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ORIGINAL PAPER

Cervine genotype is the major *Cryptosporidium* genotype in sheep in China

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Abstract To identify *Cryptosporidium* species/genotypes in sheep in China and to elucidate the endemic transmission of cryptosporidiosis, a total of 1,701 fecal samples from five farms in four prefectures in Henan Province (central China) were examined. Eighty-two *Cryptosporidium*-positive samples were analyzed by polymerase chain reaction (PCR)–restriction fragment length polymorphism analysis of the small subunit (SSU) rRNA gene and PCR analysis of the 60 kDa glycoprotein (gp60) gene, and 41 were further analyzed by DNA sequencing of the PCR products. The SSU rRNA-based PCR identified two *Cryptosporidium* species and one genotype, including the *Cryptosporidium* cervine genotype (74/82), *Cryptosporidium andersoni* (4/82), and *Cryptosporidium xiaoi* (4/82). The cervine genotype was found in all age groups, *C. xiaoi* in lambs, and *C. andersoni* in ewes. There were intragenetic differences in the SSU rRNA gene sequences of the *Cryptosporidium* cervine genotype and *C. xiaoi*. No *Cryptosporidium parvum* was detected by both SSU rRNA- and gp60-based PCR assays. These findings suggest that sheep are a potential source for zoonotic infections of the *Cryptosporidium* cervine genotype.

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Introduction

Cryptosporidium species are important zoonotic parasites. It has a wide spectrum of hosts including humans, other mammals, birds, reptiles, amphibians, and fish. It is one of the common causes of diarrhea in humans and domestic animals and exerts significant public health and economic impact (Xiao et al. 2004; Fayer and Xiao 2008). So far, at least 20 *Cryptosporidium* species are considered valid, and more than 50 host-adapted genotypes with undetermined species status have been described (Fayer 2009; Fayer et al. 2008; Fayer and Santín 2009).

Previous studies have indicated that some animals are important zoonotic reservoirs of *Cryptosporidium* in humans (Xiao and Fayer 2008). In particular, calves have attracted extensive attention, as they are widely infected with *Cryptosporidium parvum*, the most important zoonotic *Cryptosporidium* species (Fayer and Xiao 2008; Xiao and Feng 2008). The role of sheep in transmitting cryptosporidiosis to human is comparatively less studied. Just like cattle, sheep are also commonly raised in many countries including China. The molecular characterizations conducted thus far have identified several species/genotypes in sheep, including *C. parvum*, *Cryptosporidium hominis*, *Cryptosporidium andersoni*, *Cryptosporidium suis*, *Cryptosporidium fayeri*, *Cryptosporidium bovis*-like genotype (recently named as *Cryptosporidium xiaoi*), *Cryptosporidium* cervine and sheep genotypes and pig genotype II, with *C. parvum*, *C. xiaoi*, and the *Cryptosporidium* cervine genotype as the major species (Majewska et al. 2000; McLauchlin et al. 2000; Chalmers et al. 2002; Ryan et al. 2005; Leoni et al. 2007; Navarro-i-

Martinez et al. 2007; Santín et al. 2007; Santín and Fayer 2007; Soltane et al. 2007; Elwin and Chalmers 2008; Geurden et al. 2008; Giles et al. 2009; Mueller-Doblies et al. 2008; Paoletti et al. 2009; Quílez et al. 2008; Fayer and Santín 2009;Yang et al. 2009). However, there are seemingly some significant differences in the distribution of *Cryptosporidium* species/genotypes in the different studies. For example, in several studies conducted in Spain, Italy, and UK, *C. parvum* was much more prevalent than other species in lambs. In contrast, *C. xiaoi* and *Cryptosporidium* cervine genotype were the most common ones in studies conducted in Australia and USA (McLauchlin et al. 2000; Ryan et al. 2005; Santín et al. 2007; Santín and Fayer 2007; Geurden et al. 2008; Mueller-Doblies et al. 2008; Quílez et al. 2008; Paoletti et al. 2009; Yang et al. 2009). Results of a recent study conducted in Australia, however, indicate that the use of small subunit rRNA-based polymerase chain reaction (PCR) tools in *Cryptosporidium* genotyping may underestimate the prevalence of *C. parvum* (Yang et al. 2009).

In China, the total sheep population was 173.9 million in 2006 (http://www.caaa.cn). Sheep play a critical role in agricultural economy, especially in grassland regions of northwestern China where rearing sheep is a major source of income for local farmers. However, there are no systematic studies of cryptosporidiosis in sheep, and there are very few genetic data on *Cryptosporidium* species in China. The few studies on the prevalence of *Cryptosporidium* in sheep were conducted using a microscopy (Wang et al. 2008b). The objectives of this study were to identify the distribution and zoonotic potential of *Cryptosporidium* species in sheep in China.

Materials and methods

Sample collection and DNA extraction

Random fresh fecal samples were collected between July 2006 and July 2007 from four breads of sheep (Small Tail Han, Poll Dorset, Dorset Down, and Rommay) on five sheep farms in four prefectures in Henan Province, China. A total of 1,701 fecal samples were used in the study, including those from preweaned and postweaned lambs, adult sheep, and ewes preparturition and 0–5 weeks after parturition sheep (Table 1). Samples were examined for *Cryptosporidium* by microscopy of fecal materials concentrated by the Sheather's sugar flotation technique and stained with the modified acid-fast stain. *Cryptosporidium*-positive samples were stored in 2.5% potassium dichromate at 4 °C.

DNA extraction

Cryptosporidium oocysts were isolated from the positive fecal samples by the discontinuous density sucrose gradient centrifugation. Genomic DNA was extracted from the purified oocysts using the Mag Extractor-Genome kit (Toyobo Co. Ltd., Osaka, Japan) based on chaotropic extraction followed by absorption of DNA onto silica-coated magnetic beads, using the manufacturer-recommended procedures. The extracted DNA was kept at−20 °C before it was used in molecular analysis.

Cryptosporidium genotyping

Cryptosporidium species and genotypes were determined by nested PCR of the small subunit (SSU) rRNA gene and restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products using restriction enzymes *Ssp*I and *Vsp*I. Primers and amplification conditions used for PCR-RFLP were adopted from previous publications (Xiao et al. 1999; Xiao et al. 2000; Xiao et al. 2001; Jiang et al. 2005; Feng et al. 2007). The diagnosis of *C. andersoni* and *C. xiaoi* were confirmed by DNA sequencing of all positive SSU rRNA PCR products, and the *Cryptosporidium* cervine genotype by DNA sequencing of PCR products from 33 samples from representative farms and age groups. To exclude the possible presence of light infections of *C. parvum* or *C. hominis*, all *Cryptosporidi-*

Table 1 Number of fecal samples examined for *Cryptosporidium* oocysts by microscopy on each farm and the distribution of *Cryptosporidium* genotypes determined by PCR-RFLP of 18S rRNA gene

Collection site	Sample size	*Cryptosporidium*-positive (%)	Cervine genotype	*C. andersoni*	*C. xiaoi*
Zhongmou	70	1 (1.4%)	1 (100%)	0	0
Yuanyang	200	5 (2.5%)	5 (100%)	0	0
Longquan	276	9 (3.3%)	9 (100%)	0	0
Dengfeng	158	3 (1.9%)	2 (66.7%)	0	1
Xinxiang (September 2006)	561	36 (6.4%)	33 (91.7%)	2 (5.6%)	1
Xinxiang (April 2007)	436	28 (6.4%)	24 (85.7%)	2 (7.1%)	2
Total	1701	82 (4.8%)	74 (90.2%)	4 (4.9%)	4 (4.9%)

um-positive samples were also analyzed by a nested PCR targeting the 60 kDa glycoprotein (gp60) gene, which does not amplify DNA of *C. andersoni*, *C. xiaoi*, and the *Cryptosporidium* cervine genotype. After purification, the secondary PCR products of the SSU rRNA gene were sequenced directly with secondary PCR primers on an ABI PRISMTM 3730 XL DNA Analyzer (Applied Biosystems, USA) by Shanghai Biotechnology Co. Ltd. (Shanghai, China), using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if necessary.

Nucleotide sequence accession numbers

The nucleotide sequences of the partial SSU rRNA gene of *Cryptosporidium* species obtained in this study were deposited in GenBank under accession numbers EU827362 to EU827363, EU827366 to EU827367, EU827369 to EU827370, EU827371 to EU827403, and GU014552 and GU014553.

Results

Prevalence of *Cryptosporidium* species in sheep

Microscopy analysis of 1,701 fecal samples showed the presence of *Cryptosporidium* oocysts in 82 (4.8%) samples (Table 1). The percentage of animals shedding oocysts was 10.8%, 4.3%, 2.1%, and 2.5% in preweaned lambs, postweaned lambs, pregnant ewes, and postparturition ewes, respectively (Table 2).

Distribution of *Cryptosporidium* species/genotype

The SSU rRNA gene of *Cryptosporidium* species in all 82 microscopy-positive samples was successfully amplified by the nested PCR. RFLP analysis of the SSU rRNA gene products revealed the presence of three *Cryptosporidium* species/genotypes, including *C. xiaoi* (4/82), *C. andersoni* (4/82), and *Cryptosporidium* cervine genotype (74/82; Tables 1 and 2). DNA sequencing of the SSU rRNA PCR products from four *C. andersoni*, four *C. xiaoi*, and 33 *Cryptosporidium* cervine genotype-positive samples confirmed the identification of these species/genotypes. There was a complete agreement between RFLP and DNA sequencing results. No amplification of the gp60 gene was achieved by a nested PCR analysis of DNA extracted from the 82 microscopy-positive samples, suggesting that *C. parvum* or *C. hominis* was not concurrently present among the three *Cryptosporidium* species/genotypes.

Age patterns of *Cryptosporidium* species/genotypes

The cervine genotype was the most commonly identified *Cryptosporidium*, responsible for 90.2% of all *Cryptosporidium* infections. It was found in all age groups examined in this study, on all five sheep farms and in all four sheep breeds. In contrast, the prevalence of *C. xiaoi* and *C. andersoni* was much lower, each responsible for 4.9% of *Cryptosporidium* infections (Tables 1 and 2). The former was only found in lambs and the latter was only found in ewes (Table 2).

Intragenotypic variations in *Cryptosporidium* cervine genotype and *C. xiaoi*

Sequence heterogeneity in the SSU rRNA gene was observed in the *Cryptosporidium* cervine genotype and *C. xiaoi*. Four types of nucleotide sequences were obtained from samples positive for the *Cryptosporidium* cervine genotype and two types for *C. xiaoi.* The sequences of the *Cryptosporidium* cervine genotype differed from each other by the presence of an A to G nucleotide substitution (at position 503 of AF442484) and AT deletion (nucleotides 691 and 692 of AF442484) with the majority (27/33) having a nucleotide sequence identical to AF442484 (Table 3; da Silva et al. 2003). The sequences of *C. xiaoi* differed from each other by the presence of T to C nucleotide substitution (at position 633 of DQ991389). Three of the samples yielded a sequence identical to EU327319 and one sequence identical to EU408317. Both were previously obtained from sheep in UK (Elwin and Chalmers 2008).

Table 2 *Cryptosporidium* species/genotypes identified among different age groups of sheep

Age group	Sample size	*Cryptosporidium*-positive (%)	Cervine genotype	*C. andersoni*	*C. xiaoi*
Preweaned lamb	378	41(10.8%)	38	0	3
Postweaned lamb	585	25(4.3%)	24	0	1
Pregnant ewe	580	12(2.1%)	10	2	0
Postparturition ewe	158	4(2.5%)	2	2	0
Total	1701	82(4.8%)	74	4	4

Table 3 Intragenotypic variations in nucleotide sequences of the partial SSU rRNA gene of the *Cryptosporidium* cervine genotype

	Nucleotide position[a]		GenBank equivalent	Sequence type designation by Santin and Fayer (2007)
Sample ID	503	691–692		
5–7, 9, 12–15, 17–32, and 35–37	A	AT	AF442484, AJ849456, AY737592, and EF362479	Cervine I
8	A	–	AF262328, AY737593, EF641017, and FJ031238	None
34	G	AT	None	None
10, 11, 16, and 33	G	–	AY458613, EF362480, and EU408313	Cervine II

[a] Nucleotide position numbers according to AF442484, with the beginning of the sequence as position number 1. The cervine III sequence type (with T to A nucleotide substitution at position 587 comparing to the cervine I sequence type) by Santin and Fayer (2007) was not seen in this study and any other entries in the GenBank

Discussion

Cryptosporidiosis was shown to be prevalent in lambs in this study. A 4.8% overall infection rate of *Cryptosporidium* species was observed. As reported in previous studies conducted in other countries (Xiao et al. 1993; Majewska et al. 2000; Causapé et al. 2002; Sturdee et al. 2003; Santín et al. 2007), the highest infection rate (10.8%) was seen in preweaned lambs. The infection rate decreased significantly in weaned lambs (4.3%) and reached low levels in pregnant and postparturition ewes (2.1% and 2.5%, respectively).

Thus far, nine *Cryptosporidium* species/genotypes have been identified in sheep, including *C. parvum*, *C. hominis*, *C. andersoni*, *C. fayeri*, *C. suis*, the *Cryptosporidium* cervine genotype, sheep genotype and pig genotype II, and a *C. bovis*-like genotype (Table 4). The *C. bovis*-like genotype appears to be a sheep-adapted *Cryptosporidium* specie and has been recently named as *C. xiaoi* (Fayer and Santín 2009). Among the species and genotypes identified in sheep, *C. parvum*, *C. xiaoi*, and the *Cryptosporidium* cervine genotype are the three common ones. The remaining ones have each been only detected in less than a handful of animals (Table 4). In this study, only two of the common species/genotypes and *C. andersoni* were detected in the 82 *Cryptosporidium*-positive sheep.

Like previously observed in cattle and recently observed in pigs (Santín et al. 2004; Kváč et al. 2009), there seemingly is an age-associated distribution of *Cryptosporidium* species in sheep. In this study, all age groups of sheep were infected by the *Cryptosporidium* cervine genotype. In contrast, *C. xiaoi* was only detected in lambs and *C. andersoni* in ewes (Table 2). Previously, few studies compared the distribution of *Cryptosporidium* species in different age groups of sheep. Although an earlier study failed to detect any differences in the distribution of *Cryptosporidium* cervine genotype and *C. xiaoi* between preweaned lambs and postparturition ewes (Santín et al. 2007), results of a recent study conducted in UK showed that *C. parvum* was only found in preweaned lambs and *C. xiaoi* almost exclusively in postweaned lambs (Mueller-Doblies et al. 2008).

There are also probably geographic differences in the distribution of *Cryptosporidium* species in sheep (Table 4). An earlier study conducted in Australia suggested that *C. parvum* was largely absent in sheep. Instead, *C. bovis* (probably *C. xiaoi*) and the *Cryptosporidium* cervine genotype were the two common parasites in sheep (Ryan et al. 2005). This has been confirmed in a subsequent study in USA (Santín et al. 2007) and two small scale studies in Belgium and Tunisia (Soltane et al. 2007; Geurden et al. 2008). In contrast, several studies conducted in UK, Italy, Poland, and Spain showed *C. parvum* as dominant species in preweaned lambs (Table 4). The *Cryptosporidium* species distribution in sheep in Henan, China obtained in this study is apparently similar to the one observed in USA and very different from the one seen in recent studies in Europe.

Other factors might have also been attributed to causing the observed differences in the distribution of *Cryptosporidium* species in published studies. One group of researchers suggested that clinically ill lambs are more likely infected with *C. parvum*; whereas, healthy lambs are more likely infected with the *Cryptosporidium* cervine genotype and *C. xiaoi* (Chalmers et al. 2002; Elwin and Chalmers 2008; Mueller-Doblies et al. 2008). However, *C. parvum* was frequently detected in apparently normal lambs in other European studies (Majewska et al. 2000; Pritchard et al. 2007; Paoletti et al. 2009), and *C. xiaoi* was detected in at least one lamb that died of apparent severe cryptosporidiosis (Navarro-i-Martinez et al. 2007). Results of a recent study in Australia have demonstrated that the genotyping technique used would also affect the observed distribution of *Cryptosporidium* species in sheep. When a genus-specific SSU rRNA-based tool was used, *C. xiaoi* was identified as the dominant species in preweaned lambs and *C. parvum* was only identified in two of the 66 positive samples. In contrast, when a PCR technique preferentially detecting *C. parvum* was used, 63 samples was positive for *C. parvum*, including ten samples coinfected with *C. xiaoi*

Table 4 *Cryptosporidium* species/genotypes in sheep in reported studies

Country	Number of positive/sample	Age group	Genetic target	Species/genotype	Reference
Poland	16/159	Old lambs and adult sheep (>3 m)	SSU rRNA	*C. parvum* (ten)	Majewska et al. (2000)
UK	16/16	Lambs	COWP	*C. parvum* (16)	McLauchlin et al. (2000)
	4/4	Lambs	COWP	*C. parvum* (four)	Leoni et al. (2007)
	56/155	Lambs (<12 weeks)	SSU rRNA	*C. parvum* (16)	Pritchard et al. (2007)
	76/99	Lambs (4–5.5 m; 90), ill sheep (nine)	COWP, HSP70, TRAP-C2, and SSU rRNA	*C. parvum* (nine; all in ill animals), cervine genotype (22), *C. bovis*[a] (five), *C. bovis*[a]+cervine genotype (four), and unidentifiable (36)	Chalmers et al. (2002); Elwin and Chalmers (2008)
	127/297	Various age groups	SSU rRNA and COWP	*C. parvum* (52), *C. bovis*[a] (seven), and cervine genotype (one)	Mueller-Doblies et al. (2008)
	1	Lamb (10 days)	SSU rRNA	*C. hominis* (one)	Giles et al. (2009)
Spain	1	Lamb (8 days)	SSU rRNA	*C. bovis*-like[a] (one)	Navarro-i-Martinez et al. (2007)
	137 positive	Lambs with diarrhea (<21 days)	SSU rRNA	*C. parvum* (137)	Quílez et al. (2008)
Belgium	18/137	Lambs (1 day to 10 weeks)	SSU rRNA and HSP70	Cervine genotype (nine) and *C. parvum* (one)	Geurden et al. (2008)
Italy	26/149	Lambs (2 weeks; 3 m)	COWP	*C. parvum* (26)	Paoletti et al. (2009)
Tunisia	10/89	Lambs (<3 m) and adult sheep	SSU rRNA	*C. bovis*[a] (three)	Soltane et al. (2007)
USA	57/189	Lambs (7, 14, and 21 days) and adult ewes	SSU rRNA	Cervine genotype (48), *C. bovis*-like[a] (seven), and *C. parvum* (two)	Santín et al. (2007)
Australia	131/500	Postweaned lambs (<12 m) and adult sheep (>12 m)	SSU rRNA/HSP70	Cervine genotype (33/20), *C. bovis*[b] (14/4), pig genotype II (4/2), *C. fayeri* (4/2), *C. suis* (2/2), *C. andersoni* (1/1) *C. hominis* (1/1), and sheep genotype (1/0)	Ryan et al. (2005)
	117/477	Lambs (<8 weeks)	SSU rRNA	*C. bovis*[b] (52), cervine genotype (ten), and *C. parvum* (two)	Yang et al. (2009)
			Unknown *Cryptosporidium* target	*C.parvum* (63)	

[a] *C. xiaoi* type of sequences are available in GenBank

[b] SSU rRNA gene sequences are not available in GenBank

(Yang et al. 2009). A similar strategy was used in the present study, but no *C. parvum* was detected in any of the microscopy-positive samples.

Among the three *Cryptosporidium* species/genotypes identified in sheep in Henan, China, only the *Cryptosporidium* cervine genotype has significant public health importance. It is ranked sixth of the most commonly distributed *Cryptosporidium* species in humans and has been reported in at least 25 sporadic cases of cryptosporidiosis. All, but one, of the cases were reported in industrialized nations (Wang et al. 2008a; Davies et al. 2009; Pollock et al. 2009). It is also one of the most common *Cryptosporidium* species found in drinking source water in USA and Canada (Jiang et al. 2005; Ruecker et al. 2007; Karanis et al. 2007; Yang et al. 2008; Jellison et al. 2009). Because of the common occurrence of this parasite in sheep and its absence in cattle, sheep are likely a major source of the *Cryptosporidium* cervine genotype in humans and source water.

In conclusion, *Cryptosporidium* cervine genotype was found to be the dominant *Cryptosporidium* in sheep in China. More studies, especially those conducted in developing countries and/or involving comparison of the distribution of *Cryptosporidium* species/genotypes in different age groups, are needed for better understanding of the differences in transmission and public health significance of cryptosporidiosis in sheep in various areas.

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