Vetsuisse-Fakultät der Universität Bern

Departement für klinische Veterinärmedizin

Pferdeklinik

(Leiter a.i.: PD Dr. med. vet. Vinzenz Gerber;

Stv. Leiter: Prof. Dr. med. vet. Reto Straub)

Arbeit unter der Leitung von

PD Dr. med. vet. Vinzenz Gerber

Seroprevalence of equine piroplasmosis in the Swiss horsepopulation

Inaugural-Dissertation

zur Erlangung der Doktorwürde

der Vetsuisse-Fakultät der Universität Bern

vorgelegt von

Liv Ursula Sigg

von Ossingen, ZH

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1. Abstract

In Switzerland, incidence and prevalence of equine piroplasmosis (EP) caused by *Theileria equi* and *Babesia caballi* are unknown. In order to obtain a first descriptive insight into the prevalence, a representative sample of 689 sera from horses kept in Switzerland was serologically investigated for antibodies against *T. equi* and *B. caballi* using the Indirect Fluorescence Antibody Test (IFAT). A total of 50 (7.3%) horses were seropositive for EP: 30 horses (4.4%) for *T. equi*, 10 (1.5%) for *B. caballi* and 10 (1.5%) had antibodies against both parasite species. Overall, the seroprevalence of *T. equi* was significantly higher than that of *B. caballi* (p=0.002). In domestic horses (animals bred and raised in Switzerland) seropositivity rate was 4.8% (11/230). Four (1.7%) of these horses were positive for *T. equi*-, six (2.6%) for *B. caballi*-antibodies and one (0.4%) had antibodies against both species. In imported horses, the EP-infection rate was 8.5% (39/459) and the prevalences were 5.7% (26/459) for *T. equi*, 0.9% (4/459) for *B. caballi* and 2.0% (9/459) had antibodies against both parasite species. Unlike in domestic horses, where no significant difference in seroprevalences could be observed between the species, the seroprevalence of *T. equi* was significantly higher (p<0.001) than that of *B. caballi* in imported horses. More than half of the imported horses that tested positive for EP originated from France. Horses imported from France, Spain and Portugal had a significantly higher and horses imported from Germany a significantly lower seroprevalence of EP compared to domestic horses. There were no associations between sex, age, weight loss, pasture time, surgery or blood transfusions with *T. equi* and *B. caballi* seroprevalences.

The overall seroprevalence of 7.3% clearly shows that EP is a threat to the health of the horses kept in Switzerland. With the presumed expansion of permissive tick vectors, EP has the potential to further increase in importance. Therefore, continuous monitoring is

indicated.

2. Introduction

Equine piroplasmosis (EP), caused by *Theileria equi* (*T. equi*) and *Babesia caballi* (*B. caballi*), ranges among the most important tick borne diseases of horses. Piroplasmosis occurs in regions and environments (pasture) where horses are exposed to vectors – ticks, especially of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* [1-7]. The disease is mainly endemic in tropic and subtropic areas [8, 9], but regions with a moderate climate may be affected as well. Parts of Africa, the Middle East, Asia, Central- and South-America, the Caribbean, but also Europe are concerned[1]. In the 1980s EP was tentatively eradicated from the United States, but focal outbreaks are regularly reported[2]. Rigorous import regulations are in place to prevent re-emergence of the infection in many non-affected countries.

Dermacentor sp. and *Rhipicephalus* sp., important vectors of both *T. equi* and *B. caballi*, have been sporadically observed in parts of Switzerland for decades and seem to progressively expand their habitat [10, 11, 12]. Several factors may play an important role in the spread of the disease: the relocation of infected horses and ticks through national and international movement together with the geographic expansion of the tick-vectors due to climate warming and respective creation of new areas ecologically permissive for these vectors. Since the first description of an autochthonous case in Switzerland in 1994 [13], EP has been considered as an 'emerging disease'[3].

To date, information on the epidemiology of equine piroplasmosis is very limited in Switzerland. The goal of this study was therefore to descriptively determine the *T. equi* and *B. caballi* seroprevalences with the use of a representative number of sera from adult imported and adult domestic horses, i.e. horses bred and raised in Switzerland.

3. Materials and Methods

3.1. Selection of the study-population and sample collection

To achieve a diagnostic detection limit of 0.5% disease prevalence with a certainty of 95% in a population of 85'000 horses in Switzerland, a minimum required sample of 596 randomly selected animals was calculated (WinEpiscope v_2, Module Disease Detection[4]). Therefore, a sample-size of 650 sera was targeted to get a sufficiently representative sample for the Swiss horse-population. For the purpose of threshold prevalence estimation, we considered factors such as breed, sex, intended use and husbandry to be irrelevant. The participation in the study was voluntary for the horse owners. The imported animals originated from countries from which horses are regularly introduced to Switzerland, including Germany, France, Spain, Portugal, Austria, Italy, Hungary, Czech Republic, Slovakia, Latvia, Lithuania, Poland, Belgium, Netherlands, Denmark and Slovenia. To select imported horses, the federal customs authority provided a list of horses imported between 2003 and 2007 with the names and addresses of horse-importers, as well as the number of imported horses and their country of origin. The goal of the sample selection of imported horses was to accurately represent the proportions of the country of origin of actual horse imports. In domestic horses, the goal of sample selection was to get a representative geographical distribution of the horse population in Switzerland. Because equidae are not registered in a central database yet, horses were identified using the databases of the equine clinic at the Vetsuisse-University in Berne and the Swiss Association for Horse Sports (Schweizerischer Verband für Pferdesport, SVPS). Eligible horse owners were phoned - and if they complied to the study – visited between December 2007 and December 2008 to collect blood samples of their animals. We collected serum

samples from 689 horses, 230 domestic, 459 imported. All horses where considered healthy by the owner at the moment of sampling. For each horse, a questionnaire was completed by the owner focussing on how long the horse has been in the owner's possession, if weight loss or other signs of disease were observed, if the horse had travelled outside of Switzerland and on housing conditions, (intended) use, feeding, vaccination and deworming practices. A detailed description of the questionnaire can be obtained from the corresponding author. The collected sera were also used in a separate study on the prevalence of equine infectious anaemia (EIA) [14].

During the visit, a 9 ml sample of blood was collected from the jugular vein and placed in sterile serum tubes (Monovette ® 9 ml Z; Sarstedt AG and Co., D-51588 Nümbrecht). The samples were stored at 4°C for a maximum of 4 days and then spun at 4°C and 1'160 x g for 11 minutes (Centrifuge 5810 R, Eppendorf). The serum was then placed in sterile 9 ml-Vacuettes ® (Z No Additive; Greiner Bio-One GmbH, A-4550 Kremsmünster) and stored at -80°C until serological analysis.

We tested 689 serum samples from 459 (66.6%) imported horses and 230 (33.4%) domestic horses. Overall, the median age of horses in the study was 11 years (Range 2-31; Figure 1). The median age of Swiss-bred horses was 18 years (Range 13-31), the median age of imported horses was 8 years (range 2-29). 4% (65) of the horses were stallions, 42.7% (294) were mares, and 47.9% (330) were geldings.

3.2. Serology (IFAT)

The serum samples were serologically analyzed with an indirect fluorescent antibody test (IFAT) as described by Tenter and Friedhoff [15]. Slides coated with either *T. equi* and *B. caballi* merozoites, respectively, were purchased at the laboratory of Dr. Böse GmbH,

Harsum, Germany. The serum bank of the Institute of Parasitology in Berne (IPB) was used to provide positive and negative controls. As secondary antibody, a flourescein-conjugated anti-horse antibody was used at a dilution of 1:90 (rabbit-anti-horse-IgG-FITC, ICN Pharmaceuticals Inc., Ohio, USA). The IFAT had been previously standardized and validated to obtain an accreditation status according to ISO 17025. Sera yielding a bright fluorescence at a dilution of 1:40 or higher for *T. equi* and 1:80 or higher for *B. caballi,* respectively, were considered as positive. In a first screening step the samples were diluted 1:40 (*T. equi*) and 1:80 (*B. caballi*), respectively. Sera that tested positive in this assay were subsequently analyzed by titration with two-fold dilutions up to 1:320 (*T. equi*) and 1:640 (*B. caballi*).

3.3.   Statistics

The seroprevalences of *T. equi* and *B. caballi* were determined with exact 95% confidence levels (CL), and compared between various groups such as parasites, horse origin (import, domestic as well as various countries of origin), gender and age classes using cross tabulation and the Chi-Square or Fishers Exact Test, whenever appropriate. Median age was compared between groups (serological status, origin) using the Mann-Whitney U / Wilcoxon Rank-Sum Test, and the association between prioplasma seropositivity and import status with age was evaluated using a 2-way analysis of variance (ANOVA) model. A *P* value <0.05 was considered significant. All analysis were preformed with the software NCSS 2007 (NCSS Statistical Software[5])

4. Results

Out of a total of 689 horses, 50 (7.3%) tested seropositive for EP. Thirty (4.4%) were positive for *T. equi*-antibodies, 10 (1.5%) for *B. caballi*-antibodies and ten (1.5%) horses were seropositive for both parasite species (Table 1). Overall, the prevalence of *T. equi* was significantly higher (p=0.002) than that of *B. caballi*.

Of 230 domestic horses, eleven (4.8%) were seropositive for piroplasmosis. Four horses (1.7%) had antibodies against *T. equi*, 6 (2.6%) against *B. caballi* and one horse (0.4%) was double-positive for both species (Table 1), differences were not significant. Of the eleven domestic horses that tested positive, five had been abroad for periods of one day up to three years, all in France. The six other horses had not been abroad for as long as the owners knew the horses, but a prior stay abroad could not be excluded.

Out of 459 imported horses, 39 (8.5%) tested seropositive for piroplasmosis. Twenty-six (5.7%) had antibodies against *T. equi*, four (0.9%) against *B. caballi* and nine horses (2.0%) had antibodies against both parasite species (Table 2). The seroprevalence for *T. equi* was significantly higher (p=0.0001) than that of *B. caballi*. From 138 horses imported from France, 24 (17.4%) were found to be seropositive for EP. Thirteen horses (9.4%) were positive for *T. equi*, four horses (2.9%) were positive for *B. caballi*. Seven horses had antibodies against both species. Thus, in horses imported from France, the seroprevalence for *T. equi* was significantly higher than that for *B. caballi* (p=0.03). Horses from Germany, Italy, Poland, Hungary, Czech Republic and Austria were found to have antibodies against *T .equi* only, whereas in horses from France, Spain and Portugal, antibodies against both species were found. Horses imported from France (p=0.0002), Spain (p=0.003) and Portugal (p=0.002) had significantly higher prevalences of EP than horses born in Switzerland. German horses had a significantly lower prevalence of EP than

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domestic horses. No horses from Belgium, Ireland, the Netherlands and Denmark had antibodies against *T. equi* or *B. caballi*.

Out of 65 stallions taking part in the study, six (9.2%) were seropositive for *T. equi*; two of them had antibodies against *B. caballi* as well. Stallions had significantly more often antibodies against *T. equi* than against *B. caballi* (p=0.046). From 294 mares, 21 (7.1%) were piroplasmosis-positive. Thirteen (4.4%) had antibodies against *T. equi*, four (1.4%) against *B. caballi* and four (1.4%) mares had antibodies against both species. Mares also had a significantly higher seroprevalence for *T. equi* than for *B. caballi* (p=0.03). Out of 330 geldings, 23 (7%) were seropositive for equine piroplasmosis. Thirteen (3.9%) had antibodies against *T. equi*, six (1.8%) against *B. caballi* and four (1.2%) were positive for both parasites. No statistically significant difference could be detected in the seroprevalences for *T. equi* and *B. caballi* in geldings. Furthermore, there was no statistically significant difference in the prevalence of EP between male and female horses, neither for *T. equi* (p=0.44) nor for *B. caballi* (p=0.97) (Table 1).

With the titration of the positive samples we found nine sera to be positive at a dilution of 1:40, nine at 1:80, seven at 1:160 and 15 at 1:320 for *T. equi*. For *B. caballi* six were positive at a dilution of 1:80, nine at 1:160, one at 1:320 and four at 1:640 (Table 2). The ten horses in which we simultaneously detected antibodies against *T. equi* and *B. caballi* all had different titers for both parasites, except horse no. 6 (Table 3).

There was no significant difference in age distribution between piroplasmosis-positive and –negative horses (Figure 1). However, the imported horses in the study were significantly younger when compared to the domestic horses. For further analysis we divided the horses into 4 age groups representing the quartiles for domestic and imported horses: 0-16 years, 17-18 years, 19-21 years and 22-31 years for domestic horse and 0-6 years, 7-8 years, 9-11 years and 12-32 years for import horses. There were no differences in prevalence of EP

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between the different age groups, neither in domestic nor in imported horses (*T. equi* p=0.13; *B. caballi* p=0.68).

Horses which were positive for EP did not show significantly more weight loss than the EP-negative horses and there was no significant difference in pasture time between the positive and the negative group either. Horses positive for EP had not undergone surgery (p=0.93) or recorded blood-transfusions (p=0.19) significantly more often than negative horses.

5. Discussion

Standard serological tests for babesiosis are the complement fixation test (CFT) and the indirect fluorescent antibody test [2]. The IFAT for *T. equi* is more sensitive than the CFT (89% vs. 63%), while the estimated specificity is the same (96%) [16]. The IFAT for *B. caballi* is more sensitive than the CFT (92% vs.28%) and slightly less specific (95% vs. 99%) [17]. In this study, we decided to use the IFAT because of its high sensitivity coupled with a good specificity.

Several studies on the seroprevalence of *T. equi* and *B. caballi* have been conducted in different countries. High infection rates were predominantly found in subtropical and tropical regions (in Brazil between 80 and 90% for both parasite species [18, 19, 20]) In Europe, however, published data reflecting larger geographical areas exist only from Spain (seropositivity 94% to *T. equi* and 90% to *B. caballi* by IFAT in Cordoba, [21]; 40% for *T. equi* and 28.3% for *B. caballi* by IFAT in Galicia, Spain [22]), and from Italy (12.4% for *T. equi* and 17.9% for *B. caballi* with IFAT for Italy [23]). In our study, of the eleven horses imported from Spain, three were seropositive for *T. equi* and one horse was positive for both parasites. This leads to a seropositivity rate for EP of 36.4%. Of the 33 horses imported from Italy, three (9.1%) were positive for *T. equi* and none for *B. caballi.* The difference between the results reported from Italy [23] and our results may be due to the limited number of Italian horses in our study and differences in the geographical distribution within Italy. No recent data about seroprevalence of EP is available of other European countries, which regularly export horses to Switzerland. The relatively high numbers of horses imported from France and Germany tested in our study (138 and 200, respectively) may provide a rough estimate of the real seroprevalences in these countries. The seroprevalence of horses imported from Germany was 1%, while it was 17.4% for the

horses imported from France. Assuming that mainly sound horses are imported to Switzerland, the real seroprevalence in these countries may be higher. However, we cannot completely rule out that the horses were infected after their importation to Switzerland.

In domestic horses, the prevalence rate for EP was 4.8%. The respective eleven horses with antibodies against one or both parasite species were distributed across various regions of Switzerland. Six of the positive horses had stayed in France for durations ranging from one day to three years. Evidence of autochthonous infections in different areas of Switzerland would be an important epidemiological information to elaborate control measures. Unfortunately, however, incomplete owner information precluded uninterrupted reconstruction of the life histories of the infected animals identified in the present study: it was not possible to confirm out that the five remaining positive horses had never left Switzerland. Autochthonous infections could thus not be conclusively proven with this study. An alternative approach, searching for *T. equi* or *B. caballi* in the domestic tick population, may be more adequate to investigate this important question.

As the age of our study population was not normally distributed and the domestic horses had a higher mean age than imported horses, we separated them to compare age groups. The comparison of 25%, 50% and 75% percentiles of both groups showed no difference in seroprevalence between age groups. Neither could we see a different age distribution between infected and non-infected horses. For the interpretation of the results, it is important to consider that *T. equi* persists as a life-long infection, whereas the expected persistence of *B. caballi* in its host is 1.5 years on average. Consequently, a *T. equi*-positive IFAT may reflect an actual infected (chronic and long persisting) status, whereas, for *B.caballi*, a horse may be seropositive without harboring the parasite [24] anymore.

This persistent infection may be the reason for increased seropositivity rates in older horses in several other studies [24, 25, 26, 27]

The comparison of titers against *T. equi* and *B. caballi* in horses that were seropositive to both species also indicated that in three of these horses a potential cross-reaction, as shown before by others [28], might have occurred, as one of the titers was only marginally positive (Table 3).

In our study the prevalences for *T. equi* versus *B. caballi* did not differ between female and male horses. This is in agreement with some other studies [26, 27], while increased prevalences in geldings and in mares were observed in Mongolia [28] and in Italy [23], respectively.

Recently, specimens of *Dermacentor reticulatus*, infected with *Babesia canis canis,* have been found in the Western part of the Lake Geneva region [12]. As these ticks also are potential vectors for EP, autochthonous cases of EP might be possible in this region. In Southern Switzerland (canton of Ticino), a study on dogs had revealed that approximately 1/3 of ticks collected on dogs belonged to the species *Rhipicephalus sanguineus* [11]. Since this tick species may also contribute to the transmission of EP, these are also relevant to horse owners and veterinarians in this canton. Climate and ecological changes in the potential habitat of ticks, increasing host- and vector-populations and increasing mobility of owners and their horses may thus advocate for an emergence situation of infection risk for *Babesia* spp. and *Theileria* spp. by time, both in horse and dog populations [12].

To our knowledge, this is the first report on a larger scale on EP using serology in Switzerland. Accurate diagnosis of equine piroplasmosis is essential for providing baseline

information about the incidence, distribution and prevalence in the affected equine population, and is thus a prerequisit for elaborating appropriate and effective control measures. The overall seroprevalence of 7.3% clearly shows that EP is an important threat to the health of horses kept in Switzerland. Due to the potential of EP to further increase in importance, continuous careful monitoring is needed. The present results together with data from the recent literature indicate that testing for EP should be recommended when horses are imported from France, Italy, Spain and Portugal.

6. Acknowledgements

7. Footnotes

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[2] http://www.thehorse.com/ViewArticle.aspx?ID=15300

[3] www.vsp-fsec.ch/boxalino/files/BXMediaOne95file.pdf

[4] www.clive.ed.ac.uk/winepiscope

[5] www.ncss.com

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9. Tables and Figures

Table 1:

Seroprevalences and 95% confidence intervals (95% CI) of equine babesiosis in horses in a sample of 230 domestic and 459 import horses in Switzerland (2008)

Group	Number of horses	Piroplasmosis positive	*T.equi* positive	*B.caballi* positive	*T.equi+ B.caballi* positive
Total	689	50 (7.3%)[a]	30 (4.4%)	10 (1.5%)	10 (1.5%)
95% CI		4.3%-7.8%	1.9%-4.4%	0.8%-2.7%	0.8%-2.7%
Switzerland	230	11 (4.8%)	4 (1.7%)	6 (2.6%)	1 (0.4%)
95% CI		2.7%-8.4%	0.7%-4.4%	1.2%-5.6%	0.0%-2.4%
Total imported horses	459	39 (8.5%)[a]	26 (5.7%)	4 (0.9%)	9 (2.0%)
95% CI		6.3%-11.4%	3.9%-8.2%	0.3%-2.2%	1.0%-3.7%
Germany	200	2 (1.0%)[b]	2 (1.0%)	0	0
95% CI		0.3%-3.6%	0.3%-3.6%	0%-1.9%	0%-1.9%
France	138	24 (17.4%)[a,c]	13 (9.4%)	4 (2.9%)	7 (5.1%)
95% CI		12%-24.5%	5.6%-15.5%	1.1%-7.2%	2.5%-10%
Italy	33	3 (9.1%)	3 (9.1%)	0	0

95% CI		3.1%-23.6%	3.1%-23.6%	0%-10.4%	0%-10.4%
Poland	20	1 (5.0%)	1 (5.0%)	0	0
95% CI		0.9%-23.6%	0.9%-23.6%	0%-16.1%	0%-16.1%
Hungary	16	1 (6.3%)	1 (6.3%)	0	0
95% CI		1.1%-28.3%	1.1%-28.3%	0%-19.4%	0%-19.4%
Czech Republic	13	1 (7.7%)	1 (7.7%)	0	0
95% CI		1.4%-33.3%	1.4%-33.3%	0%-22.8%	0%-22.8%
Spain	11	4 (36.4%)[c]	3 (27.3%)	0	1 (9.1%)
95% CI		15.2%-64.6%	9.7%-56.6%	0%-25.9%	1.6%-37.7%
Austria	11	1 (9.1%)	1 (9.1%)	0	0
95% CI		1.6%-37.7%	1.6%-37.7%	0%-25.9%	0%-25.9%
Portugal	4	2 (50%)[c]	1 (25%)	0	1 (25%)
95% CI		15.6%-85%	4.6%-70%	0%-49%	0.8%-10.5%
Stallion	65	6 (9.2%)[a]	4 (6.2%)	0	2 (3.1%)
95% CI		4.3%-18.7%	2.4%-14.8%	0%-5.6%	0.8%-10.5%
Mare	294	21 (7.1%)[a]	13 (4.4%)	4 (1.4%)	4 (1.4%)
95% CI		4.7%-10.7%	2.6%-7.4%	0.5%-3.5%	0.5%-3.5%
Gelding	330	23 (7.0%)	13 (3.9%)	6 (1.8%)	4 (1.2%)
95% CI		4.7%-10.2%	2.3%-6.6%	0.8%-3.9%	0.5%-3.1%

a: significant difference between *T. equi-* and *B. caballi-*seroprevalence

b: significantly less piroplasmosis-positive horses than in domestic horses

c: significantly more piroplasmosis-positive horses than in domestic horses

Table 2:

Number of horses at different dilutions/concentrations in a sample of 230 domestic and 459 import horses in Switzerland (2008)

Theileria equi	<1:40[a]	1:40[b]	1:80[b]	1:160[b]	1:320[b]
Number of horses	649	9	9	7	15
Babesia caballi	<1:80[a]	1:80[b]	1:160[b]	1:320[b]	1:640[b]
Number of horses	669	6	9	1	4

a: negative sera
b: positive sera

Table 3:

Titers of horses seropositive for both parasite species (*B. caballi* and *T. equi*) in a sample of 230 domestic and 459 import horses in Switzerland (2008)

	Theileria equi	*Babesia caballi*
Horse 1	1:40	1:80
Horse2	1:40[a]	1:160
Horse 3	1:40[a]	1:320
Horse 4	1:80	1:160
Horse 5	1:160	1:80[a]
Horse 6	1:160	1:160
Horse 7	1:160	1:640
Horse 8	1:320	1:160
Horse 9	1:320	1:160
Horse 10	1:320	1:640

a: crossreaction as possible reason for seropositivity

Figure 1: Age distribution of domestic horses seronegativ (-) and seropositiv (+) for equine piroplasmosis and of imported horses seronegativ (-) and seropositiv (+) for equine piroplasmosis in Switzerland (2008). In the box plot, open dots indicate moderate and filled dots strong outliers.