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Occurrence of *Borrelia burgdorferi* s.l. in different genera of mosquitoes (Culicidae) in Central Europe

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ABSTRACT

Lyme disease or Lyme borreliosis is a vector-borne infectious disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex. Some stages of the borrelial transmission cycle in ticks (transstadial, feeding and co-feeding) can potentially occur also in insects, particularly in mosquitoes. In the present study, adult as well as larval mosquitoes were collected at 42 different geographical locations throughout Germany. This is the first study, in which German mosquitoes were analyzed for the presence of *Borrelia* spp. Targeting two specific borrelial genes, *flaB* and *ospA* encoding for the subunit B of flagellin and the outer surface protein A, the results show that DNA of *Borrelia afzelii*, *Borrelia bavariensis* and *Borrelia garinii* could be detected in ten Culicidae species comprising four distinct genera (*Aedes*, *Culiseta*, *Culex*, and *Ochlerotatus*). Positive samples also include adult specimens raised in the laboratory from wild-caught larvae indicating that transstadial and/or transovarial transmission might occur within a given mosquito population.

1. Introduction

In the second half of the 20th century, infectious diseases became increasingly important. About 15 million (>25%) of 57 million annual deaths worldwide are estimated to be directly related to emerging and re-emerging infectious diseases (Morens et al., 2004). Of these, an accelerating number is transmitted by obligate haematophagous arthropods with mosquitoes and ticks as the most crucial vectors.

Lyme borreliosis is a multisystemic infectious disease caused by distinct spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, which are transmitted by hard ticks (Ixodidae). Early signs of the disease often include unspecific symptoms such as headache, fever and fatigue. After 3–30 days, 70–80% of infected individuals developed a ring-like skin rash at the ticks bite site (erythema migrans). If left untreated, the pathogens can disseminate, leading to more severe symptoms involving diverse organs, e.g. the heart, the joints, and the central nervous system. The *B. burgdorferi* s.l. complex comprises a group of at least 20 genospecies worldwide (Casjens et al., 2011; Skuballa et al., 2012; Ivanova et al., 2014). Regarding their potential to cause the disease, the 20 genospecies can be divided into two groups of which the first group consists of five genospecies frequently isolated from patient samples while the second group comprises genospecies with either uncertain pathogenic potential or that have never been detected in human samples (Rudenko et al., 2011). Among human pathogenic genospecies, *Borrelia garinii*, *B. burgdorferi*, and *Borrelia afzelii*, the three main European ones are maintained by birds or small mammals. *Borrelia bavariensis*, a serotype formerly regarded as *B. garinii* OspA type 4 is also adapted to rodents (Skuballa et al., 2012; Margos et al., 2013). Of these, different genospecies can be present simultaneously in a single tick (Rauter and Hartung, 2005; Wodecka et al., 2010). This is of particular interest as genospecies can be associated with different clinical manifestations, e.g. *B. burgdorferi* sensu stricto (s.s.) is often associated with arthritis, *B. garinii* with neurological manifestation, *B. afzelii* almost always induces acrodermatitis chronica atrophicans (Skotarczak, 2014) and *B. bavariensis* has been linked to neuroborreliosis (Margos et al., 2013). Therefore, an infection with various genospecies may result in the manifestation of multiple disease symptoms. Although ticks are the prominent transmission vectors of these particular pathogens, borreliae have also been detected in other arthropods, especially

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in mosquitoes. Mosquitoes have been known since the late 19th century to be the vectors for a number of several human diseases, such as malaria, dengue, chikungunya or yellow fever. Recently, it has been demonstrated that human cases of tularemia caused by *Francisella tularensis holarctica* are transmitted by mosquitoes (Lundström et al., 2011; Thelaus et al., 2014).

Especially in Germany, there is a lack of knowledge which pathogens can be successfully transmitted by diverse mosquito species. In mosquitoes, Spirochetes were found for the first time, in a *Culex* species in 1907 (Jaffé, 1907). Concerning spirochetes of the *B. burgdorferi* s.l. complex, different genospecies could be identified in mosquitoes already (Halouzka et al., 1999; Žákovska et al., 2004). So far the maximal periods of survival for borreliae in naturally infected mosquitoes are still unknown (Magnarelli and Anderson, 1988). In experimentally fed mosquitoes borreliae could be detected up to 14 days after a blood meal in two species (*Aedes aegypti* and *Aedes triseriatus*) (Magnarelli et al., 1987). Additionally, there is one report of a patient who developed erythema migrans after a mosquito bite (Hård, 1966).

Owing to the very different feeding time and frequency of mosquitoes and ticks, Matuschka and Richter (2002) postulated that transmission of *Borrelia* species by mosquitoes appears to be impossible, with the result that research on this topic stopped nearly completely for over a decade. This is astonishing, as it is still unknown whether these insects may be able to play a small role influencing the ecology and epidemiology of Lyme borreliosis.

Aim of this study is to check whether borreliae can be found in different species or even genera of mosquitoes with different feeding habits. Therefore, anthropophilic, mammalophilic as well as ornithophilic species have been screened for the presence of borreliae. In addition, we sought to examine, whether borreliae can also be detected directly after the metamorphosis in mosquitoes, as transstadial transmission is known in ticks, which is a crucial step for a vector-borne pathogen to be transmitted later on.

2. Material and methods

2.1. Collection of samples

Mosquitoes were sampled from April to October 2013 at 42 sampling sites in Germany during a mosquito monitoring program, which included the detection of possible pathogens. Most of these sites were in Saxony ($n = 9$) followed by Brandenburg ($n = 6$) and Hesse ($n = 15$) (SI 1). Individuals were collected using EVS-traps with dry ice, BG-Sentinel™ traps (Biogents AG, Regensburg, Germany) with CO_2 as an attractant, hand nets and by human bait (Supplemental Table 1). Larvae were collected in natural (e.g. puddles, ponds, tree holes) as well as in artificial (e.g. vases, rain barrels) water pools using hand nets. After capture, larvae were kept alive and raised to adults. For this study, no specific permissions were required and no endangered or protected species are involved.

Supplementary Table 1 related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.10.018.

2.2. Morphological identification

Morphological identification of mosquito species was carried out with a stereomicroscope. The morphological characters of each specimen were analyzed using two identification keys (Mohrig, 1969; Becker et al., 2010).

2.3. DNA-extraction

For DNA-extraction, morphologically identified mosquitoes were pooled by species, collection date and collection site, each pool contained up to ten individuals. Samples were homogenized individually with a tissue mill (MM400, Retsch GmbH, Germany) and 2 stainless steel beads (3 mm, VWR, Germany) for 2 min at 25 Hz. Subsequent DNA-extraction was carried out with glass fiber plates (Pall GmBH, Dreieich, Germany) following the protocol of Ivanova et al. (2006).

2.4. Detection of borrelial DNA by nested PCR

Nested PCR was applied to detect two different borrelial genes, *flaB* and *ospA* in the collected samples. The *flaB* fragments were amplified using primers FL3/5 and FL6/7and primer pairs N1/C1 and N2/C2 were used to identify the *ospA* sequences (Table 1) (Picken, 1992; Rijpkema et al., 1997; Žákovska et al., 2004). The reaction mixtures for primers FL3/5 as well as N1/C1 (both 25 μl) consisted of 12.5 μl Master Mix (Peqlab Biotechnology GmbH, Erlangen, Germany) containing 0.4 mM dNTP, 4 mM $MgCl_2$, 40 mM Tris–HCl, 32 mM $(NH_4)_2SO_4$, 0.02% Tween 20 and 1.25 U Taq-Polymerase, 1 μl of each primer (10 pmol μl^{-1}) 9 μl ddH_2O and 1.5 μl genomic DNA. The reactions for the FL6/7 nested PCR contained 12.5 μl Master Mix, 1 μl of each primer (10 pmol μl^{-1}) 5.5 μl ddH_2O and 5 μl PCR-product of FL3/5. The amplification of N2/C2 was performed with 12.5 μl Master Mix, 1 μl of each primer (10 pmol μl^{-1}) 9 μl ddH_2O and 1.5 μl PCR-product of N1/C1. For FL6/7 the cycle parameters followed the protocol of Picken et al. (1996): FL3/5 = 1 cycle of 94 °C, 12 min; 30 cycles of 94 °C, 1 min; 70 °C, 2 min and 72 °C, 3 min followed by terminal extension of 72 °C, 7 min and a final ramping to 8 °C; FL6/7 = 1 cycle of 94 °C, 12 min; 30 cycles of 94 °C, 1 min; 54 °C, 2 min and 72 °C, 3 min, final extension at 72 °C, 7 min and a final ramping to 8 °C; N1/C1 = 1 cycle of 37 °C, 5 min and 94 °C for 10 min; 30 cycles of 94 °C, 1 min; 45 °C, 1 min and 72 °C (extended for 5 s with each cycle), 1 min, final extension at 72 °C, 5 min and a final ramping to 8 °C; N2/C2 = 1 cycle of 94 °C, 10 min; 25 cycles of 94 °C, 1 min; 43 °C, 1 min and 72 °C, 1 min, and a final extension at 72 °C for 5 min and a final ramping to 8 °C (slightly modified after Rijpkema et al. (1997)). In each PCR attempt, a positive as well a negative control were performed to rule out the possibility of laboratory contamination. Quality and yield of PCR products was analyzed by Midori Green (Nippon Genetic EUROPE GmbH) staining and agarose gel-electrophoresis. For subsequent Sanger-sequencing reactions, with product specific forward primers, positive samples were purified using the peqGOLD Cycle-Pure Kit (Peqlab Biotechnology GmbH, Erlangen, Germany). Each obtained sequence was edited using BioEdit (Hall, 1999) and compared with sequences deposit in GenBank using the BLAST algorithm (Altschul et al., 1997).

The sequences obtained were deposited in EMBL Nucleotide Sequence Database under accession numbers LN650604–LN650631.

3. Results

In total 3615 adult mosquitoes were morphologically identified (Supplemental Table 2) and analyzed, including 74 adults reared from field-caught larvae in the laboratory. Specimens which could not be identified morphologically to species level because of their poor state of preservation were analyzed individually.

Supplementary Table 2 related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.10.018.

For all analyses, no distinction was made between *Culex pipiens* and *Culex torrentium* and specimens were treated as pools of "*Cx. pipiens*/*torrentium*" as well as between *Ochlerotatus annulipes* and *Ochlerotatus cantans*, which were treated as pools of "*Oc. annulipes*/*cantans*". The analyzed adult mosquitoes belong to the genera *Aedes* ($n_{pools} = 265$), *Anopheles* ($n_{pools} = 12$), *Coquillettidia*