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ARTHROPODS AND MEDICAL ENTOMOLOGY - SHORT COMMUNICATION

Bartonella species in medically important mosquitoes, Central Europe

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Abstract
Here, we provide the first mass molecular screening of medically important mosquitoes for *Bartonella* species using multiple genetic markers. We examined a total of 72,115 mosquito specimens, morphologically attributed to *Aedes vexans* (61,050 individuals), *Culex pipiens* (10,484 individuals) and species of the *Anopheles maculipennis* complex (581 individuals) for *Bartonella* spp. The initial screening yielded 63 *Bartonella*-positive *A. vexans* mosquitoes (mean prevalence 0.1%), 34 *Bartonella*-positive *C. pipiens* mosquitoes (mean prevalence 0.3%) and 158 *Bartonella*-positive *A. maculipennis* group mosquitoes (mean prevalence 27.2%). Several different *Bartonella* ITS sequences were recovered. This study highlights the need for molecular screening of mosquitoes, the most important vectors of arthropod-borne pathogens, for potential bacterial agents.

Keywords *Bartonella* · Mosquito · *Culex* · *Anopheles* · *Aedes*

Introduction

Mosquitoes represent a major arthropod-borne health threat to humans and wild and domestic animals. In particular, malaria jeopardises the health of millions of people worldwide (WHO 2019). Among others, mosquitoes also transmit many arboviruses associated with human and animal disease, such as dengue, West Nile, yellow fever, Zika and chikungunya (Hubálek and Rudolf 2011). Thus far, mosquitoes have not yet been equally implicated in the biological transmission or maintenance of bacterial pathogens of vertebrates. To date, only the zoonotic pathogen *Francisella tularensis* was recovered from aquatic and adult stages of *Aedes* spp. mosquitoes in Sweden (Thelaus et al. 2014). Lundström et al. (2011) demonstrated transstadial transmission of the tularaemia agent *F. tularensis holarctica* in mosquitoes and that mosquitoes may acquire the bacterium already as larvae. Furthermore, spirochaetes of *Borrelia burgdorferi* sensu lato have been detected in *Culex* spp., *Aedes* spp. and *Anopheles* spp. mosquitoes in Central Europe (e.g. Melaun et al. 2016). A number of potentially new spotted fever group rickettsiae have also been documented in mosquitoes of the genera *Culex*, *Anopheles* and *Aedes* in China (Zhang et al. 2014; Guo et al. 2016). Based on these studies and detections, it is conceivable that mosquitoes could serve as mechanical vectors of bacteria in nature. Interestingly, pathogenic *Rickettsia felis* has also been detected in *Anopheles gambiae* mosquitoes in Africa (Socolovschi et al. 2012). Experimental modelling which showed detectable rickettsiemia in healthy mice bitten by *R. felis*-infected mosquitoes (Dieme et al. 2015) might change our perception on mosquitoes as vectors of bacterial agents

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and challenge new research directions in this neglected area. In Australia, an association between Buruli ulcer outbreaks and mosquitoes, harbouring *Mycobacterium ulcerans*, was observed (Johnson et al. 2007).

We argue that the ability of mosquitoes to transmit bacteria between animals and from animals to humans has been poorly investigated thus far (Laroche et al. 2018). To increase the knowledge about possible mosquito-borne bacterial pathogens, we focused on intracellular bacteria of the genus *Bartonella*. This genus of bacteria has been isolated from a wide variety of haematophagous arthropods and vertebrates (including wild and domestic animals and humans) and causes a wide range of pathological manifestations in humans ranging from fever, rash, headache and regional lymphadenitis to bacillary angiomatosis or severe endocarditis (Hubálek and Rudolf 2011). Commonly, *Bartonella* infections cause persistent intraerythrocytic bacteraemia within the specific mammalian hosts, which potentially facilitates its transmission between mammals by arthropod vectors. Currently, vector competence of some groups of arthropods (e.g. fleas, lice and ticks) has been proven experimentally for *Bartonella* transmission (Chomel et al. 1996; Seki et al. 2007; Morick et al. 2011; Reis et al. 2011; Bouhsira et al. 2013), while additional groups of arthropods are suspected as *Bartonella* vectors (e.g. biting midges, keds, bat bugs) based on the detection of specific DNA or epidemiological observations (Chung et al. 2004; Mosbacher et al. 2011; Lappin et al. 2013; Rudolf et al. 2016; McKee et al. 2018). However, it must be acknowledged that the transmission of *Bartonella* species by some arthropods remains unproven. Of note, non-pathogenic *Bartonella* species were described in arthropods in addition to pathogenic ones (e.g. Kešnerová et al. 2016).

Although the detection of *Bartonella* DNA within haematophagous insects does not prove transmission or the presence of viable bacteria, such observations are important to provide direction of specific research for investigating the role of specific arthropods in the transmission of *Bartonella* infections. Thus, we present the first molecular survey of medically important mosquito species from Central Europe for the presence of *Bartonella* species using multiple genetic markers.

Material and methods

Mosquito collection

Adult mosquitoes were collected at 12 study sites across the Czech Republic and Slovakia during the 2010–2017 mosquito seasons (Blažejová et al. 2018; Rudolf et al. 2014). Detailed information on the habitats of the collected mosquitoes is provided by Blažejová et al. (2018). Briefly, mosquitoes were trapped using CO_2-baited CDC light traps and BG sentinel traps. The mosquito specimens were immediately transported in chilled containers to the laboratory, where they were stored at − 20 °C before identification and then at − 60 °C until further processing. Mosquitoes were morphologically identified on a chilled table under an Olympus SZ-40 binocular microscope according to entomological keys (Becker et al. 2010).

DNA extraction

Mosquitoes were surface-sterilised by immersion into 70% ethanol for 5 min, then rinsed in sterile H_2O and homogenised in 200 μL sterile PBS (Oxoid, England) individually or in pools (sorted by species and consisting of max. 50 individuals) using a pestle. Genomic DNA was extracted from 100 μL of mosquito homogenate using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and following the manufacturer's protocol.

PCR procedure

PCR as well as all post-PCR procedures were performed on each mosquito individual or pool according to the modified protocol by Urushadze et al. (2017) with amplification of the *ITS*, *gltA*, *rpoB*, *nuoG* and *ssrA* genes of *Bartonella* species. Each reaction tube contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.001% Tween 20, 2.5 mM $MgCl_2$, 200 mM mixture of dNTPs, 2.5 U Taq purple DNA polymerase (Top-Bio, Prague, Czech Republic) and 25 pmol of each primer. PCR was performed using an Eppendorf Mastercycler 5341 (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation 5 min at 94 °C, followed by 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 66 °C and 50 s extension at 72 °C and final extension of 5 min at 72 °C. The PCR products were separated on 2% agarose gels, stained with GelRed (Biotium, Fremont, CA, USA) and visualised under UV light. The processing of mosquito pools, DNA extraction, PCR setup (preparation of Mastermix, addition of primers, PCR reaction) and post-PCR procedures (agarose gel electrophoresis) were carried out in separate rooms to avoid cross-contamination.

Cultivation attempts

A total of 100 μL of homogenate from samples PCR positive for *Bartonella* species was put on brain-heart-infusion agar supplemented with 10% of rabbit blood and cultivated at 35 °C in 10% CO_2 atmosphere up to 3 weeks as previously described (Urushadze et al. 2017).

Sequencing and phylogenetic analysis

Sequencing of PCR products and bioinformatic analyses were done according to a previous study (Rudolf et al. 2016). The

PCR products were purified using the DNA Clean & Concentrator-5 Kit (Zymo Research, Irvine, CA, USA). Cycle sequencing of the purified PCR products was performed using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The ZR DNA Sequencing Clean-up Kit (Zymo Research) was used for the rapid removal of post-cycle sequencing reaction contaminants. The sequencing was carried out on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). PCR amplicons were bidirectionally sequenced to ensure high-quality reads. The DNA sequences were edited and aligned using the Seqman module within Lasergene v. 6.0 (DNASTAR Inc., Madison, WI, USA) and also checked manually. The FASTA format and BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches. Phylogenetic analyses were conducted using maximum likelihood (ML) algorithm and the Kimura-2 model (MEGA 6.0).

Statistical evaluation

Prevalence rates for *Bartonella* species were calculated for *A. vexans*, *C. pipiens* and *A. maculipennis* complex, and differences among them were evaluated using chi-square test SOLO (BMDP statistical software). Differences were considered significant at $P < 0.001$.

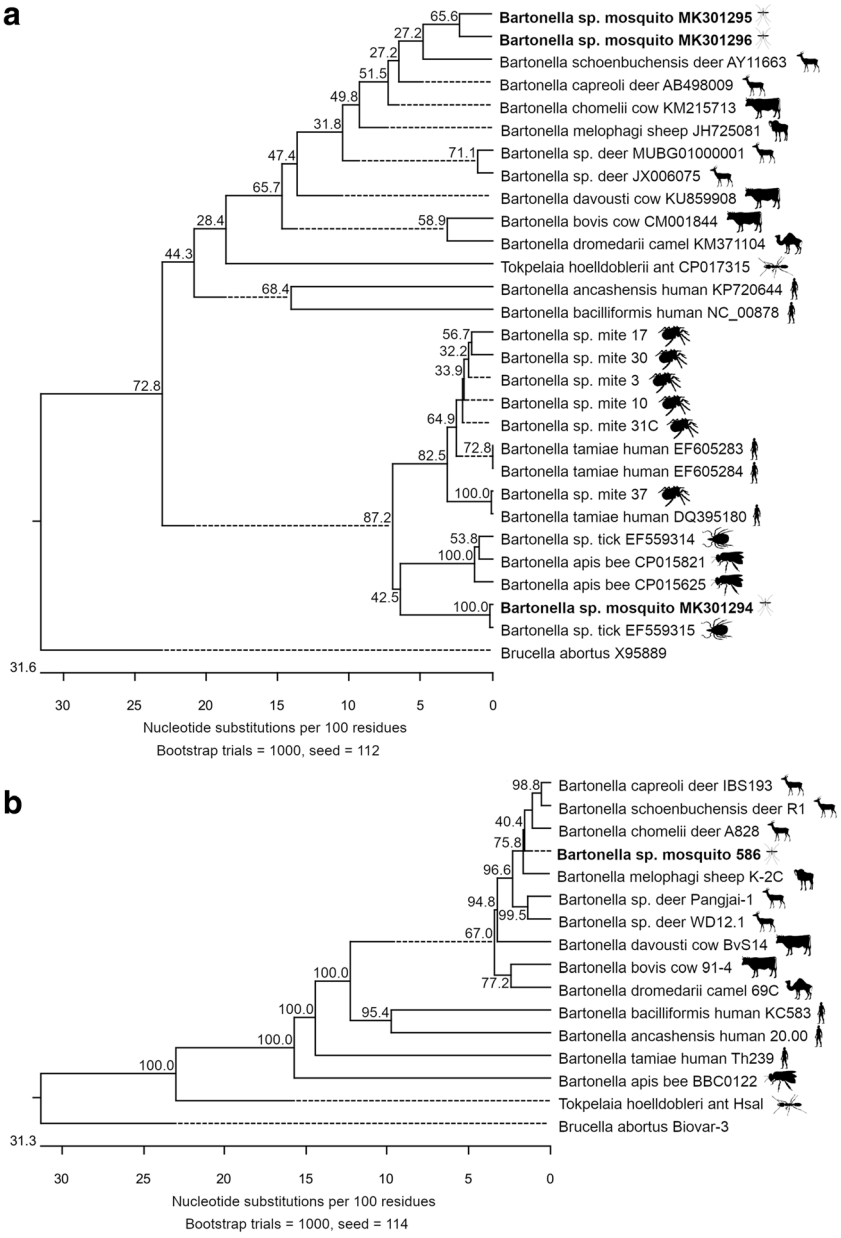

Fig. 1 Phylogenetic relationships between *Bartonella* strains found in mosquitoes in this study and *Bartonella* species originating from haematophagous arthropods and vertebrates. Neighbour-joining trees were generated from (**a**) ITS sequences and (**b**) concatenated ITS, *gltA*, *rpoB* and *nuoG* sequences. Phylogenetic trees were produced using maximum likelihood (ML) algorithm and the Kimura-2 model (MEGA 6.0). The robustness of trees was tested by bootstrap resampling of 1000 replicates with values being listed near the nodes. The horizontal bar represents the genetic distance

Results and discussion

We screened a total of 72,115 mosquito specimens morphologically attributed to *A. vexans* (61,050 individuals), *C. pipiens* (10,484 individuals) and taxa belonging to *A. maculipennis* complex species (581 individuals) for *Bartonella* species targeting the ITS gene sequence (Kosoy et al. 2018). The initial screening yielded 63 *Bartonella*-positive *A. vexans* mosquitoes (mean prevalence 0.10%; 95% C.I. 0.08–0.13%), 34 *Bartonella*-positive *C. pipiens* (mean prevalence 0.32%; 95% C.I. 0.22–0.43%) and 158 *Bartonella*-positive mosquitoes of the *A. maculipennis* complex (mean prevalence 27.19%; 95% C.I. 23.58–30.81%). Significant differences for *Bartonella* prevalences were found between particular mosquito species (χ^2 32.30; $P < 0.001$ for *A. vexans* vs. *C. pipiens*; χ^2 11,821; $P < 0.001$ for *A. vexans* vs. *A. maculipennis* complex; χ^2: 2331; $P < 0.001$ for *C. pipiens* vs. *A. maculipennis* complex).

Several different *Bartonella* ITS sequences were recovered in the study (Fig. 1a). One *Bartonella* ITS sequence (deposited in GenBank under accession number MK301294) was detected in all collected mosquito species and shared 99.0% sequence identity with *Bartonella* sp. clone Hill-02-66 from *Amblyomma americanum* ticks collected in the USA (Billeter et al. 2008). PCR detection and sequencing failed to detect sequences related to this *Bartonella* clone in all other target genes tested (namely *gltA*, *rpoB*, *nuoG* and *ssrA*). However, sequences of this *Bartonella* clone were recovered from the sensitive ITS marker (a very effective tool for *Bartonella* spp. screening in arthropods (Kosoy et al. 2018)). We can hypothesise that this sequence represents a yet undescribed *Bartonella* sp. closely related to the *B. tamiae*-like group (Billeter et al. 2008) and the human pathogenic *B. tamiae*, which has been isolated from febrile patients and rodent ectoparasites in Thailand (Kosoy et al. 2008; Kabeya et al. 2010). According to a recent study (Segers et al. 2017), *Bartonella tamiae* has retained many of the ancestral genome characteristics reflecting an evolutionary intermediate state toward a host-restricted intraerythrocytic lifestyle. These results also suggest that the ancestor of the pathogenic *Bartonella* species was a gut symbiont of insects and that the adaptation to blood-feeding insects facilitated colonisation of the mammalian bloodstream. In the light of our findings, we can hypothesise that mosquito *Bartonella* spp. from the *B. tamiae*-like group might act as such an intermediate relative standing between the symbiotic and pathogenic states.

Two additional *Bartonella* ITS sequences (deposited in GenBank under accession numbers MK301295 and MK301296) were detected only in *A. maculipennis* complex mosquitoes, and these grouped with 98–99% identity to *Bartonella capreoli* (GenBank accession no. AB498009), *B. schoenbuchensis* (AY11663), *B. chomelii* (KM215713), *B. melophagi* (JH725081) and *B. bovis* (CM001844). In the case of the *Bartonella*-specific ITS sequence derived from the *A. maculipennis* complex (GenBank accession number MK301295), we were also able to amplify parts of the *gltA* (deposited in GenBank under accession number MK301297), *rpoB* (MK301298) and *nuoG* (MK301299). These sequences (Fig. 1b; no. 586) also showed highest similarity to a *Bartonella* species closely related to agents isolated from wild and domestic ruminants, namely *B. schoenbuchensis* (CP01978) and *B. chomelii* (KM215718). We speculate that these sequences represent bacterial DNA from a mammalian blood meal (deer, cow) acquired during feeding, since mosquitoes of the *A. maculipennis* complex are strictly mammalophilic and commonly feed on domestic cattle and wild ruminants (Becker et al. 2010).

Attempts to cultivate *Bartonella* species failed in all mosquito specimens. We provide the first evidence of *Bartonella* species in medically important mosquitoes, raising concerns about the possible role of mosquitoes in the transmission of these agents. Additionally, this study highlights the need for molecular screening of mosquitoes, the most important vectors of arthropod-borne pathogens, for bacterial pathogens. Unfortunately, full characterisation of detected *Bartonella* species as well as consideration of their pathogenic status and any experimental transmission studies will depend on their successful cultivation. In the light of these findings, we can only speculate about the still unclear role of mosquitoes in *Bartonella* species maintenance. In summary, there is no evidence of a public health importance of mosquito-borne bartonellae at this point. We believe, however, that our findings should encourage further investigations considering the potential significance of mosquitoes for the transmission of pathogenic and non-pathogenic *Bartonella* species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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