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SHORT COMMUNICATION

Anaplasma phagocytophilum seroprevalence in equids: a survey in Sicily (Italy)

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Abstract This study was undertaken to determine the prevalence of *Anaplasma phagocytophilum* infection in Equidae and investigate the possibility of exposure to the organism in Sicily (Southern Italy). During the study blood samples were collected in horses and donkeys housed in five of the nine provinces of Sicilian Island. Of 133 horses and 100 donkeys tested, respectively 9.0% and 6.0% were seroactive (IFAT) with *A. phagocytophilum* antigen. In only 4.7% of the horses, specific *A. phagocytophilum* DNA was recorded; in donkey, *Anaplasma* DNA was not found. Our results indicate a low prevalence of *A. phagocytophilum* in Sicilian equids. This condition does not justify the exclusion of equids from prophylactic plans for this multihost pathogen infection, a zoonosis with a wide distribution in other European countries. However, further studies are necessary to elucidate the possible mechanisms that involve the Equidae as host of this pathogen.

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Introduction

Anaplasma phagocytophilum, a multihost pathogen, is the emended name replacing three species of granulocytic bacteria, *Ehrlichia phagocytophila* (cattle and small ruminants), *Ehrlichia equi* (horses) and the agent of human granulocytic ehrlichiosis, after a reorganization of the families Rickettsiaceae and Anaplasmataceae under the order Rickettsiales (Dumler et al. 2001). Equine granulocytic anaplasmosis (EGA) was for the first time recognized as a disease of horses in California (Gribble 1969) and later found in other parts of the USA, Europe (Woldehivet 2010) and South America (Madigan 1993). The incidence and severity of the disease are largely dictated by the genetic variant of *A. phagocytophilum*, the host involved and the vectors present in a particular region or area (Ogden et al. 2002; Teglas and Foley 2006; Tuomi 1967).

In the horse EGA is recognized as an acute disease with an incubation period of <14 days, characterized by high fever, lethargy, partial anorexia, staggering or ataxia, distal limb oedema and haematological alterations, such as thrombocytopenia, neutropenia, lymphopenia and mild anaemia (Franzén et al. 2009). Persistence of infection by *A. phagocytophilum* has been observed for up to 4 months after experimental infection in horses (Franzén et al. 2009). Clinical observation and results of experimental infections suggest that horses less than 4 years old tend to develop mild forms of the disease. In horses less than 1 year of age, the clinical signs are subtle, with reports of only mild pyrexia (Pusterla and Madigan 2007).

The analysis for detection of IgG against *A. phagocytophilum* offers an excellent screening method to explore the geographical distribution of the bacteria. The detection of an increasing titre during the acute and convalescent phase of the illness may be helpful in establishing a diagnosis in the absence of microscopic and molecular analysis; in contrast, seroconversion cannot prove a status of active infection but only an exposure to the pathogen, if the interval between the samples is longer (Passamonti et al. 2010).

In Italy, limited information is available regarding the epidemiology of *A. phagocytophilum* in equids. Considering that Sicily is a large island in which environmental conditions, particularly climate and vegetation, are suitable for tick life cycle, the purpose of the present study was to explore the prevalence of *A. phagocytophilum* within Sicily, by testing asymptomatic horses and donkeys by indirect immunofluorescence antibody test (IFAT) and polymerase chain reaction (PCR).

Materials and methods

Animals and sample collection

Our study was carried out in Sicily on 133 horses of various breeds (62 male and 71 female, 6 months to 14 years old) and 100 Ragusana breed or cross-breed donkeys (13 male and 87 female, 6 months to 20 years old) randomly chosen within various farms and horse centres located in five of the nine Sicilian provinces (Fig. 1). From spring to autumn, during the tick season, blood samples were collected from all animals by jugular vein puncture into vacutainer tubes (Terumo Corporation, Japan) with or without anticoagulant (EDTA) and maintained at 4°C until arrival at the laboratory. Blood samples with no additive were centrifuged at 3,000 rpm for 10 min, and the obtained sera were separated. Blood and serum samples were stored at −20°C until analysis.

Serological tests

Samples included in the study were analysed by the Centro Nazionale di Referenza per *Anaplasma*, *Babesia*, *Rickettsia* and *Theileria* at the Istituto Zooprofilattico Sperimentale della Sicilia. IFAT kits for *A. phagocytophilum* (Fuller Laboratories, Fullerton, CA, USA), based on *A. phagocytophilum* HGE-1 isolate antigens derived from HL-60 cells, were used following the manufacturer's recommendations. Fluorescein isothiocyanate-conjugated anti-host equine immunoglobulins IgG (Sigma, St Louis, MO, USA) were used as secondary antibodies. Samples were considered negative when no fluorescence was detected at 1:80 dilution of the test serum, as recommended by the manufacturer. Infected blood was used as positive control (de La Fuente et al. 2005a).

DNA test

DNA was extracted from blood samples of 52 animals (42 horses and 10 donkeys) using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma). The DNA was resuspended in sterile distilled water and stored at −20°C until used. PCR analysis of *Anaplasma* spp. 16S rDNA, *A. phagocytophilum* 16S rDNA or *msp4* were conduced, and *msp4* gene

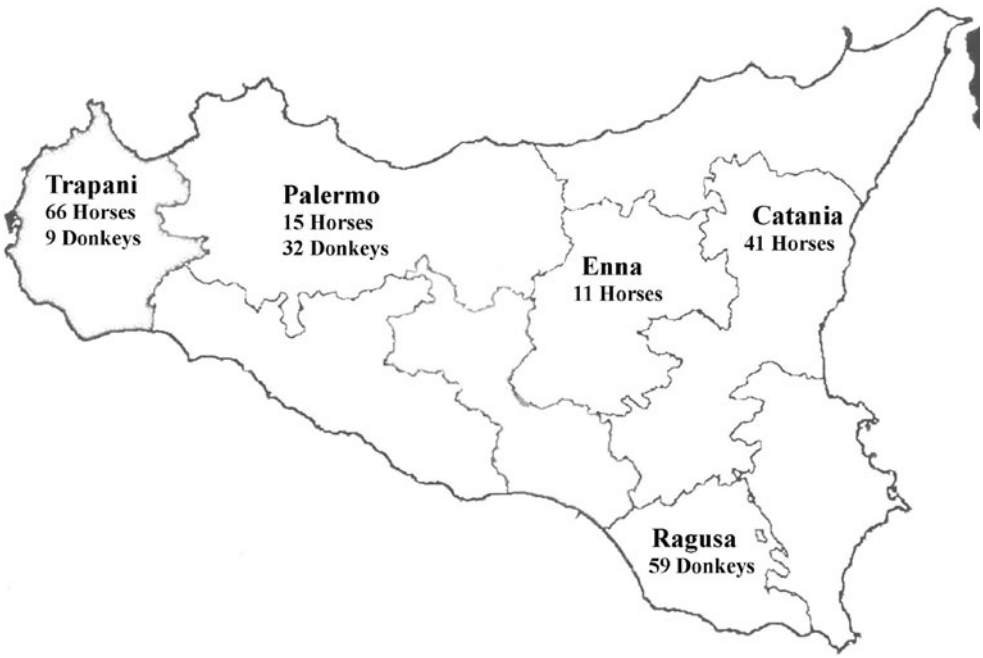

Fig. 1 Geographical location of sampling in Sicilian provinces

Table 1 Primers and temperature cycles during PCR run

Species	Genes	Primers		PCR first programme	PCR nested programme	bp	References
Anaplasma spp.	16SrRNA	16SANAF 5′-CAGAGTTTGATCCTGGCTCAGAACG-3′		94°C×5′ 94°C×1′		467 bp	Stuen et al. (2003)
		16SANAR 5′-GAGTTTGCCGGGACTTCTTCTGTA-3′		69°C×50″ 72°C×1′			
				30 cycles 72°C×5′			
A. phagocytophilum	16SrDNA	EE1 5′-TCCTGGCTCAGAACGAACGCTGGCGGC-3′		94°C×4′ 94°C×1′	94°C×4′ 94°C×1′	First 1,435 bp, nested 928 bp	Bown et al. (2003)
		EE2 5′-GTCACTGACCCAACCTTAAATGGCTG-3′		55°C×50″ 72°C×1′	55°C×50″ 72°C×1′		
		EE3 5′-GTC GAA CGG ATT ATT CTT TAT AGC TTG C-3′		35 cycles 72°C×7′	35 cycles 72°C×7′		
		EE4 5′-CCC TTC CGT TAA GAA GGA TCT AAT CTC C-3′					
A. phagocytophilum	*msp4*	MSP4AP5 5′-ATGAATTACAGAGAATTGCTTGTAGG-3′		95°C×5′ 95°C×30″		849 bp	de la Fuente et al. (2005e)
		MSP4AP3 5′-TTAATTGAAAGCAAATCTTGCTCCTATG-3′		53°C×30″ 72°C×1′			
				30″ 35 cycles 72°C×5′			

was sequenced (Table 1). PCRs were performed with 1 μl DNA using 10 pmol of each primer and the Ready-To-Go PCR beads (Amersham, Piscataway, NJ, USA). Reactions were performed in an automated DNA thermal cycler for 35 cycles. PCR products were separated by electrophoresis on 1% agarose gels to check the size of amplified fragments by comparison with a DNA molecular weight marker (1 kb DNA ladder; Promega Madison, WI, USA). Control reactions were done without the addition of DNA to the reaction to exclude the possibility of contaminations during PCR (Bown et al. 2003; de la Fuente et al. 2005a, b, c, d, e).

Results

By means of IFAT, IgG antibodies for *A. phagocytophilum* were detected in 18 out of 233 equids tested (7.7%): specifically, 12 out of 133 horses (9.0%) and 6 out of 100 donkeys (6.0%). Table 2 summarises the distribution of *A. phagocytophilum* in infected horse and donkey in the Sicilian provinces tested.

By means of PCR, *Anaplasma* spp. DNA was detected in 17 (40.5%) of 42 horses tested, and in none of donkeys; *A. phagocytophilum* DNA was detected in only 2 out of 42 (4.7%) blood samples from horses. No other positive animal was found. *A. phagocytophilum* strains had *msp4* sequences identical to the MRK isolated from a horse in the USA (de la Fuente et al. 2005a). The two PCR-positive horses belonged to the same farm housed in Catania (Eastern Sicily) and showed neither clinical signs of anaplasmosis nor haematological and blood biochemical abnormalities. These horses were also seropositive. In the other four seropositive horses and three seropositive donkeys, in which the PCR test was performed, *A. phagocytophilum* DNA was not detected (Table 3).

Discussion

Positive serological testing and DNA detection by PCR are both evidence of *A. phagocytophilum* in Sicily. Our results showed that *A. phagocytophilum* infection occurs in Sicily with a higher prevalence in horses (9.0%) than in donkeys (6.0%). A similar low seroprevalence of *A. phagocytophilum* in equids has been previously observed in studies conducted both within Italy and other European countries: in particular, the prevalence was 0.3–7.8% in Italy (Lillini et al. 2006; Torina et al. 2007), 11.3% in France (Leblond et al. 2005) and 6.5% in Spain (Amusategui et al. 2006). The highest seroprevalence has been found recently in horses of Central Italy and Sweden (17.0%) and in Denmark (22.0%) (Ebani et al. 2008; Engvall and Engvall 2002; Hansen et al. 2010; Passamonti et al. 2010).

Table 2 *A. phagocytophilum* infection in equids from Sicily

Location	Horse			Donkey			Total
	Negative	Positive	Tested	Negative	Positive	Tested	
Catania	36	5	41	–	–	–	41
Enna	11	–	11	–	–	–	11
Ragusa	–	–	–		2	59	59
Palermo	11	4	15	28	4	32	47
Trapani	63	3	66	9	–	9	75
Total	121 (90.9%)	12 (9.02%)	133	94 (94.0%)	6 (6.0%)	100	233 (7.7%)

Results of IFAT applied on 133 horses and 100 donkeys

The low frequency of seropositivity found in the present study could be attributed to a limited distribution of the bacterium in this region, to a low susceptibility of equids to the *A. phagocytophilum* variant present in this area or to the use of a non-cross-reacting antigen in the serological test. Considering that we found a horse variant, we can suppose that horses in Sicily have a low exposure to *A. phagocytophilum*. This could be due to: (1) a different exposure behaviour of the horses, which live in a paddock on the farm, by comparison to other species that live with other types of vegetation, and (2) a low prevalence of the tick vector *Ixodes ricinus* that in Sicily finds a suitable habitat only in forest areas (Torina et al. 2008). In fact, the two PCR-positive horses lived in foothills of Mount Etna (Catania), with free access to the paddock area. With respect to the antigen used in the serological test, even though it is a human antigen (NS1 isolate derived from a human case in Massachussetts), no significant differences in titres were found when the prototype *E. equi* from a horse was used as antigen (Fuller Laboratories data). Nevertheless, we cannot exclude the possibility of a reduced sensitivity.

The number of PCR-positive samples (2 out of 52, 3.8%) is much lower than the IFAT-positive samples (6 out of 52, 11.5%). This underlines the significant discrepancy between the two testing methods. IgG production becomes detectable 2–4 weeks after the infection date, and reaches its peak approximately 8 weeks after the infection, when it tends to become serologically negative unless reinfections occur (Pusterla and Madigan 2007; Van Andel et al. 1998). PCR becomes positive between days 1 and 21 post-infection; after day 21 only sporadically PCR positivity can be observed, particularly when horses are subjected to stress intervention (Franzén et al. 2009). Therefore, in the two horses with simultaneous serological and PCR-positive results, one can estimate that the infection has occurred about 3–8 weeks earlier.

Seropositive horses could have a subclinical EGA, reflecting the situation in the endemic area in which the *A. phagocytophilum* seroprevalence may be elevated, but the clinical signs of disease are mild or absent (Madigan 1993). The existence of a subclinical persistent EGA in horses was also hypothesized by Chang et al. (1998).

The discrepancy between the results of PCR for *Anaplasma* spp. (40%) and the *A. phagocytophilum*-specific PCR (4.7%) is attributable to the lower sensitivity of the former test that amplifies the 16S gene. Since this gene is highly conserved, it cannot be excluded in that it amplifies other *Anaplasma* and *Ehrlichia* species whose 16SrRNA gene was not yet submitted to GenBank. In our experience, its primers can be used as a screening test, but their specificity does not detect pathogens with certainty; this is the reason why specific more sensitive nested PCR is also performed on positive samples.

Although anaplasmosis is a zoonosis with a wide distribution in European countries, our results indicate a low prevalence of *A. phagocytophilum* in Sicilian equids. This condition does not justify the exclusion of equids from prophylactic plans for this multihost pathogen infection, particularly in forest areas where the vector lives. In addition, the transport of horses among regions and sporting events could favour the diffusion of the infection in other areas. Further studies are

Table 3 PCR test in 52 blood samples (42 horses and 10 donkeys) and comparison with IFAT

Test	Horse			Donkey			Total equids
	IFAT+	IFAT−	Subtotal	IFAT+	IFAT−	Subtotal	
PCR+	2	–	2 (4.7%)	–	–	–	2 (3.8%)
PCR−	4	36	40 (95.2%)	3	7	10 (100%)	50 (96.1%)
Subtotal	6 (14.2%)	36 (85.7%)		3 (30.0%)	7 (70.0%)		
Total			42			10	52

necessary to elucidate the mechanisms that make the Equidae the host of this pathogen.

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