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RESEARCH ARTICLE

The first serological evidence of *Anaplasma phagocytophilum* in horses in Slovakia

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ABSTRACT

Anaplasma phagocytophilum is the causative agent of granulocytic anaplasmosis. It affects humans and several wild and domesticated mammals, including horses. The aim of our study was a preliminary survey of the occurrence of these re-emerging pathogens in horses in Slovakia. The sera from 200 animals of different ages and both sexes were tested for the presence of *A. phagocytophilum* antibodies by indirect immunofluorescence assay. Subsequently, detection of the *16S* rRNA gene fragment of *A. phagocytophilum* was attempted by polymerase chain reaction (PCR) in each blood sample. Our results confirmed the presence of specific antibodies in 85 out of 200 individuals (42.5%), but no significant changes were found between the animals of different ages and sexes. However, the PCR analysis did not detect any positive animals. Our data represent one of the highest values of seropositivity to *A. phagocytophilum* in horses in Central Europe. These results may contribute to a better understanding of the circulation of *A. phagocytophilum* in this region, thus indicating a potential risk to other susceptible species.

KEYWORDS

equine granulocytic anaplasmosis, indirect immunofluorescence assay, Central Europe, vector-borne diseases

INTRODUCTION

As a result of the climatic and urban changes in the environment, tick-borne diseases are becoming an emerging problem in the temperate regions of Europe (Parham et al., 2015; Yang et al., 2018). *Anaplasma (A.)* spp. are one of the important bacterial pathogens transmitted by ticks. In Europe, *Ixodes ricinus* (Kiewra et al., 2014; Tomassone et al., 2018) was described as the main vector of this pathogen. Worldwide, other tick representatives of the genus *Ixodes* as well as the genera *Dermacentor*, *Rhipicephalus*, *Amblyomma* and *Haemaphysalis* play a key role in the transmission of anaplasmosis (Rymaszewska and Grenda, 2008). Until now, there are several known species of bacteria from the genus *Anaplasma*, such as *A. phagocytophilum*, *A. marginale*, *A. bovis*, *A. platys*, *A. ovis*, *A. centrale, A. caudatum, A. odocoilei* as well as the newly discovered *A. capra* (Tate et al., 2013; Yang et al., 2015; Dantas-Torres and Otranto, 2017; Mullen and Durden, 2018). Furthermore, a few strains were newly detected and named as '*Candidatus* Anaplasma boleense', '*Candidatus* Anaplasma camelii', '*Candidatus* Anaplasma corsicanum', '*Candidatus* Anaplasma ivorensis', '*Candidatus* Anaplasma mediterraneum', '*Candidatus* Anaplasma rodmosense', and '*Candidatus* Anaplasma sphenisci' (Bastos et al., 2015; Ehounoud et al., 2016; Guo et al., 2016;

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Dahmani et al., 2017; Lu et al., 2017; Vanstreels et al., 2018). The zoonotic potential of the most important species *A. phagocytophilum* and *A. capra* may represent a risk for public health in Europe (Greene, 2012). Until now, only *A. phagocytophilum* has been detected in horses. This agent invades the granulocytes of various mammalian species and is the causative agent of the tick-borne fever in ruminants, horses, dogs and humans, known as granulocytic anaplasmosis (Lotrič-Furlan et al., 2006; Passamonti et al., 2010; Król et al., 2016). The disease is characterised by high fever, depression, anorexia, icterus, ataxia, lower limb oedema, thrombocytopenia, anaemia and leukopenia in both naturally and experimentally infected horses (Bermann et al., 2002; Franzén et al., 2005). The acute infection is typical in horses, humans and mice models, while persistent infections occur in sheep, rodents and dogs (Rejmanek et al., 2012).

After reorganisation in the order *Rickettsiales* based on *16S rRNA* and *GroESL* gene analysis, the species *A. phagocytophilum* substituted the species *Ehrlichia (E.) phagocytophila*, *E. equi* and the agent of canine and human granulocytic ehrlichiosis (Dumler et al., 2001; Woldehiwet, 2010; Pishmisheva et al., 2016). *A. phagocytophilum* is the most prevalent species of the genus in various parts of the world depending on tick occurrence (Eisen, 2018). It was also described in animals and humans in many countries of Europe (Jahfari et al., 2014). Some studies observed co-exposure to *A. phagocytophilum* with other serious zoonotic pathogens, especially *Borrelia burgdorferi* (Derdáková et al., 2011; Butler et al., 2016; Tsachev et al., 2018).

In Slovakia, specific *A. phagocytophilum* antibodies were detected in humans for the first time by Kalinová et al. (2009). The first clinical case of human granulocytic anaplasmosis was described by Nováková et al. (2010) in a 54-year-old man; subsequently, Kalinová et al. (2015) confirmed specific antibodies to *A. phagocytophilum* in 22 patients with suspected tick-borne encephalitis. However, the presence of *A. phagocytophilum* has been reported in Slovakia in 8.3% of ticks (Derdáková et al., 2003) and 3.9% of sheep (Derdáková et al., 2011). Also, Smetanová et al. (2006) observed *A. phagocytophilum* in 4.4% of ticks, 5.5% of wild boars, 1/2 of roe deer, 1/3 of red deer and in 6% of rodents tested. Later on, Svitálková et al. (2015) demonstrated a higher rate of *A. phagocytophilum* infection in *I. ricinus* in an urban habitat in south-western Slovakia. The authors also suggested that rodents are not the main reservoirs of this pathogen.

Until now, only little information has been available regarding the prevalence of *A. phagocytophilum* in horses. For example, Slivinska et al. (2016) tested 39 horses from Slovakia by PCR and observed only one positive case. Since the infection is characterised by short-term bacteraemia (Passamonti et al., 2010), the detection of specific antibodies facilitates an understanding of the disease circulation.

The aim of this study was to determine and follow up the seroprevalence of *A. phagocytophilum* and to perform the molecular identification of this agent in horses in Slovakia.

MATERIALS AND METHODS

Ethics statement

The study was performed in compliance with the institutional guidelines for animal welfare issued by The Ethics Committee of the University of Veterinary Medicine and Pharmacy in Košice. All animal samples in this study were examined with the assistance of their owners. Blood samples were collected by a veterinarian.

Blood sampling

Ten-ml samples of venous blood were collected from the jugular vein of 200 horses without clinical signs consistent with equine granulocytic anaplasmosis at the time of sampling. The blood was collected into sterile coagulant-free tubes that facilitated coagulation and into sterile tubes with an anticoagulant. The coagulated blood was centrifuged and the obtained sera and unclotted blood were stored at –80 °C for further tests. The horses included in this study were of both sexes (108 females and 92 males), 22 different breeds and their age ranged from a 10 days old foal to a 26 years old mare. The horses originated from 17 studs (Table 1).

Characterisation of the sampling sites

The horse studs were selected from various regions of Slovakia (Fig. 1). A large portion of Slovakia is part of the Carpathian Mountains region (Kozak et al., 2013). The average annual temperature in Slovakia is 10 °C, while during the summer the average temperature increases to 26 °C. In association with an annual average relative humidity of 60% and rainfall varying from 500 to 2000 mm (Onderka et al., 2020), the whole territory of Slovakia represents a very suitable biotope for tick occurrence (Bazovska et al., 2005).

Serological analysis

The sera were tested for IgG against *A. phagocytophilum* using the commercial *A. phagocytophilum* IFA Equine Antibody Kit (Fuller Laboratories, USA) based on *A. phagocytophilum* HE-1 isolate antigens derived from HL-60 cells. The test was performed according to the manufacturer's instructions. Briefly, all samples were tested at a titre of 1:80 as a starting dilution in phosphate-buffered saline solution (PBS) of pH 7.2. The samples giving a positive reaction at a titre of 1:80 were tested also at 1:160, 1:320 and 1:640. The diluted sera were placed onto the slides with *A. phagocytophilum* antigen and incubated for 30 min at 37 °C in a humid chamber. After washing with PBS, anti-horse IgG conjugate was added and the slides were incubated under the same conditions. After the final wash, the PBS samples were mounted to buffered glycerol. The results were analysed using a NIKON Labophot 2A fluorescence microscope at ×400 magnification. The reaction was scored positive when *A. phagocytophilum* morulae giving bright green fluorescence were shown, indicating the presence of specific antibodies. Samples giving a positive reaction at 1:640 were considered positive.

Table 1. Characterisation of the horse studs

Stud number	District	Location	Altitude above sea level	Season	Management method
1	Trenčín	48°58′1.27″N 18°07′8.11″E	226 m	Spring	day pasture
2	Trenčín	48°48′59.99″N 17°47′59.99″E	254 m	Summer	day pasture
3	Rožňava	48°49′14.63″N 20°22′11.57″E	456 m	Summer	day/night pasture
4	Trenčín	48°58′59.99″N 18°08′60.00″E	230 m	Autumn	day pasture
5	Košice – okolie	48°35′59.99″N 21°20′59.99″E	181 m	Autumn	day/night pasture
6	Zlaté Moravce	48°25′09.8″N 18°24′49.1″E	206 m	Autumn	hours outing
7	Košice	48°43′21.8″N 21°13′25.9″E	297 m	Autumn	day pasture
8	Brezno	48°39′59.99″N 19°38′59.99″E	900 m	Autumn	day/night pasture
9	Lučenec	48°19′56.96″N 19°40′1.49″E	187 m	Summer	day pasture
10	Liptovský Mikuláš	49°07′60.00″N 19°30′59.99″E	574 m	Summer	day/night pasture
11	Liptovský Mikuláš	49°05′52.91″N 19°36′20.09″E	577 m	Summer	day pasture
12	Ružomberok	49°04′29.28″N 19°18′27.04″E	481 m	Summer	hours outing
13	Liptovský Mikuláš	49°05′21.3″N 19°39′00.6″E	624 m	Summer	day/night pasture
14	Košice – okolie	48°36′51.41″N 20°59′58.45″E	209 m	Summer	day pasture
15	Košice	48°39′54.6″N 21°12′22.9″E	273 m	Summer	day pasture
16	Nitra	48°19′60.00″N 18°12′60.00″E	200 m	Summer	day pasture
17	Levoča	49°00′60.00″N 20°45′59.99″E	463 m	Autumn	day pasture

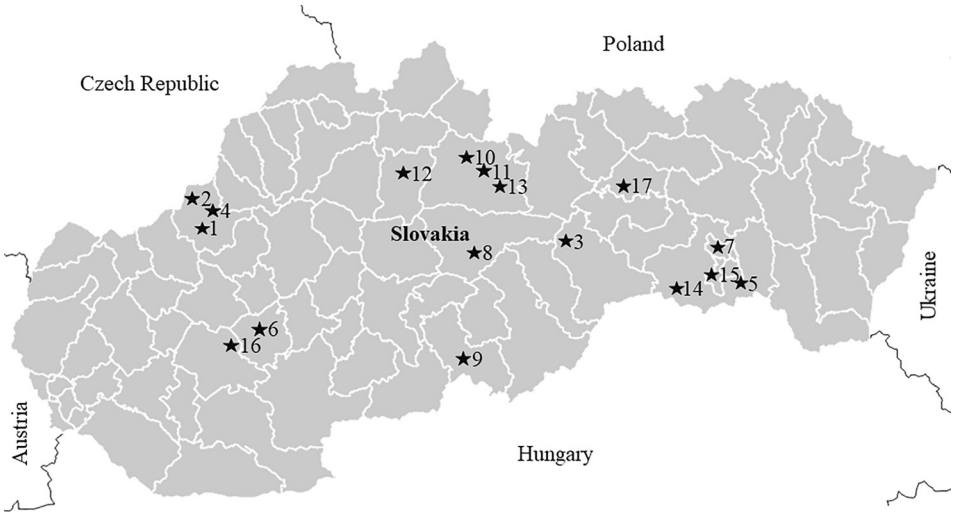

Fig. 1. Locations of the sampling sites. 1–17: numbers of horse studs

PCR analysis

The molecular detection of *A. phagocytophilum* was attempted, based on an 839-bp fragment of the 16S rRNA gene, using specific primers designed in the Primer3plus software (F: 5′GCATGTAGGCGGTTCGGTAAGTT3′ and R: 5′ATGGCGTGACGGGCAGTGT3′). The PCR reaction was performed in a total volume of 50 µL of the reaction mixture containing 2 µL of the tested DNA, 1.2 µL of primers, 5.5 µL of the PCR Master Mix (Jena Bioscience, Germany), 0.2 µL of the Taq Polymerase (Jena Bioscience, Germany) and 41.5 µL of the PCR Ultra H_2O (Top Bio, Prague, Czech Republic). In each PCR assay, positive and negative controls were used. The PCR protocol consisted of the following steps: initial DNA denaturation for 2 min at 94 °C followed by the next 30 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 51.2 °C for 30 s and extension at 72 °C for 1 min, ending with a final extension at 72 °C for 10 min and the subsequent rapid cooling to 4 °C. The PCR product was visualised on 1% agarose gel with Sybr Gold (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

All statistical analyses were performed in the statistical analysis software GraphPad Prism, version 5.01 (GraphPad Software, Inc., San Diego, California, USA). The statistical comparison of categorical variables was carried out with the chi-square (χ^2) test or the Fisher's exact test, and *P* values of less than 0.05 were considered significant. The differences in prevalence observed for individual sex and age categories of mares, stallions and geldings, respectively, were tested by the chi-square (χ^2) test.

RESULTS

Our results (Table 2) confirmed the presence of specific antibodies to *A. phagocytophilum* in 85 out of the 200 horses tested (42.5%). The seropositivity rates identified in individual studs varied from 15.4% to 83.3%. The differences between studs in antibody prevalence were not statistically significant ($\chi^2 = 17.40$, df = 16, $P = 0.360$).

The results of serological analyses by sex and age are shown in Table 3.

In our study, specific antibodies were observed in 46/108 mares, 10/22 stallions and 29/70 geldings. The comparison of seroprevalence in animals by sex did not show significant differences ($\chi^2 = 0.1128$, df = 2, $P = 0.946$). The comparison of seroprevalence in individual age categories relative to sex did not show significant differences either. However, different results were obtained in the various age categories. In the category of less than three years, the sample size was too small to evaluate the chi-square test; for 3–10 years old animals, an insignificant difference, i.e. less than 0.05 ($\chi^2 = 0.755$, df = 2, $P = 0.686$), was demonstrated. For the age category of more than 10 years, an insignificant prevalence, i.e. a *P* value higher than 0.05 ($\chi^2 = 1.265$, df = 2, $P = 0.531$), was detected as well.

No positive PCR result was obtained at all.

Table 2. Results of screening for anti-*Anaplasma phagocytophilum* IgG antibodies by the indirect immunofluorescence assay in horses from selected regions in Slovakia

Stud number	Number of horses tested	Finally negative titre ≤1:320		Tested titre			Finally positive titre ≥1:640	
				1:80	1:160	1:320		
		Number	%	Positive/total animals	Positive/1:80 positive	Positive/1:80 positive	Number	%
1	15	10	66.7	7/15	7/7	5/7	5	33.3
2	10	5	50.0	6/10	5/6	5/6	5	50.0
3	20	11	55.0	13/20	10/13	10/13	9	45.0
4	12	8	66.7	4/12	4/4	4/4	4	33.3
5	13	9	69.2	7/13	5/7	5/7	4	30.8
6	19	14	73.7	9/19	7/9	5/9	5	26.3
7	10	6	60.0	5/10	5/5	4/5	4	40.0
8	20	10	50.0	15/20	15/15	12/15	10	50.0
9	5	3	60.0	2/5	2/2	2/2	2	40.0
10	6	1	16.7	5/6	5/5	5/5	5	83.3
11	11	5	45.5	8/11	6/8	6/8	6	54.5
12	13	11	84.6	3/13	3/3	3/3	2	15.4
13	4	2	50.0	3/4	2/3	2/3	2	50.0
14	9	6	66.7	7/9	4/7	3/7	3	33.3
15	21	8	38.1	16/21	13/16	13/16	13	61.9
16	7	4	57.1	5/7	5/5	3/5	3	42.9
17	5	2	40.0	3/5	3/3	3/3	3	60.0
Total	200	115	57.5	118/200	101/118	90/118	85	42.5

Table 3. Results of the serological analysis by sex and age[a]

Sex	IFA, total		IFA by age category					
			<3 years		≥3 < 10 years		≥10 years	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Mares	62	46	4	2	23	13	35	31
Stallions	12	10	2	3	7	2	3	5
Geldings	41	29	0	1	13	8	28	20
Total	115	85	6	6	43	23	66	56

[a] PCR-positive samples were not found at all; IFA = indirect immunofluorescence assay.

DISCUSSION

In this paper we present the first multiregional study focused on the seroprevalence of *A. phagocytophilum* in the horse population of Slovakia. We confirmed a 42.5% prevalence of antibodies to *A. phagocytophilum*. Consistently with the results obtained by Rolim et al. (2015), no predisposition for infection based on the animal's sex or age was observed in our study, and no molecular evidence of *A. phagocytophilum* was found in the animals tested.

In Europe, a seropositivity higher than this was observed only in the Czech Republic (Praskova et al., 2011). In other European countries, seropositivity to *A. phagocytophilum* in horses has ranged between 16.7 and 22.75% until now (Egenvall et al., 2001; Leblond et al., 2005; Hansen et al., 2010; Passamonti et al., 2010; Ebani, 2019; Tsachev et al., 2019).

Similarly, different results were observed in Brazil as well. Nogueira et al. (2017) screened 97 blood samples from horses and 11.34% of them were seropositive to *A. phagocytophilum*. Similar results were presented by Dos Santos et al. (2019), with the seropositivity reaching 17.4%. A higher seropositivity rate (65%) was observed by Salvagni et al. (2010) in Brazilian horses.

In contrast to the previous data, a very low seroprevalence for *A. phagocytophilum* was detected in horses in Korea – 3.1% (Lee et al., 2015) and in Canada – 0.53% (Schvartz et al., 2015). There may be several reasons for these differences. One of them is the geographical variability of equine granulocytic anaplasmosis dependent on the tick-friendly environment (Janzén et al., 2019). Another reason may be the growing occurrence of this re-emerging disease worldwide. For example, Andersen et al. (2019) observed approximately twice as high *A. phagocytophilum* prevalence in the roe deer population as compared to the results obtained 14 years previously in Denmark (Skarphédinsson et al., 2005). Furthermore, such variation in the seropositivity levels may be caused by the use of different serological tests or horse management methods (Salvagni et al., 2010).

We suggest that the negative results obtained by molecular detection in this study may have been due the fact that none of the tested clinically healthy horses was in the acute phase of infection (Rejmanek et al., 2012). The acute phase is characterised by limited and short-term bacteraemia, while the peak antibody titre occurs between days 19 and 81 of infection and humoral immunity can persist for at least two years (Van Andel et al., 1998). On the other hand, while applying the PCR method, Passamonti et al. (2010) observed 11 horses positive for *A. phagocytophilum* in a group of 120 animals without any clinical signs, and none of the horses showed clinical or haematological changes typical of this disease. This can be regarded as one of the reasons why clinical anaplasmosis is still underdiagnosed.

The positive results of our serological analysis prove the circulation of *A. phagocytophilum* in Slovakian horses. Although it seems unlikely for the infected horses to serve as effective reservoirs of *A. phagocytophilum* (Sellon and Long, 2014), the infection was found to persist in experimentally infected horses for at least 129 days. Our data contribute to a better understanding of the potential occurrence and spread of this disease and facilitate the identification of new sites with a higher risk of *A. phagocytophilum* infection.

Anaplasmosis is an re-emerging zoonotic disease with a natural cycle. Due to the non-specific clinical signs and/or the frequently subclinical course of anaplasmosis in both animals and humans, it is important to include this disease in the differential diagnosis of vector-borne encephalitis for animals as well as humans. The results of this serological survey indicate that anaplasmosis can be common in horses. In view of the One Health concept, the results can significantly contribute to improving the knowledge of the epidemiological situation and serve as a basis for successful diagnosis and risk assessment in this region of Central Europe.

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