Frequent Occurrence of Mixed *Enterocytozoon bienersi* Infections in Humans

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Enterocytozoon bienersi (phylum *Microsporidia*) is a human pathogen with a broad host range. Following the sequencing of 3.8 Mb of the estimated 6-Mb *E. bienersi* genome, simple sequence repeats (micro- and minisatellites) were identified. Sequencing of four such repeats from various human and animal *E. bienersi* isolates identified extensive sequence polymorphism and enabled the development of a multilocus genotyping method to study the epidemiology of this pathogen. We genotyped *E. bienersi* DNA extracted from 197 fecal samples originating from children with diarrhea who were residing in Kampala, Uganda. Three newly identified microsatellite markers and the internal transcribed spacer were PCR amplified, and multiple cloned amplicons for each marker were sequenced from each individual. Most microsatellite sequences were unique to the Ugandan population. Significantly, polymorphism not only was present among isolates but was also found within isolates. This observation suggests that infections with heterogeneous *E. bienersi* populations are common in this region. However, the data do not exclude that some of the polymorphism originates from divergent paralogs within the genome. The frequent occurrence of multiple sequences within an isolate precluded the identification of multilocus genotypes. This observation raises the possibility that in a region in which the prevalence of *E. bienersi* is high, sequencing of uncloned PCR products may not be adequate for multilocus genotyping.

Enterocytozoon bienersi is a unicellular microsporidian fungal pathogen transmitted by environmentally resistant spores. Among eukaryotes, microsporidia stand out for their small size, a compact genome, and a unique mechanism of host cell invasion involving a polar tube (1). The first microsporidian genome to be sequenced was that of *Encephalitozoon cuniculi*, which was found to be only 2.9 Mb in length (2). At 2.3 Mb, the genome of *E. intestinalis* is even smaller and is viewed as the smallest eukaryotic genome (3).

Among the numerous microsporidian species identified to date, *E. bienersi* is the most important human pathogen. In immunocompromised individuals, the infection can cause severe diarrhea and malabsorption (4). Until recently, genetic markers to study the epidemiology of microsporidiosis caused by *E. bienersi* were limited to a single marker, the internal transcribed spacer (ITS). The sequence of the single ITS located between the large- and small-subunit rRNA genes was first reported 20 years ago (5). Since then, numerous surveys have used this marker to classify *E. bienersi* isolates originating from humans and animals (6, 7). ITS sequencing has revealed that the large majority of genotypes belong to a group of closely related zoonotic isolates referred to as group I (8). In addition, more-divergent ITS sequences (groups II, III, and IV) were found in raccoons, dogs, a marmoset, baboons (9), and rarely in humans. These ITS genotypes are also referred to as host adapted (10). Alternative classifications of ITS diversity recognizing five and seven groups were also proposed (11, 12).

Genotyping using a single marker can be useful for studying the epidemiology of clonally reproducing organisms. For sexually reproducing organisms, for which genetic recombination can produce large numbers of recombinant haplotypes, single-locus methods are unreliable. In such species, clusters obtained on the basis of a specific marker may not be representative of the genome. Although multilocus genotypes are typically more polymorphic than single loci, it is their ability to identify genetic recombinants

that makes them essential in molecular epidemiology. Although it is unknown whether *E. bienersi* undergoes sexual recombination, the taxonomic proximity of this species with sexually reproducing fungi implies that this possibility needs to be considered. The publication of a draft sequence of the *E. bienersi* genome (13) has led to the identification of several simple sequence repeats (14). Feng and coworkers initially identified seven tandem repeats, five of which were repeats of two- and three-nucleotide motifs and two of which had longer repeats. Consistent with the genetic divergence of *E. bienersi* isolates originating from certain animal species, primers flanking some of these repeats failed to amplify the repeat sequence from *E. bienersi* isolates from some host species (14). Initial surveys of human and animal isolates using four microsatellite markers detected high levels of sequence polymorphism (15). In this study, we used three microsatellite markers to genotype *E. bienersi* isolates from symptomatic children from Uganda. By cloning and sequencing multiple clones derived from microsatellite amplicons, we found that a majority of the isolates were genotypically mixed.

MATERIALS AND METHODS

Study population. Fecal samples were collected anonymously from 197 children up to 60 months of age who were admitted to the Mulago Hospital (Kampala, Uganda) between August 2011 and February 2012. Stool

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FIG 1 Rarefaction analyses of MS1, MS3, and MS7 diversity from Uganda and other countries. (A) Analysis of Ugandan microsatellite genotypes and comparison with ITS sequence diversity. MS1 is significantly more diverse than other markers. (B) Diversity of Ugandan microsatellite sequences exceeds that observed in published sequences from a geographically diverse collection of sequences. Error bars indicate 95% confidence intervals.

samples were collected in sterile disposable plastic tubes, refrigerated at 4°C, and shipped to Tufts University.

Sample processing. DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH) and stored at −20°C. *E. bieneusi*-positive stool samples were identified by PCR using nested primers (EBITS3, EBIT34, EBIT31, and EBIT32.4) that amplify the internal transcribed spacer region plus the flanking large- and small-subunit rRNA genes (16). The Platinum PCR SuperMix High Fidelity (Life Technologies, Grand Island, NY) was used as described previously (16). Negative and positive controls were included throughout. A control DNA extract containing no stool sample was also included for each set of DNA extractions to check for DNA contamination. Twenty-eight samples of fecal DNA positive by ITS PCR were further analyzed using three nested primer pairs that amplify the microsatellite sequences MS1, MS3, and MS7 (14). The approximate sizes of the primary amplicons are 670 bp, 535 bp, and 470 bp, respectively. Secondary amplicons of the expected size (~345 bp) were cloned into the pCR2.1-TOPO TA vector (Life Technologies, Grand Island, NY). A minimum of three clones per amplicon were randomly selected, and the inserts were sequenced. DNA sequences were visualized with Sequencher (Gene Codes, Ann Arbor, MI).

Data analysis. Rarefaction curves were plotted using the program EstimateS (17). Intrasample sequence diversity (H) was calculated for each marker using the Shannon diversity index according to the formula $H = -\sum [p_i \times \log(p_i)]$, where p_i is the frequency of sequence i and the sum is over the number of alleles observed for each locus and isolate. To calculate pairwise distances between microsatellite alleles, MS1, MS3, and MS7 sequences were aligned with ClustalW and the alignments were imported into mothur (18). Because most polymorphisms among alleles are indels located in the repeat region, pairwise genetic distances were calculated twice, once using the onegap algorithm and once using the eachgap algorithm. In the former method, each gap is considered a single position, regardless of its length, whereas in the eachgap method, each nucleotide difference is counted individually such that alignments with large deletions will generate higher distance values. To visually display distances between sequences originating from our survey and previously published sequences (14, 15, 19), onegap and eachgap matrices for each marker were imported into GenAlEx (version 6.5) (20) and distance matrices were visualized using principal coordinate analysis (PCoA).

The Mantel test (21) was used to test for correlation between genetic and geographic distance. A matrix of pairwise genetic distances was obtained for each of three markers using the eachgap method described above. Isolates were grouped by country, and the latitude/longitude coordinates of the capital were used. A geographic distance matrix was computed with the geographic distance calculator in GenAlEx. The correlation coefficient derived from pairs of data matrices, calculated with the same program, ranges from −1 to +1. Statistical significance of the correlation between matrices was tested by random permutation. In this test,

the observed value is compared to the distribution of correlation values obtained after random permutation of one matrix (20).

IRB approval of research involving human subjects. This research was granted exempt status by the Tufts Institutional Review Board (IRB) because the fecal samples were anonymized and no patient personal information was provided to us.

Nucleotide sequence accession numbers. All sequences have been deposited in GenBank under the accession numbers [KF261732](#) to [KF261987](#).

RESULTS

Microsatellite allele diversity. We initially used primers specific for the markers MS1, MS3, MS4, and MS7, as published by Feng and colleagues (14), but MS4 was dropped because of unexpected amplification results. Rarefaction analysis was used to compare the diversity of the three microsatellite markers with that of the frequently used ITS sequence (6). To capture the entire microsatellite diversity, each microsatellite sequence was included. Because multiple microsatellite genotypes were present in most isolates, the number of sequences included in the rarefaction analysis exceeded the number of isolates. MS1 was by far the most diverse marker (Fig. 1A). Rarefied to the number of MS3 sequences ($n = 84$), 74.6 (95% confidence interval [CI], 67.3 to 81.9) MS1 sequences were estimated to be present, compared to 46 MS3 sequences, 34.1 MS7 sequences, and 35.4 ITS sequences. MS3 was the second-most-diverse locus, followed by MS7 and ITS, which were equally diverse. The rarefaction analysis was extended to previously published isolates originating mostly from Peru (15). As illustrated in Fig. 1B, a comparison of homologous microsatellites from Uganda and from other countries showed that *E. bieneusi* in Uganda is more diverse than the populations sampled by Li et al. even though they reported only one sequence per patient.

Many microsatellite PCR amplicons amplified from Ugandan patients were indicative of the presence of multiple sequences (see Fig. S1 in the supplemental material). A striking feature of the sequence chromatograms obtained from uncloned PCR products was the presence of at least one additional tracing downstream of the microsatellite sequence. The abrupt appearance of multiple tracings downstream of the repeat suggests the presence of multiple alleles differing in the length of the repeat. To investigate this possibility, PCR products were cloned into the pCR2.1-TOPO TA vector and multiple clones were sequenced. Consistent with the interpretation of the chromatograms generated from uncloned

FIG 2 Microsatellite and ITS sequence diversity in 25 Ugandan isolates. Diversity was calculated using the Shannon index and is plotted on the z axis. The shortest bars, as visible for isolate 7 MS3 and MS7, represent zero diversity (all sequences identical). Blank fields indicate no data.

amplicons, multiple sequences differing in the length of the repeat were commonly detected. To quantify intraindividual diversity, the Shannon diversity index was calculated for each isolate and each locus (Fig. 2). As readily apparent in this figure, for most isolates, multiple microsatellite alleles were found, resulting in a Shannon diversity H of >0 . Only 14 of 84 isolate-marker combinations revealed no polymorphism ($H = 0$). These results indicate that genotypically mixed infections are common in the patient population we studied. Because isolates with more than two microsatellite alleles were common in our sample (Table 1), we postulate that this observation cannot be explained exclusively by the presence of duplicated loci.

Analysis of global microsatellite diversity. In addition to the *E. bieneusi* population from Uganda genotyped in this study, MS1, MS3, and MS7 genotypes were previously reported by Li et al. (12, 15). Since these surveys used the same PCR primers, the sequences published by Li and those from Uganda can be directly compared. This enabled us to assemble a geographically diverse data set comprising isolates from eight countries (Table 2). We used this expanded data set to reexamine whether *E. bieneusi* microsatellite alleles segregate by country. As most polymorphisms are indels in the repeat regions, pairwise distance matrices were calculated using the onegap and eachgap method (see Materials and Methods) (18). For each distance metric, distance matrices were visualized by PCoA to assess whether microsatellite genotypes segregate by country. An initial analysis with 158 MS1 alleles revealed three highly divergent sequences originating from a human patient

TABLE 2 Number of isolates analyzed by locus and by country

Country ^a	No. of isolates for each locus ^b			
	MS1	MS3	MS7	ITS ^c
Brazil	1	1	3	
India	3	16	5	
Kenya	2	5	1	
Nigeria	5	16	4	
Peru	50	75	81	
Portugal	1		1	
Uganda	23 (104)	19 (84)	20 (87)	24 (107)
United States	2		1	

^a Except for those from Uganda, sequences were obtained from GenBank.

^b The number of PCR clones sequenced is shown in parentheses.

^c Only ITS sequences from Uganda were analyzed.

from Nigeria (JQ991401 [19]), from a raccoon from the United States (HQ615884 [14]), and from a marmoset from Portugal (HQ615883 [14]). To avoid compression of data points in PCoA plots, these three alleles were removed and the MS1 analysis was performed with 155 alleles, 92 of which are from the present study (Table 1). The PCoA for the three microsatellites (Fig. 3) showed no evidence of geographical segregation, not only when different countries were considered but also when comparing isolates from different continents. As expected from the large number of indels of various lengths, the eachgap PCoA plots showed a more dispersed topology. Similar observations were made with MS3 and MS7 (Fig. 3).

Only a small number of microsatellite sequences were shared between Uganda and other countries (14, 15, 19). For MS1, no shared sequences were found, whereas for MS3 and MS7, only three and four sequences, respectively, were found in Uganda and elsewhere. This observation prompted us to test for geographical segregation of microsatellite genotypes that may not be apparent on PCoA plots. As the frequent occurrence of genotypically mixed isolates precludes the identification of multilocus genotypes, each microsatellite (MS1, MS3, MS7) was tested separately. We used the Mantel test (21) to compare a matrix of isolate-versus-isolate eachgap genetic distance with an analogous matrix of pairwise geographic distances. For MS1, PCoA and Mantel were in agreement in showing no correlation between geographic distance and genetic distance. In contrast, for MS3 and MS7, a slight but significant correlation with distance was detected (Table 3). Although the correlation is clearly significant, the linear regression predicts a very small increase of 5×10^{-4} and 7×10^{-4} distance units per 10,000 km. The small correlation coefficient, together with the large amount of unexplained variation (Fig. 4) and the different test results for the three markers, indicates the need for a geographically more diverse sample.

DISCUSSION

The interpretation of intraindividual microsatellite diversity is relevant to the use of microsatellite markers to study the epidemiology of *E. bieneusi*. We do not know whether the observed sequence polymorphism originates exclusively from the presence of genetically distinct alleles or whether distinct paralogous copies are found in the genome. Duplication is known to have shaped the evolution of many fungal genomes (22–24). Segmental duplications in the microsporidian species *Nosema bombycis* have also been reported (25), raising the possibility of similar rearrange-

TABLE 1 Number of sequences analyzed by locus

Group of sequences or isolates	No. for each locus			
	MS1	MS3	MS7	ITS
Total no. of sequences from Uganda	104	84	87	107
No. of unique sequences from Uganda	92	47	35	41
No. of isolates with >1 sequence	21	16	18	17
Total no. of sequences analyzed ^a	158	168	143	107

^a Except for ITS, sequences from 8 countries are included.

FIG 3 Principal coordinate analysis for three microsatellite markers by country. Pairwise distances between sequences of cloned PCR products from Uganda and published sequences from seven countries were included in this analysis. The distances were calculated using two different models (onegap and eachgap) as detailed in Materials and Methods. A more dispersed topology in the eachgap analysis is consistent with most of the sequence differences being length polymorphisms.

ments in *E. bieneusi*. Because of the fragmented nature of the only published *E. bieneusi* genome (13), it is difficult to ascertain whether any duplication events have created paralogous copies of the loci we genotyped. The identification of multicopy genes in *E.*

bieneusi (13) and the detection of extensive karyotype polymorphism between a human *E. bieneusi* isolate and an isolate from a captive macaque (X. Feng, D. E. Akiyoshi, Q. Zhang, and S. Tzipori, unpublished data) are consistent with the possibility of chro-

TABLE 3 Mantel test results

Locus	Rxy ^a	P (Rxy _{rand} ≥ Rxy _{obs}) ^b	b ^c
MS1	0.02	0.42	10 ⁻⁵
MS3	0.125	0.01	5 × 10 ⁻⁴
MS7	0.193	0.01	7 × 10 ⁻⁴

^a Mantel correlation coefficient.^b Probability (Rxy of randomly permuted data ≥ observed Rxy).^c Regression coefficient (genetic distance/1,000 km).

mosomal duplication events in this species. Many of the isolates that we genotyped contain more than two genotypes; duplication events by themselves, however, cannot explain the observed microsatellite diversity. The ploidy of *E. bienersi* has not been determined, but evidence of diploidy in the taxonomically related microsporidian *Encephalitozoon cuniculi* has been reported (26). As for chromosomal rearrangements, diploidy cannot explain our results, leaving mixed populations as a plausible explanation of microsatellite heterogeneity. While we cannot discriminate the individual effects of each of these three possibilities on the observed microsatellite polymorphism, based on the multiplicity of microsatellite sequences found in many isolates, we conclude that mixed *E. bienersi* populations are frequent in Ugandan patients. Importantly, this observation implies that assembling multilocus genotypes from individual *E. bienersi* markers may be problematic. Therefore, analyses based on multiple genetic markers, such as linkage disequilibrium, may be difficult to apply to this pathogen.

To gain insight into the global population structure of *E. bienersi*, we initially visualized genetic distances between individual microsatellite sequences using PCoA. Color-coding of sequences according to the country of origin (Fig. 3) did not reveal any apparent geographical clustering. A Mantel test was then applied to further test for any significant correlation between genetic and geographical distances. For the reasons stated above, the test was applied separately to each of the three microsatellite markers. The detection of a slight but significant correlation for two of the markers (MS3 and MS7) raises the possibility that geographically distant genotypes are more diverse than those originating from the same country. We realize that the small regression coefficient

(Table 3) and the large amount of variation not explained by linear regression raise many questions regarding the biological significance of this result. However, having tested the data, we felt compelled to include the results, even if the interpretation is ambiguous. Contributing to the uncertain significance is the observation that only two loci show any correlation, perhaps an indication that the three microsatellite markers are subject to different evolutionary forces. MS1 is the only marker located in a functionally annotated gene. It is intriguing that this microsatellite, the most diverse of the three markers, is located in the tRNA (5-methylamino-methyl-2-thiouridylate) methyltransferase gene, a gene which has to our knowledge not been reported to be under selection for increased divergence. In contrast, MS3 and MS7 are located in proteins annotated as hypothetical, precluding us to speculate on the evolutionary forces driving genetic polymorphism of these loci. Together, these results emphasize the need for additional markers and a geographically more diverse sample to gain a clearer insight into the global population structure of *E. bienersi*. A previous analysis of the global distribution of a smaller sample from four countries did not detect any geographical segregation (19). Based on the arguments expressed above, it should not be a surprise that the analyses of multilocus and single-locus genotypes lead to different conclusions.

The fact that the diversity of the microsatellite alleles from Uganda exceeded that of previously analyzed isolate collections (14, 15, 19) was unexpected. The rarefaction curves for Uganda shown in Fig. 1 were drawn based on all sequenced PCR clones. As discussed above, for many isolates, this sequence collection included multiple identical sequences, which should have depressed the level of diversity. In addition, previously published microsatellite sequences originate from several countries whereas our sequences originate from a geographically restricted patient population. These differences between data sets would be expected to reduce the diversity of the Ugandan microsatellite collection, which was not the case. If diversity correlates with prevalence, as observed with *Plasmodium falciparum* (27, 28) and *Cryptosporidium* spp. (29), the remarkable diversity of microsatellite sequences in Uganda may indicate a high prevalence of microsporidiosis. Epidemiological surveys of Ugandan children with persistent diarrhea are in agreement with this prediction (30), but comparable epidemiological data are needed from different regions to support this view.

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FIG 4 Regression analysis of genetic diversity on geographic distance. According to the Mantel test, only the MS1 genetic distance is independent of geographical distance. Red, MS1; blue, MS3; green, MS7. Linear regression lines are dashed.

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