Development and Application of a gp60-Based Typing Assay for *Cryptosporidium viatorum*

C. R. Stensvold,^a K. Elwin,^b J. Winiecka-Krusnell,^c R. M. Chalmers,^b L. Xiao,^d M. Lebbad^c

Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark^a; Cryptosporidium Reference Unit, Public Health Wales Microbiology, Singleton Hospital, Swansea, United Kingdom^b; Department of Microbiology, Public Health Agency of Sweden, Solna, Sweden^c; Division of Foodborne, Waterborne, and Environmental Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA^d

The apicomplexan intestinal parasites of the genus *Cryptosporidium* take a major toll on human and animal health and are frequent causes of waterborne outbreaks. Several species and genotypes can infect humans, including *Cryptosporidium viatorum*, which, to date, has only been found in humans. Molecular characterization of *Cryptosporidium* spp., critical to epidemiological analyses, is commonly based on gp60 gene analysis, which appears to require bespoke species- or group-specific PCR primers due to extensive genetic diversity across the genus. In this study, we amplified, sequenced, and characterized the gp60 gene of *C. viatorum* for the first time. Moreover, we developed and validated a gp60 typing assay for this species and applied it to 27 isolates originating from Asia, Africa, and Central America. A single subtype family, XVa, was identified containing multiple alleles.

Cryptosporidium is one of the leading causes of infant and childhood morbidity (1). Nearly 20 species and genotypes of *Cryptosporidium* have been isolated from human stool (2). While *Cryptosporidium parvum* and *Cryptosporidium hominis* account for the majority of human cases of cryptosporidiosis, many other species and genotypes, most of which are acknowledged as zoonotic, appear to occur sporadically or rarely in outbreaks all over the world (3–7). The availability of relevant, precise, and robust typing tools is therefore critical to outbreak investigation, surveillance, and control of the parasite.

Molecular characterization of *Cryptosporidium* based on gp60 gene data has proved to have high epidemiological relevance (8). To date, the resolution of the gp60 locus has enabled a subtle classification of species and genotypes into subtype families, which in turn can be differentiated at the subtype level. However, the high resolution and genetic variability existing in the gp60 locus also entails a challenge in terms of developing primers applicable to the many species and genotypes of *Cryptosporidium* (4, 9–11). The primers commonly used for gp60 subtyping of *C. parvum* and *C. hominis* (12) do not reliably amplify many other *Cryptosporidium* species. For example, new primers have been published for *Cryptosporidium meleagridis* (9) and *Cryptosporidium ubiquitum* (11). Similarly, the *C. parvum*/*C. hominis* primers do not apply to *C. viatorum* (4, 10), one of the panoply of *Cryptosporidium* species infecting humans and first described in 2012 (10); the 10 cases identified in the United Kingdom at that point all had a history of recent return from traveling to the Indian subcontinent. Later, *C. viatorum* was identified in patients in Nigeria (13) and Ethiopia (14) and in Swedish patients who had travelled to Kenya or Guatemala (4, 5).

The aims of the present study were, first, to amplify, sequence, and characterize the gp60 gene of *C. viatorum*, and second, to develop, validate, and apply primers appropriate for gp60 typing of *C. viatorum*, thus complementing the panel of *Cryptosporidium* gp60 primers and contributing to *C. viatorum* gp60 data useful for epidemiological research.

MATERIALS AND METHODS

Samples. As of January 2015, 28 human fecal samples positive for *C. viatorum* had been reported following sequence analysis of an ~830-bp fragment of the small-subunit (SSU) rRNA gene (3–5, 10, 13, 14), and DNA extracts from 27 of these were available for this study (Table 1). To evaluate primer specificity and to test for mixed species/subtype infections, DNAs from 90 samples previously subtyped at the gp60 locus using published gp60 primers (9, 11, 12, 17) and representing a range of 10 non-*viatorum* *Cryptosporidium* species/genotypes were included (Table 2); 77/90 of these samples were from patients who contracted infection in Africa, Asia, or South America, i.e., regions to which *C. viatorum* appears endemic. To further investigate the capacity of the primers to specifically amplify *C. viatorum* DNA in mixed templates, serial dilutions (1:10 or 1:100) of *C. viatorum* DNA with *C. parvum* and *C. hominis* DNA were included in the analysis.

The 27 *C. viatorum*-positive samples represented cases of cryptosporidiosis initially diagnosed by either PCR-based screening of fecal DNAs (14) or microscopy using Ziehl-Neelsen or auramine phenol staining of fecal smears (10). All Ethiopian samples were from HIV-positive individuals included in a study by Adamu et al. (14) in which 520 HIV-positive individuals were screened for *Cryptosporidium* by PCR. The United Kingdom samples originated among *Cryptosporidium*-positive stool samples from diarrhea patients attending primary health care physicians, submitted routinely to the *Cryptosporidium* Reference Unit by primary diagnosis.

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Address correspondence to C. R. Stensvold, run@ssi.dk.

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TABLE 1 DNAs from fecal samples positive for *C. viatorum* reported to date^a

Isolate or sample	Laboratory to which identification is attributed	Host age	Gender ^b	Geographic origin of infection	Mo/yr of collection	Subtype	Initial diagnosis of <i>Cryptosporidium</i> infection	Gene product(s), GenBank accession no.	Reference or source
W14532	<i>Cryptosporidium</i> Reference Unit, UK	2 yrs	F	India	1/2007	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	gp60, KP115936	3 , 10
W16767	<i>Cryptosporidium</i> Reference Unit, UK	16 yrs	M	Pakistan	4/2008	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	NA	10
W22242	<i>Cryptosporidium</i> Reference Unit, UK	10 mos	M	Bangladesh	4/2010	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	NA	10
W24580	<i>Cryptosporidium</i> Reference Unit, UK	11 yrs	M	India	11/2011	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	SSU rRNA, JN846708	10
W25121	<i>Cryptosporidium</i> Reference Unit, UK	24 yrs	M	India	1/2011	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	NA	10
W25123	<i>Cryptosporidium</i> Reference Unit, UK	25 yrs	M	India and Dubai	2/2011	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	NA	10
W25244	<i>Cryptosporidium</i> Reference Unit, UK	17 yrs	F	India	3/2011	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	SSU rRNA, JN846705 ; HSP70, JN846706 ; actin, JN846707	10
W25259	<i>Cryptosporidium</i> Reference Unit, UK	12 yrs	M	India	3/2011	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	NA	10
W27485	<i>Cryptosporidium</i> Reference Unit, UK	20 yrs	F	India	1/2012	XVaA3a	Modified Ziehl-Neelsen or auramine phenol staining	NA	Present study
SC322	Swedish Institute of Infectious Disease Control	30 yrs	M	Kenya	2/2013	XVaA3b	Modified Ziehl-Neelsen	gp60, KP115937	Present study
Swec066	Swedish Institute of Infectious Disease Control	26 yrs	F	Guatemala	3/2007	XVaA3c	Modified Ziehl-Neelsen	gp60, KP115938 ; COWP, JX984441 ; HSP70, JX978274	4 , 5
W27691	<i>Cryptosporidium</i> Reference Unit, UK	68 yrs	F	NA	3/2012	XVaA3d	Modified Ziehl-Neelsen or auramine phenol staining	NA	Present study
W29326	<i>Cryptosporidium</i> Reference Unit, UK	32 yrs	F	Barbados	9/2012	XVaA3d	Modified Ziehl-Neelsen or auramine phenol staining	NA	Present study
Swec025	Swedish Institute of Infectious Disease Control	25 yrs	F	Kenya	7/2006	XVaA3d	Modified Ziehl-Neelsen	gp60, KP115939 ; SSU rRNA, JX978271 ; HSP70, JX978273 ; actin, JX978269	4 , 5
35465	CDC	27 yrs	M	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
35467 ^c	CDC	1 yrs	M	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
35479 ^c	CDC	2 yrs	M	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
35484	CDC	5 yrs	F	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
35487	CDC	5 yrs	M	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
35503	CDC	7 yrs	F	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
35999 ^c	CDC	2 yrs	M	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
36004 ^c	CDC	5 yrs	F	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
36016 ^d	CDC	8 yrs	M	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
36066	CDC	7 mos	F	Ethiopia	NA	XVaA3d	PCR on fecal DNA	NA	14
W23461	<i>Cryptosporidium</i> Reference Unit, UK	21 yrs	M	Nepal	9/2010	XVaA3e	Modified Ziehl-Neelsen or auramine phenol staining	gp60, KP115940	10
W25284	<i>Cryptosporidium</i> Reference Unit, UK	20 yrs	M	India	3/2011	XVaA3f	Modified Ziehl-Neelsen or auramine phenol staining	gp60, KP115941	10
W27718	<i>Cryptosporidium</i> Reference Unit, UK	49 yrs	F	India	3/2012	XVaA3f	Modified Ziehl-Neelsen or auramine phenol staining	NA	Present study
31332 ^e	University College Hospital (UCH), Ibadan, Nigeria	28 yrs	F	Nigeria	NA	NA	PCR on fecal DNA	SSU rRNA, JX644908	13

^a Twenty-seven of 28 samples were available for analysis. NA, not available.

^b F, female; M, male.

^c Negative by primer set I but positive by primer set II ([Table 3](#)); see text for details.

^d Negative by both primer set I and primer set II ([Table 3](#)). We did not obtain a PCR product for this sample.

^e This DNA sample was unavailable for analysis but was included in the table so that the table represents all published *C. viatorum* DNAs identified to date.

TABLE 2 *Cryptosporidia* used to test the primers designed for amplification of *C. vitorum* gp60 DNA for specificity and for occurrence of mixed species^a

<i>Cryptosporidium</i> species or genotype	No. of isolates	No. of isolates that originated from:				Reference for primers used to amplify gp60 gene
		Africa	Asia	South America	Sweden/Europe	
<i>C. parvum</i>	19	5	9	5	—	12
<i>C. hominis</i>	41	19	13	9	—	12
<i>C. meleagridis</i>	13	1	12	—	—	9
<i>C. ubiquitum</i>	2	—	—	—	2	11
<i>C. cuniculus</i>	4	—	—	—	4	12
Chipmunk genotype I	1	—	—	—	1	17
<i>C. felis</i>	6	—	2	—	4	gp60 gene not determined
<i>C. canis</i>	1	1	—	—	—	gp60 gene not determined
Horse genotype	1	1	—	—	—	12
<i>C. erinacei</i>	2	—	—	—	2	12
Total	90	27	36	14	13	

^a A total of 90 samples were tested, of which 83 were previously positive for the gp60 gene, while 7 were negative. No primers were available for the *C. felis* and *C. canis* gp60 genes. —, not applicable.

tic microbiology laboratories for typing for epidemiological purposes (10); *C. vitorum* has so far been identified in samples from patients returning to Great Britain from the Indian subcontinent or Barbados. All three Swedish samples were from Swedish tourists returning from abroad with diarrheal symptoms.

Molecular characterization of the gp60 gene and subtyping of samples. Initially, ~1,440 bp of the gp60 locus of *C. vitorum* were amplified from fecal DNA sample SC322 (GenBank accession number KP115937) using the primers outFmod 5'-CAC ATC TGT AGC GTC A-3' and outR 5'-TCC TCA CTC GAT CTA GCT CA-3' (9). The sequence obtained (~1,240 bp), covering the entire open reading frame (ORF) plus about 360 additional bases, was used to design primers for a nested PCR targeting the *C. vitorum* gp60 gene. PCR amplification was performed using the primers CviatF2 and CviatR5 for primary reactions and CviatF3 and CviatR8 for secondary reactions (Table 3, primer set 1), with the PCR products expected to comprise 1,192 and 950 bp, respectively. Nested PCR was carried out using Maxima hot start PCR master mix (Thermo Scientific), 0.5 μM each primer, and 1 to 2 μl of extracted DNA. For the secondary PCR, 1 μl of the primary PCR product was used. The reaction conditions for all PCRs included an initial denaturation of 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 7 min.

For specimens failing to amplify, a second primer set was used, CviatF2 and Cviat6.2R for primary reactions and CviatF3 and CviatR3 for secondary reactions (Table 3, primer set 2), with the PCR products expected to be 918 and 805 bp, respectively. The amplified DNAs were purified using ExoSAP-IT (Thermo Fisher) and subjected to bidirectional sequencing (BigDye chemis-

try; Applied Biosystems) using the primers employed in the secondary PCR. DNA sequences were aligned and edited, and phylogenetic analysis was performed using MEGA 6 (15).

Prediction and analysis of protein sequences. ORFs were predicted using the ORF Finder at the NCBI database (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Signal sequence prediction was performed using SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/). Nucleotide sequences were translated, and structural sequences (signal peptide and glycosylphosphatidylinositol [GPI] anchor) and N-glycosylation and O-glycosylation sites predicted using PSORT II (<http://psort.hgc.jp/form2.html>), the PROSITE database at the ExPASy Bioinformatics Resource Portal (<http://web.expasy.org/>), and NetOGlyc 4.0 (<http://www.cbs.dtu.dk/services/NetOGlyc/>), respectively. Furin proteolytic cleavage sites were predicted using the ProP 1.0 server available at <http://www.cbs.dtu.dk/services/ProP/>. Deduced amino acid (aa) sequences were aligned using the MUSCLE algorithm in MEGA6 or the T-Coffee program (<http://www.ebi.ac.uk/Tools/msa/tcoffee/>), available at the site of the European Bioinformatics Institute, and analyzed manually. For aa sequence alignment, species and genotypes for which entire ORFs were available were included, comprising (with GenBank accession numbers in parentheses) *C. vitorum* SC322 (KP115937), *C. parvum* (AF022929), *C. hominis* (ACQ82748), *C. meleagridis* (AHW45799), *Cryptosporidium fayeri* (FJ490059), and *C. ubiquitum* (L. Xiao, unpublished data).

Nucleotide sequence accession numbers. Nucleotide sequences representing the different *C. vitorum* subtype variants identified in the study were deposited in GenBank under the accession numbers KP115936 to KP115941.

RESULTS

The complete gp60 ORF was available for SC322 and comprised 873 bp, encoding 290 aa. At the aa sequence level, the gp60 gene of *C. vitorum* has the greatest overall similarity to that of *C. fayeri* (NCBI BLASTP, 60% identity to the sequence with accession number ACN87713), confirming the phylogenetic relationship previously inferred by nucleotide sequence analysis of the SSU rRNA gene (4, 10). Analysis of the deduced aa sequence from gp60 revealed the presence of an N-terminal signal peptide, a relatively short polyserine domain, multiple predicted O-glycosylation sites, two potential N-glycosylation sites, and a hydrophobic region (transmembrane domain and GPI anchor) at the C terminus (Fig. 1). A furin cleavage site (16) was clearly identified in all *C. vitorum* aa sequences, consistently reading "IVKR" (only shown for SC322 in Fig. 1).

TABLE 3 Primers used for gp60 subtyping of *Cryptosporidium vitorum*

Primer	Primer sequence (5'→3')	Product size (bp)
Set I		
CviatF2	5'-TTCATTCTGACCCCTTCATAG-3'	1,192
CviatR5	5'-GTCTCCTGAATCTCTGCTTACTC-3'	
CviatF3	5'-GAGATTGTCACTCATCATCGTAC-3'	950
CviatR8	5'-CTACACGTAATAATTCGCGAC-3'	
Set II		
CviatF2	5'-TTCATTCTGACCCCTTCATAG-3'	918
Cviat6.2R	5'-CAAAAACAGAAGGGATGATGTATC-3'	
CviatF3	5'-GAGATTGTCACTCATCATCGTAC-3'	805
CviatR3	5'-GGAAGAAGAAGCTGCATCC-3'

FIG 2 Evolutionary relationships of 22 *C. viatorum* sequences (nearly complete ORFs). The evolutionary history was inferred using the neighbor-joining method (21). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method, and the unit of measure is the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 2). The analysis involved 22-nucleotide sequences. The codon positions included were as follows: 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 858 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (15). Bootstrap values lower than 50 have been removed.

The expected PCR product of ~950 bp was obtained from 22/27 *C. viatorum*-positive DNAs with nested PCR using primer set I, and 4/5 samples that were PCR negative by primer set I tested positive by primer set II. Subsequent sequence analysis was successful for all 26 *C. viatorum* gp60 PCR products. Of note, single cases of double peaks were noted in 5 of the 26 successfully amplified samples; however, these double peaks did not occur at any specific position and could not be reproduced by repeat PCR and sequencing (see Fig. S1 in the supplemental material).

None of the *Cryptosporidium* non-*viatorum* samples previously characterized at the gp60 gene locus generated bands of the expected size. *C. viatorum* DNA diluted 1:10 and 1:100 in *C. parvum* and *C. hominis* DNA, respectively, generated specific amplicons by PCR and *C. viatorum* sequences without any trace of mixed infections.

A total of 26 *C. viatorum* sequences were available for intraspecies comparative analysis. Four of the Ethiopian sequences (35467, 35479, 35999, and 36004) were ~130 bp shorter than the remaining four CDC sequences. Since all eight CDC sequences otherwise appeared 100% identical and since genetic variation was observed in some of the non-CDC sequences in the region not covered by the shorter CDC sequences, the shorter CDC sequences were not included in the phylogenetic analysis. The 22 remaining sequences, representing the nearly complete ORF, segregated into six different clusters (Fig. 2) comprising two major groups. The following gp60 subtype terminology for *C. viatorum* is proposed. According to the terminology of Ryan et al. (2), 13 species/genotypes of *Cryptosporidium* have been allocated the

subtype names I to XIII. The subtype designation XIV will be allocated to *Cryptosporidium* chipmunk genotype (17), and so subtype designation XV is proposed for *C. viatorum*. Due to the presence of three contiguous TCA trinucleotides in each sequence, all sequences included in this study are suggested to be designated XVaA3. Finally, since some genetic variation existed in the post-repetitive region, we suggest that this variation be denoted using lowercase letters along the lines of what has been proposed for *C. parvum* IICa5G3 (18). Accordingly, sequences included in the present study each belonged to one of six subtype variants, XVaA3a to XVaA3f (Fig. 2, Table 1). Nineteen of the 22 isolates formed a large cluster, comprising subtype variants XVaA3a to XVaA3d, separated from a second cluster, XVaA3e to XVaA3f, by a bootstrap value of 100.

DISCUSSION

This study is the first to characterize the gp60 of *C. viatorum*. The protein is linked in *C. parvum* to host cell invasion (19), and the main features previously described for other species also apply to *C. viatorum*. *C. ubiquitum* and, possibly, *Cryptosporidium* chipmunk genotype I (17) appear to lack the furin cleavage site common to other species of *Cryptosporidium*. In *C. viatorum*, a furin cleavage site was predicted, reading “IVKR” in all of the *C. viatorum* isolates for which gp60 sequences are currently available (data not shown). All *Cryptosporidium* gp60 genes sequenced to date except for those belonging to *C. ubiquitum* (11) contain a serine tract. In most species, the serine tract is of variable length, depending on the number of trinucleotide repeats (20); indeed,

the very essence and most of the genetic resolution of the gp60 subtyping system is based primarily on variation in the trinucleotide repeats coding for the serine residues. Analysis of the 26 *C. viatorum* sequences confirmed the presence of a serine tract in this species; however, in all 26 sequences, this varied neither in number (three) nor composition (TCA). Therefore, it appears to be the post-repetitive region that provides the variation on which subtyping of *C. viatorum* is based, similar to the situation seen for *C. parvum* IIc. However, due to the limited number of *C. viatorum* isolates identified to date, further accumulation and comparison of sequence data are required to validate this terminology.

C. viatorum is remarkable in the sense that it is currently the only species of *Cryptosporidium* that has been found exclusively in humans, although extensive studies of animals in the same areas where the human infections originated are lacking. The species was reported only a couple of years ago (10) despite the likelihood of the parasite having a more or less global distribution; indeed, data collected in this and previous studies indicate the presence of *C. viatorum* in Asia (Nepal, Bangladesh, India, Pakistan, and potentially Dubai), Africa (Nigeria, Ethiopia, and Kenya), and Central America and the Caribbean (Guatemala and Barbados). gp60 sequence analysis of the samples included in this study shows that the genetic variation observed appears to be linked to differences in geographical origin. Hence, the nine samples positive for XVaA3a were from patients who contracted cryptosporidiosis while visiting Asia, primarily India. Only single cases of XVaA3b and XVaA3c were identified, originating in Kenya and Guatemala, respectively. Meanwhile, all the samples from Ethiopia that could be successfully subtyped ($n = 9$) belonged to XVaA3d, and although another XVaA3d sample was from a patient who contracted the infection in Kenya, this subtype variant appears not to be a strictly African subtype, since a United Kingdom patient with a history of traveling to Barbados was also positive for XVaA3d. XVaA3e and XVaA3f, representing a separate clade, were seen in one and two cases, respectively, and exclusively in cases who visited Asia. With novel primers now available for gp60 subtyping of *C. viatorum*, it is expected that accumulating data will add to our understanding of the phylogeography, host specificity, and clinical significance of this little-studied parasite. Although no animal reservoir has been identified, it is possible that the few cases of *C. viatorum* infections identified so far could point toward cases of rare zoonotic transmission; however, at present, the situation remains unclear. Indeed, in those regions where *C. viatorum*-positive patients may have contracted the infection, sampling from nondomesticated animals with a view to screening for *Cryptosporidium* remains scarce.

Especially given the profound implications for the routine typing of *Cryptosporidium*, it is interesting to try and speculate as to the reason why *C. viatorum* was discovered only recently. Restriction fragment length polymorphism (RFLP) analysis of SSU ribosomal DNA (rDNA) PCR products has been widely used to differentiate species of *Cryptosporidium*, being a rapid and inexpensive alternative to sequencing that also obviates the problem of sequencing the homopolymers characteristic of at least some *Cryptosporidium* SSU rRNA genes and because mixed *C. parvum*/*C. hominis* infections may be easier to disentangle by RFLP analysis than by sequence analysis. However, RFLP patterns resulting from digestion of amplicons representing *C. parvum* and *C. viatorum* using the widely used endonucleases SspI and VspI are practically indistinguishable (4, 14), and so cases of *C. viato-*

rum may have been confused for *C. parvum* by SSU rDNA RFLP. Moreover, until recently, the primers targeting the *Cryptosporidium* oocyst wall protein (COWP) were used as a first-line typing tool, and these primers appear not to work well with a number of species, including *C. viatorum* (4, 5, 10). Consequently, the recognition of *C. viatorum* followed only after sequencing of SSU rRNA genes, prompted by the absence of a positive COWP PCR and the presence of an SSU rDNA RFLP pattern characteristic of *C. parvum* (10). This example shows the relevance of sequencing SSU rDNA.

The PCR primers developed to characterize *C. viatorum* appeared to be robust in that they were 100% specific for *C. viatorum* and that all available *C. viatorum*-positive DNAs except one could be successfully characterized by bidirectional sequencing of PCR products obtained by at least one of the two available primer pairs. As our primers appear highly specific for *C. viatorum* DNA and not affected by the coexistence of high concentrations of *C. parvum* and *C. hominis* DNA, we suggest that they could be useful not only for molecular characterization of *C. viatorum* but also in situations when screening for mixtures of *Cryptosporidium* spp., including *C. viatorum*, is carried out.

Even though we did not detect any cases of mixed *Cryptosporidium* infections involving *C. viatorum* in our panel of 90 non-*viatorum* samples used for primer specificity testing and mainly originating from patients infected in Africa, Asia, or south America, such mixtures (*C. viatorum* plus *C. meleagridis*) have been reported (10).

Nonreproducible and apparently random double peaks (see Fig. S1 in the supplemental material) noted in 5/26 samples in the present study have not been recognized in gp60 sequences obtained from samples positive for *C. hominis*, *C. parvum*, or *C. meleagridis*. Sequence artifacts are difficult to rule out, but the fact remains that this phenomenon has not previously been witnessed according to our knowledge. Future whole-genome sequencing of different species of *Cryptosporidium*, including *C. viatorum*, might give an explanation for this observation.

In conclusion, we characterized and annotated the gp60 gene of *C. viatorum*. We developed primer pairs for subtyping the species, developed a subtype terminology, and contributed molecular epidemiological data on *C. viatorum* infections identified so far. Finally, we discussed some of the limitations and caveats in the procedures typically involved in molecular epidemiological surveillance of *Cryptosporidium*.

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