SUMMARY

Key words: phylogenetic analysis, antirabies antibodies, NGS, rabies, sequencing.

The PhD thesis with the title ***"Research regarding diagnostic methodology, surveillance, prevention and control in rabies"*** comprises 199 pages and according to the rules in force it is structured in two main parts: the first part, entitled *"Current Stage of Knowledge"*, comprising 18 pages and the second part *"Personal Contributions"*, extended on 133 pages, to which is added the content, the introduction, the summary, the annexes and the related bibliography.

The first part, ***„Current state of knowledge",*** consists of two chapters, which briefly describe the etiological and epidemiological characteristics, but also the methodology for diagnosis, surveillance, control and eradication of rabies infection in animals, with the latest updates in the field of literature from abroad, as well as from our country.

The second part ***„Personal Contributions"*** is structured in 9 chapters, each detailing the results obtained during doctoral studies, the latter summarizing the final conclusions of the researches.

In performing the doctoral thesis, 59 brain samples (38 from north-eastern Romania and 21 from Republic of Moldova), 72 fox mandibles from Moldova region counties, 392 samples of fox thoracic liquid and 312 samples (sera and thoracic liquid) from wild boar were studied.

CHAPTER III, ***„PhD thesis aim and objectives and the organizational framework of the researches"*** shortly describes the institutions and the organizational frames in which the PhD researches were conducted next to the purpose and the proposed objectives.

Taking into consideration the importance of the disease, the small amount of researches conducted until the present in our country and also the lack of information regarding Republic of Moldova (the second country taken into study) the researches performed during the PhD thesis had the purpose to realize an epidemiological statistic of the rabies cases from the north-est of Romania and also Republic of Moldova, for a period of 7 years in order to gain a better understanding of the actual situation and also to establish an accurate prevalence of the disease for both domestic and wild animals. Another important objective was to perform an molecular epidemiological study, using a phylogenetic analyses of the rabies virus isolates from the study area, with the purpose to follow the circulation, distribution and evolution of the rabies virus next to the molecular viral adaptation mechanisms.

CHAPTER IV entitled ***„Epidemiological investigations regarding the presence and prevalence of rabies infection in animals in north-east of Romania and Republic of Moldova",*** presents the results obtained after the epidemiological statistic conducted for the

period 2010-2016 for the 8 counties of Moldova region and Republic of Moldova, obtaining a complete view regarding the situation of positive rabies cases.

The prevalence of positive rabies cases in domestic animals from the total of 781 samples received at the laboratory with rabies suspicion, collected between 2010-2016 from the north-est of our country was 24,07%. The most affected domestic species was bovines with a total of 103 cases (13.19%); most of them confirmed in Iasi county area with a total of 48 cases (6,14%). The prevalence of positive rabies cases in wild animals from the total of 1314 samples collected in the same period was 23,05%. The most affected were foxes with a total of 284 cases (21,61%); the highest number of positive cases in wild animals was reported in Suceava county with a total of 93 cases (7,07%).

Regarding the rabies cases situation in the Republic of Moldova, from the total of 1150 samples collected from domestic animals during 2010-2016, 573 were found positive with a prevalence of 49,82%. The highest number of confirmed cases were from bovines with a total of 255, representing 22,17%. From the total of 305 samples collected from wild animals from Republic of Moldova, 191 were confirmed to be positive with a prevalence of 62,62%. The most affected wild species was the fox with a total of 158 positive cases (51,8%).

CHAPTER V ***„Rabies diagnostic confirmation by direct fluorescent antibody test"*** had the purpose to reconfirm the rabies diagnostic of the samples collected for our study, before their molecular characterization, in order correlate the results but also to highlight the optimal conjugate for obtaining precise and valid results, in this way being tested 3 different conjugates.

Thus, out of the total of 59 retested samples, 43 were found positive and 16 negative by the FAT technique. The samples DR1016, DR1199, DR1202, DR1344, DR1354 and DR1355, considered to be positive by the laboratories from where they were collected, after our testing they were found to be negative using FAT and also other techniques (RTCIT, RT-PCR conventional, Real time qRT-PCR, no viral RNA being detected).

Our results underline the importance of using a good quality conjugate in order to obtain a correct and precise diagnostic. In the majority of cases, the quality of the tested samples together with that of the conjugate have a major importance in obtaining a valid result. Not taking this factors into consideration can lead to the possibility of obtaining false positive or false negative results. Even if a false positive result doesn't have too severe consequences a false negative one can have tragic outcomes if human victims are involved.

During the comparative study regarding the 3 types of conjugate used, the best results were obtained using the Bio-Rad conjugate, the results being far superior to the other two, offering a high intensity fluorescent signal next to a dark background, being capable to identify the viral antigen in 100% of the microscopes fields examined.

CHAPTER VI ***„Rabies diagnostic confirmation by rabies tissue culture infection test (RTCIT)"*** is structured in 3 subchapters and had the purpose to confirm the rabies diagnostic using RTCIT as an alternative method to the mouse inoculation test, to highlight the optimal cell line and also the ideal system for the isolation and multiplication of the rabies virus.

The researches performed during the subchapter ***„Isolation of rabies virus on cell cultures (RTCIT) as alternative to the mouse inoculation test"*** were done for all the 59 samples taken into study using the cell line N2a, with a concentration of 8x105 cells/ml, in Labtek chamber, using the Bio-Rad conjugate, the results being in accordance with those obtained by FAT. Taking into consideration the fact that rabies has a major medical impact for humans, even if false negative results are rare, an quick alternative method has to be available as a back-up, RTCIT meting all the criteria.

The second subchapter, ***„Comparative study regarding the sensitivity of two different cell lines N2a and BHK-21 used for isolation of rabies virus on cell cultures"*** was realized with the purpose to evidentiate and confirm the optimal cell line for isolating and multiplication of the rabies virus on cell cultures. For this, a total of 20 samples were taken into study, two different cell lines being used: N2a and BHK-21. Regarding the cell line N2a, out of the 20 tested samples all (100%) were positive for a incubation period of 48h and 19 (95%) for 72h. In comparison for the cell line BHK-21, 19 (95%) samples were positive for a incubation period of 48 h and only 14 (70%) samples for 72 h.

Evaluating the results obtained for the 2 cell lines, we conclude that N2a is more sensitive for the multiplication of the rabies virus that BHK-21, being preferred the incubation period of 48 h. Although both cell lines are used as a reference for the rabies virus, the N2a cell line is more sensitive, the results being superior to those obtained for BHK-21 cell line.

The last subchapter **„The use of two different systems for the isolation of rabies virus on cell cultures"** had the purpose to achieve RTCIT using two different systems, namely: titration microplates (n= 96 wells) and Labtek glass chambers (n = 8 boxes) in the idea of observing and determining whether the rabies virus multiplied as well in both types of support. These investigations were performed on a total of 16 samples using the N2a cell line with different concentrations for the two systems mentioned above.

Thus, in the LabTek glass system, a single cell concentration of $8x10^5$ cells/ml was used, with a 48 h incubation period, all 16 samples (100%) tested being positive, this system being typically used in the technique RTCIT to confirm the diagnosis of rabies.

The second ELISA microplate procedure was performed using 2 different cellular concentrations: $5x10^5$ cells/ml and $2,5x10^5$ cells/ml, with a 72h incubation period, each sample being inoculated in duplicate. Thus, for the cell concentration of $5x10^5$ cells/ml, out of the 16 samples tested, 6 (37.5%) were negative and 10 (62.5%) positive, the same situation being recorded for the concentration of $2.5x10^5$ cells/ml. The DR1025, DR1027, DR1028, DR1030 and DR1032 samples were negative for both cellular concentrations. For two more samples, the results were different. Thus, DR1029 was positive for the first cell concentration, but negative for the second. In contrast, DR1031 was negative for $5x10^5$ cells/ml, but positive for $2,5x10^5$ cells/ml.

The best results were obtained in the LabTek system, the cell concentration of $8x10^5$ cells/ml being considered to be optimal for good isolation and multiplication of the rabies virus. With respect to plastic microplates, the results were inconsistent, yielding satisfactory results for cell concentrations per ml of $5x10^5$ and $2.5x10^5$, but not for those below $2x10^5$ cells/ml, since the small number of cells did not allow isolation, respectively multiplication of

the virus, and their use is not recommended. The results obtained for microplates may have been influenced by repeated freezing and defrosting, along with storage conditions, resulting in a loss of infectivity.

CHAPTER VII entitled ***„Rabies diagnosis confirmation by real-time PCR and conventional RT-PCR techniques"*** was performed with the purpose to confirm the results obtained using the reference techniques as an additional lyssavirus detection tool.

Thus, RNA extractions of all of the 59 samples taken in the study were realized, followed by quantification of nucleic acids by *One step Real time SYBRGreen qRT-PCR* technique using a real-time cicler, obtaining a total of 43 positive and 16 negative samples, results consistent with those obtained from reference techniques. In the case of the samples tested, the determination coefficient R2 had a value of 0.98-0.99, considered satisfactory and the PCR efficiency was ranged between 1-1.04 and was ideal for the standard curve. The fluorescence signal emitted by SYBRGreen coupled with the PCR product of the test samples was emitted between cycles 6-14, the Ct values obtained varied between 13.71 and 23.64 for confirmed positive samples, and the number of copies / reaction ranged between 1,37E + 05 and 1.34E + 08. These values indicate that some samples are more concentrated in the rabies virus than others, the concentration of samples being also reflected in the difference of number of cycles needed to obtain the fluorescent signal.

The next step consisted in partial amplification of the rabies virus N gene by means of a *conventional Heminested RT-PCR* using primer pairs JW12 / JW6 and JW12 / JW10, which allow specific amplification of the nucleoprotein for all 15 rabies virus species, a 606-bp amplification product was obtained. Following testing of 47 samples from the total of 59 included in our study, 43 were positive and 4 negative, consistent with IFD and RTCIT.

For complete N gene sequencing, it was amplified using also a *conventional Heminested RT-PCR* using JW12 / PVN8bis and M13-20JW12 / M13RevPVN8bis primers on a number of 43 samples, yielding a total of 41 positive and 2 negative samples.

The final step in this chapter consisted in performing the *TaqMan Real time qRT-PCR technique*, which aimed at framing samples in one of the genotypes 1 (RABV), 5 (EBLV-1) and 6 (EBLV-2) found in Europe by the use of LysGt1, LysGt5 and LysGt6 fluorescence probes, which allow specific amplification of rabies nucleoprotein genotypes.

For the detection of the first genotypecRABV, the technique was performed on 47 samples (DR1016-DR1036, DR1187-DR1202, DR1331-DR1336, DR1343-DR1358), 43 of which were positive and 4 negative, Ct ranging between 19.57 and 26.97. For the detection of EBLV-1 and EBLV-2 genotypes 5, the technique was performed for the first samples studied in 2015, namely DR1016-DR1036, all samples being negative, except for positive controls that matched the standard.

The positive results obtained for the first genotype and negative for genotypes 5 and 6 confirm and frame the rabies virus isolates within the first RABV genotype.

CHAPTER VIII ***„Molecular characterization of rabies virus strains from north-east of Romania and Republic of Moldova"*** was performed with the purpose of realize sequencing and phylogenetic analysis of the partial and complete gene N. Of the total samples

sent for sequencing at the Genoscreen Company in Lile, France, the sequence of 39 samples for the partial N gene and 37 samples for the total N gene was succeeded.

For the phylogenetic analysis, the genetic variability of the various isolates included in our study was assessed using the Neighbour Joining (NJ) method along with other programmes and softwares.

A phylogenetic tree was made for the partial and complete N gene in order to determine whether the generated tree show the same topology. Thus, it was performed by comparing the sequences obtained of the partial gene (n=39) and full (n=37) N gene with reference nucleotide sequences extracted from the GenBank international database. It was evaluated for all trees generated by the NJ method, tree confidentiality. The most commonly used statistical method, bootstrap, was calculated in the phylogenetic analysis program (MEGA6, the bootstrap option).

Following the phylogenetic analysis carried out in our study on samples from north-eastern Romania, but also from the Republic of Moldova, was showned a single phylogenetic group: NEE (North-Eastern Europe).

This group has a broad geographic distribution and has been identified over the years in various countries such as Poland, Estonia, Lithuania, Bulgaria, Eastern Slovakia, Ukraine and Russia, as demonstrated by Bourhy et al., 1999, Kuzmin et al., 2004, McElhinney et al., 2006, Picard et al., 2012 and Robardet et al., 2013. In these countries, the isolates came from both foxes and ratons, which highlights the fact that both species are reservoirs for this variant of the virus. In our study, the NEE group was identified in all tested species, namely: dog, cat, bovine, fox, wolf, deer and ferret, which demonstrates that this variant can adapt to different animal species.

Until now, the complete sequencing of rabies virus nucleoprotein has not been performed in our country, implicitly a related phylogenetic analysis, these being the first data reported so far. Regarding the results obtained for the studied samples from the Republic of Moldova, these are singular, so far no partial and full N nucleoprotein sequencing, respectively a phylogenetic analysis, has been carried out, with no knowledge of the variants of the rabies virus circulate on the territory of that country.

Regarding the phylogenetic analysis carried out for the N-E part of our country, the NEE phylogenetic group was identified, being first signaled for its presence in Botosani, Iasi, Vaslui, Galati and Vrancea counties. The complete sequencing of the nucleoprotein was performed for the first time on samples collected from our study areas, no complete sequences being reported until now.

CHAPTER IX, entitled ***„The complete sequencing of rabies virus full genome”***, aimed to characterize the entire genome of the rabies virus by using a new generation of sequencing on a selective number of samples to identify possible pathogenicity changes at the animal species level and for a better understanding of the diversity of lyssaviruses.

Next generation sequencing was performed for the first time in our country and Republic of Moldova on samples from both domestic and wild animals.

Following the bioinformatic analysis undertaken, our samples showed a similarity percentage of the five rabies genes sequences between 96-100% for N, P and M genes, 97-100% for G gene and 97-99% for L gene.

As a consequence of studying the amino acid positions of the rabies virus gene G, known to be involved in pathogenicity, no changes have been identified that may suggest differences in pathogenicity at the animal species level.

For a better understanding of the diversity of lyssaviruses and pathogenicity differences, in-depth studies involving a larger geographic area and a larger number of samples are required.

CHAPTER X ***„Researches regarding the surveillance and control strategy of animals rabies infection in the north-east of Romania"*** aimed at verifying the effectiveness of surveillance campaigns in the north-east of our country by determining the post-vaccine antirabies antibodies and determinate the vaccine marker in foxes. Also, in order to confirm or not the hypothesis that wild boar would be potential competitors for these baits, a number of samples have been collected and tested in this respect.

Within the first subchapter ***„Detection of antirabies antibodies and determination of tetracycline in foxes",*** the study area was represented by the eight counties in the Moldavian region of our country, together with the Maramureş and Buzău counties, where oral rabies vaccination was performed in foxes, 392 samples of thoracic liquid and 72 mandible samples were tested.

Detection and titration of antirabies antibodies for 392 samples was performed by ELISA method, 345 of which were processed using the BioPro Rabies ELISA Ab kit (BioPro, Prague, Czech Republic) and the remaining 47 using the Platelia ™ Rabies II kit for veterinary use (BioRad, France) kit.

Thus, in the first kit, out of the 345 samples of foxes, 98 were positive (28.40%) and 247 negative (71.60%). Of the 345 samples of foxes processed, 247 (71.60%) recorded a PB <40%, 47 (13.62%) were in the category of a PB of 40-70% and the remaining 51 (14.78%) in the latter category with a PB> 70%. A sample is considered positive if PB is equal to or greater than 40% and negative if PB is less than 40%, in order to evaluate the effectiveness of vaccination campaigns, with a PB value of ≥40% recommended.

Following testing of the 47 samples via the Platelia Rabies kit, 14 of these were positive and 33 negative for antirabies detection. Out of the total of 47 samples tested using this kit, none of the samples were in the first category of > 4 EU / ml, and for 24 of them there was no evidence of seroconversion, being below 0.125 EU / ml. An insufficient level of antirabies antibodies was detected in 9 samples, ranging from 0.125-0.5 EU / ml, considered to be nonprotective in the case of rabies virus infection. Only 14 samples ranged from 0.5-4 EU / ml, with an antibody titer sufficient to contracting a rabies virus infection.

As for the determination of the tetracycline vaccine marker in the teeth or fox mandibles, out of a total of 72 samples tested, 60 of these were positive and 12 negative. In our samples, fluorescent lines in the cement were detected only in 2 samples out of the total of 72, in dentine 33 samples, and 60 in the bone. Regarding the intensity of the detected

fluorescence signal, it ranged from + for 17 samples, ++ for 13 samples and +++ for 30 samples.

The second subchapter ***„Detection of antirabies antibodies in wild boars"*** was made in order to determine whether wild boar interfere with the effectiveness of these campaigns by the uncontrolled consumption of vaccine baits, in this sense being tested 312 samples (serum and thoracic liquid) resulting from the hunting campaigns carried out during the period 2014-2016.

Thus, of the 312 samples of wild boars tested, 132 were positive (42,30%) and 180 negative (57,70%) by ELISA serological method. To assess the sensitivity of the ELISA, 56 of the positive confirmed samples in this test were tested in parallel and using the FAVN reference technique, with a sensitivity of 96.43%. For 2 of the 56 samples tested by FAVN, the antibody titre was 0,02 IU / ml and 0,22 IU / ml respectively, with results inconsistent with those obtained in ELISA.

Detection of antirabies antibodies from wild boar samples by ELISA revealed a surprising number of positive samples (132/312), these being the first data reported in this species to date in our country. Our results highlight the fact that these wild animals represent a real danger to the good outbreak of fox oral vaccination campaigns, being the main competitors for bait consumption.

In conclusion of these researches, a number of 24 final conclusions are summarized.