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ORIGINAL PAPER

Prevalence and distribution of *Cryptosporidium* spp. in dairy cattle in Heilongjiang Province, China

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Abstract Few data are available on the molecular characterization of *Cryptosporidium* spp. in cattle in China. In the present study, a total of 507 fecal specimens from six dairy farms in Heilongjiang Province were examined for *Cryptosporidium* spp. by light microscopy of concentrates from the formalin-ethyl acetate sedimentation method (for less than 2-month-old calves) or Sheather's floatation method (more than 3-month-old dairy cattle). Twenty-seven post-weaned calves on five farms were positive for *Cryptosporidium* oocysts. PCR and DNA sequence analysis of the 18S rRNA, actin, and 70 kDa heat shock protein genes identified *Cryptosporidium andersoni* and *Cryptosporidium. ryanae*, with *C. andersoni* as the dominant species (26 out of 27). In comparison with other regions of the world, the distribution of *Cryptosporidium* species in the areas appears to be unique.

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Introduction

It has been demonstrated recently that four species of *Cryptosporidium* are mostly responsible for bovine cryptosporidiosis, with an age-associated distribution of them. The two most common species are *Cryptosporidium parvum* and *Cryptosporidium andersoni*, with the former commonly seen in neonatal calves and the latter commonly in adult cattle. The two other more recently described species, *Cryptosporidium bovis* and *Cryptosporidium ryanae*, usually infect weaned calves and yearlings, with *Cryptosporidium bovis* is more commonly seen than *C. ryanae*. In addition, a small number of infections with *Cryptosporidium felis* and *Cryptosporidium suis* have been also reported in cattle (Bornay-Llinares et al. 1999; Fayer et al. 2006; Geurden et al. 2006). Of the four common *Cryptosporidium* spp. in cattle, *C. parvum* is the only recognized zoonotic species, although a few cases of *C. andersoni* infections were reported in humans (Leoni et al. 2006).

In China, the first report of cryptosporidiosis in dairy cattle was in 1986 based on microscopy of acid-fast stained oocysts in feces (Chen et al. 1986). Since then, bovine cryptosporidiosis has been reported in various areas in China. The prevalence ranged from 0% to 75.4% depending on the geographical areas of the studies and methods used in detection. The *Cryptosporidium* spp. identified in the studies included *Cryptosporidium muris*-like, *C. parvum*-like, and *C. andersoni.* However, all the diagnoses were based on either morphological identification of oocysts in feces alone or immunofluorescence microscopy. Although the identification of oocysts based on morphology and immunofluorescence provides evidence for the presence of *Cryptosporidium*, neither method is capable of accurately identifying the species involved.

Two recent studies characterized a small number of *Cryptosporidium* specimens from cattle in China and identified three species, *C. andersoni*, *C. bovis*, and *C. ryanae* in Xuzhou and Shanghai, (Liu et al. 2007; Feng et al. 2007). In this study, we genetically characterized *Cryptosporidium* spp. in different age groups of dairy cattle on six farms in Heilongjiang Province, in China. The results obtained suggested that there might be a unique distribution of *Cryptosporidium* spp. in cattle in the study area.

Materials and methods

Specimen collection and examination

Between April and August 2008, 507 fecal specimens were randomly collected from six dairy cattle farms (A through F) located in Heilongjiang Province, China (Table 1). In order to increase the sensitivity of microscopy detection, at least 25 g fecal specimens were used for concentration of oocysts. Oocysts in fecal specimens from pre-weaned calves (less than 2-month-old) were concentrated by formalin-ethyl acetate sedimentation method in order to remove the fats in the feces specimen and examined by modified fast-acid staining technique (McNabb et al. 1985). In contrast, Sheather's floatation method was used to concentrate oocysts in specimens from post-weaned calves and older cattle (McNabb et al. 1985). The concentrates were examined by bright-field microscopy under ×400 and ×1,000. *Cryptosporidium* oocysts were purified from positive fecal specimens by discontinuous sucrose gradient centrifugation as described previously (Arrowood and Donaldson 1996). Oocysts were stored in 2.5% potassium dichromate solution at 4°C before were used in DNA extraction.

Oocyst DNA extraction

DNA was extracted from the purified oocysts using the Mag Extractor-Genome kit (Toyobo, Osaka, Japan), based on chaotropic extraction followed by absorption onto silica-coated magnetic beads. Briefly, 50 μl of oocyst suspension was resuspended in 750 μl of lysis buffer. After five cycles of freeze–thaw (−80°C for 5 min and 37°C for 5 min), 40 μl of silica-coated magnetic beads were added to the oocyst lysate, and the tube was vortexed for 10 min. The magnetic beads were then separated from the suspension using a magnet, and washed twice in 900 μl of washing buffer and once in 900 μl of 70% ethanol. Afterwards, the magnetic beads were resuspended in 100 μl of reagent water, and the tube was vortexed for 10 min. The bead suspension was then centrifuged at 2,000×*g* for 3 min, and the supernatant was collected. The supernatant containing DNA was kept at −20°C before it was used in PCR analysis.

18S rRNA, HSP70 and actin gene amplification and sequencing

Primers and amplification conditions used in nested-PCR analysis of the partial 18S rRNA, 70 kDa heat shock protein (HSP70), and actin genes were previously described (Xiao et al. 2001; Sulaiman et al. 2000, 2002). DNA sequencing of the PCR products was done by the TaKaRa Biotechnology (Dalian, China) on an ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Some 18S rRNA PCR products and all actin PCR products were sequenced after they were cloned into the pGEM® T Easy vector as recommended by the supplier (Promega, Madison, USA), and three positive clones per product were sequenced. Nucleotide sequences obtained were aligned with reference sequences using the ClustalX 1.83 (Thompson et al. 1997). The nucleotide sequences obtained in the present study were deposited in the GenBank database under accession numbers FJ463171-463197 (18S rRNA), FJ463198-463201(HSP70), and JF463202-463206 (actin).

Table 1 Prevalence of *Cryptosporidium* spp. in dairy cattle on six farms in Heilongjiang Province, China

Farm	No. of positive/No. of examined (%)					*Cryptosporidium* species
	Age group (months)				Total	
	<2	3-11	12-24	>24		
A	0/1 (0)	6/25 (24.0)	0/3 (0)	8/78 (10.3)	14/107 (13.1)	*C .andersoni*
B	0/13 (0)	1/64 (1.6)	0/40 (0)	1/78 (1.3)	2/195 (1.0)	*C. andersoni*
C	0/1 (0)	0/8 (0)	1/5 (20)	2/21 (9.5)	3/35 (8.6)	*C. andersoni*
D	0/1 (0)	0/5 (0)	3/9 (33.3)	4/30 (13.3)	7/45 (15.6)	*C. andersoni*
E	0/6 (0)	0/24 (0)	0/12 (0)	0/60 (0)	0/102 (0)	*C. andersoni*
F	NE	1/19 (5.3)	0/4 (0)	NE	1/23 (4.4)	*C. ryanae*
Total	0/22	8/145	4/73	15/267	27/507(5.3)	

NE not examined

Results

Prevalence and clinical signs

Twenty-seven of the 507 fecal specimens (22 from pre-weaned and 485 post-weaned cattle) from the six farms were diagnosed as *Cryptosporidium*-positive by microscopic examinations. All 27 *Cryptosporidium*-positive dairy cattle had no obvious clinical signs at the time of sampling. On the farms A through F, the infection rates were 13.1% (14/107), 1.0% (2/195), 8.6 (3/35), 15.6% (7/45), 0% (0/102), and 4.4% (1/23), respectively. We did not identify any *Cryptosporidium* infection in calves younger than 2 months (the pre-weaned), while the overall prevalence of cryptosporidiosis was constant in older calves and adult cattle (5.5% in 3-11 month-old calves, 5.5% in 12-24-month-old cattle, and 5.6 in cattle older than 24 months) (Table 1).

Distribution of *Cryptosporidium* spp.

Sequences of the 18S rRNA gene were obtained from all 27 *Cryptosporidium*-positive specimens. Among them, 26 were identified as *C. andersoni*. Nine of the 26 sequences of the 18S rRNA gene showed 100% identity to the *C. andersoni* Japan cattle isolate reported by Koyama Y et al. in 2005 (AB089285), while 17 sequences showed 99.8% identities with an insertion of T at the nucleotide 634. One specimen (no. 23) appeared to be *C. ryanae,* as the 18S rRNA gene sequence was identical to that of reported by Fayer et al. 2008 (EU410344) with 100% of similarity.

Four *C. andersoni*-positive specimens, no. 1, 6, 12, 18, were selected for DNA sequencing of the HSP70 and actin genes. The four specimens shared an HSP70 gene sequence that is identical to sequences obtained from bovine *C. andersoni* isolates in Australian and Japan (AF221542 and AB089288, respectively). However, there were two mutations at nucleotides 1,873 and 1,920 in the sequences compared to a *C. andersoni* isolate in a camel in China (DQ989577). Similarly, at the actin locus, there were no sequence differences between the four *C. andersoni* specimens from the study. However, there were three or five nucleotide differences in the partial actin gene sequences between the bovine specimens from this study and the camel isolate in China (DQ989575) or the cattle isolate in United States (AF382352). A partial actin gene sequence was also obtained from the *C. ryanae*-positive specimen (no. 23); it was identical to that of a *C. ryanae* isolate in the United States (EU410345).

Discussion

Molecular biologic characterizations of *Cryptosporidium* spp. in recent years have led to a reevaluation of our understanding of the epidemiology of cryptosporidiosis in

Table 2 The prevalence of *Cryptosporidium* in cattle in China by microscopy

Location	Sample size	Age	Average prevalence (%)[a]	Species. identified	Reference
Anhui	124	Calves and cows	60.2 (33.3–72.7)	*C. parvum*-like,	Li et al. 1998
	814	Calves and cows	6.4 (0–20.7)	*C. parvum*-like, *C. muris*-like	Li et al. 1999
	502	Calves and cows	5.2 (0–14.8)	*C. parvum*-like, *C. muris*-like	Xu et al. 2007a, b
Beijing	176	Calves	10.2 (0–19)	Unspecified	Jiang et al. 1989
Guangdong	105	Calves	26.7	*C. parvum*-like, *C. muris*-like	Guo and Lian 1999
	1087	Calves	8.5 (0–12.8)	*C. muris*-like	Xiang et al. 2004
Henan	70	Calves and cows	40 (16.7–52.4)	*C. muris*-like	Ning et al. 1997
	582	Calves and cows	11 (0–58.3)	*C. andersoni, C. parvum*-like	Jian et al. 2005
	582	Calves	26.1 (7.7–36.4)	*C. andersoni, C. parvum*-like	Lu et al. 2008
Jiangsu-Nanjing	55	Calves and cows	29.1 (19.2–40)	Unspecified	Liang and Huang 2000
Jiangxi	181	Buffalo (1-7 month)	2.3–18.3	*C. parvum*-like, *C. muris*-like	Xie et al. 2002
Jilin	300	Calves and cows	14.3 (4.5–41.2)	*C. parvum*-like, *C. muri*-like	Tian et al. 2000
	51	Calves and cows	47.1 (50–75)	Unspecified	Xu et al. 2002
Neimenggu	71	Calves and cows	22.5 (0–66.7)	*C. muris*-like	Yang et al. 2004
Qinghai	55	Calves	12.7 (6.7–20)	*C. parvum*-like, *C. muris*-like	Chen et al. 1989
	281	Calves and cows	36.7 (32.7–39.7)	*C. parvum*-like, *C. muris*-like	Zhang 2007
Shandong	154	Calves and cows	25.3 (4.8–57.5)	Unspecified	Guo et al. 1993
Shanxi	722	Calves and cows	6.3–75.4	Unspecified	Zhang et al. 1991
Shanghai	586	Calves and cows	36.5	Unspecified	Xu et al. 2007a, b

[a] The numbers in the parenthesis indicate the range of different regions in the area

cattle. In China, despite the presence of many studies on the prevalence of *Cryptosporidium* infection in cattle (Table 2), the precise identification of *Cryptosporidium* species had not been done.

Results of the study clearly showed that *C. andersoni* is the predominant species in the geographical area investigated. Since *C. andersoni* oocysts are significantly larger than oocysts of the other three bovine species, we believe that the previous observations on high occurrence of *C. muris*-like oocysts in cattle reflect the high prevalence of *C. andersoni* in China (Table 2). Some of the previous studies also identified a high occurrence of *C. parvum*-like oocysts (Table 2). Because oocysts of *C. parvum* are morphologically similar to those of *C. bovis* and *C. ryanae*, the true prevalence of *C. parvum* in cattle in China remains unclear.

Recent studies suggest that the occurrence of the four *Cryptosporidium* spp. in cattle are age-related (Santín et al. 2004; Fayer et al. 2005, 2006, 2007; Feng et al. 2007; Langkjaer et al. 2006; Geurden et al. 2006). In the present study, only two species, *C. andersoni* and *C. ryanae*, were found in dairy cattle. The absence of *C. parvum* in the present investigation might result from the low prevalence of the species in this area or the small number of specimens examined from the pre-weaned calves. Previous studies in the United States indicated that *C. parvum* was responsible for about 85–97% of the *Cryptosporidium* infections in pre-weaned calves but only 1–4% of the *Cryptosporidium* infections in post-weaned calves and heifers (Fayer et al. 2006; Santín et al. 2004). Extensive investigations with large number of the specimens should be considered in the future.

There was an absence of *C. bovis* in the study. It is the most common *Cryptosporidium* species found in post-weaned calves in United States and other countries, and cattle of all ages are probably susceptible to infections with the species (Fayer et al. 2005, 2006; Santín et al. 2004, 2008; Feng et al. 2007; Brook et al. 2009; Feltus et al. 2008; Keshavarz et al. 2009; Sakai et al. 2003; Burenbaatar et al. 2008). A previous study of *Cryptosporidium*-positive specimens from five pre-weaned calves and one post-weaned calf in Shanghai, China identified *C. bovis* in four pre-weaned calves and one post-weaned calf (Feng et al. 2007). In the United States, an average *C. bovis* prevalence of 4.2% (0–23.8%) was reported (Fayer et al. 2006). This species was the predominant species infecting 2- to 11-month-old dairy calves (Santín et al. 2004). Recently, *C. bovis* was found in 8.3–46.7% (5/5 of herds) of 6- to 8-month-old calves and 0–3.3% (1/6 of herds) of cows in beef cattle (Feltus et al. 2008). A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age showed a 80% cumulative prevalence of *C. bovis* in the United States (Santín et al. 2008). The prevalence of *C. bovis* in other countries varied, from 0% to 6.7% in Japan (Sakai et al. 2003), 7.8% in Iran (Keshavarz et al. 2009), to 26.4% in Mongolia (Burenbaatar et al. 2008). The fact that the infection of *C. bovis* in dairy cattle was not found in the present study may reflect the low prevalence of the species in this area.

The *Cryptosporidium* species in cattle are linked to different clinical manifestations. Among the species found in cattle, *C. parvum*, which can infect the small intestine and colon of pre-weaned calves and humans, often causes diarrheal disease, therefore, it is the species of public and veterinary health significance (Xiao and Feng 2008). *C. andersoni* infects the abomasums of juvenile and mature cattle and induces no apparent clinical signs, but has been implicated as a cause of reduced milk production (Olson et al. 1997; Anderson 1998; Lindsay et al. 2000; Santín et al. 2004; Fayer et al. 2006; Feng et al. 2007; Langkjaer et al. 2006). Its occurrence in humans has been reported only in three cases (Leoni et al. 2006). Infection of cattle with *C. bovis* and *C. ryanae* has not been associated with any signs of disease (Fayer et al. 2005, 2008). Therefore, identifying factors that contribute to the occurrence of different species in cattle is critical to the understanding of economic and public health importance and transmission of cryptosporidiosis in cattle.

In the present study, the low prevalence (5.3%) of *Cryptosporidium* infection was found despite the analysis of a large amount of fecal specimens (25 g). We should consider that the present prevalence based on morphology (with relatively low sensitivity comparing to the molecular methods) may be underestimated compared to the actual prevalence. Kvác et al. found that zero to seven and three to 18 oocysts of *C. parvum* could be detected, from 1×10^5 and 1×10^6 oocysts in the stool specimen, respectively. Sheather's flotation may increase the sensitivity to eight to 53 oocysts from 1×10^5 oocysts (Kvác et al. 2003). Oocyst number in the sample from young post-weaned calves with the lowest infection intensity was 3.75×10^4/25 g after the oocyst concentration in the present study. More thorough and extended studies are needed before we can have a better picture of bovine cryptosporidiosis in China.

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