SUMMARY

Keywords:*Mycobacterium avium ssp. avium, Mycobacterium avium ssp. paratuberculosis*, IS900, IS901, LSPs, MIRU-VNTR

Doctoral thesis: **Epidemiology and diagnosis in *Mycobacterium avium* infections** was develped within the Doctoral School of "Ion Ionescu de la Brad" Univerisity of Agicultural Sciences and Veterinary Medicine from Iaşi as a part of the project "*Sprijinirea participării doctoranzilor la programele doctorale*" - POS-DRU8/1,5/S/52176 that was financed from the European Social Fund through the Ministry of Labour, Family and Social Protectionrespectively the Managing Authority for the Sectoral Operational Programme Human Reserves(AM POSDRU) under the Sectoral Operational Programme Human Resources Development 2007-2013.

The paper was written during four years of study, 01.10.2009-01.10.2013 and it is structured in accordance with current legal provisions in two main parts: the first part, represented by **The current state of knowledge** which includes 45 pages and represents 33% of the content of the paper and the second part, **Personal contributions** with 80 pages whichrepresents 66%.

The content of the first part of the **Current state of knowledge** is divided into three chapters that briefly reporte the information from national and international literature on the subject of this paper work.

The first chapter entitled **General characteristics of mycobacteria**presents first the **Taxonomy and classificatio of mycobacteria** with diferent classifications. The Bacteriology classifys the mycobacteria in slow growing mycobacteria and rapidly growing mycobacteria, based on pathogenity in pathogenic, saprophytic or opportunistic mycobacteria and after the well known classification of Runon in photochromogens mycobacteria, scotochromogens, nonchromogenic and rapid growers. In the next subchapter are presented general characteristics of mycobacteria with the morphological structure, physiology and biological features that distinguish them from other bacteria.

Taking into account the pathogenic importance of mycobacteria for human and veterinary medicine: *Mycobcaterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium*

paratuberculosis, the subject of the **Chapter II, Main methods of detection and diagnosis of mycobacterial infections**, describes the diagnostic methods used in practice in order to identify diseases caused by causative agents mentioned above. Delayed hypersensitivity testing is used the steps of diagnosis of tuberculosis in humans, bovine tuberculosis and in the identification of infectios caused by *M. paratuberculosis*and*M. avium* in ruminants. As any diagnostic test, the tuberculin test has advantages and disadvantages: it is a standard test method for in vivo cell-mediated immune response with a low cost but it can not be automatized, it is sometimes difficult to read the reaction and does not assess the state of infection. What gives intradermal test uncertainty is cross-reactivity by the appearance of false-positive reactions and in extensive infections, false negative reactions (anergy).

An evaluation of an infected individual can be made with the help of serological tests such as ELISA or immunochromatographic rapid tests with the detection of various humoral markers (IgM, IgG and IgA anti-A60). Serological tests versus culture have a lower sensitivity (1-60%) and specificity (53-99%) but presents advantages as: a result within a few hours (even minutes) are practical for children (eliminates difficult the sputum) and patients suspected with extrapulmonary tuberculosis [WHO, 2008 Steingart et al, 2011].

Strategic surveillance and control of the National Sanitary Veterinary and Food Safety Authority (ANSVSA) by collecting campaigns of sera from animals facilitate the diagnostic by using s tests ELISA in the laboratories for the detection of paratuberculosis. Serological test used in the diagnosis of bovine tuberculosis is based on the detection of gamma-interferon in combination with allergic test.

Isolation and identification of the etiologic agent in mycobacterial infections is an important step in establishing a certaint diagnosis . In section "Bacterial culture" it describe the techniques and special culture media for isolation of mycobacteria . Lately,it has been automatised this step of diagnosis using liquid culture media and detection systems for bacterial growth as MB / BacT, BACTEC-MGIT 960, ESP Culture System II and MB Redox [Bemer et al., 2004]. Regardless of the culture medium used the bacteriological examination is the "golden standard techique" for the diagnosis of mycobacterial infections [Aziz et al., 2007, Mueller et al., 2008].

The progress that have been made in molecular biology allows facilitation of the diagnosis in mycobacterial infections by reducing the time worked, the waiting time required in clasical cutural examination. The response in a molecular diagnostic test is achieved within a few days. Nowadays, there are commercial tests which permit the identification of specific molecular targets specific for the species / subspecies of searched mycobacteria. The understanding of the

spread and the persistence of certain mycobacterial strains at local or global level is conducted using typing methods as RFLP or MIRU-VNTR[Smith et al., 2006, Sahraoui et al., 2009, Collins, 2011].

Chapter III, entitled "Mycobacterium avium group" presents the general characteristics the group and describes four members of the group: M. avium subsp avium, M. avium subsp paratuberculosis, M. avium and M. avium subsp silvaticum ssp hominissuis with the similarities and differences between them and their pathological implications.Among the best known diseases we mention avian tuberculosis, ruminant paratuberculosis and Crohn's disease in humans.The fact that intrigues the most is the zoonotic implication of each of these mycobacteria with the determination of specific granulomatous lesions in particular affecting immunodeficient persons, inter-species barrier being easily overcome. If in the animals is found a disease caused by a mycobacteria there is not justify a treatement, in humans the identification of species/subspecies of mycobacteria is essential in order to establish a diagnosis and an appropriate treatment based on antibiotic sensitivity.

The second part of this thesis, **Personal contributions,** is composed of five chapters. The Chapter **Aim and objectives of the research** motivates the research topic of this thesis through the importance of the study and the use of modern diagnostic methods in infections due to *Mycobacterium avium ssp. avium* and *Mycobacterium avium ssp. paratuberculosis*.

In the chapter V entitled **Epidemiological investigation of infection with *Mycobacterium avium ssp. paratuberculosis* in Romania**, in the section **Material and methods** are presented the types of epidemiological studies,harvesting and storage of the samples forserology evidence and principle of the indirect ELISA method. In this study we used two commercial ELISA diagnostic tests "*Pourquier ® paratuberculosis antibody ELISA verification. Institute Pourquier*" and "*LSIVet ™ Advanced Ruminant paratuberculosis-Serum ELISA kit* ".In the **Results and discussion** section is firstly a statement of *Mycobacterium paratuberculosis* infection in Romania based on surveys carried out in the country and based on the official website of the International Organisation for Animal Health OIE. Further Study was focused then on the seroepiedemiologic situation of paratuberculoase infection in counties of Iaşi and Neamt between 2005-2010.**Seroprevalence survey of paratuberculosis in ruminants in eastern Romania** consisted of a study conducted over two years, from 2009 to 2011 on 318 animals, cattle, sheep and goats in order to identify individuals serologically positive for infection with *Mycobacterium paratuberculosis*.Analyzing the data from the serological examination it is observed a prevalence of 6,25% for paratuberculosis infection in cattle, 19,54%

in sheep and 28,2% in goats. Regarding the age of the animals with positive serological response to infection with MAP, sheep and goats are in the same range, respective 3-4 years.

In Chapter VI entitled **Etiological investigations in the infections with *Mycobacterium avium ss. avium* and *Mycobacterium avium ssp. paratuberculosis*** we aimed to identify the causative agent by conventional laboratory methods: delayed hypersensitivity testing, bacterioscopy, histopathological and bacteriological exams.The biological material consisted of 38 cases: 12 birds, 21 sheep and four cattle. Some poultry and small ruminants(four sheep and three goats) were tested for the allergic reaction eith avian tuberculin, reading of response at 48 hours after inoculation.

Histopathological examination was performed in the histopathology laboratory of the Faculty of Veterinary Medicine Iaşi. The sampling aimed processing the tissue samples for hematoxylin-eosin staining (HE), hematoxylin-eosin-methylene blue staining (Masson's trichrome stain) and to view the etiologic agent the Ziehl - Neelsen (ZN) stain.In case of poultry the histopathology was performed on nine samples of tissue: liver, spleen and intestine from four subjects and the lesions described in subsection histopathological findings were characteristic to infection with *Mycobacterium avium ssp avium*: tuberculous granulomas in various stages in the liver, spleen and intestine, the presence of Langhans giant cells and specific tubercle bacilli showed by Ziehl-Neelsen (ZN) staining.Regarding the ruminants it has not been observed characteristic lesions of infection with MAP but the histopathology examination revealed cellular changes in samples from a cow and a ram: macrophage infiltration and the presence of Langhans giant cells in intestinal sections ileo-cecal valve and rectal wall. The staining Z-N showed AFB rods in sections taken from mesenteric lymph node and the rectal wall and from a ram.

At the **Bacteriological examination** thedecontamination of tissue and fecal samples was done with hexadecilpiridinium (HPC) 0.75% as described in the work protocol proposed by OIE and culture media used were specific for mycobacteria: Löwenstein-Jensen and Herrold with egg and mycobactin J.The bacteriological examination in hens did not encounter difficulties, after about 7 days of incubation on Löwenstein-Jensen and HEYM media with mycobactin J were observed the first specific colonies of *M. av. ssp avium*. At this step the confirmation was performed by making a Zhiel-Neelsen smear directly from culture.

The tissue samples from ruminants were also processed for sowing the culture media HEYM with mycobactin J. After incubation of 2 months at 37^0C under an atmosphere with CO_2 on HEYM with mycobactin J was observed colonies similar to those of*Mycobacterium av. ssp. paratuberculosis*from bovines isolates.The confirmation of the development of colonies of *M. paratuberculosis* was based on mycobactin-dependece, the slow growing character and on acid-

fastness.For the sheeps isolates we sowed on the HEYM media with mycobactin J and there were not observed any specific colonies of *M. av. ssp. paratuberculsis* even after 8 months of incubation at 37^0C in a CO_2 atmosphere.

Further, in the Chapter VII **Molecular identification and characterisation of *Mycobacterium avium* strains isolated from animals** we aimed the use of molecular biology methods to confirm the diagnosis of paratuberculosis and avian tuberculosis, previously established by the usual techniques: ELISA, allergic test, bacterioscopic exam, culture and histopathologic. The great advantage of molecular techniques is therapidity of obtaining a result and the posiblity to characterise the identified species / subspecies, the discover of new patterns of a geographical distribution, the description of different profiles (MIRU-VNTR) and establish the degree of pathogenicity by highlighting certain gene sequences (LSPs). The extraction method of mycobacterial DNA wasthe one specified in the commercial kit (Macherey Nalgel) with silicon membrane and for the cultures isolated we used the lisys by thermal shock.

Mycobacterium avium ssp avium isolates from birds have been identified by the amplification of specific sequences IS 901S and IS 1245. The biological material used was represented by samples of tissue (liver) and culture. From 12 birds studied, eight of them had specific lesions and S 901 and IS 1245 PCR confirmed the infection with *M.avium ssp.avium.*

Isolates of *Mycobacterium avium ssp. paratuberculosis* from ruminants, sheep, cows and goats were identified by performing a PCR reaction to determine the sequence IS900. The biological material used was represented by tissue samples (portions of intestine, mesenteric lymph node) and faeces. The culture isolates from cattle were confirmed by a IS 900 PCR.

The next step was to perform for the *Mycobacterium paratuberculosis* and *Mycobacterium avium* isolates two molecular typing methods for determination of the LSPs(Large Sequence Polymophisms) and determination of MIRU-VNTR (Mycobacterial Interspersed Repetitive Units and Variable Number of Tandem Repeat).

Recent studies argue that the large sequence polymorphisms (LSPs) have a major role in genetic diversity, among the MAC organisms are important sources of genetic variability. Microarray comparative tests conducted by Semret et al. (2004) using *M. avium ssp. avium* reference strain 104 identified regions that he called LSP^As specific to *M. av.ssp. avium* isolates but absent in *M. av. ssp. paratuberculosis* isolates. In this study we used the primers described by Semret et al. (2006) for the detection of LSP^A4and LSP^A20. The genetic material analyzed here consists of DNA extracts from samples of three sheeps and four cattle initially identified as IS900 positives. The use of LSP typing allowed the differentiation of MAP strains type "S" byMAP strains type "C".

The LSP4 plays an important role in the mycobacteria metabolism, particularly in the synthesis of mycobactin. Lack of the sequence LSP 20 determines the slowly growth of *M. paratuberculosis* cultures.

MIRU-VNTR typing method was performed using a PCR reaction that amplifies multiple tandem repeats loci that allowed to obtain a numeric code in the following order: MIRU 292, X3, VNTR 25, 47, 3, 7, 10 and 32. A number assigned to each strain profile was followed by INMV (INRA Nouzilly Miru VNTR). This notation allows the exchange of results between laboratories and avoids transcription errors in the number of repetitions. In order to perform this typing method we used DNA extracts from samples of eightbirds, DNA extracts from samples of4 cows and 3 sheeps serologically positives for paratuberculosis infection (intestine and mesenteric lymph node). The results for the avian isolates of the 8 amplified MIRU-VNTR markers described three different profiles: INMV 106 (25,131,127) INMV 99 (24,131,117) and INMV 100 (24,131,127), their variability is marked by repetition of gene markers MIRU X3 and VNTR 10. From seven isolated from ruminants, at four of them the tandem repeats of these primers were not carried out amplification of specific segments. After analysing the samples from ruminants we obtained three completely profiles MIRU-VNTR: 2 cattle profiles IMNV 1 (242 332 228) and a sheep profile 52131118 , the last one being different from the profiles described by now. MIRU-VNTR variability in isolates of *M. avium. ssp. paratuberculosis* was marked by repetition of genes VNTR 22 and MIRU X3.

In the end of this study we noted 12 final conclusions, from which we point out the following:

- The fact that, in our research, the delay hypersenzitivity test showed a value of 71% for the serological positive animals, necropsic exam of the serological positive ruminants did not revealed charcateristic lezions, the histopathological exam revealed few tissue lesions, just in the case of two serological positive animals and bacteriological exam provided conclusive data just in bovines, reconfirm the orientiative value of serological and delay hypersenzitivity tests, methods that must be used, mandatory together or in combinations with other methods, bringing up in discussion the dificulty of establishing a certainty diagnostic in paratuberculosis of ruminants.
- The diagnostic in avian tuberculosis did not encounter difficulties, collating the results from necropsy, bacterioscopic examination and histopathology with highlighting the acid-fast bacilli, these are methods of certainty in establishing a *M. avium ssp. avium* infection in birds. All cases of hens with specific lesions of tuberculosis were subsequently confirmed by culture examination and PCR.

- By using molecular biology techniques (PCR), with amplification of specific subspecies sequences of the group *M. avium* (IS 900, IS 901 and IS 1245), it was confirmed the infection with *M. avium ssp. avium* in birds samples and the infection with *M. avium ssp. paratuberculosis* in ruminants samples. These techniques can be considered as reference.
- The molecular techniques for identification and characterisation (LSP, MIRU-VNTR, etc.) are rapid and useful diagnostic tools, especially for understanding and explaining fundamental issues still unclear of bacterial infections within the group *Mycobacterium avium*.