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SUMMARY

EPIDEMIOLOGICAL RESEARCH REGARDING WEST NILE VIRUS IN ROMANIA

Doctoral thesis for the acquirement of the scientific title " Doctor in Veterinary Medicine"

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SUMMARY

West Nile encephalitis (fever) is an arboviral, re-emerging infection with a zoonotic character, affecting equine, human and birds causing clinical manifestations which varying regarding from asymptomatic cases to severe meningoencephalitis, sometimes fatal. The doctoral thesis entitled ***"Epidemiological research regarding West Nile virus in Romania"*** is extended to 202 pages and is structured in accordance with the current legal requirements in two main parts: the first part entitled ***"Current state of knowledge"*** is elaborated over 55 pages and contains 5 tables and 14 figures and the second ***"Personal contribution"*** includes 120 pages, 36 tables and 46 figures, for better representation and synthesis of content.

The first part consists of three chapters in which information on the thesis subject, from the reference literature, are summarized and used later in the second part as references for the interpretation and discussion of results.

In the first chapter entitled ***"West Nile virus - history and importance"*** data from the references are presented dealing with the disease first descriptions and etiological agent, the historical development and importance of West Nile virus that causes disease. The etiologic agent of West Nile encephalitis is the homonym virus, named after the place where the virus has been isolated for the first time West Nile district, Uganda (Eastern Africa). Currently the its distribution is vast, WNV infections being reported in Africa, Europe, Middle East, Central and West Asia, Oceania and more recently in North America. Since 1996, the year in which its presence was reported in Romania the virus has become a matter of public health and veterinary surveillance in Europe, the Mediterranean and later in USA, where epidemics and outbreaks reported in human and horse populations at the first introduction of the virus in the Western Hemisphere had dramatic effects.

The second main chapter entitled ***"Etiology pathogenesis and epidemiology"*** has three chapters in which the data on morphology and phylogenetic classification, virus particularities, pathogenic and immune response mechanisms, emphasizing on the importance of factors

influencing the occurrence of disease. Also the chapter presents an overview of the epidemiology of infections caused by West Nile virus in the world and in Romania aiming at all aspects of the epidemiological characteristics.

Regarding the development of infections caused by West Nile virus in Romania, from July to October 1996 an outbreak of West Nile fever occurred in the South (in Bucharest and the Danube Valley), causing hundreds of disease cases in humans as manifested by progressive neurological - West Nile encephalitis. The registered a mortality rate of this outbreak was approximately 10%. In October 1996 epidemiological, entomological, ornithological and environment surveys were conducted in Bucharest and in the adjacent rural areas showing that *Culex pipiens P.* mosquitoes were the main vector of the epidemic, a fact confirmed by the subsequent isolation of WNV, serotype RO97-50, following collection of *Culex pipiens P.* by suction from the walls and ceiling of a wooden house located in the city center.

In the last chapter of the first part entitled ***"Clinical aspects, diagnosis, treatment and prevention"*** are described the clinical forms of West Nile virus infection, currently available diagnostic methods and the prevention and control measures adopted, focusing on the description of specific prevention methods, the study of the vector based vaccine used in vaccinology particularly on lentiviral vector based vaccines. Thus it was found that the severity of clinical signs of infections caused by West Nile virus is influenced by several factors: age, growth conditions, viral strain virulence and host genetic susceptibility (Perianu T., 2006) and clinical manifestations of the disease situates the disease in the cadre of the arbovirosis with flulike manifestations but also in those with visceral or neurological tropism (encephalitis and encephalomyelitis). In birds the disease progresses in most cases asymptomatic, while symptoms in humans and horses may vary from insidious progression or influenza syndrome to a fatal meningoencephalitis. The absence of characteristic clinical signs is making the diagnosis and disease alert difficult (Steinman et al., 2002).

Apart from birds or horses, other vertebrates are susceptible to West Nile virus infection. WNV antibodies were detected at least 30 different species: farm animals, pets and wildlife. Seroprevalence appears to be higher in regions where the virus circulation was detected earlier.

Laboratory diagnosis is necessary because of often subclinical or asymptomatic infection and nonspecific clinical signs development and serology remains the main method of diagnosis of WNV infection. The standard serological test is the plaque seroneutralization assay, used both for confirmation of infection and titration of neutralizing antibody specific to West Nile virus from serum or cerebro-spinal fluid. West Nile virus can be isolated by cell culture and plaque titration and identification is achieved by „reverse-transcription polymerase chain reaction" (RT-PCR), real-time RT-PCR antigen detection tests, immunohistochemical methods.

Until 10 years ago, control of mosquito invasion was the only strategy applied to prevent and control the dissemination of the virus, but the increased frequency and severity of the infection during the past decade has led to the elaboration of several vaccines against WNV are in different testing phase or licensed for marketing (veterinary vaccines - U.S. only). Although vaccination can control dissemination of the infection in equine populations in Romania there is not a commercially available veterinary vaccine.

New generations of vaccines the vector-based vaccine including the lentiviral vectors based vaccines become popular in vaccinologie, because their ability to induce an immune response is an alternative for the prevention of many diseases in animals. Vector based vaccines (lentiviral or poxviral vector) is able to induce a protective immunity in mice after experimental infection with West Nile virus (Iglesias et al. 2006; Despres et al, 2005).

During Chapter IV entitled ***"Purpose and objectives of the research"*** are underlined the premises of the research. Research goal is the evaluation of the epidemiological situation on the presence and dissemination of West Nile virus in Romania, monitoring of emerging risk areas of infection, the better understanding of the immune status of horses and other species that are indicators of the presence of virus West Nile and a first sentinel surveillance for the virus in human populations and the establishment of effective methods for diagnosis and monitoring of infection in order to be available in case of suspicion.

The main objectives pursued during the research are the implementation of laboratory diagnostic techniques and working protocols commonly used in surveillance of West Nile virus circulation in other countries as well as testing and validation of new diagnostic methods, a sero-epidemiological survey on West Nile virus infection in horses in counties located in the lower basin of the Danube and in wild and domestic birds in the Danube Delta the development of a new method for indirect ELISA serological diagnosis of infections caused by West Nile virus in horses and developing and testing of a vector-based vaccine capable of protecting horses lentivirali against infections caused by West Nile virus.

Chapter V entitled **"*Research regarding the methodology of diagnosis in West Nile virus infections*"** is divided into two subsections. The first section extensively describe the serological methods used for routine diagnosis of West Nile virus infections (West Nile test indirectly ID Screen ®, MAC ELISA test, neutralization techniques) and the research carried out to assess the reproducibility of indirect ELISA. Thus, testing a number of 76 test samples using the *ID Screen ® West Nile* indirectly made in two different laboratories (USAMV Iaşi and UMR 1161Alfort France) showed a 94.45% reproducibility of the tests conducted in Romania. It also describes research carried out for validation of two commercial tests: a rapid diagnostic test product for horses *ImmunoComb* made by *Biogaled Laboratories* and competitive ELISA (*ID*

Screen ® West Nile Competition) for the serological diagnosis in birds. Tests using rapid diagnostic kit ELISA, **"**WNV-ImunnoComb-Equine West Nile Virus Antibody Test Kit**"**, made by Biogal Galed applied on previously tested samples by virus neutralization tests showed a sensitivity of 97.67% and a specificity of 95.55 %.

Regarding ID Screen ® ELISA kit Competition West Nile was specifically, the test results of bird sera is consistent with neutralization test results showing that the method lends itself to examination in laboratories with high accuracy and facilitate safe serological diagnosis of infections caused by West Nile virus in birds in Level I laboratories.

The second chapter presents the research on virological methods for diagnosis of West Nile virus infections highlighting the citopatogen effect of the virus on cell culture. The presence of West Nile virus infections in animals and birds in the region studied, initially reported by serological investigation was confirmed by virological examination in cell cultures with results that showed citopatogen effect for four samples derived from crows organs and from one mosquito sample.

In Chapter VI, entitled ***"Observations regarding the presence and dissemination of West Nile virus in Romania"*** are described methods of work, materials used and results obtained from epidemiological surveys carried out on West Nile virus infection in the period 2006-2008, in the counties located in the lower Danube basin in samples from horses and domestic and wild birds (considered natural reservoirs of the virus) and other species naturally unresponsive to infection with this virus but are able to support specific West Nile virus seroconversion. The research was taken on a number of 1701 blood samples from horses from which a number de1488 samples were tested serologically for the detection of anti-West Nile IgG and 69 samples were analyzed for detection of the specific IgM.

Results showed that seroprevalence remains at elevated levels in horse populations from counties located in areas where the presence of mosquito vectors and climatic conditions facilitates the maintenance of virus such as Braila, Galati and Tulcea. After testing samples from poultry using virus neutralization tests and competition ELISA, we were able to appreciate an average prevalence of 2.7% (12 positive samples from a total of 378 analyzed). The largest number of positive samples was obtained in sera from *Corvidee*, and *Passeriformes* which is consistent with data from literature. Tests conducted in ruminants have shown an overall seroprevalence of 5.10%.

Chapter VII entitled "***Development and testing of a new ELISA method for the serological diagnosis of West Nile virus infections***", presents the materials and methods used to develop a new indirect ELISA method based on using as a microplate coating antigen recombinant genetic sequences coding for the West Nile virus envelope protein (E), protein with

the most important immunogenic role. The following describes the results obtained, the research for the improvement of the new protocol and the estimation of the intrinsic values. Intrinsic values of the new indirect ELISA methods were related to the test results obtained using serum neutralization test estimating a sensitivity of 92.3% and a specificity of 100%.

Chapter VIII, entitled ***"Research on the development of a vector based vaccine for the use in immunoprophylaxis of West Nile virus infection in horses"*** lays the description of the research made for obtaining a lentiviral vector in which a transgene coding for E protein of West Nile virus is inserted. The vector was used subsequently to develop a new vaccine for West Nile virus infection immunoprophylaxis**.** In the same chapter, preliminary *in vitro* and *in vivo* tests made to evaluate the quality of the vaccine and their immunogenic capacities are described. The response obtained after mice immunization with the new lentiviral vector-based vaccine provided a strong and stable protection in time without endangering the normal health and physiological status of laboratory animals, issues that are comparable to those described in the literature.

Finally, in Chapter IX, entitled ***"Research regarding the immunogenicity testing for the vector-based vaccin by experimental inoculation in horses"*** the protocol and results obtained in the cadre of a kinetics study assessed to evaluate the immune response following experimental inoculation of the vaccine in horses it is described. Testing was performed initially in a pilot phase on a group of four horses aiming the determination of the optimal inoculation dose. In a later stage an experiment for the evaluation of the postvaccinal response of the new lentiviral vector based vaccine compared with the commercially vaccine *Recombitek ® Equine West Nile virus vaccine* was made. The results show that vaccinated animals developed a humoral immune response against West Nile virus, with a trend corresponding to the inoculated dose. Regarding the comparison with the immune response obtained from horses immunized with the commercial vaccine *Recombitek ® Equine West Nile virus vaccine*, from the 14th day post inoculation has been registered. a superior immune response in animals immunized with the new version of the lentivirali vactor based vaccine

In Chapter X ***"Final conclusions"*** a number of 25 conclusion learned from the completion of investigations are formulated.

In conclusion it can be said that infection caused by West Nile virus has to be continuously monitored and the rapid movement of the pathogen has to be detected in order that health authorities can implement protective measures considering the implications of infection in animal health and also in public health as an emerging zoonosis.