SUMMARY OF THE DOCTORAL THESIS

A RETROSPECTIVE AND PROSPECTIVE STUDY ON H5 AND H7 AVIAN INFLUENZA VIRUSES IN POULTRY AND WILD FROM THE AREA DOBRUDJA

Scientific guide:

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Key words: HPAI and LPAI viruses, avian flu hotbeds, RT-PRC (Reverse Transcription Polymerase Chain Reaction).

Summary

Currently, the avian influenza is a major problem for the avian pathology, with a major risk for human health with economic and social implications worldwide. A permanent improvement of control projects on avian influenza which targets wild birds—considered as the main source of avian influenza virus—will contribute to the understanding of the factors involved in the maintenance and transmission of the avian influenza virus from the natural reservoirs to the domestic poultry and consequently to the human beings, and, also, to the elaboration of the strategies for the prevention of eventual pandemics and to the development of some strategies to prevent the spread of the virus not only in poultry but also in human beings. The avian influenza is a huge danger for the entire world, including Romania, because of its geographical particularities, respectively for the Danube Delta and the phenomenon of the bird migration, birds which are considered the natural reservoir of the avian influenza virus.

Thesis Structure

The doctorate thesis with the title “A retrospective and prospective study on H5 and H7 avian influenza viruses in poultry and wild birds in Dobrudja territory” is extended on 339 pages and it contains 2 parts, in accordance with the current legislation: the first part, “The knowledge stage”, contains a number of 75 pages and the second part, “Personal research” is extended on 233 pages.

Bibliography

The bibliography contains a number of 180 scientific titles from the autochtonous and foreign specialized literature.

The first part is formed of 75 pages and illustrated through 6 figures and 4 tables, being structured in 8 chapters in which data from the specialized literature are presented,
data about the avian influenza. This part also contains a systematic approach on the current knowledge regarding:

- the taxonomy of bird flu viruses and the importance of the genetic recombination phenomena;
- the zoonotic risk represented by the avian influenza viruses;
- epidemiologic and ecologic aspects regarding: the circulation of the avian influenza viruses, the importance of the bird species as reservoirs and transmitters, the possibilities of diffusing of the bird flu viruses, the resistance to the environment factors;
- the ethiologic characterization of the avian influenza viruses;
- the methods of isolation and identification of the avian influenza virus (virusological and serological examinations);
- measures regarding the surveillance and fighting the avian flu in poultry and wild birds in accordance to the European legislation;
- aspects regarding immunoprophylaxis;
- aspects regarding avian influenza in poultry;

The second part of the doctorate thesis formed of 233 pages contains personal research carried off in the period 2005-2012, being structured in twelve chapters, each chapter of this part being formed of subchapters, where the material and the method, obtained results and partial conclusions are presented. In the second part there are also presented 43 tables, 41 graphs, 99 figures.

The target of the surveillance of avian influenza in wild birds is to identify the risk of introduction of the avian influenza virus (with weak pathogen strains or high pathogen strains) in the populations of poultry. The morbidity and mortality cases in poultry were first investigated, especially the species presenting a high risk, such as the Anseriformae order (swans, geese and ducks), the Charadriipformae order (seagulls and other shore birds), The Gruiforme order (moor hens), the Pellicaniformae order (cormorants, pelicans) and so on.

The prevailed samples for the isolation of the virus and molecular detection (PCR) were represented by: cloacal and tracheal/oral tampons and/or tissues (the brain,
the heart, the lungs, the trachea, the kidneys and the intestines) from the ill, dead or shot wild birds (shot for diagnosis).

The serological surveillance was not carried off on samples prevailed from wild birds because the serological RIHA exam cannot distinguish between HP and LP strains, and the results connected to the emphasizing of the antibodies do not allow the correlation regarding the location where the birds had been infected.

**The target of the surveillance of the avian influenza in poultry** is to identify LPAI infections with the subtypes H5 and H7 of avian influenza virus strains, especially in the target populations which presented a specific risk to infection because of the home raising system. The surveillance also targeted the birds raised in the commercial stations.

All the mortality cases or the being taken ill cases in poultry were investigated in the direction of avian influenza diagnosis. All the poultry which presented clinical signs of disease and anatomopathological modifications which resembled those produced by avian influenza were investigated; the blood samples for the serological examination were prevailed from all the poultry species (not only from commercial stations but also from the ones raised in systems of outdoor raising), the drawing of the samples being planned in Constantza county and Tulcea county, so that the samples could be representative for those territories.

The study was carried off in the period of time 2005-2012; due to objective reasons I structured the presentation of the obtained data in two distinct periods, namely:

- the results obtained during the period October 2005-April 2006 when there were registered hot beds of avian influenza in our country - **retrospective study**;
- the results obtained during the period June 2006-2012 - **a prospective study** - considered a period of epidemiologic silence in which, except two hot beds of avian influenza diagnosed in Tulcea county, the bird flu virus was no longer isolated.

In chapter XI I presented the results of the epidemiological, clinical and anatomopathological research carried off to diagnose and monitor avian influenza in wild birds in the period 2005-2012 and which led to the following conclusions:

- the wild birds are carriers of a LPAI viral subtype and represent the main source of infection in poultry;
the lab examinations carried on samples drawn from wild birds in Dobrudja area showed that a very small number of birds was infected with H5Ni virus; during the period October 2005-May 2012, from 1798 wild birds examined, only from 18 birds was the avian virus isolated, from which 6 birds in Constantza county: one wild goose, 2 swans, 1 moor hen, 1 wild pigeon and 1 owl and in Tulcea county from 12 wild birds: 7 swans, 3 wild geese, 1 gallinule and 1 moor hen, resulting a percentage of about 1%.

- the clinical signs in wild birds are more weakly expressed compared to those manifested by the poultry. The clinical signs were observed especially in swans and consisted in the existence of atypical behavior of a healthy bird: they had trouble feeding themselves, could not fly or took off with great difficulty and flew over short distances manifesting signs of tiredness; some exemplaries manifested nervous signs, such as head and neck trembles, spasmodic contractions of limbs, ataxia, torticollis;

- the lesion picture in wild birds is less expressed compared to the one recorded in poultry, the hemorrhagic lesions being predominant;

- from all the wild birds diagnosed with avian influenza in our country, the H5N1 avian influenza virus was isolated in its high pathogen variant.

In chapter XII there can be found the results of the epidemiological research regarding bird flu in the poultry from the Dobrudja area. The main conclusion which arises from this study is that in the majority of avian influenza cases diagnosed in Dobrudja area and the Danube Delta, respectively in the 26 declared hot beds of bird flu, the main source of contamination was considered to be the wild birds, the way of transmission being a direct one (the contact between the poultry and the wild birds) and also an indirect one represented by the habitat (water surface, ponds, lakes, rivers on which infected birds rested). By raising the poultry near the water surfaces (especially the palmipeds) or on the pastures (gallinaceae and palmipeds) the possibilities of contact with different species of wild birds which could be infected are increased.

In chapter XIII there can be found the results regarding the clinical aspects recorded in poultry in the avian hotbeds from Dobrudja area, the study being carried off during October 2005-March 2006 (a retrospective study), when, in the 24 avian hotbeds which evolved in Dobrudja area clinical signs of illness were observed in 1157 birds,
from which: hens-963 (83%), turkey hens-77 (7%), guinea fowls-15 (1%), ducks-67 (6%) and geese-35 (3%). From all the species, the hen is the more affected, while the palmipedes are more resistant and manifest clinical signs of illness in a small proportion. The main clinical signs noticed in hens were: ingluvial indigestion, leakage of mucosities from the beak and nostrils, diarrhea, crest and wattle cyanosis and nervous manifestations. The clinical symptoms of domestic palmipedes were less marked, the evolution of the disease being longer and the mortality rate was much more reduced compared to gallinaceae.

Chapter XIV contains the morphopathological aspects produced by the avian influenza and the main lesions in the poultry examined during the study are described. Thus:

- during 2005-2006 - a retrospective study – 1323 samples were examined, represented by dead bodies and organ samples (brain, intestine, trachea, lung, liver, spleen, kidney etc) from the poultry in the avian hotbeds declared in Dobrudja area;
- during 2007-2012 - a prospective study - 560 samples were examined;
- the 1883 examined samples came from: 1455 from hens (77.3%), 133 from turkey hens (7%), 130 from ducks (6.8%), 128 from geese (6.8%) and 37 from guinea fowls (2%);
- from the affected species the gallinaceae manifested more obviously clinical signs of illness;
- in the forms of hyperacute evolution, the lesions were more weakly expressed, consisting in the cyanosis of the crest or wattles, general congestive lesions, without other characteristic modifications; in the acute phase the dead bodies were dehydrated and there were noticed the cyanosis of the crest and wattles, muscular and visceral congestion; in the forms with prolonged evolution there could be observed on the dead birds spots and echimoses, especially in the larynx, trachea, proventriculitis and hemorrhagic enteritis, hemorrhagic in the adipose tissue of the epicard and on the serous surfaces contiguous to the stern. A very important lesion which can be considered pathognomonic for the avian influenza was represented by the subcutaneous edema,
especially in the head and neck area, sometimes including the chest area, the
subcutaneous conjunctive tissue being strongly infiltrated with a citrin, serous liquid;
- in the case of palmipedes, the lesions were more weakly expressed compared to
those in hens, and consisted in congestions, hemorrhages, the cyanosis of the beak, spots
and echimosis on the serous, sinusitis, cataral-hemorrhagic tracheitis.

In chapter XIV I presented the methodology of the laboratory exams regarding the
diagnosis of avian influenza. For an accurate laboratory diagnosis, besides the using of
the most efficient methods (the latest kits and reagents for diagnosis), the trained staff, it
is also important the samples which are to be examined, a reason for which a great
attention must be paid to the prelevation of samples, storage and conservation, as well as
to the respecting of the temperature during the transportation of the samples to the
laboratory. The prelevation, packaging, labeling and transportation of the samples must
respect the provisions of Chapter IV from “General Procedures for the Prelevation and
Transportation of Samples” from the Decision of The European Comission no.
437/2006/E.C.

The diagnosis of avian influenza was carried off through virusological
examinations described in Chapter XVI and which consisted in : the rapid
immunochromatographic test, polymerase chain reaction (PCR) carried off at the
laboratories in Constantza and Tulcea, the isolation of the virus on 9-11 days old
embrionated hen eggs and the intravenous pathogenity test (IPIV) conducted at IDSA
Bucharest and through serologic examinations described in Chapter XVII and represented
by: ELISA tests, the immunodifusion reaction in agar (AGID) and the hemoaglutination
and inhibition of hemoaglutination reaction (RIHA) carried off in the laboratories in
Constantz and Tulcea.

The research regarding the virusological diagnosis of avian influenza conducted
during the period 2005-2012 led to the following conclusions:
- during the period 2005-2007 with the aid of the immunochromatographic test
  15330 samples were analysed, out of which 12926 samples were drawn from poultry and
  2404 came from wild birds;
- from the 12926 samples extracted from poultry, 237 samples (1.83%) had a
positive result and 42 samples (0,32%) a false positive result, and in the case of the wild
birds, out of 2404 examined samples a positive result was obtained for 18 samples
(0,75%) and a false positive result was obtained for 6 samples (0,26%);  
- using the PCR test, out of 303 examined samples during the period October 2005
April 2006 (279 samples prelevated from poultry and 24 samples from wild birds), a
number f 255 samples reacted positively (84,15%) and 48 samples had a negative result
(15,84%).
- during the period May 2006-June 2012 a number of 394 samples were examined
using the PCR test (82 samples coming from poultry and 312 samples from wild birds), the
obtained results being negative for all examined samples.

The research regarding the serologic diagnosis of avian influenza carried off
during the period 2005-2012 on samples drawn from poultry led to the following
conclusions:

- out of 47235 serum samples examined with ELISA test during the period 2005
2007, 907 samples had a positive result (1,92%) and 46238 samples had a negative result
(98,08%). Out of the 907 positive samples only 76 (8,38%) were also confirmed using
the IHA or ID test, and 831 samples reacted negatively, being considered false positive
results;
- using the immunodifusion test in agar a number of 291 samples coming from
gallinaceae was examined, and 79 samples coming from d the palmipedes and the results
obtained were all negative;
- the hemoaglutination and inhibition of hemoaglutination reaction was used during
the period 2006-2007 only as a method of confirmation for the positive reactions to
ELISA test. Out of the 397 serum samples examined, a number of 76 samples were
positive (19,14%) and 321 samples reacted negatively (80,86%) being considered fals
positive reactions to ELISA test;
- during the period 2008-2012 a number of 12155 samples were tested using the
hemoaglutination inhibition test, out of which 7810 samples drawn from gallinaceae and
4345 samples drawn from palmipedes. The results were negative for all investigated
samples.
The prevention of appearance and fighting avian influenza can be accomplished only through an international cooperation and collaboration, to be aware at any times of the epidemiologic situation and the pathogen capacity of H5/H7 viruses, and, taking into account the situation in our country, through the support for the Sanitary-Veterinary and for Food Safety Authority granting material and financial support.

For a good surveillance of avian influenza in the studied area and especially in the Danube Delta villages there have been taken measures for a complex monitoring and the main goal is to ensure an early detection of avian influenza produced by H5 and H7 subtypes and to intervene promptly to limit the diffusion and to eradicate the illness.

A continuous surveillance of the avian influenza virus in wild birds is very important, because of the fact that this surveillance offers us an “early warning” on the introduction of the virus in new regions, being able to carry on proper studies to evaluate the risk. It is also important to understand the behavior of wild birds, the way of transmission of the virus among wild birds and poultry, the categories of age of wild birds frequently affected, the routes of migration. It is needed a good understanding of the transmission and pathogenesis of H5N1 virus in wild birds, as well as the identification of the receptive species and their probability to develop the illness.

At the same time, it is very hard to prevent the outbreak of bird flu in poultry because of the fact that we cannot establish a control strategy for wild birds, the only solution to prevent the appearance of the illness being the maximum limitation of the contact between wild birds and poultry. In our country, where in a relatively short period of time (approximately 6 months) significant hotbeds of avian influenza evolved in poultry, the prevalence of the virus in wild birds was very low (1%), thus the role of these wild birds in spreading the illness was not very well understood. It is clear that the main problem refers to poultry and that the hotbeds which appeared and their spreading from a geographical point of view cannot be stopped without applying the adequate surveillance and control measures.

The carried off research as well as the obtained results during the period of epidemiological silence, when there were not detected strains of the avian influenza virus, may contribute to the proving of indemnity status in the studied area.