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Short communication

First report of *Cryptosporidium canis* in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) and identification of several novel subtype families for *Cryptosporidium* mink genotype in minks (*Mustela vison*) in China

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ABSTRACT

Despite the rapid and extensive advances in molecular epidemiology of *Cryptosporidium* in humans and a variety of animals, the prevalence and genetic traits of the parasite in wildlife bred in captivity and the role of the neglected hosts in zoonotic transmission of human cryptosporidiosis are rarely understood. This study investigated the prevalence, species/genotype, and subtype of *Cryptosporidium* in farmed fur animals in China and assessed the possibility of zoonotic transmission. Three of 191 (1.6%) foxes (*Vulpes vulpes*), 17 of 162 (10.5%) raccoon dogs (*Nyctereutes procyonoides*), and 48 of 162 (29.6%) minks (*Mustela vison*) were positive for *Cryptosporidium* by nested PCRs targeting the small subunit rRNA gene. Sequence analysis indicated the presence of only *Cryptosporidium canis* in foxes and raccoon dogs. There is no significant difference in prevalence between young and adult foxes (or raccoon dogs). Three *Cryptosporidium* species or genotype including *C. canis*, *Cryptosporidium meleagridis*, and mink genotype were determined in minks aged five to six months. Subtyping based on nucleotide and amino acid sequence polymorphisms of the 60 kDa glycoprotein facilitated identification of three novel subtype families named as Xb to Xd for *Cryptosporidium* mink genotype. The presence of zoonotic *C. canis*, *C. meleagridis*, and *Cryptosporidium* mink genotype in captive-bred fur animals is of public health concerns. The findings expanded the host ranges of *C. canis* and *C. meleagridis* and confirmed genetic diversity at the subtype level in *Cryptosporidium* mink genotype. This is the first study reporting *Cryptosporidium* infections in foxes and raccoon dogs in China.

1. Introduction

Cryptosporidium is a genus of apicomplexan protozoans that colonize humans, domestic animals, and wild vertebrates (Xiao and Fayer, 2008). Infections of *Cryptosporidium* spp. are an important cause of diarrhea and mortality in immunosuppressed hosts, notably AIDS patients (Xiao and Fayer, 2008). Clinical presentations vary based upon the age of the immunocompetent hosts and nature of the infection (Xiao and Fayer, 2008). Because of the lack of in vitro culture approaches and the difficulty in morphological distinctness of the oocysts isolated from various hosts, molecular typing tools based on DNA sequence polymorphisms of the small subunit (SSU) rRNA and the 60 kDa glycoprotein (gp60) are now widely applied for species determination, genotyping, or subtyping of *Cryptosporidium* spp. (Xiao, 2010). The parasite was identified to harbor almost 30 valid species and over 60 genotypes of uncertain species, and most of these species and genotypes are host adapted and have a narrow host range (Slapeta, 2013; Xiao, 2010; Xiao and Fayer, 2008). Among them, *Cryptosporidium hominis* and *Cryptosporidium parvum* are the most prevalent species documented in human infections (Xiao, 2010). Nevertheless, the occasional occurrence of some species (*C. canis*, *Cryptosporidium felis*, *C. meleagridis*, among others) and genotypes (horse genotype, skunk genotype, monkey genotype, among others) with host specificities in human infections suggested the potential for zoonotic transmission of cryptosporidiosis (Xiao and Fayer, 2008; Xiao and Feng, 2008). Most previous surveillances largely concerned *Cryptosporidium* infections in humans and a wide range of domestic and wild animals (Xiao, 2010) and little is known about the epidemiology of cryptosporidiosis in captive-bred

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Table 1
Prevalence and species/genotype of *Cryptosporidium* in foxes, raccoon dogs, and minks in China.

Host	Age (month)	No. of animal	No. of positive (prevalence)	*Cryptosporidium* species or genotype (no.)
Fox	5 to 6	117	2 (1.7%)	*C. canis* (2)
	17 to 18	74	1 (1.4%)	*C. canis* (1)
Raccoon dog	5 to 6	114	14 (12.3%)	*C. canis* (13)
	17 to 18	48	3 (6.3%)	*C. canis* (2)
Mink	5 to 6	162	48 (29.6%)	*C. canis* (19), *C. meleagridis* (3), mink genotype (18)

wild animals. The objective of this study is to investigate the prevalence and genetic characteristics of *Cryptosporidium* in farmed foxes, raccoon dogs, and minks in China and evaluate the zoonotic potential of the species or genotype identified.

2. Materials and methods

Fresh fecal specimens were randomly collected from 191 foxes and 162 raccoon dogs on a farm and 162 minks on another farm in suburban Harbin, northeast China in November 2014. One specimen per animal was used herein. Specimens from foxes and raccoon dogs were grouped as follows: young (5 to 6 months) and adult (17 to 18 months), while those from minks all fall into the young group. Numbers of specimens in various age groups were shown in Table 1. All fecal samples were donated by the farm owners, who granted permission to include those in the survey. The samples were collected immediately after natural defecation, then packaged in disposable plastic bags individually and stored frozen at −20 °C until use. DNA extraction was done within one week after sampling from approximately 0.2 g of each frozen specimen using the StoolGen DNA Kit (Spin-column) (CWBIO, China) and the manufacturer's recommended procedures. The samples of DNA were stored at −20 °C.

Nested PCRs that amplified an approximately 830 bp fragment of the SSU rRNA gene and the subsequent sequence analysis were employed for the determination of *Cryptosporidium* species or genotype as previously described (Xiao et al., 1999). The SSU rRNA-positive isolates were further subtyped by nested PCRs and sequence analysis of the gp60 gene as previously documented (Alves et al., 2003). Each specimen was analyzed twice using 2 μl of the DNA extract per PCR. All PCRs were performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA). PCR products were visualized by electrophoresis in 1.5% agarose containing ethidium bromide.

The PCR amplicons that displayed the specific DNA fragments were sequenced with the secondary primer set in both directions at Beijing Genomics Institute, China. All raw sequencing data were viewed and proofread in Chromas Pro version 1.33 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia). The resulting sequences were compared to reference sequences to determine *Cryptosporidium* species/genotype and subtype by BLAST analysis (http://www.ncbi.nlm.nih.gov/), their similarity was determined based on the degree of sequence identity. A significant difference in prevalence between host groups or age groups was scored when the p value was <0.05 using chi-square test. All tests were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

PCR identified 3 of 191 [1.6%, 95% confidence interval (CI): −0.002–0.033] foxes, 17 of 162 (10.5%, 95% CI: 0.055–0.155) raccoon dogs, and 48 of 162 (29.6%, 95% CI: 0.212–0.380) minks positive for *Cryptosporidium* (Table 1). The prevalence of *Cryptosporidium* in minks on a farm is significantly higher than that in raccoon dogs ($p < 0.01$, $\chi^2 = 18.5$) or foxes ($p < 0.01$, $\chi^2 = 55.8$) on another farm (Table 1). Young foxes (1.7%, 2/117) have a slightly higher prevalence than adults (1.4%, 1/74; $p > 0.05$) (Table 1). The difference in prevalence between young (14/114, 12.3%) and adult (3/48, 6.3%) raccoon dogs is not significant as well ($p > 0.05$) (Table 1). As seen in Table 2, previous studies have reported the prevalence rates of *Cryptosporidium* in wild foxes from Ireland (1.6%, 2/124), USA (7.9%, 6/76), and Slovakia (38.7%, 24/62) (Nagano et al., 2007; Ravaszova et al., 2012; Zhou et al., 2004). There was only one case report on *Cryptosporidium* infection in a wild raccoon dog in Japan (Matsubayashi et al., 2004), this study represents the first large-scale *Cryptosporidium* survey in farmed raccoon dogs (Table 2). Several previous reports described the existence of *Cryptosporidium* in farmed minks in central China (1.3%, 6/469) and Spain (15.7%, 8/51) and wild minks in Ireland (4.9%, 4/81) (Gomez-Couso et al., 2007; Stuart et al., 2013; Wang et al., 2008), our study presented a significantly higher prevalence (29.6%, 48/162) in farmed minks from northeast China (Table 2).

Sequencing of the SSU rRNA gene was available for three PCR-positive fox isolates, 15 of 17 raccoon dog isolates, and 40 of 48 mink isolates (Table 1). *C. canis* was detected to be the unique species present in foxes and raccoon dogs by BLAST analysis, while a diversity of *Cryptosporidium* spp. including *C. canis* ($n = 19$), *C. meleagridis* ($n = 3$), and mink genotype ($n = 18$) were seen in minks (Table 1). Nucleotide sequences of the three *C. canis*-positive fox isolates (CHF8, CHF70, and CHF157) are identical to a GenBank sequence KP890053 (recorded in dog from France) except for an insertion of 2-bp (TG) between positions 495 and 496 and an unreported substitution (T to A) at position 548 compared to KP890053 (with the beginning of the first nucleotide as position no. 1). Compared to KP890053, an unreported T to C mutation was observed in a raccoon dog isolate (CHR26) at position 534. The

Table 2
Distribution of *Cryptosporidium* species/genotype in foxes, raccoon dogs, and minks worldwide.

Host	Region	Prevalence (no. +/total)	Genetic locus (GenBank accession no.)	*Cryptosporidium* species or genotype (no.)	Reference
Fox	USA	7.9% (6/76)	SSU rRNA (AY120904, AY120908)	*C. canis* (5), muskrat genotype I (1)	Zhou et al. (2004)
	USA	100% (3/3)	SSU rRNA (AY120907, AY120908), HSP70 (AY120920), Actin (AY120926)	*C. canis* (2), fox genotype (1)	Xiao et al. (2002)
	Ireland	1.6% (2/124)	SSU rRNA (AY508962, AY508963)	*C. parvum* (2)	Nagano et al. (2007)
	Slovakia	38.7% (24/62)			Ravaszova et al. (2012)
	China	1.6% (3/191)	SSU rRNA (KU608308)	*C. canis* (3)	This study
Raccoon dog	Japan	100% (1/1)	SSU rRNA (AF108864), HSP70 (AB104730), COWP (AB104731)	*C. parvum* (1)	Matsubayashi et al. (2004)
	China	10.5% (17/162)	SSU rRNA (KU608307)	*C. canis* (15)	This study
Mink	USA	40.0% (2/5)	SSU rRNA (EF641015)	Mink genotype (2)	Feng et al. (2007); Feng et al. (2011a)
	Ireland	4.9% (4/81)	SSU rRNA (EF641015, EU245042)	*C. andersoni* (3), mink genotype (1)	Stuart et al. (2013)
	Spain	15.7% (8/51)	COWP (AF266267)	*C. parvum* (8)	Gomez-Couso et al. (2007)
	China	1.3% (6/469)	SSU rRNA (EF428186), HSP70 (EF428198), COWP (EU197210), Actin (EF428192)	Mink genotype (6)	Wang et al. (2008)
	China	29.6% (48/162)	SSU rRNA (EF641015, HM116382, KU608299)	*C. canis* (19), *C. meleagridis* (3), mink genotype (18)	This study