Parasitol Res (2015) 114:773–776
DOI 10.1007/s00436-014-4290-1

SHORT COMMUNICATION

Prevalence of *Cryptosporidium* infection in captive lesser panda (*Ailurus fulgens*) in China

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Received: 10 December 2014 / Accepted: 23 December 2014 / Published online: 7 January 2015

Abstract *Cryptosporidium* is a global epidemic parasite and one of the most important intestinal pathogens causing diarrhea in animals and humans. Despite extensive research on this parasite group, little is known about rates of *Cryptosporidium* infection in lesser pandas. In this study, we use molecular diagnostic tools to detect *Cryptosporidium* infections and identify *Cryptosporidium* species in the lesser panda. Using a PCR approach, we sequenced the 18S rRNA gene in fecal samples collected from 110 captive lesser pandas held throughout China (approximately one third of the captive population). We determined *Cryptosporidium* species via a BLAST comparison of our sequences against those of published *Cryptosporidium* sequences available in GenBank and subsequent phylogenetic analysis. We report that captive lesser pandas were infected with a single *Cryptosporidium* species, *Cryptosporidium andersoni*, at a prevalence of 6.36 % (7/110). The present investigation revealed the existence of *C. andersoni* infection in captive lesser panda and suggested that proper control measures should be taken carefully to protect the welfare of zoo workers and visitors.

Keywords *Cryptosporidium andersoni* · 18S rRNA · PCR · Lesser panda · Prevalence

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Introduction

Cryptosporidium is a worldwide epidemic parasite that has been identified as one of the most important intestinal pathogens causing diarrhea in animals and human beings (Xiao 2009). Many animals can be infected with *Cryptosporidium* spp. including mammals, birds, reptiles, amphibians, and fish (Xiao 2009). Currently, at least 30 *Cryptosporidium* species have been recognized and more than 40 genotypes have been described (Slapeta 2013). Of these, 13 species and 7 genotypes are responsible for most human cryptosporidiosis cases (Connelly et al. 2013; Kvac et al. 2009; Waldron et al. 2010; Wang et al. 2013), while 11 species and over 30 *Cryptosporidium* genotypes are known to infect wild animals (Fayer et al. 2005; Feng 2010; Ryan et al. 2004; Xiao et al. 2004).

Despite extensive research on this parasite group, little is currently known about rates of *Cryptosporidium* infection in lesser panda. So far, only one *Cryptosporidium* species, *Cryptosporidium parvum* mouse genotype, has been identified in two lesser pandas in Qinghai, China (Karanis et al. 2007). In an effort to gain more insight into the *Cryptosporidium* infection in lesser pandas to inform public health actions in China, we used molecular diagnostic tools to detect *Cryptosporidium* infection and identify *Cryptosporidium* species in captive lesser panda in China.

Materials and methods

Ethics statement

Samples were collected under the permission of the relevant zoos and institutions. All procedures were reviewed and approved by the Wildlife Management and Animal Welfare

Table 1 Prevalence of *Cryptosporidium andersoni* in captive lesser panda sampled from different regions of China

Location	Sichuan	Chongqing	Yunnan	Henan	Shanxi
Number of samples	93	2	9	3	3
Positive	6	1	0	0	0

Committee of China. During fecal collection, animal welfare was taken into consideration.

Fecal sample collection

Fecal samples were obtained from 110 lesser pandas held in captivity in five areas throughout China (Table 1). The samples were labeled and placed immediately in disposable plastic bags before being shipped to the laboratory of Sichuan Agricultural University for purification and processing.

Sample processing and DNA extraction

Oocysts were concentrated from feces as previously described (Current 1990). Briefly, each fecal sample was mixed with proper amount of dH_2O in specimen cups. The suspension was then transferred to another clean specimen cup through a sieve with an 80-mm pore size to remove larger fecal debris. The filtrate was then centrifuged at 1,800×*g* for 15 min, and the supernatant was removed. Total DNA was extracted from 200 mg of each oocyst precipitation using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The eluted DNA was stored at −20 °C prior to PCR analysis.

Gene amplification and sequencing

A PCR amplification approach was used to detect *Cryptosporidium* in DNA samples. We amplified a fragment (~830 bp) of the 18S ribosomal RNA (rRNA) gene as described previously (Xiao et al. 1999). During the PCR, the primer pairs (forward: 5′-TTCTAGAGCTAATACATGCG-3′ and reverse: 5′-CCCATTTCCTTCGAAACAGGA-3′) were used. The PCR mixture contained 12.5 μL Taq PCR MasterMix (Tiangen Biochemical Technology Co., Ltd.), 0.5 μL BSA (0.1 g/10 ml), 8 μL ddH_2O, 2 μL DNA, and 1 μL of each forward and reverse primer (working concentration 10 pmol/L) in a 25-μL reaction volume. Each of the 35 PCR cycles consisted of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 1 min after an initial hot start at 94 °C for 3 min, and ending with 72 °C for 7 min. PCR products were analyzed on a 1 % agarose gel and stained with GoldenView for visualization. Since the weak target gel bands, all the PCR products were then purified and cloned into a pMD19-T vector and sent for commercial sequencing (Invitrogen, Shanghai).

Phylogenetic analysis

The obtained sequences were aligned with each other and published 18S rRNA gene sequences of *Cryptosporidium* spp. using the software ClustalX (http://www.clustal.org) to determine *Cryptosporidium* species. Phylogenetic relationships of *Cryptosporidium* spp. were reconstructed using the neighbor-joining (1,000 replicates) analysis implemented in MEGA 5.0 (http://www.megasoftware.net/) based on genetic distances calculated by the Kimura 2-parameter model. The sequence generated from PCR analysis of the

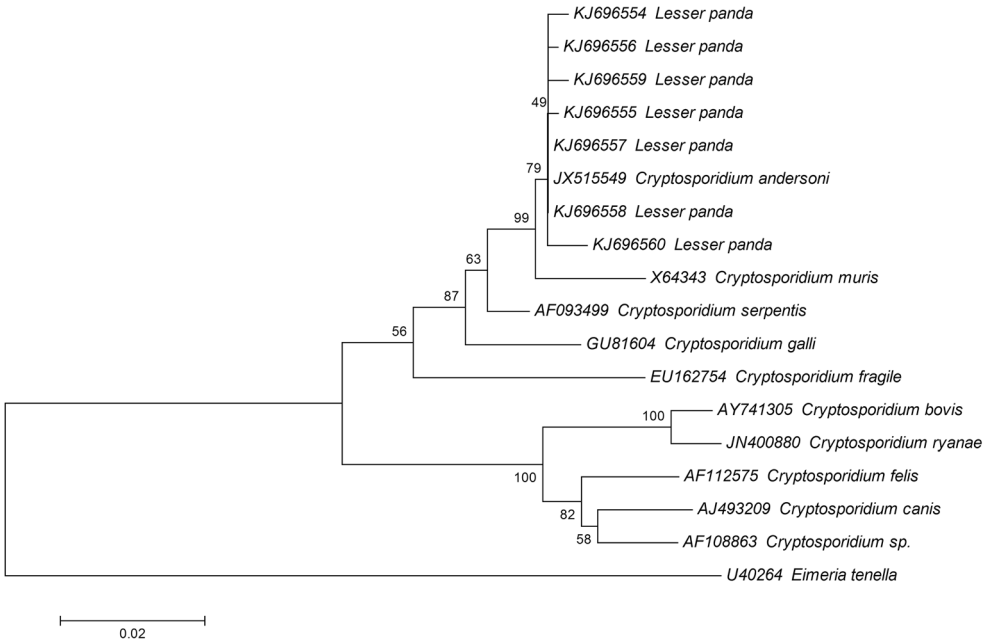

Fig. 1 Phylogenetic analysis of *Cryptosporidium* spp. using neighbor-joining method based on sequences of 18S rRNA genes. The *numbers at clades* indicate bootstrap values

~830 bp fragment of the 18S rRNA gene was used in the phylogenetic analysis. *Eimeria tenella* (GenBank accession number U40264) was used as the out-group.

Results

A total of 110 fecal samples were screened for the presence of *Cryptosporidium* by PCR amplification of the 18S rRNA gene. Of samples, 6.36 % (n=7) from captive lesser panda were identified as positive for *Cryptosporidium* spp. (Table 1). Interestingly, all the *Cryptosporidium* positive sample came from Sichuan and Chongqing provinces.

According to obtained phylogenetic tree (Fig. 1), the *Cryptosporidium* isolate analyzed in this study cluster together with the strain described as *Cryptosporidium andersoni* (with 99–100 % homolog) genotype originated from dairy calves. The nucleotide sequences of *Cryptosporidium* spp. from lesser pandas in this study were deposited in GenBank under accession numbers KJ696554 to KJ696560.

Discussion

The results of this study clearly demonstrate the presence of *Cryptosporidium* infection in captive lesser pandas in China. As we were able to sample around a third of the captive population of lesser panda in China (110 out of in total 321 lesser pandas kept in 71 zoos or institutions), this study provides the genetically characterized *Cryptosporidium* on a large geographic scale for the first time.

C. andersoni was first discovered in the abomasum of cattle in 2000 (Lindsay et al. 2000) and has been subsequently isolated from Bactrian camel (*Camelus bactrianus*), bobak marmot (*Marmota bobak*), European wisent (*Bison bonasus*) (Ryan et al. 2003), and Mongolian gerbils (*Meriones unguiculatus*) (Koudela et al. 1998). In China, *C. andersoni* has been identified to be the predominant species responsible for cryptosporidiosis in cattle (Wang et al. 2011). Here, our results revealed the lesser panda is a new host of *C. andersoni* and extended the range of host species known for this parasite. However, it is still too early to arrive at any conclusion whether the *C. andersoni* infections in the captive lesser pandas were derived from any popular agricultural species or from the wild lesser pandas in spite of their low density in nature.

Nonetheless, regardless of the original source, the *C. andersoni* infections in captive pandas should be considered as a public health concern, given to the fact that *C. andersoni* can infect humans under certain circumstances (Xiao and Feng 2008) and has been isolated from humans suffering from diarrhea in England (Leoni et al. 2006) and from pediatric patients in Malawi (Morse et al. 2007). In particular, the zoo workers should be aware of the health hazard of the less pandas they contact regularly. Proper control measures should be taken carefully to protect the welfare of zoo visitors as well.

Acknowledgments This work was supported by grants from the Science & Technology Ministry, China (No. 200910188) and the Research Fund for the Chengdu Giant Panda Breeding (No. CPF-2012-13). We would like to thank Yike Kang for comments on the draft manuscript.

Conflict of interest None declared.

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