Original Article

The Identification of the *Cryptosporidium ubiquitum* in Pre-weaned Ovines from Aba Tibetan and Qiang Autonomous Prefecture in China*

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Abstract

Objective *Cryptosporidium* spp. are prevalent globally and sheep are an important zoonotic reservoir. Little data regarding the rates of *Cryptosporidium* infections in ovines in China are available. This study assessed the prevalence of *Cryptosporidium* spp. in pre-weaned ovines from Aba Tibetan and Qiang Autonomous Prefecture in the Sichuan province of China.

Methods A total of 213 fecal samples were collected from pre-weaned ovines and were examined microscopically (following modified acid fast staining). In addition, 18S rRNA genetic sequences were amplified from fecal samples by nested PCR and phylogenetically analyzed.

Results The prevalence of *Cryptosporidium* in the collected samples was at 14.6% (31/213) and four isolates identified by PCR belonged to the *Cryptosporidium* cervine genotype (*Cryptosporidium ubiquitum*) demonstrating that this species was the primary sheep species found in sheep in China.

Conclusion The present study suggested that the high incidence of *Cryptosporidium* in sheep poses a significant public health threat and that surveillance practices must be established to prevent zoonotic disease of humans.

Key words: *Cryptosporidium ubiquitum*; Ovines; Aba; China; *Cryptosporidium* cervine genotype

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INTRODUCTION

The protozoan parasite genus *Cryptosporidium* has been linked to humans and to more than 240 species of animals worldwide and is considered one of the most important causative agents of diarrhea. Infections with this parasite have significant impacts on public health and animal husbandry, and *Cryptosporidium* spp. have been considered potential biowarfare agents in addition to causing severe infections in immunocompromised individuals. To date, at least 20 *Cryptosporidium* spp. have been confirmed[1], including *C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni*, *C.*

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ubiquitum, with more than 60 genotypes of an undefined species[1-4], including horse, rabbit, pig genotype II and the skunk and chipmunk I genotypes that have been identified in humans, and therefore considered zoonotic pathogens[5-10]. This broad spectrum of *Cryptosporidium* species with the potential of affecting humans suggests that a better understanding of the reservoir species involved in zoonoses will be needed for the development of effective public health preventive strategies. Furthermore, since effective drug treatments or vaccines against cryptosporidiosis have not been developed, a clear understanding of the epidemiology of this parasite will be essential to the development of control and prevention strategies.

Most *Cryptosporidium* studies have focused on assessing water contamination and disease outbreaks affecting humans and cattle but little is known about the role of ovines in the context of cryptosporidiosis in China. The present study was designed to estimate the prevalence of this parasite among pre-weaned ovines from Aba Tibetan and Qiang Autonomous Prefecture.

MATERIALS AND METHODS

Sampling

The study was carried out in the Aba Tibetan and Qiang Autonomous Prefecture in the Sichuan Province, China. Fecal samples were collected from 213 pre-weaned lambs selected randomly from eight farms between July 29 to August 4, 2009. Animal demographic data including sex, age, and sampling sites were recorded at the time of collection.

Oocyst Detection

Approximately 20 g of each stool specimen was collected, 1 to 2 drops of each specimen was smeared onto a glass slide and stained using the modified acid-fast staining technique prior to microscopic examination. The remaining sample was stored in a 2.5% aqueous potassium dichromate solution at 4 °C until use.

DNA Extraction

Oocyst-positive samples were washed three times with deionized water and centrifuged at1 800× g for 10 min. Genomic DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions with one minor adjustment where the fecal suspension was heated for 10 min at 95 °C. DNA was stored at -20 °C before it was used in polymerase chain reaction (PCR) amplifications.

PCR Amplification and Analysis

Nested PCR was used to amplify an approximately 840 base pair (bp) long fragment of the 18S rRNA gene locus using two sets of oligonucleotide primers: 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3' for primary PCR and 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-CTCATAAGGTGCTGAAGGAGTA-3' for secondary PCR[11-13]. Amplification reactions were carried out in 25 µL volumes consisting of 12.5 µL Taq Green Master Mix (2X), 1 µL of each primer (10X), 9.5 µL nuclease-free water and 1 µL DNA. Reaction conditions were comprised of a hot start at 94 °C for 1 min followed by 35 cycles at 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplified regions were separated using 2% agarose gel electrophoresis and vizualized following ethidium bromide staining.

DNA Sequencing

Amplified secondary PCR products were subjected to bi-directional sequencing with secondary primers performed by Invitrogen (Shanghai, China).

Sequence Analysis

Sequences were compared by homology (basic local alignment search tool; BLAST) against sequences present in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/) and multiple alignments were performed using ClustalX (version 1.83). Phylogenetic relationship analyses were performed using MEGA software (version 4.1; Biodesign Institute, Tempe, AZ, USA).

RESULTS

Study Animals and Sample Analysis

Samples collected from pre-weaned ovines from eight farms (A-H) are described in Table 1. All animals presented in good health despite collected fecal samples being loose. Following light microscopic examination, 31 oocyst-positive samples (14.6% incidence from eight farms) were identified using a modified acid fast staining technique (Figure 1).

Table 1. Ovine Population Demographics

Site	Female	Male	6 mo.*	7 mo.*	8-9 mo.*	Total
A	38	44	38	36	8	82
B	38	32	19	37	14	70
C	12	11	8	5	10	23
D	6	3	4	5	0	9
E	3	2	1	0	4	5
F	4	4	8	0	0	8
G	3	2	5	0	0	5
H	5	6	0	11	0	11
Total	109	104	83	94	36	213

Note. *Age of sheep at the respective sites.

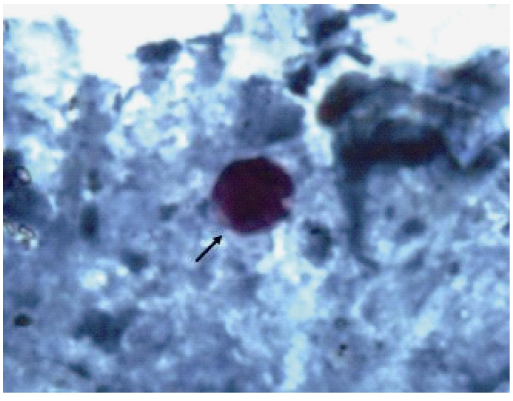

Figure 1. *Cryptosporidium* occyst from sheep feces stained using a modified acid-fast staining technique for light microscopic examination (1 000×).

After microscopic examination only 31 samples were available for DNA extraction and only 4 secondary PCR products were positive following gel electrophoresis analysis (Figure 2) that were successfully sequenced. Following BLAST analysis of these four sequences against GenBank sequences (using the BLASTN algorithm) no exact matches were identified, however, two of the sequences identified in this study were 99% identical to accession number EU827421.1, one was 99% identical to accession number EU827413.1, and the remaining sequence was 99% identical to accession number EU827413.1. Phylogenetic analysis revealed that all sequences belonged to the *Cryptosporidium* cervine genotype (i.e. *Cryptosporidium ubiquitum*[10]) (Figure 3).

DISCUSSION

Cryptosporidium species have been recognized as major enteropathogens affecting a wide spectrum of mammals, including humans. Most data available describe the prevalence of *Cryptosporidium* contaminated water or infected calves with little or no information regarding the levels of infection in sheep or goats. Although *Cryptosporidium* infections of sheep and goats have been identified in several countries where the prevalence was reported to be 4.8%-77.4% using microscopy or molecular characterization, few studies had been carried out in China. This present study identified an incidence rate of 14.6% *Cryptosporidium* infected animals after analyzing sheep from eight different farms.

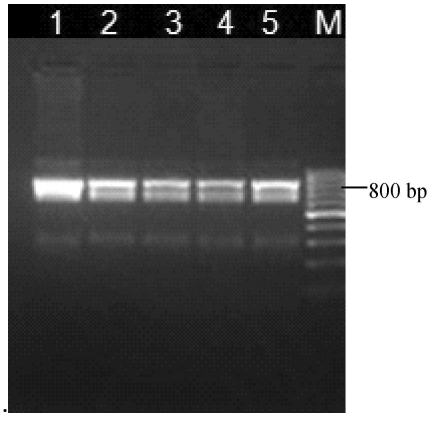

Figure 2. Agarose gel electrophoresis analysis of the secondary amplification products of 18S rRNA gene. PCR amplification products of respective *Cryptosporidium* isolates were subjected to 2% agarose gel electrophoresis. Lane 1: positive control; lanes 2-5: test samples; and M: 100 bp DNA ladder. The 18S rRNA locus is approximately 835 bp.

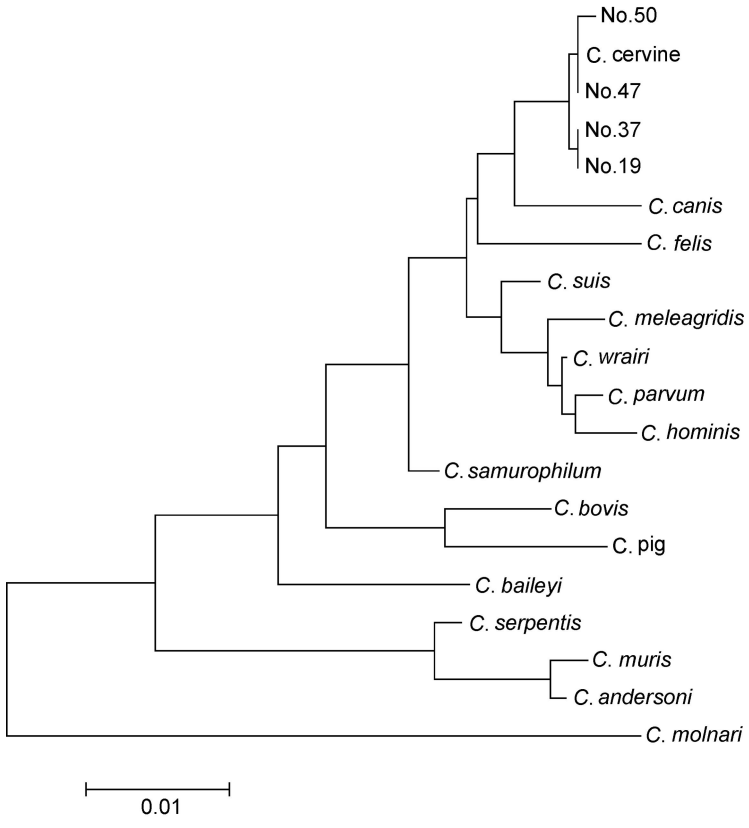

Figure 3. Phylogenetic analysis of isolated sequences. Available 18S rRNA sequences from the respective *Cryptosporidium* isolates, including four identified in this study, were phylogenetically compared. Samples 19, 37, 47, and 50 were *Cryptosporidium*-positive samples from Aba Tibetan and Qiang Autonomous Prefecture identified in the present study.

C. parvum, *C. hominis*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni, C. ubiquitum*, *Cryptosporidium* horse, rabbit, skunk, and chipmunk I genotypes have been recognized as zoonotic pathogens, especially cervine genotype infections in sheep and goats, suggesting that these animals may play larger roles as reservoir species than that previously believed, posing a significant public health threat[14]. Causape et al. previously used a modified Ziehl-Neelsen technique to examine the incidence of *C. parvum* infection by screening fecal samples from 583 lambs (aged 1 day to 3 months) and 205 ewes (>one year of age) in northeastern Spain[15]. The Spain study identified that 59% of lambs and 7.8% of ewes were *Cryptosporidium*-positive and statistical analysis demonstrated that the prevalence of this parasite in lambs was significantly associated with younger age and the form of diarrhea[15]. Further, Bomfim et al. examined 105 stool samples from dairy goats in Rio de Janeiro, Brazil, using centrifuge- flotation techniques and safranine-methylene blue staining, and found a *Cryptosporidium* incidence of 4.8%[16].

Karanis et al. found 15 *Cryptosporidium*- positive goats in 42 farms in the Qinghai province of China using immunofluorescence; one sample was identified as a *C. bovis*-like genotype and a second isolate was novel[17]. Geurden et al. launched a cross-sectional epidemiological study to define the prevalence of *Cryptosporidium* in lambs and goat kids in Belgium and, using quantitative immunofluorescence, identified 18/137 positive lambs and 14/148 positive goat kids. Furthermore, molecular characterization demonstrated that the cervine genotype was predominant in lambs but only *C. parvum* in goat kids[18]. Wang et al. microscopically examined stool samples concentrated using Sheather's sugar flotation technique and stained using the modified acid-fast stain sheep samples from the Henan Province (China). They demonstrated that a 4.8% *Cryptosporidium* oocyst incidence and that the cervine genotype was the major *Cryptosporidium* genotype identified[19], which was similar to that reported in this present study. The Henan Province

data combined with the data presented here suggested that the cervine genotype may be the main *Cryptosporidium* genotype in China.

To date, most *Cryptosporidium* prevalence studies in sheep and goats focused on microscopic examination diagnostic techniques and lacked much more sensitive assays. Except for the above-mentioned districts in China, other countries in different regions of the world, for example, Turkey[20-23], Mexico[24-25] and the west-central region of Poland[26] also used these techniques, suggesting that more sensitive PCR-based assays are need for assessing *Cryptosporidium* infection rates.

Paoletti et al. combined ELISA and PCR assays to detect *Cryptosporidium* in lambs in Italy and reported that *C. parvum* was the major species identified (17.45% prevalence), suggesting that surveillance of sheep populations for *Cryptosporidium* infections would be an important public health prevention strategy[27]. Fayer and Santin isolated a new species, *C. xiaoi*, with a prevalence of 6.96% (5/72) from sheep following a multi-locus analysis of SSU-rDNA, HSP-70 and actin gene sequences[28]. In Western Australia, Yang et al. screened 477 stool samples using PCR to amplify the 18S rRNA locus and successfully identified 10 *Cryptosporidium* cervine genotype isolates, suggesting that the *C. parvum/C. hominis* qPCR assay was more sensitive than the nested 18S PCR analysis[29]. Ryan et al. genotyped *Cryptosporidium* collected from sheep also at the 18S rRNA locus and identified three *Cryptosporidium* species and five different genotypes, including 33 *Cryptosporidium* cervine isolates[30]. In Maryland, Santin et al. utilized PCR to identify *Cryptosporidium* in the feces of pregnant ewes after parturition and from each of their lambs at three different times after birth and demonstrated that *Cryptosporidium* was present in 25% in the ewes and 77.4% in lambs. In addition, they also identified the cervine genotype and lambs with mixed *Cryptosporidium* spp. infections[3].

Several limitations in this study included: (1) insufficient stool for molecular characterization; (2) if the oocyst counts were below the microscopic detection thresholds, those samples would not have been screened by PCR; and (3) due to lack of sheep younger <6 months of age (and limited number of samples), additional research will be needed to establish a correlation between prevalence and age in addition to the effect of climate on infection status.

The prevalence of *Cryptosporidium* in pre-weaned ovine populations from Aba Tibetan and Qiang Autonomous Prefecture was 14.6% using a modified acid-fast staining technique. All *Cryptosporidium* isolates identified in the present study belonged to the *C. ubiquitum*, similar to a Henan Province study which identified the *Cryptosporidium* cervinve genotype (74/82), *C. andersoni* (4/82), and *C. xiaoi* (4/82) using an 18S rRNA-based PCR assay[19]. Taken together, these data suggested that sheep are an important reservoir for the *C. ubiquitum* and other *Cryptosporidium* spp, suggesting that surveillance of these animal populations for the presence of *Cryptosporidium* is important to public health.

REFERENCES

1. Plutzer J, Karanis P. Genetic polymorphism in *Cryptosporidium* species: an update. Vet Parasitol, 2009; 165, 187-99.
2. Xiao L, Fayer R, Ryan U, et al. *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev, 2004; 17, 72-97.
3. Santin M, Trout JM, Fayer R. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species and genotypes in sheep in Maryland. Vet Parasitol, 2007; 146, 17-24.
4. Fayer R, Santin M, Macarisin D. *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet Parasitol, 2010; 172, 23-32.
5. Xiao L, Fayer R. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int J Parasitol, 2008; 38, 1239-55.
6. Chalmers RM, Robinson G, Elwin K, et al. *Cryptosporidium* sp. rabbit genotype, a newly identified human pathogen. Emerg Infect Dis, 2009; 15, 829-30.
7. Xiao L, Ryan U (2008). Molecular epidemiology. In *Cryptosporidium* and Cryptosporidiosis (Fayer, R., Xiao, L., Eds.), pp. 119-163. CRC Press and IWA Publishing, Boca Raton, FL.
8. Kvac M, Kvetonova D, Sak B, et al. *Cryptosporidium* pig genotype II in immunocompetent man. Emerg Infect Dis, 2009; 15, 982-3.
9. Xiao L, Feng Y. Zoonotic cryptosporidiosis. FEMS Immunol Med Microbiol, 2008; 52, 309-23.
10. Xiao L. Molecular epidemiology of cryptosporidiosis: an update. Exp Parasitol, 2010; 124, 80-9.
11. Xiao L, Escalante L, Yang C, et al. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol, 1999; 65, 1578-83.
12. Xiao L, Alderisio K, Limor J, et al. Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. Appl Environ Microbiol, 2000; 66, 5492-8.
13. Jiang J, Alderisio KA, Xiao L. Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Appl Environ Microbiol, 2005; 71, 4446-54.
14. Santin M, Fayer R. Intragenotypic variations in the *Cryptosporidium* sp. cervine genotype from sheep with implications for public health. J Parasitol, 2007; 93, 668-72.
15. Causape AC, Quilez J, Sanchez-Acedo C, et al. Prevalence and analysis of potential risk factors for *Cryptosporidium parvum* infection in lambs in Zaragoza (northeastern Spain). Vet Parasitol, 2002; 104, 287-98.
16. Bomfim TC, Huber F, Gomes RS, et al. Natural infection by

Giardia sp. and *Cryptosporidium* sp. in dairy goats, associated with possible risk factors of the studied properties. Vet Parasitol, 2005; 134, 9-13.
17. Karanis P, Plutzer J, Halim NA, et al. Molecular characterization of *Cryptosporidium* from animal sources in Qinghai province of China. Parasitol Res, 2007; 101, 1575-80.
18. Geurden T, Thomas P, Casaert S, et al. Prevalence and molecular characterisation of *Cryptosporidium* and *Giardia* in lambs and goat kids in Belgium. Vet Parasitol, 2008; 155, 142-5.
19. Wang Y, Feng Y, Cui B, et al. Cervine genotype is the major *Cryptosporidium* genotype in sheep in China. Parasitol Res, 2009; 106, 341-7.
20. Nalan Ozdal PT, Yasar Goz, Serdar Deger, et al. Parasitic protozoans (*Eimeria, Giardia*, and *Cryptosporidium*) in lambs with diarrhoea in the van province (Turkey). Bull Vet Inst Pulawy, 2009; 53, 47-51.
21. Ferda Sevinc AS, Ugur Uslu. Massive *Cryptosporidium parvum* Infection Associated with an Outbreak of Diarrhoea in Neonatal Goat Kids. Turk J Vet Anim Sci, 2005; 29, 1317-20.
22. Ferda Sevinc UU, Ozelm Derinbay. The Prevalence of *Cryptosporidium parvum* in Lambs around Konya. Turk J Vet Anim Sci, 2005; 29, 1191-4.
23. Bulent Ulutas HV. Cryptosporidiosis in Diarrhoeic Lambs on a Sheep Farm. Turkiye Parazitoloji Dergisi, 2004; 28, 15-7.
24. Alonso-Fresan MU, Garcia-Alvarez A, Salazar-Garcia F, et al. Prevalence of *Cryptosporidium* spp. in asymptomatic sheep in family flocks from Mexico State. J Vet Med B Infect Dis Vet Public Health, 2005; 52, 482-3.
25. Alonso-Fresan MU, Vazquez-Chagoyan JC, Velazquez-Ordonez V, et al. Sheep management and cryptosporidiosis in central Mexico. Trop Anim Health Prod, 2009; 41, 431-6.
26. Majewska AC, Werner A, Sulima P, et al. Prevalence of *Cryptosporidium* in sheep and goats bred on five farms in west-central region of Poland. Vet Parasitol, 2000; 89, 269-75.
27. Paoletti B, Giangaspero A, Gatti A, et al. Immunoenzymatic analysis and genetic detection of *Cryptosporidium parvum* in lambs from Italy. Exp Parasitol, 2009; 122, 349-52.
28. Fayer R, Santin M. *Cryptosporidium xiaoi* n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (Ovis aries). Vet Parasitol, 2009; 164, 192-200.
29. Yang R, Jacobson C, Gordon C, et al. Prevalence and molecular characterisation of *Cryptosporidium* and *Giardia* species in pre-weaned sheep in Australia. Vet Parasitol, 2009; 161, 19-24.
30. Ryan UM, Bath C, Robertson I, et al. Sheep may not be an important zoonotic reservoir for *Cryptosporidium* and *Giardia* parasites. Appl Environ Microbio, 2005; l 71, 4992-7.