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Lyme disease and the detection of *Borrelia burgdorferi* genospecies in *Ixodes ricinus* ticks from central Italy

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Summary

The Province of Pesaro-Urbino, situated in the Marche Region of central Italy, can be considered to be an area at risk for Lyme disease because of its ecological features. Field data are not yet available although the disease is known to be present in neighbouring areas. During a field study lasting twelve months, ticks were collected from the vegetation, from wild cervids and also from humans who reported a tick bite at the local hospital. All ticks were identified and *Ixodes ricinus* specimens were tested using three different polymerase chain reaction tests for the detection of *Borrelia burgdorferi sensu lato* (*sl*). To identify the genospecies of *B. burgdorferi sl*, a fragment of the 5S-23S ribosomal rRNA intergenic spacer of the positive samples was amplified and then sequenced. Sequencing of the 5S-23S intergenic spacer led to the identification of two different genospecies, namely: *B. burgdorferi sensu stricto* and *B. lusitaniae*, both of which are involved in cases of human infection. Findings on the host-tick relationships and on the genospecies involved in the cycle of borreliosis confirm the suitable conditions for Lyme disease in the study area. The results concur with previous findings reported in the Mediterranean region.

Keywords

Borrelia burgdorferi, Genospecies, Italy, *Ixodes ricinus*, Lyme spirochetes, Polymerase chain reaction, Public health, Sequencing, Tick.

Introduction

Lyme disease is the most widespread tick-borne disease in Italy. The highest prevalence levels in ticks are recorded in north-eastern Italy where many reports of clinical symptoms in humans are also reported (10). The Province of Pesaro-Urbino, as well as the northern areas of central Italy, can be considered to be areas at risk for Lyme disease on account of the ecological features of these regions. Field data are not yet available for this area, despite the fact that the disease is known to be present in neighbouring areas. The principal vector in Europe is the tick *Ixodes ricinus* (Linnaeus, 1758), which is widespread throughout Italy and its presence is strictly associated with wild cervid populations and, in particular, roe deer (*Capreolus capreolus*, Linnaeus, 1758).

The highest densities of roe deer populations have been recorded in the western section (the highest area above sea level) of the Province where several cases of human tick bites are reported each year.

This field study was performed to assess the presence of the vector and the relationship between the vector of Lyme disease and its hosts, to establish the presence of the aetiological agent of Lyme disease, *Borrelia burgdorferi sensu lato* (*sl*), and to identify the genospecies present.

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Materials and methods

The study was conducted from September 2001 to October 2002. The western area of the Province (territory of 'Azienda Sanitaria Locale No. 2 – Urbino' now referred to as 'Zona Territoriale No. 2 Urbino, ASUR (*Azienda Sanitaria Unica Regionale*) Regione Marche') was selected for its ecological features (orography, high density of wild cervids, vegetation, presence of extensive wooded and grazing areas). During this period, ticks were collected by the employees of the public veterinary service of the 'Zona Territoriale N°2 Urbino, ASUR Regione Marche' from all wild cervids that were killed in car accidents. During the hunting season, ticks were also collected from hunted cervids by the hunters of the *Unione Regionale Cacciatori Appennino* (URCA: Regional association of Apennine hunters) in the Province of Pesaro-Urbino. In both cases, the place and date of collection were noted. From November 2001 to September 2002, ticks were also collected from people who consulted the local hospital after a tick bite; the date of removal of the tick and the supposed site of the tick bite were recorded. During this study, a retrospective analysis was also conducted at the emergency department of the local hospital to identify patients who had been admitted for a tick bite. This analysis included those patients who visited the hospital from 1 May 2001 to 29 April 2002. All samples collected from hosts were kept in 70% ethanol. Furthermore, using the dragging method, free-living ticks were collected from the vegetation once a month from May to September 2002 along a path across the woods (called a 'dragging session' during which free-living ticks are collected from the vegetation by dragging a blanket along the vegetation). This particular wooded area is representative of this geographic area (northern Apennine Region) which is characterised by mesophilous vegetation.

In addition, the samples collected by dragging were registered and kept in 70% ethanol. All specimens were identified by observation using a stereoscope at increasing magnifications (Fig. 1). A light microscope and scanning electron microscope (SEM) (Fig. 2) were used to identify *Rhipicephalus turanicus* (Pomerantzev, 1940) from anal plates of males. After the tick species had been identified, different polymerase chain reaction (PCR) tests for the detection of *B. burgdorferi sl* were performed.



Figure 1
Ventral view of a female *Ixodes ricinus*

Twenty pools of four non-engorged or semi-engorged *I. ricinus* ticks collected from the animals, four pools of four *I. ricinus* collected in the environment during the same 'dragging session' and 16 individually selected *I. ricinus* ticks collected from humans were tested.



Figure 2
Medial spines of anal plates of a *Rhipicephalus turanicus* male
Scanning electron microscope
(5 kilovolts 100×)

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DNA was extracted from tick pools and from individual ticks using the following method: the samples were homogenised in TES buffer (50 mM Tris buffer, 50 mM ethylenediamine-tetraacetic acid [EDTA], 15% saccharose, pH 8) and incubated for 3 h at 45°C. After phenol/chloroform (1:1) treatment, DNA was precipitated in 95% ethanol with 0.3M sodium acetate (14).

Three different PCR methods for the detection of *B. burgdorferi sl* were performed, as follows: a PCR method which amplifies a 185 bp fragment of *ospA* gene (11) using the method described by Marconi and Garon (8) with a PCR test targeting a 350 bp region of 16S rRNA sequence.

To identify the genospecies of *B. burgdorferi sl*, a fragment of approximately 230 bp of the spacer region between 5S-23S ribosomal RNA intergenic spacer of the positive samples from all origins was amplified using a couple of primers, as described by Rijpkema *et al.* (13). Three amplicons were then sequenced using an ABI Prism 3100 Avant genetic analyser and the sequences were aligned with the sequences of *B. burgdorferi sl* genospecies deposited in GenBank.

The positive control DNAs extracted from different genospecies of *B. burgdorferi sl* were provided by Marina Cinco from the Spirochetae Laboratory at Trieste University.

The estimated *B. burgdorferi sl* infection rate was calculated as follows, in line with a binomial distribution:

$p\hat{}=1-([n-/N]^{*}\ 1/k)$

where:

$p\hat{}$ is the estimated probability that a single tick is infected

$n-$ is the number of negative pools

N is the number of samples examined

k is the number of ticks in each single pool.

Since the detection of *B. burgdorferi sl* in ticks collected on humans was performed on each tick, the prevalence was calculated by beta distribution considering an infinite population of ticks, as follows:

beta distribution $(e+1, n-e+1)$ where:

e, the number of events, is the total number of positives ticks

n, the number of tests, is the total number of ticks examined.

The peak represents the most probable percentage of positive ticks, while its breadth gives information on the uncertainty of the estimate due to the sample size.

The confidence interval was 95%.

Results

Tick collection

During the study, 198 ticks from 131 samples were collected from wild cervids, 27 were from a stray dog and 93 ticks were removed from humans at the local hospital in Urbino. Samples collected from animals included more than one tick species per host, whereas all the samples collected from humans at the local hospital revealed the presence of only one tick species per individual (Table I).

Table I
Identification of ticks collected from various hosts

Species	Roe deer (*Capreolus capreolus*)	Fallow deer (*Dama dama*)	Stray dogs (*Canis lupus familiaris*)	Humans	Total	Percentage
Ixodes ricinus	148	18	0	79	245	77%
Dermacentor marginatus	0	3	0	3	6	2%
Rhipicephalus turanicus	29	0	0	0	29	9%
Rhipicephalus sanguineus	0	0	27	0	27	8%
Hyalomma marginatum	0	0	0	4	4	1%
Not suitable	0	0	0	7	7	2%
Total	177	21	27	93	318	100%

Using the 'dragging' method, 78 ticks were collected from the environment. The highest number of ticks was found during the month of May. Figure 3 shows the number of ticks collected during each 'dragging session'.

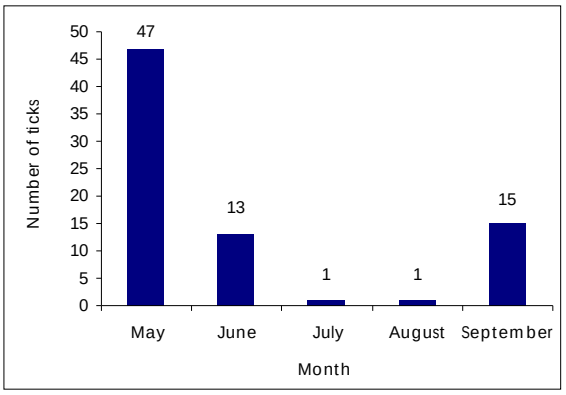


Figure 3
Ticks collected from the environment in each monthly dragging session

Retrospective analysis

The retrospective analysis on human tick bites has revealed 166 tick bite cases. Figure 4 shows the temporal distribution of these cases. There are two high values, the highest occurs in the late spring and the lowest in autumn.

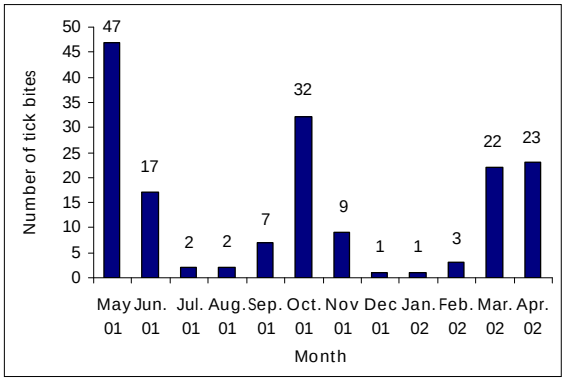


Figure 4
Temporal distribution of cases of human tick bites at the local hospital

Identification of ticks

Of the 318 specimens sampled from hosts, only 311 were identified after observation at increasing magnification under a stereoscope because 7 ticks collected from humans were not suitable for identification purposes. A total of 225 ticks collected from animals were identified at species level (Table I). The species the most frequently encountered was *I. ricinus*.

All ticks belonging to the species *R. turanicus* were collected from roe deer found around the town of Urbino, while all ticks belonging to the species *R. sanguineus* (Latreille, 1806) were collected from the stray dogs involved in this study. Of 93 ticks collected from humans at the local hospital, a total of 86 were identified (Table I). Among the ticks collected from both animals and humans, the species most frequently found was *I. ricinus* ($n = 245$).

All 78 ticks collected by dragging were identified and, in this case, the most prevalent species was *I. ricinus* (total: 43) but the number of specimens belonging to *R. turanicus* (total: 33) was also high (Table II).

Detection of *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* ticks by polymerase chain reaction

Of 20 pools of four *I. ricinus* collected from wild cervids, two were positive using all PCR methods. Both positive pools came from roe deer involved in car accidents in two different areas of the Province.

Table II
Identification of ticks collected from vegetation

Species	No. of ticks	Percentage
Ixodes ricinus	43	55.1%
Dermacentor marginatus	1	1.3%
Rhipicephalus turanicus	33	42.3%
Haemaphysalis punctata	1	1.3%
Total	78	100%

All 16 samples collected from humans selected from the good conditions of the tick gave negative results for *B. burgdorferi sl* using two PCR methods (8, 11), whereas when performing the PCR method described by Rijpkema *et al.* (13), one tick was positive.

The use of PCR techniques in four pools of four ticks sampled from vegetation by 'dragging' gave different results using the method of Marconi and Garon (8) and that of Olsen *et al.* (11), namely: the first detected only

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one positive pool, whereas the method of Olsen *et al.* revealed three positive pools which were confirmed using the PCR method described by Rijpkema *et al.* (13). Results of the amplification of the spacer region between the 5S-23S ribosomal rRNA gene from these two samples using PCR (8, 13) are presented in Figure 5.

The map of the study area and the municipalities in which positive pools from humans, animals and vegetation were found is presented in Figure 6.

The total estimated *B. burgdorferi sl* infection rate is 8.26%, with the highest level for ticks collected from vegetation reaching 29.9% and the lowest level for ticks collected from humans 6.3%.

The sequencing of a fragment of 5S-23S intergenic spacer region over three positive samples was performed. It led to the identification of the *B. burgdorferi sl* genospecies, as follows:

- *B. burgdorferi sensu stricto* (*ss*) 100% homologous with the strain 5LM218 isolated in France (GenBank Accession No.: DQ393299) in a pool of *I. ricinus* collected from vegetation
- *B. lusitaniae* from a pool of *I. ricinus* collected from a roe deer; in this case, the sequence was 100% homologous with the strain PoHL1 (GenBank Accession Number: AY209179)
- *B. lusitaniae* from a tick collected from a human, strictly related (99% homologous) to PoHL1.

The latter two strains of *B. lusitaniae* were identified in the same municipality.

Conclusions

The number of samples collected from the animals was not distributed uniformly among different districts during the period of the study which explains why a spatial or temporal analysis was not possible, but the number of samples is adequate to reveal the relationship between the species of ticks and their hosts. In roe deer, the most prevalent wild cervid in the Province and the most frequent in our collection, the principal vector

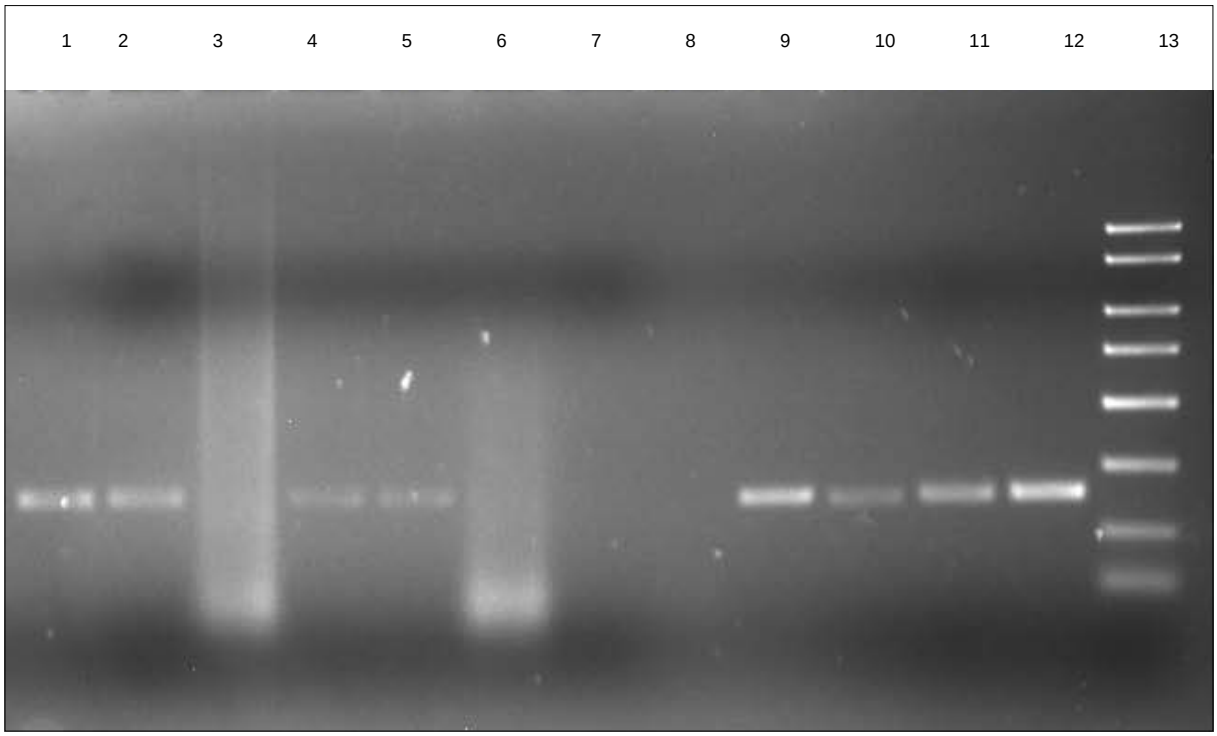


Figure 5
Results of the amplification of the spacer region between 5S and 23S ribosomal rRNA genes (13)
Lanes 1 and 2: DNA from pools of ticks collected from vegetation
Lanes 4 and 5: DNA from pools of ticks collected from roe deer
Lanes 9, 10, 11, 12: DNA from *Borrelia burgdorferi sensu lato* 'reference strains'

(a) Study area (red) and other provinces of the Marche region (pink)

(b) Municipalities in which positive samples were found (red)

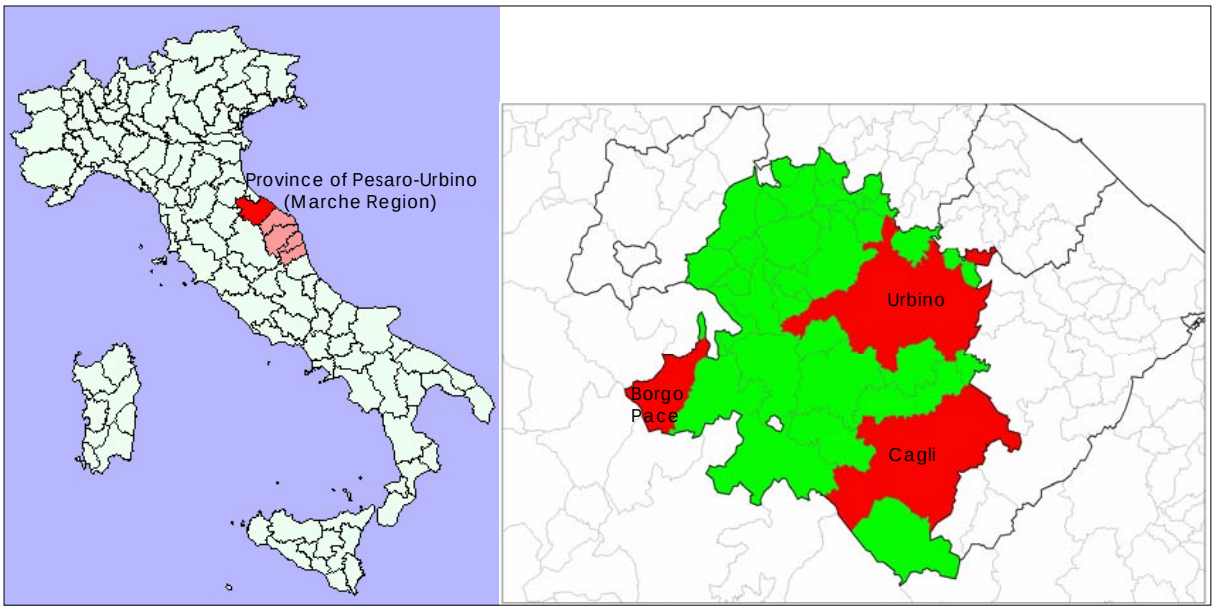


Figure 6
Study area and municipalities in which positive samples were found

of Lyme disease in Europe (*I. ricinus*) (4, 9) was the tick most often encountered. This relationship has been described previously (3). The reasons for this close association are found in the feeding habits and behaviour of this cervid which usually seeks food in grazing areas neighbouring woodlands; these areas are usually wet and protected from the sunlight, conditions that favour the development of the life-cycle of *I. ricinus* (7, 9).

Some authors link the explosion of the *I. ricinus* population to the recent increase in the roe deer population of central Italy (3).

Our data on human tick bites confirm data in the literature on tick bites in humans in central Europe, namely, that *I. ricinus* is the tick most frequently encountered among humans. This association is not only due to the abundance of this species, but also to the wide host range of *I. ricinus* (7). The number of annual cases of tick bites attended to at the local hospital is high and the annual distribution of these cases is compatible with biphasic seasonality of *I. ricinus* described in central Italy, having the highest peak of activity in late spring and the lowest in autumn (7, 9).

It should be also considered that these peaks of activity of *I. ricinus* take place at the same time when woods and grazing areas are most frequented by people (ecotourists, people collecting mushrooms or hunting, etc.), thereby increasing the risk of tick bites in humans.

The first finding of *B. burgdorferi sl* in the Province of Pesaro-Urbino confirms that there are suitable conditions for the development of the cycle of Lyme disease. The apparent lower levels of infection rates in ticks collected from cervids and humans in comparison with the ticks collected from vegetation should be investigated. The small quantities of blood in semi-engorged ticks collected from the cervids could have interfered with the PCR reaction, causing a number of false-negative results (15). Therefore, the prevalence level of *B. burgdorferi sl* could possibly be more widespread in the environment than the extent revealed by these methods.

The presence of *B. burgdorferi ss* in free-living ticks confirms that this genospecies is involved in the cycle of Lyme disease in Italy as described previously (4). It is most likely present and widespread in wild rodent populations. To confirm this statement, it will be necessary to perform further research to estimate the prevalence in wild rodents and in

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other vertebrates known to act as reservoirs (6).

The finding of *B. lusitaniae* in central Italy concurs with the recent description of this genospecies in the Mediterranean region (1, 2, 5). The simultaneous finding of this pathogenic genospecies in ticks from roe deer and from human patients required additional information on the ecology of *B. lusitaniae*, of which the wall lizard (*Podarcis muralis*, Laurenti, 1768) has been demonstrated to be the principal reservoir (1, 2, 12).

Although these results are not exhaustive, it can be confirmed that Lyme disease risk factors are present in the Province of Pesaro-Urbino based on the presence of pathogenic genospecies of *B. burgdorferi sl*, on the relative abundance of vectors (*I. ricinus*), a roe deer population that spreads these ticks in the environment and the high frequency of contacts between humans and ticks.

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