

## Genotyping *Encephalitozoon hellem* Isolates by Analysis of the Polar Tube Protein Gene

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**To develop an alternative genotyping tool, the genetic diversity of *Encephalitozoon hellem* was examined at the polar tube protein (PTP) locus. Nucleotide sequence analysis of the PTP gene divided 24 *E. hellem* isolates into four genotypes, compared to two genotypes identified by analysis of the internal transcribed spacer of the rRNA gene. The four PTP genotypes differed from each other by the copy number of the 60-bp central repeat as well as by point mutations. A simple PCR test was developed to differentiate *E. hellem* genotypes based on the difference in the size of PTP PCR products, which should facilitate the genotyping of *E. hellem* in clinical samples.**

*Encephalitozoon hellem* is one of the four most common human microsporidian parasites. Thus far, humans are the only identified mammalian hosts, although microsporidiosis caused by *E. hellem* is probably common in birds (2, 23, 32, 34, 36). Because all human cases, except the single case mentioned here, have been reported in AIDS patients, it has been suggested that *E. hellem* infection in humans is opportunistic, and the parasite may be of zoonotic origin. Concurrent *E. hellem* infection of both humans and their companion birds, however, has not yet been documented (4).

Molecular tools have been developed and employed to delineate the transmission of human microsporidiosis. Characterization of the internal transcribed spacer (ITS) of the rRNA gene has identified three genotypes of *Encephalitozoon cuniculi* based on the number of GTTT repeats present: genotype I (originally isolated from a rabbit) containing three repeats, genotype II (originally isolated from a mouse) containing two repeats, and genotype III (originally isolated from a dog) containing four repeats (11). Both genotypes I and III of *E. cuniculi* have been found in humans, indicating that *E. cuniculi* of animal origin may be a source of human infection (6, 10, 25, 27, 33). ITS sequence differences of *Enterocytozoon bieneusi* have also been reported among different humans infected with this parasite (16, 26). Additionally, ITS sequence differences have also been shown in *E. bieneusi* infecting different species of domestic animals (3, 5, 6, 16, 19, 24–26). However, the zoonotic potential of *E. bieneusi* from animals is not yet clear.

Genetic diversity probably also exists in *E. hellem*. A recent ITS sequence characterization of five human isolates from Europe and Africa has identified three genotypes of *E. hellem*

(21). The extent and significance of genetic diversity in *E. hellem*, however, are not yet clear. There is also a need for the development of simpler genotyping tools targeting other genes to define the epidemiology of human *E. hellem* infection. Recently, a gene coding for the polar tube protein (PTP) of *E. hellem* has been reported (15). Because the gene has long central repeats of 60 bp and the number of repeats in repetitive proteins tends to vary in other parasites, such as *Plasmodium* spp., we examined the sequence diversity of the PTP gene among various isolates of *E. hellem*.

### MATERIALS AND METHODS

**Parasite isolates and DNA extraction.** The *E. hellem* isolates used in this study included 24 human isolates from 20 patients in the United States, Puerto Rico, Italy, Switzerland, and Spain (Table 1). *E. hellem* diagnosis was made by a combination of electron microscopy and species-specific PCR analysis. All isolates were maintained in E6 and HLF cell cultures (37) after inoculation with patient samples, including biopsy, bronchoalveolar, sputum, and urine samples. DNA was extracted from cultured parasites using a phenol-chloroform method previously described (12). Nucleic acid from each sample was resuspended in 50  $\mu$ l of distilled water and stored at  $-20^{\circ}\text{C}$  before being used in PCR.

**PCR and sequence analysis.** The complete genes coding for the SSU rRNA and ITS and a 1,253-bp fragment of the PTP gene were amplified from DNA of each sample by PCR. The primer sets used were MICRO-F (5'-CACCAGTTGATTCTGCCTGA-3') and 1492N4 (5'-CCAACTGAAACCTTGTACGACTT-3') for SSU rRNA and ss1061f (5'-GGTGGTGCATGGCCG-3') and ls212r1 [5'-GTT(G/A)GTTTCTTTTCTC-3'] for the ITS (39). A fragment of the PTP of 1,253 bp was amplified from DNA of all *E. hellem* isolates by PCR using primers 5'-ATGAAAGGTATTTTGAAGAT-3' (nucleotides 124 to 143) and 5'-GCCTCCATGGCATACTGC-3' (nucleotides 1359 to 1376), based on a PTP sequence (AF044915) previously published by Keohane et al. (15). The PCR products were sequenced in both directions on an ABI377 autosequencer (Applied Biosystems, Foster City, Calif.). The sequences obtained were aligned with each other and the published sequence using the Wisconsin package (version 9.0; Genetics Computer Group, Madison, Wis.).

**Genotyping by direct PCR analysis of PTP.** Based on the results of PTP gene sequencing, a simple length polymorphism-based PCR genotyping technique was developed. A fragment of the PTP of 461 to 611 bp was amplified from *E. hellem* DNA by PCR using primers 5'-CATGCTTGCCAACACAGG-3' (nucleotides 764 to 781 of AF044915), and 5'-TGGAGGCATTGCAATAGG-3' (nucleotides

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TABLE 1. Human *E. hellem* isolates used in this study and genotyping results from analyses of ITS, SSU rRNA, and PTP genes

Isolate	Origin <sup>a</sup>	Sex/age (yr) <sup>b</sup>	Isolation (mo/yr)	Country/state	ITS genotype <sup>c</sup>	SSU rRNA genotype <sup>d</sup>	PTP genotype	Reference(s)
CDC:V278	Urine	M	11/92	Puerto Rico	1A	1A	1A	37
CDC:V242	Urine	M/26*	01/92	USA/Ga.	1A	1A	1A	31, 37
CDC:V274	BAL	M/26*	10/92	USA/Ga.	1A	1A	1A	31, 37
Le	Conjunctiva	M/46	12/89	USA/N.Y.	1A	1A	1A	7, 13
CDC:V257	Urine	F/35 <sup>†</sup>	06/92	USA/N.Y.	1A	1A	1A	31, 37
CDC:V258	Sputum	F/35 <sup>†</sup>	06/92	USA/N.Y.	1A	1A	1A	31, 37
CDC:V281	Nasal	F/35 <sup>†</sup>	12/92	USA/N.Y.	1A	1A	1A	31, 37
Wo	Conjunctiva	M/37	1994	USA/Mass.	1A	1A	1A	7, 9
Ye	Cornea	M/29	12/89	USA/Tex.	1A	1A	1A	7, 40
Ly	Cornea	M/30	1990	USA/Ohio	1A	1A	1A	Unpublished data
He	Conjunctiva	M/33	12/89	USA/N.Y.	1A	1A	1B	7, 13
CDC:V213	Urine	M/30	02/91	USA/Ga.	1A	1A	1B	31, 37
CDC:SP-1	BAL	M/41	1996	Spain	1A	1A	1B	22
LEPV-2-93	BAL	M/27	10/93	Italy	1A	1A	1B	30, 37
PV-3-93	BAL	M/30	12/93	Italy	1A	1A	1B	29, 37
PV-8-95	Sputum	F/34	03/95	Italy	1A	1C	1C	14, 37
VRPV-1-93	Urine	M/33	09/93	Italy	1A	1C	1C	14, 37
PV-5-95	BAL	M/38	01/95	Italy	1A	1C	1C	29, 37
PV-11-98	Nasal	M/34	01/98	Italy	1A	1C	1C	14, 37
PV-10-97	Ocular	M/12 <sup>●</sup>	01/97	Italy	1A	1C	1C	14, 37
PV-10-97EI	Sputum	M/12 <sup>●</sup>	01/97	Italy	1A	1C	1C	14, 37
MIPV-6-95	BAL	F/33	10/95	Italy	1A	1C	1C	14, 37
PV-9-96	BAL	M/38 <sup>e</sup>	07/96	Italy	1A	1C	1C	28
CDC:V261	Urine	M/49	06/92	Switzerland	2B	2B	2B	37, 38

<sup>a</sup> BAL, bronchoalveolar lavage.

<sup>b</sup> Superscript symbols indicate multiple samples from the same patient with the same symbol.

<sup>c</sup> Genotypes 1A, 1B, and 1C had an identical ITS sequence.

<sup>d</sup> Genotypes 1A and 1B had an identical SSU rRNA sequence.

<sup>e</sup> HIV negative.

1207 to 1224 of AF044915). The PCR products were differentiated by electrophoresis in agarose gel, using 100-bp ladders (Life Technologies, Grand Island, N.Y.) as molecular size markers.

**Nucleotide sequence accession numbers.** The SSU rRNA, ITS, and PTP nucleotide sequences of *E. hellem* were deposited in the GenBank database under accession no. AF33836 to AF338368 and AY024342.

## RESULTS

**Sequence analysis of SSU rRNA.** The complete gene coding for SSU rRNA was sequenced for each *E. hellem* isolate. Three types of sequences were obtained from the 20 isolates studied. Fifteen isolates had SSU rRNA sequences identical to the genotype 1 sequence (genotype 1A or 1B in Table 2) reported before (20). Eight isolates had a similar sequence except for an insertion of G at position 162 (genotype 1C in Table 2). One

isolate (CDC:V261) had an SSU rRNA sequence identical to the genotype 2 (genotype 2A or 2B in Table 2) sequence previously described (20), which had seven nucleotide base differences from genotype 1 (Tables 1 and 2).

**Sequence analysis of ITS.** All *E. hellem* isolates used in this study were also sequenced for the ITS gene. Nucleotide sequences obtained for 23 of the 24 isolates were identical to the genotype 1 sequence previously reported (genotype 1A, 1B, or 1C in Fig. 1) (21). One isolate (CDC:V261), however, had an ITS sequence similar to those of genotypes 2 (genotype 2A in Fig. 1) and 3 (genotype 2C in Fig. 1) reported before (21). Differences between these and other *E. hellem* genotypes are shown in Fig. 1.

**Sequence analysis of PTP.** All *E. hellem* isolates were also analyzed at the PTP locus. Although a PCR product of 1,253

TABLE 2. Sequence differences in the SSU rRNA among *E. hellem* genotypes<sup>a</sup>

Genotype	Nucleotide at position: <sup>b</sup>									
	119 (119)	160 (160)	— (162)	171 (172)	505 (506)	517 (518)	538 (539)	539 (540)	952 (953)	1013 (1014)
1A	A	G	—	G	A	G	T	A	A	G
1B	A	G	—	G	A	G	T	A	A	G
1C	A	G	G	G	A	G	T	A	A	G
2A	A	T	—	T	T	A	G	G	A	A
2B	A	T	—	T	T	A	G	G	A	A
2C	T	T	—	T	T	A	G	G	G	A

<sup>a</sup> Genotypes 1A and 1B = genotype 1 of Mathis et al.; genotype 2A and genotype 2B = genotype 2 of Mathis et al.; genotype 2C = genotype 3 of Mathis et al. (21).

<sup>b</sup> Position in 1C shown in parentheses.

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Genotype 1A TGGTTGATTGATTATTTT---GTGGGGATTTTTAGTTTTTTAGTT-----TTTCTTTCT
Genotype 1B .....
Genotype 1C .....
Genotype 2A .....G.....-T...GAG.....TTTTTCTTTC.....
Genotype 2B .....G.....-T...GAG.....TTTTTTC.....
Genotype 2C .....G...GTTT...-T...GAG.....TTT-----
    
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FIG. 1. Sequence differences in the rRNA ITS among *E. hellem* genotypes. Dots denote sequence identity to genotype 1A, and dashes depict nucleotide deletions. Genotypes 1A, 1B, and 1C = genotype 1 of Mathis et al.; genotype 2A = genotype 2 of Mathis et al.; genotype 2C = genotype 3 of Mathis et al. (21).

bp was expected from each isolate, this was only the case for 10 isolates. Other isolates had PTP PCR products larger than 1,253 bp. Four different sizes of PTP PCR products were detected, as reflected in the different migration rates in agarose gel electrophoresis (data not shown).

DNA sequencing analysis of PCR products confirmed the presence of four PTP genotypes. Genotype 1A was generated from the smallest PCR products from the 10 *E. hellem* isolates, 1,253 bp in length, and each was identical to the published sequence (AF044915). In contrast, genotypes 1B, 1C, and 2B

were 1,313-, 1,373- and 1,421-bp long and found in five, eight, and one isolate, respectively (Table 1 and Fig. 2). Differences in PTP sequence length among genotypes 1A, 1B, and 1C were due to variations in the number of a 60-bp tandem repeat: each of them had six, seven, and eight copies of the 60-bp repeat, respectively. Genotype 2B had three copies of the 60-bp repeat and five copies of a 66-bp repeat. In the 66-bp repeat, a 6-bp sequence (GGAAGC or GGAAGT) was repeated once at the beginning of the 60-bp repeat (Fig. 2). In addition, genotype 2B also had an 18-bp insert prior to the repeat region. Se-

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1A GTTTATTCTGCAGTTCCGCTTTGCAGTAATACTTATGATCCCTCGCAACAGCAGCCATCATATGTGCTGATTCCCAGTACTCCTGAGGCTATAACAAACTGTGCATACAGCCCAAGAAT 300
1B .....
1C .....
2B .....C.....G.....C.....C.....

1A GCATATGTCCTTCTTCTCCTACTACATCGTCTTCAACCCAGGGACAAACAACGATAACGAAACATCTCCTACTACAGAGGATGTAGGAACATGCAAGATTTCCGTTGTAAAGCATTGC 420
1B .....
1C .....
2B .....A.....

1A GACACACCAGGTGCATCATCAACACCTTGGCAGCCTGAACAACCATCCAGCACAGCCTGTTACAATGGCTACAGTTACACCAGCAATCATTGCTTCTGTTCAACACCCATCCGTTGTG 540
1B .....
1C .....T.....
2B .....G.....T.....

1A TCTGTTATTCTGTAACACAGAAGTTATTACGCTGCAACAATGATTGTGCCACTTCTTCGATTATTCC-----GGGGTATTATCCTAATGGAACGCCTGCAGCA 642
1B .....
1C .....
2B .....TGCTAACCGATGATTCC.....

1A CCTGGACAGCAAGGGCAAATTTCTTCAGGTAGCGTTCTTGCACTGGCCGCACTCATGCCAGTTGGTTCTCGGAAATACTCCAGGCCAAATGCTTCTGGAATGACGCCGGGTGTTTCT 762
1B .....
1C .....
2B .....T.....T.....

1A CCATGCTTGCCAACACAGGGCGGGGATGGAAGCAACAGACCATTCGCGGCATTGTTTATCCATGCCAGCCAGGACAGGGCGGAAGTGAAGTAACCAGACCATTCAGGTGTTATCTCC 882
1B .....T.....C.....G...A...G.T.AT
1C .....
2B .....A.....AAT...GA...C.....A...G...CA...G.T.AT

1A CCATGCCAGCCAGGACAGGGCGGAAGT-----GGAAGCAACAGACCATTCGCGGTATTGTTTATCCATGCCAGCCAGGACAGGGCGGAAGT----- 969
1B .....G...AAT...GA.....T.....A...G...A.C.CC.....GGAGGCAATCAGACCATTCCA
1C .....T.....G...AAT...GA.....T.....C.....G...A.C.CC.....GGAAGTAACCAGACCATTCCA
2B .....AAT...GGAAGC...A...C.....AAT...GGAAGCGGAAGCAACAGACCAATCCG

1A -----GGAAGTAACCAGACC 984
1B GGTGTTATCTCCCATGCCAGCCAGGACAGGGCGGAAT-----GGAAGTAACCAGACCATTCGCGGTATTGTTTATCCATGCCAGCCAGGACAGGGCGGAAGT-----T.....
1C GGTGTTATCTCCCATGTCAGCCAGGCGAGAATGGAGAT-----GGAAGTAACCAGACCATTCGCGGCATTGTTTATCCATGCCAGCCAGGACAGGGCGGAAGT.....AG.....
2B GGCATTGTTTATCCATGCCAGCCAGGACAGAATGGAAGTGAAGTGGTAGCAACCAGACCACTCCAGGCATTGTTTATCCATGCCAGCCAGGACAGAATGGAAGT...T.C.T.....

1A ATTCCAGGTGTTATCTCCCATGCCAGCCAGGACAGGGCGGAAGT-----GGAAGCAACAGACCATTCGCGGTATTGTTTATCCATGCCAGCCAGGCGAGAATGGAGAT-----GGA 1092
1B .....G...T.....A...G...A.C.CC.....A...GGC...AG.....
1C .....T.....T.....A...G...A.C.CC.....A...GGC...AG.....
2B .C...G...CA...G.T.AT.....AAT...GGAAGT...T.....C...A...C.....A...AG...GGAAGC...

1A AGTAACCAGACCATTCAGGTGTTATCTCCCATGCCAGCCAGGACAGGGCGGAATGGGAATGGCACTACTGGCCAGCCTGGGAGTGGCTCTGTTCTCTCAAATCCAAACCTATT 1212
1B G.C.T.....G.....
1C .C.T.....A.....
2B .C.G.....C.....G.....

1A GCAATGCCCTCAAATTTCCAGGTATCAGTGGAAACGGGTATCCTACTTCTACTACATACACTCAAAGCCTGGGCAGCTGGGCTTGCATTGACGTACAAAAGCCAACATCTTCATGTGAA 1332
1B .....
1C .....
2B .....A.....
    
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FIG. 2. Sequence diversity among *E. hellem* genotypes in the PTP gene. Dots denote sequence identity to genotype 1A (AF044915), and dashes depict nucleotide deletions. The repeat region is underlined, and the primer sequences used in direct genotyping PCR are double underlined. The numbers at the ends of lines are nucleotide positions in AF044915.

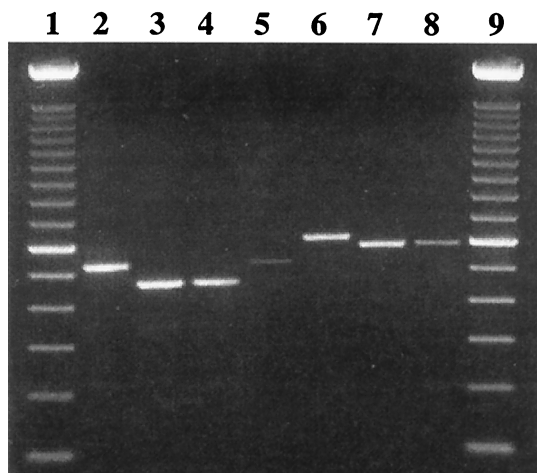


FIG. 3. Genotyping *E. hellem* isolates by PCR analysis of the PTP gene. Lanes 1 and 9, 100-bp ladders; lanes 2 and 5, genotype 1B; lanes 3 and 4, genotype 1A; lane 6, genotype 2B; and lanes 7 and 8, genotype 1C.

quence variations among genotypes were also seen in the repeat and nonrepeat regions.

**Differentiation of *E. hellem* genotypes by direct PCR.** Because of the length polymorphism among *E. hellem* genotypes in the PTP gene, a set of primers (5'-CATGCTTGCCAACA CAGG-3' and 5'-TGGAGGCATTGCAATAGG-3') was developed for the detection and differentiation of human *E. hellem* by direct PCR analysis. This primer set was designed to generate PCR products of predicted sizes of 461, 521, 581, and 611 bp for genotypes 1A, 1B, 1C, and 2B, respectively. Testing of this PCR primer set with *E. hellem* DNA of known genotypes produced PCR products concordant with the expected sizes, which were easily differentiated from each other in agarose gel electrophoresis (Fig. 3).

## DISCUSSION

Genotyping microsporidian parasites in clinical samples is helpful to the understanding of the transmission of human microsporidiosis. Since the discovery of three genotypes of *E. cucuruli* in 1995 (10), there has been increasing interest in genotyping of human-pathogenic microsporidian parasites, including *E. cucuruli*, *E. hellem*, *Encephalitozoon intestinalis*, and *E. bienersi* (1–3, 6, 8, 10, 16–19, 21, 24–26). With the exception of the use of pulsed-field gel electrophoresis and karyotyping (1, 35), most of the genotyping studies targeted the ITS. Different genotypes have been found in *E. bienersi* and *E. hellem* in addition to *E. cucuruli* (16, 19, 21, 24–26). Because most of the genotyping techniques involve DNA sequencing, these techniques are time-consuming, expensive, and not widely used in diagnostic laboratories. Therefore, alternative techniques and genetic loci are needed for better characterization of the molecular epidemiology of microsporidiosis and population and genetic structure of microsporidia.

Results of this study suggest that the PTP gene may be a good target for genotype analysis. Four genotypes of *E. hellem* were found in the 24 isolates analyzed at this genetic locus. This typing resolution is much higher than that produced by

sequence analysis of the ITS, which yielded two genotypes. In fact, the typing resolution at the ITS locus was even lower than the sequence analysis of SSU rRNA, which divided the 24 isolates into three genotypes. The PTP gene had an additional advantage of having length polymorphism. Thus, genotypes 1A, 1B, and 1C had six, seven, and eight copies of the 60-bp central repeat. Genotype 2B also had eight copies of the central repeat, but five copies of the repeat were 66 bp in length, with a smaller 6-bp repeat at the beginning of the 60-bp repeat. This length polymorphism in PTP enabled the differentiation of the *E. hellem* genotypes by electrophoresis of PCR products without restriction digestion or sequence analysis.

DNA strand slippage during parasite replication probably plays a role in the evolution of length polymorphism in the PTP gene. First, this length polymorphism in PTP occurred in the repeat region, and each genotype differed from the others by the deletion or insertion of one or more copies of the repeat, indicating that strand slippage by DNA polymerase during genome duplication some time in the long evolution of *E. hellem* was probably responsible for the length polymorphism. Second, the strand slippage theory was also supported by the insertion of a 6-bp sequence (GGAAGC or GGAAGT) in some copies of the central repeat of genotype 2B. This 6-bp sequence itself was a repetitive element, which was present at the beginning and end of each 60-bp repeat in tandem. Thus, genotypes 1A, 1B, and 1C had two copies of GGAAGC or GGAAGT at the junction of the 60-bp repeat, whereas genotype 2B had two or three copies of GGAAGC or GGAAGT tandem repeat at the junction of the 60-bp or 66-bp repeat. Third, the strand slippage theory was further supported by the insertion of an 18-bp sequence (TGCTAACCAGATGATT CC) in the nonrepeat region in genotype 2B. This 18-bp insert occurred after the sequence GATTATTCC, a variant of which (underlined) was present at the 3' end of the insert. Again, duplication error was likely the cause of an additional insert in genotype 2B.

Among the four *E. hellem* genotypes found in the 24 samples, genotypes 1A, 1B, and 1C are apparently more related to each other genetically than to genotype 2B. This was reflected by the genetic distances among the four genotypes at the ITS, SSU rRNA, and PTP genes. Genotypes 1A, 1B, and 1C had identical ITS sequence, a 1-nucleotide difference in the SSU rRNA gene, and very limited sequence differences in the non-repetitive region of the PTP gene. In contrast, genotype 2B had much different ITS and SSU rRNA sequences and more extensive changes in both the repeat and nonrepeat regions of the PTP gene (Table 2 and Fig. 1 and 2).

More *E. hellem* genotypes are apparently present. A previous characterization of *E. hellem* at the ITS and SSU rRNA loci by Mathis et al. revealed the presence of three genotypes in five isolates from humans: genotype 1 in one isolate, genotype 2 in three isolates, and genotype 3 in one isolate (21). Genotype 1 of Mathis et al. (21) had the identical ITS as genotypes 1A, 1B, and 1C and an SSU rRNA sequence identical to 1A and 1B in this study. Genotypes 2 and 3 of Mathis et al. (21), however, had ITS and SSU rRNA sequences similar but not identical to genotype 2B in this study. Thus, there are at least six *E. hellem* genotypes. Multiple alignment of all ITS and SSU rRNA sequences indicates that there are two groups of *E. hellem* parasites: genotypes 1A, 1B, and 1C are related to

each other and can be grouped together, whereas genotypes 2 and 3 of Mathis et al. (21) and genotype 2B in this study are related to each other and form a second group (Table 2 and Fig. 1). Although PTP sequences are not available from genotypes 2 and 3 by Mathis et al., judged by the sequence divergence from genotypes 1A, 1B, and 1C by 2B, they are also likely to be more divergent from these genotypes. We therefore suggest renaming genotypes 2 and 3 of Mathis et al. as genotypes 2A and 2C, respectively, to reflect their relatedness to genotype 2B as described here.

The significance of the genotypic diversity in *E. hellem* is unclear. The only nonhuman hosts for *E. hellem* known are birds. Two of the avian *E. hellem* isolates reported have been sequenced for ITS (34, 36) and produced sequences identical to genotype 1 in humans, indicating that human *E. hellem* infection could be of zoonotic origin under certain circumstances. Currently, the number of *E. hellem* isolates genotyped is very limited and does not allow a meaningful comparison of genotype distribution between humans and birds. The data accumulated so far do suggest the presence of possible geographic segregation of certain genotypes. For example, the eight genotype 1C *E. hellem* isolates found in this study were all from Italy, and 10 genotype 1A isolates were all from the United States and its protectorate Puerto Rico. Similarly, the rarer *E. hellem* genotypes 2A (two of the three isolates in reference 21), 2B (one isolate in this study), and 2C (one isolate in reference 21) identified so far were from patients in Switzerland, with the exception of one genotype 2A isolate from Tanzania.

In summary, results of this study indicate the existence of extensive genetic diversity in *E. hellem* isolates from humans. This genetic diversity was previously underestimated by the analysis of ITS sequence, but now can be assessed easily by analysis of the repetitive region of the PTP gene. More extensive epidemiologic studies and characterizations of large number of isolates from humans and birds are needed to evaluate the significance of the genetic diversity and the role of birds in human *E. hellem* infection. These studies are now more feasible with the development of a simple *E. hellem* genotyping technique in this study using direct PCR.

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#### ADDENDUM IN PROOF

A recent report by Peuvél et al. (I. Peuvél, F. Delbac, G. Metenier, P. Peyret, and C. P. Vivares, *Parasitology* **121**:581–587, 2000) showed that two human *Encephalitozoon hellem* isolates differed from each other in the polar tube protein gene sequences.

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