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Short Communication

Prevalence and molecular characterization of *Cryptosporidium* spp. in Père David's deer (*Elaphurus davidianus*) in Jiangsu, China

Prevalência e caracterização molecular de *Cryptosporidium* spp. no cervo de Père David (*Elaphurus davidianus*) em Jiangsu, China

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Abstract

Cryptosporidium is a zoonotic parasite that causes diarrhea in a broad range of animals, including deer. Little is known about the prevalence and genotype of *Cryptosporidium* spp. in Père David's deer. In this study, 137 fecal samples from Père David's deer were collected between July 2017 and August 2018 in the Dafeng Reserve and analyzed for *Cryptosporidium* spp. by nested-PCR based on the small subunit ribosomal RNA (*SSU* rRNA) gene, followed by sequence analyses to determine the species. The 60 kDa glycoprotein (*gp60*) gene was used to characterize *Cryptosporidium* spp. Among 137 samples, 2 (1.46%) were positive for *Cryptosporidium* spp. according to *SSU* rRNA gene sequencing results. Both samples belonged to the *Cryptosporidium* deer genotype, with two nucleotide deletions and one nucleotide substitution. The prevalence data and molecular characterization of this study provide basic knowledge for controlling and preventing *Cryptosporidium* infections in Père David's deer in this area.

Keywords: *Cryptosporidium*, Père David's deer, 60 kDa glycoprotein (*gp60*) gene, the small subunit ribosomal RNA (*SSU* rRNA).

Resumo

Cryptosporidium é um parasita zoonótico que causa diarreia em uma ampla gama de animais, incluindo veados. Pouco se sabe sobre a prevalência e o genótipo de *Cryptosporidium* spp. no cervo de Père David. Neste estudo, 137 amostras fecais do cervo de Père David foram coletadas entre julho de 2017 e agosto de 2018, na Reserva Dafeng, e analisadas para *Cryptosporidium* spp. por nested-PCR baseado no gene do RNA ribossômico da subunidade pequena (*SSU* rRNA), seguido de análises de sequências para determinar as espécies. O gene da glicoproteína de 60 kDa (*gp60*) foi utilizado para caracterizar *Cryptosporidium* spp. Dentre as 137 amostras, 2 (1,46%) foram positivas para *Cryptosporidium* spp. de acordo com os resultados do sequenciamento gênico de *SSU* rRNA. Ambas as amostras pertenciam ao genótipo do cervo *Cryptosporidium*, com duas deleções nucleotídicas e uma substituição nucleotídica. Os dados de prevalência e a caracterização molecular deste estudo fornecem conhecimentos básicos para controlar e prevenir infecções por *Cryptosporidium* nos cervos de Père David nessa área.

Palavras-chave: *Cryptosporidium*, cervos de Père David, Gene de glicoproteína de 60 kDa (*gp60*), RNA ribossômico da pequena subunidade (*SSU* rRNA).

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Introduction

Cryptosporidiosis is caused by *Cryptosporidium* spp., which is an important enteric apicomplexan parasite of zoonosis in the world (Parsons et al., 2015; Tanriverdi et al., 2007; Zhang et al., 2016). It is a critical emerging infectious disease in humans and animals that can lead to diarrhea or other serious symptoms (Zhao et al., 2015). In general, cryptosporidiosis is transmitted through the fecal-oral route, when ingesting food or water contaminated with infective oocysts. Currently, there is no effective drug or vaccine to cure or prevent cryptosporidiosis. Therefore, this disease has caused significant economic losses in animal husbandry. In addition, infected animals may be a source of secondary infection, because they can serve as potential carriers for human and other animal infections via excreting feces, including oocysts, that contaminate food and water (Deng & Cliver, 1999).

Père David's deer (*Elaphurus davidianus*), also called Milu deer, native to the Yangtze River Basin of China, is an endangered deer species in the world and listed as Extinct in the Wild by the International Union for Conservation of Nature (IUCN). It became extinct in the wild in China at the end of the 19th century. Fortunately, from 1985 to 1987, two groups of 40 and 39 Père David's deer were reintroduced to China from the UK and raised in the Nanhaizi Nature Reserve and Dafeng Reserve, respectively. The largest population in the world lives in the Dafeng Reserve, which is historically synonymous with Père David's deer (Ding et al., 2018).

Currently, more than 30 *Cryptosporidium* species and genotypes have been identified (Baroudi et al., 2018; Ryan et al., 2014). Eleven of them, *C. muris, C. parvum, Cryptosporidium* muskrat II genotype, *C. hominis*-like genotype, *Cryptosporidium caribou* genotype, *C. hominis, C. bovis, C. ryanae, Cryptosporidium deer* genotype, *C. ubiquitum* and *Cryptosporidium suis*-like genotype, have been identified in cervids, including red deer, sika deer, white-tailed deer, roe deer, caribou and moose in China, Czech Republic, Japan, the United Kingdom, Spain, the United States, Norway and Poland (Garcia-Presedo et al., 2013; Huang et al., 2018; Jellison et al., 2009; Kato et al., 2016; Kotkova et al., 2016; Siefker et al., 2002; Wang et al., 2008; Wells et al., 2015). Little information is available about *Cryptosporidium* infections in Père David's deer (Huang et al., 2018). In this study, the prevalence of *Cryptosporidium* infections and molecular characteristics were investigated in Père David's deer in the Dafeng Reserve, China.

Materials and Methods

Specimen collection and preparation

A total of 137 fecal samples of Père David's deer were collected between July 2017 and August 2018 in the Dafeng Reserve, Jiangsu Province, China. The samples were collected immediately after excreted onto the ground using sterile gloves and placed in individual plastic bags. No visible clinical signs were observed in these deer. The samples were pretreated in the laboratory in the following steps: 50 g of feces were placed in a beaker, diluted with normal saline, and stirred evenly with a glass rod. Then, the suspension was filtered with a 200-mesh sieve. The filtrate was loaded into a 50-mL centrifuge tube, centrifuged at 3,000× *g* for 10 min, and the precipitate was collected and stored at -20°C for further study.

DNA extraction and PCR amplification

Genomic DNA was extracted from each sample using the E.Z.N.A. Stool DNA Kit (OMEGA, USA) according to the manufacturer's directions and stored at -20°C or immediately used for PCR. *Cryptosporidium* species and genotypes were examined by nested-PCR based on the small subunit ribosomal RNA (*SSU* rRNA) gene, as previously described (Zhao et al., 2013). For further identification and subtype detection, the samples positive for *SSU* rRNA were further analyzed by nested-PCR targeting the 60-kDa glycoprotein (*gp60*) gene (Alves et al., 2003; Feng et al., 2012; Li et al., 2014). The cycling conditions were as follows: 5 min at 95°C, followed by 35 cycles, each composed of 45 s at 94°C, an annealing step at a suitable temperature (Table 1) for 45 s, and 1 min at 72°C, and the final extension at 72°C for 10 min. Positive and negative controls were included in each reaction. The products were observed under UV light after electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Table 1. Primers used in the study, annealing temperatures used in the PCR, and expected sizes of the PCR products.

Primers names	Primers Sequence(5'-3')	Annealing temperature (°C)	Amplicon length(bp)	References
SSU rRNA F1	CCCATTTCCTTCGAAACAGGA	55	830	Zhao et al. 2013
SSU rRNA R1	TTCTAGAGCTAATACATGCG			
SSU rRNA F2	AAGGAGTAAGGAACAACCTCCA	58		
SSU rRNA R2	GGAAGGGTTGTATTATTAGATAAAG			
gp60 F1	ATAGTCTCCGCTGTATTC	50	800 to 850	Alves et al. 2003
gp60 R1	GGAAGGAACGATGTATCT			
gp60 F2	TCCGCTGTATTCTCAGCC	50		
gp60 R2	GCAGAGGAACCAGCATC			

Sequencing and phylogenetic analyses

All PCR products were sequenced by the GenScript Company (Nanjing, China). The sequence accuracy was confirmed by bidirectional sequencing. To determine *Cryptosporidium* species and subtypes, the sequencing results were aligned with known reference sequences of *Cryptosporidium* available in GenBank using BLAST . MEGA 5.0 was used to construct the phylogenetic trees using neighbor-joining (NJ) analysis of the SSU rRNA sequences, based on the Kimura-2-parameter model, and bootstrapping was performed using 1000 replicates. The nucleotide sequences obtained in this study were deposited in the GenBank under accession number MK571183.

Results and Discussion

Prevalence of *Cryptosporidium*

In this study, 2 of 137 fecal samples were positive for *Cryptosporidium* infection. The overall prevalence of *Cryptosporidium* was 1.46% in Père David's deer in the Dafeng Reserve. The result was similar to 3.7% seen in wild red deer, European leisure deer, white-tailed deer and mouflon sheep in the Czech Republic (Kotkova et al., 2016), but lower than that in the red deer, Père David's deer and sika deer in Henan and Jilin, China (6.8%), the Hokkaido sika deer in Japan (7.8%) and white-tailed deer in central Maryland (12.5%) (Huang et al., 2018; Kato et al., 2016; Santin & Fayer, 2015). Although one study indicated that *Cryptosporidium* was found in Père David's deer, there was no detailed information about prevalence. Thus, it is difficult to compare the prevalence with that in other studies. In addition, due to the influence of ecological conditions, age distributions, seasons, management systems, sample sizes and other factors, explaining the discrepancies in the prevalence of *Cryptosporidium* among different studies is challenging (Huang et al., 2014).

Cryptosporidium species and genotypes

Two *Cryptosporidium*-positive samples were sequenced and genotyped by the sequence analysis of the *SSU* rRNA gene. According to the results of the BLAST (NCBI) analysis, both isolates represented the *Cryptosporidium* deer genotype. The *Cryptosporidium* deer genotype (GenBank accession numbers: KX259129), which was recently reported in red deer in Henan and Jilin, China, has two nucleotide deletions (-/G position 8, -/T position 16) and one nucleotide substitution (G/T position 11) (Huang et al., 2018) in the two isolates in the present study. In the *Cryptosporidium* genome, the *gp60* gene was used for *C. parvum* and *C. ubiquitum* subtype analysis due to its heterogeneity and biological correlation. Although no *C. parvum* and *C. ubiquitum* were detected, the two positive samples were analyzed by nested-PCR targeting the *gp60* gene (Feng et al., 2007b). The results were negative; no PCR amplicon was amplified. Currently, there are several reports on cervid infections with the *Cryptosporidium* deer genotype in England, Australia, Czech Republic, China, and Japan (Cinque et al., 2008; Feng et al., 2007a; Koehler et al., 2016; Perz & Le Blancq, 2001; Robinson et al., 2011; Xiao et al., 2002). However, there is little genotype information about *Cryptosporidium* in Père David's deer. In the present study, the genotype identified in the Père David's deer

is similar to the *Cryptosporidium* deer genotype reported before; however, compared with the *Cryptosporidium* deer genotype (GenBank accession numbers: KX259129), there were three mutants. More information and future studies are needed to determine whether this genotype represents a new genotype.

Phylogenetic analyses

Phylogenetic relationships were established by the NJ method; the *Plasmodium cathemerium* sequence was used as the outgroup, and the sequence similarity between *Cryptosporidium* species and genotypes available in GenBank was observed based on *SSU* rRNA (Figure 1). *Cryptosporidium* forms two main groups, one of which includes *C. muris, C. serpentis, C. galli* and *C. andersoni*, previously known as parasitic gastrosporidium. The other group includes *C. bovis, C. ryanae, C. scrofarum, Cryptosporidium* pig genotype, *C. avium, C. baileyi, C. canis, C. suis, C. lemurs, C. wrairi, C. meleagridis, C. parvum, Cryptosporidium* deer genotype, and the isolated strain of *Cryptosporidium* derived from Père David's deer (MK571183). The results indicated that *Cryptosporidium* spp. Père David's deer (the newly generated sequences in this study) was clustered in the *Cryptosporidium* deer genotype branch. The genotype shares a branch with isolates from the United States, Japan and China and is closely related to *C. ryanae* and *C. bovis*.

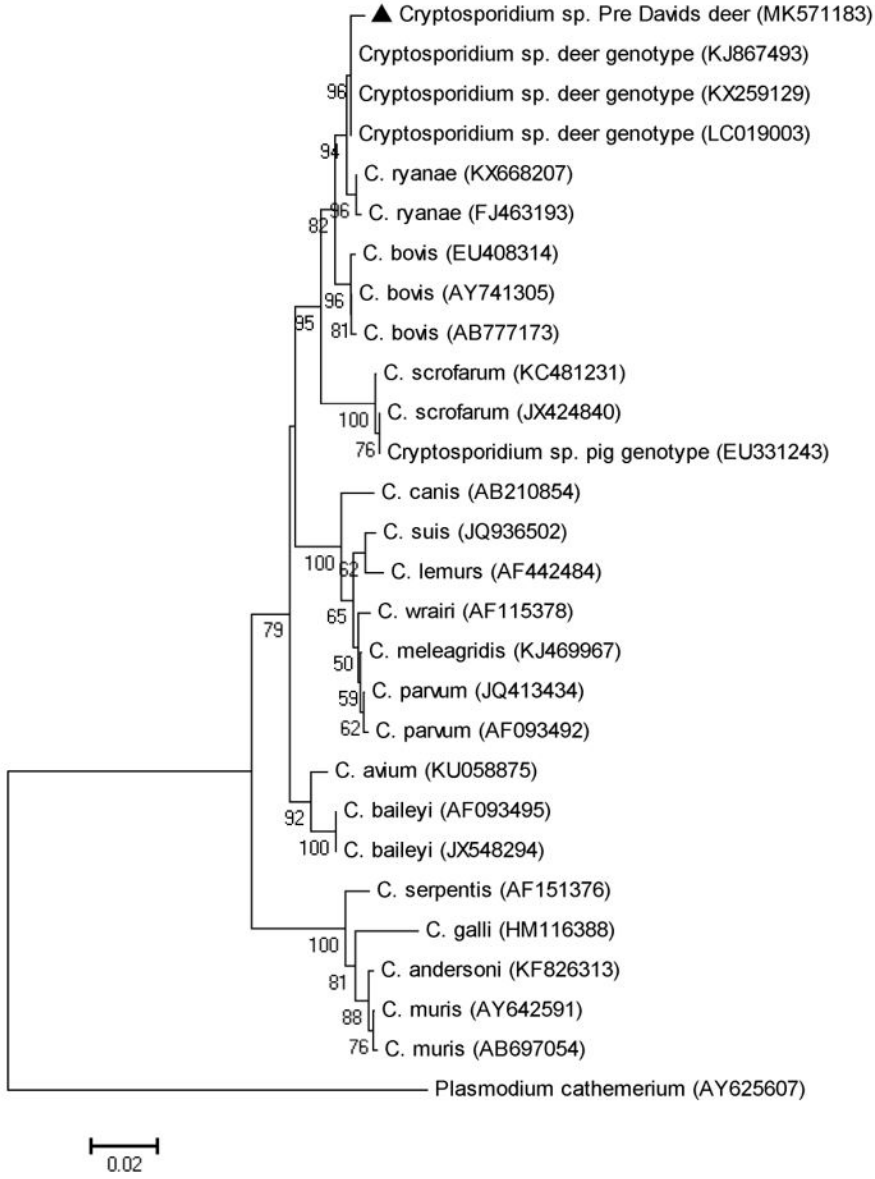

Figure 1. Phylogenetic relationship between *SSU* rRNA sequences of *Cryptosporidium* was analyzed using the Kimura-2 parametric model of Neighbor-Joining (NJ). The numbers on branches was the percentage of the bootstrap values in 1000 replicates. The *Cryptosporidium* isolates identified in this study are represented by *black triangles*.

These results indicate that the *Cryptosporidium* isolated from the Père David's deer in this study were close to the *Cryptosporidium* deer genotype. This study reported the prevalence (1.46%, 2/137) of *Cryptosporidium* infection in Père David's deer in the Dafeng Reserve, China, for the first time. The genotype identified in Père David's deer in the Dafeng Reserve was *Cryptosporidium* deer genotype with 3 mutants, which was closely related to *C. ryanae* and *C. bovis*. Further investigation into the transmission dynamics of these pathogens should be continued.

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