Experimental Parasitology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Prevalence, molecular characterization and zoonotic potential of *Cryptosporidium* spp. in goats in Henan and Chongqing, China

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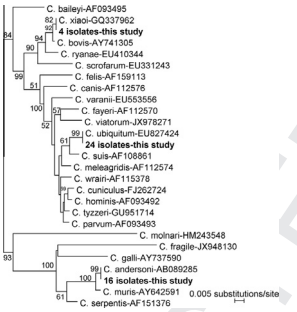
HIGHLIGHTS

- The overall prevalence of *Cryptosporidium* in goats was 3.48% (44/1256).
- *C. ubiquitum*, *C. andersoni* and *C. xiaoi* were identified.
- The zoonotic XIIa subtype 2 of *C. ubiquitum* was found.
- *C. ubiquitum* and *C. andersoni* were the first identified species in goats.
- Age-associated distribution of *Cryptosporidium* was noticed in goats.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 January 2014
Received in revised form 27 March 2014
Accepted 1 April 2014
Available online xxxx

Q3 *Keywords:*
Cryptosporidium
Goats
C. ubiquitum
C. andersoni
C. xiaoi
SSU rRNA
gp60

ABSTRACT

To estimate the prevalence and public health significance of cryptosporidiosis in goats in China, 1265 fecal samples from seven farms in Henan province and Chongqing city were examined for *Cryptosporidium* oocysts. The overall infection rate of *Cryptosporidium* spp. was 3.48% (44/1256). Significant difference was observed among age groups, with the post weaned kids having the highest infection rate (4.58%; $\rho < 0.01$). *Cryptosporidium* spp. were characterized by PCR–restriction fragment length polymorphism (RFLP) analysis and DNA sequence analysis of the small subunit (SSU) rRNA gene. The SSU rRNA-based PCR identified three *Cryptosporidium* species, including *Cryptosporidium ubiquitum* (24/44) in Henan and Chongqing, and *Cryptosporidium andersoni* (16/44) and *Cryptosporidium xiaoi* (4/44) in Henan. Among which, the *C. ubiquitum* and *C. andersoni* were first identified in goats thus far and were found in all age groups except no *C. andersoni* being found in the postparturition nannies, whereas the *C. xiaoi* was detected in pre-weaned kids and pregnant nannies. Subtyping *C. ubiquitum* by DNA sequence analysis of the 60 kDa glycoprotein (gp60) gene suggested the isolates identified all belonged to zoonotic XIIa subtype 2. Thus, the dominant *C. ubiquitum* found in this study and the XIIa subtype 2 has been found in humans indicated goats are a potential source for zoonotic infections with the *C. ubiquitum*. More studies are needed for better understanding of differences in the transmission and public health significance of cryptosporidiosis in goats.

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http://dx.doi.org/10.1016/j.exppara.2014.04.001
0014-4894/

1. Introduction

Cryptosporidium spp. are important gastrointestinal protists in a wide spectrum of hosts, including humans, other mammals, birds, reptiles, amphibians, and fish. Infection is acquired following the ingestion of infective oocysts by the fecal-oral route, via either direct contact or ingestion of contaminated food or water (Xiao and Ryan, 2004). It is one of the common causes of diarrhea in humans and animals. Thus far, twenty-six *Cryptosporidium* species are considered valid, and more than 70 host-adapted genotypes with undetermined species status have been described (Fayer, 2009; Elwin et al., 2012b; Ren et al., 2012; Kváč et al., 2013).

The first *Cryptosporidium* infection case in goat was described in Australia in a 2-week-old kid with diarrhea (Mason et al., 1981). Since then, the infection has been reported in several continents, including Europe (Spain, Italy, Switzerland, Poland, Hungary, Netherland, Belgium, UK, France, and Czech Republic), Asia (India, Mongolia, Turkey, Korea, Iran, Cyprus, Sri Lanka, Iraq, Sultanate of Oman, and China), Africa (Egypt, Tunisia, Malawi, and Zambia), and America (USA, Brazil, and Trinidad & Tobago), with the infection rate ranging from 0% to 100% (average 17.92%, 980/5468) based on point prevalence data at different geographical areas (Noordeen et al., 2000; Geurden et al., 2008; Quílez et al., 2008; Robertson, 2009; Drumo et al., 2012; Giadinis et al., 2012; Marreros et al., 2012; Jafari et al., 2013; Maurya et al., 2013; Rieux et al., 2013). However, few studies have genotyped *Cryptosporidium* spp. from goats in the world. Previous studies indicated that *Cryptosporidium parvum* was the dominant *Cryptosporidium* species, as well as *Cryptosporidium xiaoi*, *Cryptosporidium hominis*, a goat genotype, and a new *Cryptosporidium* genotype have also been detected in goats (Ryan et al., 2005; Karanis et al., 2007b; Robertson, 2009).

Goats play an important role in the agricultural economy of China. In 2011, the total goat population was 142.23 millions, ranking first in the world (http://kids.fao.org/glipha/). Unfortunately, little is known about the distribution and zoonotic transmission risk of *Cryptosporidium* spp. in goats, except for a few prevalence studies published in the Chinese language and two isolates identified as *C. xiaoi* (previously named as *C. bovis*-like genotype) and a new *Cryptosporidium* genotype (Karanis et al., 2007a,b). More recently, Li et al. (2014) developed a subtyping technique by whole-genome sequencing to characterize gp60 gene of *Cryptosporidium ubiquitum* from humans, various animals, and drinking source water, which produced six subtype families, XIIa–XIIf. Thus, the objective of this study was to identify the distribution and zoonotic potential of *Cryptosporidium* spp. in goats in Henan province and Chongqing city, China at the genotype and subtype levels.

2. Materials and methods

2.1. Sample and microscopy examination

Fresh fecal sample for each animal was collected immediately after being defecated on the ground using a sterile disposal latex glove, and then placed in a disposable plastic bag individually. A total of 1265 fecal samples were obtained between July 2006 and July 2007 from four goat farms in two areas of Henan province and from three goat farms in three areas of Chongqing city in December 2011, including those from pre-weaned and post-weaned kids, adult goats, pregnant nannies, and postparturition nannies (Table 1). *Cryptosporidium* oocysts were examined by microscopy after approximately 20 g fecal samples each animal were concentrated by the Sheather's sugar flotation technique and were stained with the modified acid-fast staining. *Cryptosporidium*-positive samples were stored in 2.5% potassium dichromate at 4 °C prior to DNA extraction.

Table 1
Number of fecal samples examined for *Cryptosporidium* oocysts by microscopy on each farm and the distribution of *Cryptosporidium* spp. determined by PCR–RFLP and sequence analysis of the SSU rRNA gene.

Collection site	Sample size	*Cryptosporidium* positive (%)	*C. ubiquitum*	*C. andersoni*	*C. xiaoi*
HN-1	790	25 (3.16%)	6 (24%)	16 (64%)	3 (12%)
HN-2	138	3 (2.17%)	2 (66.67%)		1 (33.32%)
HN-3	65	0			
HN-4	24	0			
CQ-1	64	1 (3.13%)	1 (100%)		
CQ-2	72	7 (9.72%)	7 (100%)		
CQ-3	112	8 (8.33%)	8 (100%)		
Total	1265	44 (3.48%)	24 (54.55%)	16 (36.36%)	4 (9.09%)

2.2. DNA extraction

For samples obtained between 2006 and 2007, *Cryptosporidium* oocysts were isolated from the positive fecal samples by the discontinuous density sucrose gradient centrifugation. Then, genomic DNA was extracted from the purified oocysts using the Mag Extractor Genome kit as described in previous study (Wang et al., 2008). As for samples collected in December 2011, genomic DNA was isolated from *Cryptosporidium*-positive samples using the E.Z.N.A.® Stool DNA Kit (OMEGA Biotek Inc., USA) and the manufacturer recommended procedures (Wang et al., 2011b). The extracted DNA was kept at −20 °C before it was used in molecular analysis.

2.3. Cryptosporidium genotyping and subtyping

Cryptosporidium spp. were genotyped by amplifying the small subunit (SSU) rRNA gene by nested PCR and restriction fragment length polymorphism (RFLP) analysis using restriction enzymes *Ssp*I and *Vsp*I (Xiao et al., 2001). The PCR products were further sequenced directly with the secondary PCR primers on an ABI 3730 DNA Analyzer 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.

C. ubiquitum was subtyped by using gp60 gene, with the sequences of primers being 5′-TTT ACC CAC ACA TCT GTA GCG TCG-3′ (Ubi-18S-F1) and 5′-ACG GAC GGA ATG ATG TAT CTG A-3′ (Ubi-18S-R1), and 5′-ATA GGT GAT AAT TAG TCA GTC TTT AAT-3′ (Ubi-18S-F2) and 5′-TCC AAA AGC GGC TGA GTC AGC ATC-3′ (Ubi-18SR2) for the primary and secondary PCR (Li et al., 2014). The PCR products of gp60 gene were sequenced using the secondary PCR primers as described above.

Sequence analysis was performed by alignment of SSU rRNA sequences obtained in this study and reference sequences downloaded from GenBank using the program ClustalX 1.83 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Phylogenetic analyses were performed using the software Phylip 3.69 (http://evolution.genetics.washington.edu/phylip.html). Neighbour-joining trees were constructed based on the evolutionary distances calculated by Kimura-2-parameter model. The reliability of these trees was assessed using the bootstrap analysis with 1000 replicates.

Sequences of the partial SSU rRNA and gp60 genes were deposited in GenBank under Accession Nos. EU926575–EU926602 and KJ622359.

2.4. Statistical analysis

The chi-square test was used to compare *Cryptosporidium* infection rates. Differences were considered significant when $\rho < 0.05$.

Please cite this article in press as: Wang, R., et al. Prevalence, molecular characterization and zoonotic potential of *Cryptosporidium* spp. in goats in Henan and Chongqing, China. Exp. Parasitol. (2014), http://dx.doi.org/10.1016/j.exppara.2014.04.001