Ticks and Tick-borne Diseases 6 (2015) 75–83

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

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

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ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form
23 September 2014
Accepted 23 September 2014
Available online 22 October 2014

Keywords:
Theileria sp. OT3
Tick-borne pathogens
Sheep
Ticks
RLB/PCR
Sequencing

ABSTRACT

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

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http://dx.doi.org/10.1016/j.ttbdis.2014.09.007
1877-959X/

(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 of infection with *Anaplasma*, *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 August they 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

In 2010, 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.

Table 1
List of genera/species of pathogens and RLB oligonucleotide probes used in this study.

Probe	Sequence 5′-3′
Ehrlichia/Anaplasma catch-all	GGGGGAAAGATTTATCGCTA
Anaplasma centrale	TCGAACGGACCATACGC
Anaplasma ovis	ACCGTACGCGCAGCTTG
Anaplasma marginale	GACCGTATACGCAGCTTG
Anaplasma phagocytophilum	TTGCTATAAAGAATAATTAGTGG
Anaplasma phagocytophilum	TTGCTATGAAGAATAATTAGTGG
Anaplasma phagocytophilum	TTGCTATAAAGAATAGTTAGTGG
Anaplasma phagocytophilum	TTGCTATAGAGAATAGTTAGTGG
Ehrlichia ovina	TCTGGCTATAGGAAATTGTTA
Ehrlichia ruminantium	AGTATCTCGTGTTAGTGGCAG
Theileria/Babesia catch-all	TAATGGTTAATAGGARCRGTTG
Theileria catch-all	ATTAGAGTGCTCAAAGCAGGC
Babesia catch-all 1	ATTAGAGTGTTTCAAGCAGAC
Babesia catch-all 2	ACTAGAGTGTTTCAAACAGGC
Babesia ovis	TGGGCGCGGCCTTTGCGTT
Babesia motasi	GAATGATGCCGACTTAAACCCT
Theileria lestoquardi	ATTCGTTGTGTCCCTCCG
Theileria ovis	TTGCTTTTGCTCCTTTACGAG
Theileria uilenbergi	TGCATTTTCCGAGTGTTACT
Rickettsia catch-all	TTTAGAAATAAAAGCTAATACCG

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

Table 2
List of 16S and 18S *Anaplasma* and *Theileria* nucleotide sequences available in GenBank from sheep in Europe.

Accession Number	Pathogens	Host	Country	Reference
DQ866841	*Theileria* OT3	Sheep	Spain	García-Sanmartín et al., 2007
EF092455	*Theileria* OT3	Sheep	Turkey	Altay et al., 2007
AY533145	*Theileria* OT3	Sheep	Spain	Nagore et al., 2004
JQ867384	*Theileria* OT3	Sheep	Turkey	Aktas et al., 2013
GQ428333	*A. phagocytophilum*	Sheep	Germany	Stuen et al., 2002
AY149637	*A. phagocytophilum*	Sheep	UK	Odgen et al., 2003
GU236652	*A. phagocytophilum*	Sheep	Germany	Scharf et al., 2011
EU436156	*A. phagocytophilum*	Sheep	Italy (Sicily)	Torina et al., 2008
AF336220	*A. phagocytophilum*	Sheep	Norway	Stuen et al., 2002
KF459965	*A. phagocytophilum*	Sheep	Turkey	Aydin et al., 2013
KC740450	*A. phagocytophilum*	Sheep	Germany	Van Loewerich et al., 2013
KC335227	*A. phagocytophilum*	Sheep	Italy (Sardinia)	Zobba et al., 2014
EF217398	*A. phagocytophilum*	Human	Czech Republic	Hulinska et al., 2002
GU236664	*A. phagocytophilum*	Human	Germany	Scharf et al., 2011
KF111754	*A. phagocytophilum*	Human	Poland	Welc-Falęciak et al., 2013
KF242614	*A. phagocytophilum*	Human	Slovenia	von Loewenich et al., 2013
DQ029028	*A. phagocytophilum*	Human	Italy (Sicily)	de la Fuente et al., 2005b
EU191232	*A. ovis*	Sheep	Turkey	Aktas et al., 2009
DQ837601	*A. ovis*	Sheep	Hungary	Hornot et al., 2006
GQ857077	*A. ovis*	Sheep	Italy	Zivkovic et al., 2010
AF318945	*A. ovis*	Sheep	The Netherland	Bekker et al., 2002
JF807995	*A. ovis*	Sheep	Turkey	Altay et al., 2011
KC335231	*A. ovis*	Sheep	Italy (Sardinia)	Zobba et al., 2014