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Cryptosporidium parvum Infections in Bergen, Norway, during an Extensive Outbreak of Waterborne Giardiasis in Autumn and Winter 2004†

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During a large waterborne giardiasis outbreak in Norway, many diarrheic patients were found to have *Cryptosporidium* infections. Gene sequencing identified these infections as *Cryptosporidium parvum* infections, although they were not identical. Whether these infections were due to a simultaneous outbreak of waterborne cryptosporidiosis or reflected background levels not normally detected is discussed.

During the winter of 2004 and 2005, a large waterborne giardiasis outbreak occurred in Bergen, Norway (7, 9). Giardiasis was diagnosed at Haukeland University Hospital (HUS), Bergen, either by microscopic detection of cysts in fecal samples or by a fecal antigen test (ImmunoCard STAT! Cryptosporidium/Giardia rapid assay; Meridian Bioscience). Some cryptosporidiosis cases were also identified. Positive fecal samples were forwarded to the Norwegian School of Veterinary Science, Oslo. Between October 2004 and June 2005, 1,135 samples were sent; 722 of these samples were sent in weeks 45 to 48. A crude purification procedure was performed, and 15-μl subsamples were examined by an immunofluorescent-antibody test (IFAT), which involved staining with dual monoclonal antibody against *Giardia* cysts and *Cryptosporidium* oocysts (Aqua-Glo, Waterborne Inc., New Orleans, La.) and 4′,6′-diamidino-2-phenylindole (DAPI) and examination by fluorescence microscopy. While the initial intention was to enumerate *Giardia* cysts, in several samples *Cryptosporidium* oocysts were observed. These oocysts were scored at a magnification of ×200 as follows: + (1 to 9 oocysts per field of view), ++ (10 to 50 oocysts per field of view), or +++ (>50 oocysts per field of view). DAPI staining (oocysts containing nuclei) was also recorded.

A total of 133 patients were originally considered to have *Cryptosporidium* infections. Thirty of these patients had been initially diagnosed by the fecal antigen test, and several were considered "borderline positive"; in 18 of these samples, oocysts were not detected by IFAT, so they were excluded. Therefore, 115 patients were considered to harbor *Cryptosporidium*; only 12 (10%) of these patients were initially diagnosed by the fecal antigen test.

Most of the 12 individuals that were diagnosed with the fecal antigen test as having *Cryptosporidium* infections which were confirmed by IFAT were relatively heavily infected; 5 (42%) were categorized as +++, 1 (8%) was categorized as ++/+++, 2 (17%) were categorized as ++, and 4 (33%) were categorized as +.

Most of the 103 individuals who were first diagnosed with *Cryptosporidium* infections by IFAT had light infections; 4 (4%) were categorized as +++, 20 (19%) were categorized as ++, and 79 (77%) were categorized as +. In some infections categorized as +, only two or three oocysts were identified. Fifty-six of these infections were detected by IFAT after introduction of the fecal antigen test for initial screening.

Most (68) of the 115 individuals were female (59%). The mean age was 31 years. Most infections were found during late October and early November 2004 (80% of the infections in weeks 45 to 48), when most giardiasis cases were diagnosed (7). Ninety-eight individuals (85%) had concurrent giardiasis. As only samples in which parasites (*Giardia* or *Cryptosporidium*) were detected at HUS were forwarded to the Norwegian School of Veterinary Science, other *Cryptosporidium*-positive samples may have been missed.

Oocysts were isolated by immunomagnetic separation (GC-Combo; Dynal Biotech ASA) as described in the accompanying paper for *Giardia* (7). As most samples had initially been diagnosed with only giardiasis and were used for *Giardia* cyst isolation, relatively few samples were available for oocyst isolation. However, for larger samples excess suspension had been stored and was retrieved for oocyst isolation.

Additionally, 5 liters of a septic tank suspension, at one time considered a possible giardiasis outbreak contamination source, was concentrated to 10 ml by centrifugation. *Cryptosporidium* oocysts were isolated by immunomagnetic separation, and 50% of the concentrate was examined by IFAT. At a magnification of ×200 approximately 20 oocysts per field of view were detected, although most (70%) were empty shells with no sporozoites or nuclei visible.

DNA was isolated using a QIAamp DNA mini kit (QIAGEN GmbH, Germany). Two genes were used for PCR, the *Cryptosporidium* wall protein (COWP) gene and the small-subunit (SSU) rRNA gene (8, 11). For the septic tank sample, there

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TABLE 1. Summary of patient and sample details for patients from whom *Cryptosporidium* oocysts were isolated and PCR was performed

Patient age (yr)	Patient sex	*Cryptosporidium* oocysts detected (% oocysts DAPI positive)	Method used for initial diagnosis	Date of sample (day/mo/yr)	*Giardia* cysts also detected	Gene(s) amplified and sequenced by PCR
26	M	+ (10)	IFAT[a]	27/10/04	++/+++	COWP
45	F	+ (50)	IFAT	01/11/04	++	SSU rRNA
30	F	+ (50)	IFAT	02/11/04	+	SSU rRNA[c]
18	F	+++ (20)	A/G[b]	12/11/04	Negative	SSU rRNA
28	F	++ (5)	IFAT	08/11/04	+++	COWP + SSU rRNA[d]
35	F	++ (20)	A/G	21/01/05	Negative	COWP + SSU rRNA
20	F	+++ (10)	A/G	24/01/05	+	COWP + SSU rRNA
4	M	+++ (50)	IFAT	18/11/04	Negative	COWP + SSU rRNA
20	F	++ (50)	IFAT	16/11/04	+	COWP + SSU rRNA
20	F	++/+++ (>80)	A/G	31/01/05	Negative	COWP + SSU rRNA
1	M	++ (50)	A/G	25/11/04	Negative	COWP + SSU rRNA
43	F	+++ (60)	IFAT	26/12/04	Negative	COWP + SSU rRNA
9	M	+++ (50)	A/G	31/05/05	Negative	COWP + SSU rRNA

[a] For the immunofluorescent-antibody test, samples were concentrated and stained with a dual monoclonal antibody against *Giardia* cysts and *Cryptosporidium* oocysts and DAPI and were examined by fluorescence microscopy. The test was conducted at the Parasitology Laboratory at the Department of Food Safety and Infection Biology at the Norwegian School of Veterinary Science.

[b] A/G, fecal antigen test using the ImmunoCard STATI Cryptosporidium/Giardia rapid assay (Meridian Bioscience). The test was conducted at the Unit for Infectious Diseases and Parasitology, Department of Medicine, Haukeland University Hospital, Bergen, Norway.

[c] One SNP compared with the submitted sequence (GenBank accession number AY204238). The sequence has been deposited in the GenBank database under accession number DQ182559.

[d] For the COWP gene, three SNPs compared with the submitted sequence (GenBank accession number AF266273). The sequence has been deposited in the GenBank database under accession number DQ187314.

was only enough DNA for investigation of the COWP gene. See Table S1 in the supplemental material for the reaction conditions used. PCR products were electrophoresed on 1% agarose and stained with ethidium bromide. Successful PCR was performed with oocyst DNA from 13 (11%) patients. The data are summarized in Table 1.

PCR products were purified (High Pure PCR product purification kit; Roche Diagnostics GmbH) and sent to MWG Biotech, Germany, for sequencing of both strands. Sequences were examined using the Chromas (http://www.technelysium.com.au/chromas.html), BioEdit (http://www.mbio.ncsu.edu/BioEdit/page2.html), or VectorNTI Invitrogen software, and searches were conducted using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

All sequences determined were *Cryptosporidium parvum* sequences. For the COWP gene, nine consensus sequences were identical to the GenBank accession number AF266273 sequence (12) (*C. parvum*; specific host, bovine; genotype, bovine). For one sequence there were three single-nucleotide polymorphisms (SNPs) compared with this sequence (GenBank accession number DQ187314). For the SSU rRNA gene, 11 consensus sequences were identical to the GenBank accession number AY204238 sequence (3) (*C. parvum*; specific host, *Bos taurus*; genotype, 2). For one sequence there was one SNP (GenBank accession number DQ182559), but there were not differences for the sample with three SNPs in the COWP gene.

For the septic tank sewage sample, the COWP gene PCR product differed from the PCR products from the patient samples and the sequence was identical to the following four sequences, although the bit scores depended upon the sequence length: the accession number AF411633 sequence (6) (*C. parvum*; specific host, *Homo sapiens*; genotype, cervine), the accession number AF411632 sequence (6) (*C. parvum*; specific host, *H. sapiens*; genotype, cervine), the accession number AF436074 sequence (1) (*Cryptosporidium* sp.; specific host, *Propithecus verreauxi coquereli*); and the accession number AJ849460 sequence (*Cryptosporidium* sp. strain SI23; specific host, *H. sapiens*; genotype, cervine).

Cryptosporidiosis is not a reportable infection in Norway, and the number of cryptosporidosis cases diagnosed is low. Between 1998 and 2002, 14 of Norway's 27 medical microbiology laboratories analyzed fecal samples for *Cryptosporidium*, and 9 reported no positive diagnoses; the remaining 5 (including HUS) reported zero to two cases annually (5). Additional data from HUS indicate that between 1992 and 2003 (excluding 1999, for which data are unavailable) there were just 67 requests for cryptosporidiosis investigation and only two positive samples, both in 2000.

The diagnosis of 93 *Cryptosporidium* infections between weeks 45 and 48 in 2004 and the approximately 100-fold increase in the number of cryptosporidiosis cases diagnosed compared with the expected number during the 9-month period from October 2004 to June 2005 fulfill accepted criteria for the definition of an outbreak (10). A two-tailed Fisher's exact probability test demonstrated that there was a significant difference ($P < 0.05$) between 2 of 67 positive samples and 93 of 722 positive samples (weeks 45 to 48 in 2004) but not between 2 of 67 positive samples and 115 of 1,135 positive samples (October 2004 to June 2005).

As *Giardia* cysts had contaminated the water, it was possible that *Cryptosporidium* oocysts, which are smaller and more robust, had also contaminated the water supply.

However, most *Cryptosporidium* infections were probably asymptomatic and would not normally be diagnosed since (i) most patients also had giardiasis, (ii) there were many more cases of giardiasis without concurrent *Cryptosporidium* infection, and (iii) in most infections there were only low numbers of oocysts.

Nevertheless, if infections with large numbers of oocysts (scores of ++/+++ or higher) or in which giardiasis was not simultaneously diagnosed are considered "true" symptomatic cryptosporidiosis infections, then 22 cases were recorded in the 9-month period (13 [59%] of which were during weeks 45 to 48 in 2004). Of these 22 cases, only 10 were diagnosed at the

initial screening. The increase in diagnosis may have been due to factors such as increased parasitological analysis, enhanced awareness in the diagnostic laboratory, introduction of the fecal antigen test, use of dual *Cryptosporidium-Giardia* IFAT, and increased occurrence.

The fecal antigen screening test sensitivity and specificity were low compared to the sensitivity and specificity in previous reports (2, 4). The sensitivity was 17.7% (56 samples detected only by IFAT; 12 samples detected by both methods), and the specificity was 40% (18 of 30 samples positive by the fecal antigen test were not found to contain oocysts by IFAT). The low sensitivity could have been because in many of the infections not identified by the fecal antigen test the oocyst concentrations were low. This test previously failed to identify 100% of the samples containing less than 175 oocysts per 10 μl (4). The low specificity could have been due to interpretation of weak lines of colloidal carbon as potential (weak) positives. Low band intensity has previously been reported to be a potential problem (4).

Our results suggest that data from the implementation of new techniques should be interpreted with caution, and validation with "gold standard" methods is essential.

The sequencing results indicated that sewage from the septic tank did not cause the infections and that the oocysts in the sewage did not originate from the patients. This parallels findings for the giardiasis infections (7).

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REFERENCES

1. **da Silva, A. J., S. Caccio, C. Williams, K. Y. Won, E. K. Nace, C. Whittier, N. J. Pieniazek, and M. L. Eberhard.** 2003. Molecular and morphologic characterization of a *Cryptosporidium* genotype identified in lemurs. Vet. Parasitol. **111:**297–307.
2. **Garcia, L. S., R. Y. Shimizu, S. Novak, M. Carroll, and F. Chan.** 2003. Commercial assay for detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J. Clin. Microbiol. **41:**209–212.
3. **Gibbons-Matthews, C., and A. M. Prescott.** 2003. Intra-isolate variation of *Cryptosporidium parvum* small subunit ribosomal RNA genes from human hosts in England. Parasitol. Res. **90:**439–444.
4. **Johnston, S. P., M. M. Ballard, M. J. Beach, L. Causer, and P. P. Wilkins.** 2003. Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. J. Clin. Microbiol. **41:**623–626.
5. **Nygård, K., L. Vold, L. Robertson, and J. Lassen.** 2003. Underdiagnostiseres innenlandssmittede Cryptosporidium- og Giardia-infeksjoner i Norge? Tidsskr. Nor. Lægeforen **123:**3406–3409.
6. **Ong, C. S., D. L. Eisler, A. Alikhani, V. W. Fung, J. Tomblin, W. R. Bowie, and J. L. Isaac-Renton.** 2002. Novel *Cryptosporidium* genotypes in sporadic cryptosporidiosis cases: first report of human infections with a cervine genotype. Emerg. Infect. Dis. **8:**263–268.
7. **Robertson, L. J., L. Hermansen, B. K. Gjerde, E. Strand, J. O. Alvsvåg, and N. Langeland.** 2006. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl. Environ. Microbiol. **72:**2212–2217.
8. **Spano, F., L. Putignani, J. McLauchlin, D. P. Casemore, and A. Crisanti.** 1997. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. FEMS Microbiol. Lett. **150:**209–217.
9. **Tveit, I., Ø. Søbstad, I. Kalland, A. Seim, R. Ø. Arnesen, and P. Fennell.** 2005. Giardia-utbruddet i Bergen. Bergen Kommune and Mattilsynet, Norway. [Online.] http://www.bergen.kommune.no/info_/ekstern/nyheter3/Giardia-rapport_akk_21.02.2005.doc.
10. **World Health Organization.** 2005. Communicable disease control in emergencies. A field manual, p. 107–129. World Health Organization, Geneva, Switzerland.
11. **Xiao, L., L. Escalante, C. Yang, I. Sulaiman, A. A. Escalante, R. J. Montali, R. Fayer, and A. A. Lal.** 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. **65:**1578–1583.
12. **Xiao, L., J. Limor, U. M. Morgan, I. M. Sulaiman, R. C. Thompson, and A. A. Lal.** 2000. Sequence differences in the diagnostic target region of the oocyst wall protein gene of *Cryptosporidium* parasites. Appl. Environ. Microbiol. **66:**5499–5502.