Parasitology Research (2018) 117:169–175
https://doi.org/10.1007/s00436-017-5686-5

ORIGINAL PAPER

Molecular detection and genetic characterizations of *Cryptosporidium* spp. in farmed foxes, minks, and raccoon dogs in northeastern China

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Received: 25 July 2017 / Accepted: 15 November 2017 / Published online: 25 November 2017

Abstract

Cryptosporidium spp. are common intestinal protozoa causing diarrhea in humans and a variety of animal species. With the recent development of fur industry, a large number of fur animals are farmed worldwide, especially in China. The existence of identical *Cryptosporidium* species/genotypes in humans and fur animals suggests zoonotic potential. In order to assess the presence of zoonotic *Cryptosporidium* species and/or genotypes in farmed fur animals, 367 fecal specimens were collected from 213 foxes, 114 minks and 40 raccoon dogs farmed in Heilongjiang, Jilin, and Liaoning provinces, northeastern China, during the period from June 2014 to October 2016. By PCR and sequencing of the partial small subunit (SSU) rRNA gene of *Cryptosporidium*, 20 of 367 (5.4%) animal samples were found to be infected, corresponding to 12 of 213 fox samples (5.6%) and 8 of 114 mink samples (7.0%) screened. Three *Cryptosporidium* species/genotypes were identified: *C. canis* ($n = 17$), *C. meleagridis* ($n = 1$) and *Cryptosporidium* mink genotype ($n = 2$). Two host-adapted *C. canis* types (*C. canis* dog genotype and *C. canis* fox genotype) were found. By PCR and sequencing of the partial 60 kDa glycoprotein (gp60) encoding gene, one mink genotype isolate was successfully subtyped as XcA5G1R1. The three *Cryptosporidium* species/genotypes identified in this study have been previously reported in humans suggesting that fur animals infected with *Cryptosporidium* spp. may pose a risk of zoonotic transmission of cryptosporidiosis, especially for the people working in fur animal farming and processing industry.

Keywords *Cryptosporidium* · Genotyping · Subtyping foxes · Minks · Raccoon dogs

Introduction

With the recent development of fur industry, the number of farmed fur animals has been increasing worldwide. The number of foxes, minks, and raccoon dogs has reached more than 104 million animal heads in China in 2015. An increasing number of people are involved in fur animal farming and fur processing industry and are in close contact with these animals. The variety of zoonotic pathogens in fur animals is not only a veterinarian issue of animal health status but may be also of importance for public health.

Cryptosporidium spp. are common intestinal protozoan parasites causing diarrhea in humans and a variety of other animal species. To date, six *Cryptosporidium* species (*C. canis*, *C. parvum*, *C. felis*, *C. ubiquitum*, *C. meleagridis*, and *C. andersoni*) and three genotypes (*Cryptosporidium* mink genotype, *Cryptosporidium* muskrat genotype I, and *Cryptosporidium* fox genotype) have been identified in foxes, minks, and raccoon dogs. With the exception of the *Cryptosporidium* muskrat genotype I and the *Cryptosporidium* fox genotype, they are also found in humans, suggesting a risk of zoonotic transmission (Feng et al. 2007; Gómez-Couso et al. 2007; Kellnerová et al. 2017; Mateo et al. 2017; Matsubayashi et al. 2004; Nagano et al. 2007; Stuart et al. 2013; Wang et al. 2008; Xiao et al. 2002; Zhang et al. 2016a, b; Zhou et al. 2004). Humans can acquire *Cryptosporidium* infections through the fecal-oral route, including direct transmissions (person-to-person and animal-to-person) and indirect transmissions (through water, food, and fomites contaminated with oocysts). Analysis of cryptosporidiosis epidemiology has revealed that contact with pets or other household animals is one of the most common risk factors (Mahmoudi et al. 2017). Thus, individuals who have close contact with animals for occupational or recreational reasons including farmers, breeders, and veterinarians but also animal caretakers and workers of the fur industry are

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Section Editor: Leonhard Schnittger

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at a high risk of *Cryptosporidium* infection. Zoonotic outbreaks of cryptosporidiosis have been reported among veterinarians and veterinary students as well as other people exposed to agricultural animals and children visiting farms (Gait et al. 2008; Hoek et al. 2008; Stantic-Pavlinic et al. 2003; Chalmers and Giles 2010). In addition, numerous outbreaks of cryptosporidiosis described in many countries and regions are related to waterborne or foodborne transmission (Karanis et al. 2007; Efstratiou et al. 2017; Insulander et al. 2013; Rimšelienė et al. 2011; Yoshida et al. 2007; Ethelberg et al. 2009). In many cases, the sources of *Cryptosporidium* in contaminated water or food remain unknown. Due to the large number of animal reservoir hosts of *Cryptosporidium* and the extremely high number of oocysts shed by them in natural environments, animals may contribute to these outbreaks (Smith et al. 2006).

Foxes, minks, and raccoon dogs are common fur animals and are important economic animals in the three provinces Heilongjiang, Liaoning, and Jilin in the northeast of China. The aim of the present study was to determine the presence of zoonotic *Cryptosporidium* species/genotypes in foxes, minks, and raccoon dogs sampled from these three provinces. These data will be helpful to avoid or reduce occurrence of cross-transmission and re-infection of this pathogen among different individuals within each farm as well as zoonotic transmission to humans.

Materials and methods

Ethics statement

All the animals involved in the present study were permitted by the owners or the managers of the farms. Here, only animal fecal specimens were collected and analyzed. During the procedure of collecting specimens, these animals were not disturbed. The study protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethics Committee of Harbin Medical University.

Specimen collection

During the period from June 2014 to October 2016, a total of 367 fresh fecal specimens were collected from 213 foxes, 114 minks, and 40 raccoon dogs in Heilongjiang, Jilin, and Liaoning provinces, northeastern China. Farms were sampled provided that the consent of owner's was given and ease of accessibility for sampling. Each of the fecal specimens was taken immediately from fresh feces on the ground after animal defecation using a sterile disposal latex glove and then placed into an individual plastic bag. They were transported to the laboratory in a cooler with ice packs within 24 h and stored at − 20 °C in a freezer until use. All the animals we investigated were 7 or 8 years old and had no clinical signs of illness at the time of sampling.

DNA extraction

Fecal specimens were three times sieved and washed followed by centrifugation for 10 min at 1500*g* at room temperature. Genomic DNA was directly extracted from 200 mg of each processed fecal pellet using a QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany). To obtain a high yield of DNA, the lysis temperature was increased to 95 °C. The procedures and reagents utilized were provided by the manufacturer. The eluted DNA (200 μl) was stored at − 20 °C until further use.

Cryptosporidium genotyping and subtyping

By a nested PCR amplification of an approximately 830 bp fragment of the SSU rRNA gene, all DNA preparations were screened for the presence of *Cryptosporidium* as previously described by Xiao et al. (1999). DNA preparations positive for the SSU rRNA locus of *Cryptosporidium* were subsequently analyzed to determine subtypes using a nested PCR to amplify an approximately 800 to 850 bp fragment of the gp60 gene as described in Alves et al. (2003). Every PCR amplification included a negative control without DNA template. TaKaRa Taq DNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all PCR amplifications. All secondary PCR products were subjected to electrophoresis in a 1.5% agarose gel and were visualized by staining the gel with Ultra GelRed (Vazyme GR501).

DNA sequence analysis

All positive secondary PCR products were directly sequenced with secondary PCR primers on an ABI PRISMTM 3730 XL DNA Analyzer (Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Accuracy of the sequencing data was confirmed by sequencing the PCR products in both directions. Meanwhile, for some DNA preparations producing nucleotide substitutions, deletions, or insertions compared to published sequences, at least two additional PCR products would be sequenced. Species/genotype identities of *Cryptosporidium* isolates were established by direct comparison of the acquired sequences with reference sequences downloaded from GenBank using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal X 1.83 (http:// www.clustal.org/).

Results

Percentage of *Cryptosporidium* positive samples

Of the fecal specimens, 5.4% (20/367) were PCR-positive for *Cryptosporidium* by amplifying the partial SSU rRNA gene. *Cryptosporidium* was only detected in foxes and minks. Twelve of 213 foxes tested positive, corresponding to 11 of 107 foxes screened in Heilongjiang (10.3%) and 1 of 66 foxes screened in Jilin (1.5%). *Cryptosporidium* was detected in 8 of 35 minks from Jilin (22.9%) but not in minks from Heilongjiang. In general, *Cryptosporidium* was more common in foxes than in minks ($p < 0.05$, $\chi^2 = 4.86$) (Table 1).

Cryptosporidium species/genotypes and subtypes

By sequence analysis of the SSU rRNA gene of 20 *Cryptosporidium* isolates, three *Cryptosporidium* species/genotypes were identified: *C. canis* ($n = 17$), *C. meleagridis* ($n = 1$) and *Cryptosporidium* mink genotype ($n = 2$). By aligning SSU rRNA gene sequences of *C. canis*, two different sequences were identified. Eleven fox-derived *C. canis* isolates were identical to a sequence identified in the fox-derived isolate (KU215430). Sequences of six mink-derived *C. canis* isolates were identical with one identified in a human subject (KT749818). *C. meleagridis* was identified in a fox from Jilin, which had the same nucleotide sequence as a human-derived isolate (HM485432). Two *Cryptosporidium* mink genotype isolates produced the same SSU rRNA sequence having two nucleotide insertions compared to that from a previously reported mink isolate (EF641015). The gp60 locus of one of the mink genotype isolates identified in this study could be amplified and successfully sequenced; it was found to have a 100% identity with the gp60 subtype XcA5G1R1 derived from a mink isolate (KU608305).

Discussion

Epidemiological data demonstrated *Cryptosporidium* infection in a variety of fur animals. *Cryptosporidium* has been identified by PCR in foxes in Spain (8.0%), the USA (7.9%), Ireland (1.6%), and China (1.6 to 23.1%) (Mateo et al. 2017; Nagano et al. 2007; Zhang et al. 2016a, b; Zhou et al. 2004), in minks in the USA (25.0%), Ireland (4.9%), China (29.6%), and the Czech Republic (6.0%) (Feng et al. 2007; Kellnerová et al. 2017; Stuart et al. 2013; Zhang et al. 2016a), and in raccoon dogs, China (10.5%) (Zhang et al. 2016a). In the present study, *Cryptosporidium* was detected in 12 of 213 foxes (5.6%) and 8 of 114 minks (7.0%). We did not detect *Cryptosporidium* in raccoon dogs. The result might be related to the small number of collected specimens and/or a low prevalence of *Cryptosporidium* in raccoon dogs in the investigated area.

Three *Cryptosporidium* species/genotypes were identified in this study, *C. canis* in foxes and minks, *C. meleagridis* in a fox, and the *Cryptosporidium* mink genotype in two minks. Molecular epidemiological surveys have identified nine *Cryptosporidium* species/genotypes (Feng et al. 2007; Gómez-Couso et al. 2007; Kellnerová et al. 2017; Mateo et al. 2017; Matsubayashi et al. 2004; Nagano et al. 2007; Stuart et al. 2013; Wang et al. 2008; Xiao et al. 2002; Zhang et al. 2016a, b; Zhou et al. 2004) (Table 2), with *C. canis* being the most common in foxes (71/80, 88.8%) and raccoon dogs (5/6, 83.3%) and the *Cryptosporidium* mink genotype in minks (40.0%, 28/70).

Table 1 *Cryptosporidium* species and genotypes identified in fox, mink, and raccoon dog

Animal species	Province	Examined no.	Positive no. (%)	Species/genotypes (*n*)
Fox	Heilongjiang	107	11 (10.3)	*C. canis* (11)
	Jilin	66	1 (1.5)	*C. meleagridis* (1)
	Liaoning	40	0	
	Subtotal	213	12 (5.6)	*C. canis* (11), *C. meleagridis* (1)
Mink	Heilongjiang	25	0	
	Jilin	35	8 (22.9)	*C. canis* (6), mink genotype (2)
	Liaoning	54	0	
	Subtotal	114	8 (7.0)	*C. canis* (6), mink genotype (2)
Raccoon dog	Heilongjiang	16	0	
	Jilin	24	0	
	Subtotal	40	0	
Total		367	20 (5.4)	*C. canis* (17), mink genotype (2), *C. meleagridis* (1)

Table 2 *Cryptosporidium* species/genotypes of natural infection in fur animals worldwide

Host	Country	No. of isolates	Species (*n*)	Genotype (*n*)	Reference
Fox	China	62	*C. canis* (62)		Zhang et al. 2016a, b, this study
	Ireland	2	*C. parvum* (2)		Nagano et al. 2007
	USA	9	*C. canis* (7)	Muskrat genotype I (1), fox genotype (1)	Zhou et al. 2004, Xiao et al. 2002
	Spain	7	*C. canis* (2), *C. parvum* (3), *C. felis* (1), *C. ubiquitum* (1)		Mateo et al. 2017
	Subtotal	80	*C. canis* (71), *C. parvum* (5), *C. felis* (1), *C. ubiquitum* (1)	Muskrat genotype I (1), fox genotype (1)	
Mink	China	54	*C. canis* (25), *C. meleagridis* (3)	Mink genotype (26)	Zhang et al. 2016a, Wang et al. 2008, this study
	USA	1		Mink genotype (1)	Feng et al. 2007
	Ireland	4	*C. andersoni* (3)	Mink genotype (1)	Stuart et al. 2013
	Spain	8	*C. parvum* (8)		Gómez-Couso et al. 2007
	Czech Republic	3	*C. ubiquitum* (3)		Kellnerová et al. 2017
	Subtotal	70	*C. canis* (25), *C. meleagridis* (3), *C. andersoni* (3), *C. parvum* (8), *C. ubiquitum* (3)	Mink genotype (28)	
Raccoon dog	Japan	1	*C. parvum* (1)		Matsubayashi et al. 2004
	China	5	*C. canis* (5)		Zhang et al. 2016a
	Subtotal	6	*C. canis* (5), *C. parvum* (1)		

C. canis is the most frequently identified species in dogs worldwide. However, it is also found in other Canidae animals (foxes and coyotes) (Trout et al. 2006; Zhang et al. 2016a) as well as the Mustelidae animals (minks) (Zhang et al. 2016a) and the Herpestidae animals (a mongoose) (Mateo et al. 2017). An experimental cross-transmission study reveals that *C. canis* oocysts from a domestic dog and an HIV-infected human have the ability to infect calves (Fayer et al. 2001). Human cases of cryptosporidiosis caused by *C. canis* have been reported in immunocompetent and immune compromised individuals (Ryan et al. 2014). In developing countries, *C. canis* is responsible for as much as 4.0% of overall cryptosporidiosis cases in children (Lucio-Forster et al. 2010). Due to the zoonotic nature of *C. canis*, people having direct and indirect contacts with fur animals, which include farmers, breeders, veterinarians, and workers of the fur processing industry, should be aware of the potential zoonotic transmission of cryptosporidiosis. Phylogenetic analysis revealed three different genotypes among *C. canis* isolates: *C. canis* dog, fox, and coyote genotypes (Xiao et al. 2002). Among them, exclusively the *C. canis* dog genotype has been reported to be pathogenic for humans (Zhou et al. 2004). Since then, the *C. canis* dog genotype has been also identified in dogs, minks, and raccoon dogs suggesting that contact with these animals can be a possible source for human cryptosporidiosis (Gil et al. 2017; Zhang et al. 2016a). *C. canis* fox and coyote genotypes are considered to be variants of the *C. canis* genotype that are host specific for foxes and coyotes, respectively, since they have only detected in these carnivore species (Xiao et al. 2002). Therefore, it is necessary to assess public health significance of different *C. canis* isolates based on comparing differences in host specificity and infectivity by cross-transmission studies in the future. Establishment of subtyping and MLST tools of *C. canis* will be helpful to track the source of infection/contamination and elucidate transmission dynamics of human cryptosporidiosis caused by *C. canis*. Currently, it remains unclear whether the three *C. canis* genotypes are actually three different *Cryptosporidium* species.

The *Cryptosporidium* mink genotype was found in minks, first in China and later in humans in Australia (Wang et al. 2008, Ebner et al. 2015, Ng-Hublin et al. 2013). So far, minks have been described as the only animal host of this genotype (Feng et al. 2011; Stuart et al. 2013; Wang et al. 2008; Zhang et al. 2016a). In the present study, we found the same genotype in two minks, one of which we could subtype as XcA5G1R1. This subtype has been previously reported in minks (Zhang et al. 2016a). To date, the four subtypes XaA5G1, XbA5G1R1, XcA5G1R1, and XdA4G1 have been identified in *Cryptosporidium* mink genotype isolates from minks (Feng et al. 2011; Zhang et al. 2016a). As the only available human-derived *Cryptosporidium* mink genotype isolates have not been subtyped (KT123176 and JX471005), the possibility of zoonotic transmission of this genotype has to be confirmed.

Besides in a variety of birds, *C. meleagridis* has been identified in other animal hosts including minks, cattle, wallabies, gorillas, and dogs as well as some bivalves (Fayer et al. 2003;

Hajdusek et al. 2004; Mariné Oliveira et al. 2016; Pagoso and Rivera 2017; Ryan et al. 2014; Sak et al. 2014; Vermeulen et al. 2015; Zhang et al. 2013, 2016a). In addition, *C. meleagridis* is recognized as the third most common *Cryptosporidium* species in humans (Ryan et al. 2016). In some areas, the prevalence of *C. meleagridis* in humans are as high as *C. parvum* (Cama et al. 2008; Cama et al. 2007; Gatei et al. 2002). Experimental cross-transmission infections of *C. meleagridis* have been performed successfully in the mammal species mice, rats, calves, pigs, and rabbits and in the bird species poults and chickens (Akiyoshi et al. 2003; Darabus and Olariu 2003; Huang et al. 2003; Tůmová et al. 2002; Sréter et al. 2000). Data on epidemiology and cross-transmission of *C. meleagridis* show a low host specificity of this parasite. In the present study, *C. meleagridis* was found for the first time in a fox, further expanding the host range of this parasite.

Conclusion

The present epidemiological data suggest that foxes and minks in the investigated areas are infected with *Cryptosporidium*. The finding of three zoonotic *Cryptosporidium* species/genotypes in the studied fur animals implies that there is a potential risk of human cryptosporidiosis for humans involved in this industry. In addition, these animals can be an infection source of human cryptosporidiosis through ingestion of water and food contaminated with infectious oocysts. Waterborne outbreaks of cryptosporidiosis attract more public attention due to more people involved in these events. Importantly, also aquatic shellfish can be contaminated by the *Cryptosporidium* infectious oocyst stage (Srisuphanunt et al. 2009). Thus, improved farm management systems are needed to prevent the occurrence of cross-transmission and re-infection of *Cryptosporidium* among the animals within each farm, and to reduce environmental contamination from animal manure.

Funding information This work was supported by the Heilongjiang Province Education Bureau (No. 12531266)

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