J. Protozool. Res. 25, 44-47 (2015)

Molecular characterization of *Cryptosporidium andersoni* isolated from Japanese black calves in Tokachi district, Hokkaido Prefecture, Japan

Aita, J.[1], Ichikawa-Seki, M.[1]*, Fukumoto, N.[2], Asada, M.[2], Nishikawa, Y.[3] and Itagaki, T.[1]

*Corresponding author: Ichikawa-Seki, M., E-mail: madoka@iwate-u.ac.jp

[1]Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan, [2]The National Livestock Breeding Center, 8-1 Komabanamiki Otohuke-cho, Kato-gun, Hokkaido 080-0572, Japan, [3]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi-2-13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan

ABSTRACT

Fecal samples from 94 Japanese black cattle (5–211 months old) on a farm in Tokachi district, Hokkaido Prefecture, were analyzed, and two calves (6 months old) were positive for *Cryptosporidium* oocysts (2.1%). The infections seemed to be asymptomatic because the feces were normal. The oocysts were morphologically similar to those of *C. andersoni* and were confirmed as this species based on the nucleotide sequences of their 18S ribosomal RNA (18S rRNA) genes. Both Type A and B were detected in the 18S rRNA sequences of the positive samples. This is the first report of *C. andersoni* Type B in Hokkaido Prefecture.

Key words: Cattle, *Cryptosporidium andersoni*, Genotyping, Hokkaido Prefecture, 18S rRNA

INTRODUCTION

Cryptosporidium spp. are protozoan parasites belonging to the phylum Apicomplexa that parasitize the gastrointestinal tracts of vertebrates (Koyama *et al*., 2005; Matsubayashi *et al*., 2004; Nagano *et al*., 2007). *Cryptosporidium parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* have mainly been reported in cattle (Fayer *et al*., 2006 and 2008; Lindsay *et al*., 2000; Santín *et al*., 2004). *Cryptosporidium andersoni* infects the abomasum of cattle and produces large oocysts. This species was previously designated *C. muris* (Lindsay *et al*., 2000; Sakai *et. al*., 2003). However, a molecular analysis based on the 18S ribosomal RNA (18S rRNA) gene demonstrated that *C. muris* detected in cattle is genetically distinct from the species isolated from rodents (Morgan *et al*., 2000). Moreover, *Cryptosporidium muris* isolated from cattle was unable to infect laboratory rodents (Lindsay *et al*., 2000; Morgan *et al*., 2000). Therefore, the new species name, *C. andersoni*, was conferred upon this protozoa (Lindsay *et al*., 2000; Xiao *et al*., 2004). *Cryptosporidium andersoni* has been detected in both post-weaned calves and adult cattle (Fayer *et al*., 2006; Koyama *et al*., 2005), and the infection is usually asymptomatic (Chalmers and Katzer 2013). It may, however, reduce the

milk production and growth rate of the host (Anderson *et al*., 1987; Lindsay *et al*., 2000). Type A, B, and C of *C. andersoni* have been defined based on the nucleotide sequences of their 18S rRNA genes. A single thymidine insertion distinguishes Type A and B (Nagano *et al*., 2007), and Type C shows a mixed nucleotide signal of both Type A and B at that position in a single sporozoite (Ikarashi *et al*., 2013). *Cryptosporidium andersoni* is widely distributed in Japan (Ikarashi *et al*., 2013; Koyama *et al*., 2005; Matsubayashi *et al*., 2004; Nagano *et al*., 2007; Saeki *et al*., 2000; Sakai *et al*., 2003; Satoh *et al*., 2003; Šlapeta, 2013) and was first reported in Hokkaido (Koyama *et al*., 2005; Matsubayashi *et al*., 2004; Nakai *et al*., 2004). However, no reports of *C. andersoni* in Hokkaido Prefecture have classified the species into Type A, B, and C. The objective of this study was to analyze *C. andersoni* at the molecular level using the 18S rRNA gene and to determine the genotypes of the isolates detected on a farm in Hokkaido Prefecture.

MATERIALS AND METHODS

Fecal samples from 94 Japanese black cattle (5–211 months old) were collected on a farm in Tokachi district, Hokkaido Prefecture in March 2014. The fecal samples were stored at 4 °C and transported to the laboratory, where their conditions were recorded. The centrifuge sucrose flotation method and microscopic examination were used to detect *Cryptosporidium* oocysts. The fecal samples were directly subjected to three cycles of freezing at −80 °C for 15 min and thawing in a 37 °C water bath for 15 min. The total DNA was then extracted from every fecal sample with the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), according the manufacturer's protocol. Fragments of the 18S rRNA gene were amplified with nested PCR, as described previously (Xiao *et al*., 1999). The secondary PCR products were purified with the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and inserted into the plasmid vector (pCR™ 2.1) with the TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA). After TOP10 competent cells were transformed with the construct, the plasmid DNA was extracted with the NucleoSpin® Plasmid QuickPure Kit (Macherey-Nagel). Ten positive colonies from each sample were sequenced in both directions with the secondary PCR primers, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the 3500 Genetic Analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

Large oocysts were detected in the feces of two 6-month-old calves of the 94 cattle tested. These oocysts were elliptical in shape and 7.1–7.5 × 5.1–5.4 μm (n = 10) in size, and were morphologically identified as *C. andersoni*. The conditions of the two positive fecal samples were normal, indicating that the infections were significantly asymptomatic. The *C. andersoni* detection rate in this study was 2.1% (2/94), similar to those in previous studies: 1.5% in Hokkaido (Koyama *et al*., 2005), 4.4% in Miyagi (Ikarashi *et al*., 2013), 2.8% in Shizuoka (Suzuki *et al*., 1998), and 1.7% in Hyogo Prefectures (Saeki *et al*., 2003).

The nucleotide sequences of the 18S rRNA genes confirmed that the *Cryptosporidium* oocysts detected in Hokkaido Prefecture were *C. andersoni*. Both Type A and B were detected in the 10 clones from the two

samples (Type A:Type B = 5:5 or 7:3; Table 1). The nucleotide sequences of Type A and B were deposited in GenBank under accession nos. LC012013 and LC012014. Only *C. andersoni* Type A has been reported in Hokkaido Prefecture (Koyama *et al*., 2005; Matsubayashi *et al*., 2004; Nakai *et al*., 2004); this is the first report of Type B in this prefecture. However, the presence of Type C cannot be eliminated because no single sporozoite (Ikarashi *et al*., 2013) was isolated in this study. Although *C. andersoni* is rarely associated with human cryptosporidiosis (Robinson *et al*., 2008), both Type A and B have been detected in patients with the disease (Jiang *et al*., 2014). As demonstrated in this study, the genetic diversity of *C. andersoni* in Japan is not yet sufficiently known. Further studies in various areas in Japan are required to determine the genetic diversity of *C. andersoni* and to investigate the pathogenicity of the individual genotypes.

Table 1. Profiles of *Cryptosporidium* oocyst-positive calves and the results of the analyses

No.	Month	Breed	Oocyst size (μm) (n=10)	18S rRNA		
				Species identification	Genotype[a]	Out of 10 clones
1	6	Japanese black	7.3−7.5 × 5.2−5.4	*C. andersoni*	Type A	5
					Type B	5
2	6	Japanese black	7.1−7.4 × 5.1−5.3	*C. andersoni*	Type A	7
					Type B	3

[a] Type A and Type B had the identical nucleotide sequence of *C. andersoni* Type A (GeneBank accession no. AB089285) and Type B (AB362934).

REFERENCES

Anderson, B. C. 1987. Abomasal cryptosporidiosis in cattle. Vet. Pathol. 24: 235-238.

Chalmers, R. M. and Katzer, F. 2013. Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. Trends Parasitol. 29: 237-251.

Fayer, R., Santin, M. and Trout, J. M. 2008. *Cryptosporidium ryanae* n. sp. (Apicomplexa:Cryptosporidiidae) in cattle (Bos taurus). Vet. Parasitol. 156: 191-198.

Fayer, R., Santin, M., Trout, J. M. and Greiner, E. 2006. Prevalence of species and genotypes of *Cryptosporidium* found in 1–2-year-old dairy cattle in the eastern United States. Vet. Parasitol. 135: 105-112.

Ikarashi, M., Fukuda, Y., Honma, H., Kasai, K., Kaneta, Y. and Nakai, Y. 2013. First description of heterogeneity in 18S rRNA genes in the haploid genome of *Cryptosporidium andersoni* Kawatabi type. Vet. Parasitol. 196: 220-224.

Jiang, Y., Ren, J., Yuan, Z., Liu, A., Zhao, H., Liu, H., Chu, L., Pan, W., Cao, J., Lin, Y. and Shen, Y. 2014. *Cryptosporidium andersoni* as a novel predominant *Cryptosporidium* species in outpatients with diarrhea in Jiangsu Province, China. BMC. Inf. Dis. 14: 555.

Koyama, Y., Satoh, M., Maekawa, K., Hikosaka, K. and Nakai, Y. 2005. Isolation of *Cryptosporidium andersoni* Kawatabi type in a slaughterhouse in the northern island of Japan. Vet. Parasitol. 130: 323-326.

Lindsay, D. S., Upton, S. J., Owens, D. S., Morgan, U. M., Mead, J. R. and Blagburn, B. L. 2000. *Cryptosporidium andersoni* n. sp. (Apicomplexa:Cryptosporiidae) from cattle, Bos taurus. J. Eukaryot.

Microbiol. 47: 91-95.

Matsubayashi, M., Kimata, I., Abe, N., Tanrai, H. and Sasai, K. 2004. The detection of a novel type of *Cryptosporidium andersoni* oocyst in cattle in Japan. Parasitol. Res. 93: 504-506.

Morgan, U. M., Xiao, L., Monis, P., Sulaiman, I., Pavlasek, I., Blagburn, B., Olson, M., Upton, S. J., Khramtsov, N. V., Lal, A., Elliot, A. and Thompson, R.C. 2000. Molecular and phylogenetic analysis of *Cryptosporidium muris* from various hosts. Parasitology 120: 457-464.

Nagano, S., Matsubayashi, M., Kita, T., Narushima, T., Kimata, I., Iseki, M., Hajiri, T., Tani, H., Sasai, K. and Baba, E. 2007. Detection of a mixed infection of a novel *Cryptosporidium andersoni* and its subgenotype in Japanese cattle. Vet. Parasitol. 149: 213-218.

Nakai, Y., Hikosaka, K., Satoh, M., Sasaki, T., Kaneta, Y. and Okazaki, N. 2004. Detection of *Cryptosporidium muris* type oocysts from beef cattle in a farm and from domestic and wild animals in and around the farm. J. Vet. Med. Sci. 66: 983-984.

Robinson, G., Elwin, K. and Chalmers, R. M. 2008. Unusual *Cryptosporidium* genotypes in human cases of diarrhea. Emerg. Inf. Dis. 14: 1800-1802.

Saeki, S., Inada, I., Fukumizu, S., Yoshioka, K., Okahata, K., Oh, S., Inamoto, F., Takekawa, A. and Uga, S. 2000. Epidemiological studies on *Cryptosporidium* spp. infection in cattle in Hyogo Prefecture. J. Jpn. Vet. Med. Assoc. 53: 25-29.

Sakai, H., Tushima, Y., Nagasawa, H., Ducusin, R. J., Tanabe, S., Uzuka, Y. and Sarashina, T. 2003. *Cryptosporidium* infection of cattle in the Tokachi District, Hokkaido. J. Vet. Med. Sci. 65: 125-127.

Santín, M., Trout, J. M., Xiao, L., Zhou, L., Greiner, E. and Fayer, R. 2004. Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. Vet. Parasitol. 122: 103-117.

Satoh, M., Hikosaka, K., Sasaki, T., Suyama, Y., Yanai, T., Ohta, M. and Nakai, Y. 2003. Characteristics of a novel type of bovine *Cryptosporidium andersoni*. Appl. Environ. Microbiol. 69: 692-961.

Šlapeta J. 2013. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int. J. Parasitol. 43: 957-970.

Suzuki, S., Sahara, K., Nishina, T., Ikehata, A., Atsumi, M., Honda, H. and Kuroki, T. 1998. Detection of *Cryptosporidium muris* from the feces of slaughtered cattle. J. Jpn. Vet. Med. Assoc. 1: 163-165.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A. A., Montali, R. J., Fayer, R. and Lal, A. A. 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. 65: 1578-1583.

Xiao, L., Fayer, R., Ryan, U. and Upton, S. J. 2004. *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin. Microbiol. Rev. 17: 72-97.