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First report of the zoonotic tapeworm *Echinococcus multilocularis* in raccoon dogs in Estonia, and comparisons with other countries in Europe

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ABSTRACT

The raccoon dog (*Nyctereutes procyonoides*) is an alien species in Europe and an important vector of zoonotic diseases. However, compared to the red fox (*Vulpes vulpes*), less attention has been paid to the raccoon dog as a potentially important host for *Echinococcus multilocularis*, the infective agent of alveolar echinococcosis, which is an emerging infectious disease with a high mortality rate. We examined the small intestines of 249 Estonian raccoon dogs and found 1.6% of individuals to be infected with *E. multilocularis*. The relatively large difference between this prevalence and that found in sympatric red foxes (31.5%) sampled during the same time period might be due to differences in diet: red foxes consume significantly more arvicolid rodents – the main intermediate hosts of the parasite – especially during the coldest period of the year when raccoon dogs hibernate. Nonetheless, given the relatively high density of raccoon dogs, our results suggest that the species also represents an important definitive host species for *E. multilocularis* in Estonia. Compared with other countries in Europe where *E. multilocularis*-infected raccoon dogs have been recorded (Latvia, Lithuania, Poland, Germany, and Slovakia), the prevalence in Estonia is low. The longer hibernation period of raccoon dogs at higher latitudes may explain this pattern. Both mitochondrial and nuclear loci were analysed for Estonian isolates: based on EmsB microsatellite genotyping the Estonian isolates shared an identical genotype with *E. multilocularis* in northern Poland, suggesting a common history with this region. The data from more than a quarter of the mitochondrial genome (3558 bp) revealed two novel haplotypes specific to Estonia and placed them into the same haplogroup with isolates from other European regions.

Considering that the raccoon dog is becoming increasingly widespread and is already relatively abundant in several countries in Europe, the role of the species must be taken into account when assessing the *E. multilocularis* related risks to public health.

1. Introduction

The raccoon dog (*Nyctereutes procyonoides*) is an invasive mammal species in Europe that is becoming increasingly widespread. It was introduced from the Far East to western Eurasia during 1928–1958, where it subsequently spread further, both naturally and with the help of humans (Heptner and Naumov, 1998). For example, it is known that 86 individuals originating from Kalinin oblast (Russia) were released in Estonia in 1950, though the first raccoon dog in Estonia was recorded already in 1938 (Aul et al., 1957). Being an opportunistic omnivorous mammal, raccoon dog is considered, especially when its numbers are high, a threat to a number of species, including protected birds and amphibians (Kauhala and Kowalczyk, 2011). The raccoon dog population in Estonia is regulated by infectious diseases such as rabies and sarcoptic mange, and by hunting. However, as the result of a vaccination campaign initiated in 2005, rabies has been eradicated from Estonia (Pärtel, 2013). In parallel, the raccoon dog population has increased (Veeroja and Männil, 2014).

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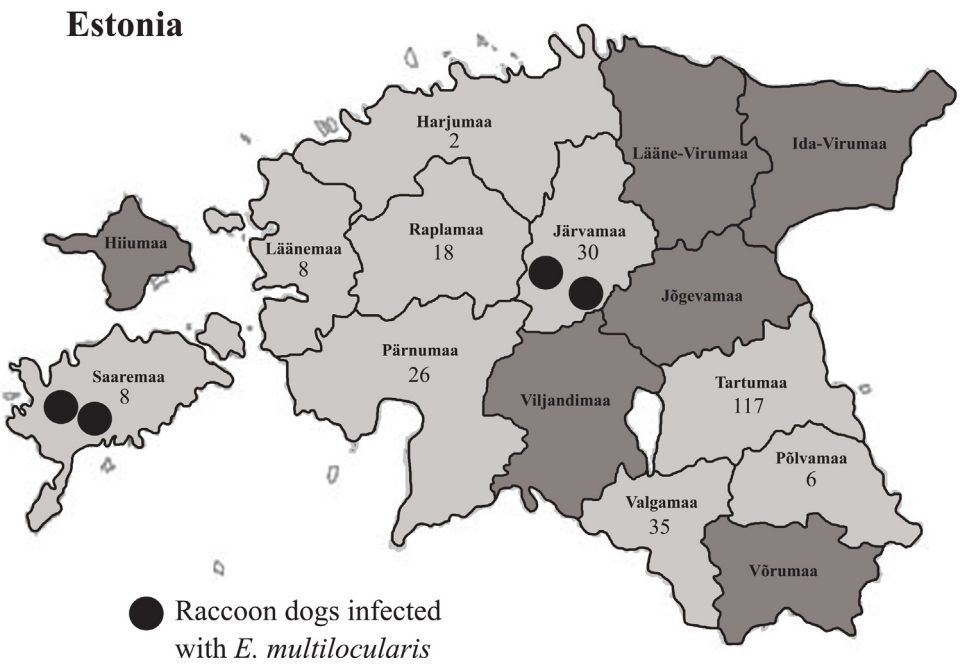

Fig. 1. Distribution of analysed raccoon dogs in different counties in Estonia (light grey). The number of animals examined is shown below the county name, and animals infected with *E. multilocularis* are shown by black circles.

The raccoon dog is an important vector for numerous zoonotic parasites, including *Echinococcus multilocularis*, a cestode that is widely distributed in the Northern Hemisphere (Sutor et al., 2014). The distribution area of *E. multilocularis* in Europe appears to be increasing, with several new endemic areas, including Estonia, identified during recent decades. The main definitive host of *E. multilocularis* in Europe is the red fox (*Vulpes vulpes*), though other wild canids and felids are susceptible, as are domestic dogs and cats (Eckert et al., 2001; Kapel et al., 2006). The other recorded wild definitive host species in Europe are the arctic fox (*Vulpes lagopus*; formerly *Alopex lagopus*), grey wolf (*Canis lupus*), golden jackal (*Canis aureus*), raccoon dog, wildcat (*Felis silvestris*) and lynx (*Lynx lynx*) (Eckert et al., 2001; Fuglei et al., 2008; Szell et al., 2013). Wild arvicolid rodents serve as the main intermediate host for *E. multilocularis*. While infection with this parasite is typically asymptomatic for the definitive host, it causes severe illness with a high mortality rate in intermediate host species, including humans. Humans can acquire *E. multilocularis* infection by ingesting parasite eggs via direct contact with a definitive host or through contaminated water, soil or food (Eckert et al., 2001).

In Europe, *E. multilocularis*-infected raccoon dogs were first reported in 2001 from Germany (Theiss et al., 2001). Subsequent reports have followed from Poland (Machnicka-Rowinska et al., 2002), Latvia (Bagrade et al., 2008), Slovakia (Hurnikova et al., 2009) and Lithuania (Bružinskaite-Schmidhalter et al., 2012). In all cases, the prevalence of *E. multilocularis* was found to be at least double the level found in the raccoon dog's native range (1.4%) in the Far-East Russia (Judin, 1977). In Estonia, both *E. multilocularis* and *Echinococcusgranulosus* have been recently reported in both rural and urban areas (Moks et al., 2005, 2006, 2008; Laurimaa et al., 2015a, 2015b). As about 30% of wild red foxes in Estonia are infected with *E. multilocularis* (Moks et al., 2005), and the parasite has also been found in urban environments in red foxes in the country (Laurimaa et al., 2015a), our aim was to estimate the parasite prevalence in raccoon dogs in order to ascertain whether it should be regarded also as a threat to public health. Moreover, we also aimed to evaluate the genetic relation of Estonian *E. multilocularis* isolates with those from other countries in Europe.

2. Materials and methods

In total, 249 raccoon dog carcasses (legally harvested by hunters for purposes other than this project) were examined for *Echinococcus* parasites. Samples were collected between September 2010 and April 2012 from different parts of Estonia, covering 9 of 15 counties (Fig. 1).

The intestinal organs were removed from carcasses and stored at −80 °C for at least 5 days as a safety precaution prior to parasitological examination (Eckert et al., 2001). Small and large intestines were separated and examined by the sedimentation and counting technique (SCT) (Hofer et al., 2000). Parasites were stored in 96% ethanol. *Echinococcus* tapeworms were identified according to their morphology after Abuladze (1964).

For genetic identification of tapeworm species, genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. A 120 bp fragment of *t*RNA-Ile/Lys in *E. multilocularis* mitochondrial DNA was amplified with species-specific primers EMfor1 and EMrev1 (Laurimaa et al., 2015a). PCR product purification and sequencing procedures followed Saarma et al. (2009). All positive samples were sequenced with the primers used for PCR.

2.1. Genetic comparisons

2.1.1. EmsB microsatellite genotyping

From each infected raccoon dog, five worms were isolated to perform EmsB genotyping as previously described (Knapp et al., 2007; Umhang et al., 2014). Briefly, after PCR reaction with EmsB primers, the PCR products were submitted to capillary electrophoresis. For each sample, the size and height of each peak constituting the EmsB profile were determined with GeneMapper 4.1. Clustering analysis was performed using the R statistical software and applying the usual genetic threshold of 0.08 in order to compare Estonian genotypes with the others EmsB profiles previously obtained from different countries in Europe: France (number of worms = 566; number of foxes = 162), Switzerland (84; 19), Germany (87; 18), Austria (98; 22), Slovakia (63; 14), Poland