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ORIGINAL PAPER

Molecular detection of *Anaplasma platys*, *Anaplasma phagocytophilum and Wolbachia sp.* but not *Ehrlichia canis* in Croatian dogs

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Abstract The bacteria *Anaplasma platys*, *Anaplasma phagocytophilum* and *Ehrlichia canis* are tick-borne agents that cause canine vector-borne disease. The prevalence of these pathogens in South Eastern Europe is unknown with the exception of an isolated case of *A. platys* detected in a dog imported into Germany from Croatia. To gain a better insight into their presence and prevalence, PCR-based screening for these bacterial pathogens was performed on domesticated dogs from different regions of Croatia. Blood samples from 1080 apparently healthy dogs from coastal and continental parts of Croatia as well as tissue samples collected from 63 deceased dogs with a history of anaemia and thrombocytopenia were collected for molecular screening by an *Anaplasmataceae*-specific 16S rRNA conventional PCR. Positive samples were confirmed using a second *Anaplasmataceae*-specific PCR assay with the PCR product sequenced for the purpose of bacterial species identification. All sequenced isolates were georeferenced and a kernel intensity estimator was used to identify clusters of greater case intensity. 42/1080 (3.8%; CI 2.7–5.0) of the healthy dogs were PCR positive for bacteria in the *Anaplasmataceae.* Sequencing of the 16S rRNA gene amplified from these positive samples revealed the presence of *A. platys* in 2.5% (CI 1.6–3.4%, 27 dogs), *A. phagocytophilum* in 0.3% (CI 0–0.6%, 3 dogs) and a *Wolbachia* endosymbiont in 1.1% (CI 0.4–1.6%, 12 dogs) of dogs screened in this study. Necropsied dogs were free from infection. Notably, no evidence of *E. canis* infection was found in any animal. This survey represents a rare molecular study of *Anaplasmataceae* in dogs in South Eastern Europe, confirming the presence of *A. platys* and *A. phagocytophilum* but not *E. canis*. The absence of *E. canis* was surprising given it has been described in all other Mediterranean countries surveyed and raises questions over the regional vector capacity of the *Rhipicephalus sanguineus* tick.

Keywords *Anaplasma platys* · *Anaplasma phagocytophilum* · *Ehrlichia canis* · Molecular survey · Dogs · Anaemia and thrombocytopenic distribution

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Background

Tick-borne diseases represent a subgroup of vector-borne diseases with high impact on animal health caused by parasites, bacteria and viruses (Otranto et al. 2009). The global geographic distribution of these pathogens is restricted by the presence of their known or proposed tick vector species. Three tick species, *Ixodes ricinus*, *Dermacentor reticulatus* and *Rhipicephalus sanguineus*, are responsible for

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transmission of the most important protozoan and bacterial species in Europe to humans and domesticated animals such as dogs (Beugnet and Marie 2009).

Among bacterial members of the *Anaplasmataceae* family, *Ehrlichia canis*, *Anaplasma platys* and *Anaplasma phagocytophilum* are three species known to infect dogs in Europe (Chomel 2011). Each of these pathogens causes a different disease in dogs, named after the mammalian cells they infect: (i) *A. phagocytophilum* causes canine granulocytic anaplasmosis, (ii) *A. platys* canine cyclic thrombocytopenia and (iii) *E. canis* is the causative agent of canine monocytic ehrlichiosis. Non-specific clinical signs such as lethargy, inappetence/anorexia, splenomegaly and fever are the most common clinical signs reported in *A. phagocytophilum* infection (Kohn et al. 2008; Eberts et al. 2011, Sainz et al. 2015). Despite descriptions which include fever, lethargy, anorexia, pale mucous membranes, petechiae and lymphadenomegaly, it is still controversial whether *A. platys* infection is responsible for these clinical signs (de Caprariis et al. 2011, Sainz et al. 1999; Dyachenko et al. 2012). For *E. canis* on the other hand, the clinical consequences of infection are better established and may include a range of clinical signs including fever, weakness, lethargy and hepato-splenomegaly with lymphadonmegaly. Pale mucous membranes, petechiae, ecchymoses, apistaxis associated with thrombocytopenia or vasculitis are also commonly present (Harrus et al. 1997; Little 2010; Sainz et al. 2015). The most common laboratory finding in ehrlichiosis and anaplasmoses is thrombocytopenia although infections often persist for long periods of time without visible clinical manifestations (Kohn et al. 2008; Santos et al. 2009; Sainz et al. 2015).

A. phagocytophilum is the most widespread pathogen within the *Anaplasmataceae* family in Europe, having been detected in ticks and mammals from nearly all European countries, the latter primarily by serology (Sainz et al. 2015; Bown et al. 2008; Nijhof et al. 2007; Strle 2004; Stuen 2007; Stuen et al. 2013). Molecular studies have also been performed in dogs in Portugal (Santos et al. 2009), Central Italy (Otranto and Dantas-Torres 2010) and Germany (Jensen et al. 2007) revealing prevalence rates between 0 and 6.3%. In Europe, *A. phagocytophilum* is transmitted by *Ixodes ricinus* ticks, although the other *Ixodes* spp. ticks have also been implicated in transmission, including *Ixodes hexagonus*, *Ixodes trianguliceps* and *Ixodes ventalloi* (Bown et al. 2008; Nijhof et al. 2007; Santos et al. 2009).

In terms of *A. platys*, the tick species, *R. sanguineus*, is suspected to be its major vector (Inokuma et al. 2000; Kontos et al. 1991; Woody and Hoskins 1991), although experimental studies failed to confirm *R. sanguineus* as a competent vector (Simpson et al. 1991). *A. platys* is thought to be endemic in southern European countries with molecular studies revealing the presence of this pathogen in Greece (Kontos et al. 1991), Albania (Hamel et al. 2016), Portugal (Cardoso et al. 2010; Maia et al. 2015; Santos et al. 2009), Spain (René-Martellet et al. 2015), France (Beaufils et al. 2002) Romania (Andersson et al. 2013) and Italy (de la Fuente et al. 2006). In contrast to *A. platys*, it is well recognised that *E. canis* can be transmitted by *R. sanguineus*. Otherwise, this pathogen has been shown to have a similar distribution as *A. platys*, having been detected in dogs in Greece (Siarkou et al. 2007), Italy (Maia et al. 2015; Solano-Gallego et al. 2006; Trotta et al. 2009), Spain (Aguirre et al. 2006; René-Martellet et al. 2015; Tabar et al. 2009), Portugal (Alexandre et al. 2009; Cardoso et al. 2010; Maia et al. 2015) and Albania (Hamel et al. 2009, 2016).

The presence and distribution of canine vector-borne pathogens for the genera *Babesia* and *Hepatozoon* have been investigated in Croatia (Beck et al. 2009; Vojta et al. 2009) as well as the prevalence of apparently healthy *Leishmania infantum*-infected dogs from coastal regions of this country (Živičnjak et al. 2005). With the exception of Albanian studies (Hamel et al. 2009, 2016), to our knowledge, the prevalence of *Anaplasmataceae* infections in dogs has not yet been systematically investigated in Croatia and South Eastern Europe, beyond a single case of co-infection of *A. platys* and *Babesia vogeli* discovered in a dog in Germany, imported from Croatia (Dyachenko et al. 2012). The aim of the current study was to investigate the prevalence and identity of *Anaplasmataceae* species infecting cohorts of healthy dogs and those with confirmed anaemia and thrombocytopenia in Croatia.

Materials and methods

Blood samples

A total of 1080 blood samples from apparently healthy dogs were collected from coastal and continental Croatian regions during the period 2007–2014. The studied population of apparently healthy dogs was chosen to provide a better insight into the species diversity infecting dogs since analysing only sick dogs could bias the survey towards pathogenic species (Beck et al. 2009). Prior to venipuncture, all the dogs included in the study had been examined by a veterinary clinician, according to a standard scheme for general clinical examination. Sampled animals showed no clinical signs of the disease. Blood samples were collected in tubes coated with EDTA, cooled and stored at 4 °C during transportation to the laboratory where they were frozen at − 20 °C until DNA extraction.

Tissue samples

A second group of samples originated from 63 deceased dogs found with gross findings consistent with anaemia (35%), or having haematological history with thrombocytopenia (21%) or anaemia with concurrent thrombocytopenia (44%). All

animals were delivered for necropsy upon the owners' request. Most dogs originated from the Croatian continental region of Zagreb County (97%) with one dog previously imported from Turkey and another single dog from southern Dalmatia. According to the available clinical history for these animals, none of the necropsied dogs were treated with doxycycline prior to death. From each carcass, tissue samples from the bone marrow, spleen, myocardium, lungs, kidney and liver were collected into a DNA/RNAase-free 2-ml tube (Deltalab, Spain) and stored at − 20 °C sampled. In addition, 10 mL of the blood from the right ventricle of the heart was sampled into an EDTA-containing tube.

DNA isolation, PCR screening and sequence analysis

The isolation of DNA from 200 μl EDTA-blood samples was primarily performed using the QIAcube automated DNA isolation system (Qiagen, Hilden, Germany) and QiaAmp DNA mini QIAcube Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). Distilled water was used as a negative control for the DNA purification and was included as a control with every 11 samples extracted. Isolated DNA samples were stored at − 20 °C. From each organ, 20 mg of tissue was used for DNA extraction with the same kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany).

All samples were screened using DNA amplification of a conserved fragment of the 16S rRNA (345 bp) gene of bacteria within the family *Anaplasmataceae* (*Ehrlichia, Anaplasma, Wolbachia* and *Neorickettsia*) by conventional PCR with specific primers EHR16SD (5 -GGT ACC YAC AGA AGA AGT CC-3) and EHR16SR (5 -TAG CAC TCA TCG TTT ACA GC-3′) for screening of all samples, as previously described (Parola et al. 2000). For the purpose of identifying presumptive *Anaplasmataceae*-positive samples, samples that were PCR positive by this initial screening were re-amplified using a second set of *Anaplasmataceae*-specific primers, EC9 5′-TACCTTGTTACGACTT-3, and reverse primer EC12 5′-TGATCCTGGCTCAGAACGAACG-3′, that amplifies a near-full length 1400-bp fragment of the 16S rRNA gene, as previously described (Kawahara et al. 2006). In samples that were found to be PCR positive for *Wolbachia* spp., molecular screening of dog filarids was conducted using primers, DIDR-F1 (5′-AGT GCG AAT TGC AGA CGC ATT GAG-3′) and DIDR-R1 (5′-AGC GGG TAA TCA CGA CTG AGT TGA-3′). Amplification was carried out according to the method developed by Rishniw et al. (2006) to amplify the internal transcribed spacer region 2 (ITS2) for *Dirofilaria immitis* (542 bp), *Dirofilaria reconditum* (578 bp) and *Dirofilaria repens* (484 bp), with the species determined by the estimation of sizes of the amplified PCR fragment following capillary electrophoresis.

DNA extracted from a blood sample of a dog confirmed to be positive for *A. phagocytophilum* by PCR, collected during routine diagnostics, was used as a positive control. RNase-Free Water (Takara Clontech) was used as a negative control. The amplification products were analysed by capillary electrophoresis on the QIAxcel System (QIAGEN, Hilden, Germany) with a 100–3000-bp size marker (QIAGEN, Hilden, Germany). For the purpose of DNA sequencing, amplified PCR products were purified using the ExoSAP-IT-PCR Clean-Up Reagent, according to the manufacturer's instructions (USB Corporation, Cleveland, USA). Sequencing in both directions was performed by Macrogen Europe with the same primers used for PCRs. Sequences were assembled using the SeqMan Pro software edited with EditSeq of the Lasergene software (DNASTAR, Madison WI, USA) and compared with available sequences using BLAST.

Mapping

Positive *Anaplasmataceae* cases were georeferenced in point form by using a household address and analysed by *ArcMap* 10.2 Spatial Analyst. A kernel intensity estimator was used to identify geographic case clusters of greatest intensity based on positive and negative samples. This latter tool is an exploratory analysis method for spatial data that makes it possible to view exposed localities and identify different degrees of intensity within a given area. For visual presentation of infection density, higher densities are shown in darker tones and lower in lighter tones.

Results

Molecular screening for the presence of *Anaplasmataceae* in dogs

Apparently, healthy dogs from Croatia were tested for the presence of *Anaplasmataceae* DNA in blood samples. Out of 1080 tested samples, 42 dogs appeared to be positive for the presence of *Anaplasmataceae* (overall prevalence 3.8%; CI 2.7–5.0%) by *Anaplasmataceae*-specific 16S rRNA screening and following visualisation of PCR products by capillary electrophoresis.

To determine the identity of these 42 *Anaplasmataceae*-positive canine blood samples, an additional round of 16S rRNA PCR amplification was performed on a larger 16S rRNA *Anaplasmataceae* gene-specific fragment. Sequencing of the resulting PCR products revealed that 27/42 of these positive samples (62.5%; CI 49.8–78.8%) or 2.5% of the animals screened in this study (CI 1.6–3.4%) were positive for *A. platys*, with the amplified 16S rRNA gene sequences identical to that of an *A. platys* sequence from a Croatian dog tested in Germany (accession no. JQ396431). Twelve of the

amplified sequences (28.5%, CI 14.9–42.2%) or 1.1% of the original dogs screened (CI 0.4–1.6%) were found to be identical to a partial 16S rRNA gene sequence from a *Wolbachia* endosymbiont of *Dirofilaria repens* (accession no. AJ276500). The three remaining amplified sequences (3/42; 7.1%, CI 0.0–14.9%) or 0.3% of the dogs screened in this study (CI 0.0–0.6%) were confirmed to be from *A. phagocytophilum*, based on 100% sequence identity to several *A. phagocytophilum* sequences present in GenBank (e.g., CP006618). Sequences obtained in this work were deposited in GenBank under the accession numbers KY114935, KY114936 and KY114937. Based on the detection of 16S rRNA gene sequences that were identical to a partial 16S rRNA gene sequence previously described from a *Wolbachia* endosymbiont of *D. repens*, we also investigated the presence of *D. repens* in these samples using a ITS2 region PCR. This PCR screening, and subsequent identification based on the discrimination of PCR products following capillary electrophoresis, revealed that 10 out of 12 (83.0%) of the *Wolbachia* PCR-positive dog blood samples were also positive for *D. repens,* while two samples remained negative.

Anaplasmataceae-specific PCR failed to detect any positivity in the tissues or post-mortem collected heart ventricular blood samples from the 63 necropsied dogs in this study (data not shown).

Geographic distribution of *Anaplasmataceae*-positive dogs in Croatia

To visualise the distribution of these positive cases throughout Croatia, samples were divided into three groups based on geographical origin: the Continental region, North Adriatic region and Dalmatia (Table 1). The highest prevalence of *Anaplasmataceae*-positive dogs were identified in the North Adriatic region (10/126; 7.9%) followed by the Continental region (11/242; 4.5%) and Dalmatia (21/712; 2.9%). Kernel density estimation revealed geographic variability of infections as visible in Fig. 1. The most prevalent *Anaplasmataceae* detected, *A. platys*, was similarly distributed in the Continental region (14/712; 2.0%) and Dalmatia (4/242; 1.7%) while the highest prevalence was recorded in the North Adriatic region (9/126; 7.1%). Kernel density calculated from this prevalence indicate that *A. platys* infection were generally distributed along coastal parts of Croatia and centred around bigger cities in Dalmatia (Split) and North Adriatic (Pula). Two more clusters of cases with lower density were present in the Eastern and Western Continental region. The *Wolbachia* endosymbiont detected in this study showed an equal prevalence of 0.8% in coastal Croatia (North Adriatic and Dalmatia) and the highest in the Continental region (5/242; 2.1%). *A. phagocytophilum* was the rarest species detected in this study and only found in the Continental region (2/242; 0.8%) and Dalmatia (1/712; 0.1%).As shown in Fig. 1, the highest density of *A. phagocytophilum* was observed in central parts of the Continental region.

Discussion

In an effort to expand our knowledge on the prevalence of different pathogens within the family *Anaplasmataceae* in Croatia, we analysed blood samples from 1080 apparently healthy dogs and 63 dead dogs by an *Anaplasmataceae*-specific PCR. Three different bacteria were detected from the blood of apparently healthy dogs including *A. platys*, *A. phagocytophilum* and a *Wolbachia* endosymbiont of *D. repens*, with an overall prevalence of 3.8% in the dogs screened.

A. platys was the most prevalent species (2.5%, CI 1.6–3.4%) in dogs from all studied regions (Table 1). This study adds to the small but growing surveillance data that suggests that *A. platys* is endemic to countries bordering the Mediterranean. In the few European studies previously performed, *A. platys* prevalence varied from 4% in randomly selected dogs from Sicily (de la Fuente et al. 2006) and up to 70.5% in young dogs from a private animal shelter from the Apulia region in Italy (de Caprariis et al. 2011). In dogs with suspected tick-borne diseases from Portugal, *A. platys* was detected in 9.3% (5/54) (Maia et al. 2015) and 75.0% (3/4) (Hamel et al. 2009) while the prevalence in dogs from veterinary medical centres and animal shelters showed a much low prevalence of 0.5% (Beaufils et al. 2002). Of the larger studies previously performed, the 2.4% prevalence of *A. platys* in random healthy dogs detected in our large study is more consistent with results from studies from Sicily (de la Fuente et al. 2006) and Portugal (Maia et al. 2015). In saying that, the highest prevalence observed in North Adriatic region (7.2%; Table 1) represents the highest prevalence in asymptomatic dogs from southern Europe. This high prevalence may be associated with the previously reported high geographic distribution of *R. sanguineus* ticks in this region (Krčmar 2012). Interestingly, *A. platys* was detected in the continental region of Croatia (1.6%) where *R. sanguineus* s.l. is not the dominant tick species but is present (Krčmar 2012) and less abundant than in Dalmatia (2.0%). The *A. platys* distribution is consistent to that described for *H. canis* in a study of Croatian dogs (Vojta et al. 2009), where by the latter parasite was also found in dogs from all examined regions including continental Croatia.

R. sanguineus is also the proven vector of *E. canis*, the causative agent of canine monocytic ehrlichiosis (CME). One of the main objectives of the current study was to confirm the presence of *E. canis* in Croatia, especially in the coastal regions where *R. sanguineus* is the dominant tick species infesting dogs (Krčmar 2012) with antibodies reported in the sera from two dogs (0.46%) (Mrljak et al. 2017). In that

Table 1 Prevalence of pathogens in examined regions

Region	No. of animals	*A. platys*	*A. phagocytophilum*	*Wolbachia sp.*	Total
Continental	242	4 (1.6%, CI 0.1–3.3%)	2 (0.8%, CI 0–2.0%)	5 (2.1%, CI 0.3–3.9%)	11 (4.5% CI 1.9–7.2%)
North Adriatic	126	9 (7.1%, CI 0.05–3.3%)	0	1 (0.8%, CI 0–2.2%)	10 (7.9%, CI 3.8–12.1%)
Dalmatia	712	14 (2.0%, CI 1.0–3.0%)	1 (0.14%, CI 0–0.42%)	6 (0.8%, CI 0.2–1.5%)	21 (3.0%, CI 1.7–4.2%)
Total	1080	27 (2.5%, CI 1.6–3.4%)	3(0.3%, CI 0–0.6%)	12 (1.1%, CI 0.5–1.7%)	42 (3.9%, CI 2.7–5.0%)

respect, the failure to detect *E. canis* infection from 848 dogs in the coastal regions surveyed in this study was surprising since all European countries bordering the Mediterranean Sea and Portugal have been considered as endemic (Sainz et al. 2015). Furthermore, the distribution of *E. canis* and *A. platys* infections tend to overlap including in countries such as Italy, Portugal and Spain where a prevalence of 11% and 8% have been described previously (René-Martellet et al. 2015), and a near-identical prevalence of 0.4% and 0.5% were found in asymptomatic dogs, respectively (Maia et al. 2015). Nevertheless, based on our current analysis, it would appear that the population of apparently healthy Croatian domestic dogs or dogs that died with anaemia and thrombocytopenia appear to be free from *E. canis* infection. One interesting possible explanation for the absence of *E. canis* infections may lie in vector competence, with studies in South America revealing that temperate lineages of *R. sanguineus* s.l. were free from *E. canis* infection while ticks from tropical lineages were infected (Cicuttin et al. 2016; Moraes-Filho et al. 2015). Indeed, the absence of *E. canis* may be explained by the seeming absence of *R. sanguineus* sensu stricto from different parts of Croatia (Hornok et al. 2017). Future surveys should focus on ticks themselves to confirm the absence of *E. canis* from the Adriatic coast of Croatia.

The detection of *A. phagocytophilum* in Croatian dogs was not surprising due to the fact that it is the most widespread

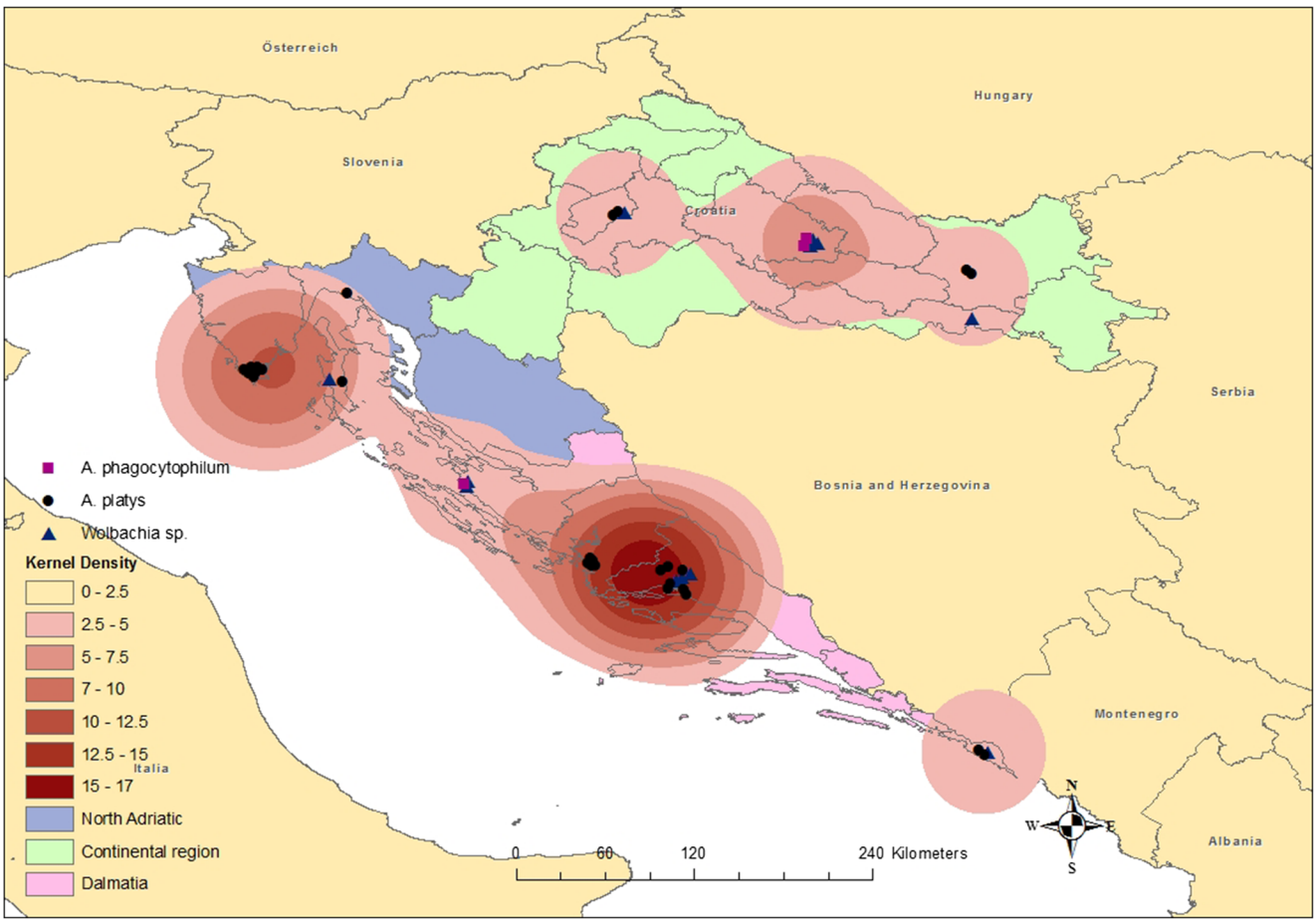

Fig. 1 Geographical distribution of all georeferenced positive dogs. Higher densities of infection are shown in darker tones and lower in lighter tones

species of the family *Anaplasmataceae* in Europe (Stuen 2007), and is transmitted by *I. ricinus* ticks which can be found in Croatia (Krčmar 2012). Nevertheless, the overall prevalence of this pathogen in the Croatian dog population was surprisingly low (0.3%). A single case in Dalmatia (0.1%) and a lack of infected dogs from the North Adriatic region corresponds with similar results from other countries with a Mediterranean climate including Southern Europe (René-Martellet et al. 2015), Portugal (Maia et al. 2015) and Italy (de la Fuente et al. 2006; Solano-Gallego et al. 2006) but, at same time, indicates the presence of *I. ricinus* in southern regions of Croatia. A higher prevalence of *A. platys* and a lower of *A. phagocytophilum* also likely indicates that antibodies detected in 6.2% (95% CI 4.1–8.9) dogs (Mrljak et al. 2017) are not all atributed to *A. phagocytophilum* as stated, since the SNAP 4Dx test is not able to discriminate between species.

The DNA of *Wolbachia* spp. endosymbionts of the nematode, *D. repens*, were detected in 12 samples (1.1%) from all studied regions which is higher than that described in a previous study that detected these bacteria in Portugal (Maia et al. 2015), although these were assigned as endosymbionts of *D. repens*. The detected *Wolbachia* sequences were identical to a single 16S rDNA sequence deposited in GenBank from Italy. As an interesting additional experiment, PCR screening of the same samples for the presence of filarid worms revealed that 83% of them were positive for *D. repens*. *D. repens* was previously shown to be the most prevalent filarid species in apparently healthy dogs in Croatia (15.5%) while *D. immitis* was detected in less than 1% of dogs from the Istrian peninsula and the southernmost coastal region (Živičnjak et al. 2007; Mrljak et al. 2017). Compared to neighbouring countries, the lack of *D. immitis* detections in *Wolbachia*-infected dogs in our study was unexpected despite the previous low prevalence reported in Croatia. Parasitological and serological surveys in dogs from Bosnia and Herzegovina revealed an overall prevalence of 5% for filarid worms with the highest prevalence in West Herzegovina (13%). Most of infections were attributed to *D. immitis* (Zahirović 2010). In Serbia, between 17 and 49% dogs were infected with *D. repens* while the prevalence of *D. immitis* microfilaremic dogs was lower (1.6–7.0%) and dependent on the area surveyed (Tasić et al. 2008, 2012). A low prevalence of *D. immitis* was also recorded in Hungary from dogs in a retrospective study with 27 out of 2622 (1%) necropsied dogs found to be infected during a sampling period from 2001 to 2015 (Bacsadi et al. 2016).

The importation and movement of domestic dogs may represent an important vehicle for pathogen and/or vector transmission from southern Europe to continental parts of Europe (Hamel et al. 2013; Dyachenko et al. 2012). As such, the current findings of *A. platys* and *A. phagocythophilum* in apparently healthy dogs support a potential need for molecular screening of dogs prior to relocation from Southern Europe. Tick infestation should also be confirmed prior to animal movement. As an aside, the detection of *A. platys* and *A. phagocytophilum* exclusively in healthy dogs and the failure to detect them in the organs and blood of dogs with evident haemolytic disease raises further questions over their pathogenic potential in dogs. On the basis of these results, we believe that, despite a previous description of anaemia and thrombocytopenia in *A. platys* and *A. phagocytophilum* infection (Sainz et al. 2015), it is unlikely that infection by these *Anaplasmataceae* species will lead to a lethal outcome.

Conclusion

Besides Albanian studies (Hamel et al. 2009, 2016), this survey represents a rare large-scale molecular study of bacteria within the *Anaplasmataceae* in South Eastern Europe. In doing so, we confirmed the presence of *A. platys* and *A. phagocytophilum* in apparently healthy dogs, but not *E. canis*. In addition to the coastal region, *A. platys* was the dominant species in the continental part of Croatia suggesting a spread of this bacteria north and away from the Mediterranean coastal regions. The failure to detect *E. canis* represents a somewhat surprising finding since it is prevalent in almost all Mediterranean countries and raises potential questions over the regional vector capacity of *R. sanguineus* s.l. ticks, a suggestion that requires further investigation. Future surveys of *E. canis* in ticks and symptomatic dogs will be required to confirm its absence from same region.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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