ABSTRACT

Mycoplasmas are the smallest free-living forms capable of self-replication discovered so far. Their size ranges from 0.2 μm to 0.8 micrometers may pass through filters used to remove bacteria. Their genome varies from 580 Kb (*Mycoplasma genitalium*) to 1358 Kb (*Mycoplasma penetrans*), leading to drastic savings of genetic resources forcing to a parasitic lifestyle. Lack of metabolic pathways, given to the small size of the genome, require medium environment for their isolation . A distinguishing feature between mycoplasmas and other bacteria is the lack of a cell wall.

Pathogenic avian *Mycoplasma* species are important causes in the losses within the intensive production of poultry (Chin et al., 2003; Kleven, 2003b; Ley, 2003). Chickens and / or turkeys are natural hosts for 12 species of Mycoplasma, meanwhile 23 species are recognized in birds (Bradbury, 1998; Kleven, 2003a). However only the species like *M. gallisepticum*, *M. synoviae*, *M. meleagridis* (only infect turkeys) and *M. Iowa* are considered to be significant pathogenic for poultry (Bradbury, 1998; Kleven, 2003).

The most important entities are: chronic respiratory disease produced by *Mycoplasma gallisepticum*, infectious sinusitis of turkeys, caused by *Mycoplasma gallisepticum*, turkey airsaculitis (caused by *Mycoplasma meleagridis*) and infectious synovitis (caused by *Mycoplasma sinoviae*).

In Romania, chronic respiratory disease caused by *Mycoplasma gallisepticum*, was first described by Stoienescu and Săndulescu in 1958 after the import of chicken from Canada.

PhD thesis entitled "**Epidemiology and diagnosis of infection with Mycoplasma spp. in birds**" covers a number of 216 pages and according to the rules in force consists of two main parts: the first part, entitled "**The current state of knowledge**" which includes 52 pages and the second part, entitled "**Personal contributions**" wide on 108 pages.

"**The current state of knowledge**" includes three chapters were are exposed briefly information from the reviewed literature regarding the PhD's subject of thesis and after were subsequently used to interpret and compare the data obtained in the "Personal Contributions".

The first chapter, entitled "**Biology and pathogenicity of Mycoplasmas**" is divided into seven chapters and summarizes the literature on phylogenetic classification, ecology, morphology and ultrastructure, metabolic factors virulence and interaction with the host immune system and the mode of transmission of mycoplasmas. Currently there are over 23 recognized

species of mycoplasmas in birds (Bradbury, 1998; Kleven, 2003) and their number is increasing. Mycoplasmas are not associated with the production of toxins, but after their metabolism is resulting hydrogen peroxide and superoxide radical. These products cause oxidative damage to the host cell membrane (Razin, 1991). The main virulence factors are represented by adhesins and neuraminidase. In Romania there are no studies up to date in terms of *Mycoplasma* species present in birds.

The second chapter, entitled "**Description of the main infections caused by *Mycoplasma spp*. in poultry**", deals with the implications of mycoplasmas in avian pathology. Infection with *M. gallisepticum* is particularly important in chickens and turkeys causing respiratory conditions and a reduction of production of meat and eggs (Bradbury, 2001; Ley, 2003). They can also cause upper respiratory tract disease in games birds. Asymptomatic manifestation is one of the main features of mycoplasmosis and especially that typical infection caused by *Mycoplasma synoviae* that induces a significant deterioration of the host's health and can cause the suppression of the immune system (Stipkovists L, Kempft I., 1994; Kleven, 2003). So far, there are no reports in the database of the Office of International Epizooties on avian mycoplasmosis in Romania.

In the last chapter of the first part, "**Laboratory diagnosis of avian mycoplasmosis**" describes in four sub chapters the main methods of detection and diagnosis of mycoplasma infections in poultry flocks. Serological detection is most commonly used. In poultry farms is used rapid slide agglutination, which is a fast and cheap method and gives information about the immune status of the flock. ELISA test is more expensive but has a high sensitivity and specificity. Immunofluorescence and immunoperoxidase tests are generally applied to suspect cultures obtained in laboratory and rarely directly or infected tissue exudates. This is because the organisms are too small to recognize conclusively under the light of the microscope and because the corresponding negative and positive samples exudate / tissue for control is unlikely to be readily available. Mycoplasmas isolation is difficult and is requiring more time being done also on special media supplemented with blood serum, yeast extract, glucose, peptone, vitamins etc .. These environments are known as the broth and agar PPLO. So far there has not been described a universal medium capable of delivering cultivation of all known species. Morphology of mycoplasma colonies should not, in any case, considered to be specific, it is dependent on many factors, including environmental composition, moisture content of the agar, inoculum concentration. An alternative to conventional cultivation are methods of detection of specific DNA. Rapid identification of these bodies is of great importance in the intensive system of

rearing chickens. MALDI-TOF mass spectrometry is a new technique fast and accurate but for not yet achieved a significant database for comparing results.

Since 2011, in Romania by adopting Decision 2011/214 / EU in the Strategic Program by the National Sanitary Veterinary and Food Safety Authority imposes through the Veterinary and State Veterinary Laboratories a surveillance of poultry (chickens and turkeys) from commercial breeding farms of reproduction for avian mycoplasmosis.

The second part of the thesis, entitled "**Personal contributions**" is divided into five chapters, each following the established structure in the last chapter presenting the final conclusions. While in Europe have done many studies on the presence and distribution of Mycoplasmas in different poultry systems, in Romania there is very little information. The research theme aims at achieving an epidemiological screening of infections with bacteria of the genus *Mycoplasma* on birds and also the isolation, identification and characterization of various species of this kind in Romania.

In **Chapter IV**, entitled "E**pidemiological investigations of infections caused by *Mycoplasma spp*. in poultry in north-eastern Romania** " was intended to carry out a survey on the prevalence of sero-epidemiological infection with *Mycoplasma gallisepticum*, *M. synoviae* and *M. meleagridis* in chickens and turkeys reared intensively and extensively in the work area.

Samples were provided from farms with intensive system of turkeys and broiler chicken, chicken breeding farms and holdings extensively (households), located in the county of Iasi, Bacau, Suceava, Botosani, Vaslui and Braşov. The investigations were conducted on a number of 90 serum samples from turkeys for meat reared in a intensively system, 384 samples from breeding chickens, 90 samples from laying hens, 120 samples from broilers reared in a intensive system and 50 samples from hens kept in a extensive system. The sample harvesting was conducted in 2011-2012, from the birds that generally are not presenting characteristic clinical signs of *Mycoplasma* infections, but also from birds with specific respiratory clinical signs.

Serum samples were analyzed using the quick slide agglutination test using coloring antigen *Nobilis® Mycoplasma gallisepticum antigen* and the *Nobilis® Mycoplasma synoviae antigen* (Intervet), and ELISA imunoassay kits using *Mycoplasma gallisepticum antibody test kit, Mycoplasma synoviae antibody test kit* and *Mycoplasma meleagridis antibody test kit* products (Affini Tech, LTD, USA). Serological response against *M. Iowa* is weak, nonspecific reactions are describing for this specie. Does not exists for this species a reliable serological test in field (Bradbury and Kleven, 2003). These tests detect antibodies from the serum of infected birds (immunoglobulin G).

From the ELISA exam test in 90 samples analyzed from turkeys for meat, non immunized, 40 samples were positive for *M. gallisepticum*, 7 for *M. synoviae* and 5 for *M. meleagridis*, which indicates the presence of mixed infection in the herd . From within 90 serum samples from non immunized hens reared in a extensively system, 71 (79%) were positive for MG and 53 (59%) were positive for MS.

In the case of samples from broilers the infection was detected in one hall of the six studied. Thus, two (11%) samples were positive for MG and 3 (16%) samples were positive for MS. Mycoplasma infections present in broiler flocks is closely related to compliance of breeding: populating with healty poultry, disinfection and general biosecurity compliance.

For samples from breeding hens immunized with attenuated vaccine and / or inactivated vaccine, the results were in close correlation with the vaccine used. Therefore the results were negative for sera of hens vaccinated with attenuated vaccine (vaccines given a cellular immunity, without the production of antibodies), while samples from hens immunized with inactivated vaccine were positive (the vaccine offers humoral immunity and it's given with the production of serum antibodies).

In the housing economic system from within all analyzed samples, all of the 50 samples were positive for *M. gallisepticum* and 46 (92%) samples were positive for *M. synoviae*. Antibody titer obtained was very high, located between 8570 and 12338. The high antibody titer is explained by repeated natural infections suffered by poultry. Rapid serum agglutination test provided similar results with ELISA test but with lower fidelity, cross-reactions can be the main cause of false positive. This test is recommended for the establishment of an immune status of an effective, with the advantage that it is cheap, fast and may performe in the farm.

The results show the presence of mycoplasma infections in all flocks examined, with a strong increase in holdings of laying hens or more age groups from within a household system.

In **Chapter V**, entitled "**Diagnosis of infections caused by Mycoplasma spp. in poultry in North-Eastern Romania**", was aimed the isolation, cultivation and identification of Mycoplasmas by cultural tests and biomolecular techniques.

A total of 135 samples of organs taken from birds that died less than 12 hours before the date of collection or sick birds, in agony, that expressed specific clinical signs specific mycoplasmosis from 54 birds reared intensively and extensively system, were placed in culture on special media. The media used were liquid medium FM4 (Frey) for mycoplasmas which ferment glucose and arginine medium for Mycoplasmas that are hydrolyze arginine. In parallel were harvested random blood samples from live birds from within the herd from which they came directly, from cadavers or dying birds that have been studied. For serological testing was

used quantitative ELISA kits *Mycoplasma gallisepticum Antibody Test Kit*, and *Mycoplasma synoviae Antibody Test Kit* (BioChek Poultry immunoassays).

By performing ELISA examination, in broiler farms were revealed for both species of *Mycoplasma* antibody prevalence between 4% and 86% for MG and 5% - 46% for MS. In the economic household system all samples (100%) were positive for both MG and MS.

For samples from birds kept in intensive system, cultures were obtained using only liquid medium FM4. Contamination was found frequently in dilution 10^{-1} on Arginine medium and was observed on almost 100% of cases. In the final, there were obtained 41 strains.

For samples sampled from birds bred in a extensively system, cultures were obtained on both liquid FM4 medium also on the Arginine medium. In comparison, the degree of contamination was much lower than in samples from the intensive system. Some strains produced a slight turbidity of the medium and the formation of a deposit that at stirring the suspension form small flakes like a "snow globe" (particularly in the case of cultures derived from pheasants). At the end were obtained 141 strains.

The main problem found was unable to obtain a culture immediately after cloning or whether this was possible, they could not get cultures at new passage on solid or liquid medium. Also, with the cloning of different morphological types of colonies obtained in the primary culture on the solid medium, were achieved uniform cultures with morphological aspect in sub-cultures different than the clone type.

For the identification by PCR there were utilised specific primers for the *M. gallisepticum, M. synoviae, M. Iowa* and *Mycoplasma spp*. (VK). Were tested in all 4 types of PCR, organ samples (forming initial suspension), primary cultures and strains obtained. DNA extract was obtained by the method described by Kellogg and Kwok. In the intensive system have been identified 4 MS positive initial suspension and were obtained 16 strains of MG and 10 strains of MS. In the extensive system have been identified 12 positive initial suspensions for MS and 7 for MG. From cloning were obtained 10 strains of MG and 11 from MS. A total of 120 strains were obtained after cloning and they were positive for PCR - VK. Our research shows that the intensive system of poultry evolves only *M. gallisepticum* and *M. synoviae* with very rare exceptions and in the household system, evolves more than two species. Was not identified any positive culture for *Mycoplasma iowae.*

Cloning is a very important step in the isolation and identification mycoplasmas, especially in mixed infections when we meet several species of *Mycoplasm*a in the same culture. It is necessary to clone a large number of colonies.

In **Chapter VI**, entitled "**Biomolecular characterization of Mycoplasma spp strains isolated from poultry in North-Eastern Romania**" it was used sequencing in order to identify and characterize strains unidentified by conventional PCR assays. Were selected in 42 strains that came from 12 birds (11 hens aged between 40 days and one year of different sex and a pheasant) of 9 different farms in the county of Iasi and Suceava. The strains were isolated from different organs (infraorbital sinus, conjunctival sac, larynx, trachea, lungs, air sacs, testis and vent). Species identification was performed by sequencing of 16S ribosomal RNA region using automated sequencer *ABI PRISM 3130 Genetic Analyzer* (Applied Biosystems). For sequencing of the purified PCR products were used *Big Dye™ Terminators* sequencing kit (Applied Biosystems). Ribosomal RNA has the same important function in the cell, regardless of species, which means that the corresponding genes are under the approximate same evolutionary pressure. These properties are well suited for the analysis of rRNA phylogenetic studies (Olsen, 1993) and evolutionary (Woese, 1987).

After sequencing phase chromatograms obtained were analyzed using the software Chromasic and GENEIOUS v. 8.0.3. Sense and antisense strands were aligned in order to obtain the chain consensus. Based on the obtained consensus chain it was managed to identify all strains studied, with a identity of 97% -100% with sequences in GenBank. Therefore 7 *Mycoplasma* species were identified, of which six have been identified, to our knowledge, for the first time in Romania: *Acholeplasma modicum, M. struthionis, M. gallinarum, M. gallinaceum, M. pullorum* and *M.iners*. Also *Acholeplasma modicum* and *Mycoplasma struthionis* species are isolated and identified from our knowledge for the first time in chicken..

By phylogenetic analysis, *M. gallinaceum* strains isolated from pheasant, differ as a separate group within the species, fact which demonstrates the introduction of a new strain different from the native by importing pheasants. Identified strains have different positions on the evolutionary tree in the fact group, which demonstrates their diversity within the species.

In **Chapter VII**, entitled "**Characterization of antibioresistance by determining the minimum inhibitory concentration (MIC) of some strains of Mycoplasma spp**." are determined MIC values of antibiotics currently used in order to treat mycoplasmosis. Mycoplasmas infection control through vaccination, is limited because there are few available vaccines and control of these infections through chemotherapy is sometimes necessary alongside biosecurity measures to reduce economic losses and the contamination on vertically and horizontally.

For testing, 10 strains were selected of Mycoplasma from different species: *M. gallisepticum*, *M. gallinarum*, *M. gallinaceum*, *M. iners* and *M. pullorum* and six antibiotics:

enrofloxacin, oxytetracycline, tylozin, tilmicosin, florfenicol, and spectinomycin. CMI was performed on liquid medium in microplates. MIC is defined as weakest concentration of antibiotic for which no color change was observedfor the medium, but with the condition that the positive control without antibiotic to change color. For this assay, cultures were titrated and standardized to a concentration of 10^{-4} CCU.

Following testing, two strains of *M. gallisepticum* isolated from a lung, and cloaca from the same bird presented different MIC values, fact which demonstrates the presence of two different strains within the same species. *M. gallinarum* strains studied, that are provided from differen farms and different geographic locations, showed different sensitivity to the used antibiotics, which demonstrates the diversity of strains within the same species. The existence of infection of two strains or with two or more different species of *Mycoplasma*, in the same individual, which have different sensitivity to various antibiotics, may explain the failure of treatment in infected flocks.

Mycoplasma gallinarum strains studied, showed the highest values of CMI. Lowest sensitivity was observed with oxytetracycline, followed by florfenicol and tilmicosin, at the opposite pole being enrofloxacin, tylozina and spectinomycin.

Coming at the end of this study, are shown a number of 24 final conclusions.