**Cryptosporidium muris**: Infectivity and Illness in Healthy Adult Volunteers

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Abstract. Although Cryptosporidium parvum and C. hominis cause the majority of human cryptosporidiosis cases, other Cryptosporidium species are also capable of infecting humans, particularly when individuals are immunocompromised. Ten C. muris cases have been reported, primarily in human immunodeficiency virus (HIV)-positive patients with diarrhea. However, asymptomatic cases were reported in two HIV-negative children, and in another case, age and immune status were not described. This study examines the infectivity of C. muris in six healthy adults. Volunteers were challenged with $10^7$ C. muris oocysts and monitored for 6 weeks for infection and/or illness. All six patients became infected. Two patients experienced a self-limited diarrheal illness. Total oocysts shed during the study ranged from $6.7 \times 10^3$ to $4.1 \times 10^7$, and the number was slightly higher in volunteers with diarrhea ($2.8 \times 10^9$) than asymptomatic shedders ($4.4 \times 10^9$). C. muris-infected subjects shed oocysts longer than occurred with other species studied in healthy volunteers. Three volunteers shed oocysts for 7 months. Physical examinations were normal, with no reported recurrence of diarrhea or other gastrointestinal complaints. Two persistent shedders were treated with nitazoxanide, and the infection was resolved. Thus, healthy adults are susceptible to C. muris, which can cause mild diarrhea and result in persistent, asymptomatic infection.

INTRODUCTION

Cryptosporidium muris was the first Cryptosporidium species discovered, and along with other cryptosporidia, it was long thought to be limited to animal hosts. Cryptosporidium infection in humans was not recognized until 1976,2,3 and it was not until the advent of molecular techniques that several Cryptosporidium species typically found in animals, including C. muris, were identified in humans, particularly immunocompromised individuals.4–10 C. muris infects the gastric rather than the intestinal mucosa.11,12 This anatomical location has been described in a number of mammalian species,13 including immunosuppressed cynomolagus monkeys.14 However, this has not been confirmed in humans. Regardless of infection site, diarrhea is the most common manifestation of C. muris infection in humans. In contrast, C. muris in animals is typically asymptomatic.13,15

The first reported case of C. muris in humans was published in 2000 (Table 1), and since that time, 10 additional C. muris cases have been reported in the literature.4–10,16,17 Most of these cases were associated with advanced human immunodeficiency virus (HIV) infection and CD4+ T-cell absolute counts of < 100/mm3. In contrast, two apparently healthy children in Indonesia were reported to have C. muris infection; however, this finding was not confirmed by definitive techniques.16 Thus, questions remain regarding the infectivity and clinical manifestations in healthy persons. To date, no outbreak of human cryptosporidiosis has been linked to C. muris.

The purpose of this study was to determine if healthy adults are susceptible to infection by C. muris oocysts and describe the clinical, microbiological, and serological outcomes of experimental challenge. These data will be useful in understanding the natural history of C. muris infection in healthy persons and will contribute to microbial risk assessment and determination of drinking water quality standards.

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PARASITE STRAIN AND OOCYST PREPARATION. C. muris isolate (RN66), originally from Waterborne, Inc. (New Orleans, LA), was verified for species by sequencing a fragment of the β-tubulin gene fragment as previously described.18,19 Approximately 5,000 oocysts were administered orally to Nu/Nu homozygous mice (Charles River, Wilmington, MA). Feces were collected starting approximately on day 30 post-infection until an adequate number of oocysts was available for purification. The approximate oocyst yield from fecal collections was $10^6$ oocysts per mouse per day.

Feces were homogenized, and the oocysts were separated from fecal material by flotation on saturated NaCl. Additional purification was achieved by sedimenting the oocysts on a 15–30% Histodenz (Sigma) step gradient as described.20 Adventitious agents were removed or inactivated in 10% bleach (0.5% sodium hypochlorite) on ice, and purified oocysts, suspended in sterile water, were immediately shipped to Houston, Texas, where they were subjected to safety testing as previously described.21

VOLUNTEER SELECTION AND ENROLLMENT. Volunteer selection and monitoring have been previously described.22 Subjects between 18 and 50 years of age were recruited and educated about Cryptosporidium and its potential effects in humans. After an initial consent, volunteers were enrolled, verified to be in sound health, and tested for evidence of serum antibodies to Cryptosporidium. In addition, volunteers were given a written test to ascertain the level of understanding about the characteristics of Cryptosporidium infection and its transmission; volunteers were required to score 100% on the examination to be enrolled in the study. A second informed consent was then obtained before challenge with C. muris oocysts.

Volunteer enrollment, challenge, and monitoring were carried out at the University of Texas Health Science Center Clinical Research Center (UCRC; Hermann Memorial Hospital, Houston, TX) in 2003. The study was reviewed and approved by the Committee for Protection of Human Subjects at the University of Texas Health Science Center at Houston, a Data Safety and Monitoring Board (DSMB), which included a Data Safety and Monitoring Board (DSMB), which included...
an ethicist who also functioned as a patient advocate, and the UCRC Scientific Advisory Committee. Five DSMB members were infectious diseases physicians or gastroenterologists familiar with cryptosporidiosis in clinical and/or research settings. The DSMB regularly met with the investigators and provided recommendations that guided the study.

**Challenge and monitoring of volunteers.** A detailed description of oocyst preparation and delivery as well as post-challenge sample collection and assessment has been described elsewhere. Briefly, a known concentration of oocysts was diluted to 1 × 10^7/mL, and six separate 10-μL aliquots were removed and counted on a hemacytometer to verify the number of oocysts present. Oocysts were delivered to volunteers within 1 hour of placement into gelatin-filled capsules; all volunteers received the same dose of 10^7 oocysts on the same date. Subjects were monitored in the clinic each day for the first 14 days and then three times per week for a total of 6 weeks or after the 6-week period, until two successive stools were negative for *Cryptosporidium* oocysts. Stool samples from subjects who experienced a diarrheal illness were evaluated for other (i.e., non-*Cryptosporidium*) pathogens as previously described. Volunteers were released from the study after 6 weeks of monitoring but only if two consecutive stools were negative for oocysts. Volunteers were recontacted for long-term follow-up stool samples approximately 4.5 months after release from the study. Definitions for the clinical and microbiological outcome measures used in this study have been published previously.

**Detection and genotyping of fecal oocysts.** Stool samples were placed in a cooler with ice immediately after passage and delivered to the laboratory within 24 hours. Samples were diluted 1:4 in buffered formalin and thoroughly mixed with fluorescein isothiocyanate (FITC)–labeled anti-*Cryptosporidium* antibody (Crypto-CEL; Cellabs Pty LTD, Australia), and oocysts were counted as described. Briefly, total oocysts per stool were calculated by multiplying oocyst concentration by stool weight, and total oocysts shed during the study was the summation of all oocyst-positive stools collected.

Polymerase chain reaction (PCR) was used for confirmation of oocyst clearing after drug treatment of volunteers with persistent colonization. Briefly, DNA from selected stool samples was extracted (QIAamp DNA Stool Mini Kit; Qiagen) and amplified by nested PCR targeting the small subunit 18S ribosomal RNA gene. PCR products were visualized on a 1.5% agarose gel containing ethidium bromide (Gel BioDoc-It Imaging System; UVP, LLC, Upland, CA). Amplicons were extracted from the gel (Qiaquick PCR Cleanup; Qiagen), digested for 90 minutes at 37°C with Vsp1 or Ssp1 restriction endonuclease, and then, resolved in a 2% agarose gel.

**Detection of serum antibodies.** Sera obtained before challenge and on days 5, 10, 30, and 45 post-challenge were assayed for the presence or absence of anti-*Cryptosporidium* immunoglobulin M (IgM) and IgG by enzyme-linked immunosorbent assay (ELISA). C. *muris* oocysts were disrupted by three freeze–thaw cycles to release antigens and then diluted in 0.15 M phosphate-buffered saline (PBS; pH 7.2) to the desired concentration. Antigen (20 ng/well) was used to coat microtiter plates. Sera were diluted 1:2 and tested in duplicate. Positive and negative control sera were included on each plate. The reaction was allowed to develop until the absorbance (414 nm) of the positive and negative controls reached the following values: positive IgG, > 0.25; negative IgG, 0.1–0.15; positive IgM, > 0.45; negative IgM, < 0.25. A positive reaction was defined as an absorbance value exceeding 1.5 times the mean negative control.

**Treatment.** All volunteers with evidence of persistent *C. muris* infection were offered antimicrobial treatment with nitazoxanide (Alinia; Romark Laboratories, Tampa, FL). Oral doses of 200 mg were given two times per day for 3 days.

**RESULTS**

The oocysts were confirmed as *C. muris* by sequencing a fragment of the β-tubulin gene before and after amplification in mice. *C. muris* oocysts were identified in volunteers’ stool samples using commercially available antibodies (Cellabs).

Six healthy adult volunteers, all of whom were negative for serum IgG antibodies to *C. parvum* antigens, were each challenged with 10^7 *C. muris* oocysts. At the time of the challenge, the oocysts were within 6 weeks of production in the murine host and yielded an excretion rate of 83%. The mean number (±SD) of oocysts delivered to each volunteer was 100,821 ± 16,571.

All six volunteers became infected as confirmed by indirect fluorescence assay (IFA) of multiple fecal samples (Figure 1 and Table 2). Only two of the infected volunteers (162 and 165) had a diarrheal illness, yielding a 33% illness attack rate. Three other volunteers passed an occasional unformed stool (166 and 167) or typically had a single soft stool per day (164) without any accompanying gastrointestinal (GI) symptoms. One volunteer (163) had no unformed stools or symptoms, although high numbers of oocysts were shed.

Both volunteers with diarrhea experienced two separate episodes of illness (Table 3). Volunteer 162 had milder illness, which was characterized by the passage of seven unformed stools (total stool weight = 1,526 g) during 3.8 days. The second volunteer with diarrhea (165) had a more prolonged course, with a total of 20 unformed stools (total stool weight = 3,637 g) during 27.3 days. Between episodes, increased gas production and occasional abdominal discomfort were noted.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Health status</th>
<th>Age</th>
<th>Country</th>
<th>Identification method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiangtip and Jongwutiwes⁵</td>
<td>HIV-positive, diarrhea</td>
<td>Adult</td>
<td>Thailand</td>
<td>Morphology, genotyping</td>
</tr>
<tr>
<td>Gatei and others⁶</td>
<td>HIV-positive, diarrhea</td>
<td>Adult</td>
<td>Kenya</td>
<td>Morphology, genotyping</td>
</tr>
<tr>
<td>Katsumata and others¹⁶</td>
<td>HIV-negative, asymptomatic</td>
<td>Children</td>
<td>Indonesia</td>
<td>Morphology</td>
</tr>
<tr>
<td>Guyot and others⁴</td>
<td>Immunocompromised</td>
<td>53 years</td>
<td>France</td>
<td>Morphology, genotyping</td>
</tr>
<tr>
<td>Palmer and others¹</td>
<td>HIV-positive, diarrhea</td>
<td>31 years</td>
<td>Peru</td>
<td>Morphology, genotyping</td>
</tr>
<tr>
<td>Al-Brikan and others¹⁷</td>
<td>Unknown, diarrhea</td>
<td>Child</td>
<td>Egypt</td>
<td>Morphology, ELISA, genotyping</td>
</tr>
</tbody>
</table>

Table 1. Human case reports of *C. muris* infections
without diarrhea. Increased gas, abdominal pain, and occasional fecal urgency were reported during diarrheal episodes.

Oocyst shedding was observed in all volunteers and first detected between days 2 and 5 (median = day 3.5). Shedding patterns revealed significant day-to-day variations in each subject, with several days where oocysts were below the limit of detection. The intensity of the infection varied among the volunteers. Total oocysts counted in collected stool samples during the study (before release) ranged from $6.7 \times 10^6$ to $4.1 \times 10^8$. The mean oocyst excretion in two volunteers with diarrhea was $2.8 \times 10^8$ versus $4.4 \times 10^7$ in four volunteers with mild to no symptoms.

Volunteers were to be released from the study after 6 weeks but only if two or more sequential stools were IFA-negative. One volunteer (162) withdrew from the study on day 64 when he moved to another state. He was apprised of his infection status, provided detailed information on hygienic practices, and advised not to have contact with elderly adults or children. He subsequently submitted intermittent stool samples from days 82 to 92, and then, he left the country for an extended period. On return (day 194), he submitted another stool sample. All of the samples were positive for *C. muris* oocysts.

Because of the persistence of the infection in volunteer 162, an attempt was made to do follow-up fecal examinations on all volunteers at 4.5 months post-challenge (Table 4). Five of six volunteers agreed to follow-up evaluation of one or more stool samples. Volunteer 162 submitted a stool sample on day 259, which was positive for oocysts, and he was treated with nitazoxanide. Volunteer 163 was contacted and in good health but declined to submit a follow-up fecal sample. It should be

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**Table 2**

Microbiological outcomes after experimental challenge with $10^5$ *C. muris* oocysts

<table>
<thead>
<tr>
<th>Volunteer number</th>
<th>Patency (days post-challenge)</th>
<th>Duration (days)</th>
<th>Total oocysts (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>2</td>
<td>192</td>
<td>8.17</td>
</tr>
<tr>
<td>163</td>
<td>4</td>
<td>41</td>
<td>7.25</td>
</tr>
<tr>
<td>164</td>
<td>4</td>
<td>54</td>
<td>8.02</td>
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<tr>
<td>165</td>
<td>3</td>
<td>46</td>
<td>8.61</td>
</tr>
<tr>
<td>166</td>
<td>3</td>
<td>211</td>
<td>7.22</td>
</tr>
<tr>
<td>167</td>
<td>5</td>
<td>211</td>
<td>6.03</td>
</tr>
</tbody>
</table>

Data include all stools collected until release from study on days 52–60.

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**Table 3**

Clinical outcomes after experimental challenge with $10^5$ *C. muris* oocysts

<table>
<thead>
<tr>
<th>Volunteer number</th>
<th>Episode</th>
<th>Onset (days post-challenge)</th>
<th>Duration (days)</th>
<th>Total number of unformed stools</th>
<th>Total stool weight (g)</th>
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</thead>
<tbody>
<tr>
<td>162</td>
<td>1</td>
<td>8</td>
<td>1.9</td>
<td>5</td>
<td>883</td>
</tr>
<tr>
<td>162</td>
<td>2</td>
<td>13</td>
<td>1.9</td>
<td>2</td>
<td>643</td>
</tr>
<tr>
<td>165</td>
<td>1</td>
<td>2</td>
<td>14.5</td>
<td>9</td>
<td>1,907</td>
</tr>
<tr>
<td>165</td>
<td>2</td>
<td>32</td>
<td>12.8</td>
<td>11</td>
<td>1,730</td>
</tr>
</tbody>
</table>
noted, however, that this volunteer was negative on three stools collected between days 47 and 52 before release from the study. Volunteers 164 (days 215 and 217) and 165 (day 232) submitted stool samples, all of which were oocyst-negative. Because these three volunteers had no evidence of infection on follow-up, no specific therapy was given. Volunteers 166 (samples provided on days 212 and 214) and 167 (samples provided on days 215 and 217) were oocyst-positive. These two volunteers returned to the clinic for nitazoxanide treatment. All three volunteers receiving treatment submitted stool samples after treatment, and all were oocyst-negative by IFA and PCR. Furthermore, all three persistent shedders (162, 166, and 167) had follow-up physical examinations, which were normal, and none reported diarrhea or other symptoms.

**Genotyping of C. muris from volunteers.** PCR amplification of 18S ribosomal RNA (rRNA) sequence was carried out on oocyst-positive stool samples from all six volunteers. All samples were PCR-positive and yielded a restriction fragment length polymorphism (RFLP) banding pattern indicative of C. muris (data not shown).

**Serological response (IgM and IgG) to Cryptosporidium antigens.** All volunteer sera were tested against the homologous antigen (disrupted C. muris oocysts) before and four time points after challenge (data not shown). Four volunteers were IgM- and IgG-negative before challenge, and three volunteers remained negative at each time point after C. muris infection. Two volunteers (164 and 165) showed positive IgM and IgG reactivity against the C. muris antigen preparation at day 0, and three volunteers (163, 164, and 165) had a modest rise (>0.1 optical density (OD) over pre-challenge absorbance) in IgG reactivity during the study.

**DISCUSSION**

The primary purpose of the study was to determine if healthy adults were susceptible to C. muris infection and evaluate the natural history of disease if infected. Because C. muris dose–response studies have not been done, a challenge dose of 10⁵ oocysts per subject was chosen as likely to vastly exceed the infectious dose. Dose–response studies with C. parvum and C. hominis indicated median infectious dose (ID₅₀) values in the range of 10 to 1,000 oocysts for healthy adults with no evidence of prior infection¹¹,¹²,¹³,¹⁹,¹⁰ and an approximately 20-fold higher ID₅₀ in those with previous exposure.³¹ Furthermore, in the multiple isolates studied, the severity of symptoms was independent of dose.

All six volunteers receiving C. muris became infected and shed large numbers of oocysts, but only two volunteers had diarrhea and GI symptoms. In a similar study, five volunteers challenged with 10⁷ C. meleagridis oocysts resulted in four cases of cryptosporidiosis (80% illness attack rate; two cases with confirmed fecal oocysts).²⁴ The total number of oocysts shed fell within the same range (10⁶–10⁷) in C. meleagridis and C. muris infections. However, the median (log) number of C. muris oocysts shed was about 10-fold higher (7.65 versus 6.45) than with C. meleagridis. Asymptomatic infections occurred with both Cryptosporidium species (C. muris, four of six [67%] versus C. meleagridis, one of three [33%]). Both the clinical illness and the intensity of infection resulting from C. muris were similar to the outcomes of challenge studies using C. parvum or C. hominis.²¹,²³,²⁹,³⁰ All parameters other than duration of shedding fell within the same range as seen with other Cryptosporidium species²³,²⁴ and C. parvum isolates.²¹,²³,²⁹,³⁰

All oocyst-positive volunteers responded to 200 mg nitazoxanide given two times per day and had no detectable oocysts in follow-up stool samples. Although nitazoxanide has been used successfully to treat children infected with C. parvum,²¹,²³,³³ we believe that this study is the first report of successful treatment of C. muris infection with nitazoxanide. It should be noted that the dosage and regimen used were based on the pediatric drug studies published at the time. Since that time, a standard nitazoxanide dose of 500 mg two times per day has been adopted for most adults.

An unexpected finding in this study was the long duration of oocyst shedding, which extended up to 194 days post-challenge and mirrors what was observed in nude mice.¹² It is unclear why the shedding period in volunteers was longer with C. muris compared with other Cryptosporidium species, and it may be influenced by environmental-, host-, or parasite-specific factors or some combination thereof. Indeed, whole-genome sequencing has revealed significant differences between C. muris and intestinal species. For instance, C. muris encodes a complete set of tricarboxylic acid cycle enzymes, which are lacking in intestinal Cryptosporidium species.³⁴ However, determinants of Cryptosporidium virulence have not yet been identified.

High oocysts doses of C. muris were used in a proof-of-concept study to show whether infection could be established in healthy adults. Lower oocysts doses may or may not have resulted in similar outcomes. This high-dose approach is reminiscent of C. hominis, which rarely infects ruminants in nature but can readily be transmitted to ruminants through high-dose challenge in the laboratory.³⁵ The high rate of C. muris infection seen in volunteer challenges may be
related to the oocyst dose. In nature, where transmission likely occurs as a result of the ingestion of small numbers of *C. muris* oocysts, atypical host species, such as humans, are rarely infected. Thus, the high oocyst doses used in the experimental challenges may override the normal *C. muris* host range and could explain why all five volunteers became infected despite the fact that human infections are very rare in nature.

In this study, *C. muris* infection resulted in little to no specific IgG response after challenge, even in the two volunteers with pre-challenge reactivity. Likewise, early studies with *C. parvum* (Iowa isolate) found no serum IgG reactivity to antigens from the homologous isolate after primary challenge and only 32% reactivity after a second challenge. Antibody reactivity, however, may vary with different *Cryptosporidium* species or isolates, because 38% of volunteers receiving a primary challenge with *C. hominis* had a specific IgG response to the homologous isolate.22

Finally, we tested several commercially available anti-*Cryptosporidium* antibody preparations to identify one that we could use to detect *C. muris* oocysts in the fluorescence assay. All of the antibodies tested recognized *C. parvum* oocysts, but only one reacted with *C. muris*. Clearly, carefully selected reagents should be used to ensure that clinical samples are adequately assessed for the variety of *Cryptosporidium* species capable of infecting humans.

In summary, we have shown that, when exposed to a large oocyst dose, healthy adults are susceptible to *C. muris* infection and that infection can be persistent. Persistence was noted for as long as 7 months but may have lasted longer if volunteers had not been treated. This finding is consistent with animal studies, which have noted asymptomatic infections lasting for months. Of note, few infected volunteers developed diarrhea, and when it occurred, the illness did not differ from diarrhea caused by *C. parvum, C. hominis*, or *C. meleagridis*. If these results are reflective of the general population, then asymptomatic *C. muris* infection may be more common than is recognized. Furthermore, the burden of disease (diarrhea) caused by *C. muris* may be underestimated, because not all commercially available antibody-based detection methods can detect this parasite species. This may contribute to the paucity of data on *C. muris* infection in humans.

Lastly, because the *C. muris* ID50 is not known, the risk of exposure is unclear. Furthermore, if immunosuppression increases susceptibility to infection and/or illness, sensitive populations could more easily acquire *C. muris* from asymptomatic contacts. Until we have a better understanding of the more unusual *Cryptosporidium* species, they must at least be considered a possible source of infection and illness, especially in those who are immunocompromised.

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