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Short communication

First detection of tick-borne pathogens of dogs from Malta

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ABSTRACT

The knowledge about the vector-borne infections in domestic dogs has been increasing worldwide. However no studies have been done on hard tick infestation and tick-borne pathogens (TBPs) of dogs present in Malta. Therefore, a total of 99 dogs was selected and inspected between March and July 2013 in 18 urban and 17 rural areas on the islands of Malta and Gozo. All ticks were removed from the dogs and identified. Blood samples were taken and tested for protozoa (*Babesia* spp. and *Hepatozoon* spp.) and bacteria (*Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp.) by conventional PCR. All of the ticks collected from 34 (34.3%; 95% CI: 26–44) dogs belong to the species *Rhipicephalus sanguineus* sensu lato. Tick infestation on the farm dogs was significantly higher than that of dogs kept outdoors as pets (OR: 15.19, 95% CI: 2.72–118.92, $p < 0.001$) or in a sanctuary (OR: 35.11, 95% CI: 3.20–1986.67, $p < 0.001$). Altogether 22 animals were infected with one or two TBPs, most of them with *Hepatozoon canis* (16/22; 72.7%). *Anaplasma platys* and *Babesia vogeli* were detected in 5 and 4 dogs, respectively. Three dogs had co-infections caused by *H. canis* and *A. platys*. To the best of our knowledge, this is the first report on tick infestation and TBPs of dogs in Malta.

1. Introduction

Dogs and wild canids can be infected with one or more tick-borne pathogens such as flavivirus, *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp., *Hepatozoon* spp. and *Cercopithifilaria* spp. (Otranto and Wall, 2008; Otranto et al., 2009; Dantas-Torres et al., 2012). Nowadays many factors including changes in climate and vegetation, increased number of wildlife hosts and pet travel contribute to the increasing incidence of canine tick-borne diseases (canine TBDs) (Beugnet and Marie, 2009; Gray et al., 2009; Irwin, 2014). Proximity of dogs to man can be regarded as risk factor for tick-borne pathogens (TBPs) affecting human health also (Otranto et al., 2009; Dantas-Torres et al., 2012; Baneth, 2014).

The dog population in Malta has increased in recent years. There are 46,700 registered dogs of which 1640 are shelter dogs (source: Veterinary and Animal Welfare Department, Ministry for Sustainable Development, the Environment and Climate Change). Dogs in Malta are mainly found to be living on farms, within homes or shelters, and rarely as strays. The European Food Safety Authority EFSA Journal (2007) reported that there was no information available on hard tick infestations and TBPs in pet animals living in Malta. Therefore the aim of this study was to get preliminary information about tick infestation and TBPs in dogs living on the Maltese islands.

2. Materials and methods

2.1. Animals and sample collection

Ninty-nine dogs were selected for sampling (57 males and 42 females). Dogs living in different habitats were inspected between March and July 2013 in 18 urban and 17 rural areas on the islands of Malta and Gozo (Fig. 1).

The age of the animals was between 1.5 months and 14 years (average of 7.7 years), and there were more purebred (60/99; 60.6%) than mixed-breed (39/99; 39.4%) dogs. The purebred dogs belonged to 16 different breeds, of which the German Pointer (n = 11), the Fox Terrier (n = 10), the German Shepherd (n = 8) and the Springer Spaniel (n = 7) were the most prevalent. Based on the owners' declaration, the dogs had been born in Malta and they had never been taken abroad. The anti-tick treatments used on these animals were also recorded.

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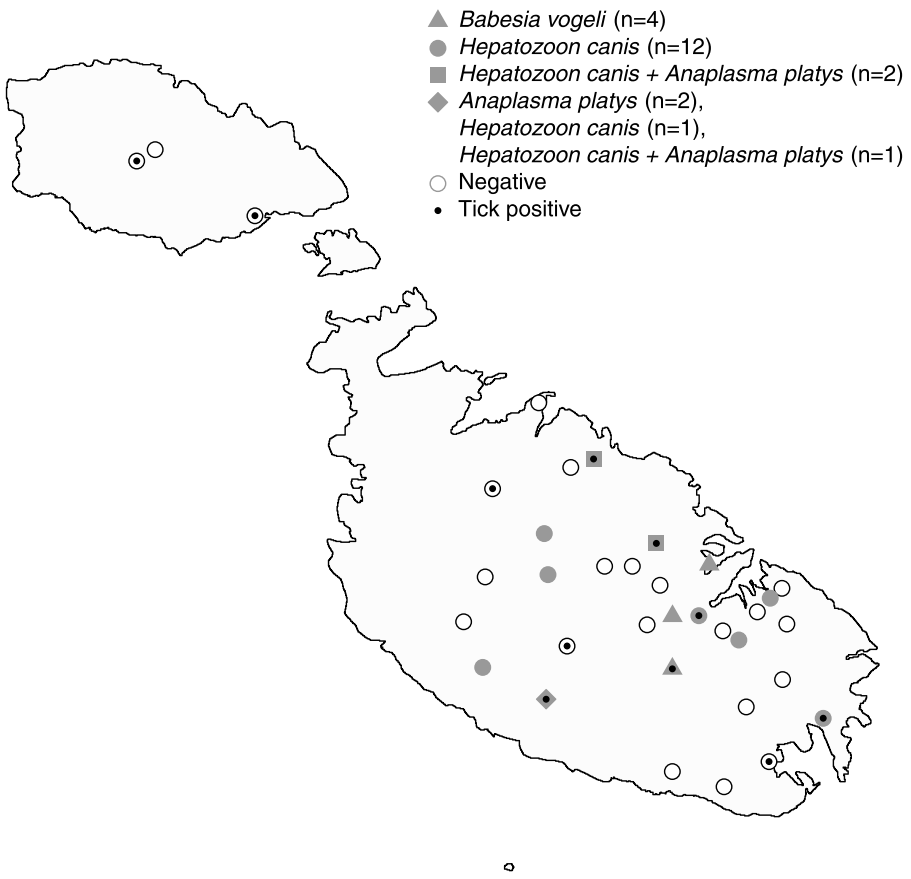

Fig. 1. Location of dogs infected with tick-borne pathogens on the islands of Malta (bigger) and Gozo.

All ticks removed from dogs were stored in tubes containing 70% ethanol and later identified on the basis of morphological features with a stereo microscope (SMZ-2T, Nikon Instruments, Japan) using standard morphological keys (Walker et al., 2000; Estrada-Pena et al., 2004; Dantas-Torres et al., 2013) and with molecular taxonomy analysis. A blood sample was collected in a tube containing EDTA from the cephalic vein from each dog and stored at −20 °C until further studies.

2.2. DNA isolation, amplification and sequencing

The single or pooled (2–5) adult and nymph ticks per dog were washed three times in detergent, tap and distilled water. They were sliced into small pieces in a 1.5 ml microcentrifuge tube containing 150 μl PBS using scissors and forceps sterilized with flame after each sample. Following an overnight incubation at 56 °C in tissue lysis buffer and Proteinase K, the DNA was extracted using the Tissue Protocol of the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). The same kit was used for extracting the DNA from the blood samples according to Blood and body fluid protocol.

A conventional PCR reaction was used with the primer pairs LCO1490: 5′- GGT CAA CAA ATC ATA AAG ATA TTG G –3′ and HCO and HCO2198: 5′ – TAA ACT TCA GGG TGA CCA AAA AAT CA- 3′ to amplify a ~710 bp long fragment from the cytochrome *c* oxidase subunit I (cox1) gene (Folmer et al., 1994.) The PCR was modified with the following conditions, 5 μl of extracted DNA were added to 20 μl of reaction mixture containing 1 U HotStar Taq Plus DNA Polymerase (QIAgen GmbH, Hilden, Germany) (5 U/μl), 0.5 μl dNTP Mix (10 mM), 0.5 μl of each primer (50 μM), 2.5 μl of 10x Coral Load PCR buffer (15 mM $MgCl_2$ included), and 15.8 μl DW. An initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min.

Conventional single-step PCR was used. The primer sets and the description of the protocols were published earlier (Farkas et al., 2014, 2015).

A conventional PCR was used with the primers EHR-16sD 5′-GGT ACC YAC AGA AGA AGT CC-3′ and EHR-16sR 5′-TAG CAC TCA TCG TTT ACA GC-3′ to amplify a 380 bp long fragment from the 5′ region of the 16S rRNA gene from various members of the family Anaplasmataceae and closely related rickettsial agents (Brown et al., 2001).

PCR products were electrophoresed in a 1.5% agarose gel (100 V, 30 min) stained with ethidium bromide and visualized under ultraviolet light. Nine PCR positive samples were purified and sequenced at Macrogen Inc. (Seoul, South Korea). The other samples were purified with GenElute™ PCR Clean-up Kit (Sigma-Aldrich, ST. Louis, USA) and sequenced by Biomi Inc. (Gödöllő, Hungary). The clean sequences were compared for similarity to sequences deposited in GenBank hosted by the National Center for Biotechnology Information (NCBI), National Institutes of Health, Bethesda, MD (www.ncbi.nlm.nih.gov/BLAST), using the BLAST program.

2.3. Statistical analysis

The association between the tick-borne infections of dogs, their gender, breed and their tick infestation was analysed using Fisher's exact test (Agresti, 2002). Logistic regression (Gelman and Hill, 2007) was used to analyze the age dependence of tick-borne infections.