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Evidence for the existence of genetically distinct strains of *Enterocytozoon bieneusi*

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Abstract *Enterocytozoon bieneusi* is the most frequently found microsporidium in human infections. In all, 3 distinct genotypes were detected in 12 stool samples from 8 patients with acquired immunodeficiency syndrome (AIDS). A total of 9 polymorphic sites were found in the 243-bp-long internal transcribed spacer (ITS) of the rDNA gene, whereas none was found in 241 bp of adjacent rRNA coding regions. The genotype was stable in samples taken during 11 weeks of infection from one of the patients. The existence of and the ability to discriminate among strains of *E. bieneusi* are important prerequisites for elucidation of the hitherto unknown reservoirs of this pathogen and the mode of its transmission and may explain its pathogenicity.

Introduction

Enterocytozoon bieneusi is the microsporidial species most often found to infect humans. It is recognized as a major etiological agent of chronic diarrhea in immunocompromised patients. However, the route of infection is currently unknown and possible environmental or host reservoirs have not yet been determined, except for a recent finding of *E. bieneusi* in pig feces (Deplazes et al. 1996a).

An important aid in the answering of these questions would be the ability to differentiate among strains of this parasite. In another microsporidium, *Encephalitozoon cuniculi*, three strains have been described that possess 2, 3, or 4 repeats of a GTTT tetranucleotide sequence in the internal transcribed spacer (ITS) of the rRNA gene; these numbers of repeats were found in 4 foxes and 2 mice, in 13 rabbits and 1 mouse, and in 2 dogs, respectively (Didier et al. 1995; Katiyar et al. 1995; Mathis et al. 1997). Interestingly, isolates from humans belonged mostly to the triple-repeat genotype (5 patients), and 3 human isolates carried 4 repeats (Katiyar et al. 1995; Hollister et al. 1996; Mathis et al. 1996). It was concluded that the organism is a zoonotic parasite (Deplazes et al. 1996b).

In *E. bieneusi* the detection of strain variation is impeded because this parasite cannot yet be cultured effectively. As a possible alternative we have recently described the culture-independent amplification of the microsporidial ITS target sequence directly from stool samples (Katzwinkel-Wladarsch et al. 1996). In the present paper we report the characterization of 12 specimens of *E. bieneusi* DNA from 8 human patients; the results demonstrate that there are at least 3 different genotypes of *E. bieneusi*.

Materials and methods

Sources of microsporidia

Microscopically confirmed stool samples with *Enterocytozoon bieneusi* originated from patients with AIDS suffering from chronic diarrhea in hospitals in Munich, Berlin, and Zurich. The identity of *E. bieneusi* was confirmed by the homology of the cloned and sequenced polymerase chain reaction (PCR) products with published DNA sequences (Zhu et al. 1994). Stool samples were stored for up to 5 days at 4 °C, transported at ambient temperature, and then either processed immediately or frozen at −20 °C until examination.

DNA isolation, amplification, cloning, and sequencing

DNA isolation from stool and nested PCR reactions were performed as previously described (Katzwinkel-Wladarsch et al. 1996). PCR products (0.5 kb) were ligated into *Eco*RI/*Hind*III-cut pBluescript II SK$^-$ vectors (Stratagene, La Jolla), with advantage being taken of the flanking restriction sites of primers MSP-3 and MSP-4B, and used to transform XL1-Blue cells (Stratagene, La Jolla) by electroporation. Sequencing was performed using a Sequenase II kit (United States Biochemical Corporation,

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Cleveland). To exclude polymerase errors and to account for possible intra-isolate polymorphisms, the DNA sequence for every isolate was determined by the identity of nucleotides for each position from three independent PCR amplifications each from the respective isolate.

Results

The 484-bp-long sequence between primers MSP-3 and MSP-4B included the last 106 bp of the small-subunit rRNA coding region, 243 bp of spacer, and the first 135 bp of the large-subunit rRNA coding region. The primer sequences themselves were not considered. The DNA amplified from the 12 stool samples contained 9 polymorphic sites. Each of the observed polymorphisms belonged to one of 3 genotypes (Fig. 1). No apparent correlation was observed among those genotypes and the origins of the samples; genotype A was found in 2 patients from Munich and 1 patient from Zurich, genotype B originated from 1 patient from Berlin and 2 patients from Zurich, and genotype C was found in 2 patients from Zurich.

Repeated PCR amplifications on the DNA isolated from the same stool sample always produced a single, invariant genotype for each of the 12 samples. All 4 stool samples taken from a single patient over a period of 11 weeks showed the same genotype (A). The same was true for 2 samples exhibiting genotype B, which were taken 9 weeks apart from another patient.

Discussion

The data obtained in the current study present evidence for the existence of genetically distinct strains of *Enterocytozoon bieneusi* and for the usefulness of the rDNA ITS region in discriminating among them. All of the 9 polymorphic sites belonged to this 243-bp spacer, whereas none was found in 241 bp of adjacent small- and large-subunit rRNA coding regions.

However, when rDNA is used to discriminate between strains, two problems must be considered. First, the term *strain* must not be confused with *individual*. The finding of a different genotype for each isolate under consideration does not warrant their consideration as different strains, just as a human fingerprint does not contain any phylogenetic information and is not indicative of any common property of their carriers other than the mere differentiation of one individual from another. Therefore, a minimal requirement for the genotypic definition of "strains" should be that at least one of the postulated strains should consist of at least two individual members. Although seemingly trivial, this requirement is not always met, especially if only two or very few isolates are investigated.

A second problem arises when multicopy genes, e.g., rDNA, are used for strain differentiation. Especially in lower taxa, the different copies may not be identical within one genome. As a consequence, the finding of different DNA sequences in different isolates may not warrant their being regarded as separate genotypes, as different copies present in each genome may have been characterized and all isolates may actually be genetically identical. This is the reason why we have not yet considered the sequence reported by Zhu et al. (1994) to be a fourth *E. bieneusi* genotype. Even in the rRNA coding regions this sequence is only 87% identical with those of the three genotypes reported herein. This value is close to the 84% identity shared between *E. cuniculi* and *Encephalitozoon hellem* in the same genomic regions (Zhu et al. 1994). This example further

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type A  TTCAGATGGTCATAGGGATG AAGAGCTTCGGCTCTGAATA TCTATGGCTAGATAAAGTAC AAGTCGTAACAAGGTTTCAG TTGGAGAACCAGCTGAAGGA  100
type B  TTCAGATGGTCATAGGGATG AAGAGCTTCGGCTCTGAATA TCTATGGCTAGATAAAGTAC AAGTCGTAACAAGGTTTCAG TTGGAGAACCAGCTGAAGGA  100
type C  TTCAGATGGTCATAGGGATG AAGAGCTTCGGCTCTGAATA TCTATGGCTAGATAAAGTAC AAGTCGTAACAAGGTTTCAG TTGGAGAACCAGCTGAAGGA  100
publ.   TTCAGATGGTCATATGGATG AAGAGCTTCGGCTCTGAATA TCTATGGCTAGATAAAGTAC CAA--GGTTCCAGGTTCCAG TGAGA---CCAGCTGAAGA-   94

                                P             P                                                 P   P
type A  TCATTTtcagtttttggggt gtgggtatcggaatgtgtgg taggtgatgtgtgtgtgtat gggggatgccgaggggacca gcggtgcggtggtgtgtgta  200
type B  TCATTTtcagtttttggggt gtgggtatcggaatgtatgg taggtgatgtgtgtgtgtat gggggatgccgaggggacca gcagtgcggtggtgtgtgta  200
type C  TCATTTtcagtttttggggt gttggtatcggaatgtatgg taggtgatgtgtgtgtgtat gggggatgccgaggggacca gcggtgtggtggtgtgtgta  200
publ.   -CA-TTtcagtttt--gggt gt-ggtatcggaatgtatgg taggtgatgtgtgtgtgtat gggg-atgccgaggg-acca gcagtgcggtggtgtgtgta  187

                          P           P             P           P                                   P
type A  ggcgtgagagtgtatctgca agggtgagggatgtgggtgc agtgagttagaggtggttcc atgtggaatagtgggattgg tacgtgatggttggatgggg  300
type B  ggcgtgagagtgtatctgca agggtgagggatgtgggtgc agtgagttagaggtggttcc atgtggaatagtgggattgg tacgtgatggttggatgggg  300
type C  ggcgtgagagtgtatctgta agggtgaggaatgtgggtgc agcgagttagaggttgttcc atgtggaatagtgggattgg tacatgatggttggatgggg  300
publ.   ggcgtgagagtgtatctgca agggtgagggatgtgggtgc agtgagttagaggtggttcc atgtggaatagtgggat-gg tacgtgatggttggatgggg  286


type A  gaatgatgtgtgtatgggtg aggaaaatcggaggttgcgg tgcgagcggCAGTAGGGTGC C-ATCAAGAGGTGTATTTGG AAATATCCCTAATACAGGAT  399
type B  gaatgatgtgtgtatgggtg aggaaaatcggaggttgcgg tgcgagcggCAGTAGGGTGC C-ATCAAGAGGTGTATTTGG AAATATCCCTAATACAGGAT  399
type C  gaatgatgtgtgtatgggtg aggaaaatcggaggttgcgg tgcgagcggCAGTAGGGTGC C-ATCAAGAGGTGTATTTGG AAATATCCCTAATACAGGAT  399
publ.   gaatgatgtgtgtatgggta g---caaacggaggttgcgg t-c--acggCAGTAGGGTGC CCATCAAGAGGTGTATTTGG AAATA-CCC-AAGACGGG--  376


type A  CACTTGGATCCGTCGGCGAT GACGAGCGCGAAAGAATGCG AAAAGTCCTCAC----GGAA TGCTCTAATTATGAGATAAT CTCATGGAT  484
type B  CACTTGGATCCGTCGGCGAT GACGAGCGCGAAAGAATGCG AAAAGTCCTCAC----GGAA TGCTCTAATTATGAGATAAT CTCATGGAT  484
type C  CACTTGGATCCGTCGGCGAT GACGAGCGCGAAAGAATGCG AAAAGTCCTCAC----GGAA TGCTCTAATTATGAGATAAT CTCATGGAT  484
publ.   CACTTGGA--CG-CG-CGAT G-CGAGCGCGAAAGAA-CCG AAAAGTCCTCTCTTGGGGAA TGCTCTAATTATG---TAAT CTCATGGAT  457
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Fig. 1 DNA sequences of the 3 *Enterocytozoon bieneusi* genotypes and comparison with a previously reported sequence (Zhu et al. 1994; GenBank accession number L20290). The internal transcribed spacer is given in *lower-case letters*; its beginning and end are shown according to Zhu et al. (1994) (*P* Polymorphic site)

stresses the need to assess intrastrain and intraspecific genetic polymorphism to decide if the previously reported *E. bieneusi* genotype actually represents a different taxon close to the species level or if it merely represents a different class of *E. bieneusi* rDNA copies, possibly common to all *E. bieneusi* strains. It also suggests that it might be problematic to compare DNA sequences of multicopy genes obtained by amplification with different sets of PCR primers because they might possess different affinities to different classes of rDNA genes.

In conclusion, when properly defined and used according to the above-mentioned terms, the availability of stable genetic strain markers may contribute to our understanding of clinically suggestive differences in pathogenicity. They can also be expected to be a valuable tool for tracing of possible sources of infection with *E. bieneusi*. This approach has previously been described for *E. cuniculi* (Deplazes et al. 1996b), even though only one polymorphic site was available in the ITS of that species. The knowledge of the sources of infection and the routes of infection could then aid in the control and possible prevention of the resulting disease.

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