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Genetic Diversity and Zoonotic Potential of Cryptosporidium parvum Causing Foal Diarrhea

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Cryptosporidium isolates from diarrheic foals in New Zealand (n = 9) were identified as C. parvum, subtyped at two polymorphic loci, and compared with human (n = 45) and bovine (n = 8) isolates. Foal C. parvum isolates were genetically diverse, markedly similar to human and bovine isolates, and carried GP60 Haa18G3R1 alleles, indicating a zoonotic potential.

Intestinal Cryptosporidium parasites, in particular C. parvum, are common causes of diarrhea in humans and animals worldwide. Cryptosporidium parvum is also a zoonotic species.

Diarrhea is a common clinical condition of newborn foals (2, 4, 10, 13). However, while some studies suggested that intestinal carriage of Cryptosporidium parasites is relatively common in horses (1–3, 8, 14, 22, 23), reports confirming the role of these parasites in foal diarrhea are rare. In 2003, we reported an outbreak of neonatal foal diarrhea in which C. parvum was identified as the sole agent (6). However, at that juncture the genetic makeup and zoonotic potential of the foal isolates were not assessed, as subtyping tools for Cryptosporidium isolates were not widely available. The aim of this study was to address these questions by using recently developed molecular tools. Therefore, Cryptosporidium isolates collected in New Zealand from diarrheic foals in 2002, 2006, and 2007 were genetically identified and subtyped by sequencing of the polymorphic regions of the sporozoite 60-kDa glycoprotein (GP60) and the 70-kDa heat shock protein (HSP70) genes. To infer on possible transmission routes and zoonotic potential, the foal isolates were compared with human and bovine isolates.

Parasites. Cryptosporidium-positive diagnostic fecal specimens collected in New Zealand from foals (n = 9), humans (n = 45), and cattle (n = 8) were used in this study. The specimens from foals were collected during the foaling seasons of 2002 (n = 3), 2006 (n = 5), and 2007 (n = 1). The specimens from 2002 originated from an outbreak of foal cryptosporidiosis in the Waikato region, which has been previously described (6). Four specimens from 2006 originated from an outbreak of foal diarrhea in a farm located on the North Island. One of these specimens originated from a foal that was hospitalized due to severe diarrhea, and the other three were collected a week later by the first author from 1- to 2-week-old diarrheic foals presented for examination during a visit to the farm. The fifth specimen from 2006 and the specimen from 2007 originated from 2- and 3-week-old diarrheic foals and were donated by a commercial diagnostic laboratory operating on the North Island. No additional information was provided on these sporadic specimens.

The human specimens were collected between 2001 and 2003 as part of a different study (11). As only C. parvum was identified in foals, C. parvum-positive human specimens were used (see below). The bovine specimens originated from diarrheic calves on eight farms and were collected between August and October 2006 by a veterinary laboratory operating on the North Island.

All fecal specimens were stored between 2 and 4°C, with no preservatives.

Cryptosporidium identification and subtyping. The identification of human C. parvum and foal C. parvum isolates collected in 2002 has been previously described (6, 11). Sequencing of a species-specific region of the Cryptosporidium 18S small subunit rRNA (18S rRNA) gene was used for the identification of foal and bovine Cryptosporidium isolates from 2006 and 2007. Genomic DNA of foal specimens from 2006 was extracted from the oocysts as described previously (6), with a modification consisting of the use of three freeze-thaw cycles with 1 minute in liquid nitrogen and water at 95°C. Genomic DNA of bovine specimens and the foal specimen from 2007 was extracted using a DNA extraction kit (DNA stool mini kit; Qiagen GmbH, Hilden, Germany). PCR was performed with a thermocycler (GeneAmp 9700; Applied Biosystems, Foster City, CA), using published forward and reverse primers and conditions (24). Molecular-grade water and a C. parvum-positive specimen from a calf were used as negative and positive controls, respectively. Amplicons were purified on columns (High Pure purification kit; Roche Diagnostics GmbH, Mannheim, Germany) and sequenced in both directions by using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Complementary sequences were aligned and edited; marginal segments that could not be accurately determined were trimmed and the final sequences aligned with Cryptosporidium 18S rRNA sequences in our database by using ClustalX soft-
ware (21). For the subtyping, genomic DNA was extracted using extraction kits as described above. Subtyping was achieved by means of sequencing of two polymorphic loci. The first is an ~830-base-pair region of the Cryptosporidium sporozoite GP60 gene. This locus was amplified using the nested PCR described by Glaberman et al. (5), except that the annealing temperature of the primary PCR of the present assay was 60°C. The second locus is an ~470-base-pair region of the HSP70 gene that comprises a polymorphic repeat (9, 15). Amplification was performed using 200 μM of the primer sequences 5′-CACCATCCAAGAACCAGG (forward) and 5′-GCCTAAAGGTAGAGTGTGCTTTTC (reverse), 1× PCR buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl2, 1 mM deoxynucleoside triphosphates (Fermentas Life-sciences GmbH), and 1 unit of Taq polymerase (Platinum Taq; Invitrogen, Carlsbad, CA) in a final volume of 20 μl. Thermocycling consisted of 1 cycle at 96°C for 2 min, 57°C for 2 min, and 72°C for 2 min; then 40 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and a final elongation step at 72°C for 2 min. Water was used as a negative control in each batch testing. GP60 and HSP70 amplicons were electrophoresed, purified, and sequenced as described above. The final sequences were aligned with published GP60 and HSP70 gene sequences (9, 19). Due to the conserved nature of the HSP70 protein among eukaryotic organisms, the possibility that HSP70 genes from other organisms were amplified was checked by clustering the sequences with similar sequences deposited in GenBank using the neighbor-joining algorithm of BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi; accessed in January 2008).

Initially, human and bovine Cryptosporidium isolates were not sequenced at the HSP70 locus. However, isolates having GP60 sequences identical to the sequences in foal isolates were chosen at random and subtyped at the HSP70 locus, allowing a comparison between bilocus sequence types (BLSTs) from different hosts.

**Cryptosporidium species and subtypes.** All the 18S rRNA gene sequences of foal and bovine Cryptosporidium isolates were indistinguishable from the *C. parvum* sequence deposited in GenBank under accession number AF093490 (24). One foal sequence from 2006 initially differed by 1 base pair, but reextracted DNA yielded a sequence indistinguishable from AF093490.

Six foal specimens were amplified at both the GP60 and the HSP70 loci, one at the GP60 locus only, and one at the HSP70 locus only. One could not be amplified at either locus. The edited GP60 and HSP70 sequences were longer than 710 and 420 base pairs, respectively, and comprised the repeat regions of both loci. All foal, human, and bovine *C. parvum* isolates had GP60 IaA18G3R1 alleles (17, 20). There were five different GP60 sequences in foal *C. parvum*, differing by single-nucleotide polymorphisms but exhibiting more than 99% similarity to each other. There were five different HSP70 sequences in foal *C. parvum*, which differed from the sequence reported by Khramtsov et al. (9) by the number of minisatellite repeats and/or single-nucleotide polymorphisms external to the repeat. With BLAST software, these sequences clustered only with *C. parvum* sequences. In each outbreak, at least one isolate differed from others at both loci. Eight subtyped foal *C. parvum* isolates defined seven genetic variants. Identical GP60 sequences were found in 3 foal, 41/45 human, and the 8 bovine *C. parvum* isolates. Ten human *C. parvum* isolates and the 8 bovine *C. parvum* isolates with this GP60 allele were also sequenced at the HSP70 gene, revealing the same BLST in 2 foal, 10 human, and 7 bovine *C. parvum* isolates (Table 1).

**Table 1. BLSTs of foal, human, and bovine *Cryptosporidium parvum* isolates in New Zealand***

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>GP60 allele designation</th>
<th>HSP70 allele designation</th>
<th>BLST designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/A (foal/Waikato/02)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2/1 (foal/Waikato/02)</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3/2 (foal/Waikato/02)</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4/74 (foal/Manawatu/06)</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5/73 (foal/Manawatu/06)</td>
<td>4</td>
<td>DN</td>
<td>ND</td>
</tr>
<tr>
<td>6/48 (foal/Manawatu/06)</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>7/72 (foal/Manawatu/06)</td>
<td>DN</td>
<td>DN</td>
<td>ND</td>
</tr>
<tr>
<td>8/47 (foal/Waikato/06)</td>
<td>DN</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>9/569 (foal/Waikato/07)</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10-19 (human)</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>20-26 (bovine)</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>27 (bovine)</td>
<td>1</td>
<td>DN</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Arbitrary numbers designate alleles and BLSTs. Isolates 9/569 and 8/47 are from the sporadic cases. DN, did not amplify; ND, not determined.

*These isolate numbers were also used in GenBank.

Based on the identification of a novel partial 18S rRNA gene sequence in a Przewalski's wild horse (*Equus przewalskii*) in a zoo (18), Xiao and Feng suggested that horses are infected with a *Cryptosporidium* “horse genotype” (25), which has never been identified in other hosts. In addition, results of recent molecular studies support the possibility of the existence of host restriction in *C. parvum* (reviewed in reference 25). Thus, the mere identification of *C. parvum* in the 2002 outbreak did not allow conclusions to be drawn about the origin or zoonotic potential of foal *Cryptosporidium* parasites. In this study, the foal isolates were identified, subtyped, and then compared with human and bovine isolates. Conforming with the superdiverse nature of *C. parvum* (7), eight foal isolates could be subdivided into seven genetic variants. Nonetheless, the dominant Ila GP60 allele and BLST were shared by the three host species. GP60 Ila alleles are also highly prevalent in human and bovine *C. parvum* isolates in other countries (12, 20). Therefore, the genetic repertoires of foal, bovine, and human *C. parvum* parasites largely overlap, and so foal *C. parvum* should be considered potentially zoonotic. Lastly, in accordance with the results of waterborne outbreak investigations with humans (5, 12, 16), we identified different *C. parvum* alleles among foals in two outbreaks, suggesting that genetically heterogeneous parasite assemblages may be involved in outbreaks of cryptosporidiosis. This feature may hamper our ability to track infection sources by using molecular tools.

**Nucleotide sequence accession numbers.** The foal *C. parvum* GP60 sequences observed in this study have been deposited in GenBank under accession numbers EU483074 to EU483080.

Our good friend and coauthor Jim Learmonth, an inspiring researcher, passed away on 4 May 2008.
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