SUMMARY

Key words: *arboviruses, zoonosis, sero-neutralisation, High-Throughput Real-Time PCR*

The present PhD thesis, entitled "*Arboviruses with zoonotic character transmitted by mosquitoes in Romania*", is structured according to the rules in force in two main parts: the first part, represented by "**Current state of knowledge**" which summarises updated information from the literature on the topic addressed and the second part, that of **„Personal Contributions"**, in which are described the working methods and the results obtained as a result of the research undertaken during the doctoral period.

The first part, "*The current state of knowledge*", structured in three chapters includes a brief systematization of information identified from the literature on the main arboviruses, clinical manifestations in humans and animals, the main insects that can act as vectors and vectorial capacity with the factors involved in transmission of pathogens.

The **first chapter** entitled "*The main zoonotic arboviral infections with emergence and re-emergence potential in Romania*" describes, as the title shows, the main families and viral genera that include viruses whose transmission involves the participation of a hematophagous insect. Besides, the clinical syndromes associated with the infection with the mentioned viruses in humans and animals are also reported. The main viral families described in this chapter are the Flaviviridae family, with the viruses: West Nile, Usutu and tick-borne encephalitis virus, the Togaviridae family, with the Sindbis virus and the Peribunyaviridae family, with the Tahyna virus.

Regarding the main species of mosquitoes involved in the transmission of viruses described above, in the **second chapter** entitled "*The main culicidae involved in the transmission of pathogens to human and animals in Romania*", describes indigenous and invasive species, which are found in our country. Out of the 55 species of mosquitoes recognized as native to Romania, this chapter describes the species: *Culex pipiens*, with the two bioforms, *Culex modestus*, *Aedes vexans* and the invasive species, *Aedes albopictus*.

Chapter III "*Vectorial capacity of mosquitoes*" focuses on vector capacity and vector competence, crucial aspects for the transmission of pathogens.

The second part of the thesis "*Personal contributions*" is structured in five chapters (IV - VIII), each of them presenting the working methods approached, the results obtained during the doctoral period and the conclusions drawn from the analysis presented.

Chapters IV and **V**, respectively "*The aims and objectives of research*" and "*Description of the organizational and institutional framework in which the research was conducted*" briefly describe the purpose and objectives pursued, as well as the place of activities necessary to achieve the proposed objectives.

Thus, this thesis resulted from the implementation of a surveillance program in the eastern part of the country. Surveillance action focused on three major aspects involved in the arboviral life cycle: (i) mosquito communities (Diptera: *Culicidae*); (ii) detection and characterization of arboviruses found in captured mosquitoes; (iii) active serological surveillance of susceptible species.

Chapter VI, entitled *„Serological investigations regarding the main arboviral infection in animals and human"*, is structured in four subchapters. The division into subchapters emphasize the broad approach of the topic and of the targeted receptive species, which represent essential links in the transmission and maintenance cycle of arboviruses in nature. Thus, the serological investigations aimed the detection and prevalence of specific antibodies, in serum samples from horses, birds, dogs and people, from the eastern part of Romania.

The aim of the first seroepidemiological investigation was the detection of antibodies against West Nile, Usutu and tick-borne encephalitis viruses, in clinically healthy horse serum samples from three counties and 14 places. For the mentioned purpose, the tested samples were harvested from Iași (n = 118), Vaslui (n = 208) and Tulcea (n = 28) and were collected simultaneously with the samples intended for testing for equine infectious anaemia, during February - April and in May and July. The individual data of the animals included in the study were obtained from the owners and were systematized in two variables: age category and gender.

Serological testing of the 354 samples was initially performed by enzyme-linked ELISA assay, for IgG detection, using the commercial ID-Screen West Nile Competition kit (IDVet Diagnostics, Montpellier, France). Subsequently, the positive samples were analysed for confirmation by the seroneutralization assay. The techniques were performed according to the internal ANSES protocols.

The results of the first serological assay revealed a prevalence of anti-WNV antibodies of 46.6% (165 positive samples). Regarding the studied variables, the largest percentage corresponded to the age category "adult" (5-14 years), while there were no noticeable differences between the sexes.

To rule out false-positive results, due to cross-serological reactions with other flaviviruses, positive and doubtful samples by ELISA technique, were analysed by micro-neutralization virus (MNT) test. Thus, neutralizing anti- WNV antibodies were detected in 136 out of the 168 tested samples, with an overall seroprevalence of 38.4% (136/354).

As a result of micro-neutralization assay testing, four positive samples were found positive for WNV and USUV. Because the condition for specific antibodies to be assigned to a particular virus is that a virus has an antibody titre four times higher than the second, the four samples were considered positive for WNV. Regarding the detection of neutralizing antibodies against tick-borne encephalitis virus, there was no antibody detection in any of the tested serum samples.

The second serological investigation aimed at the detection of IgG antibodies in serum samples from wild birds from four different locations in the Danube Delta Biosphere Reserve (DDBR): Caraorman, Furtuna, Murighiol and Sălcioara. The status (migratory or resident) of the captured birds was established as well as the age category: youth or adult.

To achieve the goal, 68 samples were collected in stages from the four places mentioned above. The samples were tested using the ID Screen® West Nile Competition Multispecies commercial kit (IDVet, Grabels, France), for the detection of anti-WNV antibodies, following the manufacturer's instructions.

Out of the 68 wild birds, 22 were grouped in the “youth” category (hatched in the 2016 season) and 46 were classified as “adult”. Regarding adult birds: 12 were females and 14 males, and for another 20 birds, sex was not determined.

Following testing by the competition ELISA assay, the presence of specific anti-WNV antibodies were detected in eight samples out of 68 tested (11.8%, CI95% 11.5% –12.1%). The confirmation of the positive samples by seroneutralization was performed on 51 samples from two sites, while for 17 samples, the initial amount of serum did not allow further testing. Thus, out of the total samples tested for the detection of neutralizing anti-WNV antibodies, six samples were confirmed [11.8%, CI95% 2.92% - 20.61%)].

The third seroepidemiological survey included samples from dogs, with various diseases or healthy, from six counties in the eastern side of the country. To achieve the proposed objective, 363 samples were collected from dogs from paddocks, private households and veterinary clinics, from: Suceava (n = 12), Iași (n = 237), Bacău (n = 24), Brăila (n = 16), Galați (n = 53) and Tulcea (n = 21).

Detection of specific anti-WNV antibodies (IgG) was performed gradually, using the competition ELISA assay, with two ready-to-use veterinary commercial kits: ID-Screen West Nile Competition (IDVet Diagnostics, Montpellier, France) and Ingezim WNV Compaq kit (Ingenasa).

In the first phase, 179 samples were serologically tested by ELISA enzyme immunoassay, using the commercial ID-Screen West Nile Competition kit (IDVet Diagnostics, Montpellier, France), following the protocol, derived from the supplier's instructions. In the second step, 184 samples were tested using the commercial kit INGEZIM West Nile COMPAC (Ingenasa, Madrid, Spain).

Depending on the availability of individual data, the animal gender, age, breed size and degree of exposure were considered as variables.

Following the testing of the 179 samples, in the first stage, anti-WNV antibodies were detected in 79 samples [(44.1%) 95% CI 36.86-51.41], while in the second stage, after testing 184 samples, we obtained a prevalence of anti-WNV antibodies of 51% [(94/184) 95% CI 43,86-58,31].

In order to verify the sensitivity and specificity of the used test, the 94 positive samples resulting from the testing in the second stage, were analysed by the competition technique, using the ID Screen WNV Competition kit. Thus, out of the 94 positive samples, depending on the amount of serum available, 86 samples were reanalysed, obtaining a degree of fidelity and a positivity of 100% between the two kits.

In order to reduce the bias given by the intrinsic values of the two screening tests used and to have statistical significance, to establish an overall seroprevalence, were considered only the results obtained from the test using the ID Screen WNV Competition kit. Thus, out of 363 tested samples, specific antibodies for WN virus and other antigenically related flaviviruses were detected in 165 samples (79 from the first stage and 86 confirmed as a result of the second analysis), with an overall seroprevalence of 45.5% (95% CI: 40.33 - 50.58).

ELISA seropositive samples were retested by micro-virus neutralization (MNT) technique for the detection of specific neutralizing antibodies for West Nile, Usutu and tick-borne encephalitis viruses. Thus, in order to confirm seropositivity and depending on the amount of sample available, 161 out of 165 positives were tested.

Specific neutralizing antibodies against WNV were detected in 131 out of 363 samples, anti-USUV antibodies (antibody titre 160) were detected in a single sample, while for TBEV there was no positive sample.

The simultaneous reveal of anti-WNV and anti-USUV antibodies was observed in 10,3% of the samples (17/165). In 16 of them, the titre of anti-WNV-specific neutralizing antibodies was significantly higher, compared to that of Usutu virus-specific antibodies.

The last step of the serological investigations aimed at the detection of IgG and IgM antibodies against West Nile virus and IgG for Usutu virus. In order to establish the prevalence of antibodies in humans, samples collected in two phases (2015 and 2019) from patients of the "Sfânta Parascheva" Infectious Diseases Hospital in Iași were analysed. Patients who agreed to take the samples filled a questionnaire designed to collect information regarding demographic and clinical dates, or the place of origin.

The analysis of 176 human serum samples, revealed a prevalence of specific anti-WNV antibodies (IgG) of 3.4% (6/176). Following the testing of 88 samples, collected in 2019, for the detection of anti-WNV IgM, a seroprevalence of 9.1% (8/88) was observed. Regarding the detection of anti-USUV (IgG) antibodies, out of 88 serum samples collected in 2019 and tested, there was no positiveness.

Chapter VII, entitled " *High-Throughput detection of moboviruses*" encompass the results obtained from vector surveillance conducted in two counties from the eastern part of the country. This

chapter includes the results obtained from the detection and isolation of viral pathogens from mosquitoes, through molecular biology techniques and in vitro culture techniques.

In the framework of this study were analysed mosquitoes captured during the transmission seasons in seven collection points, distributed within two counties in the eastern part of Romania (Iași and Tulcea), in 2018 and 2019.

Mosquitoes were captured using four types of automatic and manual devices. The collected specimens were stored at -20 ° C for 5 minutes and subsequently identified based on morphological characteristics and gonotrophic stage (fed / not fed, pregnant) on ice plates. Subsequently, the specimens were grouped into pools according to the date of collection, sex, species, collection point and gonotrophic stage. For arboviruses detection, fed females were grouped in pools of 1 to 19 specimens, and unfed females were analysed in monospecific pools containing 20 up to 50 specimens per pool.

The total RNA from monospecific mosquito pools was extracted automatically. To obtain complementary DNA, reverse transcription of extracts was performed using the Reverse Transcriptase Master Mix kit (Fluidigm Corporation, USA) while the pre-amplification of reactions was performed using the PreAmp Master Mix Kit (Fluidigm Corporation, USA).

Eventually, pre-amplified cDNA samples were tested for the detection of 41 mosquito-borne viruses by the microfluidic real-time PCR assay, using the BioMark real-time PCR system (Fluidigm, USA) developed by Moutailler and others, 2019.

The corresponding samples to the positive signals in microfluid real-time PCR assay were subsequently tested for confirmation, by a series of techniques that followed each other in cascade. Thus, the positive samples were retested by real-time TaqMan PCR, isolated on cell cultures (Vero and C6 / 36), specific sequences were obtained, by Pan-flavivirus RT-PCR and sequencing.

Out of the 17.694 mosquitoes identified, 16.827 mosquitoes were analysed and grouped in 365 pools, and subjected to viral detection.

In total, 11 species of mosquitoes and an unspecified taxon were collected and identified. Of these, six species and the unidentified taxon were analysed for the presence of possible arboviruses.

As a result of microfluidic real-time PCR method, out of 365 tested mosquito pools, 11 pools belonged to three taxa (*Culex pipiens* s.l, *Culex modestus* and *Aedes vexans*) were positive for arboviruses. The most commonly detected virus was West Nile - in ten samples and one pool was positive for Sindbis virus.

The WNV positive samples by real-time microfluidic PCR technique were confirmed performing a duplex real-time PCR, targeting for amplification the 3'NC and ProC regions of the genome.

Confirmation of the positivity of molecular results, followed by in vitro isolation and disseminated of the virus on mammalian cell cultures and insect-derived cell cultures, revealed the appearance of rounded and refractive cells in Vero-type cell cultures four days post-infection. The cytopathic effect of degenerative changes was noticed in three flasks of infected cell cultures. A first passage on mosquito-derived cells was performed for samples initially inoculated on mammalian cell cultures. The supernatant from both types of cell cultures was subjected to viral genome detection by real-time PCR, using as targets, the 3'NC and ProC regions of WNV.

The virus was detected in the cell culture supernatant from a single pool, using the ProC region as a target.

Subsequently, amplification by Pan-flavivirus PCR (NS5 region of interest), two sequences could be obtained, and the bioinformatics analysis of the fragments indicated that the isolates belong to WNV, genetic line 2.

For the sample positive for Sindbis virus, the interaction between the inoculum and the cell monolayer did not reveal the appearance of any morphological changes of the cells, their detachment or individualization.

This thesis brings a significant contribution and emphasizes the dynamics of WNV circulation, supported by a wide range of receptive vectors and hosts, in the areas covered in framework of the study.

The **last chapter**, "*Final Conclusions*" focuses on the essential conclusions drawn from the actions of vectors surveillance and four seroepidemiological investigations for the detection of specific antibodies against flaviviruses.

Viral amplification by classical PCR, for Sindbis virus envelope protein "E", for mosquito RNA, from Vero cell supernatant and C6 / 36 line supernatant, did not confirm the positive signal obtained after the initial amplification technique.

The novelty of this thesis is given by the first molecular reporting of Sindbis virus and Usutu virus, by serological tests.

Regarding the investigations on the two viruses mentioned above, synchronized entomological, molecular and serological researches are needed, over a wider area, in order to succeed the isolation and characterisation of Sindbis and Usutu viruses.

However, the results obtained serve as basic data for further studies that will focus on the study of vector populations and the implementation of an interdisciplinary system of active and passive surveillance for the control of arboviruses with emergency potential in Romania.