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Equine piroplasmosis in Austria – a serological pilot study

Esther Dirks[1], Irina Preining[1], Roman Peschke[2], Phebe de Heus[1], Anja Joachim[2] and Jessika-M. V. Cavalleri[1]

[1] Clinical Unit of Equine Internal Medicine, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria
[2] Institute of Parasitology, University of Veterinary Medicine Vienna, 1210 Vienna, Austria

Summary: Equine piroplasmosis (EP) is a tick-borne disease of horses, donkeys and wild equids. Chronically infected animals, which often only show reduced performance or are clinically unremarkable, constitute a potential risk for parasite spread. In Europe, currently no transboundary preventive measures for identifying infected animals or preventing their cross-border transportation are in place. In Austria, as in the neighbouring countries of Switzerland and Germany, EP has only been detected sporadically. By contrast, Austria's eastern neighbour, Hungary, is considered endemic for EP. The geographic vicinity to Hungary and a recently described autochthonous case of EP induced the present pilot study on the serological status of horses in eastern Austria. Serum samples of 173 clinically healthy horses of different age and sex from 43 different locations in Vienna and Lower Austria, without known stays abroad within the last 12 months, were examined by cELISA. Sampling had been conducted during a prevalence study on West Nile infections in 2017. Four horses (2.3%) aged 7–28 years from three different locations were positive for anti-*T. equi* antibodies. Two of the positive horses had a Hungarian passport, and two horses an Austrian passport. One of the horses with an Austrian passport, however, was born in Romania. No horse was positive for anti-*B. caballi*-antibodies. The results of this study show that animals infected with *T. equi* occur in the east of Austria. Equine practitioners should consider EP as a possible cause of febrile diseases and haemolytic anaemia, but also be aware of chronic or subclinical courses of infection and the risk of parasite transmission from inapparent carriers. In addition, EP should be monitored in areas with known occurrence of *Dermacentor* as a vector-competent tick genus.

Keywords: piroplasmosis, *Theileria equi*, *Babesia caballi*, cELISA, vector-borne diseases, prevalence

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Correspondence: Prof. Jessika M. Cavalleri, University of Veterinary Medicine Vienna, Equine University Hospital, section of equine internal medicine, Veterinaerplatz 1, 1210 Wien; jessika.cavalleri@vetmeduni.ac.at

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Introduction

Equine piroplasmosis (EP) is a tick-borne disease that affects horses, donkeys, and wild equids. It is caused by the apicomplexan haemoprotozoa *Babesia caballi*, *Theileria equi* (formerly *Babesia equi*) and *Theileria haneyi*. *Theileria haneyi* was recently described in the USA as a novel species, but has not been reported in Europe (*Onyiche* et al. 2019). After transmission with an infected tick's blood meal, the parasites infect primarily erythrocytes and, in the case of *Theileria*, also peripheral blood mononuclear cells, i.e. lymphocytes and macrophages. The infection causes erythrolysis and, consequently, haemolytic anaemia (*Onyiche* et al. 2019, *Ramsay* et al. 2013). The reservoir for *T. equi* is represented by chronically infected horses, while it is so far unclear whether the tick or the mammalian host is the reservoir for *B. caballi* (*Ueti* et al. 2008).

The infection is frequently challenging to diagnose due to often vague and variable clinical signs with various levels of severity. Common clinical findings are recurrent high fever, anaemia, pale or icteric mucus membranes, malaise, pigmenturia and peripheral oedema (*Dunkel* 2018).

The course of the disease can vary from peracute infections, documented mainly in neonatal foals (*Adam* et al. 2016, *Butler* et al. 2005), to acute cases with high, recurrent fever and anaemia, and chronic infections where affected animals often show poor performance or no clinical signs at all (*Wise* et al. 2014).

The detection of intraerythrocytic parasite stages in stained blood smears provides direct diagnosis but shows only a low sensitivity (*Ambawat* et al. 1999, *Malekifard* et al. 2014, *Pikalo* et al. 2016, *Sumbria* et al.,2014). To increase detection sensitivity and to unequivocally determine the parasite species involved, microscopic assessment of the blood smear should always be combined with molecular methods such as PCR and, if possible, sequence determination (*Alhassan* et al. 2007, *Ueti* et al. 2003). Despite its high sensitivity when compared to the evaluation of blood smears, PCR may not detect infection in chronically infected horses (*Allred* 2003). In those cases, detection of specific antibodies should be performed to determine or rule out infection. The capture enzyme-linked immunosorbent assay (cELISA) is the method of choice for the screening of horse populations (*Ybañez* et al. 2018). Some countries, e.g., Canada and the USA, request a negative antibody test for horses entering the country (https://www.aphis.usda.gov/animal_health/animal_diseases/piroplasmosis; 30.03.2022).

When infected with *T. equi*, horses seroconvert within 7 to 11 days post-infection and titres peak at 30 to 45 days (*Wise* et al. 2014). Titres may be positive for at least one year even after an infection with *T. equi* was successfully treated (*Wise* et al. 2018), and are continuously elevated in persistent infections, even when parasite detection by PCR renders negative results because of parasite sequestration in the spleen (*Ribeiro* et al. 2013a). Horses infected with *B. caballi* seroconvert after 3–21 days post infection (*Camino* and *Cruz* 2017). Nothing is known about antibody titre persistence in infections with this species.

The occurrence of the disease is strongly linked to the presence of its vector ticks (*Tirosh*-Levy et al. 2020). In Europe, depending on the geographic location, members of the genera *Dermacentor*, *Rhipicephalus* and *Hyalomma* must be considered (*Scoles* and *Ueti* 2015). *Dermacentor reticulatus* and *Dermacentor marginatus* are considered to be vector-competent ticks and occur in large parts of Europe including Austria and its neighbouring countries (*Drehmann* et al. 2020, *Rubel* et al. 2016, *Sands* et al. 2021). Equine piroplasmosis has an almost worldwide distribution (*Onyiche* et al. 2019, *Tirosh-Levy* et al. 2020, https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.08_EQUINE_PIROPLASMOSIS.pdf; 30.03.2022). Prevalences determined in recent studies from Europe ranged from 7.3% in Switzerland tested by IFAT (*Sigg* et al. 2010) to 25.4% in the Romanian Danube Delta tested by PCR (*Gallusová* et al. 2014) and were 8.9% for *B. caballi* and 39.8% for *T. equi* in Italy tested by cELISA (*Bartolomé Del Pino* et al. 2016). A study conducted in Hungary in 2012 using PCR, IFAT or cELISA determined that out of 27 tested farms, 17 held horses positive for *T. equi* (*Farkas* et al. 2013). Austria is so far considered to be non-endemic for EP, although in 2021 a case of an autochthonous infection with *T. equi* was reported from Burgenland, the most easterly province of Austria (*Dirks* et al. 2021). To obtain more information on the presence of EP in eastern Austria, a pilot study was carried out on the current serological status of horses in the area of greater Vienna (Austria), applying commercial cELISA.

Materials and Methods

Sample collection

Convenience blood samples were taken from 173 horses in Vienna and its surroundings. The sampling was conducted in 2017 for a study on West Nile Virus prevalence (*de Heus* et al. 2021). The owners responded to a questionnaire with specific attention to possible transports or stays abroad of the animals. Information included were the current address and the time of living there; breed; age; coat colour; stable type; water source; if the horse was imported and if so, how long ago and from which country; vaccination status; history of chronic illness; usage of insect repellent physical and chemical. Only horses that had not travelled outside of Austria during the last 12 months were included in the study. Clinical and neurological examinations were carried out, and only horses without signs of neurological or acute infectious disease were included. Stays abroad were investigated in more detail in the four seropositive horses. Blood was taken from a jugular vein and collected into EDTA and serum tubes. The samples were centrifuged, plasma was removed, aliquoted and frozen at -20 °C until further analysis.

Preparation of samples

After storage samples were defrosted at room temperature in May 2021.

For the determination of antibodies, cELISA assays for the detection of antibodies against *B. caballi* or *T. equi* (*Babesia caballi* Antibody Test Kit and *Theileria equi* Antibody Test Kit, Veterinary Medical Research & Development, Washington DC, USA) were used according to the manufacturer's instructions. According to the manufacturer the assay has a sensitivity and specificity of 100% when cELISA protocols developed by the national veterinary service laboratory of the world organisation of animal health (OIE) are used as gold standards (http://inel-mt.hr/wp-content/uploads/2018/07/VMRD-Portfolio-2018.pdf; 30.03.2022). A FilterMax F5 Multimode microplate reader with SoftMaxPro® 6.5.1 software (Molecular Devices, LLC, San Jose, CA, USA) was used for read-out at 620 nm wavelength.

Inhibition was calculated according to the manufacturer's instructions as percent inhibition

$$(\%I) = 100 \times (1 - (OD_{sample} \div OD_{negative\ control})]$$

and samples were classified as positive when $\%I > 40$ and as negative when $\%I < 40$ as recommended by the manufacturer (https://vmrd.com/test-kits/equine;30.03.2022).

Results

Samples were obtained from 43 different geographic locations (stables) and two different federal states (Vienna and Lower Austria). They included horses of 10 different breeds (Arabian, Haflinger, Icelandic Horse, Lipizzaner, Noriker, Quarterhorse, Shetland Pony, Standardbred, Warmblood, Welsh Pony) as well as other ponies and horses of no specified breed. Horses were between one and 31 years of age, with a median age of 13.4 years. Of the 173 tested animals 66 were mares, 87 geldings and 20 stallions. Physical (e.g., blankets) or chemical insect control was applied to 109 horses and not applied to 57 horses, for seven horses this was unknown. The use of chemical insect repellents was reported for 63 horses; 103 horses were reported as untreated; for seven horses this information was not documented. Regarding stabling conditions, 136 horses were mainly kept in a boxstall and 36 mainly outdoors. In one horse this information was not given.

Of the 173 samples none was seropositive for *B. caballi* and four (2.3%) were seropositive for *T. equi*. The four seropositive horses were located on three different farms (Figure 1). The most important details of the horses that tested positive for *T. equi* are shown in Table 1 and Table 2. The respective stables were all located within 40 km of the city of Vienna. All four positive tested horses were housed in boxstables and their main water source was inside. On one of the positive horses a chemical insect repellent (icaridin) was used. Three horses had a dark coat one had a two-tone coat. Three horses originated from countries other than Austria (Hungary and Rumania). Two of the positive tested horses had Hungarian passports; of these animals one also had a Hungarian chip number. The other two seropositive horses had Austrian passports, but one horse was born in Romania. The horses lived at their current address between 1.5 and 13 years, during this time no transports outside of Austria were reported by the owner.

Discussion

The present study shows that 2.3% of the horses tested in eastern Austria are positive for antibodies against *T. equi*. One of the positive tested horses has never left Austria. In this case an autochthonous infection is likely. No antibodies against *B. caballi* were found. Although this study is not representative for the whole country, it gives an indication of the occurrence of equine piroplasmosis in Austria. Vector-borne diseases are frequently considered as highly important emerging diseases, and due to globalisation and climate change, they have become an increasing concern worldwide (*Caminade* et al. 2019, *Mandal* 2018). Due to the globalisation of horse trading and equestrian competitions, cross-border transport of horses is common and increasing, leading to the spread of diseases such as equine piroplasmosis, and this is a growing concern for veterinarians worldwide (*Timoney* 2000). While Austria has so far been considered a non-endemic country for EP, recent findings of autochthonous infections (*Dirks* et al. 2021) with *T. equi* pointed to a change in this situation, and the present study supports the hypothesis that Austria may be or soon become endemic for equine piroplasmosis, as potential vector ticks (*D. reticulatus*, *D. marginatus*) are already present in Austria (*Rubel* et al. 2016). Sporadic or endemic infections of equids in other central European countries (*Butler* et al. 2012, *Farkas* et al. 2013, *Gallusová* et al. 2014, *Laus* et al. 2013, *Moretti* et al. 2010, *Ribeiro* et al. 2013b, *Sigg* et al. 2010, *Slivinska* et al. 2016) and frequent horse travel could increase the number of infected horses in Austria, and support the development of endemic foci, especially in eastern Austria with its warm climate and long summers. Although the limited study population does not permit prevalence rate determination, the finding of 2.3% in the examined horses clearly hints at the presence of infected horses in Austria. In line with the course of infection, *B. caballi* which causes transient infections in untreated animals could not be detected by testing single convenience samples, while long-lasting infections with *T. equi* maintain antibody levels for sufficiently long periods of time to detect putative carriers by serology. Consequently, serological testing is considered the most sensitive assay for the detection of infections *with T. equi* (*Wise* et al. 2014, *Ybañez* et al. 2018). Even though the horses in the current study have not travelled outside of Austria in the last 12 months, they may have been infected before that time. After detailed research into possible stays outside Austria, three horses originated from endemic European countries (Hungary and Romania) so autochthonous infections could not be confirmed in these cases. One horse had no documentation of ever having left the country as judged by the owner questionnaire, vaccination record as well as country codes derived from the chip and passport. This horse could represent the only autochthonous case of this series, although the animal's birthplace could not unequivocally be determined. In addition, another horse from the same location was also found seropositive. Even though one of these two originated from Hungary, encountering multiple seropositive animals on the same farm raises suspicion for

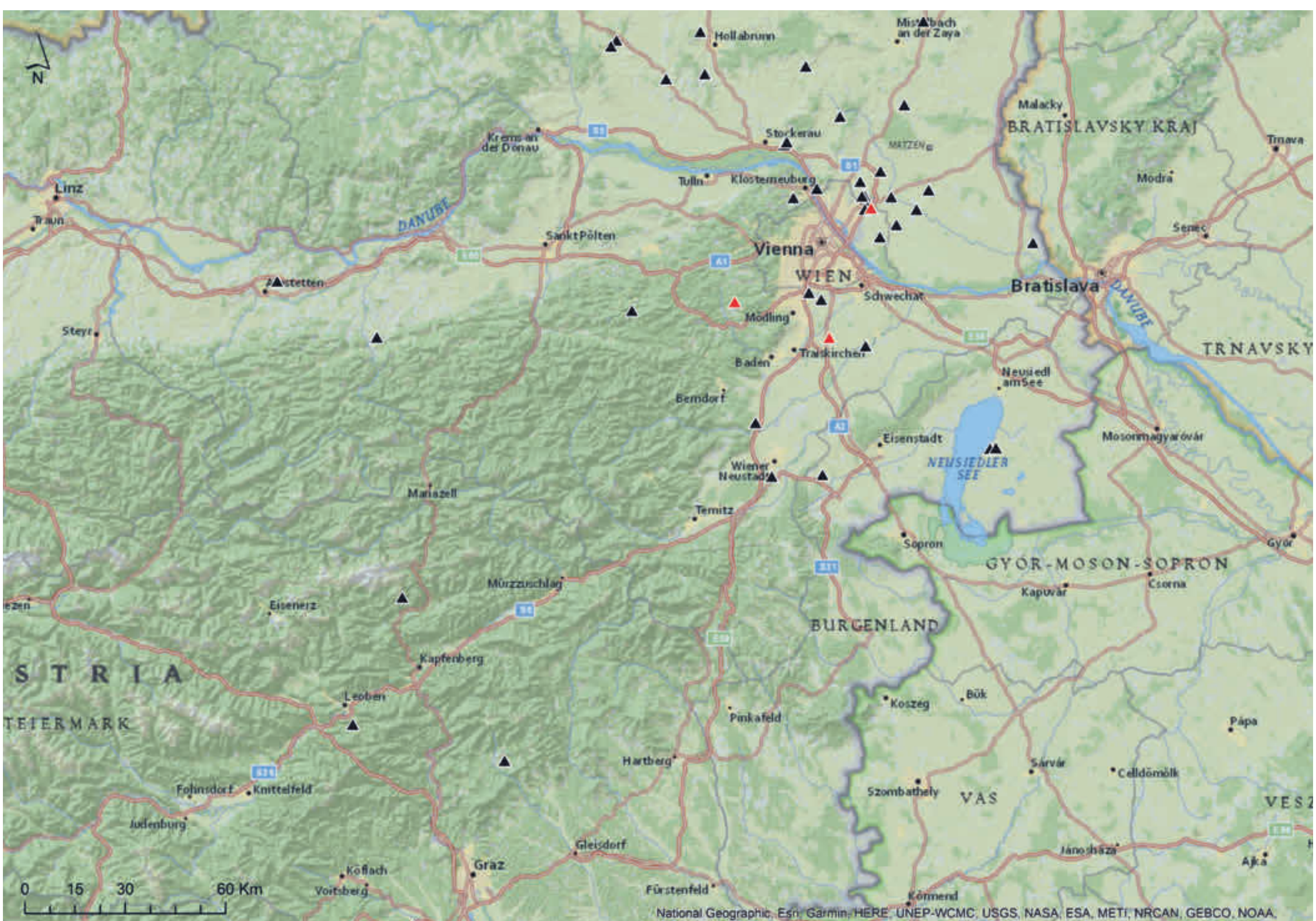

Fig. 1 Locations of all tested equids. Black triangles: locations with negative horses; red triangles: Location with positive horses. | *Standorte aller getesteten Equiden. Schwarze Dreiecke: Standorte mit negativ getesteten Pferden, rote Dreiecke: Standort mit positiv getesteten Pferden.*

a connection between these cases, possibly involving infected vector ticks in the area.

The four seropositive horses had been in Austria for at least one year and could therefore act as reservoirs for *T. equi*. They appeared clinically healthy and were not reported to have received antiprotozoal treatment. Occult infections of clinically healthy carriers are specifically important in cases of *T. equi* infections which could remain undetected over a long time and therefore contribute to the transmission of the parasite to other horses at the same location or the spread of the infection in case of transport.

Besides tick-borne transmission, iatrogenic as well as intrauterine (transplacental) transmission (*Wise* et al. 2014) must not be neglected in this context. An infected breeding mare may transmit *T. equi* to the foetus with the consequence of either disease or permanent carrier status in the foal (*Wise* et al. 2014). The treatment of equine piroplasmosis is performed using Imidocarbdipropionate. Dosing and dosing intervals depend on the type of the treated haemoparasite (*Belloli* et al. 2002). To confirm the success of a treatment a series of PCR testing must be performed. Preferably three tests should be performed, each one month apart. If the treatment is not carried out successfully, equids can become silent carriers and serve as reservoirs which is frequently the case in *T. equi* infections. Currently, no drug is approved for tick repellence in Europe. Permethrin is approved as a repellent against flies and could also be effective against ticks, but dosage and dosing intervals are not specified. Physical repellents such as blankets can be used, but their effectiveness is not investigated.

To determine specific risk areas or endemic foci for EP in Austria, a cross-sectional investigation of horses from all areas of Austria should be undertaken, including serological as well as PCR-based detection of piroplasms to determine not only infection but also carrier status of the Austrian horse population. While the presence of *B. caballi* could not be confirmed in the present study, its presence in Austria currently cannot be ruled out, as imported cases have already been detected in Austria and neighbouring countries (*Joachim* et al. 2022).

It could be assumed that in areas where horses are pastured the risk of autochthonous infections is higher than in the sampled area where, due to a shortage of pasture in the urbanised surroundings of Vienna, most horses are stabled. However, obviously also mostly stabled horses can encounter infected ticks (e.g., through outdoor riding activities), as it was observed for the *T. equi*-seropositive horses in this study. In addition, greater Vienna is not an area of high horse density; most horses are kept in the pre-alpine areas in the west of the country (https://www.statistik.at/web_de/statistiken/wirtschaft/land_und_forstwirtschaft/viehbestand_tierische_erzeugung/viehbestand/index.html; 30.03.2022). It is conceivable that infectious diseases such as piroplasmosis can spread more easily in regions with high host population density, and that the prevalence for the Austrian horse population is therefore underestimated in the present study. For tick-borne diseases, the density of the tick vector is also decisive for transmission and endemicity of infectious agents in an area; however, tick density cannot readily be determined and is highly variable over the year (*Vogelgesang* et al. 2020, *Weiler* et al. 2017), so this critical risk factor cannot easily be quantified in the context of EP.

Conclusion

While Austria is currently considered non-endemic for EP, the results of the current study question this, as, in addition to a previously described autochthonous infection, horses serologically positive for *T. equi* were detected in a small number of convenience samples from horses in eastern Austria. To obtain a better understanding of the actual prevalence of piroplasmosis in Austria, further studies are needed, applying a cross-sectional study design with a higher number of sampled animals all over Austria, with a focus on regions with high horse densities. Molecular analysis of whole blood samples by PCR in addition to serology could be beneficial to detect active infections and determine genotypes by amplicon sequencing.

As currently no screening programmes are implemented, it is left to the attending veterinarians to decide how to proceed in identifying and treating an infected horse. It is crucial to increase the overall understanding of the disease and the importance of correct treatment to minimise the number of chronically in-

Table 1 Results and details regarding the horses tested positive for T. equi. antibodies. | *Angaben zu Signalement und Management der vier Pferde mit nachweisbaren Antikörpern gegen T. equi.*

ID	Breed	Age*	Sex	Housing	Insect control	I (%)
1	Pony	7.2	Mare	Box	No	59.5
2	Warm-blood	19.4	Gelding	Box	Yes	77.58
3	Lippizaner	14.3	Gelding	Box	No	80.18
4	Pony	28.8	Gelding	Box	No	53.79

*age in years

Table 2 Detailed research into (foreign country) stay regarding the horses tested positive for T. equi. | *Detaillierte Nachforschung zum (Auslands) Aufenthalt zu den positiv auf T. equi getesteten Pferden.*

ID	Birth place (Country)	UELN Country Code	Microchipnr. Country Code	Vaccination outside AUT	Duration of stay at current stable**	Current stable*
1	no data	HUN	HUN	no	5	A
2	ROU	AUT	no data	no	13	B
3	no data	HUN	no data	no	6	C
4	no data	AUT	AUT	no	1.5	B

*stable data anonymized for privacy reasons; UELN universal equine life number; ROU Romania; HUN Hungary; AUT Austria / **in years fected animals that can act as reservoirs for piroplasms and to prevent or at least limit the establishment of this infection in currently non-endemic areas. Reporting of tick-borne disease risks on a national level, such as in Switzerland (https://www.ch.ch/de/zeckenschutz;30.03.2022), or the use of digital media to identify an elevated risk for tick bites, like a smartphone app developed in the Netherlands for dog owners to report tick infestations (*Jongejan* et al. 2019) could be used to minimise infection risks for horses, too. In addition, border controls for horses entering the country could be implemented to prevent the import of infected animals if elevated risks must be suspected.

Equine practitioners should be aware of piroplasmosis, not only as the cause of acute illness with high recurrent fever and anaemia, but also regarding the chronic or subclinical course of infection where horses may act as reservoirs. Since the vector-competent tick genus *Dermacentor* is native to Austria, caution is advised, and both prophylactic measures and appropriate treatment should be initiated to prevent the disease from further spread.

Conflict of Interest statement

The authors have no conflicts of interest to declare. All co-authors have seen and agreed with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Animal health statement

The collection of serum samples and questionnaire data in anonymised form was approved by the Ethics Committee of the University of Veterinary Medicine Vienna, the Austrian Federal Ministry of Labor, Social Affairs, Health and Consumer Protection and the Austrian Federal Ministry of Education, Science and Research (study reference number BMWF-68.205/0125-WF/V/3b/2017).

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