ABSTRACT

***Keywords*:** IS901, IS1245, IS6110, INH, *Mycobacterium avium subsp. avium*, *Myocobacterium tuberclosis*, NTM, PAW

PhD thesis entitled ***"Research regarding phenotypic and genotypic changes induced by physical and chemical agents on mycobacteria isolated from humans and animals"*** is spread over a number of pages 265 and according to the regulations in force, consists of two parts and highlights: first part, entitled "The current state of knowledge" 69 the pages comprising the second part, entitled "Personal contributions" extended 115 pages.

Investigations on the topic, were held in seven institutions: Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad", Iaşi, Pneumology Hospital, Laboratory of Bacteriology of Iasi, Institute of Macromolecular Chemistry "Petru Poni" Iasi, Faculty of Medicine Pierre et Marie Curie, Paris, CHU Pitié Salpêtrière, Paris, functional testing center in the Faculty of Medicine Pierre et Marie Curie, Paris, Pasteur Institute, Paris, during four years of study in the period 1 October 2010 - September 1, 2014.

"**The current state of knowledge**" includes four chapters that are exposed briefly reviewed the literature regarding the subject thesis and which were subsequently used to interpret and compare the data obtained in the researches.

The first chapter, entitled "**General characteristics and structural features of mycobacteria**" is divided into five chapters and synthesizes information from the literature on the classification of mycobacteria after the main characteristics of mycobacteria as: acid-alcohol-resistant, mycolic acids, high content of guanine cytosine in DNA and based on their pathogenicity. At present, plenty species were added to *Mycobacterium* genus, all characterized by acid resitency, considering that there are 178 species, of which some are pathogenical for animals other for humans. More than 130 species have been identified as nontuberculosis species

(NTM), and about 60 species are suspected or known to be pathogenic. Mycobacteria has 5 representative groups: *Mycobacterium tuberculosis complex, Mycobacterium avium* complex, *Mycobacterium fortuitum* complex, *Mycobacterium chelonae-abscessus* complex and *Mycobacterium terrae* complex.

In this chapter are presented and studied information on mycobacterium cell on ultrastructural level and thus cell wall construction that brought solid clarifications related to compounds from this level. Following glycolipides: cord factor (trehalosis 6,6'-dimycolate; TDM) lypoarabinomanan (LAM); mycolic acids, major components and essential for intracellular survival of *Mycobacterium* members. It was discussed also about the permeability of the cell wall which is 40-60% of the dry weight of bacteria with porines, the proteins which cross the membrane and forms hydrophilic channels that permit the passive passage of small hydrophilic molecules. There are also given information on the main features of the mycobacterium genome, the antigenic structure of the existence of a "mosaic" of antigens some common to genus other specific to species and other pathogenic factors from mycobacterium.

The second chapter entitled "**Diagnosis of mycobacterium infections describing the main Phenotypic and genotypic methods**" to identify the mycobacterium. The bacterioscopic exam which uses the acid-alcohol resistance of mycobacterium has been described here, also known as a presumptive exam in tuberculosis; the bacteriological exam with it's most important medium used and the most frequent decontamination methods used. Information regarding the chemosensibility of *M. tuberculosis* strains to anti tuberculosis substances were synthesized. Only standard methods are included (the proportions method, (Canneti, France) and absolute concentration method, (Meissner, Germany); description of main molecular identification technique, genotyping *Mycobacterium* strains by RFLP (Restriction Fragment Lenght Polymorfrism), MIRU-VNTR (Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat)16S rRNA sequencing and phylogenic (Kirschner şi colab., 1993); classification using LSPs technique (Large Sequence Polymorphisms Classify).

Chapter III, entitled "**Mycobacterium resistance to physical and chemical agents**" describes the main physical agents with mycobacterium killing scope. The antybiotics against mycobacteria were clasified in antitubeculostatics grupe 1 (isoniazid, rifampicin, pyrazinamide and ethambutol), 2nd groupe (streptomycin, kanamycin, amikacin and capreomycin), fluoroquinolones, thioamides (ethionamide protionamida), cycloserine, paraaminosalicilic acid; and the specification of ongoing antibiotics development. This chapter summarizes information about the natural resistance of mycobacteria characterized by resistance to a number of antibiotics and a rapid selection of mutations of resistance to antibiotics that are naturally

susceptible; acquired resistance is always linked mutants of chromosomal genes and is not transferable from one strain to another. In a spontaneous mutant bacterial population can be referred to as "normal" or "wild".

In the last chapter of the first part, "**Experimental Chemotherapy in tuberculosis**" are given the experimental models used in this study. Chemotherapy has been used in experimental murine model of current research is most commonly used. Experimental Chemotherapy of Tuberculosis allowed evaluation of in *vivo* antimicrobial activity of drugs by comparison with other existing drugs; Synergistic or antagonistic activity of an antibiotic when administered with other antibiotics and the ability to sterilize lesions in experimentally infected animals.

There are also described the main available mice lines (Balb/c, C57Bl/6, C3H, KO, SCID, and C3HeB/EFJ), where the most frequently used in model mice tuberculosis are the non-inbred types, a Swiss named heterogeneity immune offering the opportunity to demonstrate differences between tested groups. To test the immunodeficiency there are recommended the athymic mice (Nude), which are kept permanently in an insulator, completely in sterile conditions.

In order to assess the effectiveness of chemotherapy on experimental murine model there was used isoniazid or hydrasid, an analog of nicotinamide, same as ethionamide and pyrazinamide which is part of Group 1 tuberculostatics (first line oral AB) according to data classification WHO. Isoniazid is a prodrug, which is activated by the catalase-peroxidase enzyme KatG (Zhang, 1992). The bactericidal activity of INH was described ; their mode of action, although it was discovered more than 50 years, is still controversial; the resistance mechanism of isoniazid and its particular complexes involving multiple genes. Approximately 80% of isoniazid-resistant strains the point mutation in the *katG* gene (causing a high level of resistance), or partial or complete deletion of the gene. Spoted mutations are the most common, particularly those affecting the serine in position Ser315Thr. According to studies carried out so far there are 70% of strains with this location (Musser et al., 1996; Ramaswamy et al., 2003; Cardoso et al., 2004; Guo et al., 2006).

Mutations in the gene promoter *inhA* (causing low resistance), and more rarely in gene *inhA* itself have been observed in 10-40% of isoniazid-resistant strains (Musser et al., 1996; Lavender et al. , 2005; Hazbón et al., 2006). Recent studies have listed other gene involved in isoniazid resistance without demonstrating resistance mechanism (*ndh*, *ahpC*, *kasA*, *mabA*, *furA*) (Ramaswamy et al., 2003). *katG* gene encoded by catalase peroxidase has been identified as being responsible for the activation of isoniasid (Zhang et al., 1992) and the *inhA* gene encoding

enoyl-ACP reductase NADH-dependent, being identified as another "target" for INH (Banerjee, 1994).

A bibliographic summary was performed on the second innovative experimental model using cold plasma as decontamination technique which is an ongoing experiment . There have been described the main types of lightning which generally with moist air, produce a burst of high-energy electrons. Knowing that moist air contains a variety of molecular species (N_2, O_2 and H_2O), NO • OH •, radicals leading non-thermal phase (Ming Du et al., 2012), get formed by the decomposition of these elements in the electric field (Benstali et al., 2002).

The second part of the thesis, entitled "**Personal contributions**", is structured in six chapters, each one of them following a well established structure and ending with conclusions.

The first chapter, **"Isolation and identification of mycobacterial strains, in humans and animals"**, consists in two subchapters. The first one, reveals investigations concerning isolation and identification of mycobacterial strains, that are pathogenic to humans. Even if the species of the *Mycobacterium tuberculosis* complex (MTC) are responsible for most mycobacterial infections worldwide, the study was oriented focused on opportunistic infections due to non-tuberculous mycobacteria (NTM), since their incidence is increasing in recent years (Kankya și colab., 2011; van Ingen și colab., 2009), representing a challenge to public health. Though in Europe were conducted studies on the presence and distribution of non-tuberculosis strains, in Romania there is no information. In this context we intend to conduct a study that involved reporting the non-tuberculous strains frequency in Pneumology Hospital, Iasi, for a period of five years, from January 2010 to September 2014. Identification of the non-tuberculous strains in humans was performed/ conducted phenotipically, by taking microscopic examinations (optical, fluorescence), cultural examinations on Lowenstein-Jensen growth medium and Middlebrook 7H9 in automated system MB/BacT and antigenically, using an immunochromatographic rapid test consisting in MPT64 protein identification. For decontamination was used the sodium hydroxide method without centrifugation (drop method, recommended by WHO).

After antigenic testing, were identified 122 non-tuberculous strains. Of these, 101 NTM (82.78%) were isolated from lung samples (sputum, bronchial aspirate, bronchoalveolar lavage (BAL), and gastric lavage) and 21 NTM (12.21%) from extrapulmonary sample (pus, biopsy, lymph nodes). The share of extrapulmonary and pulmonary cases was 82.79% and 17.21%. NTM strains isolation rate was 13.93% in 2010, 20.49% in 2011, 21.31% in 2012, 25.40% and 18.85% 2013 2014. The data obtained Pneumology Hospital, Iasi, indicates a clear increase in the frequency of NTM isolates in the last 4 years with 11.47%.

The second section aimed the **isolation and the antigenic, phenotypic and genotypic identification of the pathogen mycobacterium isolate from mink and hens**. For this reason it was done a necropsy, a histopathology exam, bacteriological and bacterioscopic examination.

The isolation of the mycobacterium strains from minks and chikens was done by inoculating de pathological product on both liquide and solid medium: Löwenstein-Jensen, Löwenstein-Jensen with mycobactin, Herrold with egg (HEYM) and mycobactin and Middlebrook 7H9. In order to isolate the strains from minks and pigs there were used the MB/BacT flasks introduced in the automatic system MB/BacT.

Identification for mycobacterium species was performed by PCR technique, by magnifying the sequences IS6110 in mink and IS901 and IS1245 in chikens; and the molecular characterization of species in mink was performed by sequencing (Sanger method) and comparing the sequences of interest present in the GenBank nucleotide sequences.

After assessing the macroscopic and histopathological changes, multiple granulomas at different stages of development in almost all organs were spotted in minks. The predominance of affected organs varied from animal to animal, but the lungs, spleen and mesenteric lymph nodes were frequently affected; disseminated lesions could be observed in almost all organs of the thoracic and abdominal.

Histopathological lesions were characteristic fot the tuberculosis infection with tuberculosis granulomas in different steps of evolution, from the the young granuloma with a macrophage reaction characterized by the Langhans type giant cells presence, and the formation of necrosis zones and granuloma oxifile composed by the Langhans giant cells type and collagen fibers.

After evaluating the results of semiquantitative microscopic examination, numerous alcoloo-acid-resistant (AFB) bacilli were observed, unable to quantify them because of the bacillary density . The AAR bacili were present in almost all systems and organs : lungs, spleen, liver, adrenal gland, ovary, the uterus, the thoracic cavity wall, mesenteric , mediastinal and epigastric lymh nodes.

On the cultural examination, all the minks were positive. After the PCR, there was found positive for *Mycobacterium tuberculosis* complex, the sample was successfully sequenced and identified as *Mycobacterium tuberculosis.*

The birds also showed gross lesions specific for avian tuberculosis. The image was dominated by specific tuberculosis nodules, varying in size, well defined and big, some with caseous and necrotic aspect, most often observed in the liver and spleen.

On the culture and bacterioscopic exams chickens were positive, first colonies could be seen after 4-7 days. After one week from sowing it was able to observe small colonies *M. avium subsp characteristic. avium*. Using IS901 and IS1245 it could be confirmed the infection with *Mycobacterium avium subsp. avium*, to all chickens suspected of tuberculosis, the bimolecular examination method seemed much faster and more accurate than conventional culture.

The chapter entitled **"Evaluation of the effectiveness of isoniasid in tuberculosis therapy experimentally induced immunodeficient and immunocompetent mice "** considered the evaluation activity on murine model isoniasid.

Depending on the action, was mentioned the tuberculostatics used to specify nature of it's action: bacteriostatic or bactericidal, if able to maintain the death of mice used in this study, especially if able to prevent the death of mice.

Genetic lines of mice used in the study were Swiss female (immunocompetent) and athymic, Nude (immunodeficient) for four weeks.

The experimental test model was the so-called "clean" so that treatment of mice was initiated 14 days after their inoculation. The purpose of this study was to compare the isoniasid resistance mutations in vivo between immunocompetent and immunodeficient mice

The type of species used in this study was *Mycobacterium tuberculosis* $H_{37}Rv$ which is a reference strain known for it's sensitivity, well characterized and with the complete sequenced genome. The method of apportionment of mice was randomization in blocks, one of the most used random assignment procedures of mice to experimental conditions used in research.

In this experimental study in vivo efficacy of isoniazid was evaluated at different time periods of treatment by measuring the following criteria: the rate of survival of the mice, mice weight, spleen weight, gross lung lesions, bacterial loads lung, the percentage of mice in which the culture were positive and selection of mutants resistant to isoniazide.

Analyzing the difference between the first month and the second month of treatment, mycobacterial load decrease for Swiss mice was equivalent to approximately 2 $\log_{10}$ CFU and for Nude mice increased by 4 $\log_{10}$ CFU following the selection of resistant mutants. For Swiss mice between the second and third month, there was also a decrease of 1.3 $\log_{10}$ CFU.

Initial bactericidal activity of isoniasid is approximately 0.5-0.7 $\log_{10}$ per day and proved to be the strongest tuberculosis killer. However, this activity falls after the second day of treatment (0.113 $\log_{10}$ per day), the rapid decline is due to the selection of resistant mutants.

The Chapter VII, entitled **"Selection of isoniazid-resistant mutants *in vivo*"** consisted in selecting and determining the type of mutations associated with resistance profile

mycobacterial strains tested against isoniazid murine model immunocompetent and immunodeficient mice.

By *in vivo* testing, the goal was to select and determine the mutations proportion , based on the following assumptions: development of resistance in a mycobacterial population resulting from the combined action of two phenomena: spontaneous mutations in the chromosome, followed by selection of mutants comming after an inappropriate chemotherapy.

Therefore the DNA coding for *katG* gene, *inhA* and its promoter has been sequenced to verify the presence or absence of mutations and their description to those two genetic lines of mice used for isoniazid. DNA extraction was performed, PCR for *total katG* gene amplification,*inhA* and *inhA* promoter and for all subjects from which mutants were selected.

In order to have a quality genetic material, purification of the amplified product was made in view of sequencing (Sanger enzymatic method), after which there was again developed a purification of the sequenced products. Thus, all the DNA fragments obtained in the study were subject for sequencing. Data were processed using "ABI Prism DNA Sequencing Analysis V 3.4.1".

The nucleotide sequences obtained were analyzed using the software V.2.5 SeqScape®. The consequences of the new position was studied and mutations detected in *katG in vivo* in both mice Swiss and Nude through crystallographic structure of the protein of *Mycobacterium tuberculosis* KatG (Zhao et al., 2006;. PDB 2CCA) with soft Pymol acquis.

To Nude mice were found 16 point mutations, and not even onnce S315T, when there are fewer macrophages to destroy mycobacterial cells, so *Mycobacterium tuberculosis* on Nude mice has less need of KatG protein with good activity of catalase -peroxidase, which is why we have identified several mutations in *katG*, which could have reduced catalase peroxidase activity. On Swiss mice, there were two types of mutation (W341R and V469V) that can preserve good activity of catalase-peroxidase which is necessary to defend against oxygen radicals of macrophages.

R498L mutation (n=4) was frequently detected in Nude mice and presented a phenotypically silent polymorphism.

Its location in KatG very far from the catalytic site containing the hem. Away from the active site mutations are typically associated with a low level of resistance to INH.

The R484C (n=3) mutation selected in Nude mice was located adjacent to water pocket from KatG protein structure. Mutations near the water grand canal are generally associated with high levels of resistance to INH.

Identified mutations could disturb KatG dimerization, reducing its overall stability or preventing interactions with other in vivo proteins.

Chapter VIII, entitled "***In vitro* selection of mutants resistant to isoniazid**" INH-R *in vitro* mutants have been selected in order to study in isogenic background, the effect of the mutations to mycobacterial strains .

Two reference strains or *Mycobacterium tuberculosis* $H_{37}Rv$ and *Mycobacterium tuberculosis* $H_{37}Ra$ were used for this study. *In vitro* selection of mutations in *Mycobacterium tuberculosis* of the two reference strains was performed on culture medium containing 7:11 Middlebrook INH.

A PCR reaction was performed to amplify the *katG* gene (total KatG), INHA and its promoter strains that have been observed to be mutant *in vitro*. For *katG* gene were amplified and sequenced 3 pairs of primers (*kata and katCas, kat2 and kat4as, katC and katEas*).

InhA and *inhA* gene promoter region was amplified and sequenced with primers *Pro1* and *Pro2*, *FabG1* and *FabG2*, *INH1* and *INH2*, and *INH3* and *INH4*. The sequences obtained were compared with sequences in GenBank under accession number X68081 for *katG* gene and accession numbers U66801 and U02492 for operon *Maba-Inha*.

The nucleotide sequences obtained were analyzed and recorded using SeqScape® V.2.5 software and changes were highlighted by comparing imported reference sequences from Genbank .

All isolates had mutations *in vitro* only in *katG*, changes which are often associated with high-level resistance to isoniazid. The studied strains were identified of having 63 mutations INH-R.. Mutations detected in *katG*, consisted of deletions (partial or total), point mutations, insertions and mutations in codon STOP. The average proportion of the mutations in the *katG* was the deletion of 28.12% (12 partial and complete 6), the STOP codon mutations were 28.87%, 18.75% are point mutations and insertions have been 3.12%, which suggests that the mutations in *katG* are more likely to be highly resistant.

For 28.12% of INH-R mutations in vitro, no mutation was found in *katG*. For all the mutants selected *in vitro*, no mutation has been identified in *inhA* and its promoter, suggesting that the normal mechanisms of resistance, *in vivo*, is not reflected by *in viro* mechanism. The fact that no mutation was not observed in *inhA* and its promoter, shows the complexity of the mechanisms of resistance to INH in *M. tuberculosis.*

In order to carry out molecular modeling of the following point mutations in the *katG* were included: G494D (n=1), and T275I (n=1), T394M (n=1), P232L (n=2), R484H (n=3) and D137Y (n=1). Among these mutations, only the presence of G494D has been described so far in

clinical isolates (Brossier, 2011), the rest being new mutations in *katG*. After molecular modeling P232L and T275I indicated a reduction in enzyme activities in agreement with molecular modeling. D137Y managed together with Ser 315, enzymatic activity (peroxidase) attenuation of *KatG*. For the remaining mutations, enzyme activities were significantly reduced and their role is difficult to explain.

In Chapter IX, entitled **"Effect of mycobactericidal water activated with cold plasma"**, it was investigated the effect of water activated with cold plasma (PAW), to some pathogenic species of the *Mycobacterium* genus.

The experimental setup used in this study consisted of a cold plasma type electrochemical reactor operating at atmospheric pressure and ambient temperature.

Unlike electrochemical reactors used in other studies in this paper we are using a new algorithm experimentally proposed and patented by Hnatiuc's team and collaborators, which is based on a multielectrod system, using a elelctrochimic reactor GlidArc type, also with auxiliary electrodes and unloading flat and rotary displacement. For PAW treatment there were used 8 reference strains of *M. avium subsp. avium* (CECT 7407), *M. abscessus* (CECT 8517), *M. chelonae* (CECT 8519), *M. kansasii* (CECT 3030), *M. peregrinum* (CECT 3023), *M. marinum* (CECT 7091), *M. smegmatis* (CECT 3017) and *M. tuberculosis* $K_{37}Ra$, Lehmann and Neumann (ATCC 25177) and 10 non-tuberculosis strains nine of which were isolated from humans (NTM) and one from chicken (*Mycobacterium avium subsp. avium*).

Given that drug-resistant tuberculosis in TB control raises serious obstacles in the world and in Romania especially that chemoresistant strains are as contagious as the sensitive, were studied five susceptible strains (WT), 10 multidrug-resistant strains (MDR) and 10 super-strong strains (XDR)

There was used a bacterial suspension with initial concentration of 108 CFU. For each series of experiments, treatment times were 5 minutes, 10 minutes, 15 minutes, 20 minutes and 30 minutes. The efficiency of inactivation of treated mycobacteria with PAW, was estimated by calculating the logarithmic reduction index (Ir) and statistical analysis of variance was performed using the software GraphPad Prism®, using ANOVA statistical test.

It was found also, a log reduction to mycobacterial after PAW contact, for treatment periods up to 30 minutes. After 15 minutes of treatment with PAW, the logarithmic reduction was upper than 5 $\log_{10}$, so that all the strains under study were inactivated certified as anti-mycobacterial effects. So, at an exposure time of 20 minutes, the degree of inactivation was complete. Bactericidal activity against mycobacteria appears to be dependent on high levels of reactive oxygen species involving impaired cell wall ultrastructure.

The last chapter titled **"Instrument analysis of mycobacteria treated with cold plasma"** investigated the way of PAW action, because so far it is not known the exact mechanism of actionf or the cold plasma or PAW (NAIT et al., 2010). In this regard, by scanning electron microscopy was used to observe the morphological changes of mycobacteria directly after treatment with PAW, UV spectrophotometry and x-ray photoelectron spectroscopy type species used in this study was *M. smegmatis* 3017 (CECT), as reference strain well characterized and with the complete sequenced genome.

The spectrophotometric quantification of the concentration of protein and DNA after the treatment was analyzed the PAW to next treatment time: 5 minutes, 10 minutes and 15 minutes. Thus, we obtained an average protein concentration of 1.01 mg/mL after 5 minutes, 3.65 mg/mL after 15 minutes and 5.24 mg/mL after 30 minutes of treatment with PAW. The average concentration of nucleic acids was 1 mg/mL after 5 minutes, 1.5 mg/ml after 15 minutes and 1.7 mg / mL 30 minutes after PAW treatment .

After treatment with PAW, the content of oxygen (O) increased from 16.91% to 25.94%, while the relative content of carbon increased (C) but not significantly from 49.80% to 57.55%. Thus, the ratio of O/C has increased by 9.03% and 7.75%, as a result of significant oxidation of DNA from *Mycobacterium smegmatis*, after treatment with the PAW. Instead, nitrogen concentration (N) decreased by 16.49% from 32.76% to 16.27%, as was the case of phosphorus (P) by 0.3%, from 0.53% to 0.23% after treatment with PAW.

Were accurately investigated the changes in the *Mycobacterium smegmatis* specific for mycolic acids (α-mycolic, α'-Micol and epoximicolaţi), essential to the survival of mycobacteria, and arabinogalactan (AG) mycolic acids attached. Thus, after treatment with PAW that the content of oxygen (O) in the top layer of the cell wall increased from 27.43% to 37.84%, while the relative content of carbon (C) decreased, but not significantly from the 66.31% to 58.52%.

The results indicated that the chemical structures of DNA and lipids in the structure of the main cell wall of *Mycobacterium smegmatis* has been modified as a result of oxidation of the PAW. Mycolic acid arabinogalactan-peptidoglycan complex, known for the structural rigidity that gives to the mycobacterial cell wall, had also included major changes.