

Molecular Characterization of Isolates of Waterborne *Cryptosporidium* spp. Collected during an Outbreak of Gastroenteritis in South Burgundy, France

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In September 2001, a waterborne outbreak of gastroenteritis occurred in eastern France. Of 31 fecal samples from symptomatic individuals, 19 tested positive for *Cryptosporidium* with two PCRs targeting the Hsp70 and the 18S rRNA genes of *Cryptosporidium*. Sequencing of the PCR fragments produced sequences identical to that of *Cryptosporidium parvum* genotype 1.

Organisms of the genus *Cryptosporidium* are widespread protozoans that develop in epithelial cells lining the digestive and respiratory tracts of vertebrates (8). The species *Cryptosporidium parvum* is the main agent of human cryptosporidiosis. Molecular typing studies have shown that the species can be differentiated into two major genotypes. The human genotype (genotype 1) primarily infects humans, and the zoonotic genotype (genotype 2) is able to infect humans, ruminants, and a variety of other mammals (5). Aside from *C. parvum*, the following *Cryptosporidium* species or genotypes have been identified in immunocompetent and immunocompromised humans: *C. meleagridis*, *C. felis*, a dog genotype or species (*C. canis*), a cervine genotype, and *C. muris* (4, 9, 11, 13, 17–19, 27).

Transmission of the parasite occurs by the fecal-oral route, through the ingestion of oocysts that are shed with the feces of infected hosts (7). Immunocompetent individuals experience short-term gastroenteritis, while immunocompromised patients may suffer from chronic diarrhea (15). Together with the resistance of oocysts to environmental conditions and chlorine (8), a large parasite reservoir and a low infective dose (6) account for the risk of transmission by water. At least 40 waterborne *Cryptosporidium* outbreaks have been described, mainly in the United States, Canada, the United Kingdom, and Japan (7, 10). Most outbreaks were caused by *C. parvum* genotype 1, even in areas where the majority of sporadic cases were caused by *C. parvum* genotype 2 (27). In European countries other than the United Kingdom, few *Cryptosporidium* outbreaks have been reported. The first outbreak occurred in 1995 in The Netherlands, but the source of transmission was not identified (3, 24). A second outbreak due to the contami-

nation of a water tank was reported in Italy in 1997 (21). In France, *Cryptosporidium* was the likely causative agent of a waterborne outbreak that occurred in the south of the country in 1998 (12).

The outbreak investigated in the present paper occurred in 2001 in Dracy le Fort, a county of 1,100 inhabitants in eastern France. On 20 September, health authorities were notified of clustered cases of gastroenteritis. Investigations showed that gastroenteritis cases and complaints about the water quality had been reported by a population served by the same water network since 14 September. Contamination of the public water supply was thus suspected, and an outbreak was declared on 20 September. *Cryptosporidium* oocysts were identified in water samples collected from the public network in Dracy le Fort on 27 September.

Thirty-one samples from symptomatic patients were collected from 28 September to 2 October 2001 and submitted for detection of parasites. The specimens were examined for *Cryptosporidium* oocysts by using the modified Ziehl-Neelsen stain (14) and an indirect immunofluorescence assay based on a *C. parvum* oocyst wall antibody (1). For molecular biology experiments, samples were subjected to a phosphate-buffered saline-ether extraction prior to extraction of DNA with a commercially available kit (QIAamp DNA stool minikit; Qiagen). When this method was applied to fecal specimens which tested negative for *C. parvum* and which were then seeded with purified oocysts, amplification of the expected PCR fragment was achieved for 10 of 10 samples seeded with 1,000 oocysts per g (data not shown). Two diagnostic PCRs were applied to the specimens collected during the outbreak. One assay targeted the region of the Hsp70 gene obtained with the CPHSP2 primer pair (22). This reaction is based on primers designed for identification of a 361-bp DNA fragment specific to *C. parvum*. However, primer-annealing regions are identical in *C. meleagridis* and *C. wrairi* (23), and this assay may also identify these two species. The second reaction targeted a hypervari-

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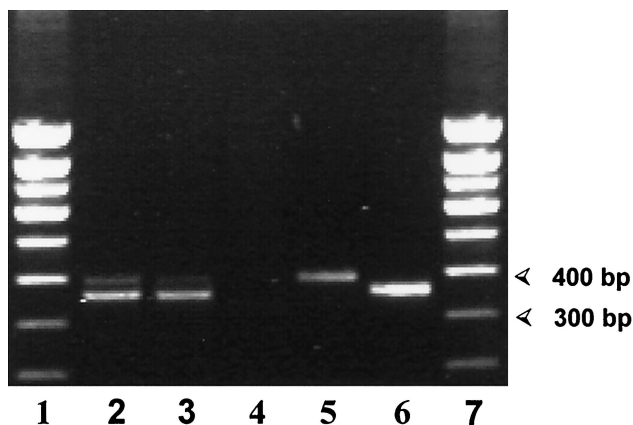


FIG. 1. Coamplification of the CPHSP2 region of the *Cryptosporidium* Hsp70 gene together with the corresponding internal control. Ten copies of the internal control were added to the amplification reactions analyzed in lanes 2 to 5. The 394-bp fragment corresponds to the internal control amplification product. The 361-bp fragment corresponds to amplified *C. parvum* DNA. Lanes 1 and 7, molecular weight markers (Smart ladder; Eurogentec) with apparent molecular sizes given in base pairs. Lanes 2 to 4, PCR products obtained from isolate 15 of the Dracy le Fort outbreak. Two and five microliters of fecal lysates were used as templates of the PCRs displayed in lanes 2 and 3, respectively. For the PCR analyzed in lane 4, 10 μ l of the fecal lysate was used as the template; the absence of PCR product is due to the increased amount of PCR inhibitors resulting from the increased fecal template. Lane 5, isolate 1 from the Dracy le Fort outbreak. Amplification of the 394-bp internal control fragment indicates that this result is a true negative. Lane 6, positive control (genomic DNA from purified *C. parvum* oocysts as a template).

able region of the 18S rRNA gene that discriminates among all *Cryptosporidium* species as well as among multiple *C. parvum* genotypes. This region is flanked by primer-annealing sequences that are common to all known *Cryptosporidium* 18S rRNA genes (16, 20). Therefore, a positive ~435-bp fragment at this locus identifies the genus *Cryptosporidium*, while secondary analysis of a PCR fragment allows for species and genotype identification. In order to detect a false-negative PCR result, we developed a positive internal control of the CPHSP2 region of the Hsp70 gene. Composite primers containing M13 mp18 phage sequences flanked by the *C. parvum* Hsp70 primer sequences 5'-AAA TGG TGA GCA ATC CTC TGC GCT ACA CTT GCC AG -3' and 5'-CTT GCT GCT CTT ACC AGT ACC AGC AGG CGA AAA TC-3' were used to amplify the corresponding region of the M13 mp18 phage DNA (2). This amplification resulted in a 394-bp DNA sequence containing the same primer-annealing sequences as the diagnostic 361-bp *C. parvum* Hsp70 region. About 10 copies of this control were added to the amplification reactions as a positive control (Fig. 1).

No discrepancy in results was observed between the two PCR methods employed. Altogether, 19 of 31 samples (61.2%) were positive for *Cryptosporidium* by PCR. Three samples tested negative for *Cryptosporidium* by microscopy and were positive by PCR. The facts that procedures to avoid false-positive results (work flow organization, a uracyl-*N*-glycosylase protocol, and negative controls) were utilized and that the two PCR assays targeting distinct genes were positive strongly sug-

gest that the three PCR-positive, microscopy-negative samples were true positives.

Direct sequencing of the PCR products was subsequently performed to identify *Cryptosporidium* isolates at the species and genotype levels. Primers cphsp2423F and cphsp2764R were used for sequencing the CPHSP2 region of the Hsp70 gene (22). Primers described by Xiao et al. (25) were used for sequencing the polymorphic region of the 18S rRNA gene. At the Hsp70 locus, all 19 samples produced sequences identical to that of *C. parvum* strain AWS 12 (GenBank accession number AF150827). Comparison of this sequence with homologous regions of *C. meleagridis* and *C. wairi* (GenBank accession numbers AF 221537 and AF 221536, respectively) (23) showed variations at 7 nucleotide positions with *C. wairi* and at 14 nucleotide positions with *C. meleagridis*. In contrast, the sequence from the Dracy le Fort isolate differed at two nucleotide positions with the *C. parvum* human isolate 497 and at four nucleotide positions with *C. parvum* bovine isolate 11 (GenBank accession numbers AF 221535 and AF 221528, respectively) (23). At the 18S rRNA locus, sequences were identical to that of *C. parvum* genotype 1 in the hypervariable regions at nucleotide positions 632 to 667 and 678 to 698 (20). These data indicate that the Dracy le Fort outbreak was due to *C. parvum* genotype 1.

In the sequencing of the hypervariable 18S rRNA locus, a dramatic reduction of signal was observed on both strands after the poly(T) region at nucleotide positions 686 to 696 (20). A similar finding was described previously for this locus in the *C. parvum* human genotype (26), and it was shown that the signal reduction resulted from heterogeneous copies of the 18S rRNA gene, which differed in the number of T repetitions at nucleotide position 686 and subsequent positions and which generated competing signals. To assess this hypothesis and to ensure that genotype 1 was the sole *C. parvum* genotype involved in the Dracy le Fort outbreak, PCR products from isolate 7 were cloned, and 10 recombinant clones were selected for sequencing of the insert. All 10 clones showed heterogeneous sequences that were essentially identical to that of *C. parvum* genotype 1 in the polymorphic regions of nucleotides 632 to 667 and 678 to 698 (20) and that differed in the number of T repetitions at nucleotide position 686 and subsequent positions (20): five clones had 11T repetitions, 2 clones had 10 T repetitions, one clone had 8 T repetitions, and two clones had 12 T repetitions (Table 1). The genotypes with 10 T and 8 T repetitions have been described previously (26). However, the genotype with 12 T repetitions has never been reported. This genotype was confirmed by two separate experiments, as well as by sequencing by an independent laboratory (Genome Express, Grenoble, France). One of the clones with 11 T repetitions also showed a change from C to T at nucleotide position 669, and one of the clones with 12 T repetitions showed a change from T to C at nucleotide position 587 (positions based on the *C. parvum* reference sequence deposited under GenBank accession number L16996). Interestingly, no reduction of the signals occurred in the poly(T) region upon sequencing of the cloned products, confirming that this phenomenon was caused by the mixture of sequences. Other than *C. parvum* genotype 1, no clone homologous to the *Cryptosporidium* species or genotype was obtained.

This was the first waterborne outbreak of *Cryptosporidium* in

TABLE 1. Sequences of the hypervariable regions of the *Cryptosporidium* 18S rRNA gene from four representative clones of isolate 7

Clone	Sequence ^a	
	Positions 632 to 667	Positions 678 to 698
1	ATATAAAATATTTTGATGAATATTTATATAATATTA	TATTACTATTTTTTTTTTTT-AGTAT
2	ATATAAAATATTTTGATGAATATTTATATAATATTA	TATTACTATTTTTTTTTTTTGTAGTAT
3	ATATAAAATATTTTGATGAATATTTATATAATATTA	TATTACTATTTTTTTTTTTT--AGTAT
4	ATATAAAATATTTTGATGAATATTTATATAATATTA	TATTACTATTTTTTTTTT----AGTAT

^a Sequence numbers based on the sequence of *C. parvum* 18S rRNA (GenBank accession number L16996).

France in which a high percentage of the clinical specimens were positive for *C. parvum* and for which molecular characterization of the isolates was possible. Epidemiologic investigations conducted by public health authorities showed that an estimated 563 individuals presented gastroenteritis symptoms (attack rate, 50.8%). The clinical incidence of patients presenting with diarrhea, vomiting, and abdominal pain spanned a long period. Consumption of tap water before 20 September was the only risk factor associated with the clinical symptoms. Genotyping of *Cryptosporidium* oocysts recovered from water samples collected in Dracy le Fort could not be performed. However, the identification of *C. parvum* genotype 1 in all fecal specimens tested, together with the presence of other human pathogens, including group A rotaviruses (20%), enteroviruses (14%), *Campylobacter jejuni* (14%), enteropathogenic *Escherichia coli* (12%), and adenoviruses 40 and 41 (6%), indicates that contamination was probably caused by human sewage. Dracy le Fort is located at the end of a water supply network, and no other city served by the same water system was affected by the outbreak. Moreover, water samples collected upstream of Dracy le Fort were negative for *Cryptosporidium* oocysts and other markers of fecal contamination. These data suggest that the source of the contamination was neither the alluvial water table supply nor the water treatment plant, but, rather, the distribution network upstream of the city of Dracy le Fort. The medical community, public health authorities, and the water treatment industry should be aware of the risk of waterborne cryptosporidiosis in continental Europe and should develop cooperative networks to prevent and treat future outbreaks.

Nucleotide sequence accession number. The nucleotide sequence for the *C. parvum* 18S rRNA clone with the 12 T genotype (designated clone 2 in Table 1) has been deposited in the GenBank database under accession number AY 177391.

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