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NEOSPOROSIS IN CATTLE AND DOG. RESEARCHES REGARDING EPIDEMIOLOGY, DIAGNOSIS AND CONTROL OF THE DISEASE

Abstract of PhD Thesis

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ABSTRACT

*Neospora caninum* is a protozoan parasite closely related to *Toxoplasma gondii* (Dubey, 1999; Holmdahl et al., 1994). This intracellular parasite may infect warm blooded vertebrates from the entire world such as dogs, cats, cattle, sheep, horses, rats, foxes, goats, alpacas, lamas, deers, camels, buffalos and others (Cabaj et al., 2004, 2005; Dubey, 2005; Dubey and Dubey, 2003). In cattle the infection with this parasite causes major economic losses due to reproductive failure associated with abortion and congenitally infected calves mortality (Anderson et al., 1995, Cabaj et al., 2000; Davison et al., 1999; Moskwa and Cabaj, 2003; Wouda et al., 1999).

Dogs (*Canis familiaris*) have an important role in epidemiology of *N. caninum* infection as they are together with coyotes (*Canis latrans*) and wolves (*Canis lupus*) the only definitive hosts that eliminate oocysts in the environment. (Gondim et al., 2004; Dubey et al., 2011).

Some epidemiological studies have demonstrated a positive correlation between the presence of dogs and bovine abortions due to *N. caninum* infection (Pare et al., 1998). Other studies have shown that dogs raised near farms that previously had abortive episodes had a higher seroprevalence than the ones from urban areas (Ferroglio et al., 2004).

In Romania researches that study this disease are very few and if they exist they involve cattle and dogs from small areas so they are not able to offer nationwide data regarding this subject. That is why in this PhD thesis we took under consideration the next objectives:

1. A detailed study of neosporosis epidemiology in cattle from centre and north-west of Romania by a) studying the seroprevalence of this disease in cattle taken under study; b) determining the *N. caninum* infection in cattle by testing milk samples and c) identification of *N. caninum* genomic DNA in bovine abortion
2. Researches regarding *N. caninum* infection in dogs by a) evaluation of seroprevalence of neosporosis in dogs from the entire country; b) detection of *N. caninum* genomic DNA in organs collected from dogs from Cluj county and c) epidemiological studies of intestinal neosporosis in dogs;
3. Assessing neosporosis infection in other intermediate hosts represented by goats and horses;
4. Identification of natural infection with *N. caninum* in wild rodents, insectivores and wolves;
5. Experimental researches concerning cultivation of *N. caninum* tachyzoites on fibroblast of animal origin.

1. **Epidemiology of neosporosis in cattle**

   a) **Seroprevalence of neosporosis in cattle from center and north-west of Romania**

   For determining the seroprevalence of *Neospora caninum* infection in cattle raised in different raising systems and in those that previously had reproductive disorders we have taken under study a number of 1746 serum samples from animals from several counties in north-west...
and centre of the country: Alba, Bistrița, Cluj, Hunedoara, Maramureș, Mureș, Sibiu and Satu Mare. Researches were developed between February 2008 – April 2011. From the total number of 1746 samples, 244 were collected from animals with reproductive failure: abortions, repeated mounting (more than 3 for a gestation) and vaginal inversions. For detection of specific anti-\textit{N. caninum} IgG antibodies we used the Indirect ELISA method by applying two commercial kits HerdCheck \textit{Neospora caninum} Antibody Test Kit by IDEXX Laboratories, Switzerland and ID Screen \textit{Neospora caninum} Indirect by ID Vet, France.

We obtained a general seroprevalence of 29.3% (95% CI 27.2-31.5%), 511 samples out of 1746 being positive. Most of the positive samples were collected from bovines with at least one gestation, 29.4% (95% CI 27.2-31.7%). For heifers seroprevalence of neosporosis was 27.5% (95% CI 20.0-36.0%). The differences obtained in different age groups were not statistically significant, \( p = 0.3 \) (\( \chi^2 = 0.1349 \)).

Seroprevalence of neosporosis was different among raising systems. For animals raised in extensive system the value was of 23.5% (95% CI 20.6-26.6%) while for animals raised in intensive systems was 34.1% (95% CI 31.1-37.2%), \( p=0.0000005 \) (\( \chi^2 = 22.9007 \)). Males were slightly more affected (30/93, 95% CI 22.9-42.7%) than females of which 481 were positive (IC 95% 27.2-31.7%). In cattle raised for milk production antibodies against \textit{N. caninum} were detected in 33.4% (95% CI 30.5-36.5%). In the ones raised for meat 22.6% (CI 95% 9.6-41.1%) were positive and 24.0% (CI 95% 21.0-27.3%) of the ones raised for both milk and meat, \( p<0.001 \) (\( \chi^2 = 18.5593 \)).

Out of 1746 collected samples, 244 (13.97%) came from cattle with reproductive failure: repetitive mounting (77/244, 31.6%), vaginal disturbances (3/244, 1.2%) and abortions (164/244, 67.2%). The overall seroprevalence of neosporosis in this category was 38.9% (95% CI 32.8-45.4%). For the ones that had at least one abortion 39.6% (CI 95% 32.1-47.6%) were positive, in ones with repeated mountings 37.7% (CI 95% 26.9-49.4%), and in those with vaginal disturbances, 33.3%.

Out of 27 samples collected from cattle that aborted in first trimester of gestation, 8 presented antibodies against \textit{N. caninum}. From 106 cattle with abortion in second trimester, 44 were positive and from 30 that aborted in third trimester, 12 were positive. Depending on month of gestation, the values of neosporosis were between 0% in second month and 58.3% in 8th month (7/12).

\textbf{b) Detection of \textit{N. caninum} infection in cattle by testing milk samples}

The aim of this research was to detect for the first time in Romania of antibodies against \textit{N. caninum} in milk samples collected from animals which after being tested in the first chapter were diagnosed as positive. Researches were made between January 2008 and May 2011. A number of 189 milk samples were collected from cattle raised in three counties: Alba, Cluj and Satu-Mare. We applied the indirect ELISA method by using the Bio-X commercial kit ELISA Kit (Bio K 192) by Bio-X Diagnostics, Belgium.

After testing, 25 samples were positive (95% CI 8.7-18.9%), and the same number were doubtful. The highest prevalence was obtained in Alba county (12/58, 95% CI 11.2-33.4%), and the lowest in Cluj county (9/99, 95% CI 4.2-16.6%). Most of the samples were collected from cows raised in intensive systems where 13.5% presented antibodies against \textit{N. caninum} (95% CI
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8.7-19.5%). In the intensive system the prevalence was 11.1 (95% CI 1.4-34.7%). Mixed breed animals were significantly more affected (16.1%) than pure breeds (12.7%).

c) Identification of *N. caninum* genomic DNA in bovine abortions

To set up a working technique necessary for neosporosis diagnose in bovine abortions and to identify *N. caninum* genomic DNA in tissue fragments and liquids collected we used 53 fetuses with gestational age between 3 and 8 months. All of them were collected from pastures or farms after spontaneous abortion and all of them came from cattle raised on farms. They were thoroughly examined and brain, lung, heart, liver, muscle and placenta (if available) were collected. All of them were tested by PCR for *N. caninum* DNA detection. The research was developed between February 2008 and March 2011.

After necropsy, most of the abortions were of 4 month of age (14/53) followed by the ones of 6 month of age (6/53). Mean gestational age (±DS) was 5.1±1.5 months. Most of the abortions had a normal aspect (43.4%), followed by ones with mummified (32.1%) and emphysema aspect (24.5%). After microbiological testing for all animals bacterial cause of abortion was ruled out. According to age of gestation, most of the abortions took place in the 2nd trimester, in 4th month (26.4%).

Positivity percentage differed depending on pair of used primers. If pair Np6/Np21 was used infectivity was diagnosed in 32.1% cases (95% CI 19.9-46.3%), and if Np4/Np7 pair was used it was 28.3% (95% CI 18.6-42.3%), (p >0.05, and $\chi^2 = 0.202$). Using Np6/Np21, 8 normal aspect, 7 mummified and 2 emphysema abortions presented *N. caninum* DNA. Most of positive cases were in 2nd trimester of gestation (64.2%), followed by 3rd (20.8%) and 1st trimester (15.1%).

After Np6/Np21 amplification most positive samples were collected from brain (13/53) and liver (6/53). Using Np4/Np7 pair positivity was higher in heart (15.1%, 95% CI 6.7-27.6%), muscle (13.2%, 95% CI 5.5-25.3%) and lung (11.3%, 95% CI 4.3-23.0%).

2. Epidemiology of *N. caninum* infection in dogs

a) Seroprevalence of *N. caninum* infection in dogs

To asses for the first time the national seroprevalence of *N. caninum* infection in dog population from Romania and to analyze possible correlations between it and some intrinsic (age, sex and breed) and extrinsic factors (origin and category) we used a number of 1114 sera samples from different regions of the country: Banat, Crișana, Maramureș, Transilvania, Oltenia, Muntenia, Dobrogea, Moldova and Bucovina. Most of them were collected from males (624 samples) and mixed breeds (932/1114, 83.7%). Dogs were divided by origin (kennels, pets, shelter, guard, raised near farms and hunting dogs) and age (less than a year old, between 1 and 5 years and older than 5 years) and environment (rural and urban).

The study was performed between February 2008 and March 2011 and we used IFAT testind at a cut-off value of 1:50. For positive samples serial dilutions were performed using *in house* IFAT using FITC Rabbit Anti-Dog IgG (H+L), Jackson ImmunoResearch, UK conjugate, and for *N. caninum* antigen, tachyzoites cultivated on VERO cells.

After reading the results 364 samples were positive (32.7%). Some samples were positive until a dilution of 1:800. At 1:100 dilution the value decreased to 14.5% (95% CI 12.6-16.8%), at
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1:200 to 8.9% (95% CI 7.3-10.8%), at 1:400 to 4.1% (95% CI 3.1-5.5%), and to 1:800, to 1.9%, p = 0.00000 ($\chi^2 = 387.01$). The highest seropr evalence was obtained in Crișana (43.5%, 95% CI 33.2-54.2%) and the lowest Moldova (16.7%, 95% CI 3.6-41.4%). Sexes were differently affected, males 29.6% and females 36.9% (p = 0.005, $\chi^2=6.205$). Dogs from second age category were more affected (206/572) than the ones from the first (59/257) and third (99/286), (p = 0.0007, $\chi^2=14.48$). In rural 34.5% (95% CI 30.2-39%) and urban 31.4% (95% CI 27.8-35.1%) dogs positivity percentage was almost equal.

b) Detection of *N. caninum* genomic DNA from organs collected from dogs

Study regarding diagnosis on *N. caninum* infection in organs collected from dogs was developed between January and June 2011 and a number of 52 brain samples from 4 cities from Cluj county (Dej, Turda, Cluj-Napoca and Dezmir) were taken under study. The animals had different ages from 6 weeks to 12 years and were divided in age categories: under 1 year old, between 1 and 5 years old and older than 5 years old. Most of the samples came from urban dogs 43/52 (82.7%). There were pets, guard dogs, stray dogs and shelter dogs. Most of them were females 55.8% (29/52) and mixed breeds 35/52 (67.3%).

After direct examination of slide samples collected from frontal lobe, a number of 8 animals (15.4%) presented structures resembling to the ones of *N. caninum* cysts. After PCR testing all of them were negative to *N. caninum* and *T. gondii* genomic DNA.

After amplification of all 52 samples with primers pairs Np6/Np21 and Np4/Np7 a number of 3 samples were positive to *N. caninum* genomic DNA. Each age category had a positive sample (p=0.9422, $\chi^2=0.1192$). None of the shelter and guard dogs were positive to *N. caninum* infection, but one of the pets and two stray dogs (p=0.5879, $\chi^2=1.9259$). Percentage of pure breed (5.9) and mixed breed (5.7) infected animals was almost equal. Males (8.7%) were more than double affected than females (3.4%). All the positive samples came from urban dogs (7%, 95% CI 1.5-19.1%).

c) Epidemiological studies of intestinal neosporosis in dogs

Our objectives were to identify the ethiological agent responsible for intestinal neosporosis in dogs by using copro-parasitological methods and to certify the diagnosis by using PCR testing.

Researches were developed between April 2—8 and June 2011 on a number of 111 fecal samples from dogs 5 counties: Alba, Cluj, Hunedoara, Mureș and Sibiu. Dogs had ages under 1 year, between 1 and 5 years old and older than 5 years old. Most of the samples were collected from males (63/111). According to origin they were divided in: farm dogs, rural, urban and zoo dogs. For fecal testing we used ovohelmintoscopic quantitative and quality samples: Heriksen colouration, sucrosis flotation and active sedimentation.

After testing, a percentage of 65.8% (73/111) of the dogs presented parasitological forms in their feces. Most of the positive samples were collected from animals with age between 1 and 5 years old (52.1%). Females were more affected than males, p = 0.015 ($\chi^2=3.9965$). Most of the positive samples came from urban (58.9%, 95% CI 46.8-70.3%) and zoo dogs (26.0%, 95% CI 16.5-37.6%).

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Identified parasite species were Flagellata (Giardia spp.); Sporozoasida: Cryptosporidium spp, Isospora canis Hamondia/Neospora and Nematoda: Toxocara canis, Ancylostoma caninum, Uncinaria stenocephala, Tricocephalus vulpis, Capillaria spp. The only sample in which an oocyst with 10.75µm size of Hammondia/Neospora came from a dog raised near a farm from Cluj county where cattle neospornosis reached a value 27.9%. The sample was positive to N. caninum DNA after PCR testing.

Parasitological burden differed in age category and parasitic species. For dogs under 1 year old the parasites with the higher frequency were Cryptosporidium spp.(66.7%), I. canis (66.7%) and T. canis (53.8%); in dogs with age between 1 and 5 years old and over 5 years old were A. caninum (62.1%), Capillaria spp. (44.4%), T. vulpis (56.8%) and U. stenocephala (60.5%).

Because most of the samples were collected from urban dogs dogs it is logical why most of the infected samples came from this category (43/73) compared with other categories: 19 probe from zoo dogs, 6 from rural dogs and 5 from farm dogs.

3. Prevalence of neosporosis in other intermediate hosts

a) Seroprevalence of neosporosis in goats from north-west, centre and south of Romania

The aim of this study was to determine for the first time in Romania the seroprevalence of N. caninum infection in goats from centre, north-west and south of Romania and its distribution according to landscape and territory divisions. Researches were developed between October 2007 - August 2010. 512 samples were collected from four regins of the country: Maramureș, Crișana, Transilvania and Muntenia. 469 were collected from adults and the rest of 42 from animals with age under 1 year old. Most of the samples came from animals raised in herds (444) and the rest from animals raised in backyard system (68). We used the indirect ELISA method.

After testing we obtained a seroprevalence of neosporosis of 2.3% (CI 95% 1.3-4.2%, 12/512). Depending of county the seroprevalence was: Bihor 0% (95%CI 0.0-20.6%), Bistrița-Năsăud 8.2% (95%CI 2.3-19.6%), Cluj 0% (95% CI 0.0-1.4%), Covasna 0% (95% CI 0.0-33.6%), Hunedoara 0% (95% CI 0.0-30.8%), Ialomița 7.5% (95% CI 1.6-20.4%), Mureș 0% (95% CI 0.0-14.2%), Sălaj 11.4% (95% CI 3.8-24.6%) and Satu-Mare 0% (95% CI 0.0-7.3%), p≤ 0.001. All seropositive animals were adults (2.6%). None of the animals raised in backyard system presented antibodies against N. caninum. Most of the positive samples came from plateau regions (5/44), followed by the ones from planes (3/105) and hills (4/344), p ≤ 0.005.

b) Seroprevalence of N. caninum infection in horses from centre and north-west of Romania

Purpose of this study was to determine the seroprevalence of Neospora spp. infection in horses from north-west of the country. Reserches were made between October 2010 and January 2011.

In total, a number of 82 blood samples were collected from both sexes: males and females. For one animals data regarding age and sex were missing. Animals came from four counties of Transilvania: Satu-Mare, Mureș, Maramureș and Sălaj and were raised in backyard systems.
According to age the animals were divided in 3 categories: from 1 to 5 years old, from 5 to 10 years old and older than 10 years old. We used the IFAt method at a cut-off value of 1:50. For positive samples repeated dilutions were made until 1:200.

After reading the results 19/82 were positive (32.2%). At 1:100 dilution decreased to 7.4%, and at 1:200 to 2.5%, \( p < 0.0003 \). The highest value was obtained in Maramureș county (40.0%), and the lowest in Sălaj county (0%). Out of the 33 females, 9 were positive and out of 48 males, 10. For animals with age between 1 and 5 years old serorevalence of \( N. caninum \) infection was 30.4% (95% CI 13.2-52.9%), in 5 to 10 age category of 17.2% (95% CI 5.8-35.8%), and in over 10 years category of 24.1% (95% CI 10.3-43.5%).

4. Neosporosis in wild rodents, insectivores and wolves

In this study we aimed to investigate if wild animals are naturally infected with \( N. caninum \) by PCR examination of brain samples collected from animals captured in different areas from Romania. Researches were made from January 2011 to June 2011. A number of 65 animals were taken under study and from each one samples of brain and heart were preserved. The animals originated from Cluj (Dumbrava, Corușu, Baciul) and Neamț counties and were of different specie: \( Sorex araneus, Mus musculus, Pitmys subterraneus, Micromys minutus, Crocidura leucodon, Crocidura suaveolens, Apodemus sylvaticus \) and \( Apodemus agrarius \). Also, samples were collected from 4 wolves (\( Canis lupus \)), 2 two from Cluj county and 2 from Neamț county.

After Np6/Np21 testing all 130 samples were negative for \( N. caninum \) genomic DNA. After Np4/Np7 amplification \( N. caninum \) genomic DNA was identified in 6 brain samples and one heart sample indicating a seroprevalence of 9.2%. The animals that had both organs positive to PCR was a wolf from Cluj county. According to species, 5 of the positive samples were collected from \( Apodemus agrarius \). Most of the positive samples were from animals originated from Dumbrava (83.3%, 95% CI 35.9-99.6%).

5. Experimental researches regarding \( N. caninum \) cultivation on fibroblasts of animal origin

Through this experiment we aimed to obtain primary cell cultures from explants originating in organs from mice one day old. This was desirable to demonstrate that under normal conditions of a laboratory cell culture can be obtained which then later to be used for maintaining intracellular parasites including \( Neospora caninum \) and \( Toxoplasma gondii \). In this sense organs were harvested under sterile conditions from newborn mice from which fragments, explants were prepared, and then pursued growth rate of fibrocytic cell line. In case of of brain fragments the degree of astrocytes development was observed.

To see which of the culture media are viable to initiate a primary cell culture two media commonly used in laboratories were selected: RPMI-1640 and IMDM. These media were enriched with fetal bovine serum, L-glutamine, amphotericin B and non-essential amino acids.

Explants were monitored daily and differences between them have been noted.

In cardiac explants fibroblast cells with typical aspect were observed more quickly (starting day 3) for cells grown in IMDM medium. The ones in RPMI-1640 showed no characteristic appearance and failed to adhere to the surface only from day 5.
Although cardiac fibroblasts were observed on the third day of the experiment, they have shown signs of multiplication until Day 5, after which he noticed their stagnation. Thus, we failed to obtain a necessary and sufficient confluence for their use in *N. caninum* infection.

In case of explants obtained from the brain, those grown in RPMI-1640 medium initially had uncharacteristic aspect which remained until day 5 of cultivation. Starting this day a small number of astrocytes was observed but they have shown signs of degeneration. In cultures maintained in IMDM medium astrocytes were observed as early as day 3 of culturing, and they continued growing so in day 5 15% of the wells was covered. Their growth has remained slow, the desired confluence was reached only at 16 days after the start of the experiment.

For obtaining primary cultures the best were ones originating from lung and skin explants. Thus, in RPMI-1640 cultures were obtained with a large number of cells that showed high growth rate. Most of the lung fibroblast surface was tripled from day 3 to day 5, and the ones from skin covered a larger area on day 5, 15%. A negative aspect of cultivation in RPMI-1640 medium was that in addition to adhering fibroblasts there was a large number of cells in suspension. Comparatively, the cultivated area in IMDM doubled the covered surface on day 5. Another difference was that there were a very small number of cells in suspension. Growth trend in these cells has remained upward. Degree of confluence at day 14 for lung fibroblasts was 70% and 90% of leather.

One aspect that marked the differences between the two types of media used was the fact that a week after the beginning of the experiment all the cultures in which we used RPMI-1640 medium were infected with *Listeria spp.* and could not be saved.

Regarding infection of primary cultures with *N. caninum* tachyzoites, in those of lung fibroblasts we observed the fastest multiplying phenomenon which appeared from day 2 of infection. On Day 3 post-infection multiplying tachyzoites have been reported in primary cultures of astrocytes, skin fibroblasts and VERO cells.

Cell destruction and identification of parasites in suspension was observed from day 8 post-infection, both primary cultures and in witness, VERO cell cultures.

Infection with *N. caninum* of cell and negative effects caused by this determines the abortion and nervous clinical signs in intermediate hosts. Because of this we tried to obtain an *in vitro* experimental model that can provide useful information regarding tissue phases of neosporosis.