Theileria sp. OT3 and other tick-borne pathogens in sheep and ticks in Italy Molecular characterization and phylogeny

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a b s t r a c t

PCR Reverse Line Blot (RLB) hybridization and sequencing were used to determine the dynamics of infection with tick-borne pathogens in one hundred apparently healthy sheep in Italy. Blood samples were tested once prior to the onset of the grazing season (June 2010) and once after the end of the grazing season (August 2010). Ticks collected from sheep and from the vegetation were also tested by PCR/RLB. Before grazing, 56% of the sheep harbored several tick-borne pathogens: Anaplasma ovis was the most prevalent (41%), followed by A. ovis co-infected with Theileria sp. OT3 (14%). After grazing, 87% of sheep were positive for A. ovis alone (41%), co-infected with Theileria sp. OT3 (8%) or co-infected with Babesia motasi (5%). Other sheep were infected with Anaplasma phagocytophilum alone (20%), co-infected with B. motasi (7%) or with Theileria sp. OT3 (5%) ($p < 0.001$). After grazing, sheep were significantly more infected with tick-borne pathogens than before grazing. Ticks collected were all Haemaphysalis punctata (n-89) and 36% were positive for A. ovis, Ehrlichia ovina and A. ovis combined with A. phagocytophilum. Phylogenetic analysis including isolates from countries in the Mediterranean Basin show circulation of the same variants of Theileria sp. OT3, whereas two different geographical origins for the isolates of A. ovis and A. phagocytophilum were identified. This is the first report from Italy of Theileria sp. OT3 in sheep, whereas the detection of Ehrlichia ovina in ticks is worth noting, and the presence of A. phagocytophilum in sheep and in ticks poses a potential public health risk.

Introduction

Tick infestations in small ruminants are of increasing concern, in particular because the epidemiology and geographical distribution is constantly changing, due to climatic change, abundance of wildlife hosts, sheep-farming economics, and management of environmental biodiversity (Taylor, 2012). Consequently, the incidence of tick-borne diseases (TBDs) is increasing (Dantas-Torres et al., 2012), and in endemic areas sheep can be simultaneously or sequentially infected with more than one tick-borne pathogen. Anaplasmosis, theileriosis and babesiosis are the most important TBDs, and cause health, economic and management-related problems in small ruminants (Uilenberg, 1999). Anaplasma phagocytophilum and Anaplasma ovis (Rickettsiales: Anaplasmataceae) are the most frequently reported species in small ruminants in the Mediterranean area; A. phagocytophilum is the most pathogenic, and is of greatest zoonotic interest particularly in Europe (Woldehiwet, 2010). In Europe, the tick vector of A. phagocytophilum appears to be predominantly Ixodes ricinus (Stuen, 2007; Woldehiwet, 2010), whereas Rhipicephalus bursa and Dermacentor marginatus are considered to be vectors of A. ovis in the Mediterranean region (Friedhoff, 1997). Theileria lestoquardi (Piroplasmida: Theileriidae) is the most pathogenic of the Theileria species, and in Southern Europe Theileria spp. are transmitted mostly by R.

bursa (Friedhoff, 1997). Theileria sp. OT1 and Theileria sp. OT3 are novel taxa and may be pathogenic (Nagore et al., 2004; Duh et al., 2008) and their vectors are unknown. Babesia ovis and Babesia motasi (Piroplasmida: Babesiidae) are the most important agents of babesiosis in small ruminants in southern Europe (Uilenberg, 2006); B. ovis is the most pathogenic in sheep and is transmitted in southern Europe mainly by Haemaphysalis punctata and R. bursa (Friedhoff, 1997). Molecular surveys on tick-borne pathogens in Italy on small domestic ruminants have been limited to the southernmost areas, i.e. Sicily (de la Fuente et al., 2005a; Torina et al., 2008a, 2010). In order to fill this gap, we (i) used PCR/RLB and sequencing to determine the dynamics ofinfection withAnaplasma, Ehrlichia, Rickettsia, Babesia and Theileria in sheep, and in feeding and questing ticks;(ii) compared the sequence data with published sequences reported from other European countries, including Italy; and (iii) performed phylogenetic analyses to explore the relationships of the isolates characterized.

Materials and methods

Study area

The survey was carried out in the Gran Sasso National Park (42.423136 N–13.3211 E) in Abruzzo Region (Italy) on a sheep farm located at Castel del Monte (AQ) (42.3645187 N–13.7257911 E). During the cold season, sheep are kept in a shed at 1100 m a.s.l., whereas from June to Augustthey are grazed on an area of 955 ha in the locality of Fonte Vetica (42.4246286 N–13.5240626 E) at about 1550 m a.s.l. Blood and tick collections In2010, one hundred, apparently healthy, sheep of four different breeds (Gentile di Puglia, Garfagnina, Cross-breeds, Sopravvisana) were randomly selected, and blood samples were taken on two occasions. One hundred blood samples were collected before the grazing season (early June) and a further one hundred samples were collected from the same sheep just after the grazing season (end of August). Blood samples were collected in EDTA and maintained at 4 ◦C until arrival at the laboratory, where they were stored at −20 ◦C until tested. All sheep were inspected for the presence of ticks for three consecutive days, and ticks were collected from infested subjects on two occasions: (i) in the first week of June before grazing began and (ii) in the last week of August at the end of grazing. Ticks were also collected on the pasture in the third week of July and in the third week of August. Feeding ticks were removed with forceps from sheep; questing ticks were collected by dragging, and the cloth was inspected for the presence of ticks every 10 m. The ticks were placed in numbered and dated vials containing 70% ethanol and ticks collected from animals and pasture were counted in the laboratory, grouped according to their developmental stage, identified according to Estrada-Pena et al. (2004) and Manilla (1998) keys, and stored at −20 ◦C before molecular processing.

DNA extraction

DNA was extracted from blood samples (n = 200) using the Nucleospin Blood Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. Ticks were washed three times in 1X phosphate buffered saline, rinsed with distilled water and dried on sterile filter paper before DNA extraction. Disruption and homogenization were performed in lysis buffer from the Nucleospin Tissue kit (Macherey-Nagel, Germany) using the TissueLyser LT (Qiagen, Venlo, Netherlands) and 5 mm stainless steel beads according to the manufacturer's instructions. DNA was extracted individually from adults, nymphs and larvae. All genomic DNA from blood and tick specimens was stored at −20 ◦C.

Molecular procedures

After DNA extraction, PCR amplification and reverse line blot hybridization (RLB) assay were used on blood and tick samples to simultaneously detect and discriminate more genera/species of pathogens i.e. those reported in Table 1 . Samples found positive only to catch-all (Ehrlichia/Anaplasma and Babesia/Theileria) were then sequenced to detect possible variants of investigated species and/or new species/strains, and a phylogenetic analysis was performed on all the obtained sequences.

PCR amplification

Ehrlichia/Anaplasma PCR was performed using the forward primer Ehr-F (5 -GGAATTCAGAGTTGGATCMTGGYTCAG-3) and Ehr-R (5 -biotin-CGGGATCCC GAGTTTGCCGGGACTTYTTCT-3) to amplify a fragment of 460–520 bp from the V1 hypervariable region of the 16S rRNA gene (Nijhof et al., 2007). Babesia /Theileria PCR was performed using primers RLB-F2 (5 -GACACA GGGAGGTAGTGACAAG-3) and RLB-R2 (biotin-5 - CTAAGAATTTCACCTCTGACA GT-3) to amplify a fragment of 460–540 bp from the 18SrRNA gene spanning the V4 region (Matjila et al., 2004). PCR reactions were performed in 25 l volumes with 1X PCR Buffer, 200 M of each dNTP, 20 pmol of each primer, 0.125 l of Phire Hot Start II Polymerase (Fisher Scientific Netherlands) and 2.5 l of genomic DNA. PCR conditions consisted of an initial denaturation step of 30 s at 98 ◦C, a touch-down PCR of 5 s at 98 ◦C, 5 s at 67 ◦ C and 7 s at 72 ◦ C for 10 cycles, in which after each of the 10 cycles the annealing temperature decreased 1 ◦C from 67 ◦C to 57 ◦C, then a second step followed of 40 cycles of 5 s at 98 ◦C, 5 s at 57 ◦ C and 7 s at 72 ◦C, and finished with a final extension step of 1 min at 72 ◦C. Positive and negative controls were included in each run and obtained PCR products were stored at −18 ◦ C until further use.

Reverse line blot hybridization (RLB) assay

The RLB assay was performed as previously described (Matjila et al., 2004). Oligonucleotide probes (Table 1) containing an N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,Ndiisopropyl phosphoramidite)-C6 amino linker (Eurogentec, Maastricht, The Netherlands) were covalently linked to the RLB membrane (Biodyne C blotting membrane; Pall Biosupport, Ann Arbor, MI, USA) using the following procedure. The membrane was activated by a 10-min incubation at room temperature in a freshly prepared 10-ml solution of 16% 1-ethyl 3-(3- dimethylaminopropyl) carbodiimide HCl (Sigma, St. Louis, MO). The membrane was briefly rinsed in distilled water and then placed in an MN45 miniblotter (Immunetics, Cambridge, MA) and residue liquid was aspirated. The oligonucleotide probes were diluted to a concentration of 400 pmol/150 l in 500 mM NaHCO 3 solution (pH 8.4), and linked to the membrane by loading onto the lanes of the miniblotter; this was followed by a 1-min incubation after which the probes were aspirated. After aspiration of the oligonucleotide probe solutions,the membrane was washed in a 100 ml freshly prepared 100 mM NaOH solution for 8 min at room temperature under gentle shaking to inactivate the membrane. After the membrane was inactivated, the membrane was washed for 5 min at 60 ◦ C in a 2 × SSPE (2 × SSPE is 0.36 M NaCl, 20 mM NaH 2PO 4 , and 2 mM EDTA [pH 7.7]) − 0.1% sodium dodecyl sulfate (SDS) solution under gentle shaking followed by a washing step using a 20 mM EDTA solution for 15-min at room temperature under gentle shaking to rinse the membrane of any residue before storing in fresh 20 mM EDTA solution at 4 ◦C until further use. The RLB membrane was hybridized with the PCR products and further developed as described by Gubbels et al., 1999).

Sequencing

Samples positive to Ehrlichia/Anaplasma and Babesia/Theileria catch-all PCR were purified using enzymes EXO I and FAST AP (Fisher Scientific, Netherlands) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions with the ABI PRIMS BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems), using the same primers as the respective PCR reactions (Ehr-F and Ehr-R for Ehrlichia/Anaplasma) and (RLB-F2 and RLB-R2 for Babesia/Theileria) according to the manufacturer's instructions. An ABI PRISM 3130 Genetic Analyzer (Applied Biosystem) was used to determine sequences, electropherograms were inspected by eye and consensus sequences were determined. Each sequence was compared to nucleotide sequences available in publicly accessible databases using BLASTn software. Subsequently, sequences were aligned using the ClustalW program (BioEdit software).

Phylogenetic analysis

The MEGA v5.1 program was used to align and compare all sequences (Tamura et al., 2011). Pairwise comparisons of nucleotide variation (p-distance) were made using the formula p-distance = 1 − (M/L), where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared. In addition, the numbers of variable, singleton and parsimony-informative sites were determined, as well as the transition/transversion (s/v) ratios. All Anaplasma/Ehrlichia and Babesia/Theileria sequences determined in the present study were aligned with homologous sequences available in GenBank from sheep in Europe, as reported in Table 2. Each alignment was analyzed by Neighbour Joining (NJ) using MEGA software, using Ehrlichia ruminantium (GenBank accession numbers NR074155) and Toxoplasma gondii (GenBank accession numbers L37415) as outgroup.

Statistical analysis

Sheep which tested positive for atleast one tick-borne pathogen by PCR combined with RLB hybridization and sequencing were defined as positive. Prevalence values were calculated as the number of positive animals/number of examined animals × 100, with the corresponding 95% confidence intervals (95% CI). Different prevalence values were compared by the chi-square test according to the time of sampling (before vs. after pasture). P values < 0.05 were considered significant and P values < 0.01 were considered highly significant. The Odds Ratio (OR) and corresponding 95% CI values were also calculated as a measure of the risk. Statistical values determined as not significant are not presented.

Results

Of 200 DNA blood samples tested by RLB, 59 (29.5%) were positive to Ehrlichia/Anaplasma catch-all (E/A), and 76 (38%) to Babesia/Theileria catch-all (B/T). Fifty-four (91.5%) of E/A amplicons and 28 (36.8%) of B/T amplicons were successfully sequenced. Of the 54 sequences obtained, 32 belong to A. phagocytophilum and 22 belong to A. ovis, with a range of similarity for both Anaplasma species of 98–99%. The 28 sequences obtained belong to Theileria sp. OT3 (99–100% of similarity). Of the 89 tick specimens collected from sheep's muzzles, heads and groin areas and on pasture (52 larvae, 17 nymphs, 3 females and 17 males), all were identified as H. punctata and 32 (36%) harbored pathogens. Using RLB, 4 (12.5%) specimens tested positive for Babesia/Theileria catch-all, and 16 (50%) positive for Ehrlichia/Anaplasma catch-all; two tested positive for A. ovis, and six for E. ovina, while 4 (1 larva from the host and 3 larvae from the pasture), showed a similarity of 98% to Anaplasma ovis/A. phagocytophilum after sequencing. Table 3 summarizes the sites (animal or pasture), number, percentage and stage of the collected ticks, and the pathogens and/or co-infections detected. Concerning the prevalence of pathogens in sheep, 56% of the animals harbored tick-borne pathogens or their associations before grazing. A. ovis was the most prevalent pathogen (41%), followed by A. ovis + Theileria sp. OT3 (14%). After grazing, tick-borne pathogens or their associations were detected in 87% of sheep and an overall total of 35 new cases of single and/or multiple infections were detected. After grazing, 87% of infested sheep were positive for A. ovis alone (41%), co-infected with Theileria sp. OT3 (8%) or co-infected with Babesia motasi (5%). Other sheep were infected with Anaplasma phagocytophilum alone (20%), co-infected with B. motasi (7%) or with Theileria sp. OT3 (5%) ($p < 0.001$). Highly significant differences were observed in the prevalence of tick-borne pathogens before and after grazing (Table 4). The 54 Anaplasma sequences obtained from sheep samples included 251 conserved (65.2%) and 134 variable sites (34.8%), of which 82 were singletons (61.2%) and 52 were parsimonyinformative (38.8%). The average nucleotide base composition was T (23.35%), C (31.51%), A (26.82%) and G (18.32%). The ratio of transitional substitution wasA/G (18.2%), T/C (13.13%), C/T (16.73%) and G/A (15.49%). The ratio of transvertional substitution was A/C and G/C (3.34%), T/G and C/G (5.74%), T/A and C/GA (4.89%), and finally A/T and G/T (4.25%). The average amino-acid frequency was 122.37 and the uncorrected p-distance between sequences was 0.05%. Of the 54 sequences, 22 clustered to A. ovis

group and 32 clustered to A. phagocytophilum group with full statistical reliability (bootstrap value, 100%). A. ovis sequences obtained in this study showed genetic closeness to the sequence detected in Sicily (GQ857077), to one fromTurkey (EU191232) and one from the Netherlands (AF318945); however,there was a clear genetic distance from a second sequence detected on sheep in Turkey (JF807995) and from one found on the island of Sardinia (Italy) (KC335231) and from another found on sheep in Hungary (DQ837601). The pairwise uncorrected p distances between these sequences was 0.04% (Fig. 1; Table 2). The A. phagocytophilum sequences obtained in this study cluster together with A. phagocytophilum sequences reported previously from sheep in Norway (GQ428333), Germany (GU236652; AF336220; KC740450), UK (AY149637) and Italy (EU436156) A. phagocytophilum sequences showed a clear genetic distance from one detected on sheep in Turkey (KF459965) and one from Sardinia (Italy) (KC335227). The pairwise uncorrected p distances between these sequences was 0.04%. Our sequences also cluster with A. phagocytophilum sequences reported from human isolates in the Czech Republic (EF217398), Germany (GU236664), Poland (KF111754), Slovenia (KF242614) and one previously detected in Italy (DQ029028). The pairwise uncorrected p distance between the two groups (from sheep and human) was 0.05% (Fig. 2; Table 2). The 28 Theileria sequences included 91 conserved (22.98%) and 305 variable sites (77.02%), of which 120 were singletons (39.34%) and 185 were parsimony-informative (60.65%). The average nucleotide base composition was T (36.44%), C (22.10%), A (26.16%) and G (14.29%). The ratio of transitional substitution was A/G (10.06%), T/C (7.08%), C/T (18.07%) and G/A (12.36%). The ratio of transvertional substitution was A/C and G/C (3.74%), T/G and C/G (5.79%), T/A and C/A (7.12%), and finally A/T and G/T (9.55%). The average amino acid frequency was 126.2 and the overall uncorrected p-distance between sequences was 0.17%. Theileria sp. OT3 sequences cluster in two main groups known as I and II; the sequences from Group I appear closely related to those from Spain (DQ866841; AY533145) and Turkey (EF092455; JQ867384) (Fig. 3; Table 2). The pairwise uncorrected p distances between them was 0.22%, while the pairwise uncorrected p distances within Groups I and II were 0.18% and 0.07%, respectively. Two sequences lay outside the two groups. Nucleotide sequences without ambiguous positions have been deposited in GenBank under the accession numbers KF270715–KF270742 for Theileria sp. OT3, KF293699–KF293720 for A. ovis and KF293667–KF293698 for A. phagocytophilum.

Discussion

RLB is an effective and practical diagnostic tool because of its ability to detect simultaneously low numbers of tick-borne bacteria and protozoans using specific oligonucleotide probes. The results of the present study show that Theileria sp. OT3, A. ovis, A. phagocytophilum, E. ovina and B. motasi are present in sheep, and that A. ovis, E. ovina and A. ovis/A. phagocytophilum are present in all stages of questing H. punctata ticks. Theileria sp. OT3 was found for the first time in sheep in Italy. Its prevalence (alone or in association) registered in this study (28%) is notably higher than that reported in small ruminants in other Mediterranean countries i.e. Turkey (Altay et al., 2007; Aydin et al., 2013), and Croatia (Duh et al., 2008). The presence of two main groups (I and II) for Theileria sp. OT3, shows that this pathogen consists of several variants, and the close relationship of our sequences with those from Spain (DQ866841; AY533145) and Turkey (EF092455; JQ867384) (Fig. 3) allows us to hypothesize thepossible circulationofthe same variants of Theileria sp. OT3 between countries of the Mediterranean Basin, possibly related to livestock movement.

A. ovis and A. phagocytophylum have been serologically and molecularly detected in sheep in southern Italy (Sicily) (Torina et al., 2006, 2008b, 2010). Their prevalence registered in this study appears much higher than that previously registered for A. ovis and A. phagocytophilum in Sicily (Torina et al., 2006, 2008b, 2010) or for B. motasi in Abruzzo (Savini et al., 1999). With regard to A. ovis, the small genetic distance between the sequences from Turkey (EU191232), and Sicily (Italy) (GQ857077) (Fig. 1; Table 2), and the great genetic distance from those from the eastern Black Sea region of Turkey (JF807995) and Sardinia (Italy) (KC335231), allow us to hypothesize two different routes of A. ovis isolate circulation within

Mediterranean countries. Analogous considerations apply to A. phagocytophilum. The presence of B. motasi is not new in Italy. This species has previously been detected microscopically and serologically in sheep in central Italy (Abruzzo region) (Savini et al., 1999), but never detected in sheep using molecular analysis.

Interestingly, Ehrlichia ovina was also detected in sheep. Information about this microorganism is very limited; it is recognized as not being highly pathogenic and is now included in the 'Ehrlichia canis group' (Faburay et al., 2007). In this study, sheep dogs may have acted as reservoirs of this Ehrlichia species; however, this epidemiological aspect of E. ovina/canis anditspathogenic role insheep needs to be investigated. The absence of clinical symptoms related to TBDs in sheep is not new (Savini et al., 1999; Giudice et al., 2011), and may be related to the resistance of autochthonous breeds, as recently demonstrated for A. ovis in Italy (Ciani et al., 2013). As expected, the total prevalence of tick-borne pathogens in sheep increases dramatically after grazing (87%, $p < 0.01$); sheep were significantly more infected with single tick-borne pathogens and/or their associations after grazing (Table 3). Ticks collected from animals and the pasture harbor most of the pathogens detected in sheep blood. The detection of E. ovina, A.ovis/A. phagocytophilum DNA in questing H. punctata – the only species of tick collected – is interesting in terms ofthe pathogens detected and the stage of detection. Although I. ricinus is considered the most important vector of A. phagocytophilum also in Italy (Capelli et al., 2012), in this study, I. ricinus was not detected in sheep or in questing ticks. Therefore, itis possible that H. punctata may play a role in the transmission of these pathogens in central Italy, as already suspected (Torina et al., 2008a). It is also worth noting that these pathogens were detected in questing larvae, some of which were apparently unfed. Trans-stadial transmission is thought to be important in the life cycles of Anaplasma spp., and transovarial transmission has been demonstrated for A. phagocytophilum in Dermacentor ticks (Baldridge et al., 2009). The present detection of A. ovis and A. phagocytophilum in unfed H. punctata larvae may imply that transovarian transmission also occurs in this tick species, and thatlarvae are consequently able to transmit the bacteria; this requires further investigation, since it is possible that the pathogen DNA could be maintained and detectable from one tick life stage to the other without any viable pathogen or transmission by the tick. Wild ruminants like deer may act as reservoirs of A.ovis and A. phagocytophilum also in Italy, where roe deer (Capreolus capreolus) (Carpi et al., 2008) and fallow deer (Dama dama) (Veronesi et al., 2011) were found to be infected by A. phagocytophilum. However, A. phagocytophilum has also been found to infect a variety of other animals (cattle, horses, foxes, dogs, cats, mice) (Lillini et al., 2006; Ebani et al., 2008; Aureli et al., 2012) in central and southern Italy. In the study area of the present research (Abruzzo Region National Park), the co-existence of domestic (dogs, horses, cattle) and wild animals (deer, small rodents) – all susceptible to rickettsia infection – may allow the maintenance and distribution of A. ovis and A. phagocytophilum and may therefore play an important role in the epidemiology. A. phagocytophilum causes human granulocytic anaplasmosis (HGA; formerly human granulocytic ehrlichiosis); detection of A. phagocytophilum in sheep and in ticks in this study indicates a potential human health threat, also becauseA. phagocytophilum isolates and those previously detected in sheep in Sicily (Torina et al., 2008b) showed a very close genetic identity to the human isolates from the Czech Republic (EF217398), Germany (GU236664), Poland (KF111754), Slovenia (KF242614) and to one previously detected in Italy (DQ029028) (Fig. 2; Table 2). In Italy, HGA seropositivity of up to 8.6% (Nuti et al., 1998; Cinco et al., 2004; Beltrame et al., 2006) and two clinical cases (Ruscio and Cinco, 2003) have been reported in humans. In conclusion, this study has confirmed that several tick-borne pathogens of sheep, including some of zoonotic importance, are highly prevalent in southern Italy. The presence of already known species (i.e. A. phagocytophilum, A. ovis and B. motasi) and of previously unknown pathogens (i.e. Theileria sp. OT3 and E. ovina) deserves further investigation.

References

Aydin, M., Aktas, M., Dumanli, N., 2013. Molecular identification of Theileria and Babesia in sheep and goats in the Black Sea Region in Turkey. Parasitol. Res. 112, 2817–2824.

Altay, K., Dumanli, N., Aktas, M., 2007. Molecular identification, genetic diversity and distribution of Theileria and Babesia species infecting small ruminants. Vet. Parasitol. 147, 161–165.

Aureli, S., Foley, J.E., Galuppi, R., Rejmanek, D., Bonoli, C., Tampieri, M.P., 2012. Anaplasma phagocytophilum in ticks from parks in the Emilia-Romagna region of northern Italy. Vet. Ital. 48, 413–423.

Baldridge, G., Scoles, G.A., Burkhardt, N.J., Schloeder, B., Kurtti, T.J., Munderloh, U.G., 2009. Transovarial transmission of Francisella-like endosymbionts and Anaplasma phagocytophilum variants in Dermacentor albipictus (Acari: Ixodidae). J. Med. Entomol. 46, 625–632.

Beltrame, A., Ruscio, M., Arzese, A., Rorato, G., Negri, C., Londero, A., Crapis, M., Scudeller, L., Viale, P., 2006. Human granulocytic anaplasmosis in northeastern Italy. Ann. N. Y. Acad. Sci. 1078, 106–109.

Capelli, G., Ravagnan, S., Montarsi, F., Ciocchetta, S., Cazzin, S., Porcellato, E., Babiker, A.M., Cassini, R., Salviato, A., Cattoli, G., Otranto, D., 2012. Occurrence and identification of risk areas of Ixodes ricinus-borne pathogens: a cost-effectiveness analysis in north-eastern Italy. Parasit. Vectors 27 (5), 61.

Carpi, G., Cagnacci, F., Neteler, M., Rizzoli, A., 2008. Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. Epidemiol. Infect. 136, 1416–1424.

Ciani, E., Alloggio, I., Petazzi, F., Pieragostini, E., 2013. Looking for prognosticators in ovine anaplasmosis: discriminant analysis of clinical and haematological parameters in lambs belonging to differently susceptible breeds experimentally infected with Anaplasma ovis. Acta Vet. Scand. 22 (55), 71.

Cinco, M., Barbone, F., Grazia ciufolini, M., Mascioli, M., Anguero Rosenfeld, M., Stefanel, P., Luzzati, R., 2004. Seroprevalence of tick-borne infections in forestry rangers from northeastern Italy. Clin. Microbiol. Infect. 10, 1056–1061.

Dantas-Torres, F., Chomel, B.B., Otranto, D., 2012. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 28, 437–446.

de la Fuente,J., Torina,A.,Caracappa, S., Tumino, G., Furlá,R.,Almazán,C.,Kocan,K.M., 2005a. Serologic and molecular characterization of Anaplasma species infection in farm animals and ticks from Sicily. Vet. Parasitol. 133, 357–362.

de la Fuente, J., Torina,A., Naranjo,V., Caracappa, S., Di Marco,V.,Alongi,A., Russo, M., Maggio, A.R., Kocan, K.M., 2005b. Infection with Anaplasma phagocytophilum in a seronegative patientin Sicily, Italy: case report.Ann. Clin. Microbiol.Antimicrob. 4, 15.

Duh, D., Punda-Polic, ´ V., Trilar, T., Avsic-Zupanc, T., 2008. Molecular detection of Theileria sp. in ticks and naturally infected sheep. Vet. Parasitol. 151, 327–331.

Ebani, V., Cerri, D., Fratini, F., Ampola, M., Andreani, E., 2008. Seroprevalence of Anaplasma phagocytophilum in domestic and wild animals from central Italy. New Microbiol. 31, 371–375.

Estrada-Pena, A., Bouattour, A., Camicas, J.L., Walker, A.R., 2004. Ticks of Domestic Animals in Mediterranean Region: A Guide to Identification of Species. Atalanta, Houten, The Netherlands, ISBN 84-96214-18-4.

Faburay, B., Geysen, D., Munstermann, S., Taoufik, A., Postigo, M., Jongejan, F., 2007. Molecular detection of Ehrlichia ruminantium infection in Amblyomma variegatum ticks in The Gambia. Exp. Appl. Acarol. 42, 61–74.

Friedhoff, K.T., 1997. Tick-borne diseases of sheep and goats caused by Babesia, Theileria or Anaplasma spp. Parassitologia 39, 99–109.

García-Sanmartín, J., Aurtenetxe, O., Barral, M., Marco, I., Lavin, S., García-Pérez, A.L., Hurtado, A., 2007. Molecular detection and characterization of piroplasms infecting cervids and chamois in Northern Spain. Parasitology 134, 391–398.

Giudice, E., Giannetto,C., Torina,A., Gianesella,M., 2011.Anaplasma phagocytophilum intragranulocytic morulae in aborting sheep: a herd case in Sicily. Transbound. Emerg. Dis. 58, 263–267.

Gubbels, J.M., De Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., De Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine Theileria and Babesia species by Reverse Line Blot Hybridization. J. Clin. Microbiol. 37, 1782–1789.

Hulinska, D., Votypka, J., Plch, J., Vlcek, E., Valesova, M., Bojar, M., Hulinsky, V., Smetana, K., 2002. Molecular and microscopical evidence of Ehrlichia spp.

and Borrelia burgdorferi sensu lato in patients, animals and ticks in the Czech

Republic. New Microbiol. 25, 437–448.

Lillini, E., Macrì, G., Proietti, G., Scarpulla, M., 2006. New findings on anaplasmosis

caused by infection with Anaplasma phagocytophilum. Ann. N. Y. Acad. Sci. 1081,

360–370.

Manilla, G., 1998. Fauna d'Italia. In: Acari – Ixodida. Ed Calderini, Bologna.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of Babesia canis vogeli in domestic dogs in South Africa. Vet.

Parasitol. 122, 119–125.

Nagore, D., García-Sanmartín, J., García-Pérez, A.L., Juste, R., Hurtado, A., 2004. Identification, genetic diversity and prevalence of Theileria and Babesia species in a

sheep population from Northern Spain. Int. J. Parasitol. 34, 1059–1067.

Nijhof, A.M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen,

L., Jebbink, F., Jongejan, F., 2007. Ticks and associated pathogens collected

from domestic animals in the Netherlands. Vector Borne Zoonotic Dis. 7,

585–595.

Nuti, M., Serafini, D.A., Bassetti, D., Ghionni, A., Russino, F., Rombolà, P., Macri, G.,

Lillini, E., 1998. Ehrlichia infection in Italy. Emerg. Infect. Dis. 4, 663–665.

Ruscio, M., Cinco, M., 2003. Human granulocytic ehrlichiosis in Italy: first report on

two confirmed cases. Ann. N. Y. Acad. Sci. 990, 350–352.

Savini, G., Conte, A., Semproni, G., Scaramozzino, P., 1999. Tick-borne diseases in

ruminants of Central and Southern Italy: epidemiology and case reports. Parassitologia 41 (Suppl. 1), 95L 100.

Scharf, W., Schauer, S., Freyburger, F., Petrovec, M., Schaarschmidt-Kiener, D.,

Liebisch, G.,Runge,M., Ganter,M.,Kehl,A., Dumler,J.S., Garcia-Perez,A.L.,Jensen,

J., Fingerle, V., Meli, M.L., Ensser, A., Stuen, S., von Loewenich, F.D., 2011. Distinct

host species correlate with Anaplasma phagocytophilum ankA gene clusters. J.

Clin. Microbiol. 49, 790–796.

Stuen, S.,Van De Pol, I., Bergstrom,K., Schouls, L.M., 2002. Identification of Anaplasma

phagocytophila (formerly Ehrlichia phagocytophila) variants in blood from sheep

in Norway. J. Clin. Microbiol. 40, 3192–3197.

Stuen, S., 2007. Anaplasma phagocytophilum – the most widespread tick-borne infection in animals in Europe. Vet. Res. Commun. 1, 79–84.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5:

Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28,

2731–2739.

Taylor, M.A., 2012. Emerging parasitic diseases of sheep. Vet. Parasitol. 189, 2–7.

Torina, A., Khoury, C., Caracappa, S., Maroli, M., 2006. Ticks infesting livestock on

farms in Western Sicily, Italy. Exp. Appl. Acarol. 38, 75–86.

Torina, A., Alongi, A., Naranjo, V., Estrada-Pena, ˜ A., Vicente, J., Scimeca, S., Marino,

A.M.F., Salina, F., Caracappa, S., de la Fuente, J., 2008a. Prevalence and genotypes of Anaplasma species and habitat suitability for ticks in a Mediterranean

ecosystem. Appl. Environ. Microbiol. 74, 7578–7584.

Torina, A., Alongi, A., Naranjo, V., Scimeca, S., Nicosia, S., Di Marco, V., Caracappa, S.,

Kocan, K.M., de la Fuente, J., 2008b. Characterization of Anaplasma infections in

Sicily, Italy. Ann. N. Y. Acad. Sci. 1149, 90–93.

Torina, A., Galindo, R.C., Vicente, J., Di Marco, V., Russo, M., Aronica, V., Fiasconaro,

M., Scimeca, S., Alongi, A., Caracappa, S., Kocan, K.M., Gortazar, C., de la Fuente,

J., 2010. Characterization of Anaplasma phagocytophilum and A. ovis infection in

a naturally infected sheep flock with poor health condition. Trop. Anim. Health

Prod. 42, 1327–1331.

Uilenberg, G., 1999. Importance of ticks and tick-borne diseases of domestic ruminants in Southern Europe. Parassitologia 41 (Suppl. 1), 107L 9.

Uilenberg, G., 2006. Babesia – a historical overview. Vet. Parasitol. 138, 3–10.

Veronesi, F., Galuppi, R., Tampieri, M.P., Bonoli, C., Mammoli, R., Piergili Fioretti, D.,

2011. Prevalence of Anaplasma phagocytophilum in fallow deer (Dama dama) and

feeding ticks from an Italy preserve. Res. Vet. Sci. 90, 40–43.

Woldehiwet, Z., 2010. The natural history of Anaplasma phagocytophilum. Vet. Parasitol. 167, 108–122.

Welc-Faleciak, ¸ R.,Werszko, J., Cydzik, K., Bajer, A., Michalik, J., Behnke, J.M., 2013. Coinfection and genetic diversity of tick-borne pathogens in roe deer from Poland.

Vector Borne Zoonotic Dis. 13, 277–288.

Zivkovic, Z., Torina, A., Mitra, R., Alongi, A., Scimeca, S., Kocan, K.M., Galindo, R.C.,

Almazán, C., Blouin, E.F., Villar, M., Nijhof, A.M., Mani, R., La Barbera, G., Caracappa, S., Jongejan, F., de la Fuente, J., 2010. Subolesin expression in response

to pathogen infection in ticks. BMC Immunol. 11, 7, http://dx.doi.org/10.1186/

1471-2172-11-7.

Zobba, R., Anfossi, A.G., Pinna Parpaglia, M.L., Dore, G.M., Chessa, B., Spezzigu, A.,

Rocca, S., Visco, S., Pittau, M., Alberti, A., 2014. Molecular investigation and phylogeny of Anaplasma spp. in Mediterranean ruminants reveal the presence of

neutrophil-tropic strains closely related to A. platys. Appl. Environ. Microbiol.

80, 271–280.