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 IIaA18G3R1 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 junction 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 and bovine isolates were compared with human *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 thermostycler (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-
were (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’-CACCATCCAAGAACAAAGG (forward) and 5’-GCCTAAGGTTAGAGTGCTTTTTT (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. The 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).

This is the first report describing the genetic diversity of Cryptosporidium isolates causing foal diarrhea. The key findings of this study were the high genetic diversity of foal C. parvum isolates and their similarity to human and bovine isolates.

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 IIA GP60 allele and BLST were shared by the three host species. GP60 IIA 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.

**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.
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REFERENCES