S U M M A R Y

of the doctoral thesis entitled:

IMMUNOLOGICAL PROFILES IN CANINE BLOOD DONORS

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The main purpose of this research was to outline the canine blood donor's immunological profile in order to contribute to the development of this department within veterinary medicine. The experiments within the thesis pursued several plans, as followed: determining the prevalence of DEA 1 blood group among canine blood donors participating in the pet blood bank in Romania; establishing the prevalence of infection with *Dirofilaria immitis*, *Anaplasma phagocytophilum/Anaplasma platys*, *Ehrlichia canis/Ehrlichia ewingii* and *Borrelia Burgdorferi* among canine blood donors participating in the pet blood bank in Romania and studying the dynamics of post-vaccination antibodies among canine blood donors participating in the pet blood bank in Romania by analyzing the anti-rabies immune response, examining the dynamics of antibodies against *Leptospira spp*, studying the dynamics of the immune response against canine parvovirus and Carré disease virus and researching the dynamics of antibodies against canine adenovirus and canine parainfluenza virus in blood donor dogs.

The doctoral thesis entitled **"Immunological profiles in canine blood donors"** was structured according to the legal stipulations in two main parts, the first part – Bibliographic study and the second part – Personal research.

The first part includes four chapters presented in 34 pages that represent approximately 28% of the entire thesis. These chapters describe the most actual data of the study theme which is sustained by past information.

The first chapter (*"Transfusion therapy in veterinary medicine"*) is composed from two sub-chapters and presents the most important details from the veterinary

transfusion medicine domain, starting from the first transfusion performed from an animal to another animal and finishing with the current veterinary transfusion practices.

Current practices in veterinary transfusion medicine focus primarily on the use of blood byproducts, blood group typing, and compatibility testing.

Current practices within the veterinary blood banks focus on how to select and monitor canine donors (vector-borne infectious diseases and blood group typing as a current practice), also highlighting the increased monitoring of blood donors compared to what was previously reported, an increase due to improved knowledge and understanding of the practices required for a safe blood transfusion.

In **the second chapter** (*"Canine blood groups and blood-typing methods in dogs"*) within the four sub-chapters there are described the blood groups in dogs, the blood typing methods by laboratory testing and the profile of a *universal canine blood donor* in terms of desirable antigens is drawn.

The DEA system is described in more detail, emphasizing the importance of the DEA 1 antigen, for which there are currently commercial tests available in hospitals and veterinary clinics. Other important antigens in veterinary transfusion are also mentioned: Dal, Kai, System D with antigens D1 and D2, type C. Current knowledge on these new and still less known antigens is still limited, and their prevalence is still undetermined and insufficiently studied to be able to appreciate their importance during transfusions. However, practicing veterinarians must be aware of the possibility of transfusion sensitization to a wide variety of red cell antigens even when transfusing DEA group 1 negative blood, the so-called universal donor blood.

The DEA 1 blood group/antigen identification methods briefly presented are tests that are no longer used in veterinary medicine, but were a starting point for the creation of current tests, such as the blood group identification method in tubes, and current methods of blood typing, such as the blood typing card method (Rapid Vet) and the immunochromatographic method (Alvedia), ending with the automatic blood typing method, the most recently developed method.

The broadest chapter of the first part is **the third chapter** (*"The selection and biological status of canine blood donors"*) consists of two sub-chapters in which the selection process of canine blood donors is described, starting from the origin of the donors and ending with their selection criteria, the emphasis being on individual characteristics, on the control of the health status of the canine donor and, not least, on the biological investigations prior to blood collection, more precisely the investigation for vector-borne infectious diseases.

Most blood banks in veterinary clinics rely on a combination of sources in terms of blood donors: the animals of the clinic employees, the clinic's patients, but also the so-

called "donor colony", specially purchased dogs to maintain the donation program from the clinic or blood bank.

The criteria for a desirable canine blood donor are age (1 – 8 year old), weight (over 22.7 kg), medical history (no previous blood transfusions, no current medication, no current pregnancy), negative status for transmissible infectious diseases vectors (*Babesia spp.*, *Ehrlichia spp.*, *Neorickettsia spp.*, *Leishmania spp.*, *Trypanosoma cruzi*, *Brucella canis*, *Dirofilaria immitis*), protected from fleas, ticks and mosquitoes, vaccinated according to the vaccination schemes agreed at the level of the country in which they live, with calm, docile temperament and with a responsible, reliable owner who is involved in providing optimal conditions for the animal's well-being.

The fourth chapter (*"The postvaccinal immune response in dogs"*) consists in three sub-chapters and briefly outlines elements of the post-vaccination immune response in the dog, focusing on the immune response and antibodies, immunological memory and immunological tolerance.

An important part of this chapter is represented by the description of the active immunization by vaccination, listing the basic criteria of vaccines, types of vaccine, revaccination and the duration of immunity provided by vaccines, while also pointing out the main adverse effects of vaccination.

The second part is divided into three chapters set out in 87 pages, representing approximately 72% of the whole thesis. The part of **personal research** includes the materials and work methods used within the thesis experiments, the results and the conclusions obtained, including 5 figures, 20 graphs and 25 tables.

The first chapter of this part, **chapter five** of the doctoral thesis, entitled *"Research on the prevalence of blood group DEA 1 among canine blood donors participating in the blood bank for animals in Romania"* aimed to study the prevalence of the DEA 1 antigen in the donor population of a blood bank for animals in Romania, exposing the fact that, at the moment, the specialized literature does not provide information about the prevalence of this antigen among dog breeds autochthonous or generic at national level, the field of transfusion medicine and conservation of canine blood within domestic or commercial blood banks being in the pioneering stage compared to Western Europe or North America.

Emphasis is placed on knowing the differences and the so-called predisposition of breeds towards a certain blood group to increase the efficiency of recruiting new canine blood donors.

The study took place between January 2015 and December 2016 in the in-house Laboratory of Hemopet Pet Blood Bank, Bucharest, Romania. Between 2015 and 2017, 105 of the blood bank's 766 canine donors were tested for DEA 1 blood typing. A total of 105 whole blood samples collected on EDTA were examined in this study using the

Lab.Test BT DEA 1 immunochromatographic test (Alvedia Veterinary Diagnostic, Lyon, France) following the manufacturer's specifications. Briefly, 3 drops of the buffer solution from the test kit were pipetted into a test tube without additives. Afterwards, 10 μL of the whole blood sample collected on EDTA was added. The sample was homogenized in the test tube by several successive pipetting. Afterwards, the immunochromatographic strip was inserted into the created suspension, red cells diffusing along the entire length of the strip. Erythrocytes that contained the appropriate antigen formed a visible line on the membrane indicating DEA 1 positive (red line next to DEA 1) or negative (no red line next to DEA 1). The waiting time for complete red cell migration and reading the result was 5 minutes.

Following the results evaluation, it emerged that the prevalence of DEA 1 positive blood group among the tested donors from the blood bank population was 56.19% (59/105), a result consistent with the results of specialized studies up to this point at the international level. Of the total canine donors tested DEA 1 positive, 37.29% (22/59) were male, 88.13% (52/59) were purebred dogs and 11.87% (7/59) were mixed breed.

Considering the results regarding blood group frequency within breeds, most Golden Retriever dogs tested DEA 1 positive (86.67%; 13/15) and most Labrador Retriever dogs (83.33%; 10/12) tested DEA 1 positive.

For DEA 1 negative blood group, the prevalence was 93.48% (43/46) in purebred dogs tested, with the specification that all Doberman dogs (100%; 6/6) tested DEA 1 negative, and all Bull Terrier dogs (100%; 7/7) tested DEA 1 negative.

The prevalence of DEA 1 negative blood group in the tested males was 56.52% (26/46), and in the tested females it was 43.48% (20/46).

Until now, there are no published data on the prevalence of the DEA 1 antigen among native dogs in Romania, both for donors and recipients, so for this reason the results of our study cannot be compared with the results of other similar studies. Blood typing within the blood bank's donor pool will minimize the risks associated with dog blood transfusion.

Chapter VI entitled *"Research of the prevalence of infection with Dirofilaria immitis, Anaplasma phagocytophilum/Anaplasma platys, Ehrlichia canis/Ehrlichia ewingii and Borrelia burgdorferi among canine blood donors participating in the blood bank"* aimed to study the prevalence of infection with vector-borne infectious diseases in the population of blood donor dogs from a pet blood bank in Romania.

The novelty of this chapter lies in the contribution it brings to the shaping of the canine blood donor's immunological profile in Romania, considering that transfusion medicine and the field of blood banks for animals are still in the pioneering period in our country compared to Western veterinary medicine.

The study was carried out between January 2015 and December 2016 in the in-house Laboratory of Hemopet Blood Bank for Companion Animals, Bucharest, Romania and included 575 dogs identified as potential blood donors between January 2015 and December 2016 in a Romanian pet blood bank (Hemopet Blood Bank, Bucharest, Romania), of which 82 dogs were already tested in 2015 and subsequently re-evaluated at each blood donation in 2016.

Only 14% of all canine donors donated blood in both 2015 and 2016 and were serologically tested in both years, while 86% were tested in 2015 or 2016. For 24 months, 1253 blood samples were collected to perform serological testing using a Point-of-care ELISA test: SNAP 4Dx Plus® (Idexx Laboratories, Fremont, CA) following the manufacturer's instructions.

All blood samples, reagents and SNAP tests were kept for 30 minutes at room temperature (18 – 22°C) before use. Four drops of the enzyme-labeled conjugate were mixed with three drops of whole blood in a tube and subsequently added to the SNAP assay well. The mixture of blood sample and conjugate flows through the matrix, interacting with the test and control points on the surface of the matrix, reaching the activation circle in approximately 30 – 60 seconds. The device is then activated by depressurization or snap activation, which results in the release of wash buffer and substrate solution from the device's reagent reservoir. Positive results are visualized by the formation of colored reaction products; the examination is complete after 6 – 10 minutes, depending on the test. Color development at the level of positive control indicates that the reagents are working properly.

Following the statistical analysis, it was found that 14.20% (50/352) dogs were positive for Dirofilaria immitis in 2015 and 12.13% (37/305) in 2016. It was also found that the prevalence of dogs infected with *Dirofilaria immitis* retested after the first donation (donors already included in the Donor's Register) was relatively low compared to the number of canine blood donors: 1.32% (4/302) in 2015 and 1.48 % (4/270) in 2016, while the prevalence of dogs infected with *Dirofilaria immitis* tested before the first donation (donors not included in the Donor's Register) was relatively high compared to the number of potential donors selected: 13.06% (46/ 352) in 2015 and 14.79% (33/223) in 2016.

Screening for *Anaplasma phagocytophilum/Anaplasma platys Ac*. revealed 1.70% (6/352) positive animals in 2015 and 1.63% (5/305) positive animals in 2016. It should be emphasized that the prevalence of dogs infected with *Anaplasma phagocytophilum/ Anaplasma platys* retested after the first donation (donors already included in the Donor's Register) was relatively low compared to the number of canine blood donors: 1.13% (4/352) in 2015 and 0.89% (2/223) in 2016. Also, the prevalence of dogs infected with *Dirofilaria immitis* tested before the first donation (donors not included in the

Donor's Register) was relatively low compared to the number of potential donors selected: 0.66% (2/302) in 2015 and 1.11% (3/270) in 2016.

None of the 575 dogs evaluated through the 1253 immunological tests performed over a 24-month period (2015-2016) generated positive results for *Ehrlichia canis/ Ehrlichia ewingii* and *Borrelia burgdorferi*.

The results of this chapter support the importance of testing the existing canine blood donors in the blood bank database as well as potential canine blood donors for vector-borne diseases to increase the safety of the transfusion act in the dog and to limit the potential diseases induced in the recipient that the transmission of these vector-borne diseases would produce.

Chapter VII entitled *"Postvaccinal immune response in dogs – research on the dynamics of post-vaccination antibodies among canine blood donors participating in the blood bank"* is structured in four sub-chapters representing four different experiments that aim to study the dynamics of the post-vaccination antibody titer of blood donor dogs registered in the database of a pet blood bank in Romania.

Compared to the data available in the specialized literature, this chapter represents an element of novelty both at the national and international level, since until now there are no experiments or studies that follow the immunological profile of the canine blood donor or studies that follow the dynamics of post-vaccination antibodies between blood donations to assess whether or not the act of donation can influence the post-vaccination antibody titer.

In **the first sub-chapter** and experiment entitled *"Dynamics of the anti-rabies immune response in canine blood donors"* the dynamics of anti-rabies antibodies in the population of donor dogs of a pet blood bank in Romania is studied.

The study was carried out on the 22nd of November 2018, in the Serology Laboratory within the Discipline of Infectious Diseases, Preventive Medicine and Clinical Lectures by Species, Faculty of Veterinary Medicine Bucharest, Romania and it analyzed 76 serum samples from 16 dogs registered as blood donors at the Hemopet Pet Blood Bank in Bucharest, Romania.

The immunological status of the canine donors taken into the study was assessed using the indirect ELISA method using the PLATELIA™ RABIES II kit (BIO-RAD, France) following the manufacturer's instructions. The results were read spectrophotometrically at wavelength of 450 nm and were then recorded for statistical analysis. Optical densities (OD) for negative, positive and standard dilution curves validated the results.

Following the statistical analysis of the results, it emerged that all the blood donor dogs participating in the study benefited from an appropriate vaccination schedule, and the titer of anti-rabies antibodies in the group of 16 registered donors of the Romanian

Pet Blood Bank were not influenced by blood donation, as follows: 7 donors (#1, #2, #4, #8, #9, #10, #13) recorded OD > ODC + 0.5 at each test; 6 donors (#6, #7, #11, #14, #15, #16) recorded OD < ODC + 0.5 at each test and 3 donors (#3, #5, #12) recorded booster OD values after vaccination.

The second sub-chapter comprises the study entitled *"Dynamics of the anti-Leptospira spp. antibodies in canine blood donors"* and aims to evaluate the immune status against *Leptospira spp.* of canine donors from a blood bank in Romania and to assess if the post-vaccination antibody titer is influenced by blood donation and/or its frequency.

The study was carried out between October 2016 and January 2020 on 17 dogs (5 males and 12 females), regular donors of the blood bank for animals in Romania, using a commercially available diagnostic kit and the logistical resources of the Laboratory of Serology at the Pasteur Institute, Bucharest, Romania, by testing blood samples taken at each successive donation and using the NovaTec VetLine Leptospira ELISA qualitative ELISA test kit, Immundiagnostica GMBH, Germany. Donors were divided into 3 groups: dogs with 6 donations, dogs with 5 donations and dogs with 4 donations.

80 blood serum samples were used in this study, aliquoted and stored in a freezer (-70 - -20 °C) until the date of testing (January 29, 2020). In accordance with the diagnostic kit manufacturer's recommendations, heat inactivation of the samples was not performed.

Subsequently, the manufacturer's recommendations were followed, and the average value of the absorbance of the Cut-off control was used to calculate the results, and the calculation formula provided by the manufacturer was applied to express the results in NovaTec Units (NTU). To interpret the results, the following values were considered: Cut-off: 10 NTU; Positive > 11 NTU; Inconclusive 9 – 11 NTU; Negative < 9 NTU.

Following the statistical analysis, it was found that the results of the study on the dynamics of the immune response in dogs donating blood revealed insignificant variations of anti-Leptospira antibodies during repeated blood donation, results consistent with those reported in the case of anti-rabies antibodies in the previous subchapter. The NTU values of anti-Leptospira antibodies obtained in the group of 17 investigated canine blood donors were not significantly influenced ($p<0.005$) by the number of blood donations, but the results showed that not all dogs benefit from an adequate vaccination schedule, and regular vaccination of dogs is of the utmost importance for the protection of animals throughout their life.

No data is available on the evaluation of the dynamics of anti-Leptospira spp. serum antibodies in canine blood donors and whether the antibody titer for both Leptospira species changes between blood donations, so the present experiment cannot

be compared with the results of other studies or experiments, emphasizing the originality and the element of novelty brought by our study in the field.

In **the third sub-chapter** from chapter VII entitled *"Dynamics of the anti-parvovirus antibodies (CPV) and anti-Distemper virus antibodies (CDV) in canine blood donors"* the evaluation of the dynamics of post-vaccination anti-CPV and anti-CDV antibodies among the same group of 17 canine blood donors from the previous chapter between blood donations was aimed at determining whether it is influenced or not by the act of donating blood.

Parvovirus titration was performed in December 2019 and Carré disease virus titration was performed in January 2020. Both studies were carried out in the Serology Laboratory of the Pasteur Institute, Bucharest, Romania. Serum samples (n = 80) were collected from healthy canine donors (n = 17; 5 males and 12 females) who are regular blood bank donors in Romania. The immune status of investigated canine donors was assessed using VetLine Canine Parvovirus (CPV) and VetLine Canine Distemper Virus (CDV) qualitative ELISA kits from NovaTec (Immundiagnostica GMBH, Germany), following the manufacturer's specifications. The results were read spectrophotometrically at a wavelength of 450 nm. To interpret the results, the following values were used as a guide: for CPV (Positive at > 11 NTU; Inconclusive at 9 – 11 NTU; Negative at < 9 NTU), and for CDV (Positive at > 7 NTU; Inconclusive at 6 – 7 NTU; Negative at < 6 NTU), the unit of measure NTU being calculated according to the method described by the manufacturer.

After analyzing the results, it was found that in the case of CPV, the overview of the NTU results revealed insignificant variations within the group of D1-D6 values ($p<0.01$, $F<F$ crit), and the NTU values were above the minimum threshold used as reference value (>9 NTU) within all groups, ranging between 10.03 NTU and 23.55 NTU. One dog (BNA11FP) had an inconclusive NTU value (9-11 NTU) for Canine Parvovirus in 3 out of 5 blood donations (10.03; 10.78; 10.55), recording a booster value of 12.78 NTU following the annual vaccine, but is it should be emphasized that the NTU value never fell below the threshold of 9 NTU for the donor to be considered unprotected from vaccine-induced antibodies. In the case of CDV, the overview of the NTU results revealed insignificant variations between and within the D1-D6 groups ($p<0.01$, $F<F$ crit), and the NTU values were above the minimum reference value used (>6 NTU) for all groups, ranging between 8.39 NTU and 44.45 NTU, with no donor having inconclusive values (6-7 NTU) in any of the blood donations in all three analyzed groups, thus suggesting optimal immunization of canine donors even with frequent blood donations.

No data are available in the field literature regarding the dynamics of anti-CPV and anti-CDV serum antibodies in the case of canine blood donors, respectively if the antibody titer for both viruses undergoes changes between blood donations, so the

present experiment cannot be compared with the results other studies or experiments, representing an element of novelty in the field both nationally and internationally.

Sub-chapter four of chapter VII entitled *"Dynamics of the anti-adenovirus antibodies (CAV) and anti-canine parainfluenza virus antibodies (CPiV) in canine blood donors"* comes to complete the results obtained in the previous subsections and thus provide an overview of the immunological profile of the canine blood donor, following the dynamics of post-vaccination anti-CAV and anti-CPiV antibodies between blood donations.

The study on the dynamics of anti-CPiV antibodies in blood donor dogs was carried out between October 2016 and March 2020 in the Serology Laboratory of the Pasteur Institute, Bucharest, Romania, using the qualitative ELISA technique (NovaTec VetLine Parainfluenza Virus ELISA, Immundiagnostica GMBH, Germany) according to the working procedure recommended by the manufacturer, and the study on the dynamics of anti-CAV antibodies was carried out between October 2016 and June 2021 within the discipline of Infectious Diseases and Preventive Medicine, Faculty of Veterinary Medicine in Bucharest, Romania, using the qualitative ELISA technique (Canine Adeno Virus Ab ELISA, DRG International, Inc., USA), according to the working procedure recommended by the manufacturer.

According to the results obtained in our study, it was observed that not all sera taken from dogs that were vaccinated 2-6 months ago had anti-CPiV antibody titers higher than 9 NTU (n=10, 12.5%), but the heterogeneity and the small number of subjects with negative ELISA results could not be associated with the number of blood donations, and the statistical analysis shows that the number of donations did not significantly influence ($p<0.05$) the interpretation of the ELISA results (i.e. , positive, inconclusive or negative).

Of 80 ELISA tests performed to assess the dynamics of anti-CAV antibody titers, 23 tests provided negative results (of which 10 in dogs vaccinated 2-6 months before blood donation and 13 in dogs vaccinated >6 months before blood donation), 8 sera provided inconclusive results (of which 6 in dogs vaccinated 2-6 months before blood donation and 2 in dogs vaccinated >6 months before blood donation) and 49 sera provided positive results (of which 30 in dogs vaccinated 2-6 months before blood donation and 19 in dogs vaccinated >6 months before blood donation). The results thus obtained reveal insignificant variations of anti-CPiV antibodies during repeated blood donation, results consistent with those reported in the case of anti-rabies, anti-CPV, anti-CDV and anti-Leptospira spp. antibodies from the previous subsections.

Chapter VIII is dedicated to the *general conclusions and recommendations* synthetically formulated based on all the experiments' results.

The bibliography of the thesis lists 210 sources, from which approximately 10% are from the last 4 years (2020-2024 inclusive) and 42% are from the last 10 years (2014-2024 inclusive).

The thesis brings up the following **original elements**:

- Determining the prevalence of DEA 1 blood group among canine blood donors participating in the pet blood bank in Romania.
- Establishing the prevalence of infection with *Dirofilaria immitis*, *Anaplasma phagocytophilum/Anaplasma platys*, *Ehrlichia canis/Ehrlichia ewingii* and *Borrelia Burgdorferi* among canine blood donors participating in the pet blood bank from Romania.
- Studying the dynamics of post-vaccination antibodies (by analyzing the anti-rabies immune response, examining the dynamics of anti-Leptospira spp., anti-CPV and anti-CDV, anti-CAV and anti-CPiV antibodies) among canine blood donors participating in the pet blood bank from Romania to assess the potential effect of repeated blood donation on it and the possible need to institute a special vaccination schedule for blood donor dogs.