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Original article

Detection of *Candidatus* Neoehrlichia mikurensis, *Borrelia burgdorferi* sensu lato genospecies and *Anaplasma phagocytophilum* in a tick population from Austria

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ABSTRACT

Candidatus Neoehrlichia mikurensis DNA was discovered in *Ixodes ricinus* ticks in 1999 and is referred to as an emerging human pathogen since its first detection in patients with febrile illness reported in 2010. In recent years, *Ca.* Neoehrlichia mikurensis has been detected in ticks from several European, Asian, and African countries. However, no epidemiological data exist for Austria, which is a highly endemic region for tick-transmitted diseases. To assess the geographic spread and prevalence of *Ca.* Neoehrlichia mikurensis sympatric with other tick-transmitted pathogens, we analysed 518 *I. ricinus* ticks collected in 2002 and 2003 in Graz, Austria. The prevalence of *Ca.* Neoehrlichia mikurensis was 4.2%, that of *Borrelia burgdorferi* sensu lato 25.7%, and that of *Anaplasma phagocytophilum* 1%. Coinfections with *Ca.* Neoehrlichia mikurensis and *B. burgdorferi* sensu lato were found in 2.3% of all ticks. Thus, the results show a relatively high prevalence of *Ca.* Neoehrlichia mikurensis in Austrian ticks suggesting a high probability for the occurrence of undiagnosed human infections in Austria.

Introduction

Ticks are second to mosquitoes as vectors of human vector-borne pathogens worldwide (de la Fuente et al., 2008). In western and central Europe, the hard tick *Ixodes ricinus* is the primary tick vector for many human pathogens (Heyman et al., 2010). The incidence of tick-borne diseases has increased over the past decades due to global warming with subsequent altitudinal and latitudinal migration and extended activity of the tick vector. This has been exemplarily shown for Lyme disease/borreliosis, which is the most prevalent tick-borne disease in temperate regions in the Northern hemisphere (Medlock et al., 2013, and references therein). It is caused by spirochaetes of the *Borrelia burgdorferi* sensu lato (s.l.) group, which includes human pathogenic genospecies such as *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto (Hengge et al., 2003). Lyme borreliosis commonly begins with an erythema migrans at the site of an infectious tick bite and with flu-like symptoms. Untreated patients may develop a multisystem disorder with affection of the nervous- or musculoskeletal system, or the heart (Hengge et al., 2003). Because of the high prevalence of Lyme borreliosis, physicians are aware of a possible infection with *B. burgdorferi* s.l. after a tick bite. Hence, they routinely perform easy-accessible diagnostic procedures and are familiar with therapy strategies. Besides *B. burgdorferi* s.l., *I. ricinus* ticks less often transmit a variety of other pathogens that can cause harmful diseases such as *Anaplasma phagocytophilum* and *Candidatus* Neoehrlichia mikurensis. Both species belong to the family of Anaplasmataceae (Dumler et al., 2007) and were recently described to cause infectious diseases with potentially life-threatening outcome. *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis, which is a moderate, self-limited flu-like illness in most cases, but fatal courses have been observed in immunocompromised individuals (Dumler et al., 2007). The first European case of human granulocytic anaplasmosis was reported from Slovenia in 1997 (Petrovec et al., 1997) and probably less than

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100 laboratory-confirmed European cases are currently known (Edouard et al., 2012; Jin et al., 2012). *Candidatus* Neoehrlichia mikurensis was first detected in *I. ricinus* ticks from the Netherlands in 1999 and referred to as an *Ehrlichia*-like species ('Schotti variant'). Since then, it was found in questing ticks from several European, Asian, and African countries (Alekseev et al., 2001; Andersson et al., 2013; Brouqui et al., 2003; Capelli et al., 2012; Fertner et al., 2012; Jahfari et al., 2012; Kamani et al., 2013; Li et al., 2012; Lommano et al., 2012; Maurer et al., 2013; Movila et al., 2013; Nijhof et al., 2007; Rar et al., 2010; Richter and Matuschka, 2012; Schouls et al., 1999; Shpynov et al., 2006; van Overbeek et al., 2008; Wielinga et al., 2006). Therefore, ticks are generally considered as the main vectors for this pathogen to humans. The first human infections with *Ca.* Neoehrlichia mikurensis were reported in 2010, and only 15 cases of human neoehrlichiosis have been published in Europe and Asia until September 2013. They were characterized by fever, malaise, weight loss, and septicaemia in previously healthy individuals as well as in immunocompromised patients (Fehr et al., 2010; Li et al., 2012; Maurer et al., 2013; Pekova et al., 2011; von Loewenich et al., 2010; Welinder-Olsson et al., 2010). Due to its recent detection in ticks and the small but growing number of diagnosed clinical cases, human infection with *Ca.* Neoehrlichia mikurensis is referred to as an emerging infectious disease (Lommano et al., 2012; Maurer et al., 2013).

Austria with its location in central Europe and its temperate climate is predestined to be a highly endemic region for tick-transmitted diseases, which is particularly proved for *B. burgdorferi* s.l. The spirochaete has been found in 16% of ticks (Leschnik et al., 2012), which explains the high rate of seropositive individuals in high-risk groups for tick bites (e.g., 54% among healthy hunters) (Cetin et al., 2006) and one of the highest Lyme borreliosis incidences in central Europe with 130 cases per 100,000 individuals (Hengge et al., 2003). *Anaplasma phagocytophilum* has been identified only occasionally in ticks from Austria (Leschnik et al., 2012; Polin et al., 2004; Sixl et al., 2003), and only 8 cases of human infection have been diagnosed in this area so far (Haschke-Becher et al., 2010; Vogl et al., 2010; Walder et al., 2006). However, we have shown that 20% of patients with erythema migrans from southeastern Austria are seropositive for *A. phagocytophilum* (unpublished data). *Candidatus* Neoehrlichia mikurensis has not been detected in Austrian ticks so far, and no case of human disease has been reported in Austria by September 2013.

In 2002 and 2003, we collected questing *I. ricinus* ticks in southeastern Austria with the aim to determine the prevalence of *B. burgdorferi* s.l. and *A. phagocytophilum*. Recent reports of a high prevalence of *Ca.* Neoehrlichia mikurensis in ticks from neighbouring countries such as Switzerland, Germany, and Italy (Capelli et al., 2012; Lommano et al., 2012; Richter and Matuschka, 2012) prompted us to investigate these ticks collected in 2002/2003 also for the presence of this pathogen. This helps to assess the chronological and geographical spread of *Ca.* Neoehrlichia mikurensis in Europe.

Materials and methods

Study area and tick sampling

This study was carried out in a mixed woodland recreational area in the city of Graz in southeastern Austria (47° 4′ 0″ N, 15° 26′ 0″ E, altitude 353 m). The climate is generally temperate with average annual temperatures around 10 °C and annual rainfalls of 800 mm. The sampling area is popular for walkers and hobby joggers and is well known for a high tick activity. Ticks were collected by the flagging method in June and September 2002 and 2003. Briefly, we dragged a 1.5-m^2 white flannel cloth over the low vegetation for a distance of 2–5 m for each drag. Then, the cloth was turned around and the attached *I. ricinus* ticks were gently removed with plastic tweezers. Groups of 10 ticks were put into a 50-ml conical tube with humidified sterile gaze and stored at 4 °C until DNA extraction. Directly before DNA extraction, determination of *I. ricinus* was reassured, and the life stages were identified based on morphological characters.

DNA extraction

Ticks were removed from the 50-ml conical tubes and separately washed twice in 70% ethanol. After mechanical crushing with a sterile steel probe, DNA was extracted from each tick individually using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), protocol 'DNA purification from tissues'. Briefly, crushed ticks were incubated with 180 μl buffer ATL and 20 μl proteinase K on a shaking water bath at 56 °C overnight to ensure tissue lysis. The next steps were performed according to the manufacturer's protocol. DNA was eluted in a total volume of 200 μl of elution buffer. Quantity and purity of DNA was measured at 260 nm and 280 nm in a Beckman DU 600 spectrometer (Beckman Coulter, Vienna, Austria). DNA was stored at −80 °C until analysis was done.

PCR for Ixodes ricinus housekeeping gene

To confirm successful extraction of tick DNA, 5 randomly chosen samples of each extraction batch consisting of 20 ticks were subjected to a PCR targeting the tick 16S mitochondrial ribosomal DNA gene as described previously (Norris et al., 1996). Briefly, the 50-μl reaction mixture contained 1× PCR, 2.5 mM $MgCl_2$, 200 mM dNTPs, 1.25 U Taq polymerase, 0.5 mM of forward primer 16S+1 (5′-CCGGTCTGAACTCAGATCAAGT-3′) and reverse primer 16S−1 (5′-CTGCTCAATGATTTTTTAAATTGCTGTGG-3′). The cycling protocol included a polymerase activation step at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 48 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min and was performed on a GeneAmp 2700 (Applied Biosystems, Vienna, Austria). PCR products were visualized on a 2% agarose gel (Sea Kem LE Agarose, Biozym, Hessisch Oldendorf, Germany) stained with 1 mg/ml ethidium bromide (Bio-Rad, Vienna, Austria). For each sample, a PCR amplicon was detected.

PCR for Borrelia burgdorferi sensu lato

Extracted DNA was subjected to a semi-nested conventional PCR that was previously developed as a reliable detection method for all European *B. burgdorferi* s.l. genospecies (Michel et al., 2004). This PCR amplifies an 818-bp fragment of the *ospA* gene. Briefly, 5 μl of DNA extract was tested in a 50-μl reaction volume containing 5 μl of 10× PCR buffer containing 1.5 mM $MgCl_2$, 200 mM dNTPs, and 0.5 U Taq polymerase. Primers used in the first PCR were V1a forward (5′-GGGAATAGGTCTAATATTAGC-3′), V1b forward (5′-GGGGATAGGTCTAATATTAGC-3′), and R2 reverse (5′-CATAAATTCTCCTTATTTTAAAGC-3′) at a concentration of 10 pmol each. For the semi-nested PCR, 5 μl of the reaction mixture of the first PCR was used with 100 pmol of primers V3a forward (5′-GCCTTAATAGCATGTAAGC-3′), V3b forward (5′-GCCTTAATAGCATGCAAGC-3′), and R2. All PCR reagents and primers were obtained from Applied Biosystems. Cycling conditions for both PCR runs were 95 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 48 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The *B. garinii* strain Pfri (In-house strain, Max von Pettenkofer Institute, Munich, Germany) was used as a positive control in each PCR experiment. PCR amplicons were visualized on a