Veterinary Parasitology 164 (2009) 192–200

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Cryptosporidium xiaoi n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (*Ovis aries*)

Ronald Fayer *, Mónica Santín

Environmental Microbial and Food Safety Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Building 173, BARC-East, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

ARTICLE INFO

Article history:
Received 13 January 2009
Received in revised form 9 April 2009
Accepted 6 May 2009

Keywords:
New species
Taxonomy
Cryptosporidiosis
Transmission
Molecular

ABSTRACT

A new species, *Cryptosporidium xiaoi*, is described from sheep. Oocysts of *C. xiaoi*, previously identified as the *Cryptosporidium bovis*-like genotype or as *C. bovis* from sheep in Spain, Tunisia, United Kingdom, and the United States are recorded as such in GenBank (EU408314–EU408317, EU327318–EU327320, EF362478, EF514234, DQ991389, and EF158461). Oocysts obtained from naturally infected sheep were infectious for a lamb and oocysts from that lamb were infectious for three other lambs. The prepatent period for *C. xiaoi* in these four *Cryptosporidium*-naïve lambs was 7–8 days and the patent period was 13–15 days. Oocysts are similar to those of *C. bovis* but slightly smaller, measuring 2.94–4.41 μm × 2.94–4.41 μm (mean = 3.94 μm × 3.44 μm) with a length/width shape index of 1.15 ($n = 25$). Oocysts of *C. xioai* were not infectious for BALB/c mice, *Bos taurus* calves, or *Capra aegagrus hircus* kids. Fragments of the SSU-rDNA, HSP-70, and actin genes were amplified by PCR, purified, and PCR products were sequenced. The new species was distinct from all other *Cryptosporidium* species as demonstrated by multi-locus analysis of the 3 unlinked loci. Based on morphological, molecular and biological data, this geographically widespread parasite found in *Ovis aries* is recognized as a new species and is named *C. xiaoi*.

Published by Elsevier B.V.

1. Introduction

Ovine cryptosporidiosis, first described in diarrheic lambs in Australia (Baker and Carbonell, 1974), has subsequently been reported in 12 other countries (reviewed by Santín and Trout, 2008). The highest prevalence of infection has been found most often in lambs (Abd El-Wahed, 1999; Majewska et al., 2000; Sturdee et al., 2003; Santín et al., 2007), especially those less than 1 month of age (Causapé et al., 2002; Misic et al., 2006). Most studies of cryptosporidiosis in sheep that were conducted before molecular testing assumed that sheep were infected with only one species, *Cryptosporidium parvum*, but with the application of molecular testing the following species and genotypes have been detected in sheep feces: *C. parvum*, *C. suis*, *C. andersoni*, *C. hominis*, *C. bovis*, *C. bovis*-like, cervine genotype, novel bovine B genotype, pig genotype II, marsupial genotype, novel sheep genotype, and a unique unknown genotype (Morgan et al., 1998; McLauchlin et al., 2000; Chalmers et al., 2002; Ryan et al., 2005; Navarro-i-Martinez et al., 2007; Santín et al., 2007; Soltane et al., 2007; Elwin and Chalmers, 2008; Geurden et al., 2008; Mueller-Doblies et al., 2008). The novel sheep genotype as well as the unidentified *Cryptosporidium* reported in sheep by Chalmers et al. (2002) were further investigated and were identified as *C. bovis*/*C. bovis*-like and as the cervine genotype (Elwin and Chalmers, 2008). The species *C. suis*, *C. andersoni*, and *C. hominis* were each found in the feces of only one or two sheep (Ryan et al., 2005) and might represent oocysts passing through the intestinal tract as opposed to actual infections. The pig genotype II and marsupial genotype

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* Corresponding author. Tel.: +1 301 504 8750; fax: +1 301 504 6608.
E-mail address: ronald.fayer@ars.usda.gov (R. Fayer).

0304-4017/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetpar.2009.05.011

that were each found in only four specimens might also represent oocysts simply passing through the intestinal tract (Ryan et al., 2005).

Oocysts identified as *C. bovis* or as the *C. bovis*-like genotype have been reported in sheep in Spain (Navarro-i-Martinez et al., 2007), Tunisia (Soltane et al., 2007), the United Kingdom (UK) (Elwin and Chalmers, 2008; Mueller-Doblies et al., 2008), and the United States (US) (Santín et al., 2007). A novel bovine B genotype (*C. bovis* was previously reported as the bovine B genotype) was reported in sheep in Australia (Ryan et al., 2005). The *C. bovis*-like genotype also was reported from a goat and a yak in China (Feng et al., 2007; Karanis et al., 2007). The *C. bovis*-like genotype and *C. bovis* are genetically very similar. Identification as the *C. bovis*-like genotype is based solely on the nucleotide sequence similarity of a fragment of the SSU-rDNA gene compared with *C. bovis* found in cattle and with the *C. bovis*-like genotype in other hosts. Oocysts of *C. bovis* isolated from cattle were not infectious for two experimentally exposed lambs less than 1 week of age and were not detected in 42 lambs 2–3 months of age, but were detected in a 2-week-old lamb (Fayer et al., 2005).

To determine if the *C. bovis*-like genotype was a slight variant of *C. bovis* or a distinct species, segments from two additional unlinked loci, the HSP-70 and actin genes, were sequenced and compared with those of *C. bovis*. Furthermore, as proposed by Xiao et al. (2004) for qualification of species status, morphometric and biological data were obtained. Data obtained in the present study indicate that the *C. bovis*-like isolate from sheep qualifies as a new species and as such is named *Cryptosporidium xiaoi* in honor of Dr. Lihua Xiao for his many contributions to the taxonomy and molecular epidemiology of *Cryptosporidium* species.

2. Materials and methods

2.1. Experimental design

Fecal specimens were collected from ewes in a commercial flock and from mixed age sheep in an experimental flock at the Agricultural Research Service, Beltsville Agricultural Research Center (BARC) to obtain the initial *C. xiaoi* oocysts for the present study. *C. xiaoi* oocysts from four naturally infected sheep were pooled and used to infect a single lamb (Lamb 1). Oocysts from that lamb were used to infect three additional lambs (Lambs 2–4). Oocysts from the three lambs were used to infect 2 calves, 3 goats, and 12 mice. Oocysts from these lambs were measured and photographed. Oocysts from every infected animal were observed by microscopy and confirmed by PCR followed by gene sequencing as described below.

2.2. Oocyst collection

To obtain oocysts for microscopic examination and for DNA extraction, feces were processed as described (Fayer et al., 2000). Fifty-three fecal specimens were collected from the ground of a farmyard pen of ewes in Union Bridge, Maryland, placed in specimen cups and transported to the laboratory for processing. Nineteen fecal specimens were collected from a pasture where mixed age sheep were held at the BARC. Feces from experimentally exposed lambs, calves, and goats were collected daily for 21–24 days beginning on the day of inoculation and placed in individual specimen cups with lids, labeled with the date and animal number, and held at 4° C until processed. Five to 15 g of feces from ewes, lambs, calves, and goats were transferred from each specimen cup to a 50 ml centrifuge tube containing approximately 35 ml distilled water (dH_2O). The tube was capped and contents were thoroughly mixed using a Vortex-Genie (Scientific Industries, Bohemia, New York). To remove large particles the fecal suspension was sieved through a 45 μm pore size wire screen. The sieved suspension was placed in another 50 ml tube and the final volume was adjusted to 50 ml with dH_2O. The tube was centrifuged at $1800 \times g$ for 15 min, supernatant was discarded, and the pellet, suspended in 25 ml dH_2O, was mixed by Vortex-Genie. Twenty-five millilitres of CsCl (1.4 g/l) was added to the tube, the contents were mixed thoroughly, and the tube was centrifuged at $300 \times g$ for 20 min. Four millilitres of supernatant were aspirated from the top of the tube and transferred to a 15 ml centrifuge tube where dH_2O was added to reach a final volume of 15 ml. The tube was centrifuged at $1800 \times g$ for 15 min and similarly washed twice with dH_2O before the final pellet was suspended in 500 μl of dH_2O. Portions of the suspension were examined by immunofluorescence microscopy (IFA), differential interference microscopy, and molecular methods as described below.

2.3. Microscopic examination of oocysts

A 2 μl aliquot of cleaned oocyst suspension was mixed with 2 μl of premixed anti-*Cryptosporidium* reagent (Mer*IFluor*^{TM}, Meridian Biosciences Inc., Cincinnati, Ohio). Two microlitres of this mixture was pipeted into a well (11 mm diameter) of a 3-well glass microscope slide and the slide covered with a coverslip. The well areas were examined using a Zeiss Axioskop equipped with epifluorescence and an FITC-Texas Red^{TM} dual wavelength filter. Twenty five oocysts were measured by ocular micrometer at 100×. For purposes of comparison, oocysts ($n = 25$) of *C. parvum* from a bovine source were measured using the same microscope. Using a Zeiss Axioskop microscope with differential interference contrast microscopy, photomicrographs of *C. xiaoi* oocysts were obtained and deposited as phototypes in the US National Parasite Collection, Beltsville, MD as accession no. USNPC 101171.

2.4. DNA extraction, PCR, and sequence analyses

DNA was extracted from each 50 μl suspension of cleaned oocysts using a DNeasy Tissue Kit (Qiagen, Valencia, California). To increase the amount of DNA that could be recovered, nucleic acid was eluted in 100 μl of elution buffer included in the DNeasy Kit.

PCR was used to amplify fragments of the SSU-rDNA (~830 bp), HSP-70 (~325 bp), and actin (~1066 bp) genes (Xiao et al., 2001). For the SSU-rDNA fragment, primary amplification employed the primers CryptoF: 5′TTC TAG AGC TAA TAC ATG CG3′ and CryptoR: 5′CCC ATT TCC TTC GAA ACA GGA3′. Secondary amplification employed the