV 100 profiles are differentiated by a single repetition at the locus 10 and between INMV 100 and INMV 67, there is a difference between the numbers of repeats determined for the X3 locus. Compared with the central clone INMV 100, INMV 2 (32332228) is distinguished by different repeats number in 6 of the 8 loci.

Discussion

Real-time PCR technique is a diagnostic tool with high efficiency and sensitivity. However, due to poor DNA quality it may provide false responses (Agdestein et al., 2011). The IS*1245* element is considered a reliable target for *M. avium* identification and it is also commonly used for RFLP (restriction fragment length polymorphism) typing. For two of the chicken lysates tested (Chicken 2 and 3) a IS*1245*– and IS*901*+ response was observed. Contradictory results for *M. avium* samples, IS*1245* negative, were previously reported (Beggs et al., 2000; Higgins et al., 2011). Two Belgian human isolates, identified as *M. avium* subsp. *hominissuis*, were IS*1245*– and IS*901*+ and further analysis showed that the IS*901* positive response was due to the presence of IS*Mav6* (Vluggen et al., 2016). A 96 % identity was discovered between the proteins encoded by IS*Mav6* and IS*901* (Ichikawa et al., 2009).

Depending on the number of samples, the diversity of their origin, the number of tested loci and, of course, the number of different profiles obtained, the DI values mentioned by various authors vary within fairly wide limits. Our overall DI value is in concordance with the values specified in other publications. Radomski N. obtained a 0.885 index in his study, testing a large pool of samples, of both animal and human origin (Radomski et al., 2010). Thibault et al. obtained a value of 0.751 (Thibault et al., 2007) and Scherrer et al., characterizing the diversity of *M. avium hominissuis* strains isolated from bovines, obtains an index of 0.972 (Scherrer et al., 2018). Lower values were mentioned in studies by Stevenson et al., 2009 and Gioffré et al., 2015, 0.64 respectively 0.63.

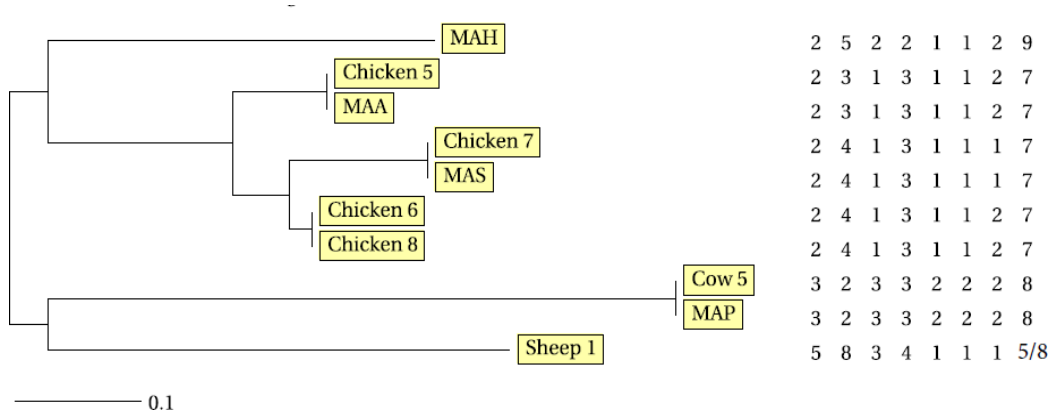

Fig 2. Neighbor joining (NJ) dendrogram based on the obtained MIRU-VNTR profiles for the tested samples and four reference strains, using the tools available in the MIRU-VNTRplus database (original)
*Reference strains: MAP-*M. avium paratuberculosis* K10 (Thibault et al., 2007), MAH-*M. avium hominissuis* ATCC898, MAA- *M. avium avium* ATCC 25291, MAS- *M avium silvaticum* ATCC 49884 (Scherrer ş.a., 2018)

The importance of the X3 locus in MIRU-VNTR typing is previously mentioned by other authors (Radomski, 2007; Imperiale et al., 2017). MIRU-X3 specific repeat units have a length of 53 bp and in the genome of *M. avium* subsp. *paratuberculosis* K10 (access number in GenBank:

NC_002944) are present in the SenX3-RegX3 region in two copies (Biet et al., 2005 B). In the MAC-INMV-PLUS database there are no *M. avium* subsp. *paratuberculosis* profiles with more than three repetitions for this locus, but in the literature are described isolate with 5, 7 or even 15 TR (Möbius et al., 2009; Castellanos et al., 2010; Macovei et al., 2013). The TR variability for loci X3 and 10, described by Macovei for bird samples, in a previous study in Romania, was also observed in the present study (Figure 2).

In Romania, the MIRU - VNTR genotyping technique was used in a previous research to identify the genetic diversity of *M. avium* isolates of animal origin, and the profiles INMV 100 and INMV 99 were identified in poultry samples and INMV1 in those from ruminants. The profiles INMV 1, INMV 2 and INMV 3 are frequently identified at European level (de Kruijf et al., 2017) but also in isolates from other countries such as Argentina or Canada (Gioffré et al., 2015, Ahlstrom et al., 2015). Correlated with the etiologic agent of paratuberculosis, INMV 2 has been described for strains isolated from both domestic and wild ruminants and even DNA extracts from amoeba (Samba-Louaka et al., 2018).

The INMV 100 profile identified in our study in two of the chicken samples was also found described for other species: swine, cattle, goats, wild birds, such as the red ibis (*Eudocimus ruber*) or domestic birds (Radomski, 2007). In the same study, INMV 99 and INMV 67 were identified in samples from various species of birds and mammals.

Conclusions

We noted the usefulness of molecular identification and genotyping method MIRU-VNTR in epidemiological investigations. Strain identification by Real time PCR targeting specific insertion sequence elements is fast and reliable, especially when dealing with sheep *M. avium paratuberculosis* types that are difficult to isolate in culture. MIRU 292, MIRU X3, VNTR 25, 47, 3, 7, 10 and 32 facilitated the characterization of samples and the comparison of our results with those obtained by other authors and those found in international databases, and NJ based analysis facilitates the observation of phylogenetic relations between samples.

Identical MIRU-VNTR profiles do not necessary reflect a close phylogenetic relationship between isolates that may be distantly related, given the fact that the use of a limited number of loci restrains the analysis only to those parts of the genome and differences may exist in other genetic elements (Scherrer et al., 2018). Identical MIRU-VNTR profiles may be exhibited by strains from different geographical regions or that were isolated from different species. In general, the loci that exhibit greater variability are useful markers for differentiation, especially when closely related isolates are genotyped, and the ones with lower variability are more suitable for phylogenetic studies of strains or clones that have evolved over a larger time period.

For a general evaluation of the genetic diversity and distribution of NTM infection in the North-Eastern region of Romania, further evaluation on a larger number of samples is required.

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