SEROPREVALENCE OF *ANAPLASMA PHAGOCYTOPHILUM*, *EHRLICHIA* SPP. AND *BORRELIA BURGDORFERI* INFECTIONS IN HORSES: FIRST REPORT FROM NORTHERN BULGARIA – SHORT COMMUNICATION

Ilia Tsachev[1], Magdalena Baymakova[2*] and Nikola Pantchev[3]

[1]Department of Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria; [2]Department of Infectious Diseases, Military Medical Academy, 1606 Sofia, Bulgaria; [3]Department of Infectious Diseases, IDEXX Laboratories, Ludwigsburg, Germany

Lyme borreliosis, granulocytic anaplasmosis and monocytic ehrlichiosis are well studied in humans and dogs. In horses, these diseases are not widely investigated and limited information is available about their occurrence. The purpose of this study was to present the first ELISA-based report on the seroprevalence of *Anaplasma phagocytophilum*, *Ehrlichia* spp. and *Borrelia burgdorferi* in horses from Northern Bulgaria. A total of 192 horses were investigated from three regions in Northern Bulgaria (Northwestern, North-Central and Northeastern Bulgaria). All equine sera were tested for *A. phagocytophilum*, *Ehrlichia* spp. and *B. burgdorferi* antibodies by a commercial rapid ELISA test. Antibodies against *A. phagocytophilum* were found in all the three regions at a mean frequency of 12% (23/192), ranging from 9.38 to 15.63% by region. Antibodies against *Ehrlichia* spp. were found in horses from one region (Northeastern) at a rate of 0.5% (1/192). Anti-*B. burgdorferi* antibodies were detected in all the three regions with a mean frequency of 15.1% (29/192), ranging from 14.06 to 17.19% by region. A co-exposure to *A. phagocytophilum* and *B. burgdorferi* was observed in 6.3% of the cases (12/192). This is the first report on the natural exposure of horses to these bacteria (*A. phagocytophilum*, *Ehrlichia* spp. and *B. burgdorferi*) in Northern Bulgaria.

Key words: Seroprevalence, *Anaplasma phagocytophilum*, *Ehrlichia* spp., *Borrelia burgdorferi*, horses, Bulgaria

Granulocytic anaplasmosis (GA) is a tick-borne infection of neutrophils caused by *Anaplasma phagocytophilum*, formerly known as *Ehrlichia equi*, *E. phagocytophila* and the agent responsible for human granulocytic ehrlichiosis (HGE agent) (Dumler et al., 2001). Human GA was first identified in 1990 (Chen et al., 1994), although the agent had been defined as a pathogen of veteri-

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*Corresponding author; E-mail: dr.baymakova@gmail.com

nary importance already 1932 (Woldehiwet, 2010). In horses, *E. equi* infection was first described as a febrile disease with inclusion bodies detectable in the cytoplasm of granulocytes, and resembling the clinical disease of ruminants in California (Gribble, 1969).

Ehrlichia spp. generally have a tropism for either macrophages or granulocytes, where they grow within cytoplasmic membrane-bound vacuoles and have ticks as vectors (Dumler and Walker, 2015).

Lyme borreliosis (LB) caused by the spirochaete *Borrelia burgdorferi* (sensu lato), occurs in temperate regions of the northern hemisphere in North America, Europe, and Asia (Steere et al., 2016). Antibodies against *B. burgdorferi* in horses were first detected in New England, USA (Marcus et al., 1985). In 1998, *B. burgdorferi* was found by PCR in urine samples of healthy horses in a Lyme disease-endemic region of Connecticut, USA (Manion et al., 1998). Antibodies against *B. burgdorferi* were found in 45.1% of the horses tested in the Northeastern United States in 2000 (Magnarelli et al., 2000).

Recently, seroprevalence data for the above-mentioned pathogens have been reported in Southern Bulgaria (Tsachev et al., 2018). The aim of this study was to conduct a similar survey in horses from Northern Bulgaria.

Draft male and female horses (n = 192) were enrolled from three northern regions of Bulgaria – Northwestern (Montana and Vratsa districts), North-Central (Pleven and Veliko Tarnovo districts) and Northeastern (Targovishte and Varna districts). The animals showed no clinical signs at the time of sampling (autumn of 2017), and their age varied from 1 to 12 years. The regions are located in areas of plains and forests (latitude: 43° 24' 30.6576'' N and 43° 12' 50.58'' N; longitude: 23° 13' 32.6244'' E and 27° 54' 53.0388'' E), and the climate is continental (mean annual temperature 10–12 ℃, precipitation approx. 630 mm/m^2).

Blood samples (up to 10 mL per horse) were taken by venipuncture from the jugular vein. All samples were collected in blood EDTA tubes without anti-coagulant and kept at room temperature (22 °C) until visible clot retraction, centrifuged at 1500 *g* for 10 min, and the serum was separated and kept at –20 °C until processing.

Serum samples of 192 horses were tested for *A. phagocytophilum*, *Ehrlichia* spp. and *B. burgdorferi* antibodies using a commercial ELISA rapid test (SNAP® 4Dx® Plus Test, IDEXX Laboratories Inc., Westbrook, ME, USA), according to the manufacturer's instructions. The SNAP® 4Dx® Plus is a test for dogs and screens for seven vector-borne pathogens (*Dirofilaria immitis*, *A. phagocytophilum*, *A. platys*, *B. burgdorferi*, *E. canis*, *E. ewingii* and *E. chaffeensis*). According to Stillman et al. (2014), the sensitivity and specificity of the test system are 93.2% and 99.2% for *A. phagocytophilum*, 89.2% and 99.2% for *A. platys*, 96.7% and 98.8% for *B. burgdorferi* (s.l.), 97.8% and 92.3% for *E. canis*, and 98.9% and 99.3% for *D. immitis*. The assay uses an all-species-conjugate

and it is not species-specific for animal species. Thus, the test has been validated for horses by Chandrashekar et al. (2008) who evaluated its performance for the detection of antibodies to *B. burgdorferi* and *A. phagocytophilum* and found 100% sensitivity and 95% specificity for the detection of antibodies to *B. burgdorferi*, and 100% sensitivity and specificity for *A. phagocytophilum*. Furthermore, Wagner et al. (2013) compared the test in regard to *Borrelia* against multiplex analysis of antibodies to OspC, OspF, and C6 peptides in 191 equine sera and found a sensitivity of 93% and a specificity of 96%. Another study from Europe evaluated the performance of the test in regard to different *B. burgdorferi* genospecies, by means of whole-cell *Borrelia* ELISA and *A. phagocytophilum* IFA (Butler et al., 2016*a*; Tsachev et al., 2018). Some studies, mainly from the South American region, used the test for the detection of antibodies to *Ehrlichia* species in horses and compared its performance with different IFA tests, some in addition to PCR (Tsachev et al., 2018).

This study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Trakia University of Stara Zagora and was conducted according to the ethical principles of animal experimentation, adopted by the Bulgarian Ministry of Agriculture, Food and Forestry.

Data on seroprevalence were compared among the different regions by Chi-square and unpaired F (Kruskal–Wallis) tests. Statistical analysis was performed by Excel 2007 (Microsoft, Redmond, Washington, USA) and SPSS Statistics 19.0 (IBM Corp., Armonk, New York, USA). A P value < 0.05 was considered statistically significant.

Antibodies against *A. phagocytophilum* were detected in 12% of the equine sera in all three Northern Bulgarian regions represented in the study (Table 1). The seroprevalence ranged between regions with a mean ± SD = 11.98 ± 3.25%. Antibodies against *Ehrlichia* spp. were detected in 0.5% of the horses. Antibodies against *B. burgdorferi* were found in all three regions in 15.1% of the tested samples and the seroprevalence varied between regions with a mean ± SD = 15.10 ± 1.80%. A co-exposure to *A. phagocytophilum* and *B. burgdorferi* was found in 6.3% of the cases.

A recent study in horses from Southeastern Bulgaria found 20% (95% CI: 14.0–27.2%) *A. phagocytophilum*-positive samples (Tsachev et al., 2018). In Strandja Nature Park, Nader et al. (2018) reported 6.2% (95/1541) PCR positivity for *A. phagocytophilum* in *Ixodes* spp. ticks. In the present study, the seropositivity of *A. phagocytophilum* in horses was found to be significantly lower in Northern Bulgaria compared to data from Southern Bulgaria (12% and 20%, respectively; P = 0.04). This could be explained by the availability of infested vectors (*Ixodes* spp.) and hosts, which is supposed to be lower in the northern part of the country than in Southern Bulgaria. Similar data are found for human GA, with more cases being reported from Southern Bulgaria. In 2008 six cases of human GA were reported in Bulgarian patients (Christova et al., 2008) and nine

years later other three cases were documented in citizens from Southern Bulgaria (Pishmisheva et al., 2017). Our results for the seroprevalence of *A. phagocytophilum* (12%) were lower than in a similar study conducted in The Netherlands, where a seropositivity of 23% was found (Butler et al., 2016*b*).

Table 1

Seroprevalence of *A. phagocytophilum*, *Ehrlichia* spp. and *Borrelia burgdorferi* antibodies in horses from Northern Bulgaria

Positive samples	Northwestern region, n (%)	North-Central region, n (%)	Northeastern region, n (%)	Total	
				n (%)	95% CI
Ap	7/64 (10.94)	10/64 (15.63)	6/64 (9.38)	23/192 (12)	7.7–17.4
Es	0/64 (0.0)	0/64 (0.0)	1/64 (1.56)	1/192 (0.5)	0.0–2.9
Bb	9/64 (14.06)	9/64 (14.06)	11/64 (17.19)	29/192 (15.1)	10.4–21.0
Co-infections					
Ap + Bb	0/64 (0.0)	7/64 (10.94)	5/64 (7.81)	12/192 (6.3)	3.3–10.7

Ap – *Anaplasma phagocytophilum*; Es – *Ehrlichia* spp.; Bb – *Borrelia burgdorferi*; CI – confidence interval

In 2006 Tsachev et al. reported an *E. canis* seroprevalence level of 30 ± 15.41% among dogs in Southern Bulgaria (Tsachev et al., 2006*a*), and found a 37.5 ± 15.73% positivity among dogs in Northern Bulgaria (Tsachev et al., 2006*b*). A study from central-southern Bulgaria performed with a similar test in dogs revealed an *E. canis* seroprevalence of 21% (95% CI: 15.1–27.9%) (Pantchev et al., 2015). Data reported in 2018 presented 3.9% (95% CI: 1.4–8.2%) seroprevalence of *Ehrlichia* spp. in horses from Southeastern Bulgaria (Tsachev et al., 2018). That was the first study which provided evidence of *Ehrlichia* spp. exposure in horses in Europe. The overall seroprevalence rate of *Ehrlichia* spp. observed in the present study in horses from Northern Bulgaria is significantly lower than that reported in Southern Bulgaria (0.5% and 3.9%, respectively; P = 0.03). The reasons for these results could be the same as those mentioned above for the serological evidence of infection with *A. phagocytophilum*, likely to be explained by the availability of a certain tick vector. A Brazilian study found 62.5% anti-*Ehrlichia* spp. antibodies in horses using ELISA (SNAP® 4Dx® Test) (Vieira et al., 2013). A study from Nicaragua reported 51% (51/92) seroreactivity to *Ehrlichia* spp. in equine sera applying the same test as in our study – SNAP® 4Dx® Plus Test (O'Nion et al., 2015). The varying data could be influenced by geographic location, climate characteristics and variation in the presence of vectors. The test conducted in the present study (SNAP® 4Dx® Plus) could not identify the *Ehrlichia* species causing the seroconversion. It remains speculative whether *E. canis* occurring in Bulgaria or a different, yet not identified *Ehrlichia*

sp. is responsible for the *Ehrlichia* spp. seroconversion in Bulgarian horses (Tsachev et al., 2018). Consequently, further molecular diagnostic assays are needed for determination of the causative agent. A recent study from South America based on phylogenetic analysis of the 16S rDNA, *sodb*, and *groEL* genes and translated amino acids found that Brazilian and Nicaraguan *Ehrlichia* isolates from horses probably belong to the same novel *Ehrlichia* species infecting horses, differing from *E. ruminantium* (Vieira et al., 2018). Still, the vector, the biology and the pathogenicity of the novel *Ehrlichia* species infecting equines are not known.

For the period 2008 to 2017, the registered cases of human Lyme borreliosis were 7.1 cases per 100 thousand population in Bulgaria (NCIPD, 2018). In 2015, Pantchev et al. reported 2.4% (95% CI: 0.7–6.0%) seropositivity of *B. burgdorferi* in dogs from central-southern Bulgaria with a similar test. In 2018, our previous study reported 23.2% (95% CI: 16.8–30.7%) *B. burgdorferi* seropositivity in South Bulgarian horses (Tsachev et al., 2018). The higher seroprevalence (antibodies to C6 peptide) in horses than in dogs can be explained by the different tick exposure and the availability of different registered acaricides for tick prophylaxis. Although the established *B. burgdorferi* seroprevalence in horses in Northern Bulgaria is lower than that reported in Southern Bulgaria (15.1% and 23.2%, respectively), this difference was statistically not significant (P = 0.05). The morbidity of human Lyme borreliosis in Northern Bulgaria is higher than that observed in Southern Bulgaria (Kunchev, 2018), which could be attributable to different *Borrelia* genospecies (Nader et al., 2018). Our results (15.1%) are similar to those reported from Germany (16.1%; Kasbohrer and Schonberg, 1990), Sweden (16.8%; Egenvall et al., 2001) and Romania (11.92%; Kiss et al., 2011), but these studies used IFA and ELISA for the detection of antibodies. Our results differ notably from those obtained in The Netherlands (44.7%; Butler et al., 2016*b*) and Denmark (29%; Hansen et al., 2010) using the same diagnostic technology as ours.

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References

Butler, C. M., Sloet van Oldruitenborgh-Oosterbaan, M. M., Stout, T. A. E., Jongejan, F., Werners, A. H. and Houwers, D. J. (2016*a*): Classification of ticks collected from horses in the Netherlands in 2008–2009 and identification of the (zoonotic) agents they contain. Pferdeheilkunde **32,** 329–334.

Butler, C. M., Sloet van Oldruitenborgh-Oosterbaan, M. M., Werners, A. H., Stout, T. A. E., Nielen, M., Jongejan, F., van der Kolk, J. H. and Houwers, D. J. (2016*b*): *Borrelia*

burgdorferi and *Anaplasma phagocytophilum* in ticks and their equine hosts: A prospective clinical and diagnostic study of 47 horses following removal of a feeding tick. Pferdeheilkunde **32,** 335–345.

Chandrashekar, R., Daniluk, D., Moffitt, S., Lorentzen, L. and Williams, J. (2008): Serologic diagnosis of equine borreliosis: evaluation of an in-clinic enzyme-linked immunosorbent assay (SNAP® 4Dx®). Intern. J. Appl. Res. Vet. Med. **6,** 145–150.

Chen, S. M., Dumler, J. S., Bakken, J. S. and Walker, D. H. (1994): Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32,** 589–595.

Christova, I., Taseva, E., Gladnishka, T., Bakardzhiev, K., Komitova, R., Ilieva, P., Andonova, L., Goranova, G., Yordzheva, K. and Balnikova, N. (2008): Human granulocytic anaplasmosis in Bulgaria: confirmed cases and review of the literature [in Bulgarian]. Med. Rev. (Sofia) **44,** 63–67.

Dumler, J. S. and Walker, D. H. (2015): *Ehrlichia chaffeensis* (Human monocytotropic ehrlichiosis), *Anaplasma phagocytophilum* (Human granulocytotropic anaplasmosis), and other Anaplasmataceae. In: Bennett, J. E., Dolin, R. and Blaser, M. J. (eds) Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 8th edition. Elsevier Saunders, Philadelphia, USA. pp. 2227–2233.

Dumler, J. S., Barbet, A. F., Bekker, C. P., Dasch, G. A., Palmer, G. H., Ray, S. C., Rikihisa, Y. and Rurangirwa, F. R. (2001): Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **51,** 2145–2165.

Egenvall, A., Franzen, P., Gunnarsson, A., Engvall, E. O., Vagsholm, I., Wikstrom, U. B. and Artursson, K. (2001): Cross-sectional study of the seroprevalence to *Borrelia burgdorferi* sensu lato and granulocytic *Ehrlichia* spp. and demographic, clinical and tick-exposure factors in Swedish horses. Prev. Vet. Med. **49,** 191–208.

Gribble, D. H. (1969): Equine ehrlichiosis. J. Am. Vet. Med. Assoc. **155,** 462–469.

Hansen, M. G., Christoffersen, M., Thuesen, L. R., Petersen, M. R. and Bojesen, A. M. (2010): Seroprevalence of *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in Danish horses. Acta Vet. Scand. **52,** 3.

Kasbohrer, A. and Schonberg, A. (1990): Serologic studies of the occurrence of *Borrelia burgdorferi* in domestic animals in Berlin (West) [in German]. Berl. Munch. Tierarztl. Wochenschr. **103,** 374–378.

Kiss, T., Cadar, D., Krupaci, A. F., Bordeanu, A., Brudasca, G. F., Mihalca, A. D., Mircean, V., Gliga, L., Dumitrache, M. O. and Spinu, M. (2011): Serological reactivity to *Borrelia burgdorferi* sensu lato in dogs and horses from distinct areas in Romania. Vector Borne Zoonotic Dis. **11,** 1259–1262.

Kunchev, A. (2018): Research on the epidemiology of the most prevalent tick-borne infections in Bulgaria: Crimean-Congo haemorrhagic fever, Q fever, Lyme borreliosis and Mediterranean spotted fever [in Bulgarian]. PhD Thesis, NCIPD, Sofia (Bulgaria).

Magnarelli, L. A., Ijdo, J. W., Van Andel, A. E., Wu, C., Padula, S. J. and Fikrig, E. (2000): Serologic confirmation of *Ehrlichia equi* and *Borrelia burgdorferi* infections in horses from the northeastern United States. J. Am. Vet. Med. Assoc. **217,** 1045–1050.

Manion, T. B., Khan, M. I., Dinger, J. and Bushmich, S. L. (1998): Viable *Borrelia burgdorferi* in the urine of two clinically normal horses. J. Vet. Diagn. Invest. **10,** 196–199.

Marcus, L. C., Patterson, M. M., Gilfillan, R. E. and Urband, P. H. (1985): Antibodies to *Borrelia burgdorferi* in New England horses: serologic survey. Am. J. Vet. Res. **46,** 2570–2571.

Nader, J., Krol, N., Pfeffer, M., Ohlendorf, V., Marklewitz, M., Drosten, C., Junglen, S. and Obiegala, A. (2018): The diversity of tick-borne bacteria and parasites in ticks collected from the Strandja Nature Park in south-eastern Bulgaria. Parasit. Vectors **11,** 165.

National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria (2018): Annual reports of the contagious infectious diseases in Bulgaria (2008–2017) [in Bulgarian]. Available at: https://www.ncipd.org/index.php?lang=bg [Accessed at: 10 October 2018].

O'Nion, V. L., Montilla, H. J., Qurollo, B. A., Maggi, R. G., Hegarty, B. C., Tornquist, S. J. and Breitschwerdt, E. B. (2015): Potentially novel *Ehrlichia* species in horses, Nicaragua Emerg. Infect. Dis. **21,** 335–338.

Pantchev, N., Schnyder, M., Vrhovec, M. G., Schaper, R. and Tsachev, I. (2015): Current surveys of the seroprevalence of *Borrelia burgdorferi*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Leishmania infantum*, *Babesia canis*, *Angiostrongylus vasorum* and *Dirofilaria immitis* in dogs in Bulgaria. Parasitol. Res. **114,** 117–130.

Pishmisheva, M., Baymakova, M., Tsachev, I. and Christova, I. (2017): *Anaplasma phagocytophilum* among Bulgarian patients: case reports [in Bulgarian]. Med. Rev. (Sofia) **53,** 49–52.

Steere, A. C., Strle, F., Wormser, G. P., Hu, L. T., Branda, J. A., Hovius, J. W., Li, X. and Mead, P. S. (2016): Lyme borreliosis. Nat. Rev. Dis. Primers **2,** 16090.

Stillman, B. A., Monn, M., Liu, J., Thatcher, B., Foster, P., Andrews, B., Little, S., Eberts, M., Breitschwerdt, E. B., Beall, M. J. and Chandrashekar, R. (2014): Performance of a commercially available in-clinic ELISA for detection of antibodies against *Anaplasma phagocytophilum*, *Anaplasma platys*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Ehrlichia ewingii* and *Dirofilaria immitis* antigen in dogs. J. Am. Vet. Med. Assoc. **245,** 80–86.

Tsachev, I., Kontos, V., Zarkov, I. and Krastev, S. (2006*a*): Survey of antibodies reactive with *Ehrlichia canis* among dogs in South Bulgaria. Rev. Med. Vet. (Toulouse) **157,** 481–485.

Tsachev, I., Pantchev, N., Marutsov, P., Petrov, V., Gundasheva, D. and Baymakova, M. (2018): Serological evidence of *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Ehrlichia* spp. infections in horses from Southeastern Bulgaria. Vector Borne Zoonotic Dis. **18,** 588–594.

Tsachev, I., Papadogiannakis, E. I., Kontos, V., Zarkov, I., Petrov, V. and Pelagic, V. (2006*b*): Seroprevalence of *Ehrlichia canis* infection among privately-owned dogs in Northern Bulgaria. J. Hell. Vet. Med. Soc. **57,** 212–216.

Vieira, R. F., Vieira, T. S., Nascimento Ddo, A., Martins, T. F., Krawczak, F. S., Labruna, M. B., Chandrashekar, R., Marcondes, M., Biondo, A. W. and Vidotto, O. (2013): Serological survey of *Ehrlichia* species in dogs, horses and humans: zoonotic scenery in a rural settlement from southern Brazil. Rev. Inst. Med. Trop. Sao Paulo **55,** 335–340.

Vieira, T. S. W. J., Qurollo, B. A., Mongruel, A. C. B., Baggio, R. A., Vidotto, O., Breitschwerdt, E. B. and Vieira, R. F. C. (2018): Potentially same novel *Ehrlichia* species in horses in Nicaragua and Brazil. Emerg. Infect. Dis. **24,** 953.

Wagner, B., Goodman, L. B., Rollins, A. and Freer, H. S. (2013): Antibodies to OspC, OspF and C6 antigens as indicators for infection with *Borrelia burgdorferi* in horses. Equine Vet. J. **45,** 533–537.

Woldehiwet, Z. (2010): The natural history of *Anaplasma phagocytophilum*. Vet. Parasitol. **167,** 108–122.