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Characterization of Genotypes of *Enterocytozoon bieneusi* in Immunosuppressed and Immunocompetent Patient Groups

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ABSTRACT. A retrospective phylogenetic analysis was performed on isolates of *Enterocytozoon bieneusi* to characterize the genotypes in different patient cohorts. Fifty-seven isolates, collected from patients living in Malawi and the Netherlands, were classified by age and immune status of the hosts. Sequence analysis of the internal transcribed spacer (ITS) region identified 16 genotypes; nine have not previously been described. Genotypes K and D were most prevalent among patient groups, whereas genotype C was restricted to transplantation patients receiving immunosupressives and genotype B showed a predisposition toward patients living with HIV/AIDS. Different genotypes showed more dispersion among isolates from Malawi compared with those from the Netherlands. A constructed map estimating the genealogy of the ITS region reveals a dynamic evolutionary process between the genotypes.

Key Words. *Enterocytozoon bieneusi*, genotypes, internal transcribed spacer, microsporidia.

THE phylum Microsporidia comprises approximately 1,200 species, 14 of which are known to infect humans. Most infections are caused by *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* (Didier 2005). Microsporidiosis has increasingly gained interest during the AIDS epidemic after being recognized as the etiological agent causing persistent diarrhea and systemic disease (Desportes et al. 1985). Highly active antiretroviral therapy significantly reduced the prevalence of intestinal microsporidiosis in HIV-infected persons (Conteas et al. 2000). However, in developing countries, enteric microsporidiosis still remains a major health problem due to high prevalence of HIV/AIDS in combination with underfinanced health care systems (Endeshaw et al. 2006). With heightened awareness, intestinal microsporidiosis has been increasingly diagnosed in transplant recipients, children, elderly, and travelers (Fournier et al. 1998; Guerard et al. 1999; Leelayoova et al. 2005; Lopez-Velez et al. 1999; Lores et al. 2002; Muller et al. 2001; Samie et al. 2007; Tumwine et al. 2002; Wanke, DeGirolami, and Federman 1996). Evidence that *E. bieneusi* can also be present asymptomatically is accumulating (Mathis, Weber, and Deplazes 2005; Nkinin et al. 2007).

Characterization of microsporidial genotypes elucidates the dynamics of microsporidial infections in different human and animal populations. Currently, *E. bieneusi* genotypes are based on the heterogeneity of the internal transcribed spacer (ITS) of the rRNA gene (Mathis et al. 2005). Only a proportion of the described genotypes were isolated strictly from humans; various other genotypes have been isolated both from humans and animals, drawing attention to the zoonotic potential of the parasite and a possible degree of host specificity for at least some genotypes (Sulaiman et al. 2003b, 2004). Differences in the prevalence of various genotypes were also recognized between HIV-negative and HIV-positive individuals (Liguory et al. 2001).

Molecular epidemiological data of infections of *E. bieneusi* in transplant recipients, children, and immunocompetent persons remain limited. More so, previous studies on isolates of *E. bieneusi* were derived from microscopy-positive stool samples and therefore are biased toward cases with high parasite loads. Therefore, we undertook a retrospective study of isolates of *E. bieneusi* from patient groups with different clinical and demographic backgrounds using a highly sensitive and specific real-time PCR detection method (Verweij et al. 2007). These isolates were subsequently genotyped by sequence analysis of the ITS region and analyzed for possible association with different patient groups.

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MATERIAL AND METHODS

Isolates of *Enterocytozoon bieneusi*. Fifty-seven fecal-DNA samples were selected in which *E. bieneusi* was detected using real-time PCR (Verweij et al. 2007). Samples from Dutch patients were collected from 1996 onwards at the Leiden University Medical Center. Additionally, samples were collected between 2003 and 2004 from patients examined at the Queen Elizabeth Central Hospital in the city of Blantyre, Malawi (Table 1). All DNA samples were from individual patients and stored at $-20\,^{\circ}C$.

DNA amplification. Genotyping primers specific for *E. bieneusi* were designed using Primer Express software (Applied Biosystems, Foster City, CA) on the ITS sequence (GenBank Accession no. AF101200). The forward primer Eb-80F (5′-GTT GGA GAA CCA GCT GAA GGT-3′) and reverse primer Eb-375R (5′-ATA CAC CTC TTG ATG GCA CCC T-3′) amplified a 296 base pair fragment. Amplification reactions were performed in 50 µl containing 2 × PCR buffer (HotstarTaq master mix, QIAgen, Venlo, the Netherlands), 5 µg bovine serum albumin (Roche Diagnostics Nederland, Almere, the Netherlands), 0.5 µM of each primer (Biolegio, Malden, the Netherlands), and 5 µl of the DNA sample. DNA amplification was carried out on an I-cycler Thermal cycler (Biorad, Herculas, CA) operated at 95 °C for 15 min, then 36 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. A last step at 72 °C for 5 min was used for final extension period.

DNA sequencing. Ten microliters of the PCR products were visualized on ethidium bromide-stained 2% agarose gels. Amplification products were purified from 40 µl of the reaction mixture with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. DNA concentration was measured with Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Delaware, CA). Both the forward and reverse strands were sequenced with the primers used for the PCR. Sequence analysis was

Table **1.** Demographic and clinical features of male (M) and female (F) patients from the Netherlands (NL) and Blantyre, Malawi (MA) with characterized *Enterocytozoon bieneusi* genotypes.

Patient no.	Source	Age	Gender	Underlying conditions	Genotype
1	NL	30	M	HIV positive	S8
2	NL	38	M	HIV positive	B
3	NL	39	M	HIV positive	D
4	NL	41	M	HIV positive	B
5	NL	43	M	HIV positive	K
6	NL	55	M	HIV positive	B
7	NL	NA	NA	HIV positive	B
8	NL	33	M	Kidney transplant recipient	C
9	NL	38	F	Kidney transplant recipient	C
10	NL	52	M	Kidney transplant recipient	C
11	NL	58	F	Kidney transplant recipient	C
12	NL	68	M	Kidney transplant recipient	C
13	NL	53	F	Colitis ulcerosa	S9
14	NL	43	M	Hypogammaglobulinemia	K
15	NL	1	F	NA (adoption child)	K
16	NL	27	F	NA (traveler)	A
17	NL	33	F	NA (resident of Cuba)	K
18	NL	49	F	NA (abdominal discomfort)	S7
19	NL	50	M	NA	K
20	NL	NA	NA	NA	D
21	MA	1	M	HIV positive	D
22	MA	1	F	HIV positive	S5
23	MA	2	M	HIV positive	K
24	MA	2	M	HIV positive	D
25	MA	2	M	HIV positive	S2
36	MA	2	F	HIV positive	D
37	MA	2	F	HIV positive	K
38	MA	3	M	HIV positive	S2
39	MA	7	F	HIV positive	K
30	MA	23	M	HIV positive	K
31	MA	23	M	HIV positive	AF502396
32	MA	24	F	HIV positive	S3
33	MA	26	M	HIV positive	S5
34	MA	27	F	HIV positive	K
35	MA	28	F	HIV positive	S5
36	MA	29	F	HIV positive	S2
37	MA	30	M	HIV positive	S2
38	MA	30	M	HIV positive	S2
39	MA	32	F	HIV positive	S5
40	MA	33	M	HIV positive	K
41	MA	35	M	HIV positive	K
42	MA	35	M	HIV positive	S4
43	MA	35	F	HIV positive	S6
44	MA	37	F	HIV positive	S2
45	MA	37	F	HIV positive	S2
46	MA	38	M	HIV positive	S3
47	MA	38	F	HIV positive	S2
48	MA	39	F	HIV positive	AY371283
49	MA	39	M	HIV positive	S2
50	MA	57	M	HIV positive	S2
51	MA	1	M	HIV negative	S2
52	MA	1	F	HIV negative	K
53	MA	2	M	HIV negative	S1
54	MA	2	M	HIV negative	S6
55	MA	1	M	NA	K
56	MA	1	F	NA	D
57	MA	3	M	NA	S1

performed by the Leiden Genome Technology Centre (Leiden, the Netherlands) on an ABI 3700 DNA Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Sequence results were analyzed using Vector Advance 10, Contig Express (Invitrogen, Breda, The Netherlands). To test the robustness of the sequencing method three stool specimens produced on subsequent days were obtained from patient no. 18 (Table 1) and subjected to sequence analysis.

Sequence analysis. A BLASTN search on NCBI database was performed with the nucleotide sequences of the isolates. Published ITS sequences representing *E. bieneusi* genotypes E, F, G, H, I, J, L, M, N, O, P, and Q, were included. All sequences were aligned using ClustalX 2.0.10 software (Larkin et al. 2007) with default parameters. The aligned sequences were mapped with TCS 1.2.1 software to estimate gene genealogies (Clement, Posada, and Crandall 2000). The cladogram was made by the TreeView program provided by Roderic D.M. Page at the Division of Environmental and Evolutionary, Institute of Biomedical and Life Sciences, University of Glasgow.

RESULTS

The amplified PCR fragments from clinical samples were sequenced directly after purification on spin columns, without prior cloning into a vector. The robustness of this method was tested using DNA isolated from three stool specimens produced on separate days by patient no. 18. The ITS nucleotide sequences of all three isolates showed 100% similarity.

The nucleotide sequences of the ITS fragment of all 57 DNA isolates revealed 16 distinct *E. bieneusi* genotypes. The polymorphisms were fixed at 22 positions in the ITS sequence (Table 2). Nine of the sequences were identified as new genotypes: six (S1–S6) were from Malawi and three (S7–S9) were from The Netherlands (Table 1). These nine genotypes were related to genotypes of already published sequences of *E. bieneusi* isolated from humans and animals (Fig. 1). Genotype C was identified in all five isolates from kidney transplant recipients and was not seen in any of the other patients. The remaining six sequences were identical with previously found genotypes A, B, D, K, and unnamed genotypes with GenBank Accession numbers AF502396 and AY371283 (Fig. 1).

The relatedness of the isolates, grouped by its sequence identity, are showed in the phylogenetic tree (Fig. 2). The GenBank accession numbers assigned to the sequences determined in this study are as follows: genotype S1, FJ439677; genotype S2, FJ439678; genotype S3, FJ439680; genotype S4, FJ439679; genotype S5, FJ439681; genotype S6, FJ439682; genotype S7, FJ439683; genotype S8, FJ439684; and genotype S9, FJ439685.

DISCUSSION

More than 50 genotypes of *E. bieneusi* described, based on the ITS region, show a certain degree of host specificity (Mathis et al. 2005). In the present study, using 57 fecal-DNA samples in which *E. bieneusi* was detected by real-time PCR (Verweij et al. 2007), 16 genotypes were identified of which nine showed novel variations fixed at 22 positions in the ITS sequence.

The increasing number of *E. bieneusi* genotypes thwarts systematic classification. Although the occurrence of polymorphisms at fixed base positions implies a stable divergence of the strains, the overall differences between the described genotypes are subtle. The interpretation of the phylogenic map based on a single locus has to be taken cautiously as this may not be representative for the genome as a whole (Anderson 2001; Constantine 2003). It can be argued that equal genotypes described in this study are formed by convergent evolution and if they can therefore be representative for the same genotypes described in previous publications. Genotypes presented on the phylogenetic map indicate a dynamic evolution of genotypes that do not always follow a linear path. Thus, analysis of at least a second, preferably transcribe

Table **2.** Polymorphic sites in ITS sequences of *Enterocytozoon bieneusi* isolates.

Position no.	92	102	112	121	133	139	146	153	157	173	177	189	190	193	201	223	235	236	237	239	251	253
K	C	C	A	C	G	C	C	C	G	G	A	G	C	C	C	C	A	C	C	C	C	C
Genotype																						
S6	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·
S9	·	A	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
AY371283[a]	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·
D	·	·	·	·	·	·	·	A	·	·	G	·	·	·	·	·	·	·	·	·	·	·
A	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
B	·	·	·	·	A	·	·	·	·	·	·	·	·	T	·	·	·	·	·	T	·	·
S5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·
AF502396[a]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	T	·	·
S1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	T	·
S4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	T	·	·
S2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	T	T	·
S3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	T	·
C	T	·	·	A	·	·	T	·	A	·	·	A	·	·	·	·	·	·	·	T	·	A
S8	T	·	·	A	·	·	·	·	A	·	·	A	·	·	·	·	·	G	·	T	·	A
S7	·	·	C	·	A	G	·	·	A	A	·	A	T	T	T	·	·	·	A	T	·	·

[a]Unnamed genotype.
ITS, internal transcribed spacer.

gene should be used to determine the reliability of genotypes described on the basis of the ITS region.

Various host groups that have been described in the present study and in the literature have been associated with particular genotypes (Table 3). We identified nine new genotypes (S1–S9), genotypes A, B, C, AF502396, and AY371283, which were described already in previous studies in humans with and without HIV infection (Breitenmoser et al. 1999; Liguory et al. 1998; Rinder, Katzwinkel-Wladarsch, and Loscher 1997; Tumwine et al. 2002), and genotypes D and K isolated from humans as

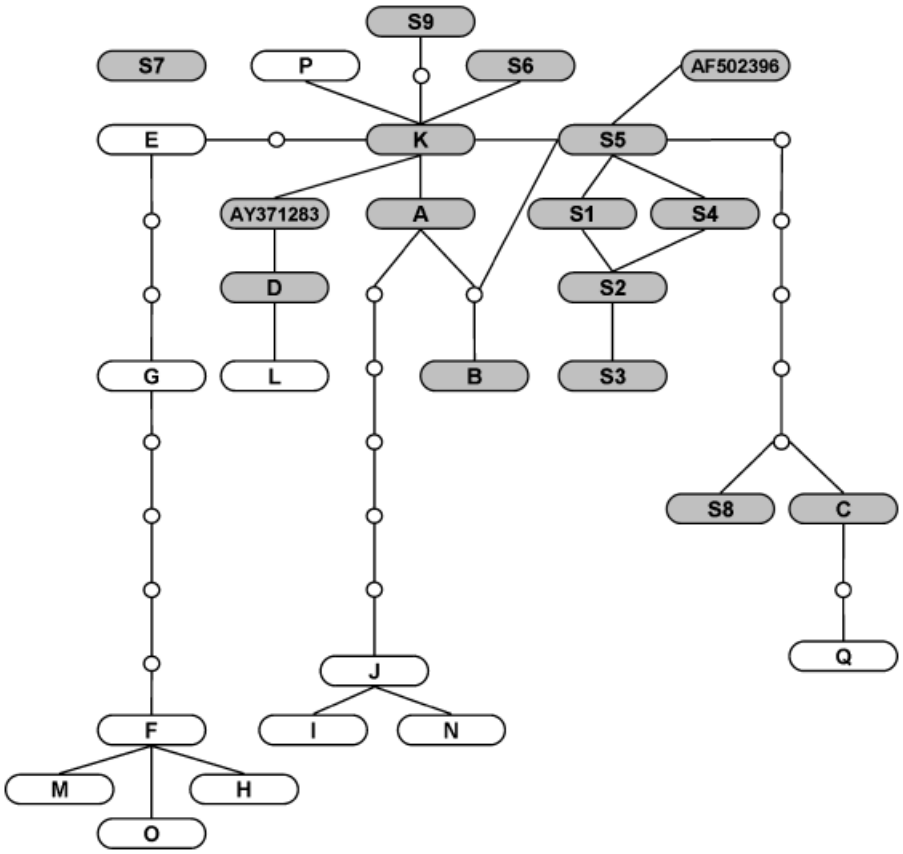

Fig. **1.** Map constructed with TCS 1.2.1 software to estimate the gene genealogies of internal transcribed spacer sequences of *Enterocytozoon bieneusi*. The mapped genotypes were derived from isolates in this study (gray boxes) and from the NCBI database (white boxes). Genotypes E, G, F, M, O, and H have been isolated from pigs and genotypes J, I, and N from cattle. Each line between ellipses and/or circles represents a single nucleotide conversion.

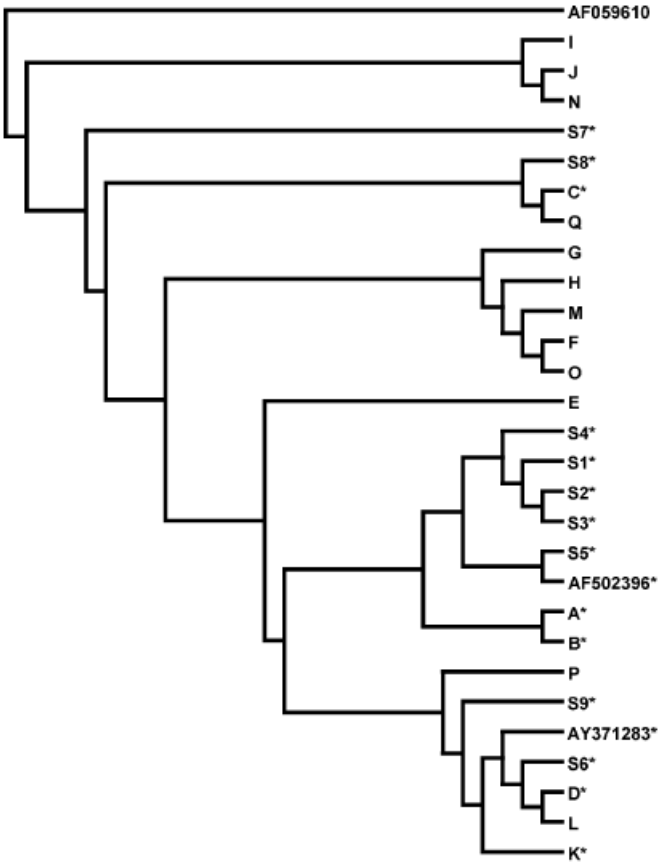

Fig. **2.** Most parsimonious tree based on the neighbor-joining analysis of the internal transcribed spacer region of the rRNA gene of *Enterocytozoon bieneusi* isolates. The genotypes derived from isolates in this study have an asterik (*). The tree was defined with an outgroup taxon (AF059610).

well as from animals in different regions throughout the world (Dengjel et al. 2001; Sulaiman et al. 2003b). Both in Malawi as well as in The Netherlands, genotypes D and K have been represented most. Domestic animals were recognized as a possible source of transmission for genotypes D and K (Dengjel et al. 2001). The presence of genotypes D and K in different host species and its high representation in the present study might be the result of a combination of low transmission barriers between hosts and high virulence of the genotypes. In the phylogenetic map of Fig. 1, genotype K is shown as a common source for the further evolution of all other genotypes, with exception to genotype S7, which showed no linkage to any of the described genotypes. In the cladogram of Fig. 2, genotype S7 is related closest to the genotypes isolated from cattle. The patient with *E. bieneusi* genotype S7 infection was a middle-aged male presenting severe diarrhea and rectal bleeding. HIV infection was considered unlikely with this patient and except of diabetes, no other noticeable characteristics were recognized.

Of note is the detection of genotype C only in stool specimens of all five kidney transplant recipients tested. The predominance of this particular genotype with this particular patient group concurs with the previously described findings, in which seven of eight HIV-negative transplant recipients in France were also infected with genotype C (Liguory et al. 2001). The possibility of a common source of infection to explain this distribution of genotype C is unlikely as the material for these two studies was collected over a period of several years. Another similarity between the two studies is the increased prevalence of genotype B in HIV-infected patients both in France and in The Netherlands.

Individuals with a decreased immunity may show susceptibility to a wider variety of different parasite (sub)species because microorganisms tend to be less host specific in relation to hosts with a disturbed immunological barrier (Chicharro and Alvar 2003; Gatei et al. 2002; Karp and Auwaerter 2007). Probably this patient group is therefore also susceptible to a wider spectrum of different genotypes of *E. bieneusi*. The number of different genotypes ob-

Table **3.** Numbers of *Enterocytozoon bieneusi* isolates characterized in this study and in previous reports, sorted by country of origin, host, and immune status if the host.

Report	Source	Host	Immune status	Genotype														
				K	S2	S6	S1	D	S5	S3	S4	AF	AY	S9	C	S8	B	A	S7
This report	Malawi	Children	Competent	1	1	1	1												
	Malawi	Children	NA	1			1	1											
	Malawi	Children	HIV positive	3	2			3	1										
	Malawi	Adults	HIV positive	4	8	1			3	2	1	1	1						
This report	The Netherlands	Child	NA	1															
	The Netherlands	Adults	Impeded	1										1	5				
	The Netherlands	Adults	HIV positive	1				1								1	3		
	The Netherlands	Adults	NA	2														1	1
	The Netherlands	NA	HIV positive														1		
	The Netherlands	NA	NA					1											
Tumwine et al. (2002)	Uganda	Children	NA	6								4							
Leelayoova et al. (2005)	Thailand	Children	Competent															9	
Leelayoova et al. (2006)	Thailand	Adults	HIV positive					6											
Sulaiman et al. (2003b)	Peru	Humans	HIV positive	18				9										35	
Breitenmoser et al. (1999)	Switzerland	Humans	HIV positive												2		8	3	
Sadler et al. (2002)	England	Humans	HIV positive	1				1									11		
Liguory et al. (2001)	France	Humans	HIV positive	9											9		66		
	France	Humans	Impeded	2											8				
	France	Humans	Competent	1											1				
Santin et al. (2006)	Colombia	Cats		2															
Lobo et al. (2006)	Portugal	Dog						1											
Sak et al. (2008)	Czech	Pigs						2											
Sulaiman et al. (2003a)	USA	Wildlife						9											
Sulaiman et al. (2004)	USA	Cattle		6															

served in HIV-positive adults in Malawi was higher than in The Netherlands. Nevertheless, the isolates originating from Malawi (i.e. genotypes AY371283, AF502396, D, K, S1, S2, S3, S4, S5, and S6) appear clustered while isolates originating from The Netherlands (i.e. genotypes A, B, C, D, K, S7, S8, and S9) are dispersed over a larger area of the phylogenetic map. Presumably, the genotypes found in patients from The Netherlands showed less homology because the clinical background of the patients with microsporidiosis is more diverse, whereas in Malawi the isolates originated mainly from HIV-positive persons. The cluster of genotypes from Malawi might also be the result of the ubiquity of spores in the environment. Age-dependant host specificity could not be demonstrated reliably in our study because of the incomplete sampling of patients: most patients in The Netherlands were adults while there were no immunocompetent adults from Malawi. The predominance of genotypes in specific populations can also be observed in animal hosts, where genotype clusters have been observed: genotypes E, G, F, M, O, and H described in pigs and genotypes J, I, and N in cattle (Fig. 1) (Dengjel et al. 2001; Sak et al. 2008).

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