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The raccoon dog (*Nyctereutes procyonoides*) as a reservoir of zoonotic diseases in Denmark

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ABSTRACT

Raccoon dogs have successfully invaded Europe, including Denmark. Raccoon dogs are potential vectors and reservoir hosts of several zoonotic pathogens and thus have the potential for posing a threat to both human and animal health. This study includes analysis of four zoonotic parasites, 16 tick-borne pathogens and two pathogen groups from 292 raccoon dogs collected from January 2018 to December 2018. The raccoon dogs were received as a part of the Danish national wildlife surveillance program and were hunted, found dead or road killed. The raccoon dogs were screened for *Alaria alata* and *Echinococcus multilocularis* eggs in faeces by microscopy and PCR, respectively, *Trichinella* spp. larvae in muscles by digestion, antibodies against *Toxoplasma gondii* by ELISA and screening of ticks for pathogens by fluidigm real-time PCR.

All raccoon dogs tested negative for *E. multilocularis* and *Trichinella* spp., while 32.9% excreted *A. alata* eggs and 42.7% were *T. gondii* sero-positive. Five tick-borne pathogens were identified in ticks collected from 15 raccoon dogs, namely *Anaplasma phagocytophilum* (20.0%), *Babesia venatorum* (6.7%), *Borrelia miyamotoi* (6.7%), *Neoehrlichia mikurensis* (6.7%) and *Rickettsia helvetica* (60.0%).

We identified raccoon dogs from Denmark as an important reservoir of *T. gondii* and *A. alata* infection to other hosts, including humans, while raccoon dogs appear as a negligible reservoir of *E. multilocularis* and *Trichinella* spp. infections. Our results suggest that raccoon dogs may be a reservoir of *A. phagocytophilum*.

1. Introduction

The raccoon dog (*Nyctereutes procyonoides*) is considered an invasive species in Denmark. Since its arrival in 1980, the population has increased continuously and at present comprises 2000–3000 individuals (The Environmental Protection Agency, n.d.). Raccoon dogs are well-known hosts of several zoonotic and pathogenic infections (Al-sabi et al., 2013; Kauhala and Kowalczyk, 2011; Malakauskas et al., 2007; Wodecka et al., 2016) posing a potential risk to human and animal health. However, neither tick-borne pathogens from raccoon dogs nor the prevalence of the zoonotic parasites *Toxoplasma gondii* and *Trichinella* spp. has previously been identified in raccoon dogs from Denmark, while *Echinococcus multilocularis* and *Alaria alata* has been identified several years ago (Al-sabi et al., 2013; Petersen et al., 2018).

Echinococcus multilocularis infections in humans are rare. Nonetheless, the clinical implications are critical and cause considerable public health concerns (Torgerson et al., 2008). Humans acquire the infection by ingesting *E. multilocularis* eggs, excreted by canine hosts, usually from contaminated food or water. *Trichinella* spp. infection can cause serious illness in humans including death (Darwin Murrell and Pozio, 2011) following consumption of *Trichinella* spp. infected raw or undercooked meat. Denmark is officially recognized with negligible risk of *Trichinella* spp. infection in farmed pigs (Danish Veterinary and Food Administration, n.d.), but organic and other outdoor pig production systems are increasing (Denmark Statistics, 2021). Currently, no systematic surveillance of *Trichinella* spp. occur in Danish wildlife, but previous surveillance projects have demonstrated *T. pseudospiralis* in a wild mink from the island of Bornholm in 2007 (Data not published), and three cases of *Trichinella* spp. in red foxes (0.1%) in the mid-1990s (Enemark et al., 2000). *Toxoplasma gondii* infection can cause toxoplasmosis, and 30–50% of the world's human population are estimated to be infected with *T. gondii* (Flegr et al., 2014). Most *T. gondii* infections in immunocompetent humans are asymptomatic or subclinical with minor symptoms (Montoya and Liesenfeld, 2004). However, ocular disease occur

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occasionally (Holland, 2003), and infection might be a contributing causal factor for developing any psychiatric disorder (Burgdorf et al., 2019). *Toxoplasma gondii* can also be transmitted to the foetus via the placenta resulting in congenital toxoplasmosis which can cause abortion, neonatal death, or foetal abnormalities (Chaudhry et al., 2014; Fallahi et al., 2018). Human *Alaria* infections are rare, but infections include clinical signs ranging from low-grade respiratory and cutaneous symptoms (Hedges, 2000) to fatal anaphylactic shock (Möhl et al., 2009). The adult *Alaria* stage which infects raccoon dogs has little relevance as a pathogen, while the mesocercariae stage found in paratenic host, e.g. wild boars (*Sus scrofa*), is pathogenic (Möhl et al., 2009). Infected raccoon dogs can act as reservoir for infection in wild boars and further transmission to humans through ingestion of wild boar meat (Möhl et al., 2009; Ozoliņa et al., 2020; Riehn et al., 2012).

Tick-borne diseases are primarily transmitted from one host to another via the tick vector (Estrada-Peña and de la Fuente, 2014) and the host can serve as a reservoir continuously infecting new feeding ticks (Keesing et al., 2012; Ostfeld et al., 2014). The disease-causing pathogens belonging to *Borrelia burgdorferi* sensu lato (s.l.) species complex commonly occur in the northern hemisphere (Kjær et al., 2020b), including species causing Lyme borreliosis in both humans and animals, in particular *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.), *B. lusitaniae*, *B. spielmanii,* and *B. valaisiana* (Klitgaard et al., 2019b; Michelet et al., 2014; Skarphédinsson et al., 2007; Vennestrøm et al., 2008; Wilhelmsson et al., 2010). Furthermore, *Anaplasma phagocytophilum*, species of the genus *Babesia* (*Babesia canis*, *B. divergens*, *B. microti*, *B. venatorum*), *Bartonella henselae, Borrelia miyamotoi*, *Coxiella burnetii*, *Francisella tularensis*, *Neoehrlichia mikurensis, Rickettsia helvetica*, and Tick Borne Encephalitis virus complex (TBEV) have been found in Scandinavia (Fertner et al., 2012; Fomsgaard et al., 2013; Jensen et al., 2017; Kjær et al., 2020a; Lundkvist et al., 2011; Michelet et al., 2014; Quarsten et al., 2017; Stuen et al., 2013; Svensson et al., 2019). *Borrelia miyamotoi*, *R. helvetica* and *N. mikurensis* rarely cause disease in humans in Scandinavia (Frivik et al., 2017; Grankvist et al., 2014; Nilsson et al., 2010; Welinder-Olsson et al., 2010). *Anaplasma phagocytophilum* and species within the *Babesia* genus are known disease-causing agents in domestic livestock (Stuen et al., 2013), but can also cause disease in humans (Heyman et al., 2010). For an overview of some of the pathogens found in Denmark and Scandinavia and their zoonotic potential, see Table 1.

Previous studies have revealed infections with *A. phagocytophilum*, *B. henselae*, *Borrelia* spp., and *N. mikurensis* in raccoon dogs (Han et al., 2017; Härtwig et al., 2014; Hildebrand et al., 2018; Kang et al., 2018; Szewczyk et al., 2019), thus investigating the raccoon dog's potential as a wildlife reservoir of zoonotic diseases is necessary to access the impact of an increasing raccoon dog population on human and animal health. We performed this study to address the raccoon dogs as wildlife reservoirs of tick-borne pathogens and serious zoonotic parasites.

2. Materials and method

2.1. Study animals

We included raccoon dogs submitted for necropsy from January 2018 to December 2018, as a part of the Danish national wildlife surveillance program. The raccoon dogs were hunted or trapped, found dead or road killed. All animals were transported in sealed plastic bags and subsequently stored at −80 °C for min. four days prior to necropsy to inactivate potential zoonotic parasites. At necropsy, faecal sample from rectum, a muscle sample from the thighs, the heart and ticks were collected and stored at −20 °C until further examination. Data of origin and sex of the raccoon dog were noted. Occasionally, samples were unsuitable for examination due to traumatic injury, predation or decomposition and were excluded.

2.2. Screening for parasites

2.2.1. Alaria alata

Alaria alata eggs were identified in individual faeces samples by sedimentation. Briefly, 4 g faeces were diluted in 36 ml tap water, filtered through gauze, the filtrate distributed into 10 ml tubes and centrifuged at 1000×*g* for 10 min. The supernatant was removed, flotation fluid added to each tube up to 8 ml and centrifuged at 53×*g* for 1 min. The supernatant was filtered through a 20 μm sieve and the sieves examined microscopically for *A. alata* eggs using a Leica DMRB light microscope (Leica Microsystems A/S, Brønshøj, Denmark).

2.2.2. Echinococcus multilocularis

Genomic DNA was isolated from 0.25 g faeces using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Samples were analysed for the presence of *E. multilocularis* eggs using the *E. multilocularis* primer set EmSP1-A'/B′

Table 1
A selection of tick-borne pathogens found in Denmark/Scandinavia included in the real-time PCR assay, their zoonotic potential, primary cause of disease in Denmark, as well as their main reservoirs.

Species	Zoonotic potential	Vector	Main reservoirs	References
Anaplasma phagocytophilum	Human granulocytic anaplasmosis	*I. ricinus*	Rodents, wild ruminants, birds	(Heyman et al., 2010; Jaarsma et al., 2019)
Babesia canis	None, but causes canine babesiosis	*D. reticulatus*	Canines	(Jongejan et al., 2015; Øines et al., 2010)
Babesia divergens	Human babesiosis	*I. ricinus*	Bovines	(Heyman et al., 2010; Karlsson and Andersson, 2016)
Babesia microti			Rodents	
Babesia venatorum			Cervids	
Bartonella henselae	Cat scratch fever	Cat fleas, *I. ricinus*	Cats	(Cotté et al., 2008; Dietrich et al., 2010)
Borrelia afzelii, B. burgdorferi s. s., *B. garinii, B. lusitaniae, B. spielmanii, B. valaisiana*	Lyme borreliosis	*I. ricinus*	Rodents, birds, lizards	(Heyman et al., 2010; Kjelland et al., 2010)
Borrelia miyamotoi	Relapsing fever	*I. ricinus*	Rodents	(Krause et al., 2015; Tobudic et al., 2020)
Coxiella burnetii	Q fever	Aerosols from infected animals, *I. ricinus*, *D. reticulatus*	Wild and domestic mammals, birds, arthropods	(Špitalská and Kocianová, 2003; Sprong et al., 2012)
Francisella tularensis	Tularaemia	Ingestion/aerosols from infected animals, *D. reticulatus*, deer flies, horse flies, mosquitoes	Rodents, lagomorphs	(Byström et al., 2005; Petersen et al., 2009; Seiwald et al., 2020)
Neoehrlichia mikurensis	Neoehrlichiosis	*I. ricinus*, *D. reticulatus*	Rodents	(Jahfari et al., 2012; Portillo et al., 2018)
Rickettsia helvetica	Tick-borne rickettsiosis	*I. ricinus*, mites, lice fleas	Ticks, rodents, deer	Sprong et al. (2009)

with forward primer 5′-GTCATATTTGTTTAAGTATAAGTGG-3′ and reverse primer 5′-CACTCTTATTTACACTAGAATTAAG-3' (Nonaka et al., 2009) targeting a fraction of the cytochrome *c* oxidase subunit I gene (cox1). For all reactions, mastermix were prepared containing all ingredients to ensure homogeneity between wells. All reactions in volumes of 22 µl, containing 0.7 µM of each primer, 5u/µl KAPA 2G robust DNA polymerase, 5.0 µl KAPA 2G buffer, 5.0 µl KAPA Enhancer (all KAPA 2G products were included in the KAPA 2G Robust PCR kit, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.2 mM dNTP, 2.0 µl DNA and dH_2O. The PCR was performed in a conventional PCR machine using the following conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s and final extension for 10 min at 72 °C. The protocol could consistently amplify DNA from two *E. multilocularis* eggs per 0.25 g faeces. A negative control without DNA was included in all tests.

2.2.3. Trichinella spp

Muscles from thighs were analysed for *Trichinella* spp. larvae by the magnetic stirrer method for pooled sample digestion according to Mayer-Scholl et al. (2017). Briefly, 20 g individual muscle samples from 10 animals were mixed with digestion fluid. The mixture was then stirred for a maximum of 60 min at 45 °C, subsequently sieved and allowed to sediment for 30 min. The sediment was collected and allowed to sediment for another 10 min. The supernatant was removed and the sediment was analysed for presence of *Trichinella* spp. larvae by stereomicroscopy at × 20 magnification using a Leica M125 stereo microscope (Leica Microsystems A/S, Brønshøj, Denmark).

2.2.4. Toxoplasma gondii

Antibodies against *T. gondii* were examined in meat juice from the heart using a commercial indirect enzyme-linked immunosorbent assay (ID Screen® Toxoplasmosis Indirect Multi-species ELISA kit, ID.vet, France). The heart was placed in a plastic funnel (CC Plast A/S, Hillerød, Denmark) at room temperature as described by Nielsen et al. (1998). Meat juice was collected in a 10 ml tube during thawing and placed at −20 °C until further analysis. The collected meat juice was subsequently examined for IgG antibodies, following the instructions of the manufacturer (ID Screen® Toxoplasmosis Indirect Multi-species ELISA kit, ID.vet, France). The samples and the controls provided in the kit were analysed in duplicate. The optical density (OD) was read at 450 nm. Results were evaluated following the instructions of the manufacturer for carnivores by calculating the S/P% (sample/positive percentage) = (mean OD of sample − mean OD of negative control)/(mean OD of positive control − mean OD of negative control) × 100. Samples with S/P% ≤40% were considered negative, 40–70% doubtful, and ≥70% positive. Raccoon dogs that tested positive with the ELISA were considered seropositive; others were considered seronegative.

2.3. Tick collection and screening for tick-borne pathogens

Raccoon dogs were macroscopically screened for ticks, particularly the inner thighs, belly and eye area. Tick larvae and nymphs were screened for pathogens commonly known or assumed present in Denmark: *A. phagocytophilum*, *Babesia* spp. *(B. divergens, B. microti, B. canis, B. venatorum)*, species belonging to the *B. burgdorferi* s.l. species complex (*Borrelia garinii, B. afzelii, B. spielmanii, B. valaisiana, B. lusitaniae, B. burgdorferi* sensu strictu), *B. miyamotoi, N. mikurensis*, *C. burnetii*, *F. tularensis*, and *R. helvetica* (Table 1) (Kjær et al., 2020a; Klitgaard et al., 2019b; Michelet et al., 2014). We focused on larvae in this study, as any tick-borne pathogens found in the larvae could indicate infection transmitted from the raccoon dog to the tick larvae. The most common tick species in Denmark, the castor bean tick (*Ixodes ricinus)* only feeds once per life cycle (larva-nymph-adult, Estrada-Peña and de la Fuente, 2014), and thus pathogens found within larvae may come from this first blood meal taken from the raccoon dogs. Naturally, this only pertains to pathogens that are not vertically transmitted within the tick vector (Han et al., 2019; Michelitsch et al., 2019; Oechslin et al., 2017; Sprong et al., 2009).

2.3.1. DNA extraction

The ticks were washed in 70% ethanol followed by 2 × 5 min in sterile water. We added 75 µl of incubation buffer (D920), 75 µl of lysis buffer (MC501) and 3 mm tungsten beads (Qiagen, Hilden, Germany), and homogenized the ticks in a TissueLyser II (Qiagen, Hilden, Germany). DNA was subsequently isolated using the Maxwell 16 LEV Blood DNA kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions with a few modifications (samples were incubated at 56 °C overnight).

2.3.2. Screening of tick-borne pathogens by real-time PCR

We examined the ticks for the presence of the above mentioned bacterial and parasitic pathogens (see section 2.2) via the BioMark real-time PCR system (Fluidigm, San Francisco, California, USA). Whereas *I. ricinus* is the most common tick in Scandinavia, the closely related taiga tick, *I. persulcatus*, has been found in Sweden and the meadow tick *Dermacentor reticulatus* has sporadically been found in both Denmark, Norway and Sweden. Thus, we additionally screened for the tick species *I. ricinus*, *I. persulcatus* and *D. reticulatus*. The method used is thoroughly described in Michelet et al. (2014), Klitgaard et al. (2019b) and in Kjær et al. (2020a). Prior to the analysis, we homogenized the ticks using a TissueLyser II (Qiagen, Hilden, Germany) in a mixture of 75 µl Incubation buffer (D920), 75 µl Lysis buffer (MC501) and with three 3-mm Tungsten beads (Qiagen) for 2 × 2.5 min at 25 Hz. We then pre-amplified the DNA in a master mix consisting of 2.5 µL TaqMan PreAmp Master Mix (2X), 1.2 µL pooled primer mix (except primers targeting tick DNA) and 1.3 µL DNA. We did not pre-amplify tick DNA, as homogenising whole ticks, should provide enough tick DNA for analysis. The samples ran one cycle at 95 °C for 10 min, 14 cycles at 95 °C for 15 s and 4 min at 60 °C. We first diluted the pre-amplified DNA 5-fold in water after which we ran the real-time PCR with FAM and black hole quencher (BHQ1)-labelled TaqMan probes with TaqMan Gene Expression Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The final cycles were performed as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and at 60 °C for 15 s. The results were obtained through the BioMark real-time PCR system and analysed using the Fluidigm real-time PCR Analysis software, and cut-off values for crossing points (CP) were set to ≤28. We included one negative water control and *Escherichia coli* primers and probes for internal inhibition control (Michelet et al., 2014). Numerous studies have used and validated the Fluidigm real-time PCR system (Klitgaard et al, 2017a, 2019a, 2019b; Michelet et al., 2014; Moutailler et al., 2016; Reye et al., 2013).

2.4. Statistical analyses

When possible, depending on sample size, the prevalence of the zoonotic parasitic infections, the sero-prevalence of *T. gondii* and the prevalence of pathogens in ticks were calculated with their respective 95% confidence intervals (CI). Also when possible, differences in prevalence were determined by a binary logistic regression model with pathogen as the dependent variable and sex and region of origin as independent variables using SAS 9.4 software (SAS for Windows, SAS Institute Inc., Cary, NC, USA). The variables are analysed independently. A p-value below 0.05 was considered significant.

3. Results

A total of 292 raccoon dogs were included in the study. Of these, 149 (47.6%) were males and 139 (51.0%) females, while sex was not recorded for four (1.4%) raccoon dogs. All raccoon dogs originated from the Jutland peninsula of Denmark (Fig. 1), with 30 (10.3%) animals originating from Northern Jutland, 121 (41.4%) from Middle Jutland

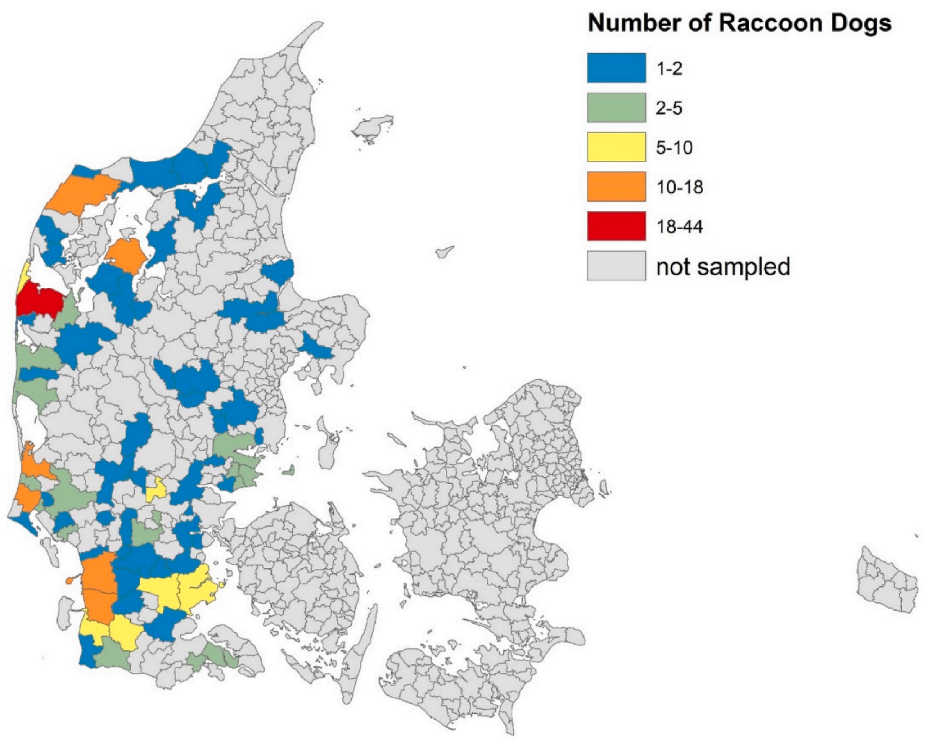

Fig. 1. Map of Denmark showing the origin of the collected raccoon dogs by county. The colour coding shows differences in the number of raccoon dogs collected. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and 141 (48.3%) from Southern Jutland.

3.1. Parasites

Table 2 gives an overview of examined raccoon dogs and raccoon dogs positive for the various parasites along with their 95% CIs. All raccoon dogs were negative for *Trichinella* spp. and *E. multilocularis*, while 32.9% excreted *A. alata* eggs and 42.7% were sero-positive for *T. gondii*. Neither *A. alata*, nor *T. gondii* was associated with sex or region of origin.

3.2. Tick-borne diseases

In total, 41 raccoon dogs were examined for ticks. Of these 41 raccoon dogs, 25 carried 392 tick larvae in total. We decided to test 10 larvae from each raccoon dog for pathogens due to time and funding available. Only 15 raccoon dogs contained 10 or more larvae, resulting in analysis of 150 individual larvae. Additionally, if any of the selected 15 raccoon dogs had ten or more nymphs, then we would screen ten nymphs from each raccoon dog as well. Only 4 of the raccoon dogs with ten or more larvae also had ten or more nymphs. From 150 larvae screened for tick DNA, 127 larvae from 13 individual raccoon dogs were successfully amplified by PCR, all of which were identified as *I. ricinus* (Table 3). *Ixodes persulcatus* and *D. reticulatus* were not detected. Failed pre-amplification of tick DNA is most likely due to insufficient crushing of the ticks prior to extraction. Insufficient crushing may also affect the extraction of pathogen DNA and these samples were therefore omitted from the analysis.

We found *A. phagocytophilum* in 3/13 remaining raccoon dogs. One raccoon dogs had 1/10 analysed larvae testing positive for *A. phagocytophilum*, one raccoon dog had 3/10 larvae testing positive, and one raccoon dog had 6/9 larvae testing positive for *A. phagocytophilum*. *Rickettsia helvetica* was found in 17 larvae distributed on 8 raccoon dogs. From the 127 analysed larvae, one tested positive for *B. venatorum*, one for *B. myiamotoi* and one for *N. mikurensis* (Table 3).

From 40 nymphs screened for tick DNA, 22 nymphs from 3 individual raccoon dogs were successfully amplified by PCR and identified as *I. ricinus* (Table 3). From the raccoon dog with six *A. phagocytophilum* positive larvae, 7/9 nymphs were additionally positive for *A. phagocytophilum*. An overview of the pathogen results are listed in Table 3.

Table 2
Prevalence and 95% confidence intervals of zoonotic parasites and tick-borne pathogens in raccoon dogs by sex and region. CI=Confidence interval.

	Total		Males[a]		Females[a]		Northern Jutland		Middle Jutland		Southern Jutland	
Pathogen	Pos/ total	% [95% CI]	Pos/ total	% [95% CI]	Pos/ total	% [95% CI]	Pos/ total	% [95% CI]	Pos/ total	% [95% CI]	Pos/ total	% [95% CI]
Alaria alata	78/ 237	32.9 [27.0–39.3]	39/ 118	33.1 [24.7–42.3]	39/ 117	33.3 [24.9–42.6]	10/ 28	35.7 [18.6–55.9]	31/ 98	31.6 [22.6–41.8]	37/ 111	33.3 [24-7-42.9]
Echinococcus multilocularis	0/ 281	–	0/ 142	–	0/ 134	–	0/29	–	0/ 118	–	0/ 133	–
Trichinella spp.	0/ 233	–	0/ 114	–	117	–	0/29	–	0/90	–	0/ 114	–
Toxoplasma gondii	97/ 227	42.7 [36.2–49.5]	53/ 110	48.2 [35.2–57.9]	43/ 115	37.4 [28.6–46.9]	16/ 28	57.1 [37.2–75.5]	33/ 98	37.1 [27.1–48.0]	48/ 111	43.6 [34.2–53.4]

[a] Sex was unknown for two raccoon dogs.

Table 3
Overview of the results from the screening of tick-borne pathogens. Only tests where the positive control for *I. ricinus* DNA was validated are depicted. # Raccoon dogs in parenthesis denote how many raccoon dogs the positive larvae and nymphs stemmed from. None of the ticks tested positive for *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *B. spielmanii*, *B. valaisiana*, *B. lusitaniae*, *B. divergens*, *B. microti*, *B. canis*, *B. henselae*, *C. burnetii* or *F. tularensis*.

Pathogen	# larvae tested[a]	#positive larvae (# raccoon dogs)	# nymphs tested[b]	# positive nymphs (#raccoon dogs)
Anaplasma phagocytophilum	127	10 (3)	22	7 (1)
Babesia canis	127	0	22	0
Babesia divergens	127	0	22	0
Babesia microti	127	0	22	0
Babesia venatorum	127	1 (1)	22	0
Bartonella henselae	127	0	22	0
Borrelia spp.	127	0	22	0
Borrelia miyamotoi	127	1 (1)	22	0
Coxiella burnetii	127	0	22	0
Francisella tularensis	127	0	22	0
Neoehrlichia mikurensis	127	1 (1)	22	2 (1)
Rickettsia helvetica	127	17 (8)	22	5 (2)

[a] Stemmed from 13 raccoon dogs.
[b] Stemmed from 3 raccoon dogs.

Prevalence and 95% CIs were not calculated due to low number of raccoon dogs. We furthermore did not conduct any statistical analyses as the statistical power given these sample sizes, would be very low.

4. Discussion

This is the first investigation of *T. gondii* sero-prevalence and tick-borne pathogens in raccoon dogs from Denmark. Moreover, we studied the prevalence of the zoonotic parasites *Trichinella* spp., *A. alata* and *E. multilocularis* to get updated information on the occurrence in the increasing raccoon dog population. The results demonstrated that a noteworthy proportion of raccoon dogs from Denmark are infected with *A. alata* (32.9%) and are *T. gondii* sero-positive (42.7%), while the zoonotic parasites *E. multilocularis* and *Trichinella* spp. were not identified. A previous Danish study identified *E. multilocularis* in 2/123 raccoon dogs from South Jutland (Petersen et al., 2018), an area where 21 raccoon dogs in our study also originated from. In contrast, *Trichinella* spp. infection remain unidentified in raccoon dogs from Denmark, although both *E. multilocularis* and *Trichinella* spp. are prevalent in raccoon dogs from other European countries (Cybulska et al., 2019; Laurimaa et al., 2015; Mayer-Scholl et al., 2016; Oksanen et al., 2016). However, the absence of *E. multilocularis* and *Trichinella* spp. positive raccoon dogs in our study cannot exclude a negligible prevalence of these parasites in Danish raccoon dogs.

Nearly half the raccoon dogs (42.7%) in our study were *T. gondii* sero-positive. Infection was unrelated to sex or region of origin, thus *T. gondii* infection is prevalent throughout the raccoon dog population. *Toxoplasma gondii* sero-positive Danish wildlife species (Christiansen and Siim, 1951; Laforet et al., 2019), livestock (Agerholm et al., 2006; Kofoed et al., 2017) and humans (Burgdorf et al., 2019) have previously been identified. Studies of *T. gondii* infections in raccoon dogs are generally scarce, and those studies existing are mostly performed on farmed domestic raccoon dogs from China (Qin et al., 2020; Zheng et al., 2017). However, *T. gondii* positive wild-living raccoon dogs from Poland have been identified (Kornacka et al., 2016; Sroka et al., 2019). Raccoon dogs consume a wide range of food items including small mammals, birds and carrions (Mikkelsen et al., 2016), all of which can carry *T. gondii* cysts (Dubey, 2002; Tenter et al., 2000) and be a source of infection for the raccoon dogs.

Almost one third (32.9%) of the raccoon dogs excreted eggs of *A. alata*. This finding is significantly lower than a previous Danish study of *A. alata* infection in raccoon dogs (69.7%) (Al-sabi et al., 2013). In other European countries, the prevalence of *A. alata* in raccoon dogs varied from 30% in Austria (Duscher et al., 2017) to 96.5% in Lithuania (Bružinskaitė-Schmidhalter et al., 2012). However, the previous study identified adult *Alaria* worm in the whole intestine, while in our study we identified eggs excreted in faeces. The differences in method used could explain the lower prevalence observed in our study compared to the previous Danish study. The adult stage of *A. alata* parasitize the small intestine of carnivores, where embryonated eggs are excreted with faeces. Subsequently, *A. alata* needs two intermediate hosts to develop into infective mesocercariae. The final host is reached by ingestion of an infected amphibian secondary intermediate host or a paratenic host harbouring mesocercariae. Numerous animal species can act as paratenic hosts (Odening 1963; Möhl et al., 2009). In Denmark, mesocercariae have been identified in 66.7% of badgers (6/9) and 3.0% of feral cats (3/99) (Takeuchi-Storm et al., 2015), whereas 34.4% of examined red foxes had adult worms in the intestine (Al-sabi et al., 2013) indicating that *A. alata* is highly prevalent in Denmark. Humans can act as paratenic hosts for *Alaria* spp., however, reports of human alariosis have only been published for the American species of the genus *Alaria* (Beaver et al., 1977; Fernandes et al., 1976; Fried and Abruzzi, 2010; Möhl et al., 2009). Thus, Odening (1963) has shown severe infestation in rhesus macaque (*Macaca mulatta*) experimentally infected with *A. alata*, proving that a host closely related to humans can become infected with this trematode.

The real-time PCR test for tick-borne pathogens only identified the tick species *I. ricinus*, which is also the most common tick species found in Denmark and Northern Europe. We also screened for the meadow tick, *D. reticulatus* and the taiga tick *I. persulcatus*. Although *I. persulcatus* has not been found in Denmark (Kjær et al., 2019), it has been found in northern Sweden (Jaenson et al., 2016), thus we included it in our screening. *Dermacentor reticulatus* has been found on a migrating golden jackal (*Canis aureus*) in Denmark (Klitgaard et al., 2017b) and several dogs on the Danish islands of Lolland and Falster have recently been diagnosed with canine babesiosis, caused by *Babesia canis* which is only transmitted via *D. reticulatus* in Denmark (data not published). This suggests that *D. reticul*atus is present in Denmark, but so far we have not been able to confirm this (Kjær et al., 2019). Furthermore, with the small sample sizes used in the screening for tick- and pathogen species in this study, we cannot rule out the presence of species not found through the real-time PCR.

We found *A. phagocytophilum*, *B. venatorum*, *B. myiamotoi*, *N. mikurensis*, and *R. helvetica* in the tick nymphs and larvae analysed from the raccoon dogs. In the larvae, we found a relatively high percentage of the raccoon dogs with *R. helvetica* and *A. phagocytophilum*. Vertical transmission from mother to offspring has been documented for *R. helvetica* and *B. myiamotoi* in *Ixodid* ticks (Breuner et al., 2017; Han et al., 2019; Socolovschi et al., 2009; Sprong et al., 2009), thus we cannot conclude that these pathogens were transmitted from the raccoon dog host to the tick larvae. Many of the *Babesia* species also exhibit transovarial transmission from female to offspring (Bonnet et al., 2007; Øines et al., 2012), and the one *B. venatorum* positive larva we found, could have obtained the pathogen through the adult female tick. However, there is no evidence for vertical transmission of *A. phagocytophilum* in *I. ricinus* (Jaarsma et al., 2019; Severinsson et al., 2010). Among the 17 ticks (10 larvae and 7 nymphs) testing positive for *A. phagocytophilum,* 6 larvae and 7 nymphs originated from the same raccoon dog, whereas 3 other larvae originated from one other raccoon dog. This high level of clustering of positive ticks on individual raccoon dogs strongly suggests that raccoon dogs may function as reservoirs of *A. phagocytophilum* and infect *I. ricinus* larvae with the pathogen. While it is still unknown whether *N. mikurensis* can be transmitted transovarially, most researchers agree that this is not the common transmission

pathway (Jahfari et al., 2012; Obiegala and Silaghi, 2018; Silaghi et al., 2012). We only found two raccoon dogs with ticks positive for *N. mikurensis* (one with one positive larva and one with two positive nymphs), thus our study cannot conclude whether raccoon dogs may serve as reservoir host for *N. mikurensis*. Studies from Poland and Germany have detected both *A. phagocytophilum* and *N. mikurensis* in raccoon dogs (Hildebrand et al., 2018; Szewczyk et al., 2019), so our findings, at least for *A. phagocytophilum* correspond well with what has been found elsewhere in Europe.

Anaplasma phagocytophilum has long been known to cause tick-borne fever in domestic ruminants and can cause high fever, anorexia, drop in milk yield, abortion in ewes and reduced fertility in rams (Stuen et al., 2013). As a zoonosis, *A. phagocytophilum* can cause human granulocytic anaplasmosis, with symptoms varying from mild fever and influenza-like symptoms to fatal infections (Heyman et al., 2010; Stuen et al., 2013). Natural reservoirs of *A. phagocytophilum* are rodents, wild ruminants and birds (Heyman et al., 2010; Jaarsma et al., 2019). *Neoehrlichia mikurensis* has only recently been discovered as a cause of human disease, and many aspect of this pathogen have not yet been fully investigated (Obiegala and Silaghi, 2018; Portillo et al., 2018). However, it is believed that rodents are the main reservoirs of this pathogen (Obiegala and Silaghi, 2018; Portillo et al., 2018). *Neoehrlichia mikurensis* mostly affects immunosuppressed people and causes mild fever, joint pain, headache, and in some cases rashes (Obiegala and Silaghi, 2018). Wildlife reservoirs of zoonotic pathogens help maintain these pathogens in the environment, where they can continuously infect animals and humans. Although the racoon dog population in Denmark is fairly limited, it seems to be continuously increasing (The Environmental Protection Agency, n.d.). Being a natural reservoir of *A. phagocytophilum* and potentially *N. mikurensis* and other tick-borne pathogens, an increasing raccoon dog population may play a role in maintaining and spreading these zoonotic pathogens, potentially increasing the health risk for livestock, wildlife and humans.

5. Conclusion

Assuming *T. gondii* sero-positivity equals hosting viable parasites, a great proportion of raccoon dogs from Denmark are potential reservoir of *T. gondii* cysts. Also, raccoon dogs are a potential reservoir of *A. alata* infection, while our findings suggest that raccoon dogs are negligible reservoir of *E. multilocularis* and *Trichinella* spp. infections. Since raccoon dogs are not consumed, the risk of acquiring *T.* gondii infection when handling them is considered negligible. However, raccoon dogs seems to be an essential factor in the continued maintenance of these infections in the environment and there is a risk of acquiring the infection when handling the raccoon dogs during pelting if the hygiene is poor. Strong clustering of *A. phagocytophilum*-positive ticks on individual raccoon dogs analysed in this study indicates that raccoon dogs may act as reservoirs of this parasite and thus may play a part in maintaining and spreading *A. phagocytophilum* in nature.

Declaration of competing interest

None.

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