SUMMARY

Key words: *non-tuberculous mycobacteria, MNT, M. avium complex, MIRU-VNTR genotyping, spolygotyping.*

The present PhD thesis entitled "*Identification, characterization and taxonomic classification of nontuberculous mycobacterial strains isolated from human and animals*" is structured into two main parts: **Part I** - "Current state of knowledge", which accumulates a number of 36 pages and includes a systematization of the information extracted from the specialized literature on the topic of study and **Part II** - "Personal contributions", comprising a total of 103 pages, in which the results of the researches undertaken during the doctoral studies are presented.

The first part, "*Current state of knowledge*", includes a systematization of the topical issues identified in the specialized medical literature on non-tuberculous mycobacteria, their characterization and pathological implications, epidemiological and ecological aspects and diagnostic methods. This part is structured in two chapters: Chapter 1 - "*Bibliographic aspects of nontuberculous mycobacteria*" and Chapter 2 - "*Main species of nontuberculous mycobacteria with implications in veterinary and human pathology*".

Part II, "Personal Contributions", is structured in 7 chapters (3 - 9), each of them describing the studies carried out on the proposed research theme, the material, the method and the results obtained, and in the last chapter are summarized the general conclusions following analysis.

Chapter 3, "*Research goal, objectives and the organizational framework*" in the opening of the second part of the thesis, includes a brief description of the purpose and objectives pursued and the organizational framework in which the researches were carried out.

Considering the importance given to nontuberculous mycobacteria in the last two decades by veterinarians and human physicians, the scarce research conducted in our country on this subject, the opportunistic pathogenic capacity, the large number of hosts and the isolation frequency of these species from various natural and man-made environmental niches, the primary purpose of this thesis was to assess the diversity of the NTM identified in animals and humans, thus contributing to a better understanding of the epidemiological aspects of the main species in this group, in the northeastern region of Romania.

A number of objectives and activities have been pursued and implemented to achieve the proposed goal: estimation of MAC seroprevalence by testing samples collected from wild boars and foxes, isolation and identification of NTM with implications in

veterinary infectious pathology, assessment of NTM diversity from human patients, phylogenetic characterization and analysis of the identified
species and comparison with the data provided by the medical literature and those recorded in the Mac INMV database.

Chapter 4, called "*Seroepidemiological investigation regarding M. avium infection in wild boars and wild foxes*", aimed at an epidemiological investigation regarding the prevalence of anti-MAC antibodies in wild boar serum samples (n = 275) from 4 counties in the eastern area of our country: Iaşi (n = 209), Botoșani (n = 11), Bacău (n = 17) and Galați (n = 27), collected during the 2017-2018 hunting seasons. A total of 92 samples of fox thoracic fluids were also tested, collected from five counties in the same study region: Iaşi (n = 30), Suceava (n = 20), Neamț (n = 19) , Vaslui (n = 11) and Galați (n = 12), previously tested for the purpose of checking the efficacy of rabies vaccines, in the 2015 season.

The distribution and identification data of the wild boar samples (hunting fund, sex and age of animals) were known and systematized only for the samples from Iași County.

Sample testing was performed using the ID Screen® *Mycobacterium avium* Indirect Multi-species (ID.vet, Innovate Diagnostics, France) kit, according to the protocol specified by the manufacturer. A single sample from foxes, from Galați County, was positive (1.08%). Lack of exposure to MAC mycobacteria is most likely the explanation for the negative results obtained and could be due to their reduced distribution in the environment as well as to a reduced number of wild animals with infections caused by these bacterial agents. The absence of seroconversion in boars and foxes demonstrates the limited role that these animals have in maintaining and spreading CMA species in the eastern region of the country.

The seroprevalence rates identified in wild animal populations, in other European countries, suggest the need to complete this preliminary study by increasing the number of tested samples from the same species and through diversification of the animal species evaluated.

Chapter 5, "*Isolation, identification and characterization of nontuberculous mycobacterial strains from animals*" includes the results obtained by applying bacteriological, histopathological and molecular diagnostic methods on various samples of tissues and pathological materials collected from animals suspected of mycobacterial infection, respectively eight hens, five cows, two sheep, one deer and two dogs. The culture media (Löwenstein-Jensen with and without mycobactin and Middlebrook 7H9) and decontamination techniques used are compliant with the guidelines described by the World Organization for Animal Health (WOAH OIE) - Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Molecular identification was performed by real-time PCR using a series of insertion elements to identify and differentiate MAC members, according to the working protocol used in the ANSES's Animal Health Laboratory, *Bacterial Zoonoses Unit*, Maisons-Alfort, *France*.

Avian tuberculosis specific granulomatous lesions could be observed for all birds in the study, disseminated in the liver, spleen, intestines and ovaries, with with sizes ranging

from a few millimeters to more than one centimeter and a yellowish-gray appearance. The histopathological examination, using modified Ziehl-Neelsen staining, facilitated the observation of Acid-Fast Bacilli (AFB) and granulomatous lesions in various evolutionary stages. On solid media, the appearance of the colonies was noticeable: smooth, small, initially non-pigmented and white-yellowish on ageing. After molecular testing of extracts from cultures and tissues, *M. avium* subsp. *avium* was identified, based on the response to the IS*1245* and IS*901* targets.

Histopathological lesions specific to mycobacterial infections were highlighted in tissue samples collected from a sheep and a cow. The presence of granulomas was observed in the pulmonary sections (in a bovine), the subsequent characterization of the identified mycobacteria being included in Chapter VIII. The histopathological examination performed on the ovine samples was also conclusive, with the presence of BAAR, granulomas (mature - type I and in the early steps of formation) in mesenteric lymph nodes, as well as inflammatory infiltration in the sections of the ileum. The corroboration of the results obtained in the conducted diagnostic procedures led to the identification of *M. avium* subsp. *paratuberculosis* for two of the tested animals (sheep 1: CT IS900 = 27.38, cow 5: CT IS900 = 27.21). The investigations carried out on two hunting dogs were able to highlight the involvement of mycobacteria in cutaneous nodular infections without establishing the diagnosis of canine leproid granuloma.

Chapter 6, "*Isolation and identification of nontuberculous mycobacteria from human samples*", presents the results of the studies conducted on the diversity of NTM isolated from human respiratory samples in the Bacteriology Laboratory of the Clinical Hospital of Pneumology, Iasi, between May 2015 and March 2017. The primary isolation followed the protocol used in the above-mentioned institution, the samples being decontaminated with 4% sodium hydroxide and the culture carried out on Löwenstein-Jensen solid medium and in the BACTEC™ MGIT960™ automated system (Becton, Dickinson & amp; Company). The differentiation from *M. tuberculosis* complex members was based on the phenotypic characteristics and on the response to the rapid immunocromatographic MPT64 antigen detection assay. For species identification, molecular testing was performed using real time PCR and sequencing targeting Hsp65, *rpoB* and 16S rRNA genes and phylogenetic analysis (BLAST NCBI, Seaview, Mega7) of the obtained sequences.

The overall isolation rate identified is 0.25%, with variations in monthly rates from 0 to 1.33%. Most NTMs were isolated from sputum samples (70%). The isolation frequency was higher among patients over 60 years, 30% between 60-70 years, and 57% of isolates were from male patients. The phenotypic characters observed were varied: smooth and rough colonies, non-pigmented or with yellow or yellow-orange pigment.

Molecular and bioinformatic analysis led to the identification of species and subspecies of 82% of isolates, 10% were identified only at CMA complex level and 8% could not be further identified, being classified as *Mycobacterium* spp. MAC and *M. fortuitum* complex members were most commonly identified (29% and 32%). Within the complex, *M. avium* subsp. *hominissuis* was the predominantly isolated species (13%), a

major opportunistic pathogen for humans and swine. Additionally, from the slow growing group *M. lentiflavum* (7%), *M. gordonae* (5%) and *M. terrae* (2%) were isolated.

Among the rapid growers, the following species were identified: *M. peregrinum* (15*%), M. chelonae* (13%), *M. fortuitum* (7%) and less frequent *M. septicum*/*M. porcinum, M. setense, M. insubricum, M. peregrinum/M. porcinum, M. mucogenicum* and *M. mageritense*.

These findings are in line with the data provided by collaborative global studies, but also with reports targeting narrower territories. Thus, in Europe, the most frequently isolated species are *M. avium*, *M. gordonae*, *M. xenopi*, *M. intracellulare* and *M. fortuitum* (van der Werf et al., 2014), with territorial variations, these results being reflected by the present study. Thus, the results obtained in this chapter make important contributions, completing the NTM epidemiological information.

Due to the presence of NTM in various environmental niches, in water distribution systems, isolation from non-sterile specimens may be due to contamination or colonization. The clinical relevance of a nontuberculous mycobacterial isolate varies according to origin, clinical context and pathogenicity. MNT infections are not systematically notified, thus making the epidemiological situation in the world unclear. Furthermore, interpretation of an isolated NTM from human respiratory sample is made considering a wide range of factors, most often based on criteria established by the American Thoracic Society (Griffith et al., 2007).

Chapter 7, "*Investigations on the molecular diversity of Mycobacterium avium mycobacteria strains*", presents the results of the investigations on the molecular diversity of NTM strains identified in animal and human samples and included in the *M. avium* complex. The molecular typing technique used was MIRU-VNTR following the working protocol and panel of eight tandem repeats loci, MIRU 292, MIRU X3, VNTR 25, 47, 3, 7, 10 and 32, described by Thibault et al. (Thibault et al., 2007).

Twenty human isolates and 6 samples from animals were selected for this study, based on the strong response (CT $\leq$ 30) to the hsp65 target obtained at real time PCR. By analyzing the obtained amplicons for each locus, numerical profiles were obtained, which were then compared with data from the on-line "Mac INMV database" - http://mac-inmv.tours.inra.fr (INMV = INRA Nouzilly MIRU-VNTR).

Analysis of the genetic diversity of human isolates led to the identification of three complete profiles: 03331158 (16 isolates), 02331128 (one isolate) and 24131127 (two isolates) and, for a single isolate, a partial profile 033311-8, without amplification specific for the VNTR10 locus. A major profile (80% of isolates) was obtained - INMV 209-03331158, corresponding to *M. avium* subsp. *hominissuis*. Profile 02331128 corresponds to INMV 165 (*M. avium* subsp. *hominissuis*) and 24131127 to INMV 100 (*M. avium* subsp. *avium*). The partially obtained profile is identical in the present number of repeats with the major profile. The discriminatory power of the method, in the context of human samples, is characterized by a DI = 0.3632 (4 profiles).

For the animals samples, five different profiles were identified: 23131127 (INMV 67, one sample), 24131127 (INMV 100, two samples), 24131117 (INMV 99, one sample)

for *M. avium* subsp. *avium* and 32332228 (INMV 2, one sample), 5834111 (5.8) for *M. avium* subsp. p*aratuberculosis*. The discriminatory power within these samples is characterized by a high DI of 0.933, the discriminating ability of the method being concentrated on three loci: X3, 292 and 32. The X3 locus (DI = 0.8) exhibited the greatest variability, compared with the analysis of human samples.

In Romania, the MIRU-VNTR technique was used by Macovei I. in 2014 to characterize the genetic diversity of *M. avium* isolates of animal origin, and INMV 100, INMV 99 and INMV1 profiles were identified. The INMV 100 profile, identified in the present study in two of the chicken samples but also in two human isolates, has been described in the literature for strains isolated from various species: swine, bovine, goats, wild or domestic birds (Radomski, 2007). The major profile identified in human isolates, INMV 209, has been described by Scherrer et al., in a study conducted in Switzerland, for a strain of *M. avium* subsp. *hominissuis* isolated from lymph nodes without pathological changes from a healthy bovine (Schrerrer et al., 2018).

Overall, the method expressed an DI of 0.612, the highest discriminatory power being expressed by the VNTR 10, MIRU X3 and MIRU 292 loci.

From the available data consulted by us to date, it appears that the use of the MIRU-VNTR technique for the characterization of NTM isolates with human origin has been carried out for the first time in our country, conferring an innovative character to the present thesis.

In **Chapter 8**, "*Identification and characterization of the CMTB mycobacteria from animal samples*" comprises the results obtained in the identification and characterization of mycobacteria included in the *M. tuberculosis* complex (CMTB) in samples from two minks and a cow. Also included in this study are the data obtained for two isolates of *M. tuberculosis* of human origin. Identification was performed by real time PCR, using the insertion sequences IS*6110*, IS*1081*, IS*1561* as well as RD4 and LepA as targets. Characterization of tuberculous mycobacteria was performed using microbead-based spoligotyping (Luminex technology).

The research has revealed the presence of *M. caprae* in the investigated animal samples (mink and bovine). After consulting the literature, we appreciate that this is the first identification of *M. caprae* in minks. The particular response observed for the RD4 target (RD4 negative) suggests the presence of a deletion in the mycobacterium genome, further studies are to be performed in order to characterize the extent of the deletion.

The same *M. caprae* profile was obtained, 200003777377600 (BOV_4-CAPRAE, SIT shared-type number 467), characterized by the absence of the 39-43 spacers compared to the profiles obtained from the human samples tested. Characteristic of the *M. caprae* is also the absence of the first spacer and the ones from 3 to 16. For the two isolates of *M. tuberculosis*, two different profiles were obtained, the one corresponding to the sample I 17-034 - 737777777760771 (T1, SIT 205) characterized by the absence of spacers 4 and 33 to 36, and the one corresponding to sample I 17-064 - 777777764020771 (H1, SIT 45) lacks spacers 24, 26-31 and 33-36.

The final chapter, entitled "*Final Conclusions*", encompasses the main conclusions drawn from the overall analysis of the obtained results. This doctoral thesis makes an important contribution to the study of the diversity and pathological implications of NTM in both animal and human pathology and is a starting point for further research, emphasizing the importance of determining the incidence of mycobacteriosis at a national level and their potential zoonotic implications.