

Seroprevalence of antibodies against *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in horses (*Equus caballus*) from northern Algeria

Abdelouahab Laamari¹, Naouelle Azzag¹, Safia Tennah¹, Salima-Yamina Derdour¹, Bernard China², Ryhan Bouabdallah¹, Farida Ghalmi¹✉

¹Research Laboratory Management of Local Animal Resources, Higher National Veterinary School, El Alia, Oued Smar, 1615 Algiers, Algeria

²Sciensano, Quality of Laboratories, B-1050 Brussels, Belgium
f.ghalmi@ensv.dz

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Abstract

Introduction: Horses (*Equus caballus*) are susceptible to tick-borne diseases. Two of them, Lyme borreliosis due to *Borrelia burgdorferi* and granulocytic anaplasmosis due to *Anaplasma phagocytophilum* were investigated in Algerian horses. The diseases have been less extensively studied in horses and results pertinent to Algeria have not been published. **Material and Methods:** Blood samples were obtained from 128 horses. IgG antibodies directed against *Anaplasma phagocytophilum* and *Borrelia burgdorferi* were detected by an indirect immunofluorescence antibody test (IFAT) and ELISA. The potential effects of age, gender, breed, and health status on seropositivity were also evaluated. **Results:** Using IFAT, 28 (21.8%) and 25 (19.5%) animals were positive for *B. burgdorferi* and *A. phagocytophilum*, respectively. Using ELISA, 19 (14.8%) and 33 (25.9%) animals were positive for these bacteria. **Conclusion:** The study shows that horses in Algeria are exposed or co-exposed to tick-transmitted zoonotic bacterial species.

Keywords: horses, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, seroprevalence, co-infection.

Introduction

Equine granulocytic anaplasmosis and Lyme borreliosis have long been recognised as multi-systemic tick-borne diseases affecting several species of wild and domesticated mammals, including carnivores, ruminants, and equines. These zoonotic agents have also been reported in dogs in northern Algeria (2). *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*) is an intracellular bacterium invading the granulocytes of horses, where it may induce a febrile disease called equine granulocytic anaplasmosis. The disease is characterised by a wide range of clinical signs including fever, lethargy, limb oedema, petechiae, reluctance to move, thrombocytopenia, leukopenia, and anaemia (23, 28). Granulocytic ehrlichiosis in horses is a seasonal disease closely associated with the activity of the tick vectors from mid-spring to the end of summer. Horses (*Equus caballus*) from endemic areas have a high seroprevalence of antibodies against *Anaplasma*

phagocytophilum, corresponding to the prevailing distribution of the ticks of the *Ixodes* genus. The disease has predominantly been reported in Europe. Clinical cases have been reported in Germany (7), Switzerland (15), Sweden (13), Great Britain (19), France (4), and Italy (32). In North Africa, a recent study (3) in Tunisia showed a seropositivity of 16.3% to *A. phagocytophilum* in horses.

Lyme disease, or borreliosis, is a bacterial illness caused by the spirochete *Borrelia burgdorferi*. The infection in horses is caused by attachment and prolonged feeding of infected adult *Ixodes* spp. ticks. The clinical signs most often associated with equine Lyme disease include stiffness and lameness in more than one limb, muscle wasting, hyperaesthesia, lethargy, and polysynovitis (8, 26). Pain over the thoracolumbar area has been reported in a few horses with high serum antibody titres (16). In several studies conducted on horse populations in endemic areas, high seroprevalence was observed and in Europe it ranged from 0% to 68%

(9). In North America, a large range of seroprevalence was also observed: 63% in Wisconsin, 0.2% in Texas or 84% in Connecticut (9). Other species of *Borrelia* (e.g. *B. parkeri*) are also involved in equine disease, transmitting tick-borne relapsing fever which causes abortion (14). The recommended diagnostic test to detect antibodies against *A. phagocytophilum* and *B. burgdorferi* in horses is the indirect fluorescent antibody assay (IFA). *B. burgdorferi* antibodies in horses may be detected by ELISA and confirmed by Western blot (17). The point-of-care SNAP 4Dx ELISA (IDEXX Laboratories, Westbrook, MN, USA) is authorised for the detection of antibodies against the *A. phagocytophilum* P44 antigen and the *B. burgdorferi* C6 antigen in dogs (17). As far as we know, the occurrence of these pathogens in horses from Algeria has never been investigated by serological methods. Therefore, using ELISA and an indirect fluorescent antibody method, the aim of the present study was to evaluate the seroprevalence of *B. burgdorferi* and *A. phagocytophilum* and the possible association of these bacteria in Algerian horses with risk factors and health status.

Material and Methods

Equine samples. A cross-sectional study was conducted from August 2015 to September 2016 on 128 horses of the Algerian Republican Guard. The sampled horses included in the study were 13 Arab-Barbs, 100 Arabians, and 15 Barbs aged from 1 to 25 years. Two groups of horses were distinguished. The first group comprised healthy horses admitted to the Republican Guard veterinary clinic for a vaccination ($n = 108$). The second group included horses presenting various clinical signs ($n = 20$) such as poor performance, lameness, oligoarthritis, fever, inappetence, colic or ophthalmological symptoms, and neurological disorders (Table 1). Practitioners in the veterinary clinics answered questions about the health status of the horses. Breed and age were also noted besides health status. Blood samples were drawn from the jugular vein into sterile dry tubes and kept at 4°C for 12 to 24 h, and then the sera were frozen at -20°C. No ectoparasites were collected from the sampled horses.

Serological testing

Immunofluorescence assay. Immunofluorescence antibody assays were performed for *A. phagocytophilum* and *B. burgdorferi sensu lato* using commercial slides (MegaScreen, Megacor Diagnostik, Hoerbranz, Austria). Rabbit anti-horse IgG conjugate was used (Jackson ImmunoResearch, Ely, UK). Sera were initially screened at a dilution of 1:50 in phosphate-buffered saline and all seropositive samples were re-diluted from 1:100 up to 1:1,600 in order to determine the antibody titres. The significant antibody titres were 1/50 for *A. phagocytophilum* and *B. burgdorferi* as stated by the manufacturer. Slides were evaluated using a fluorescence microscope at 400× magnification, comparing each

sample to the visual intensity and appearance of the bacteria fluorescence pattern seen in the positive and negative controls included in the kit.

ELISA. For *B. burgdorferi* serology, a commercial ELISA developed and validated (9) for the examination of dog or horse sera (*Borrelia burgdorferi* Veterinary ELISA, Virotech diagnostics, Rüsselheim, Germany) was used according to the recommendations of the producer.

Table 1. Characteristics of the study group considering gender, age, and breed of sampled horses

Variable	Healthy group (n = 108)	Affected group (n = 20)
Gender		
Male	62	14
Female	46	6
Age (years)		
1–11	54	14
12–16	33	3
17–25	21	3
Breed		
Arabian	85	15
Barb	13	2
Arab-Barb	10	3

For the detection of IgG antibodies against *A. phagocytophilum*, a commercial ELISA (*Ehrlichia equi* IgG Antibody Kit, Helica Biosystems, Inc., Santa Ana, CA, USA) was used according to the manufacturer's recommendations.

Statistical analysis. Statistical differences in the proportions of antibody levels were compared using the chi-squared (Yates corrected) or Fisher's exact test. Also, the agreement between ELISA and IFAT was assessed by the McNemar test and calculation of the value of κ . Its value was interpreted as meaning no consistency where $\kappa \leq 0.20$, poor agreement in the range of $0.21 \leq \kappa \leq 0.40$, moderate agreement for $0.41 \leq \kappa \leq 0.60$, good agreement at $0.61 \leq \kappa \leq 0.80$, and very good agreement in the case of $\kappa > 0.80$ (22).

Relative sensitivity = (number of positive results for both methods/number of positive results for the reference method) \times 100. Relative specificity = (number of negative results for both methods/number of negative results for the reference method) \times 100.

The software used was Win episode 2.0 (36), MedCalc (MedCalc Software Ltd., Ostend, Belgium) and XL stat (Addinsoft, Paris, France). The level of statistical significance assigned was 5%.

Results

Serological evaluation of sera by IFAT and ELISA. Out of 128 serum samples, 28 (21.9%) were positive for *B. burgdorferi* by IFAT and 19 (14.8%) by ELISA (Table 2). Nevertheless, there was no significant difference in the seroprevalence calculated by the two

methods ($P > 0.05$). In the same way, 25 sera (19.53%) were positive for *A. phagocytophilum* by IFAT and 33 (25.9%) by ELISA. But once again, the prevalence was not significantly different ($P > 0.05$) in regard to the method used (Table 2). The incidence of double infection was investigated. By IFAT, 10 sera were positive for both pathogens. It implies that 15 sera were positive for *A. phagocytophilum* only and 18 for *B. burgdorferi* only. By ELISA, 13 sera were positive for both pathogens. This in turn implies that 20 sera were positive for *A. phagocytophilum* only and 6 were positive for *B. burgdorferi* only (Table 2). Among horses with clinical signs, nine were positive for *A. phagocytophilum* antibodies by IFAT: five with fever and inappetence, two with poor performance, one with ophthalmological symptoms, and one with a neurological disorder. Among eleven horses positive in IFAT for *B. burgdorferi* antibodies and presenting clinical signs, three were performing poorly, three had ophthalmological symptoms, one fever and inappetence, two oligoarthritis, and two neurological disorders.

Risk factors. The association between the seroprevalence and several factors was investigated using the chi-squared (Yates corrected) or Fisher's exact test (Table 3).

For *A. phagocytophilum*, age was a risk factor by both methods. The younger animals (1–11 years) were more frequently positive than the older ones. Gender was not a risk factor by IFAT but was by ELISA, where the females were more frequently positive. Breed was not a risk factor but the presence of clinical signs was, since in the affected group of animals, the prevalence was higher than in healthy animals.

For *B. burgdorferi*, risk was associated with age when the method was IFAT, with a higher prevalence in the youngest animals (1–11 years), but not when the method was ELISA. Gender was not material to risk for either method. IFAT testing showed varying predisposition by breed but ELISA testing did not. The presence of clinical signs is a significant associative factor since the prevalence is higher in animals which are not healthy.

Comparison of methods. For *B. burgdorferi*, the percentage of agreement between ELISA and IFAT was 86.7% (111/128) and the κ index was 0.56, corresponding to moderate agreement, while for *A. phagocytophilum*, the percentage of agreement was 85.9% (110/128) and the κ index was 0.6, corresponding to substantial agreement (111/128). The McNemar test indicated that the disagreement between the methods could not be attributed to chance ($P < 0.01$) (Table 4).

Table 2. Seroprevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* by ELISA and IFAT

Pathogen	IFAT (n = 128)		ELISA (n = 128)	
	Positive	Seroprevalence (%) (95% CI)	Positive	Seroprevalence (%) (95% CI)
<i>B. burgdorferi</i> only	18	14.1 (9.1–21.1) ^{&}	6	4.7 (2.2–9.9) ^{&}
<i>A. phagocytophilum</i> only	15	11.7 (7.2–18.4) [£]	20	15.6 (10.4–22.9) [£]
<i>A. phagocytophilum</i> and <i>B. burgdorferi</i> (co-infection)	10	7.8 (4.3–13.8) [£]	13	10.2 (6.0–16.7) [£]
<i>B. burgdorferi</i> (total)	28	21.87 (13.79–28.20)*	19	14.84 (7.86–20.13)*
<i>A. phagocytophilum</i> (total)	25	19.53 (12.06–25.93)*	33	25.87 (17.34–32.65)*

* – The prevalence was not significantly different between methods ($P > 0.05$)

£ – The prevalence was not significantly different by IFAT and ELISA ($P > 0.05$)

& – The prevalence was significantly different by IFAT and ELISA ($P < 0.05$)

Table 3. Seroprevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* regarding breed, sex, age, and health status

Variable	<i>A. phagocytophilum</i>		<i>B. burgdorferi</i>	
	IFAT + (%; 95% CI)	ELISA + (%; 95% CI)	IFAT + (%; 95% CI)	ELISA + (%; 95% CI)
Age (years)				
1–11 (n = 54)	22 (40.7; 28.7–54.0)	26 (48.1; 35.4–61.2)	22 (40.7; 28.7–54.0)	10 (18.5; 10.4–30.9)
12–16 (n = 33)	3 (9.1; 3.1–23.6)	4 (12.1; 4.8–27.3)	3 (9.1; 3.1–23.6)	6 (18.2; 8.6–34.4)
17–25 (n = 21)	0 (0)	3 (14.3; 5.0–34.6)	3 (14.3; 5.0–34.6)	3 (14.3; 5.0–34.6)
P value	<0.01 SS	<0.01 SS	<0.01 SS	0.9 NS
Gender				
Male (n = 62)	12 (19.4; 11.4–30.8)	9 (14.5; 7.8–25.4)	15 (24.2; 15.2–36.2)	14 (22.6; 13.9–34.4)
Female (n = 46)	13 (28.3; 17.3–42.6)	24 (52.2; 38.1–65.9)	13 (28.3; 17.3–42.6)	5 (10.9; 4.7–23.0)
P value	0.3 NS	<0.01 SS	0.6 NS	0.1 NS
Breed				
Arabian (n = 85)	20 (23.5; 15.8–33.6)	22 (25.9; 17.8–36.1)	20 (23.5; 15.8–33.6)	14 (16.5; 10.1–25.8)
Barb (n = 13)	1 (7.7; 1.4–33.3)	5 (38.5; 17.7–64.5)	1 (7.7; 1.4–33.3)	1 (7.7; 1.4–33.3)
Arab–Barb (n = 10)	4 (40; 16.8–68.7)	6 (60; 31.2–83.2)	7 (70; 39.7–89.2)	4 (40; 16.8–68.7)
P value	0.2 NS	0.07 NS	<0.01 SS	0.1 NS
Clinical signs				
Yes (n = 20)	9 (45; 25.8–65.8)	10 (50; 29.9–70.1)	11 (55; 34.2–74.2)	7 (35; 18.1–56.7)
No (n = 108)	16 (14.8; 9.3–22.7)	23 (21.3; 14.6–29.9)	17 (15.7; 10.1–23.8)	12 (11.1; 6.5–18.4)
P value	<0.01 SS	<0.05 S	<0.01 SS	<0.05 S

Table 4. Agreement analysis between IFAT and ELISA tests

<i>B. burgdorferi</i>		IFAT			Statistics
ELISA	Sera	Positive	Negative	Total	$\kappa = 0.56$ McNemar $P < 0.01$ SS % agreement = 86.7 Relative sensitivity = 53.6% Relative specificity = 96%
	Positive	15	4	19	
	Negative	13	96	109	
	Total	28	100	128	
<i>A. phagocytophilum</i>		IFAT			Statistics
ELISA	Sera	Positive	Negative	Total	$\kappa = 0.61$ McNemar $P < 0.01$ SS % agreement = 85.9 Relative sensitivity = 80% Relative specificity = 87.4%
	Positive	20	13	33	
	Negative	5	90	95	
	Total	25	103	128	

Table 5. Seroprevalence of *A. phagocytophilum* around the world

Country	Year	Technique	n	Positive	Prevalence (%)	Lower limit	Upper limit	Reference
Algeria	2018	IFAT	128	25	19.53	12.66	26.40	this study
Bulgaria	2018	ELISA	155	31	20.00	13.70	26.30	37
Canada	2015	SNAP 4Dx	376	2	5.31	0	1.27	33
Czech Republic	2011	IFAT	92	67	72.8	63.74	81.91	30
Denmark	2010	SNAP 4Dx	390	87	22.31	18.18	26.44	30
France	2005	ELISA	424	48	11.32	8.30	14.33	30
France	2009	ELISA	408	55	13.48	10,17	16.79	30
Guatemala	2005	IFAT	74	10	13.51	5.72	21.30	30
Italy	2003	IFAT	561	2	3.56	0	8.50	30
Italy	2008	IFAT	793	134	16.90	14.29	19.51	30
Italy	2008	IFAT	154	12	7.79	3.56	12.03	30
Italy	2010	IFAT	135	23	17.04	10.69	23.38	30
Italy	2019	IFAT	479	109	22.76	19.00	26.51	12
Mongolia	2018	IFAT	216	91	42.13	35.54	48.71	39
Tunisia	2014	IFAT	343	56	16.33	12.41	20.24	3

Table 6. Seroprevalence of *B. burgdorferi* in the world

Country	Year	Method	n	Positive	Prevalence (%)	Lower limit	Upper limit	Reference
Algeria	2019	IFAT	128	28	21.88	14.71	29.04	this study
Brazil	2018	ELISA	367	214	58.31	53.27	63.36	34
Canada	2015	SNAP 4Dx	376	6	1.60	0.033	2.86	33
France	2010	SNAP 4Dx	408	134	32.84	28.29	37.40	25
French Guiana	2010	SNAP 4Dx	49	0	0	0	0	25
Italy	2013	IFAT	300	21	7.00	4.11	9.89	20
Italy	2012	SNAP 4Dx	98	15	15.31	8.18	22.43	38
Italy	2012	IFAT	386	94	24.35	20.07	28.63	11
Korea	2016	ELISA	727	40	5.50	3.84	7.16	22
Mexico	2001	IFAT	100	34	34.00	24.72	43.28	31
Poland	2008	ELISA	395	101	25.57	21.27	29.87	35
Romania	2011	ELISA	260	31	11.92	7.98	15.86	18
Sub-Saharan Africa	2010	SNAP 4Dx	113	0	0	0	0	25
Turkey	2008	ELISA	300	18	6.00	3.31	8.69	5
USA	2012	ELISA and WB	2100	175	8.33	7.15	9.52	40

Discussion

Horses are large warm-blooded animals commonly exposed to ticks. They can be directly affected by tick-borne infections or can play the role of reservoir for further transmission. The aetiological agent of one such infection, *Anaplasma phagocytophilum*, has medical as well as veterinary importance because of its zoonotic nature. The main tick-borne diseases in horses are equine piroplasmiasis caused by *Babesia caballi* and *Theileria equi*, Lyme borreliosis caused by *Borrelia burgdorferi*, and equine granulocytic anaplasmosis caused by *Anaplasma phagocytophilum* (30). The last of these has symptoms of loss of appetite, lethargy, haemorrhages, and lameness (29).

Serological testing using IFAT revealed a total of 25 (19.5%) horses out of the 128 analysed to be seropositive for *A. phagocytophilum*. The seroprevalence of the microbe obtained in this study (19.5%) confirms the presence of this tick-borne pathogen in Algeria (2) and suggests its possible association, in terms of endemic patterns, with *B. burgdorferi sensu lato*. This seroprevalence also coincides with data previously reported in other countries, including France, Spain, and Sweden (1, 13, 21) and coheres with the endemicity of equine granulocytic anaplasmosis reported in different parts of the world. The seroprevalence of *A. phagocytophilum* ranged from 0.3% to 73% (29). Table 5 shows the observed prevalence in this study in comparison with other serological studies from around the world. Our study returned a percentage approximating the average of those studies. The most interesting comparison can be made with the study from neighbouring Tunisia, where the seroprevalence of 16.53% (56/343) was not significantly different to our data ($P = 0.4$) (3). The differences in prevalence in the studies could be related to the samples, which may have been selected at random or with more focus on animals with symptoms. Nevertheless, horses in stables from which cases had previously been reported tended to have higher average titres (29).

IFAT was used as the reference method for serology, but ELISA gave similar results with no significant differences. ELISA is an easier technique that can therefore be recommended for *A. phagocytophilum* serology. Since serology is an indirect diagnostic method attesting contact between an animal's immune system and the pathogen, a more direct method such as PCR is recommended in a clinical context (29).

The differences between seroprevalence values described in this work may be explained by inconstancy in the factors which determined them, including the horse populations surveyed and, more impactfully, the frequency of exposure to the vector *Ixodes ricinus*.

The seroprevalence rate for *B. burgdorferi* reported in this study is 21.9%. The recent seroprevalence studies showed a prevalence ranging from 0% (Sub-Saharan Africa and French Guiana) to 58% (Brazil). Results of

other studies from around the world are presented in Table. 6.

The comparison of the two serological methods indicated that IFAT gave more positive samples than ELISA. Thirteen sera were positive by IFAT and negative using ELISA. Since IFAT was considered the reference method, it means that in our hands ELISA showed low sensitivity (53.6%). The ELISA used is a validated commercial kit (9). Two possible explanations exist: either the ELISA method lacks sensitivity or the IFAT lacks specificity. The examination of further serum dilutions in IFAT indicated that only 16 out of 28 positive sera were still positive at 1:100, 9 at 1:200, and 3 at 1:400. Therefore, if the comparison between IFAT and ELISA is based on 1:100 serum dilution in IFAT rather than 1:50, the agreement between the methods is much better. The general agreement is 96.1%, the relative sensitivity is 93.75%, and the relative specificity is 96.4%. The Cohen's κ coefficient is 0.83, corresponding to an almost perfect agreement.

The study of risk factors surprisingly indicated a higher prevalence both for *A. phagocytophilum* and *B. burgdorferi* in young animals in comparison with older animals. It is in contradiction to other studies indicating that older animals were more frequently infected than young animals (15, 20). There is no difference in prevalence according to gender, with data from this study being confirmed by the literature (18, 25). Breed is not a risk factor for *A. phagocytophilum* prevalence, but for *B. burgdorferi*, crossbred animals were more susceptible to being infected. Nevertheless, the small number of animals in several subgroups renders the statistics difficult to interpret due to the large range of values (see confidence intervals). Finally, as expected, the animals presenting symptoms were more frequently positive for both *A. phagocytophilum* and *B. burgdorferi* than the healthy animals. One major risk factor that was not investigated in this work is the presence of ticks on experimental horses (13). Indeed, in our study, we were unable to identify ticks on the sampled animals. Nevertheless, discussion with stablehands indicated that ticks were sometimes observed on the animals in their charge. It is of course coherent with our serological results. Moreover, the horses had been preventively treated with anti-parasitic drugs (organophosphate insecticides), which explains the absence of ticks during the sampling (personal communication).

One of the aims of our study was to investigate if there are any associations between the presence of serum antibodies against either of these two bacteria and clinical signs of the disease. In the healthy group ($n = 108$), 16% were seropositive for *A. phagocytophilum* and 17% for *B. burgdorferi sensu lato*. The proportions that were seropositive in the group of horses that were not considered healthy were statistically significantly different from the healthy horses and were similar for

both agents. Therefore, we have shown that antibody titres to *B. burgdorferi* or *A. phagocytophilum* were associated with clinical problems. Our observations are similar to those described by Büscher *et al.* (7), which were that some horses seropositive for *B. burgdorferi* showed lameness and swollen joints. In another study, an association between *B. burgdorferi sensu lato* serological status and myalgia in horses was observed (24). Regarding *A. phagocytophilum*, only one study reported that horses presenting clinical signs showed antibody titres to the bacterium; 6 out of 12 of these animals tested positive in IFAT and received tetracycline treatment for suspected granulocytic ehrlichiosis disease (personal communication). In conclusion, antibodies against *A. phagocytophilum*, *B. burgdorferi sensu lato*, and both pathogens (co-infection) were found in horses from northern Algeria. Whether a co-exposure to pathogens would also increase clinical signs in horses needs further investigation. From the technical point of view, even if IFAT remains the reference method, ELISA gave similar results and is easier to use.

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