First detection of tick-borne "*Candidatus* Neoehrlichia mikurensis" in Denmark 2011

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This is the first reporting of the tick-borne zoonotic bacterium "*Candidatus* Neoehrlichia mikurensis" in Denmark. A total of 2,625 *Ixodes ricinus* ticks from 58 locations in Denmark were collected and analysed for "*Ca.* Neoehrlichia mikurensis". A nested PCR revealed the presence of the bacterium at three geographically separate locations, which indicates that it is widely established in ticks.

Background

Since 2009, "*Candidatus* Neoehrlichia mikurensis" has been correlated with severe disease in immunocompromised people. A total of six human cases originating from Sweden [1], Germany [2], Switzerland [3] and the Czech Republic [4] have been described in the literature. In 2011, the bacterium was also isolated from a post-operative dog in Germany [5]. General clinical features in human patients have included recurrent fever, erysipelas-like rashes, arthralgias and thromboembolisms [1-4]. The infection responds well to doxycycline [1]. The pathogenic potential of "*Ca.* Neoehrlichia mikurensis" may be correlated with its putative tropism for endothelial cells [2,4].

So far little is known on the distribution, risk areas and reservoir of "*Ca.* Neoehrlichia mikurensis". If the infectious cycle resembles the other *Ehrlichia* bacteria, it has its reservoir in wild mammals and is transmitted by ticks. Accidentally humans may become infected [6]. In this study we examined *Ixodes ricinus* ticks in Denmark for the presence of "*Ca.* Neoehrlichia mikurensis" using PCR. This is the first survey for "*Ca.* Neoehrlichia mikurensis" in Denmark and in ticks in northern Europe.

Sampling methods and analysis

The analysed ticks originated from two different sampling procedures (Table): ticks collected by flagging (n=1,552) and a tick DNA/RNA archive (n=1,073).

Flagging was performed during September 2011 at four distinct localities known for an abundance of ticks and a recent history of human cases of tick-borne encephalitis (TBE). A white flannel flag was dragged over the vegetation and 1,552 ticks collected into plastic containers. These were frozen a few hours after collection and stored at -20°C for up to one month before DNA extraction. The flagging for ticks was carried out as part of a project investigating TBE virus. However, with the emergence of "*Ca.* Neoehrlichia mikurensis" as a public concern in our neighbouring countries, the DNA was additionally screened for the presence of this potentially emerging pathogen in December 2011 and January 2012.

Furthermore, a tick archive was investigated for the presence of "*Ca.* Neoehrlichia mikurensis". During 2010 and 2011, the Veterinary Institute's National Center for Wildlife Health collected 1,073 ticks from roe deer submitted for diagnosis and routine surveillance from 53 locations in Denmark. A sample of 40 ticks from a domestic sheep flock was additionally included in the archive. After removal, ticks were stored in ethanol for up to 1.5 years. DNA and RNA were extracted and stored as a tick archive of genetic material.

Before laboratory analysis, ticks from sites with large sample sizes were distributed into smaller pools (Table). Ticks were crushed and homogenised in 1 ml

Table

Ticks collected by two different sampling procedures in Denmark in 2010 and 2011 (n=2,625)

Sampling procedure	Number of locations	Number of pools	Mean number of ticks per pool (range)	Nymphs	Adult males	Adult females	**Total ticks**
Flagging	4	21	74 (3–106)	1,444	52	56	**1,552**
Tick DNA/RNA archive	54	58	19 (1–62)	30	399	644	**1,073**
Total	**58**	**79**		**1,474**	**451**	**700**	**2,625**

phosphate buffered saline (PBS). The homogenate was centrifuged and supernatant collected and stored at -80°C until DNA was extracted from 200 µL homogenate in a MagNA Pure 96 robot using MagNa Pure 96 DNA and Viral Nucleic Acid Small Volume Kit version 4.0 (Roche) according to the manufacturer's instructions.

The 16S rRNA gene was amplified with the universal bacterial primers 519F (5'-CCA GCA GCC GCG GTA ATA C-3') and 1054R (5'-ACG AGC TGA CGA CRR CCA TG-3') [7]. This was followed by a nested PCR with newly designed 16 S rRNA gene primers specific for "*Ca.* Neoehrlichia mikurensis": micurensis729F (5'-GGC GAC TAT CTG GCT CAG-3') and micurensis1016R (5'-GCC AAA CTG ACT CTT CCG-3'). The positive PCR amplicons were sequenced on an ABI PRISM 373 DNA Sequencer (PE Biosystems, Foster City, United States) and aligned with published 16S rRNA gene sequences using SEQMATCH in the Ribosomal Database Project (http://rdp.cme.msu.edu).

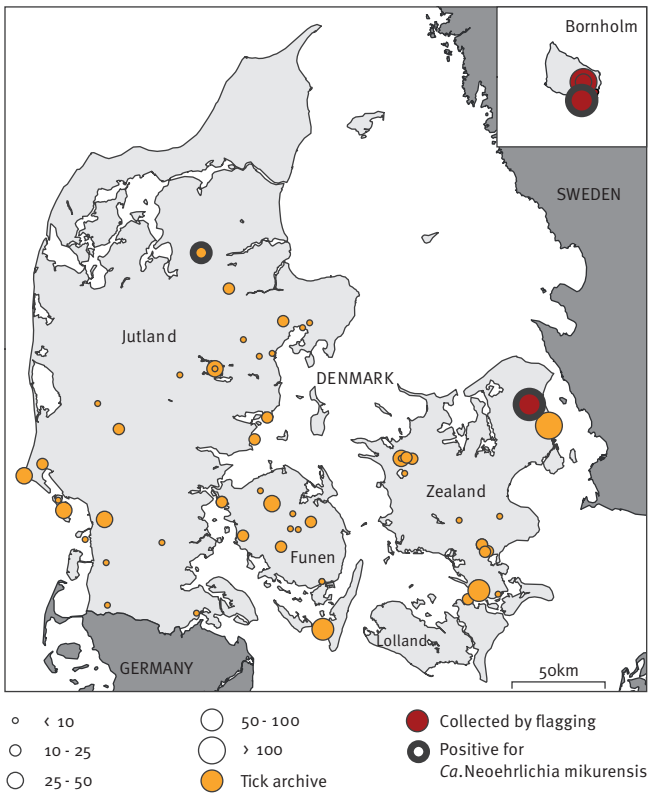
Results

Three of the 79 pools contained ticks positive for "*Ca.* Neoehrlichia mikurensis"; they originated from three locations separated from each other by the sea (Figure).

The first positive sample came from flagging in Øster Sømarken on the island of Bornholm. From this location six pools with a total of 467 ticks were collected. One pool, containing 100 nymphs, was found positive for "*Ca.* Neoehrlichia mikurensis". The second positive pool was found after flagging in the forest of Tokkekøb Hegn in Northern Zealand in the same one-hectare area where emerging TBE in both *I. ricinus* and humans were reported in 2009 [8]. At this location eight pools with a total of 736 ticks were collected, of which one, containing 100 nymphs, was found positive. The third positive sample originated from a pool of 12 male and 28 female ticks collected for the tick archive from domestic free-grazing sheep in the area of Viborg on the Danish mainland. All three isolates were verified to be 100% similar to 16S rRNA gene sequences from "*Ca.* Neoehrlichia mikurensis".

Figure

Location of tick collection, Denmark, 2010 and 2011 (n=1,552)



Discussion

"*Ca.* Neoehrlichia mikurensis" belongs to the family *Anaplasmataceae* [9] which comprises a variety of emerging tick-borne human pathogenic bacteria [10]. Former studies have suggested a potential widespread occurrence of "*Ca.* Neoehrlichia mikurensis" in the wild fauna of Asia and Europe, including our neighbouring countries [9,11-14], but it has never been reported in Denmark. In this study we examined 2,625 *I. ricinus* ticks divided in 79 pools and identified the presence of "*Ca.* Neoehrlichia mikurensis" at three distinct locations, indicating that the bacterium is widely distributed in the Danish tick population.

The recorded minimum prevalence of three of 2,625 was, however, substantially lower than that found in studies from the Netherlands in 2006, the Baltic regions of Russia in 1997–98 and a recent central European study, which all estimated 6–7% of the ticks to carry the bacterium [12,14,15]. The latter study investigated ticks from the Czech Republic, France, Germany, Poland and Portugal and found a prevalence ranging from 0% to 10% that was highest in the Czech Republic and Germany [14]. In southern Sweden, "*Ca.* Neoehrlichia mikurensis" was in 2008 found to be widespread in the wild rodents of this region with a prevalence ranging from 0% to 12.5% in the investigated locations [11].

An increase and spread of other tick-borne infections such as Lyme borreliosis and TBE, has been reported in Denmark and neighbouring countries. This trend has been attributed to increased awareness, climate change and increasing tick populations [16,17]. In this study the tick-borne pathogen was found at a known TBE site on Bornholm and at an emerging TBE site in Tokkekøb Hegn forest [8]. The recent appearance of several human clinical cases of "*Ca.* Neoehrlichia mikurensis" infection in Europe, as well as the findings in the wild fauna, indicate that this is an emerging tick-borne pathogen. However, lack of knowledge and a diagnostic test combined with a low pathogenic potential may have hindered previous detection in Denmark. Whether or not the newly reported cases are the result of previous misdiagnosis or a true emerging risk, it is important that medical doctors in the affected areas are aware of the risk for immunocompromised patients. The State Serum Institute will now establish a

diagnostic assay. Finding the pathogen on production animals suggests there may be veterinary risk as well.

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