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ORIGINAL PAPER

Molecular characterization of the *Cryptosporidium* cervine genotype from a sika deer (*Cervus nippon* Temminck) in Zhengzhou, China and literature review

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Abstract A total of 124 fecal specimens were collected from four deer farms in Zhengzhou City, China and examined for *Cryptosporidium* by Sheather's sugar flotation technique. *Cryptosporidim* oocysts were detected in two 1-year-old sika deer, and one of the two specimens was genotyped by sequence and phylogenetic analyses of the small subunit ribosomal RNA (rRNA) (18S rRNA), 70-kDa heat shock protein (HSP70), actin, and *Cryptosporidium* oocyst wall protein (COWP) genes. Results obtained suggested that the *Cryptosporidium* studied belonged to *Cryptosporidium* cervine genotype, although slight sequence differences were noticed at the three loci. The similarities between this isolate and other *Cryptosporidium* cervine genotype isolates were 99.1–99.8%, 9.8%, 99.7%, and 100% at the 18S rRNA, HSP70, actin, and COWP loci, respectively. This study is the first report of *Cryptosporidium* infection in sika deer in China.

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Introduction

Cryptosporidiosis is a significant cause of diarrheal diseases in human worldwide, usually by routes of waterborne or food-borne transmission. So far, at least seven *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, and *C. suis*) and one genotype (*Cryptosporidium* cervine genotype) have been shown to be responsible for human infections (Xiao et al. 2004).

Cryptosporidium cervine genotype has a wide host and geographic range. It has been found in sheep in Australia and USA (Ryan et al. 2005; Santin et al. 2007), an ibex in China (Karanis et al. 2007), one blesbok, mouflon sheep, and nyala each in Czech Republic (Ryan et al. 2003), humans in Canada, England, Slovenia, and USA (Blackburn et al. 2006; Feltus et al. 2006; Leoni et al. 2006; Ong et al. 2002; Soba et al. 2006; Trotz-Williams et al. 2006), wild white-tailed deer, eastern gray squirrels, eastern chipmunk, beaver, red squirrel, oodchuck, deer mice, and raccoon in New York (Feng et al. 2007; Perz and Le Blancq 2001), lemurs in USA (da Silva et al. 2003), and water samples in various geographic locations (Feltus et al. 2006; Jiang et al. 2005; Nichols et al. 2006; Ruecker et al. 2007; Xiao et al. 2000).

There is no report of *Cryptosporidium* cervine genotype infection in sika deer in China. In the present study, we have characterized a *Cryptosporidium* cervine genotype isolate from a sika deer (*Cervus nippon* Temminck) by sequence and phylogenetic analyses of four genes. Results suggest that the Chinese sika deer isolate had minor sequence difference from other known isolates.

Table 1 Deer specimens examined for *Cryptosporidium*

Animal species	Age group	No. examined	No. of positive specimens
Sika deer	4 months	18	0
	1 year	30	2
	3 years	21	0
	5 years	14	0
Red deer	3 years	5	0
	10 years	3	0
Pere David's deer	2 years	33	0

Table 2 Reported cases of human infections with the *Cryptosporidium* cervine genotype

Location	No. of positive samples	Reference
British Columbia, Canada	9	Ong et al. (2002)
Ontario, Canada	1	Trotz-Williams et al. (2006)
Ohio, USA	1	Blackburn et al. (2006)
Wisconsin, USA	1	Feltus et al. (2006)
England, UK	1	Leoni et al. (2006)
England and Wales, UK	6	Nichols et al. (2006)
Ljubljana, Slovenia	1	Soba et al. (2006)
Lima, Peru	1	Xiao and Cama, unpublished

Materials and methods

A total of 124 fecal specimens from sika deer (*C. nippon* Temminck), red deer (*C. elaphus*) and Pere David's deer (*Elaphurus davidianus*) were obtained directly by rectal collection from four deer farms in Zhengzhou City, China between 2002 and 2007 (Table 1). No clinical sign was observed when the fecal specimens were collected. The fecal specimens were concentrated by the Sheather's sugar flotation technique and examined by bright field microscope under 400× and 1,000×. Two fecal specimens from two 1-year-old sika deer were *Cryptosporidium*-positive. One hundred twenty-four fecal specimens were all stored in 2.5% potassium dichromate at 4°C. After purifying the oocysts from the two positive specimens by the discontinuous density sucrose gradient centrifugation, genomic DNA was extracted using Mag Extractor-Genome kit (Toyobo, Osaka, Japan). The DNA was kept at −20°C before it was used in molecular analysis.

Primers to amplify 18S ribosomal RNA (rRNA; ~1,700 bp), HSP70 (~1,950 bp), actin (~1,066 bp), and *Cryptosporidium* oocyst wall protein (COWP; ~530 bp) genes were adopted from previous studies (Xiao et al. 2004). Polymerase chain reaction (PCR) products of the actin gene were cloned, and four recombinant plasmids were sequenced by TaKaRa Biotechnology Co. Ltd (Dalian, China) with an ABI PRISM™ 3730 XL DNA Analyzer (Applied Biosystems, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). PCR products of the 18S rRNA, HSP70, and COWP genes were sequenced directly without cloning. The sequences were deposited in GenBank under accession numbers DQ898159, DQ898163, EF030519, and EF030518. Phylogenetic analyses were performed using the software PHYLIP version 3.67 (Felsenstein 1989).

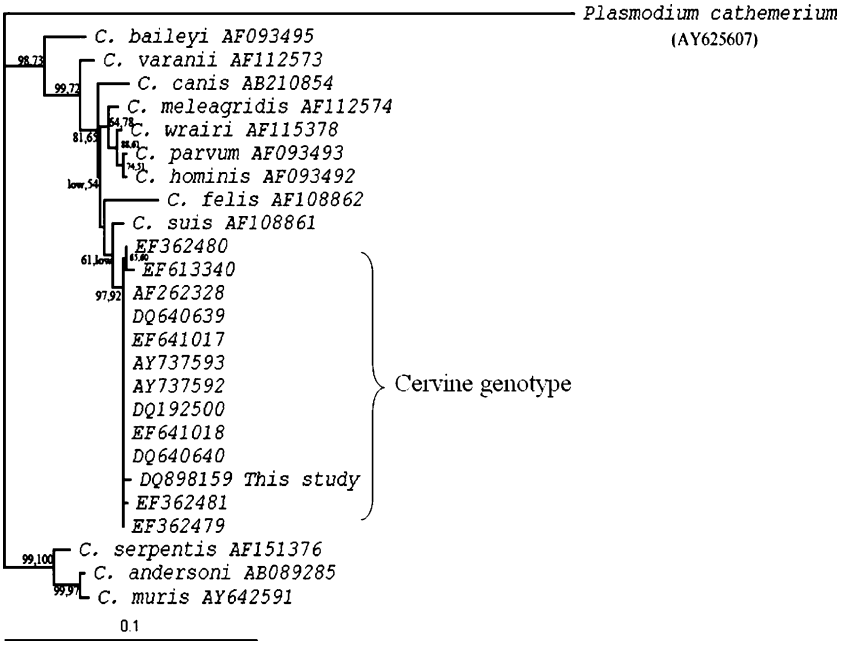

Fig. 1 Phylogenetic relationship of *Cryptosporidium* parasites inferred by neighbor-joining analysis of the 18S rRNA gene based on Kimura two-parameter model. Bootstrap values (in percentage) above 50% from 1,000 pseudo-replicates are shown for both the neighbor-joining (the *first value*) and maximum parsimony analyses (the *second value*). *Low* = node with bootstrap values lower than 50%. *Scale bar* indicates an evolutionary distance of 0.1 substitutions per site in the sequence

Table 3 Reported natural infections of the *Cryptosporidium* cervine genotype in animals

Animals	Location	No. of positive samples	Reference
Lemurs	North Carolina, USA	7	da Silva et al. (2003)
Sheep	Maryland, USA	28	Santin et al. (2007)
Sheep	Western Australia	33	Ryan et al. (2005)
Ibex	Qinghai, China	1	Karanis et al. (2007)
Blesbok	Prague, Czech Republic	1	Ryan et al. (2003)
White-tailed deer	New York, USA	4	Perz and Le Blancq (2001)
Sika deer	Henan, China	1	DQ898159
Mouflon sheep	Prague, Czech Republic	1	Ryan et al. (2003)
Nyala	New York, USA	1	Ryan et al. (2003)
Eastern gray squirrel	New York, USA	3	Feng et al. (2007)
Eastern gray squirrel	New York, USA	1	Ziegler et al. (2007)
Eastern chipmunk	New York, USA	2	Feng et al. (2007)
Eastern chipmunk	New York, USA	3	Ziegler et al. (2007)
Red squirrel	New York, USA	1	Feng et al. (2007)
Red squirrel	New York, USA	2	Ziegler et al. (2007)
Woodchuck	New York, USA	1	Feng et al. (2007)
Beaver	New York, USA	1	Feng et al. (2007)
Deer mice	New York, USA	1	Feng et al. (2007)
Raccoon	New York, USA	1	Feng et al. (2007)

Results and discussion

Sequences of the 18S rRNA, HSP70, actin, and COWP genes were obtained from one of the two *Cryptosporidium*-positive specimens (another specimen failed to amplify the specific fragment of four genes studied). They were aligned with sequences from other known *Cryptosporidium* species and genotypes.

In the 18S rRNA gene, there were two to seven nucleotide differences out of 667 bp with other *Cryptosporidium* cervine genotype isolates (EF362480, EF613340, AF262328, DQ640639, EF641017, AY737593, AY737592, DQ19250, EF641018, DQ640640, EF362481, and EF362479). The similarity (99.1%) between this sika deer isolate and the Chinese ibex isolate (EF613340) was the lowest, and the highest similarity (99.8%) was observed with a USA water sample isolate (EF641018). The similarities with other USA water sample, sheep isolates, and Canada human isolates varied from 99.2% to 99.7%. Five copies of the 18S rRNA gene are present in *Cryptosporidium* genome, and previous studies have suggested that there is slight sequence heterogeneity in some of these copies (Xiao et al. 1999). Therefore, some of the sequence differences could be due to variations among different copies of the rRNA gene.

In the HSP70 gene, comparing to DQ389176, two nucleotide changes of T to C and one change of A to C were observed at nucleotides 594 and 1062 and nucleotide 1809, respectively. The sequence similarity between the two was 98.8%. This might be due to the single nucleotide polymorphy (SNP) of HSP70 gene. For example, three SNPs were present between *C. hominis* sequences AF221535 and XM_661662. Similarly, in the actin gene, there were two nucleotide changes between the current isolate and DQ389175 at nucleotide 322 and 580, with a sequence similarity of 99.7%. Further comparison was limited because of the lack of more *Cryptosporidium* cervine genotype actin sequences. On the contrary, no sequence difference was observed in the COWP gene.

Phylogenetic trees were constructed, and two methods (neighbor-joining and parsimony) produced similar topolo-

Table 4 The occurrence of the *Cryptosporidium* cervine genotype in the environment

Sample type	Location	No. of positive samples	Reference
Source water	Ontario, Canada	1	Ruecker et al. (2007)
Source water	Maryland, USA	2	Xiao et al., unpublished
Storm water	New York, USA	63	Xiao et al. (2000); Jiang et al. (2005)
Raw wastewater	Milwaukee, USA	6	Zhou et al. (2003)
Raw wastewater	Milwaukee, USA	9	Xiao et al. (2006a)
Raw wastewater	Tunis, Tunisia	1	Xiao and Ben Ayed, unpublished
Stream sediment	New York, USA	1	Xiao et al., unpublished

gy for each of the four loci. In each of the analysis, sequence from sika deer grouped together with other *Cryptosporidium* cervine genotype isolates (a neighbor-joining tree of the 18S rRNA gene is showed in Fig. 1).

Cryptosporidium cervine genotype is the only species/genotype with noticeable broad host range (Tables 2 and 3). Since its initial finding in storm runoff in a wilderness area, it has been found in domestic and wild ruminants (sheep, mouflon sheep, ibex, blesbok, nyala, white-tailed deer, and Pere David's deer), rodents (squirrels, chipmunks, woodchucks, beavers, and deer mice), carnivores (raccoons), and primates (lemurs and humans; Xiao et al. 2000; Perz and Le Blancq 2001; Ong et al. 2002; da Silva et al. 2003; Ryan et al. 2003, 2005; Blackburn et al. 2006; Feltus et al. 2006; Leoni et al. 2006; Nichols et al. 2006; Soba et al. 2006; Trotz-Williams et al. 2006; Feng et al. 2007; Karanis et al. 2007). The generalist nature of the host specificity of the parasite and habitat sharing are probably responsible for the wide occurrence of the cervine genotype in animals.

According to available data, the cervine genotype has been found in various environmental samples such as source water, stream storm runoff, stream sediment, and raw wastewater in several countries (Table 4). In studies conducted in wilderness areas of the New York City drinking water supply watershed, the cervine genotype was the most common *Cryptosporidium* species/genotype found in river/stream water (Xiao et al. 2000, 2006b; Jiang et al. 2005) and was the only year-round *Cryptosporidium* in storm water (Xiao et al. 2000, 2006b; Jiang et al. 2005). The finding of the cervine genotype in these areas suggest the potential for spreading among wildlife species that routinely drink from streams.

An increasing number of human cases have been associated with the *Cryptosporidium* cervine genotype (Table 2). It has been reported in ten patients in Canada, seven in the UK, three in the USA, and one in Slovenia (Ong et al. 2002; Blackburn et al. 2006; Feltus et al. 2006; Leoni et al. 2006; Nichols et al. 2006; Soba et al. 2006; Trotz-Williams et al. 2006). Likewise, the increasing number of humans infected with the cervine genotype might be related to its wide range of mammalian hosts (Feng et al. 2007). Almost all the human cases are reported in industrialized nations where it ranked number four in *Cryptosporidium* spp. in humans (more human cases have been attributed to the cervine genotype than *C. canis* in industrialized nations), indicating that zoonotic transmission is probably responsible for most of the cases with cervine genotype. However, it has been recently seen in one child in a shantytown in Lima, Peru where cryptosporidiosis is known to be transmitted main via the anthroponotic route (Xiao and Cama unpublished).

In this study, four deer farms were screened for *Cryptosporidium* oocysts for 5 years, but only two fecal specimens were positive, with an average infection rate of 1.61% (2/124). The low infection rate suggests that deer studied here are not very susceptible to *Cryptosporidium* and are not an important reservoir of cryptosporidiosis. Moreover, the four farms are all far from the drinking water supply system. Therefore, the deer populations on farms may be not a major source of cryptosporidiosis in humans in Zhengzhou region.

This study broadens the host and geographic ranges of the *Cryptosporidium* cervine genotype and provides new sequence data. In conjunction with previous reports, it is expected that the *Cryptosporidium* cervine genotype will be named as a new species in future.

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