ORIGINAL ARTICLE

Bartonella vinsonii subsp. *arupensis* infection in animals of veterinary importance, ticks and biopsy samples

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Abstract

Testing for vector-borne pathogens in livestock is largely reliant upon blood and tissue. The role of biopsy samples remains poorly explored for detecting tick-borne bacteria in animals.

In a 2-year survey, animals of veterinary importance from farms throughout the northern part of Greece were routinely checked for the presence of biopsy samples. Where detected, either a portion or a biopsy was collected together with whole blood samples and any ticks at the site of the biopsy sample. Molecular testing was carried out by real-time PCR targeting the internal transcribed spacer gene of *Bartonella* species. A total of 68 samples (28 blood samples, 28 biopsy samples and 12 ticks (nine *Rhipicephalus bursa* and three *Rhipicephalus turanicus*)) were collected from goats (64 samples) and cattle (four samples). Eight (11.8%) of the 68 samples were positive for *Bartonella* species. Of the biopsy and whole blood samples, four (14.3%) of each type were positive for *Bartonella* species. None of the ticks tested positive for *Bartonella* species. All pairs of positive biopsy samples/whole blood samples originated from the same animals. Positive samples were identified as *Bartonella vinsonii* subsp. *arupensis*. Although many more samples from a much wider spectrum of animal species is required before concluding upon the merit of biopsy samples in the study of tick-borne diseases, the significance of our finding warrants further study, both for clinical consequences in small ruminants and for those humans who are farming infected animals.

Keywords: Animals of veterinary importance, *Bartonella*, biopsy sample, tick

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Introduction

Bartonella are considered as emerging pathogens, being increasingly associated with a number of diseases both in humans (trench fever, Carrion's disease, bacillary angiomatosis, endocarditis, cat scratch disease and neuroretinitis) [1], as well as, in animals (including ruminants, cattle, cats, rodents, dogs and a wide range of wild animals) [2]. In vertebrates, *Bartonella* parasitize erythrocytes and endothelial cells [3], typically for protracted periods [4].

Established and proposed new members of *Bartonella* species have increased exponentially over recent years. Over 30 species have been recognized with some having global distribution and infecting a wide variety of vertebrates [5]. A wide variety of vectors are involved in the transmission of *Bartonella* species, including body lice, fleas, ticks, mites and sandflies [6]. Examples of bacteria of the genus of *Bartonella* associated with vector transmission are *Bartonella bacilliformis*, which is transmitted by sand flies, *Bartonella henselae* (transmitted by cat fleas) and *Bartonella quintana* (transmitted by the human body louse). The role of ticks in the ecology of *Bartonella* is hypothesized [7–9], despite their notable ability to serve as arthropod vectors/reservoirs of various agents of medical and veterinary health significance [10], and an upsurge in the incidence of tick-borne diseases in many regions of the world [11].

The association between *Bartonella* and their mammalian hosts is varied, with some strictly limited whereas others are less restricted [12]. Cats play the role of the main reservoir for *B. henselae* causing cat-scratch disease. Furthermore, several strains have been isolated from various rodent [13,14] and ruminant [15,16] species throughout the world. Ruminants can also become infected with *Bartonella schoenbuchensis*, *Bartonella*

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chomelii and *Bartonella bovis*, which have been isolated from blood in Europe, Africa and North America [15,17,18]. Among cattle, *B. bovis* has been implicated in causing bovine endocarditis [19], and *B. chomelii* has also been isolated from the same animal species [20], although no clinical consequence has been demonstrated for the latter species. Moreover, *Bartonella rochalimae* causes infection in domestic animals, wild carnivores and humans [21].

In cases where vertebrate hosts, vectors and wild animal species interact with each other, deciphering the transmission cycles of zoonotic agents seems quite challenging [22]. Proper sampling plays a crucial role in the accurate approach to the study of a zoonotic disease. Serological analysis has been used extensively, especially in epidemiological studies, but is limited in its ability to discriminate closely related pathogen genotypes. Moreover, detection of antibodies does not necessarily conclude bacteraemia or even infection of the host; whereas detection of the pathogen in the host's blood or from a direct sample (biopsy sample for example) would seem a more secure approach.

The purpose of the current study was to compare biopsy samples (removed scab) with whole blood or tick vectors for detection of tick-borne bacteria in livestock to assess the diagnostic merits of various sample types for the detection of *Bartonella* species.

Materials and methods

Sampling

In a 2-year survey carried out in the laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of Crete (Greece) in conjunction with the Veterinary department of the Aristotle University of Thessalonica (Greece), animals of veterinary importance (sheep, goats, cattle) from farms throughout the northern part of Greece were routinely checked for the presence of biopsy samples. Where detected, either a portion or a biopsy was collected together with whole blood samples and any ticks at the site of the biopsy sample. Data on animal species, farm location, time of collection, etc. were recorded.

Ticks removed from animals were placed in separate 1.5-mL tubes with 70% ethanol and were uniquely coded according to individual animal, livestock and region; then transported to the Aristotle University of Thessalonica where they were kept at −80°C before testing. Each tick was identified to species using existing taxonomic keys [23] at the laboratory of Clinical Bacteriology of the University of Crete, in Greece.

Blood samples and biopsy samples were similarly removed, transferred into individual 1.5-mL tubes, labelled and stored frozen until assessed.

Molecular analysis

DNA extraction from whole blood samples (QIAamp DNA blood mini kit; Qiagen, Hilden, Germany) or biopsy samples and ticks (QIAamp Tissue extraction kit; Qiagen) was undertaken according to the manufacturer's instructions at the laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of Crete. Each tick and biopsy sample was washed in 70% alcohol, rinsed in sterile water and dried on sterile filter paper. Consequently, samples were triturated individually into sterile tubes and a portion of them was used for further DNA extraction. Once extracted, DNA samples were kept at −20°C until further analysis.

Molecular testing was undertaken at the University of East London using an initial real-time PCR targeting the internal transcribed spacer (ITS) gene of *Bartonella* species to screen as previously described [24]. Master mix was prepared containing PCR buffer, dNTPs (0.2 mM each), $MgCl_2$ (5 mM), *Taq* DNA polymerase (0.06 mM; Invitrogen, Carlsbad, CA, USA), as well as primers (1 μM each) and probe (0.1 μM; (Sigma Genosys, St Louis, MO, USA) at a final volume of 25 μL. Agilent 96-well plates and cap strips were used. Nucleotide-free sterile H_2O was used as negative control. At least four randomly selected wells in each plate were used as negative controls. A single well was used as the positive control in each assay, the positive control being a verified positive *B. quintana* DNA isolated from human blood. The master mix preparation room, the DNA addition room and the amplification room were all separated from each other to avoid any chance of contamination. All positive and/or ambiguous samples were re-tested at least once in order to demonstrate reproducibility using conditions similar to those described above. Only samples producing cT values of <35 were considered to be positive. All amplifications were performed using an Agilent Aria Mx cycler.

Positive samples were further tested by conventional PCR (targeting ITS) to obtain amplicons that were further used for sequencing as previously described [25]. All primers and probes used both for real-time PCR and for the conventional PCR are summarized in Table 1. Amplicons were purified using the PCR product purification kit (QIAquick Qiagen) and sequenced in both directions by Sanger sequencing (Durham) using the same primers used for PCR. All sequences obtained were aligned using ClustalW. Sequences were compared for similarity with those at GenBank using the nucleotide BLAST program (National Centre

TABLE 1. Primers and probes used to target the internal transcribed spacer gene either by real-time PCR or by conventional PCR

	Sequence	Gene targeted
Real-time PCR		ITS
Primer forward	GGGGCCGTAGCTCAGCTG	
Primer reverse	TGAATATATCTTCTCTTCACAATTTC	
Probe	6-carboxyfluorescein-CGATCCCGTCCGGCTCCACCA-6-carboxytetramethylrhodamine	
PCR		ITS
Primer forward (438s)	GGTTTTCCGGTTTATCCCGGAGGGC	
Primer reverse (1100as)	GAACCGACGACCCCCTGCTTGCAAAGC	

for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST) the ClustalW online software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the MEGA v. X software.

Results

A total of 68 samples ($n = 28$ blood samples; $n = 28$ biopsy samples; and $n = 12$ ticks) were collected and tested for *Bartonella* species. Livestock included goats (12 ticks, 26 eschars, 26 blood samples) and bovine animals (two eschars and two blood samples).

Of the 12 ticks collected, nine were characterized as *Rhipicephalus bursa* and three as *Rhipicephalus turanicus*. Ticks were collected from goats only.

Eight (11.8%) of the 68 samples revealed the presence of *Bartonella* species with Ct values ranging from 29.07 to 34.44 (see Table 2). All positive samples were verified by a second amplification. Of the biopsy sample and whole blood samples, eight (four from each sample type; 14.3%) were positive for *Bartonella* species. All pairs of positive biopsy samples/whole blood samples originated from the same animals. All remaining samples were negative. Of the eight positive samples, we amplified and sequenced a 408-bp portion of ITS from six samples (sample numbers 11–16) that revealed identical sequence in both directions. All positive samples despite their origin were identified as *Bartonella vinsonii* subsp. *arupensis* showing 100% (408/408 bp) similarity to the already published sequence AF312504 and 99% (404/408) similarity to the already published sequence AF442952. To further explore the extent of the relatedness of our sequences with published ones, partial ITS sequences for another 32 *Bartonella* species were aligned to construct a phylogenetic tree (Fig. 1) in which the position of our sequences against the sequences of other *Bartonella* species was demonstrated.

All bovine samples and all ticks tested were negative for *Bartonella* species. The results are summarized in Table 2.

Discussion

Interest in zoonotic tick-borne diseases has increased in the last few decades, because these are considered as important zoonoses in Europe [26]; among them are *Bartonellaceae*.

Bartonella vinsonii was described as the Canadian vole agent back in 1946 [27], and almost four decades later (1982) Weiss and Dasch further characterized the agent and named it after *Rochalimaea vinsonii* [28]. Fifteen years later (1999), its first isolation was recorded, from a 62-year-old man with bacteraemia [29].

A number of genes are used as targets for the identification of *Bartonella* species, including the 16S rRNA and citrate synthase (*gltA*) [30], the 16S/23S rRNA intergenic spacer region (ITS) [31], which shows a high degree of interspecies variability among *Bartonella* species, the *ftsZ* [32] and the *GroEL* [33] genes. In our case, we did not have enough DNA to go through the amplification of further genes, nevertheless, the successful detection of *Bartonella* in four animals, in both biopsy and blood samples, demonstrates the robustness of our findings. Control samples were included in all assays and verified correct performance of the tests reported. Sanger sequencing revealed that in all cases we had detected *B. vinsonii* subsp. *arupensis*, close to *B. vinsonii* subsp. *vinsonii*, which is rodent-associated, and to *B. vinsonii* subsp. *berkhoffii*, which has been described in dogs.

Rodent infections caused by *Bartonella* tend to be asymptomatic; however, whether they could serve as a pathogen in other vertebrates is a cause for concern. As far as ruminants

TABLE 2. Sample types and origins tested for *Bartonella* species

Animals		Ticks		Biopsy samples		Blood samples		Blood sample and eschar (pairs)[a]		Blood sample, tick and eschar (triad)[b]	
Species	*n*	*n*	Pos. (%)	*n*	Pos. (%)	*n*	Pos. (%)	*n*	Pos. (%)	*n*	Pos. (%)
Bovine	2	0	0	2	0	2	0	2	0	0	0
Goat	26	12	0	26	4 (15.4)	26	4 (15.4)	26	4 (15.4)	12	0
Total	28	12	0	28	4 (14.3)	28	4 (14.3)	28	4 (14.3)	12	0

[a] Corresponds to cases where both eschar and whole blood samples were collected from the same animal.
[b] Corresponds to cases where biopsy sample, whole blood sample and a tick were collected from the same animal.

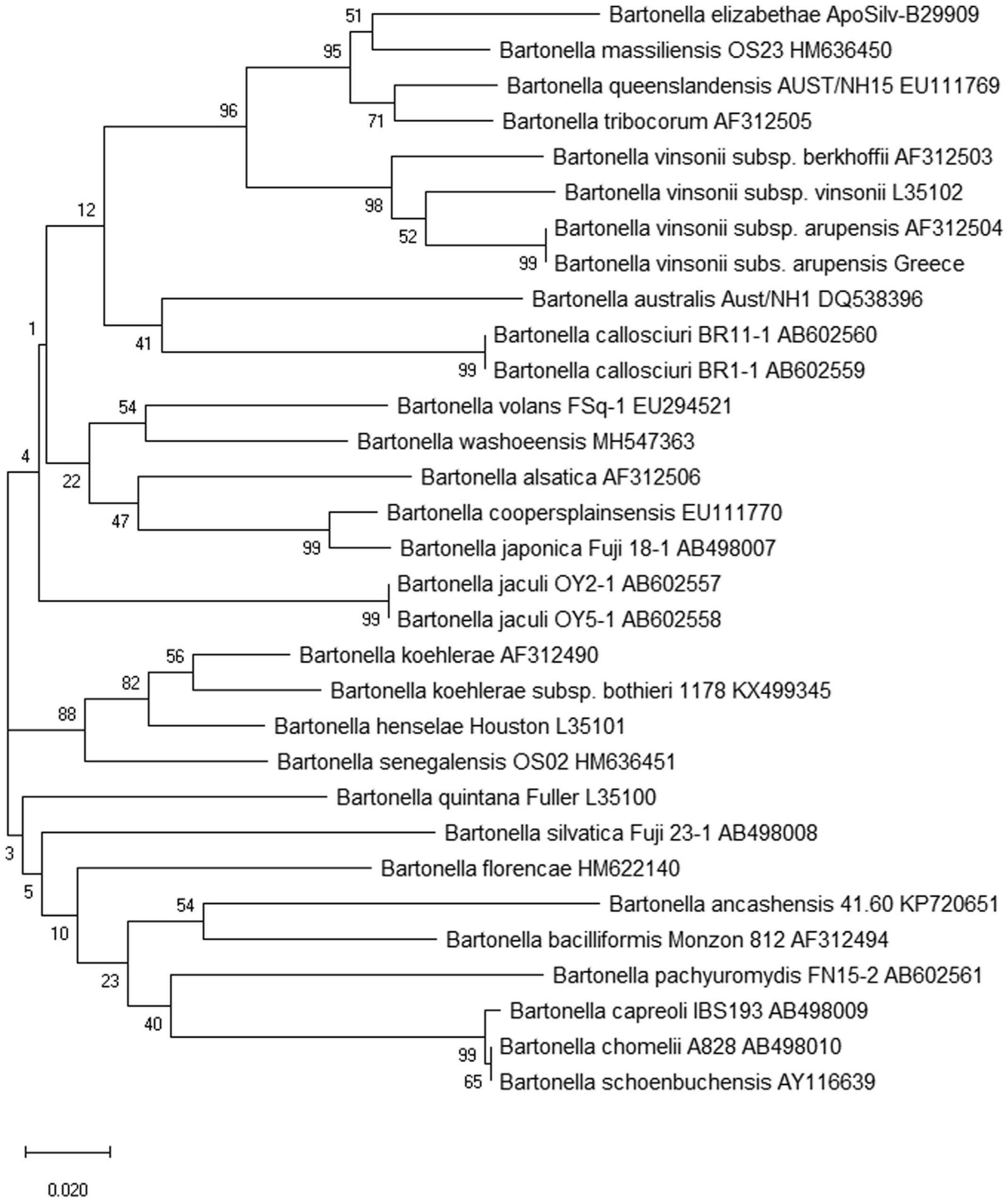

FIG. 1. Internal transcribed spacer (ITS) phylogeny for a 408-bp fragment of the 16S–23S intergenic linker region of 33 *Bartonella* species. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length 1.67495836 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option) [61].

(including water buffalo, several deer species, cattle, camels and moose) and animals of veterinary importance are concerned, a number of *Bartonella* species have been associated with these animal species, such as *B. bovis*, *B. capreoli*, *B. chomelii*, *B. dromedarii* and *B. schoenbuchensis* [15,16,34]. In contrast to the large ruminants above, the isolation of *Bartonella* species from small ruminants (including sheep and goats, which we studied herein) has been more puzzling. Indeed, several studies have failed to detect any *Bartonella* species from sheep or goats [35,36], whereas others have detected *B. melophagi* from domestic sheep samples [37] despite the great difficulties in the isolation of this group of bacteria.

The natural reservoirs of *B. vinsonii* subsp. *arupensis* are small rodents with mice believed to show persistent infection [34]. Further reports have detected this agent in deer mice in North America [38], in rodents in Mexico [39,40], in Brazil [2] and in the USA (California) [41]. Its zoonotic potential was revealed by its isolation from a human with endocarditis [42], in pre-enriched blood of four individuals in Thailand [43] and in a child where it caused hepatic granulomatous lesions [44]. *Bartonella vinsonii* subsp. *berkhoffii* is now established as a canine pathogen with the ability to cause endocarditis [45]. Interestingly, *B. vinsonii* subsp. *arupensis* has also been detected in the blood of stray dogs in Thailand [46]. The role of this organism as a pathogen in other vertebrate species remains to be clarified. Our detection of *B. vinsonii* subsp. *arupensis* in goats is intriguing. Whether it has pathogenic potential in this small ruminant is worthy of further exploration.

Importantly, this study reports the validity of biopsy samples for detection of *Bartonella* infection in livestock. Infection was confirmed by the demonstration of *Bartonella* in the blood of all biopsy-sample-positive animals. To the best of our knowledge, this is the first time that the presence of *Bartonella* DNA in veterinary biopsy samples has been recorded; on the other hand, simultaneous detection of the same *Bartonella* species in ruminants and in the vectors they carry (deer keds and cattle tail louse), has been described [37]. A biopsy sample or cutaneous necrosis is caused by vasculitis at the tick-bite site of inoculation, known as tache noire ('black spot') and usually it is pathognomonic for infection by *Rickettsia*. The presence of an eschar plays a significant role in both human clinical and laboratory diagnosis [47–50]. In contrast to humans, the role of biopsy samples in animals of veterinary importance has not been studied. Epidemiological surveys for tick-borne diseases infecting animals are generally restricted to use of serum and whole blood alone. The limitation presented with serum antibodies is that, if present, they might correspond to past infection; furthermore, only IgG antibodies can be used as a screening method. Furthermore, whole blood often fails to yield a positive PCR because bacteraemia is rare in this case in animals and is not always a feature of vector-borne pathogens.

It seems that ticks may have the potential to act as vectors of *Bartonella* species [51]. *Bartonella* has been detected in questing ticks (*Ixodes pacificus*, *Dermacentor* and *Rhipicephalus sanguineus*) in the USA [16], while other European studies (the Netherlands, France, Poland and Austria) have demonstrated the presence of *Bartonella* in *Ixodes ricinus* ticks obtained from vegetation, either by molecular means [52] or following isolation of the pathogen (*B. henselae* in *Ixodes ricinus*) [53].

Although *R. turanicus* is considered as a species frequently associated with sheep [54], it is *R. bursa* that is considered a major ectoparasite of sheep in the Mediterranean basin [54]. In our study, although we collected ticks belonging to both these species, we failed to detect any *Bartonella* DNA in any of them. Nevertheless, although the total number of ticks collected in the current survey was low (12 samples), our finding agrees with previous studies [54–59] that failed to detect pathogenic species in *R. turanicus*. In an earlier study carried out in Palestine, the DNA of *Bartonella* species was detected in *R. sanguineus* collected from dogs and from camels; however, all ticks collected from sheep or goats were negative [60]. A study of *R. bursa* ticks removed from goats reported limited detection of *Bartonella* species from Sardinia [54].

The limitations of our study are that our numbers and range of livestock and ticks tested was small. Furthermore, insufficient material was available to enable exhaustive molecular typing to confirm the identity of the *Bartonella vinsonii* subsp. *arupensis* present in small ruminants.

Conclusion

We report the presence of *B. vinsonii* subsp. *arupensis* species in goats from Greece, with four animals showing positive blood and biopsy samples. The significance of this finding warrants further study, both for clinical consequences in small ruminants and for those humans farming infected animals. Certainly, many more samples from a much wider spectrum of animal species are required before concluding upon the merit of biopsy samples in the study of tick-borne diseases; however, we provide valuable proof-of-concept data that should promote future research.

Conflicts of interest

None.

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