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Occurrence of multiple infections with different *Borrelia burgdorferi* genospecies in Danish *Ixodes ricinus* nymphs

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Abstract

The pathogen *Borrelia burgdorferi* causes Lyme Borreliosis in human and animals world-wide. In Europe the pathogen is transmitted to the host by the vector *Ixodes ricinus*. The nymph is the primary instar for transmission to humans. We here study the infection rate of five *Borrelia* genospecies: *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae* in nymphs, by IFA and PCR. 600 nymphs were collected in North Zealand of Denmark. Each nymph was first analysed by IFA. If positive for spirochaetal infection, the genospecies was determined by PCR. The infection rate of *B. burgdorferi* sensu lato was 15.5%, with the primary genospecies being *B. afzelii* (64.3%), *B. garinii* (57.1%), and *B. lusitaniae* (26.8%). It is the first time *B. lusitaniae* is documented in Denmark.

Even though, the highest infection rate was discovered for *B. afzelii* and *B. garinii*, mixed infections are more common than single infections. Fifty-one percent (29/56) of these were infected with two genospecies, 7.1% (4/56) with three, and 5.3% (3/56) with four. We try to explain the high infection rate and the peculiar number of multiple infections, with a discussion of changes host abundance and occurrence of different transmission patterns.

Keywords: *Borrelia burgdorferi*; *B. lusitaniae*; *Ixodes ricinus*; Infection rate; Multiple infections; Co-feeding

1. Introduction

Lyme Borreliosis (LB) is a tick-borne disease of humans and animals in temperate climates of the Northern hemisphere. The causative agent is the spirochaete belonging to the genus *Borrelia* [1,2]. Six genospecies *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii*, and *B. burgdorferi* sensu stricto, are known to be prevalent in Europe [1,3]. These genospecies may co-circulate in local tick populations, suggesting that the local diversity of *B. burgdorferi* sensu lato can be as high as the regional or continental diversity [4]. Individual ticks may be infected with multiple genospecies of *B. burgdorferi* sl; however, mixed infections in individual ticks may reveal important principles of the biology and ecology of *B. burgdorferi* sl, but only a few studies provide information on this aspect [5].

The pathogens are transmitted and maintained by a complex zoonotic transmission cycle, constituting a vector and several hosts. Ticks from the genus *Ixodes* are primary vectors for LB spirochaetes, where *I. ricinus* is the most frequent species in Europe [6,7]. This tick species has a very wide geographical distribution and is described within the latitudes 65° and 39° and from Portugal to Russia [8]. *I. ricinus* is a three host tick with each parasitic stage (larva, nymph and adult female) feeding on different hosts [9,10]. *I. ricinus* feeds on a highly broad array of hosts, from small, medium and large sized mammals (including humans) to birds and reptiles [9,10]. However, only some of these vertebrate hosts have been

Abbreviations: LBg, Lyme Borreliosis; PCR, Polymerase Chain Reaction; IFA, Immunofluorescence Assay.

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currently identified as reservoir hosts for *B. burgdorferi* sl [11]. A vertebrate acting as a reservoir is a susceptible vertebrate, able to amplify the pathogen more or less.

Spirochaetes are transmitted orally while the tick is feeding on the host. Because of this dependence of a vector for transmission, between the different hosts, *Borrelia* is indirectly dependent on the life cycle of its vector [12]. The spirochaete is transmitted between the ticks by four known pathways: transstadial, transovarial, sexual, and co-feeding [6,16]. Even though there are different ways for transmission between ticks the infection rate of *Borrelia* in nymphs is, generally, lower compared to adults. This is because the adult tick has parasitised two hosts and the nymph only one; as transstadial transmission leads to accumulation of the spirochaetes in later instar, the adult will have a higher infection rate [3,13]. Results from different districts in Denmark (Jutland, Funen County, Northern Zealand and Bornholm) have observed infection rates of *Borrelia* in nymphs and adults, which seem to differentiate from this pattern [13–15]. Here the nymphs had either the same or higher infection rate of *B. burgdorferi* sl compared to the adults. There is no direct explanation for this phenomenon, but different parameters might have an impact e.g. the transmission pattern, climatic conditions, tick questing behaviour, host behaviour during the day, distribution of hosts in the particular habitat, soil composition of the habitat, and seasonal variation, just to mention a few studied in the last decades [3,6,15,16].

Besides the differing infection rates in Denmark, the infection rate of *Borrelia* in ticks has also increased during the last decade in of all Europe, resulting in more cases of LB [3]. We, therefore, examine the infection rate of *Borrelia* in nymphs to observe, whether it has increased in the particular habitat in Northern Zealand from app. 5–10% in the 1990's [15].

The aim of this paper is to analyse infection rates of *B. burgdorferi* sl in nymphs collected in the summer season 2005. The study includes only nymphs, as it is the main instar parasitising humans [3,10]. Furthermore, we determine the *Borrelia* genospecies, *B. afzelii*, *B. garinii*, *B. burgdorferi* ss, *B. valaisiana*, and *B. lusitaniae*, to study the occurrence of the particular genospecies in Denmark, and for the first time to examine seasonal variation in the genospecies. Last, we try to explain the occurrence of single, double, and multiple infections.

2. Materials and methods

2.1. Collection of nymphs

The sampling was performed from May to August 2005 in Grib Skov, a forest in the Northern part of Zealand (app. 56°N, 12.5°E). The flagging area was a beech-forest, where ticks, from former studies, were shown to be abundant [16]. The forest has a rich fauna including a high density of roe deer (*Capreolus capreolus*), numerous small mammals (mice, voles, hedgehog, fox) and birds. The location was chosen because it has been included in several studies, and represents a general beech-forest at Zealand. Second, the type of forest is often chosen for recreational purpose [17]. 600 nymphs were collected by flagging the vegetation with a 100 × 60 cm linen cloth, fixed on a bamboo stick. Collection was performed one time each month at 10 a.m and persisted for app. 1 h. The ticks were stored in Petri dishes sealed with tape, and placed in a plastic box with wet foam in the bottom-retaining high humidity until analysis for *Borrelia* sl infection.

2.2. Determination of physiological age

The physiological age was determined as in [18]. The nymphs were divided within four classes, i.e. age class 1 to 4, according to the observation of reserve nutrition in the nymphal gut and cuticula characteristics, determined in a stereo microscope: The size of the gut is reduced with ageing. To prevent subjective analysis of the physiological age, the determination was performed by two independent technicians.

2.3. Immunofluorescence Assay (IFA)

Each nymph was crushed in 25 μl PBS, releasing tick gut content and hemolymph. Five micro-litre of each sample was added on a slide, containing 10 samples. The slides were air dried, and fixed in acetone for 10 min. For detection, purified polyclonal dog anti-sera (Statens Veterinaermed, Anstalt, Uppsala, Sweden)– diluted 1:80– was deposited at each sample, and left in a humidity-box at 38–40 °C for 30 min. The washing procedure was performed for 10 min in PBS. Twenty micro-litre of FITC conjugate (Dog IgG antibody, 1:200; Bethyl laboratories Inc.) was added and incubated in a humidity-box at 38–40 °C for 30 min. The washing procedure was repeated. The samples were read with an objective 40×/0.75 Plan-Nefluar, an ocular 10×/20.P1, and filter 09 blue excitation 450–490 nm. The number of spirochaetes was counted in each sample, counting in 25 fields of vision. Again, to prevent subjective analysis, the determination was performed by two independent technicians, who were highly skilful in counting spirochaetes.

As we are interested in the absence of infected nymphs special precautions were taken to avoid false negative results. Detection of *Borrelia* spirochaetes requires the presence of surface antigen. When the tick dies, different degrading processes may induce lysis of the spirochaetes. Examination of a dead tick can in this way lead to false negative results. Therefore the analysis only included active nymphs, which responded to the breath or other manipulations of the technician at the time of analysis.

2.4. Polymerase Chain Reaction (PCR)

All IFA-positive was analysed by PCR. Ten micro-litre of the supernatant was added to 40 μl NH_4OH, boiled at 99.9 °C for 17 min. The samples were left with the seal open in 10 min, to remove the NH_3.

Positive controls were kindly delivered from Statens Serum Institute (Copenhagen, Denmark). MilliQ water was applied as negative control. Positive and negative controls were included in all the analyses, to secure the certainty of the results.

The primers used in the experiment are shown in Table 1. The primers for *B. burgdorferi* sl, *B. afzelii*, *B. garinii* and