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

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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
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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 isolates.
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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>
<thead>
<tr>
<th>No.</th>
<th>Month</th>
<th>Breed</th>
<th>Oocyst size (μm) (n=10)</th>
<th>18S rRNA species identification</th>
<th>Genotype</th>
<th>Out of 10 clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Japanese black</td>
<td>7.3–7.5 x 5.2–5.4</td>
<td>C. andersoni Type A</td>
<td>Type A</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C. andersoni Type B</td>
<td>Type B</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Japanese black</td>
<td>7.1–7.4 x 5.1–5.3</td>
<td>C. andersoni Type A</td>
<td>Type A</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C. andersoni Type B</td>
<td>Type B</td>
<td>3</td>
</tr>
</tbody>
</table>

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

REFERENCES
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Microbiol. 47: 91-95.


