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Molecular Study of Microsporidiosis Due to *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* among Human Immunodeficiency Virus-Infected Patients from Two Geographical Areas: Niamey, Niger, and Hanoi, Vietnam▽†

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Microsporidiosis cases due to *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are emerging opportunistic infections associated with a wide range of clinical syndromes in humans. The aim of this study was to specify microsporidial epidemiology in two different geographical areas. From November 2004 to August 2005, 228 and 42 stool samples were collected in Niamey, Niger, and Hanoi, Vietnam, respectively. Screening for microsporidia was performed using UV-light microscopy. Detection was confirmed by molecular biology using two methods specific for *E. bieneusi* and *E. intestinalis*. All samples positive for *E. bieneusi* were subjected to genotyping. In this study, we found high prevalences of microsporidiosis among human immunodeficiency virus-infected patients, 10.5% and 9.5%, respectively, in Niamey and Hanoi. These levels of prevalence are similar to those recorded in European countries before highly active antiretroviral therapy was introduced. In the samples positive for *E. bieneusi*, we found seven distinct genotypes, including two genotypes not previously described. The *E. bieneusi* genotype distributions in the two geographical areas suggest different routes of infection transmission, person-to-person in Niger and zoonotic in Vietnam.

Microsporidia are widespread obligatory intracellular eukaryotic parasites, recently reclassified within the fungi (14), infecting a diversity of hosts, including invertebrates and vertebrates. Of the 15 species infecting humans, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are currently the two most common causes of human infection (7). Microsporidia are recognized as a major agent of chronic diarrhea in severely immunocompromised patients, especially in human immunodeficiency virus (HIV)-infected patients with $CD4^+$ T-cell counts below 100 cells per mm^3. Since the advent of highly active antiretroviral therapy (HAART), opportunistic infections such as microsporidiosis have substantially decreased in Europe (10). This contrasts with the situation in developing countries, where the rapid expansion of AIDS, associated with limited access to HAART, is responsible for an increase in the incidence of microsporidiosis.

Molecular methods, especially those based on the sequencing of the internal transcribed spacer (ITS) of the rRNA genes, have highlighted a considerable genetic diversity within *E. bieneusi* isolates (19, 23). At least 50 distinct genotypes of *E. bieneusi* based on the nucleotide sequence polymorphism of this 243-bp region have been described, and 34 have been reported in humans (17, 21). Nevertheless, even if only limited information is available on the geographical distribution of human-derived genotypes of *E. bieneusi*, the current knowledge on the epidemiology of microsporidiosis suggests that a distinct mode of transmission exists.

Epidemiological studies of microsporidiosis and the genotyping of *E. bieneusi* have been conducted in only a few countries in Africa (1, 9, 24–26) and Asia (15, 16, 28), and only limited information has been available from Niger and Vietnam (4, 18). Therefore, our aim was twofold: to assess the prevalence of microsporidiosis among HIV-infected patients in the two countries and to compare the genotypes of the *E. bieneusi* samples recovered to highlight possible differences in epidemiological features.

MATERIALS AND METHODS

Stool samples. Between November 2004 and August 2005, 270 stool specimens from HIV-infected patients from the two distinct geographical areas were analyzed. Informed consent was obtained for each subject. Samples from Niamey ($n = 228$) were collected at the Centre de Traitement Ambulatoire (day care treatment center) and the National Hospital of Niamey from patients before the introduction of HAART. Samples from Hanoi ($n = 42$) were collected from hospitalized patients at the following six hospitals: Bach Mai, Dong Da, Saint Paul, the Army hospital, Bavi hospital, and the French hospital.

Screening for microsporidian spores. All stool samples were analyzed for microsporidian spores using UV-light microscopy and the fluorescent dye Uvitex 2B (Uvibio; LDBIO Diagnostics, Lyon, France) (27). Each slide was analyzed by two different examiners. Confirmation of infection by *E. bieneusi* and *E. intestinalis* was performed using two specific real-time PCR methods.

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DNA extraction and PCR amplification. Stools specimens were stored at +4°C until DNA extraction. Total genomic DNA was extracted using a QIAamp DNA stool kit (QIAGEN, Courtaboeuf, France) in accordance with the manufacturer's instructions.

Two real-time PCR methods for detection of *E. bieneusi* and *E. intestinalis* genomic DNA targeting the small-subunit rRNA (SSU rRNA) were applied using a Rotor Gene 3000 platform (Corbett Life Science, Mortlake, Sydney, Australia). To ensure that no PCR inhibitor was present, an internal positive control (IPC) was included with each sample and amplified using specific primers and probes (TaqMan exogenous internal positive control; Applied Biosystems, Foster City, CA). Negative controls (distilled water) were included in each set of experiments.

Specific detection of *E. bieneusi* in stool specimens was performed using primers Eb1 (CGACAGCCTGTGTGTGAGAATAC) and Eb5 (CAACGAAT GACTTGACCCTGGTAA) as previously described (18). Briefly, detection of the 180-bp product was ensured by use of the specific TaqMan probe EbS2 (TGCTTAATTTAACTCAACGCGGGAAAA). PCR was performed with a 25-μl volume containing 6 mM $MgCl_2$, 1× TaqMan buffer, a 200 μM concentration of each deoxynucleoside triphosphate, 0.3 μM Eb1, 0.2 μM Eb5, 0.2 μM EbS2, 0.5 U of uracil DNA glycosylase (Invitrogen, Carlsbad, CA), 1× Exo Mix IPC and 0.5× IPC DNA (Applied Biosystems), 0.5 U of Platinum*Taq* DNA polymerase (Invitrogen), and 5 μl of DNA extract. The PCR conditions were as follows: after 2 min at 50°C and initial denaturation at 95°C for 10 min, amplification consisted of 45 cycles of 15 s of denaturation, followed by 60 s of annealing and extension at 60°C. One positive control (DNA extract from one *E. bieneusi*-positive sample) was included in each experiment.

Specific detection of *E. intestinalis* was performed as previously described (22). Briefly, amplification of 127 bp of the SSU rRNA was ensured by using primers FEI1 (GCAAGGGAGGAATGGAACAGAACAG) and REI1 (CACGTTCAG AGGCCCATTACACAGC) and the TaqMan probe S.INT (CGGGCGGCAC GCGCACTACGATA). PCR was performed with a 25-μl volume containing 6 mM $MgCl_2$, 1× PCR Rxn buffer (Invitrogen), a 200 μM concentration of each deoxynucleoside triphosphate, 0.2 μM FEI1, 0.2 μM REI1, 0.4 μM S.INT, 0.5 U of uracil DNA glycosylase (Invitrogen), 1× Exo Mix IPC and 0.5× IPC DNA (Applied Biosystems), 0.5 U of Platinum*Taq* DNA polymerase (Invitrogen), and 5 μl DNA extract. PCR conditions were as follows: after 2 min at 50°C and initial denaturation at 95°C for 10 min, amplification consisted of 45 cycles of 15 s of denaturation followed by 60 s of annealing and extension at 63°C. One positive control (DNA extract from one *E. intestinalis*-positive sample) was included in each experiment.

Both methods showed high sensitivity (data not shown), as determined by serial 10-fold dilutions of a plasmid DNA carrying the SSU rRNA fragment, which allowed the detection of less than 20 copies/μl of *E. bieneusi* and *E. intestinalis.*

Genotyping of *E. bieneusi* isolates. The genotyping of *E. bieneusi* was performed through nucleotide sequence analysis of the ITS region of the rRNA genes as previously described (13). For this purpose, amplification of 508 bp containing the 243 bp of the ITS was performed with the primer set MSP3 (GGAATTCACACCGCCCGTCRYTAT) and MSP4B (CCAAGCTTATGCT TAAGTCCAGGGAG). Cycling parameters were as follows: denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. A 4-min extension at 72°C was included at the end of the last cycle.

Each PCR product was then sequenced using the BigDye terminator sequencing kit on an ABI PrismR 3130 genetic analyzer (Applied Biosystems). Nucleotide sequences were analyzed using the Sequence Navigator software (Applied Biosystems). All genotypes were analyzed to establish possible homology to previously published genotypes by comparing them to those of the GenBank database (http://www.ncbi.nlm.nih.gov).

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to the sequences determined in this study are as follows: for genotype HAN1, EF458627; for genotype NIA1, EF458628.

RESULTS

Of the 228 stool specimens from Niamey, 62 (27%) were positive by microscopy. Of these 62 samples, 24 (38.7%) were positive by real-time PCR for *E. bieneusi*. No *E. intestinalis* organisms were identified. Therefore, the prevalence of microsporidiosis due to *E. bieneusi* was 10.5%. Of the 24 patients infected with *E. bieneusi*, all but 1 were adults. The average age was 34 years (range, 7 to 50 years of age). Low $CD4^+$ cell counts ($CD4^+$ cell count, <100/mm^3) were significantly associated with *E. bieneusi* infection ($P < 0.01$), and 67% had $CD4^+$ cell counts below 100/mm^3. The genotyping of *E. bieneusi*-positive samples was performed using all 24 PCR-positive specimens, but it was successful for only 17. Of these 17 samples, 14 belonged to previously described genotypes: genotype A (100% homology; $n = 10$), genotype D (100% homology; $n = 1$), genotype K (100% homology; $n = 1$), and the recently described CAF1 (100% homology; $n = 2$). The remaining genotype, NIA1, is new and very close to genotype A (99.6% homology; G→A, nucleotide 77; $n = 3$).

TABLE 1. Results of microscopy, real-time PCR, and genotyping for the 270 stool specimens analyzed

Assay and species or genotype identified	No. of stool samples positive from[a]:	
	Niamey ($n = 228$)	Hanoi ($n = 42$)
UV-light microscopy	62	21
PCR	24	4
E. bieneusi	24	3
E. intestinalis	0	1
Genotyping	17	3
Genotype A	10	0
Genotype D	1	1
Genotype E	0	1
Genotype K	1	0
Genotype CAF1	2	0
Genotype NIA1	3	0
Genotype HAN1	0	1

[a] *n*, total number of samples tested.

Among the 42 stool specimens collected in Hanoi, 21 (50%) were positive by microscopy. Of these 21 samples, only four were positive by real-time PCR: one for *E. intestinalis* and three for *E. bieneusi*. These results correspond to a 9.5% prevalence of microsporidiosis among HIV-infected patients. The genotyping of the three *E. bieneusi* PCR-positive samples and comparison with sequences from the GenBank database revealed that two belong to previously described genotypes: genotype D (100% homology; $n = 1$) and genotype E (100% homology; $n = 1$). The third genotype, HAN1, is new and very close to genotype E (99.6% homology; G→A, nucleotide 117; $n = 1$). The distribution of the genotypes according to the geographical area is illustrated in Table 1 (also see Table S1 in the supplemental material).

DISCUSSION

This is the first comparative study of the molecular epidemiology of microsporidiosis among HIV-infected patients in two separate geographical areas, Niamey, Niger, and Hanoi, Vietnam. To our knowledge, only little information on microsporidiosis is available in these countries, and limited epidemiological investigations have been performed to date (4, 18). We wanted to use UV-light microscopy and molecular methods to investigate the prevalence of microsporidiosis due to *E. bieneusi* and *E. intestinalis*, the two main agents of microsporidiosis in humans in both countries. Furthermore, we performed the genotyping of *E. bieneusi*-positive samples with the

assumption that microsporidiosis from these two separate geographical areas could reveal distinct epidemiological features.

In this study, we found a major difference between the results of microscopic examination and PCR. Of the 270 samples analyzed, 83 (62 of 228 and 21 of 42 in Niamey and Hanoi, respectively) were positive by microscopy using Uvitex 2B. Finally, only 28 (24 and 4 in Niamey and Hanoi, respectively) of the 83 (32.7%) were positive by real-time PCR either for *E. bieneusi* or *E. intestinalis.* Several reasons could explain this difference. First, the microscopic examination of microsporidian spores is quite difficult and highly dependent on the expertise of the examiner (12). Furthermore, fluorescence-based methods like the Uvitex 2B method are known to increase sensitivity but could lead to false-positive results due to small fungi or some artifact material that may fluoresce (11). Hence, Uvitex 2B is suitable for screening but requires confirmation with a method like the molecular method. Finally, even if *E. bieneusi* and *E. intestinalis* were the only species identified in fecal samples (8), we cannot rule out a possible infection by species other than *E. bieneusi* and *E. intestinalis* that could not be detected by our molecular methods. A PCR allowing the detection of all microsporidian species infecting humans would be useful to further investigate this difference. Unlike a recent study reporting the occurrence of more than one species in human clinical samples (29), no case of mixed infection with *E. bieneusi* and *E. intestinalis* was recorded in the present study.

Twenty-four out of 228 (10.5%) and 4 out of 42 (9.5%) HIV-infected patients were positive by PCR for *E. bieneusi* and *E. intestinalis* in Niamey and Hanoi, respectively. Therefore, the prevalences of *E. bieneusi* and *E. intestinalis* in HIV-infected patients in Niger and Vietnam are in line with the levels recorded in European countries before the introduction of antiretroviral therapies (2, 3, 27). Our findings are in agreement with previous studies reporting similar prevalences among HIV-infected patients in African and Asian countries (9, 15, 20, 24, 30).

The genotyping of *E. bieneusi* isolates is a valuable tool for epidemiological investigation. To date, at least 34 different genotypes of *E. bieneusi* have been reported to infect humans, and new genotypes have often been found. Of the 20 samples positive for *E. bieneusi* in Niamey ($n = 17$) and Hanoi ($n = 3$) reported in this study, 16 of them, according to the genotyping of the ITS rRNA, belong to previously described genotypes: genotype A ($n = 10$), genotype D ($n = 2$), genotype E ($n = 1$), genotype K ($n = 1$), and the recently described CAF1 ($n = 2$). The remaining genotypes were new and have been named HAN1 ($n = 1$), which is close to genotype E (99.6% homology; G→A, nucleotide 117), and NIA1 ($n = 3$), which is close to genotype A (99.6% homology; G→A, nucleotide 77).

Some genotypes like genotypes A and B are considered host specific because they were recovered exclusively from humans. In contrast, many genotypes have a zoonotic potential (like genotypes D, E, and K) because they are non-host specific and encountered in various mammals, including humans (21). As several studies indicate that the genotypes of *E. bieneusi* can differ in different geographical locations (6), it has been recently proposed that predominant genotypes in different geographical sites could be related to distinct sources of transmission (17). The comparative analysis of *E. bieneusi* genotypes recovered in Niamey and Hanoi produced interesting results. In Hanoi, of the three samples positive for *E. bieneusi*, two correspond to non-host-specific genotypes: genotype D ($n = 1$) and E ($n = 1$). Both genotypes have previously been recovered in humans but also in various animals, including pigs, muskrats, and beavers. A predominance of genotypes D and E, which are associated with a zoonotic potential, has already been described in Thailand (17). In this study, authors reported a higher frequency of genotypes D (36.4%) and E (15.2%) among HIV-infected patients. Together these findings support a zoonotic potential for *E. bieneusi* in Thailand and Vietnam. In other words, it suggests that transmission between animals and humans could probably occur in Vietnam. Zoonotic transmission can be explained by exposure to animals or by contamination of surface water by discharged domestic wastewater or from animal sources.

On the contrary, the Niamey data highlighted a higher frequency of genotypes known to be human specific (genotype A, $n = 10$ samples) than of non-host-specific genotypes (genotype D, $n = 1$; genotype E, $n = 1$; and genotype K, $n = 1$). Further investigations are necessary to determine whether the remaining isolates (genotype NIA1; $n = 3$), very close to genotype A, are human-derived genotypes. These results suggest that in Niger, the transmission of microsporidiosis could occur through person-to-person contact. This mode of transmission could be facilitated by enteric carriage of *E. bieneusi*, as described in previous studies (4, 5). Nevertheless, taking into consideration the relatively small numbers of genotypes recovered from both areas, we must handle these preliminary results with care.

Taken together, these findings could suggest different transmission modes of *E. bieneusi* in the two countries. We hope that further investigations will help provide a better understanding of the sources of transmission and host reservoirs of *E. bieneusi*.

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