S U M M A R Y

EPIDEMIOLOGICAL RESEARCH ON *Anaplasma phagocytophilum* INFECTION IN HORSES IN ROMANIA

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Anaplasma phagocytophilum – a tick-borne pathogen transmitted by ticks (Acari: Ixodidae) shows a broad host specificity and zoonotic potential, infecting a varied range of domestic and wild animals. This pathogen has been known in veterinary medicine for more than 80 years, and recently has been also recognized in human pathology (Gordon și col., 1940; Chen și col., 1994). *A. phagocytophilum* infection causes tick-borne fever in ruminants and granulocytic anaplasmosis in animals (horses, dogs and cats) and humans (Heikkilä și col., 2010; Stuen și col., 2013). Considering the zoonotic potential of *A. phagocytophilum* it is necessary to investigate its occurrence in animals that have frequent contact with people, horses included.

Equine granulocytic anaplasmosis can evolve from a disease with nonspecific clinical signs, to a disease with fatal complications, being possible to register economic losses due to complex investigations and treatments (Boni și col., 2009; Nowicka și col., 2022). Research regarding this infection in horses from different geographical regions and different raising systems is of great importance in veterinary practice, as it would allow the appropriate diagnosis of cases with nonspecific symptoms, so that an adequate treatment to be administered to avoid complications.

In Romania, *A. phagocytophilum* has been molecularly detected in ticks and some domestic and wild animals. However, to date, there are no reported studies regarding *A. phagocytophilum* infection in Romanian horses. Thus, knowing the great interest in horses in Romania, these animals being raised both in households and in state and private stud farms (horse riding clubs, hippotherapy), it is necessary and of real interest to study tick-borne diseases that could affect the health status of these animals, but also of humans, in the case of diseases with zoonotic risk.

Horses have a close relationship with humans, and by surveillance of infections with zoonotic risk in these animals, some of the diseases that impact also human health would also be monitored.

In this context, the research included in this doctoral thesis aimed to contribute to the study of the epidemiology of *A. phagocytophilum* infection in horses in Romania, with the following specific objectives: (i) the investigation of exposure to *A. phagocytophilum* infection in horses from different geographical regions of Romania and different raising systems (stud farms and households of the population) by serological screening; identification of potential associated risk factors; (ii) study regarding clinico-pathology, diagnostic techniques and therapeutic management in clinical forms of granulocytic anaplasmosis in horses; (iii) optimization and testing by molecular biology methods based on the PCR technique, for the confirmation of *A. phagocytophilum* infection in Romanian horses.

The structure of the doctoral thesis follows the recommendations and norms of the Doctoral School of Veterinary Medicine – USAMV of Bucharest and comprises seven chapters included in the two parts: Part I – *Bibliographic study*, consisting of three chapters (representing approximately 25%) and Part II – *Personal Research*, presented in four chapters (representing aproximately 75%). The bibliographic list contains 250 titles relevant to the thesis, and the thesis annexes include 23 tables and 40 figures.

In **the first part** of the doctoral thesis (chapters I-III), updated information on the species *A. phagocytophilum* and the infection with this pathogen in domestic animals is synthesized. Thus, in chapters I and II, data regarding the taxonomy and morpho-biological characteristics of *A. phagocytophilum* are presented, including also a brief synthesis of the prevalence of this pathogen in vectors and wild animals in Romania. In chapter III are synthesized current information regarding epidemiology, pathogenesis and clinical evolution of *A. phagocytophilum* infection in domestic animals, including general data and clinico-epidemiological characteristics of this infection in equids, bovines, ovines, canids, and felines. This information contained in the *Bibliographic Study* fundamented the research plan related to the doctoral thesis, as a basis for conducting the studies, as well as for the analysis and interpretation of the results obtained.

In **the second part**, personal research is presented in four chapters (IV-VII) that describe the studies conducted according to the research plan and the proposed objectives. Thus, *Materials and methods,* the *Results* obtained*, Discussion,* partial and general *Conclusions* were described, which are presented synthetically in chapters.

Chapter IV presents the serological investigations carried out in order to evaluate the epidemiology of *A. phagocytophilum* and other tick-borne pathogen infections in horses from different geographical regions of Romania (North, Center and South-East) and different raising systems. The study included 223 horses from five counties (Bistriţa-Năsăud – in the North; Braşov, Sibiu – in the Center; Buzău, Călăraşi – in the South-East), raised in stud farms (n=118) and households of the population (n=105). The animals were aged between 8 months and 26 years (mean 9 years; SD=5,4); allocated in 3 age categories, like: <5 years (n=65); 5-10 years (n=81); >10 years (n=77).

Regarding sex and breed, the serological screening was performed both in females (n=135), and males (n=88), of pure-breed (n=113) and mixed-breed (n=110).

Serological investigations were carried out using an ELISA Kit (IDEXX Laboratories, Inc.), that allows simultaneous detection of antibodies against *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato and *Ehrlichia* spp. – tick-borne pathogens. The results of serological tests showed an overall seroprevalence of 26,0% (58/223 horses were positive for at least one of the tested pathogens; 95% CI: 20,38-32,29%); 22,9% presented singular infections, and 3,1% - mixed infections, of which 2,7% with 2 pathogens (*A. phagocytophilum* și *B. burgdorferi* s.l.), and 0,5% (one horse) with all 3 tested pathogens.

Regarding *A. phagocytophilum*, 10,3% (23/223; IC 95%: 6,7-15,1%) horses were seropositive, of which 7,2% (n=16) presented singular infections, and 3,1% (n=7) – mixed infections. The mean values of seroprevalence registered in animals from the three regions varied from 5,0% (in the North) to 11,7% (in the South-East). The horses from households presented a higher seroreactivity (14,3%; 15/105), compared to those from stud farms (6,8%; 8/118). Also, the rates of infection were higher in horses over 10 years old (13,0%), compared to younger horses (7,7% for horses aged <5 years), in males compared to females (13,6% versus 8,1%) and in mixed-breed horses compared to those of pure-breed (13,6% versus 7,1%), but with no statistically significant differences.

In addition, 18,8% (42/223; IC 95%: 13,92-24,6%) of the tested horses were seropositive for *B. burgdorferi* s.l., and 0,5% (1/223; IC 95%: 0,01-2,5%) for *Ehrlichia* spp. Seroreactivity of horses to infection with *B. Burgdorferi* s.l. was correlated with raising system, sex and breed, higher infection rates being recorded in horses from households, in males (29,5% versus 11,9%, in females) and in mixed-breed horses (27,3% versus 10,6%, in pure-breed horses), probably after a longer exposure to ticks and the pathogens they harbour.

Chapter V presents a meta-analysis study regarding clinico-pathology, diagnostic techniques and therapeutic management in the clinical forms of granulocytic anaplasmosis described in equids. Thus, 189 clinical cases (reported between 1994-2022, in 31 publications) for which *A. phagocytophilum* infection was confirmed by different diagnostic methods, were investigated. Clinical cases of equine granulocytic anaplasmosis were diagnosed in Europe (67,20%), Asia (15,34%), America (12,70%) and Africa (4,76%). Most cases were reported in Sweden (23,81%), followed by USA (11,11%), Spain (9,52%), Germany and Pakistan (8,47%, each).

Clinical forms of granulocytic anaplasmosis were reported in horses (94,71%; n=179), donkeys (3,17%; n=6) and mules (2,12%; n=4), in animals aged from 4 months to 30 years (94/189). Regarding sex and breed, both males (55/83; 66,27%), and females (28/83; 33,73%), of pure-breed (33/38; 86,84%) and mixed-breed (5/38; 13,16%), presented clinical forms of *A. phagocytophilum* infection.

The animals showed a complex symptomatology in *A. phagocytophilum* infection. The most commonly reported clinical signs were fever (90,30%; 121/134), limb edema (48,51%), anorexia (41,79%), depression (32,84%), icterus (22,39%), ataxia (17,91%), tachycardia (16,42%) and lethargy (15,67%). Rhabdomyolysis and cavitary effusion were observed in one and two cases, respectively, and death of a horse was reported in a case with chronic cerebral anaplasmosis. The main pathological findings shown by laboratory investigations included thrombocytopenia (90,32%; 84/93), anemia (75,00%; 30/40), decreased hematocrit (70,59%; 24/34), leukopenia (55,88%; 19/34), lymphopenia (58,14%; 25/43), neutropenia (41,67%; 10/24), hyperbilirubinemia (68,97%; 20/29), hyperfibrinogenemia (13/15; 86,67%), and hyponatremia (10/10; 100,00%). In the case with rhabdomyolysis, myoglobinuria and proteinuria were observed.

A. phagocytophilum infection in cases with clinical forms was confirmed by microscopy, serological tests, isolation and molecular methods. After microscopic examination, 88,44% (153/173) samples tested positive, in which morulae were observed mainly from blood (75,16%) and buffy coat smears (20,92%), stained more frequently by Giemsa method (58,17%). Serological tests revealed antibodies against *A. phagocytophilum* in 66,67% (56/84) of clinical cases, both in the acute stage of the disease – 60,71% (62,75% by IFAT, 23,53% by IFAT and Western Blot, 5,88% by ELISA and 5,88% by ELISA and IFAT) and in the late stage – 5,95% (2/5 positive by IFAT). The pathogen was isolated in only one case (1/189; 0,53%). Using molecular techniques, 98,36% (120/122) samples tested positive, by: conventional PCR (77,50%), real-time PCR (17,50%) and PCR-RLB (5,00%); genetic markers used were, mainly, the *16S rRNA* şi *msp2* genes .

In terms of therapeutic management, 77,01% (67/87) of the cases were treated for anaplasmosis, 12,64% received nonspecific treatment before diagnosis, and 10,34% recovered without therapy. For treatment, oxytetracycline (52,24%; 35/67) and tetracycline (14,93%) were most frequently administered. Clinical improvement was observed at 10-12h after treatment (16/45; 34,56%), wihin 24-48 h (19/45; 42,22%) and at or after 3 days (2/45; 4,44%). Complete recovery was reported for 54,55% (24/44) of clinical cases, at 12 h to 8 months after treatment.

Chapter VI describes the optimization and testing of a molecular biology method based on the Real-Time PCR technique for the molecular detection of the *A. phagocytophilum* DNA in blood samples from horses (Romania). For this, samples of genomic DNA extracted and purified from horses whole blood, were tested by Real-Time PCR technique for the molecular detection of the three biovariants of *A. phagocytophilum* (*phagocytophilum, equi* and the causative agent of human granulocytic anaplasmosis). In a first stage, the method was optimized. Following the application of the amplification protocol (s1 x 1: 50°C - 2 min; s2 x 1: 95°C - 10 min; s3 x 45: 60°C - 1 min), amplification curves were obtained for both positive controls, with the value Ct-CP1= 26,9092 and Ct-CP2=27,4285, while no amplification curves were

obtained for both negative controls. These results allowed the validation of the method, since for both positive controls the value Ct<45, in accordance with the manufacturer's recommendations for the validation of the method. Subsequently, the method was used to test 23 samples from equids seroreactive to *A. phagocytophilum* infection. Following the RT-PCR reaction, amplification was obtained, in a first step, in one sample, but the amplitude of the amplification curve was below the CP amplitude. For confirmation, genetic analysis is required, which is why amplification by conventional PCR and sequencing, using specific genetic markers, will be carried out at a later stage. The method thus optimized may be useful in further clinical studies to confirm *A. phagocytophilum* infection, especially in acute infections in animals with clinical signs of suspicion.

Chapter VII includes the *General conclusions and recommendations* expressed as an outcome of the analysis of the results obtained after completing the studies included in this doctoral thesis. Thus, the results of the studies confirm the natural exposure to *A. phagocytophilum* infection in horses in the investigated areas, from Romania, underlining the importance of serological screening studies regarding infections with tick-borne pathogens, as well as the importance of implementing appropriate surveillance and control programs for tick-borne diseases. In addition, since equids can develop varied symptomatology and pathological findings in natural infection with *A. phagocytophilum*, stands out the importance of a good knowledge, based on evidence, regarding clinico-pathology, diagnosis and therapeutic management in equine granulocytic anaplasmosis, the need to include this disease in the differential diagnosis in animals with suspicious symptoms, and the application of appropriate measures for therapy and prophylaxis.

In conclusion, through the results of the studies related to this doctoral thesis, contributions are brought to scientific knowledge regarding the eco-epidemiology of *A. phagocytophilum* infection in horses in Romania – the first time study in Romania - as well as contributions with practical-applicative value for practician veterinarians, namely providing evidence-based solutions for the challenges related to the confirmation of the diagnosis, the therapeutic approach and the appropriate control measures in granulocytic anaplasmosis in horses. In addition, these results contribute to the development of new research directions in the field of tick-borne diseases in horses in Romania, field of great national and international relevance, in accordance with the current context of climate change influencing the spread of vectors and pathogens transmitted by vectors.