Short Communication

Molecular Evidence of Cryptosporidiosis in Farmed Blue Foxes (*Vulpes lagopus*) and Raccoon Dogs (*Nyctereutes procyonoides*) in Heilongjiang, China

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Abstract

Cryptosporidium, an important zoonotic protozoan, causes Cryptosporidiosis in humans as well as in animals. This study aimed to identify Cryptosporidiosis in farmed fur animals (Blue Fox and Raccoon Dogs). A total of 283 specimens (223 foxes, 60 Raccoon dogs) were screened through nested-PCR by targeting the actin gene. The results showed the overall prevalence of cryptosporidiosis was 9.1% where the prevalence in foxes and raccoon dogs was 9.1% and 8.3%, respectively. The prevalence in males (15%) was significantly higher than that in females (5.7%). The findings of this study portrayed new knowledge that foxes and raccoon dogs are likely to play an important role in the spreading of this zoonosis.

Keywords: Cryptosporidiosis, nested-PCR, Norwegian Blue Fox, Raccoon Dog, China

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Introduction

Cryptosporidium species is an important zoonotic protozoan parasite and causes Cryptosporidiosis (Qin *et al*., 2014; Kaupke and Rzeżutka, 2015; Liu *et al*., 2015). Cryptosporidiosis is an important *diarrheic* infection that leads to mortality in immune-suppressed hosts, especially AIDS patients and has been reported in humans, domesticated animals and zoo animals (Xiao and Fayer, 2008). Until now, more than 38 valid species and almost 70 genotypes of undetermined spp. have been identified (Feng *et al*., 2018). Having a very narrow host range, these species and genotypes are mostly host familiarized (Xiao and Fayer, 2008; Xiao, 2010; Šlapeta, 2013). Cryptosporidiosis in humans is usually caused by *Cryptosporidium parvum* and *Cryptosporidium hominis* (Xiao, 2010). Although, some spp. like *Cryptosporidium canis*, *Cryptosporidium felis* and *Cryptosporidium meleagridis,* and some genotypes like the horse genotype, monkey genotype and skunk genotype are almost host specified in human infections but also considered to be zoonotic (Xiao and Fayer, 2008; Xiao and Feng, 2008). Previous studies of Cryptospridiosis have been mostly related to human beings and a large number of domesticated and wild animals (Xiao, 2010) but very few studies have talked about captive-bred animals.

In China, foxes and raccoon dogs are usually reared to get furs for humans. These farmed fur animals have a close relationship with the farmers. That is why many pathogens like T. *gondii* can be transferred to humans directly and indirectly (Prestrud *et al*., 2008). Internationally, some studies to investigate cryptosporidiosis in foxes and raccoon dogs have been reported in Canada (Elmore *et al*., 2013), Germany (Leśniańska *et al*., 2016), Italy (Papini and Verin, 2019), Japan (Matsubayashi *et al*., 2004; Matsubayashi *et al*., 2005), Norway (Hamnes *et al*., 2007), the UK (Sturdee *et al*., 1999; Nagano *et al*., 2007) and the USA (Davidson *et al*., 1992; Martin and Zeidner, 1992; Foster *et al*., 2004; Zhou et al., 2004) similarly a very few studies about Cryptosporidiosis in foxes and raccoon dogs are also available in China (Zhang *et al*., 2016; Zhang *et al*., 2016).

Molecular identification of cryptosporidiosis is giving new research directions for the future (Feng and Xiao, 2017). This research aimed to examine the prevalence of cryptosporidiosis in farmed blue foxes and raccoon dogs.

Materials and Methods

A total of 283 fresh fecal samples were collected from 223 blue foxes and 60 raccoon dogs on two farms (Farm A and Farm B) in the surroundings of Harbin, China in 2018-19. These specimens were grouped as juveniles (< 1year) and adults (> 1year). Out of the 283 samples, 107 were collected from males and 176 from females after natural defecation; fresh specimens were collected from the ground and brought to the laboratory within 12 hours and stored in the refrigerator at 4°C. Stool samples were processed within 24 hours, and oocysts were isolated through a discontinuous sucrose gradient as previously described (Arrowood and Sterling, 1987).

DNA was extracted from these isolated oocysts (200ml) using a QIAmp DNA Stool Kit (QIAGEN, Hilden, Germany) with minor modifications to the manufacturer's protocol. For lysis of the thick-walled *Cryptosporidium* oocyst and release of DNA, oocysts underwent ten freeze-thaw cycles (freeze in -80°C refrigerator and thawing at 65°C within the water bath). Final elution volume was adjusted to 70 µl of AE buffer to increase DNA concentration. All the DNA samples were kept at -20°C until PCR analysis.

Nested PCR was done using a thermal cycler (SEDI G thermal cycler, Wealtec Corp. Japan). Amplification of the actin gene for *Cryptosporidium* was carried out in a 25 µl reaction mixture of primary PCR containing 7 µl DNA, 1 µl of forward and reverse primers, 12.5 µl of ExTaq premix (Takara Bio Group, Japan) and 4.5 µl of dd H_2O. In the primary PCR, a product of approximately 830 bp was amplified by using AII F1 (5′-ATGCCVGGWRTWATGGTDGGTATG-3′) as the forward primer actin and Act6R (5′-GGDGCAACRACYTTRATCTTC-3′) as the reverse primer actin. The amplification was performed with an initial preliminary cycle of 94°C for 2 mins, 58°C for 1 min, and 72°C for 2 mins, followed by 50 amplification cycles (94°C for 30s, 58°C for 30s, 72°C for 30s), with a final extension of 72°C for 7 mins. With primary PCR product, a fragment of 818 bp was amplified in secondary PCR using forward actin AII F2 (5′-GAYGARGCHCARTCVAARAGRGGTAT-3′) and reverse actin AII R1 (5′-TTDATYTTCATDGTHGAHGGWG C-3′) primers (Ng *et al*., 2006). Positive and negative controls were included in every PCR amplification. Finally, secondary products were visualized on 1.5 % agarose gels containing GoldView™ (Solarbio, China).

Statistical Package for Social Services (SPSS) version 20.0 was used to analyze the data applying the Chi-square test. The P-value was calculated, where 0.05 was considered significant at the 95% C.I (Confidence Interval) level.

Results and Discussion

A total of 283 (Blue Fox 223, Raccoon Dogs 60) fecal samples were screened: 107 male, 176 female and, by age, 91 Juveniles and 192 adults. Nested PCR detected 26 of 283 (9.18%) examined stool samples. The bands observed on 2% agrose gel indicated positivity for cryptosporidiosis (Figure 1). The results obtained for breed, age and gender are displayed in Table 1, along with the statistical significance for the analysis of association among various risk factors for the pattern of the disease's prevalence, using a 95% confidence interval. No significant association was found between the presence of *Cryptosporidium* and age (p = 0.29); on the other hand, gender showed significant results with a p-value of 0.0088 and the male with more risk of having cryptosporidiosis (OR = 2.92). Among 26 positive samples, 5.7% (10/176) obtained for females and the 15% (16/107) for males, were recorded from the total 283 samples. In the occurrence of *Cryptosporidium*, no statistically significant differences were noted between the breed having a p-value of 0.76 and foxes were evidenced to be more likely to be related to the presence of the *Cryptosporidium*.

Considering age or type of breed, the highest prevalence of the *Cryptosporidium parasite* was for foxes (9.1%), followed by raccoon dogs (8.3%) and adults (10.4%) followed by Juveniles (6.6%).

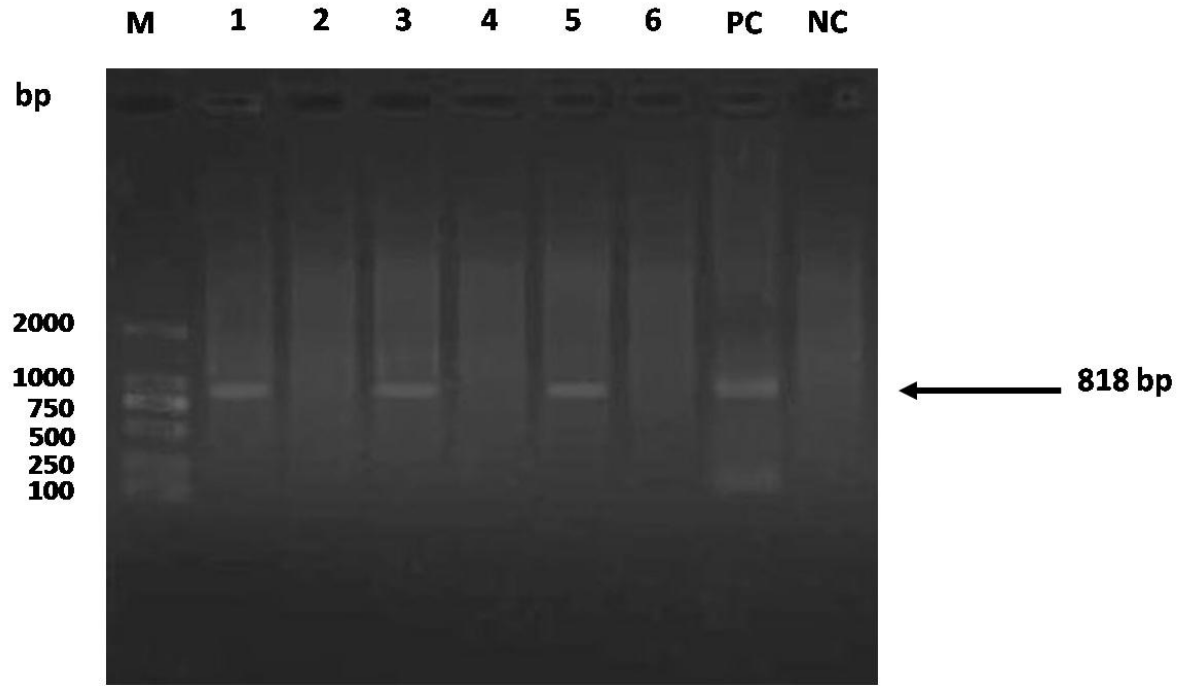

Figure 1 Results of nested PCR representing the efficient detection of *cryptosporidium spp.* using specific primers targeting the actin gene. Lane M: Marker of 2000 bp, Lane 1-6 samples used for the detection of cryptosporidium spp., PC: Positive control and NC: negative control

Table 1 Prevalence of Cryptosporidiosis in farmed foxes and raccoon dogs in Heilongjiang, Northeastern China

Category		Positive	Negative	Frequency	P-value	Odd Ratio	CI (95%)
Gender	Male (107)	16	91	15	0.0088*	2.92	1.272- 6.696
	Female (176)	10	166	5.7			
Age	Juveniles (91)	06	85	6.6	0.29	0.61	0.235- 1.568
	Adults (192)	20	172	10.4			
Breed	Fox (223)	21	202	9.1	0.79	1.14	0.412- 3.171
	Raccoon Dog (60)	05	55	8.3			

*Statistically significant difference by Chi-square test

In this study, the overall prevalence obtained for cryptosporidiosis was 9.18% in blue foxes (*Vulpes lagopus)* and raccoon dogs (*Nyctereutes procyonoides*) while it was 9.1% (21/223) in blue foxes and 8.3% (05/55) in raccoon dogs. The prevalence in foxes was higher than that in the farmed Arctic foxes (*Vulpes lagopus*) in Northeast China (1.6%) (Zhang *et al*., 2016), red foxes (*Vulpes vulpes*) in Italy (1.4%) (Papini and Verin, 2019), Norwegian wild red foxes (*Vulpes vulpes*) in Norway (2.2%) (Hamnes *et al*., 2007), gray foxes (*Urocyon cinereoargenteus*) in the USA (1.9%) (Davidson *et al*., 1992), foxes in Maryland, USA (7.9%) (Zhou *et al*., 2004), foxes (*Vulpes vulpes*) in mainland Britain (9%) (Sturdee *et al*., 1999), Arctic foxes (*Vulpes lagopus*) in Canada (9%) and, lower than that in farmed Arctic foxes (*Vulpes lagopus*) in northern China (15.9%) (Zhang *et al*., 2016) and in red foxes in the Slovak Republic (38.7%) (Ravaszova *et al*., 2012) while all foxes (*Vulpes vulpes*), fecal samples from grassland adjacent to water reservoirs located north and east of Melbourne's central business district (CBD), were negative for *Cryptosporidium* (Koehler *et al*., 2016). The report of Cryptosporidiosis in farmed fox may be responsible for infection among the involved people because of the zoonotic importance of this parasitic disease. Farm workers close to calves and camels are at risk as reported in Iran (Sazmand *et al*., 2012; Izadi *et al*., 2014) but no one was infected out of 68 Sicilian farmers from Palermo area, Italy (Di Piazza *et al*., 2013). Previously cryptosporidiosis has been reported in animals and humans along with several other vertebrates (O'Donoghue, 1995).

The first report of Cryptosporidiosis was in a raccoon dog (*Nyctereutes procyonoides viverrinus*) in Japan (Matsubayashi *et al*., 2004). The prevalence in raccoon dogs (*Nyctereutes procyonoides*) was higher than that in Maryland, USA (4%) (Zhou *et al*., 2004) and lower than that in Northeast China (10.5%) (Zhang *et*

al., 2016) and, Poland and Germany (34.7%) (Leśniańska *et al*., 2016), while all fecal samples from raccoon dogs in northern China were negative for *Cryptosporidium*.

Unlike a recent study, (Leśniańska *et al*., 2016) from Germany that reported the highest prevalence among females (38.9%) followed by males (32.3%) another study from Norway showed lower prevalence in females (1.1%) than that in males (4%) (Hamnes *et al*., 2007). In China, (Zhang *et al*., 2016) showed 1.7 % and 1.4% in juvenile and adult foxes respectively and 12.3% and 6.3% in juveniles and adult raccoons, respectively.

There is a possibility that these farmed fur animals were not suffering from infection but had simply received ocysts by eating infected chicken. Previously chicken has been reported as a *Cryptosporidium* reservoir in China, Syria, Jordan and Brazil (Kassouha, 2014; Hijjawi *et al*., 2016; Liao *et al*., 2018; Santana *et al*., 2018). A thorough molecular screening is required for poultry farms so that the spread of cryptosporidiosis can be controlled.

In this study, we found nearly the same prevalence of *Cryptosporidium* relative to most other population findings reported but the actual prevalence of *Cryptosporidium* cannot be ruled out without sequencing of the positive samples. Most of the studies preferred sequencing to identify *Cryptosporidium* ssp. and genotypes in fecal specimens. Without genetic studies, the species and genotypes could not be assessed in the farmed fur animals and the public health consequences also remain undetermined. The results showed that farmed fur animals may spread by shedding zoonotic isolates.

The findings of this study show that farmed fur animals can spread cryptosporidiosis through the shedding of zoonotic isolates. The prevalence in males was significantly higher than that in females. The present data suggests that a thorough molecular epidemiological survey is required to develop control strategies for *Cryptosporidium* in foxes and raccoons.

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