SUMMARY

Key words: *antigenic shift, influenza conserved proteins, vaccin, lentiviral vector, laboratory mouse C57BL/6, immunitary cell response*

Influenza is a real public health concern, being an original infection compared to the other viral infections. Acute respiratory disease of a zoonotic nature, highly contagious, flu affects mankind and the animal world since ancient times. If flu often passes for a common seasonal disease, genetic shift phenomenon or genetic reassortment can lead to transformation of an ordinary influenza strain in a truly international threat generating a dramatic pandemia. Seasonal influenza vaccines are successfully used in specific influenza prophylaxis if circulating viral strains singly mutations are anticipated by different specialists as: epidemiologists, bioinformaticiens, researchers and physicians. This study aims the anticipation of the unpredictability of influenza and the phenomenon of antigenic shift. The actual aproach was a completely different one, therefore, instead of targeting the proteins often suffering genetic mutations the internal conserved proteins of influenza A type were considered in developing a new influenza vaccine. The stability of proteins and proteic segments inside the vaccine was provided by cloning them into lentiviral vectors, valuable tools in biology, currently not very known in Romania.

The relevance of this project lies in the timeliness and importance of strategic, technical and scientific theme. This thesis was subject to a complex collaboration between the research team of USAMV Epidemioly and Infectious Diseases Department and a research team from the Pasteur Institut within a type PNII CNMP project with the theme: ‚The fight against recombinant influenza viruses: test of new vaccines based on lentiviral vectors in swine and poultry' ROFLU 52 180 / 2008. The partners had numerous exchanges with both teams aiming the two-way improvement for the topic aproach in a fair and compensative manner for both parties with the primary intention to develop an universal panserotype influenza vaccine.

Doctoral thesis: **Elaborating and testing of new influenza vaccines based on lentiviral vectors in swine and poultry** contains 281 pages and is structured in accordance with current legal provisions into two main parts: the first part is entitled ***„Current state of knowledge"*** being structured in 55 pages and the second part, ***„Personal contributions"*** contains 171 pages.

The structure of the first part includes four chapters that summarizes information from the literature on the subject of the thesis, the methodology approach of the topic and other important

elements that help accurately understanding and decryption of the second part as references interpretation and discussion of results.

In the first chapter: ***„Type A influenza viral infection”*** flu is presented as an infectious agent, the disease caused by influenza A viruses is described in detail, with precise analysis of it's classification, presenting its structure and properties, viral description of the immune response against influenza disease, resistance of infectious agent at different chemical or physical agents, genetic elements important for influenza infection and finally an epidemiological perspective is aproached and the simptomatology is described in mammals and bird species affected by this infection. Currently prophylaxis in order to prevent potential influenza A infections is equaly aproached.

The second chapter ***„Lentiviral vectors and their use in biotechnology”*** approaches the subject of vectorology respectively the existing molecular cloning vectors and their applicability. Later more detailed description of these vectors is exposed and lentiviral vectors are presented as long as future benefits of using these tools.

Part of the thesis, ***„Animal Experimentation in biomedical research”*** makes the subject of the third chapter generally presenting two animal species used in the development of influenza vaccine, the laboratory mouse and conventional pig among with guinea pig and poultry, species that were originally meant for vaccine testing. We deciced to choose these two species (inbred mouse and conventional pig) considering the final universal applicability of the vaccine in human and veterinary medicine.

The fourth chapter, ***„Research in country and abroad on the theme project”*** recontextualizes the topic, presenting original research conducted internationally, respectively epidemiological data, influenza vaccines on the market. Romanian researches are focalised on epidemiology and specific prophylaxis performed on the country.

The second part of the thesis, namely: **Personal contributions** meet experiments to address and complete the thesis, precisely as detailed below.

The fifth chapter of the thesis: ***„Aims and objectives of research”*** treats the flu like an illness eternally valid, citing the oldest and the most recent pandemic episodes with an important impact on human health, passing in review: Spanish influenza, Asian flu and avian influenza, but also the most recent swine flu.

The major goals of the project are identified, namely:

- group of researchers working in the field of epidemiology and influenza A vaccines based on lentiviral vectors reunited for to the development of new and innovative vaccine;

- implementing a serological surveillance of natural infections with influenza viruses in pigs and birds (including wild birds), to achieve a true epidemiological study;
- create new methods and an animal model study: standardization and animal model would allow evaluation of a new vaccine.

Research methods are cited chronologically, namely: bibliographic research followed by a thorough documentation regarding the proposed topic to detect all aspects of influenza virus infection nationally and internationally, epidemiological seroprevalence survey of influenza A natural infectios in pigs and poultry from South-East of Romania, the choice of new influenza A immunogenic proteins, design and construction of antigens, construction and production of lentiviral vector, evaluation of vaccine immunogenic properties in murine model, testing protection in murine model, immunogenetic test in pigs, evaluate the toxicity and other side effects of M1_NP lentiviral vector on conventional pigs and evaluating 'in vivo' expression of transgene M1_NP using lentiviral vector expressing luciferase and confirmation of immunogenicity of this vector (ELISPOT and pentamer staining).

Also perspectives and methods of validation and recovery outcomes measured were also treated in this chapter.

The sixth chapter***: „Epidemiological investigation of influenza A virus infection seroprevalence at certain domestic animals in the East of Romania”*** was carried out on several categories of animals within an area expanded in oder to have an overview of the expansion of infections caused by influenza A viruses in the East and South-East of Romania. Samples were taken from swine, equine and poultry, mainly from households, samples that have been analyzed using ELISA kit Influenza Blocking monocupule serum screening for antibodies specific to influenza nucleoprotein.

„M1_NP antigen design” is the subject of the seventh chapter and sets the initial choice of influenza A subtypes that served to antigens construction. As was previously exposed if basic idea was to use two highly conserved influenza proteins as: NP and M1, subtypes choice has been difficult in vaccine developement, arbitration being made between subtypes of influenza A evolving in human populations, and animal populations: avian, swine and equine, reconsidering vaccine double application: veterinary and human one. Consecutive to interested subtypes election complex and numerous bioinformatics programs were used to determine potentially immunogenic segments that might be used in molecular cloning.

Chapter 8: ***„Molecular cloning”*** combines molecular biology techniques that are used in order to introduce immunogenic protein segments: M1 and NP in FLAP plasmid, the transgene carrier. PCR techniques had been used along with: migration on agarose gel, enzymatic digestion of plasmids

expressing transgenes, DNA purification, cloning of a DNA insert into a vector, plasmids transformation into competent bacteria, amplification and purification of plasmid clones.

The effectiveness of these experiments was successfully confirmed by checking the constructions purity at Theravectys and using as well a service provider who conducted construction sequencing , confirming their purity.

„Production of lentiviral vector particles” on cell cultures (cell culture techniques) trough transfection method (co-expression of three partners: transcriptional unit encoding the vector genome, including therapeutic gene or transgene encapsidation system encoding for trans proteins, useful to particles forming and to conduct early stages (retrotranscription, integration) and the system of envelope expression) were presented in the ninth chapter. The particles assembled on this way were subsequently concentrated by ultracentrifugation.

Stock vector particles validation was performed by quantifying producted particles following a cellular transduction of the particles performed on the same type of cell culture used in the production of lentiviral vectors. Two methods of measurement were used, quantification of p24 capsid protein in vector stocks allowed us to determine the number of produced physical particles and a quantitative PCR on lysates of cells transduced with lentiviral vectors was performed in order to measure vector effective particles in produced stocks. By titration M1_NP vectors according to qPCR method correct values were obtained, correlative with those obtained by p24 method.

After lentiviral vector production several studies of immunogenicity were conducted in murine model, knowing that M1 and NP synthetized clusters with specific immunogenic potential in C57BL/6 mice were previolusly introduced in M1_NP transgene sequence. As cellular immunogenicity assessment ELISPOT technique was used. These immunogenicity studies have been the subject of tenth chapter: ***„Testing of M1_NP lentiviral vector immunogenicity in murine model”***. Immunogenic potential of these vectors was confirmed by comparing the cellular immune response obtained in immunized mice with the response obtained in the control group of mice that were injected with buffer solution used for lentiviral vectors formulation. In addition to confirming immunogenicity of these vectors present chapter aimed the choice of optimal different elements of vector that can serve to the double applicability of the vaccine: human and veterinary.

Following immunogenicity studies performed to determine the immunogenic potential of the 2 lentiviral vector constructions (two different transgenes), 2 vector constructions have been chosen before for the latter vaccine protection studies. In Chapter 11, a vaccination strategy was proposed: prime-boost vaccination, followed by a viral challenge for a better characterization of the immune response obtained after vaccination and infection. Immune response evaluation and studies regarding vaccinal efficacy have been conducted on C57BL/6 laboratory mouse after intramuscular

immunization followed by intranasal infection to mimic natural influenza infection. All the results regarding vectors immunogenicity and clinical signs of infection were meant to allow identification of possible protection provided by the two vaccine candidates following infection with 2 highly pathogenic mouse adapted influenza viruses H1N1 and H3N2. Despite the good immunogenicity of the 2 tested lentiviral vectors, vaccine safety studies have been compromised by the use of a very strong dose of influenza virus, dose that led to the early death of mice and the difficult assessment of vectors effective protection as vaccines.

Chapter 12: **„In vivo *titration of H1N1 and H3N2 viral strains”*** allowed the determination of minimum lethal doses of each virus used in part. Six serial dilutions were performed from 10^{-1} to 10^{-6} of pure virus and the C57BL/6 laboratory mice were inoculated l (40 µL on the 2 nostrils) under light anesthesia. Mice were observed daily until clinical signs were setted. The results obtained following 'in vivo' virus titration of mouse adapted viruses: Puerto Rico/8/34 H1N1 and H3N2 Scotland were considered satisfactory for protection study after a prime-boost1-boost2 vaccination protocol followed by a viral challenge.

„The protection provided by lentiviral vectors M1_NP and M1 against some type A flu viruses H1N1 and H3N2 in laboratory mouse C57BL/6 following a prime-boost1-boost2 strategy and infection with ‚in vivo’ mouse adapted viral strains on unimmunized mice”, respectively the thirteenth chapter resumed the study initiated in Chapter 11 to assess vaccine protection assured by lentiviral vectors in the context of influenza infection specifically adapted in mice and titrated 'in vivo'. Once again the immunogenicity of the 2 vectors used for boost 2 as vaccines was confirmed. Contrary to the results obtained in Chapter 11 this time encouraging results confirming the protective potential of lentiviral vector encoding for protein synthesis M1_NP were obtained.

In order to estimate the true protective panserotype potential of lentiviral vaccine encoding for M1_NP synthetic protein another study was conducted in chapter 14 against highly pathogenic H5N1. This time H5N1 virus could not be titrated on mouse because access to this type of experiment was extremely difficult (Level A3 Laboratory) and initial planning did not take this into account. Apparently, establishing infectious dose without 'in vivo' titration of this virus led to the evolution of an insidious pathogenesis without causing clinical signs. Mice showed no signs of disease. Peak infection was estimated at the 5th day after infection despite the absence of any clinical signs and few of the mice were sacrificed. Lesion score was not performed but a quick necropsy revealed the presence of hypertrophic pulmonary lesions and early lung hepatization, despite the absence of clinical signs.

Chapters 15 and 16 approached M1_NP lentiviral vector evaluation in terms of another animal model: mixed breed conventional pig Landrace-Big White-Pietrain. A true toxicology study was conducted to confirm the safety of using lentiviral vector, followed by a study of immunogenicity.

Following this analysis we could conclude that none of the 3 doses of lentiviral vector was toxic and that the administration of lentiviral vectors in vaccinology doesn't have a negative effect on swine herd health. In addition except the non-toxic effects of lentiviral vector immunogenic capacity was equally confirmed.

To deepen the knowledge concerning the functioning of our vaccine 'in vivo' trials of distribution of transgene expression over time were made. These studies have allowed biodistribution understanding, kinetics of vaccine immune response induced by our vaccination and it's excretion out of transduced cells of an organism. A bioluminescent technique was used with a pentamer staining strategy. Chapter 17, ***„Evaluation of* in vivo *expression of M1_NP transgene following the use of a lentiviral vector expressing luciferase, vector's immunogenicity confirmation (ELISPOT, pentamer staining)"*** was made with the intention to reassess the immunogenic potential of a veterinary vaccine by aproaching innovative techniques that have not been used previously on the other chapters.

In the last chapter, ***„General conclusions and recommendations"*** were drawn a number of 21 conclusions that resulted from carrying out researches, supporting the confirmation of the start of this project and supporting scientific value and their recovery experiments and few recommandations.