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Subgenotype Analysis of *Cryptosporidium* Isolates from Humans, Cattle, and Zoo Ruminants in Portugal

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***Cryptosporidium parvum* and *Cryptosporidium hominis* isolates from human immunodeficiency virus-infected patients, cattle, and wild ruminants were characterized by PCR and DNA sequencing analysis of the 60-kDa glycoprotein gene. Seven alleles were identified, three corresponding to *C. hominis* and four corresponding to *C. parvum*. One new allele was found (IId), and one (IIb) had only been found in Portugal. Isolates from cattle and wild ruminants clustered in two alleles. In contrast, human isolates clustered in seven alleles, showing extensive allelic diversity.**

Human cryptosporidiosis is mainly caused by *Cryptosporidium parvum* and *Cryptosporidium hominis* (previously known as the *C. parvum* human genotype) (7, 13). *C. hominis* is found almost exclusively in humans, whereas *C. parvum* is found in domestic livestock, wild animals, and humans (1, 3, 6, 9, 20, 21, 23, 24). The occurrence in humans of both *Cryptosporidium* parasites has provided evidence that both anthroponotic and zoonotic cycles can occur in human infections (12, 14, 17).

The GP60 gene (also known as *Cpgp15/45*) encodes a precursor protein that is proteolytically cleaved to yield mature cell surface glycoproteins gp45 and gp15 (also known as Cp17), both of which are implicated in zoite attachment to and invasion of enterocytes (4, 16). An important feature of this gene is its high degree of sequence polymorphism, particularly among *C. hominis* isolates, which is far greater than any other *Cryptosporidium* genetic loci examined to date (8, 18, 19). Recently, Strong et al. described five alleles based on extensive sequence polymorphisms in the GP60 gene, one containing all *C. parvum* isolates and four containing *C. hominis* isolates (18). Within each allele, there are different subgenotypes based on the number of a trinucleotide repeat. These results highlight the usefulness of the subgenotype analysis for fingerprinting *Cryptosporidium* isolates.

In this study we characterized *Cryptosporidium* isolates from human immunodeficiency virus (HIV)-infected patients, cattle, and zoo ruminants from Portugal by PCR restriction fragment length polymorphism (RFLP) and DNA sequencing analysis of the small-subunit rRNA (SSU rRNA) and GP60 genes. Results of the study showed extensive genetic diversity in *C. parvum* and *C. hominis* isolates from humans but only limited genetic diversity in *C. parvum* from cattle and zoo ruminants.

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***Cryptosporidium* isolates.** A total of 75 *Cryptosporidium* isolates from human HIV-infected patients from the Lisbon area with diarrhea, calves from four different geographic areas in Portugal (central, central/southern, southern, and Azores Islands), and wild ruminants from the Lisbon Zoo were used in this study (Table 1). *Cryptosporidium* oocysts were detected in stool specimens by modified Ziehl-Neelsen staining.

Molecular analysis. Oocysts were concentrated from whole feces by a modified water-ether sedimentation method (2). Genomic DNA was isolated from concentrated oocysts by a KOH/QIAamp DNA stool mini kit protocol (QIAGEN, Valencia, Calif.) as previously described (22). *Cryptosporidium* species and the *C. parvum* genotype were determined by nested PCR of a SSU rRNA gene fragment and RFLP analysis with the endonucleases *Vsp*I and *Ssp*I, as described previously (24).

Subgenotyping was achieved by sequence analysis of the GP60 gene. A fragment of the GP60 gene (800 to 850 bp) was amplified by a nested PCR, with the primers AL3531 and AL3535 (5′-GGA AGG AAC GAT GTA TCT-3′) in the primary PCR and AL3532 and AL3534 in the secondary PCR (15). PCRs were performed as previously described (5). PCR products were sequenced in both directions on an ABI Prism

TABLE 1. *Cryptosporidium* isolates and PCR RFLP analysis at the SSU rRNA locus

Isolate source	Total no. of isolates studied	No. of isolates of species:			
		C. parvum (bovine genotype)	*C. hominis*	*C. felis*	*C. meleagridis*
HIV-infected[a] humans	30[b]	16	7	3	3
Calves	35	35	0	0	0
Zoo ruminants[c]	10	10	0	0	0

[a] One isolate was a sporadic case from a non-HIV-infected person.
[b] Due to amplification failure, no RFLP data was obtained for 1 isolate.
[c] The 10 ruminants were 4 Arabian oryxes, 3 gemsboks, and 3 addaxes.

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IIa-BI344    DVPVEGSSSS SSSSSSSSSS SSS------T STVAPAN-KA RTGEDAE--- ----------
IIb-CH5      ..S....... .........- ---------S T.....SN.. .....T.--- ----------
IId-CH86     ..S....... .......... ...SSSSSS. .....TPK.E ....EV---- ----------
Ib-CH58      ..S..S.... .........-- ----------. T.P...PK.. .EADGG---- ------EEKN
Ic-CH22      ..S..S.... ....------ ----------. T.P...PK.V .ES.EG---- --------KN
Ie-CH10      ~~~.....   .......... ..-------S T.P...SK.V .EA.GS.EKD SEEKDSEEKG
If-CH73      ~~~~.S.... .......... .--------. T.P...PK.. .EA.GK.AEG KEEEGKEEEG

IIa-BI344    -----GSQDS SGT------- --EASGSQGS EEEGSEDDGQ TSAASQPTTP AQSEGATTET
IIb-CH5      -----...V. G..P------ --.S....DT ..--...GS. ..TV.ES... .....TI...
IId-CH86     -----.NPG. E.Q------- ----D.KGDT ..--T..NQT E.TV..N-.. ..T..T....
Ib-CH58      NEE-SQTPA. P.SGGVSEGQ DTQGGSKGDA ..GTEDNEQA DES.T..S.. G.GSVK.E-S
Ic-CH22      SED-SQTPA. P.S------- DSQD.SKGD- -------EAV DG...GSS.. T.AAEKEP..
Ie-CH10      SEEGSQTPA. P.GGGVSEGD T-QGDSKGDG VSSDENQSQG GD.TPGSS.Q T.ATEKEPGS
If-CH73      SEE-SQGPT. ..SGVGSEGN D-QGDSKGDG AS.DDNKNQD GDTS.ESV.. ----------

IIa-BI344    IEATPKEECG TSFVMWFGEG TPAATLKCGA YTIVYAPIKD QTDPAPRYIS GEVTSVTFEK
IIb-CH5      T..A..K... .......... ..V......D ..M....E.. K...E..... ..........
IId-CH86     T..A..K... .......... V.V.S....D ..M....E.. K......... ..........
Ib-CH58      T.T....K.. ........Q. V.V......D ..M....E.. K......... ....T...D.
Ic-CH22      P.S....... ...I...... ...T.....G .......E.. NKE....... .D.KA.....
Ie-CH10      S......... ........Q. V.VV.....G ..M....ENG K......... .K.ST.D...
If-CH73      TQ........ ...I...... ...T.....G .......E.N NRE....... ...KA.....

IIa-BI344    -SDNTVKIKV NGQDFSTLSA NSSSPTENGG -SAGQASSRS RRSLSEETSE AAATVDLFAF
IIb-CH5      -Q.S..T... .NVA.....T S.......N. -.ES.VA... ........G. T.........
IId-CH86     -QES..T... .NVE.....T S.......S. -....VP... .......A.. T-........
Ib-CH58      -QES..T... .NVE.G...T S..K....K. E.SD.VG... ....T..-TS ET........
Ic-CH22      GE........ D.KE.....S S.......N. -.T..VA... .......N.. T.........
Ie-CH10      -Q.S...... ..VE.....T S..N....S. -.ES..Q... ....A.DGT. T......I..
If-CH73      GN...I.... D.KE.....T S.....V.N. G.DV..K.I. K...T..-.D EV........

IIa-BI344    TLDGGKRIEV AVPNVEDASK RDKYSLVADD KPFYTGANSG TTNGVYRLNE NGDLVDKDN
IIb-CH5      .....Q.... ...S....A. ........N. .......... N......... .........
IId-CH86     .......... ...SD..... .NQ....... ......S... A.D.IF.... D........
Ib-CH58      .......... ...SD..V.. .N......N. .T........ N.D.I....D .......N.
Ic-CH22      .....R.... ...S....T. ........NG .......... .......... .........
Ie-CH10      ..Q....... ...SD..V.. .N......G. .T........ N.D.I....D D......N.
If-CH73      .......... ...S.D.V.. .........G .S....S... AE..I.K.DS D.N......
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FIG. 1. Amino acid sequence diversity among seven GP60 alleles of *Cryptosporidium* in humans and animals from Portugal. Sequences are labeled as alleles followed by sample identifications. Dots denote sequence identity to the allele IIa sequence in isolate BI.344, dashes denote nucleotide deletions, and ~ indicates that sequence information was unavailable. IIa, IIb, IId, and Ic are *C. parvum* alleles, and Ib, Ie, and If are *C. hominis* alleles.

3100 automated sequencer (Applied Biosystems, Foster City, Calif.) with the primers AL3532 and AL3534. Nucleotide sequences obtained from various isolates were aligned with each other and published sequences by using the GCG program (Genetics Computers Group, Madison, Wis.) with manual adjustment. A neighbor-joining tree was constructed by using the Treecom W program, based on genetic distance calculated by Kimura two-parameter model (25). The reliability of the groupings was assessed by bootstrapping analysis, with 1,000 pseudo replicates.

Nucleotide sequence accession number. The GP60 nucleotide sequences of seven alleles were deposited in the GenBank database under accession no. AY166804 to AY166810.

***Cryptosporidium* species.** PCR products of the SSU rRNA locus were obtained for all the animal isolates (35 calves and 10 wild ruminants) and for 29 of the 30 human isolates. RFLP analyses with *Ssp*I and *Vsp*I showed that all animals were infected with *C. parvum* bovine genotype while humans were infected mostly with *C. parvum* bovine genotype (16 of 29 isolates) and *C. hominis* (7 of 29 isolates). Three patients were infected with *Cryptosporidium felis*, and another three were infected with *Cryptosporidium meleagridis* (Table 1).

C. parvum bovine genotype parasites account for most of the cases of cryptosporidiosis in Portuguese HIV-infected patients. Other authors have also reported in Europe (United Kingdom, Switzerland, and France) that the *C. parvum* bovine genotype is responsible for more human infections than *C. hominis* (3, 6, 9, 10). In the United States, Australia, Kenya, Thailand, and South Africa, anthroponotic parasites are responsible for the majority of cases of human cryptosporidiosis (8, 10, 11, 20, 21). Whether these results reflect the existence of some geographic variation in humans in the distribution of *C. parvum* and *C. hominis* is unknown, as only small numbers of isolates have been analyzed in most studies.

***C. parvum* and *C. hominis* subgenotypes.** All *C. parvum* and *C. hominis* isolates plus the isolate for which no SSU rRNA data was obtained (CT.1) were analyzed at the GP60 locus. PCR amplification was obtained for all 69 isolates, only 63 of which yielded clean GP60 sequences. The remaining six isolates had underlying signals in the electropherogram, which prevented the read-out of accurate sequences. Sequence alignment revealed extensive differences in the nucleotide sequence through the entire length of the fragment and divided all *C. parvum* and *C. hominis* isolates into seven alleles (Fig. 1). The

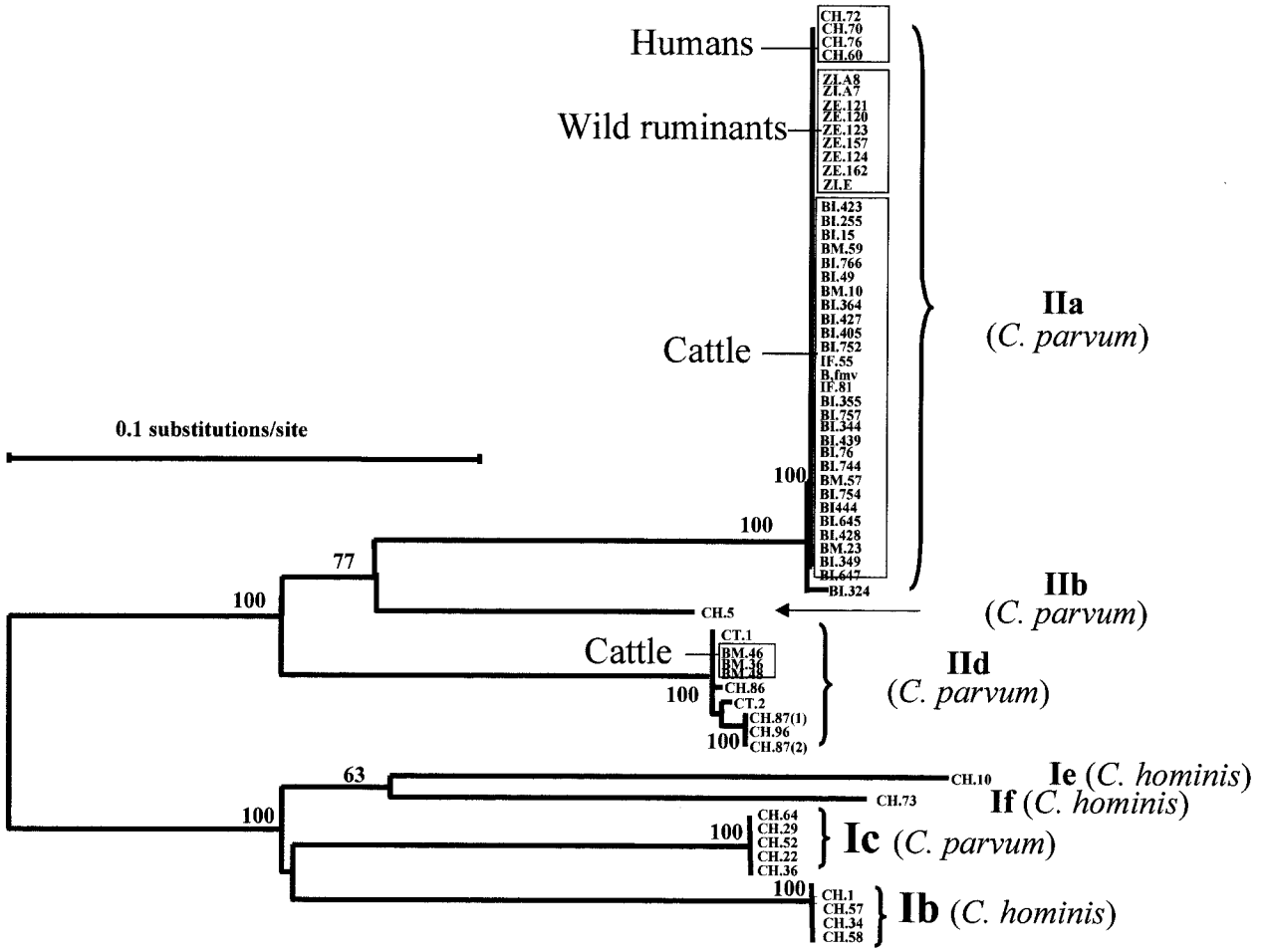

FIG. 2. Distribution of GP60 alleles of *C. parvum* and *C. hominis* from humans, calves, and wild ruminants as revealed by a neighbor-joining analysis of nucleotide sequences. Isolates from calves are named BI, BM, IF, and B.fmv. Human isolates are named CH and CT; isolate CH.60 is from a sporadic case of a non-HIV-infected person.

results of phylogenetic analysis of the nucleotide sequences supported the formation of these seven alleles (Fig. 2). Three alleles corresponded to *C. hominis* (Ib, Ie, and If) and four alleles corresponded to *C. parvum* (IIa, IIb, Ic, and IId). Six of them corresponded to previously described alleles, Ib, Ic, and IIa (18); Ie and IIb (15, 19); and If (same as Ie of Leav et al. [8]), and one was a new allele (IId). Alleles IIb and IId had, so far, only been found in Portugal. None of the isolates exhibited the alleles Ia and Id described before (8, 15, 18, 19).

Within *C. parvum* alleles, allele IIa contained all the isolates from the zoo's wild ruminants, 29 of the 32 isolates from calves, and 4 human isolates. All these isolates within this allele exhibited the same subgenotype, with the exception of one isolate from a calf, which showed a different subgenotype. Similarly, allele IId also had isolates from both cattle and humans. However, this allele had four subgenotypes, with three bovine isolates belonging to a single subgenotype and six human isolates belonging to four subgenotypes. In contrast, allele Ic had only *C. parvum* bovine genotype isolates from humans.

Most isolates exhibited GP60 alleles concordant with the SSU rRNA results. However, allele Ic, which originally was described as a *C. hominis* allele (18) and contained five *C. parvum* bovine genotype isolates from humans in this study, had the *C. parvum* bovine genotype sequence in the SSU rRNA gene. Peng et al. (15) and Leav et al. (8) also found similar results with *C. parvum* bovine genotype isolates from humans in Guatemala, Portugal, and South Africa. Thus, allele Ic should be renamed IIc. Even though it is a *C. parvum* parasite, allele Ic has so far only been found in humans. Two recently identified new alleles have been named Ie, even though they are genetically distinct (8, 15, 19). Thus, the allele Ie identified in South African patients has been renamed allele If in the present study.

Epidemiological significance. None of the Portuguese isolates displayed alleles Ia and Id, and alleles IIb and IId had so far only been found in Portugal. On the contrary, alleles Ib, Ie, If, IIa, and Ic seem to have a wide geographic distribution. Thus, geographic differences in the distribution of specific alleles may exist. However, due to the small number of isolates analyzed so far, the epidemiological significance of these results remains to be determined. Additional studies, with a larger number of isolates from various geographic areas, should be conducted to confirm these observations and to extrapolate the significance of the differences.

The isolates from animals showed limited genetic heterogeneity, with the parasites from cattle exhibiting three subgenotypes in two alleles (IIa and IId) and those from the zoo ruminants displaying only one subgenotype of one allele. Twenty-nine of 32 bovine isolates exhibited the same allele (IIa), and from these, only 28 had an identical subgenotype. Only three bovine isolates had the allele IId. The dominance of one subgenotype in calves in Portugal was probably the result of the frequent exchange of animals among farms, the relatively small size of the country, or the genetic fitness of this *Cryptosporidium* strain.

In contrast to the limited genetic diversity in *Cryptosporidium* from animals, the human isolates were found in 10 subgenotypes and in all seven alleles, including three *C. parvum* alleles (IIa, IIb, and Ic). These results suggest that transmission of human cryptosporidiosis in Portugal is complicated and multiple routes of transmission of human cryptosporidiosis were probably responsible for the high genetic heterogeneity of *Cryptosporidium* parasites seen in humans. This was also supported by the fact that *C. parvum* parasites from humans even had a higher genetic diversity than those from animals. Also, two of the *C. parvum* alleles (IIb and Ic) had only been found in human isolates. These results, though limited by the small number of isolates studied, also suggest that the occurrence of the *C. parvum* bovine genotype in humans is frequently not the result of zoonotic infections.

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