Anaplasma phagocytophilum *infection in thrombocytopenic dogs*

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Keywords
Anaplasmosis,
Blood,
Dog,
Thrombocytopenia.

Summary
Anaplasma and *Ehrlichia* spp. are tick-transmitted bacteria of clinical relevance in European dogs. The diagnosis of infection is often difficult due to the wide spectrum of disease caused by them. During infection, reduction in platelet count is considered the most common haematological abnormality, frequently representing the sole alteration in asymptomatic dogs. In this study, the presence of bacteria belonging to the genera *Anaplasma* and *Ehrlichia* was investigated in Northern Italy in blood samples from 159 thrombocytopenic dogs using a polymerase chain reaction (PCR) assay amplifying a portion of the heat shock gene (*gro*EL). Obtained amplicons were sequenced and analysed. Two dogs were positive for *A. phagocytophilum*, while *A. platys* and *E. canis* were not detected. None of the PCR-positive dogs were diagnosed at the time of hospital admission, even in the presence of clinical signs and clinicopathological abnormalities potentially related to *A. phagocytophilum* infection. Nucleotide sequence analysis showed that the 2 detected strains belonged to the cluster Europe 1 and were different from each other. This study confirms the presence of *A. phagocytophilum* infections in dogs of Northern Italy, causing clinical signs and laboratory abnormalities that could not be properly diagnosed and treated.

Infezione da Anaplasma phagocytophilum *in cani trombocitopenici*

Parole chiave
Anaplasmosi,
Cane,
Sangue,
Trombocitopenia.

Riassunto
I batteri appartenenti ai generi *Anaplasma* ed *Ehrlichia* sono responsabili di infezioni nel cane trasmesse da zecche e di notevole rilevanza in Europa. L'infezione causa sintomatologia molto variabile con conseguenti difficoltà diagnostiche. La riduzione nel numero di piastrine circolanti è l'alterazione ematologica più comune e spesso rappresentata l'unico reperto riscontrabile nei cani sintomatici. In questo studio, la presenza di batteri appartenenti ai generi *Anaplasma* and *Ehrlichia* è stata indagata in Nord Italia nei campioni di sangue di 159 cani trombocitopenici utilizzando una metodica di PCR (reazione a catena della polimerasi) che amplifica un tratto del gene *heat shock* (*gro*EL). Gli ampliconi ottenuti sono stati sequenziati e analizzati. Due cani sono risultati positivi per *A. phagocytophilum*, mentre non sono state rilevate positività per *A. platys* ed *E. canis*. A nessuno dei cani risultati positivi in PCR era stata precedentemente diagnosticata l'infezione da *A. phagocytophilum*, pur presentando sintomi clinici o anomalie clinico-patologiche ad essa potenzialmente riferibili. L'analisi delle sequenze nucleotidiche dei 2 ceppi identificati ha permesso di correlarli al cluster Europe 1. Questo studio conferma che *A. phagocytophilum* è presente nei cani in Nord Italia ed è responsabile di forme cliniche non adeguatamente diagnosticate e curate.

Bacterial species belonging to the genera *Anaplasma* and *Ehrlichia* (family *Anaplasmataceae*) are gram-negative, obligate intracellular alphaproteobacteria that replicate in the cytoplasm of eukaryotic cells (Dumler *et al.* 2001). In particular, *A. phagocytophilum*, *A. platys*, and *E. canis* are tick-transmitted parasites of great importance to canine health in Europe. During infection, they form colonies called morulae in monocytes, granulocytes, and platelets (Allison and Little 2013). Dogs infected by these pathogens can develop a wide spectrum of disease, ranging from subclinical infection to potentially life-threatening illness characterized by fever, lethargy, anorexia and, in most severe cases, bleeding diathesis. Reduction in platelet count is considered the most common haematological abnormality during *Anaplasma* and *Ehrlichia* infection in dogs, frequently representing the sole alteration in asymptomatic cases (Little 2010, Allison and Little 2013). Nevertheless, data on the relevance of these infections as causes of thrombocytopenia are lacking.

The aim of the present study was to investigate the presence of bacteria belonging to the genera *Anaplasma* and *Ehrlichia* in blood samples from thrombocytopenic dogs.

The study was conducted in a veterinary teaching hospital, in Northern Italy, on pet dogs. All dogs referred to the veterinary hospital during a 19-month period (January 2013 - July 2014) which underwent ethylenediaminetetraacetic acid (EDTA)-blood sampling for complete blood count (CBC) (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown NY, USA) and had thrombocytopenia, were included in the study. Thrombocytopenia was defined as a platelet count (PLT) of less than 160,000 platelets/µL or less than 75,000 platelets/µL in subjects with evidence of platelet clumps on microscopic examination of a blood smear. Data related to signalment, clinical presentation, and laboratory findings (in particular the PLT values) of sampled dogs were recovered from medical records. Genomic DNA extraction from stored (-20 °C) EDTA-blood samples was carried out using the NucleoSpin Tissue Mini Kit (Macherey-Nagel, Düren, Germany). Diagnosis of infection was performed using a previously described polymerase chain reaction (PCR) assay (Barber *et al.* 2010). The 2 degenerate primers used in the present study, *gro*EL-643s (5'-ACT GAT GGT ATG CAR TTT GAY CG-3') and *gro*EL-1236as (5'-TCT TTR CGT TCY TTM ACY TCA ACT TC-3'), were able to detect DNA from all known *Anaplasma* spp. and *Ehrlichia* spp. by amplifying an approximately 600 bp fragment of the heat shock gene (*gro*EL). The PCR assay was performed as previously described (Dondi *et al.* 2014). To confirm the reproducibility of the results, DNA extraction and PCR were repeated for the samples tested positive.

The obtained amplicons were purified and sequenced directly using both forward and reverse primers. The assembled nucleotide sequences were analysed using the BLAST web interface[1]. Multiple alignments between obtained and reference sequences available from GenBank were generated using the ClustalW method implemented in BIOEDIT sequence alignment editor version 7.2.5. Phylogenetic relationships of obtained sequences with *A. phagocytophilum* reference sequences were evaluated using the maximum likelihood method implemented on MEGA version 6.0.6. Kimura 2-parameter nucleotide substitution model with gamma distribution was used. Bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern and values > 50% were reported. The following reference *A. phagocytophilum* strains detected in several hosts from various parts of the world were obtained from GenBank and included in the molecular analysis: America lineage, accession numbers: AF172163; AY219849; AY848749; AY848750; AY848751; AY848752; DQ680012; Europe1 lineage, accession numbers: U96735; AY281849; AF033101; AF478563; AF482760; AF548386; AY529490; EU381150; EU381151; EU381152; EU982549; GQ452227; HM057224; KF778380; Europe2 lineage, accession numbers: AY220468; EU552912; EU552915; EU552919; EU552921; EU552923; Europe3 lineage, accession numbers: AF383227; AF478561; AY281818; EU552922.

Results were analysed using descriptive statistics. Data comparison among subgroups (breed, sex, age, and geographical origin) was performed by chi-square test; statistical significance was set at $P < 0.05$.

One hundred fifty-nine dogs were included in the study, and EDTA-blood samples were tested by PCR. DNA amplification products of the expected size were obtained from the blood samples of 2 dogs (862/2014 and 901/2014), producing an infection rate of 1.26%. Positive results were obtained by repeating the DNA extraction and PCR on blood samples of the 2 dogs. The assembled nucleotide sequences obtained for dogs 862/2014 and 901/2014 were 561 bp in length, and BLAST analysis allowed us to align them with the reference sequences of *A. phagocytophilum* (GenBank accession numbers: KT970678 and KT970679). Nucleotide alignment showed differences between the 2 detected strains (Annex 1, Supplementary Figure 1). The nucleotide sequences of 862/2014 and 901/2014 showed an identity of 99% between them and an identity of 99.5% with several reference strains detected in various hosts and countries (Dog/IT/EU982549, Dog/SI/EU381150, Dog/SI/EU381151,

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi.

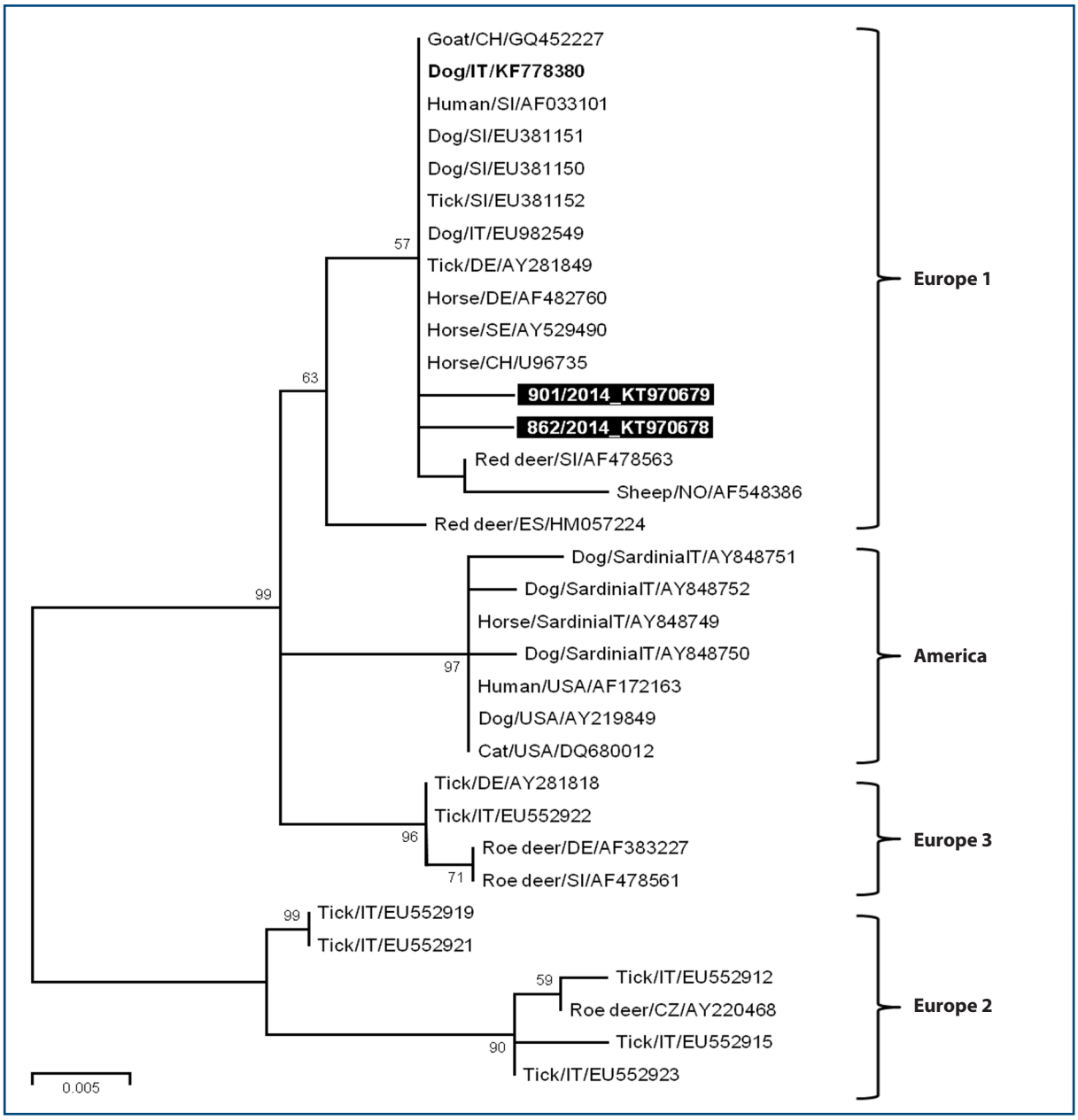

Figure 1. *Maximum likelihood tree based on the* gro*EL gene alignment.* Phylogenetic relationships were evaluated using the maximum likelihood method implemented on MEGA version 6.0.6. Kimura 2-parameter nucleotide substitution model with gamma distribution was used. Bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern and values > 50% were reported. *A. phagocytophilum* reference strains were named with: host species, acronym of nation, and GenBank accession number. Highlighted in black: sequences generated in this study. In bold: reference strain 393 (KF778380) identified in a dog in the Veterinary Teaching Hospital of the University of Bologna, Italy, in 2012.

Goat/CH/GQ452227, Horse/CH/U96735, Horse/DE/AF482760, Horse/SE/AY529490, Human/SI/AF033101, Tick/DE/AY281849, Tick/SI/EU381152), including an *A. phagocytophilum* strain previously identified in a dog in the Veterinary Teaching Hospital of the University of Bologna, Italy (Dog/IT/KF778380) (Dondi *et al.* 2014). The bacteria strains 862/2014 and 901/2014 showed 2 peculiar amino acid substitutions (data not shown). Phylogenetic analysis revealed 4 main clusters, supported by significant bootstrap values, consistent with the accepted nomenclature (Figure 1); 862/2014 and 901/2014 formed 2 independent monophyletic branches into cluster Europe 1. No significant difference in *A. phagocytophilum*-positive subjects between subgroups was detected.

In this study, the blood samples of 2 out of 159 thrombocytopenic dogs were PCR-positive for *A. phagocytophilum*. No dogs positive for *A. platys* or *E. canis* were detected. Dog 901/2014 showed no specific signs of *A. phagocytophilum*

infection, with the exception of thrombocytopenia; whereas dog 862/2014 had clinical signs and clinicopathological abnormalities potentially related to *A. phagocytophilum* infection, such as anaemia, increased hepatic enzymes, hypoalbuminemia, thrombocytopenia, positive Coombs test, and abortion. Nevertheless, anaplasmosis had not been suspected at the time of hospitalization in either dog, and neither had been treated with specific therapy. Data on the prevalence of *Anaplasma* and *Ehrlichia* infections in Northern Italy are lacking. Moreover, comparison between the data obtained in this study and those described in previous epidemiological investigations is difficult due to the different inclusion criteria and diagnostic tools used. Nevertheless, the identification of 2 dogs infected by *A. phagocytophilum* and the absence of dogs infected by *E. canis* is in contrast with other epidemiological studies conducted in Italy, which have detected a high prevalence of *E. canis* and no cases of *A. phagocytophilum* infection (Solano-Gallego *et al.* 2006, Trotta *et al.* 2009). However, a case of *A. phagocytophilum* infection in a dog had already been reported in the province of Bologna (Dondi *et al.* 2014). Contrarily, the absence of *A. platys* in blood samples from dogs is in agreement with a previous molecular survey performed in the same geographic area (Trotta *et al.* 2009), suggesting a low circulation of this bacterium in Northern Italy. The discrepancies between *A. phagocytophilum* and *E. canis* infection rates evidenced in this study and those of other studies may be due to the choice of thrombocytopenia as the only clinicopathological finding used as inclusion criteria in the present study. In this way, a greater number of dogs infected by *A. phagocytophilum* may have been selected because this bacterium is often responsible for asymptomatic infections in dogs that show only thrombocytopenia (Egenvall *et al.* 2000, Little 2010).

In conclusion, this study allows us to confirm the presence of *A. phagocytophilum* infections in dogs of Northern Italy, causing clinical signs and laboratory abnormalities that could not be properly diagnosed and treated. Furthermore, even if the number of infected dogs on the total number of thrombocytopenic dogs analysed is relatively low, our study confirm that *A. phagocytophilum* should be considered as a potential cause of thrombocytopenia in dogs.

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Annex 1

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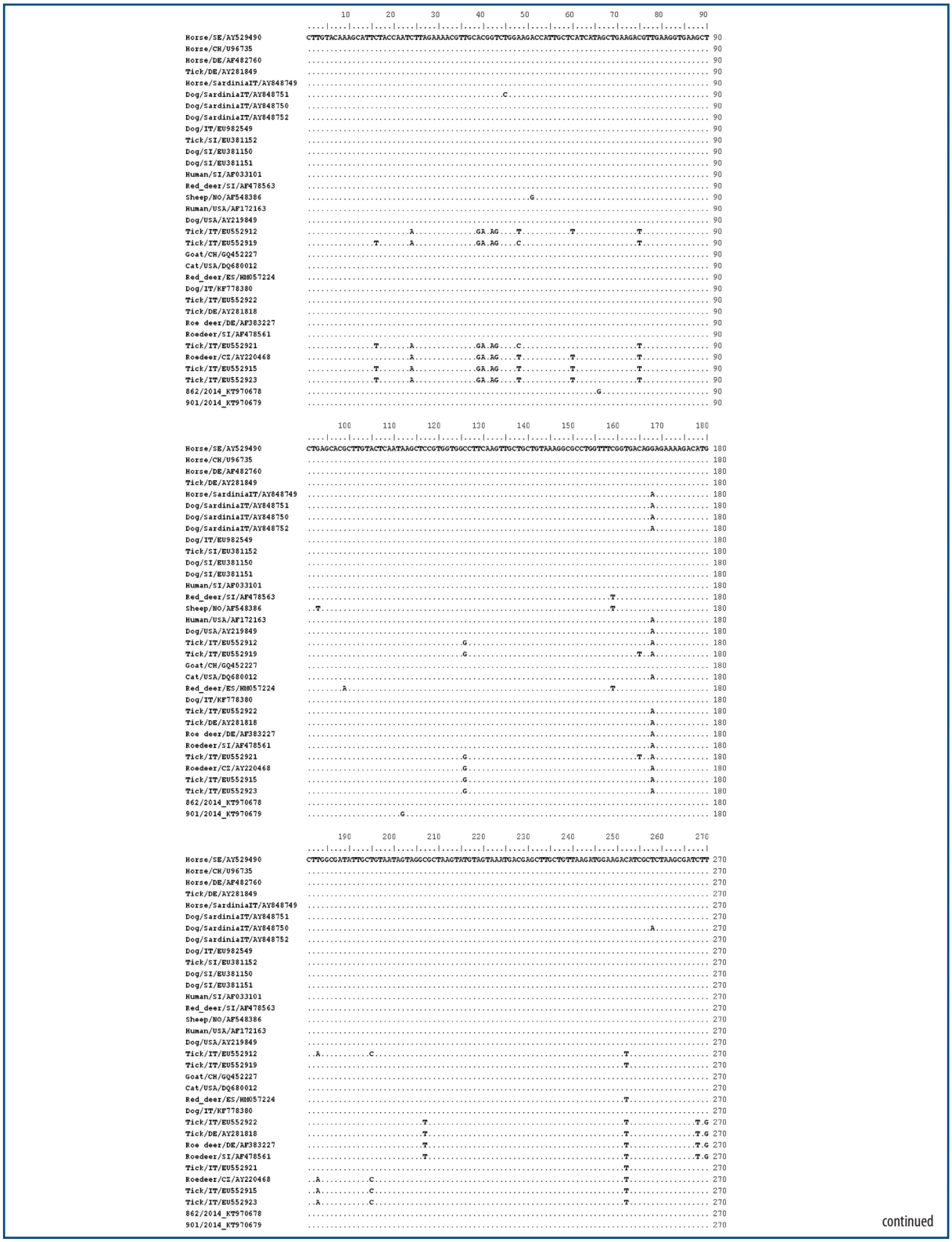
Supplementary Figure 1. *Nucleotide sequence alignment of partial* gro*EL gene between obtained and reference sequences.* Multiple alignments between obtained and reference sequences available from GenBank were generated using the ClustalW method implemented in BIOEDIT sequence alignment editor version 7.2.5. Alignment was generated with sequences of 437 bp in length, because not all the reference sequences covered the 561 bp of the two sequences obtained in this study. The ruler at the top shows the nucleotide positions. Dots represent nucleotide positions identical to the upper reference sequence. —cont'd



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