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ORIGINAL PAPER

Echinococcus multilocularis in Denmark 2012–2015: high local prevalence in red foxes

H. H. Petersen[1] · M. N. S. Al-Sabi[1,2] · H. L. Enemark[1,3] · C. M. O. Kapel[4] · J. A. Jørgensen[4] · M. Chriél[1]

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Abstract

In Western Europe, the *Echinococcus multilocularis* lifecycle is predominantly sylvatic, typically involving red foxes (*Vulpes vulpes*) as the main definitive hosts with *Microtus* spp. and *Arvicola* spp. as intermediate hosts. During a 4-year surveillance study (2012–2015), Danish red foxes and raccoon dogs ($n = 1345$) were examined for *E. multilocularis*. Moreover, 134 insectivores and rodents collected in South Jutland during spring and summer 2016 were examined for the presence of metacestodes. The sedimentation and counting technique and molecular typing were used to identify *E. multilocularis* infections in the carnivores, while the rodent livers were examined macro- and microscopically for parasite lesions. Following morphological identification of *E. multilocularis* adult worms, the identity was verified by sequence analysis of the 12S rRNA gene in most cases ($n = 13$). *Echinococcus multilocularis* infection was demonstrated in 19 red foxes (*Vulpes vulpes*) originating from only two specific areas of South Jutland, namely Højer and Grindsted, and in two raccoon dogs (*Nyctereutes procyonoides*), originating from Højer. In Højer, 28.5% (CI 95% 11.7–45.3) of the examined red foxes were *E. multilocularis* positive per year. Moreover, positive red foxes were identified each year from 2012 to 2015, while *E. multilocularis* positive red foxes were only identified in Grindsted in 2013 (4.0%) and 2014 (6.4%). In contrast, all collected rodents were negative for *E. multilocularis*. We conclude that *E. multilocularis* is locally endemic in South Jutland with a high local prevalence in Højer.

Keywords *Echinococcus multilocularis* · Denmark · Red fox (*Vulpes vulpes*) · Raccoon dog (*Nyctereutes procyonoides*) · Surveillance · Post mortem

Background

Echinococcus multilocularis is a small tapeworm belonging to the family Taeniidae. In Europe, the typical life cycle is sylvatic, including red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) as main final hosts (Oksanen et al. 2016), and the rodent species *Microtus* spp. and *Arvicola* spp. as main intermediate hosts (Woolsey et al. 2015, 2016; Beerli et al. 2017; Romig et al. 2017). Adult *E. multilocularis* are localised in the small intestine, from where eggs are excreted with faeces to the environment. The intermediate hosts become infected when ingesting infective eggs from the environment. Following hatching of the eggs, the oncospheres migrate to internal organs and develop into cyst-forming metacestodes that produce protoscolices. When the final host eat an infected rodent, the metacestode stage develops to adult intestinal tapeworms. If humans accidentally ingest infective eggs, they may develop alveolar echinococcosis, a severe zoonotic disease in Europe (Bouwknegt et al. 2018). Infections in humans are rare; nonetheless, the clinical implications are critical and cause considerable public health concern due to treatment costs and high mortality if left untreated (Torgerson et al. 2008). In Denmark, no autochthonous human cases have hitherto been reported (Statens Serum Institute 2017). However, as human echinococcosis is not notifiable in Denmark, the actual number of cases is unknown.

✉ H. H. Petersen
hhpet@vet.dtu.dk

[1] Section for Diagnostics and Scientific Advice, National Veterinary Institute, Technical University of Denmark, Kemitorvet, DK-2800 Kongens Lyngby, Denmark

[2] Department of Pharmacy, Faculty of Pharmacy, Al-Zaytoonah University of Jordan, Queen Alia Airport St. 594, P.O. Box 130, 11733 Amman, Jordan

[3] Department of Animal Health and Food Safety, Norwegian Veterinary Institute, P.O. Box 750, Sentrum, 0106 Oslo, Norway

[4] Department of Plant and Environmental Sciences, Faculty of Science, Section for Organismal Biology, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

Echinococcus multilocularis in wildlife is endemic in large parts of Europe, and infections in red foxes are reported in countries neighbouring Denmark (Lind et al. 2011; Denzin et al. 2014). In North-Western Germany, the estimated *E. multilocularis* prevalence in red foxes has increased from 12 to 20% between 1991 and 2005 (Berke et al. 2008), and in Central Germany from 12 to 42% between 1990 and 2009 (Staubach et al. 2011). The increasing red fox populations probably influenced *E. multilocularis* expansion across Europe (Davidson et al. 2012; Liccioli et al. 2015). Furthermore, raccoon dogs are observed as final hosts for *E. multilocularis* throughout Europe (Schwarz et al. 2011; Bružinskaitė-Schmidhalter et al. 2012; European Food Safety Authority 2015; Laurimaa et al. 2015). The raccoon dog originates from East Asia but is now widespread in Northern and Eastern Europe including Denmark (Wilson and Reeder 2005). The presence of red foxes and raccoon dogs in Denmark and the mild and humid climate favours the survival of *E. multilocularis* eggs and the spread of infection (Takeuchi-Storm et al. 2015), as does the presence of intermediate hosts (Winge 1908; Baggøe and Jensen 2007).

The first wildlife *E. multilocularis* infection in Denmark was diagnosed in 1999 in an epidemiological study of helminth infections in 1040 red foxes (Saeed et al. 2006). The infected red fox was road killed west of Copenhagen and harboured 53 adult worms. Additional two red foxes from the Copenhagen area were diagnosed with *E. multilocularis* in 2000 by the sedimentation and counting technique (SCT) and confirmatory PCR (Saeed et al. 2006). Throughout the following decade, only few red foxes were screened for *E. multilocularis* infections, i.e. from 2006 to 2008; 118 red foxes from the greater Copenhagen area and South Jutland were examined for *E. multilocularis*, none were positive (Al-Sabi et al. 2014); and from 2009 to 2012, 99 raccoon dogs and 384 red foxes from all regions of Denmark were examined for *E. multilocularis* (Al-Sabi et al. 2013). In the latter study, one red fox shoot in November 2011 in South Jutland was *E. multilocularis* positive, harbouring 20 adult worms (Enemark et al. 2013; Al-Sabi et al. 2013). This finding led to a 4-year surveillance program for *E. multilocularis* in Danish wildlife initiated in 2012 and a survey of intermediate hosts collected in South Jutland in 2016. The results are presented in this paper.

Materials and methods

Study design

A national *E. multilocularis* surveillance program was implemented for wild carnivores in Denmark 2012–2015. The surveillance program was designed by the National Veterinary Institute, The Technical University of Denmark (DTU-VET) and financed by the Danish Veterinary and Food Administration. Selection of carnivores for the study was-risk based with a special focus on Mid- and South Jutland due to close proximity of this region to the endemic area of North Germany. This sampling strategy implied a variation in the number of examined animals from each region. The foxes were road-killed or hunted wild carnivores collected by the Danish Nature Agency or by local hunters. The geographical origin of the carnivores was recorded. However, if the exact location was not available, the coordinates of the region were used instead.

Intermediate hosts were collected in 2016 in Margrethe Kog, a marshland in the Højer area, in the Southwestern part of Jutland adjacent to the German border. The rodents were trapped alive (Uggland-special, Grahnab, Sweden) or by using mechanical traps (Topcats, Andermatt Biocontrol AG, Switzerland).

Sampling

A total of 1345 wild carnivores, collected 2012–2015, and 134 rodents and insectivores collected during spring and summer 2016 were included in the study. Of the carnivores, 1073 were red foxes (*V. vulpes*) and 272 raccoon dogs (*N. procyonoides*).

Of the rodents, eight were common voles (*Microtus arvalis*), 15 field voles (*Microtus agrestis*), 31 wood mice (*Apodemus sylvaticus*) and 15 harvest mice (*Micromys minutus*). Of the insectivores, 47 were common shrews (*Sorex araneus*), and 16 pygmy shrews (*Sorex minutus*).

Examination of wild carnivores

The dead carnivores were transported in sealed plastic bags at − 20 °C to DTU-VET and left at − 80 °C for a minimum of 5 days to inactivate potential infective parasites before necropsy. The small intestines were examined for *E. multilocularis* worms. When the intestine was unavailable, faeces were examined for *E. multilocularis* eggs by real-time PCR.

The examination for *E. multilocularis* worms in the small intestine was carried out as described by Hofer et al. (2000) using the sedimentation and counting technique (SCT). The sediment from the SCT was examined in small portions under a stereomicroscope, and adult tapeworms were counted and identified as *E. multilocularis* by morphologic features (Eckert et al. 2001) at DTU-VET. Faeces for analysis of *E. multilocularis* eggs was analysed by a semi-automated magnetic capture probe-based DNA extraction and real-time PCR method by the Swedish Veterinary Institute (SVA) (RT-PCR) (Isaksson et al. 2014). The number of animals analysed by SCT and RT-PCR, respectively, are listed in Table 1.

Examination of rodents and insectivores

Upon dissection, each animal liver was examined macroscopically for suspect parasite lesions by placing the liver in a plastic bag and compressing it between two microscope slides. Examination for internal lesions or cysts was performed by

Table 1 Occurrence of *E. multilocularis* in wild carnivores by host species, geographical origin, sampling year and examination method (*SCT* sedimentation and counting technique, *PCR* semi-automated magnetic capture probe based DNA extraction and real-time PCR)

Region	Species	Method	2012		2013		2014		2015		Total	
			Positive	*n*	Positive	*n*	Positive	*n*	Positive	*n*	Positive	*n*
North-East Zealand	Foxes	SCT	0	17	0	18	0	6	0	1	0	42
		RT-PCR	–	–	–	–	–	–	0	3	0	3
South-West Zealand	Foxes	SCT	0	103	0	27	0	27	–	–	0	157
		RT-PCR	–	–	–	–	–	–	–	–	–	–
Funen[1]	Foxes	SCT	0	8	0	5	0	18	–	–	0	31
		RT-PCR	–	–	–	–	–	–	–	–	–	–
North Jutland	Foxes	SCT	0	30	0	73	0	26	0	1	0	130
		RT-PCR	–	–	–	–	–	–	0	1	0	1
	Raccoon dogs	SCT	0	13	0	8	0	6	0	1	0	28
		RT-PCR	–	–	–	–	–	–	0	4	0	4
Mid Jutland	Foxes	SCT	0	172	0	157	0	37	0	5	0	371
		RT-PCR	–	–	–	–	0	4	0	5	0	9
	Raccoon dogs	SCT	0	38	0	31	0	2	0	6	0	77
		RT-PCR	–	–	–	–	–	–	0	40	0	40
South Jutland	Foxes	SCT	4	122	3	104	9	85	0	6	16	317
		RT-PCR	–	–	–	–	0	4	3	8	3	12
	Raccoon dogs	SCT	0	34	0	34	2	9	0	4	2	81
		RT-PCR	–	–	–	–	0	4	0	38	0	42
Total			4	537	3	457	11	228	3	123	21	1345

[1] Funen is an island in the Region of Southern Denmark. Data is presented individually

back-illumination of the compressed liver under a dissection microscope at × 10–40 magnification. Any white or grey spots and lesions were collected and inspected microscopically, placed in 70% ethanol and stored at 4 °C. Parasitic materials were examined by inspecting the tissue and fluid from freshly cut lesions by light microscopy. Each suspected lesion was subjected to multiplex PCR analysis initially for *Echinococcus* (Stieger et al. 2002) and subsequently broader for taeniids (Trachsel et al. 2007).

Molecular analysis of adult worms

DNA from adult worms collected from 11 red foxes and two raccoon dogs was isolated using Qiamp DNA mini kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Amplification and sequencing of the 12S rRNA gene from each individual animal was done according to Stefanic et al. (2004). Using the software MEGA6 (Tamura et al. 2013), consensus sequences were established, and related sequences in GenBank were retrieved using BLASTn analysis.

Results

Wild carnivores

Most animals were sampled in Mid Jutland and South Jutland and only few originated from other regions, namely Funen, South-West Zealand and North-East Zealand (Fig. 1).

Most animals were examined by SCT (1234, 91.7%), while 111 (8.3%) animals were examined by RT-PCR (Table 1).

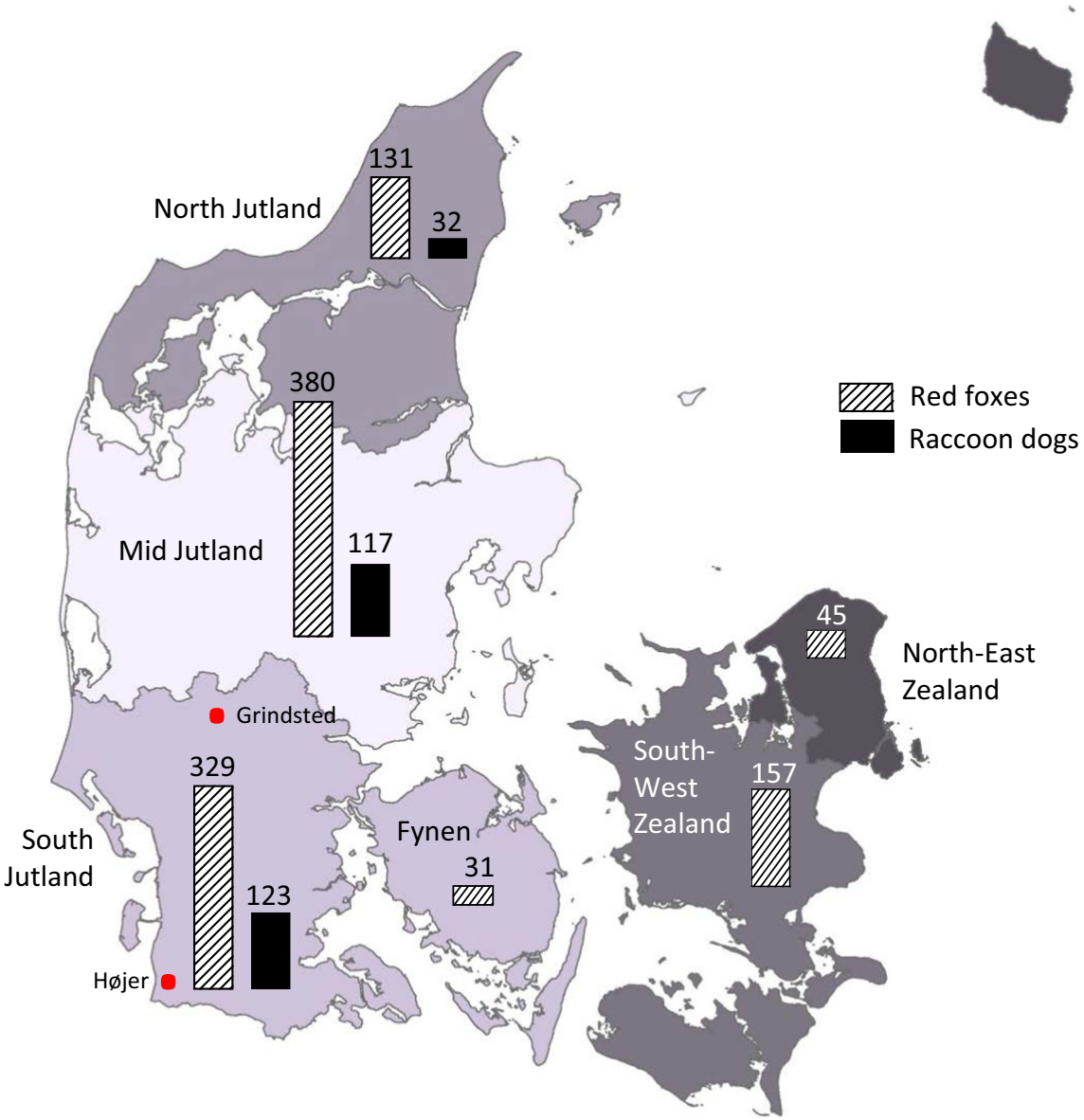

Fig. 1 Map of Denmark illustrating the number of red foxes and raccoon dogs examined for *Echinococcus multilocularis* per region

In total, 21 animals were positive for *E. multilocularis*. Of these, 18 were identified using the SCT and three by RT-PCR (Table 1). All *E. multilocularis* positive animals originated from South Jutland, bordering Germany, more specifically from the two localities Højer and Grindsted. From Højer, 28.5% (CI 95% 11.7–45.3) of the examined red foxes were *E. multilocularis* positive per year, and positive red foxes were identified every year of the 4-year surveillance period. Positive raccoon dogs were only identified in Højer in 2014. From Grindsted, 4.0% of the red foxes were *E. multilocularis* positive in 2013 and 6.4% in 2014. No positive raccoon dogs were detected in Grindsted (Table 2).

The *E. multilocularis* infection intensity in red foxes ranged from 2 to 1527 worms per animal (mean ± S.E 215.6 ± 383.6, median = 33), while the two positive raccoon dogs harboured 53 and 109 worms, respectively. Worm quantity in the three red foxes identified as positive by RT-PCR was not recorded.

The PCR amplification and sequencing of worms from 11 red foxes and two racoon dogs revealed a 198 bp product, which had 100% nucleotide match within the Danish isolates, and 99.5–99% nucleotide match to other *E. multilocularis* sequences available in GenBank (analysis date 23 July 2017). Phylogenetic analysis was inferred using the Neighbor-Joining method (Saitou and Nei 1987) and grouped the Danish isolates of *E. multilocularis* close to isolates from Germany, the UK and Polen (data not shown). Nucleotide sequence data from isolate no. A214 reported in this paper is available in GenBank™ under the accession number: MG593258.

Rodents and insectivores

Suspected lesions from 23 of 123 examined rodents and insectivores were negative for *E. multilocularis*. The PCR analysis identified five samples positive for cestodes. Further sequencing of the PCR products revealed one cyst of *Taenia mustelae* (field vole), two cysts of *Taenia polyacantha* (field vole and common vole), one cyst of *Mesocestodied* sp. (wood mouse) and one cyst (with clear protoscolices) of *Cladotaenia* sp. (field vole).

Discussion

In this study, *E. multilocularis* infection was investigated in 1345 Danish red foxes and raccoon dogs throughout 4 years (2012–2015), with special focus on Mid- and South Jutland (Fig. 1). Infections with *E. multilocularis* were demonstrated in 21 wild carnivores from only two localities in South Jutland, one of which (Højer) borders the endemic area of North Germany (Fig. 1). Infections were primarily identified in red foxes ($n = 19$), but for the first time in Denmark, we observed two *E. multilocularis* positive raccoon dogs. Likewise, none of the examined rodents or insectivores sampled in the high endemic area was positive for *E. multilocularis*.

In the present study, animals from the Copenhagen area (North-East Zealand) were all negative, confirming the low occurrence (0.7%) of *E. multilocularis* infection demonstrated in red foxes in 1999. From 2000 to 2010, no *E. multilocularis* positive animals were identified in Denmark, most likely due to the absence of an active surveillance program. However, in 2011, one *E. multilocularis* positive red fox was detected in Højer (Enemark et al. 2013; Al-Sabi et al. 2013), followed by consistent identification every year during 2012–2015. This may indicate recent invasion of infected red foxes from adjacent endemic areas or detection of established infections of low incidence in suspected areas.

Table 2 Local occurrence of *E. multilocularis* in South Jutland

		Højer[a]			Grindsted[b]		
Year	Host	Animals examined	Positive	Prevalence (CI 95%)	Animals examined	Positive	Prevalence (%)
2012	Foxes	11	4	36.	46	0	0.0
	Raccoon dogs	4	0	0.0	0	–	–
2013	Foxes	26	2	7.7	25	1	4.0
	Raccoon dogs	6	0	0.0	1	0	0.0
2014	Foxes	19	6	31.6	47	3	6.4
	Raccoon dogs	2	2	100.0	0	–	–
2015	Foxes	7	3	42.9	0	–	–
	Raccoon dogs	6	0	0.0	0	–	–
Mean prevalence	Foxes			28.5 (11.7–45.3)			3.5 (−0.7–7.6)
	Raccoon dogs			22.2 (−32.1–76.5)			0.0

[a] Including the postal codes 6240 Løgumkloster, 6261 Bredebro, 6270 Tønder and 6280 Højer

[b] Including the postal codes 7200 Grindsted

Molecular analysis of the 12S rRNA gene confirmed the morphological diagnosis of the Danish isolates of *E. multilocularis*. However, the origin of the infections could not be established using the 12S rRNA gene, and further molecular analysis using for example microsatellite markers is advised for exploring the origin of the Danish infections (Knapp et al. 2007), which is beyond the scope of this study.

In Højer, 28.5% (CI 95% 11.7–45.3) red foxes were positive for *E. multilocularis* per year, and positive foxes were identified all 4 years. The consistent detection of the parasite in this area demonstrates that *E. multilocularis* is endemic in Højer with a high local prevalence, although infected intermediate host remains unidentified. The sample size of the rodents was probably too low to detect any positive animals, as seen in other studies. For example in an endemic area in Belgium with a fox prevalence of 24.55%, only 2 out of 1249 rodents were positive (Hanosset et al. 2008). Hence, future studies must include a larger sample size aiming at increasing the likelihood of detecting positive intermediate hosts and focus on the most important intermediate hosts in Europe like the common vole (*M. arvalis*), the water vole (*Arvicola terrestris*) (which was recently separated into *Arvicola scherman* and *Arvicola amphibius* (Wilson and Reeder 2005)) and the field vole (*M. agrestis*) (Stieger et al. 2002; Pleydell et al. 2004; Romig et al. 2006). In the present study, no *Arvicola* species were examined, and only eight *M. arvalis* and 15 *M. agrestis* were investigated. Despite infected intermediate hosts remain unidentified in Denmark, the *E. multilocularis* lifecycle can only be completed if infected intermediate hosts are present.

Højer is localised in South Jutland, adjacent to the German border and The Wadden Sea. The area consists of lakes, streams, reeds and marshlands; fields grazed by domestic animals and cultivated farmland. The Danish *E. multilocularis* infection highly likely originated from Germany, since the infection has been endemic in red foxes in North Germany for years (Berke et al. 2008; Staubach et al. 2011). However, dogs returning to Denmark after visiting other endemic European areas could have contributed to the introduction of the parasite, if they were not treated with prophylactic anthelmintics according to the guidelines of the Danish Veterinary and Food Administration and the European Food Safety Authority (No author, 2016).

In Grindsted, *E. multilocularis* was identified in a red fox in 2013, followed by additionally three cases in red foxes in 2014. Grindsted is located approx. 100 km north of Højer, and since red foxes can disperse over long distances (Meia and Weber 1995), it is likely that the transmission of parasites to Grindsted originate from Højer or other endemic areas, e.g. in North Germany. Alternatively, the parasite might have been introduced with infected dogs or the infection could have been present with low prevalence in the area or nearby areas all along, although undetected. Since *E. multilocularis* was identified in red foxes in Grindsted for two consecutive years, the environment has most likely been contaminated with infective eggs, increasing the risk of an establish parasite lifecycle in the area. However, parameters like presence of suitable intermediate hosts are also needed for the lifecycle to be fully established, and the density of these hosts play a crucial role in the transmission, establishment and maintenance of the life cycle in a given habitat. The continuous observation of positive animals in only two specific areas in Jutland could be linked to the distribution or absence of intermediate hosts in the remainder of Jutland. Giraudoux et al. (2013) have demonstrated that key rodent species can be used to describe the ranges of *E. multilocularis* over large areas. Moreover, in a study from southern Switzerland, an analysis of rodent communities revealed that *M. arvalis* was present in the *E. multilocularis* endemic area and completely absent from the non-endemic areas (Guerra et al. 2014). Hence, the endemic focus in Switzerland is believed to coincide with the availability of rodents. Unfortunately, our study is limited by the lack of sampling of rodents/voles from other areas than Højer, which prevents the analysis of a possible relationship between the distribution of potential intermediate hosts and the observed spread of *E. multilocularis* in wild animals in Denmark. According to the Danish mammal atlas (Baggøe and Jensen 2007), *M. arvalis* is most prevalent in the southern part of Jutland with a decreasing frequency northwards in Jutland and absents on the majority of the Danish Islands. Contrasting, *M. agrestis* and *A. arvicola* are widespread in Denmark, while *A. schermann* is absent in Denmark (Baggøe and Jensen 2007).

In total, 512 red foxes originating from the central and northern part of Jutland all tested negative for *E. multilocularis*, which may indicate either absence or very low incidence of infection in these areas. Nonetheless, the probability of detecting *E. multilocularis*-infected animals in low endemic areas depends on the sample size. For example, approx. 3000 red foxes were examined before the first positive red fox was identified in Sweden (Lind et al. 2011).

The finding of *E. multilocularis* positive raccoon dogs in Denmark is described in other epidemiological and experimental studies from Europe (Bagrade et al. 2016; Enemark et al. 2013; Kapel et al. 2006; Laurimaa et al. 2015; Schwarz et al. 2011). Together with the high biotic potential for *E. multilocularis* (Kapel et al. 2006), the raccoon dog can potentially contribute to the establishment and spread of *E. multilocularis* throughout Denmark.

In conclusion, the cluster of *E. multilocularis* positive red foxes in two distinct areas in South Jutland demonstrates high local prevalence, especially in Højer. This high local prevalence of *E. multilocularis* in red foxes in Højer suggests an established full lifecycle including both rodents and carnivores, entailing a high risk of spread to dogs and humans.

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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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