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Zoonotic Potential of *Enterocytozoon bieneusi*

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The reservoirs and the modes of transmission of the most frequent microsporidial species in humans, *Enterocytozoon bieneusi*, are still unknown. We have examined fecal samples of 26 humans and 350 animals from 37 species to find 18 samples containing this parasite from humans, cats, pigs, cattle, and a llama. Genotypic characterization of the internal transcribed spacer of the rRNA gene resulted in 14 different genotypes, 6 of them previously undescribed. Phylogenetic analysis revealed the lack of a transmission barrier between *E. bieneusi* from humans and animals (cats, pigs, and cattle). Thus, *E. bieneusi* appears to be a zoonotic pathogen.

Microsporidia are newly emerging pathogens of humans and animals. Due to the small size of their spores and uncharacteristic staining properties they are difficult to detect by light microscopy. As a consequence, *Enterocytozoon bieneusi*, the species now known to be the most frequent in microsporidial infections of humans, was not discovered until 1985 (5). It is now recognized as a true pathogen, causing diarrhea especially in immunocompromised patients (2, 19).

E. bieneusi has recently been found in the feces of animals, including pigs, rhesus macaques, cats, and cattle (4, 11, 13, 18). However, the potential reservoirs and the mode of transmission of this pathogen are still unknown. Traditional epidemiological studies to address the zoonotic potential of this pathogen, for example, case control studies to identify risk factors such as contact with certain animals, are hampered by the small number of diagnosed microsporidial infections. Experimental infections of humans are prohibited for ethical reasons.

As an alternative, this problem could be solved by a differentiation of strains within this species and a comparison of the strains found in humans with those detected in animals. Unfortunately, because the spores of *E. bieneusi* strains are morphologically indistinguishable and since this species cannot be cultured, traditional morphological, biochemical, and immunological methods are unavailable for strain differentiation. Instead, a genotypic method has been described to differentiate characteristic genotypes of the internal transcribed spacer (ITS) of the rRNA gene (rDNA) (16).

Before this report, 14 ITS genotypes were known from humans (5 genotypes), pigs (6 genotypes), a cat (1 genotype), and cattle (2 genotypes) (1, 13, 15–18), but since no identical ITS genotypes of *E. bieneusi* were found in humans and animals its zoonotic potential was controversially discussed. In this report we investigated diarrheal fecal samples of another 26 humans and 350 animals from 37 species. Molecular epidemiological analysis of these data now offers convincing evidence for a zoonotic potential of *E. bieneusi*.

MATERIALS AND METHODS

Origin of stool and fecal samples. Fecal samples from 34 primates (26 humans, one chimpanzee, four gorillas, two baboons, and one mandrill), 122 carnivores (one mustelid, one polar bear, 60 cats, and 60 dogs), 147 even-toed ungulates (one wild boar, 50 domestic pigs, four fallow deer, one roe deer, six moose, six gaurs, three bantengs, one aurochs, 60 head of cattle, two yaks, three American bisons, one European bison, one chamois, one markhor, two ibexes, one sheep, one musk ox, one llama, and two two-humped camels), 43 odd-toed ungulates (one tapir, one zebra, one kiang, and 40 horses), one edentate (one ant-eater), three lagomorphs (three rabbits), three rodents (one mouse and two guinea pigs), two ratites (two rheas), and one fowllike bird (one peacock) were investigated. Inclusion criteria for the study were abnormal stool or feces consistencies (liquid or unformed, depending on the species) or clinically diagnosed diarrhea. The material was collected at the German diagnostic laboratories of the University of Munich's Institute for Comparative Tropical Medicine and Parasitology; the Veterinary Clinics of the Universities of Berlin, Giessen, Hannover and Munich, the Munich Zoo; and the University of Munich's Institute for Animal Pathology. Stools of human patients with diarrhea were from the Institute of Biomedicine, Caracas, Venezuela, and from the Department of Infectious Diseases and Tropical Medicine, University of Munich, Munich, Germany.

DNA isolation, amplification, cloning, and sequencing. DNA isolation from stool and nested PCRs were performed as previously described (9). PCR products (0.5 kb) were ligated into *Eco*RI/*Hin*dIII-cut pBluescript II SK(−) vectors (Stratagene, La Jolla, Calif.), taking advantage of the flanking restriction sites of primers MSP-3 and MSP-4B, and used to transform XL1-Blue cells (Stratagene) by electroporation. Sequencing was done using a Sequenase II kit (United States Biochemical Corporation, Cleveland, Ohio). At least two clones from independent PCR amplifications were used to determine each individual isolate's consensus sequence. All discrepant positions were resolved either by identity with all other isolates investigated (conserved position) or, in the case of a nonconserved position, by a third, independently generated clone. Mutations at a given position found in a single clone of a single isolate only were classified as polymerase errors or, indistinguishable from them, as rare genotypes and were not included in the consensus sequence.

Sequence analysis. DNA sequence alignments of the ITS of the rDNA were obtained using the program CLUSTAL (8) in the PC/GENE software package (Intelligenetics, Mountain View, Calif.). Computation parameters were set to a K-tuple value of 5, a gap penalty of 5, a window size of 10, and a filtering level of 2.5. Phylogenetic analyses were done using the PHYLIP phylogeny package (version 3.5c) (7) employing distance matrix, maximum-parsimony, and maximum-likelihood methods. For distance analysis, a neighbor-joining tree was generated from a Kimura two-parameter distance matrix with the algorithms DNADIST and NEIGHBOR. Maximum-parsimony analysis was performed us-

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TABLE 1. Currently known genotypes of *E. bieneusi*

Genotype	Host(s) (*n*)	GenBank accession no.	Source or reference(s)
A	Human (6)	AF101197	1, 16
B	Human (12)	AF101198	1, 16
C	Human (5)	AF101199	1, 16; this report
D	Human (1), macaque (?[a])	AF101200	17; unpublished data (GenBank accession no. AF023245)
E	Pig (11)	AF135832	1, 4
F	Pig (18), cattle (1)	AF132833	1, 18; this report
G	Pig (1[b])	AF135834	18
H	Pig (1[b])	AF135835	18
I	Cattle (2)	AF135836	18
J	Cattle (3)	AF135837	18; this report
K	Cat (2)	AF267141	This report
L	Cat (1)	AF267142	This report
M	Cattle (1)	AF267143	This report
N	Cattle (1)	AF267144	This report
O	Pig (1)	AF267145	This report
P	Llama (1)	AF267146	This report
Q	Human (1)	AF267147	15
EbfelA	Cat (1)	AF118144	13
EbpD	Pig (3)	AF076043	1
EbpB	Pig (6)	AF076041	1

[a] ?, number not published.
[b] Same animal.

ing the DNAPARS algorithm, with gaps counted as one event each. Support for phylogenies derived from distance and parsimony algorithms was measured by bootstrapping more than 1,000 replicates with the programs SEQBOOT and CONSENSE. The maximum-likelihood analysis was done with the program DNAML, and the tree with the lowest natural logarithm likelihood score was chosen. Bootstrapping is not recommended for maximum-likelihood analyses, which are statistical methods themselves. Trees were drawn with the program DRAWGRAM from the same PHYLIP package and using the ITS sequence of a taxonomically unresolved species related to *E. bieneusi* and described in dogs (13) as the outgroup. GenBank entries AF076041 (genotype EbpB) and AF076043 (genotype EbpD), both from pigs, and AF118144 (genotype EbfelA), from a cat, were included for completeness.

RESULTS

E. bieneusi DNA was detected in two humans (genotypes C and Q), seven head of cattle (genotypes F, I, J [$n = 3$], M, and N), five pigs (genotypes F [$n = 3$], G, H, and O; genotypes G and H are from the same animal), three cats (genotypes K [$n = 2$] and L), and one llama (genotype P). Genotypes K, L, M, N, O, and P have been observed for the first time (Table 1). The number of polymorphic sites in the ITS of *E. bieneusi* could be extended to 27 (Table 2).

As shown in the Fig. 1, the phylogenetic analyses using maximum-likelihood, distance matrix (neighbor-joining), and maximum-parsimony algorithms all failed to demonstrate the existence of monophyletic groups consisting only of *E. bieneusi* genotypes from humans. Instead, no segregation could be demonstrated among a group of genotypes from humans (genotypes A, B, and D), pigs (genotypes E and G), cats (genotypes K, L, and EbfelA), and llama (genotype P) with any of the three methods. This group also included *E. bieneusi* genotypes from cattle (genotypes I, J, and N) in the maximum-likelihood and maximum-parsimony analyses. The neighbor-joining method supported monophyly of this group with only a moderate bootstrap value of 85%. Similarly, monophyly of a group containing genotypes F, H, M, O, and EbpB from pigs and cattle suggested by maximum-likelihood analysis was only weakly supported by maximum-parsimony and neighbor-joining analyses, with bootstrap value of 78 and 64%, respectively.

DISCUSSION

In another human-pathogenic microsporidium, *Encephalitozoon cuniculi*, a zoonotic potential was initially suggested because this species was found both in humans and in rabbits (3). After it was possible to differentiate between three strains of *E. cuniculi* by polyacrylamide gel electrophoresis of spore proteins, Western blotting, and DNA sequencing of the ITS (6), host preferences were attributed to these strains (14). It was claimed that the discovery of the existence of *E. cuniculi* strains substantiated the argument that this parasite is of a zoonotic nature, because two of the strains had been detected in humans as well as in animals (12).

In contrast, with the exception of genotype D, found in a patient with AIDS and simian immunodeficiency virus-infected rhesus macaques, identical ITS genotypes have not been detected in *E. bieneusi* from humans and animals. It was concluded that (in pigs) "no [identical] genotypes with a possible zoonotic transmission were identified" (1) and that "in humans, *E. bieneusi* seems to be a natural infection not dependent on an animal reservoir as all genotypes from animals identified so far . . . are different from the genotypes found in AIDS patients" (12).

However, upon closer examination, the ITS of *E. bieneusi* is quite different from that of *E. cuniculi*. First, the ITS of *E. cuniculi* is only 33 to 41 bp in length, whereas the *E. bieneusi* ITS contains 243 to 245 bp. Second, only a single polymorphic site has been detected in the ITS of *E. cuniculi*, whereas *E. bieneusi* contains 27 polymorphic sites in its ITS (Table 2). Therefore, it appears to be merely less probable to find two identical genotypes when 27 polymorphic sites have to match instead of only one. Differently put, if only 33 to 41 bp of ITS were considered, as with *E. cuniculi*, numerous identical geno-

TABLE 2. Polymorphic sites in ITS of *E. bieneusi*

Genotype	Nucleotide position in ITS[b]																										No. of GT repeats following position 43
	17	31	34	76	77	81	93	95	97	113	117	118	124	129	130	131	134	136	137	141	143	147	149	158	178	156	
Consensus	G	G	G	C	G	T	T	G	C	T	G	G	G	G	G	G	C	G	C	T	A	G	G	T	G	A	5
A	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5
B	.	A	.	.	A	C	.	.	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	5
C	T	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	T	.	A	.	5
D	.	.	.	.	.	C	C	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5
E	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	5
F	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	G	5
G	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	5
H	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	G	5
I	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	T	.	.	A	.	A	.	.	5
J	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	T	.	.	A	.	A	.	.	5
K	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5
L	.	.	.	.	.	C	A	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5
M	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	T	.	G	.	.	.	.	G	5
N	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	C	.	.	T	.	.	A	.	A	.	.	5
O	.	A	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.	A	.	.	G	.	.	.	.	G	5
P	.	.	A	.	.	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5
Q[a]	T	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	T	.	A	.	6
EbfelA	.	.	.	.	.	C	C	.	.	C	T	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	5
EbpD	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	5
EbpB	.	A	.	T	.	.	.	T	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	G	5

[a] Due to a sixth GT repeat the ITS length of genotype Q is 245 bp instead of the 243-bp consensus length, but the numbering of the nucleotides shown following the GT repeats is analogous to the consensus numbering.
[b] Sources of the genotypes shown are the same as those indicated in Fig. 1. A dot designates the same nucleotide as in the consensus sequence.

types could be found in *E. bieneusi* from humans and animals, too (Table 3). It must thus be realized that the identity of genotypes in this context refers only to a limited portion of the genome. As a case in point, one of the three *E. cuniculi* genotypes found both in a human and in a rabbit was no longer identical when additional parts of the genome (16S rDNA) were characterized, and the genotypes from a human and a rabbit were distinct and could thus be differentiated (14). But even if the requirement for identical ITS genotypes is to be upheld, it must be pointed out that genotype D is no longer the

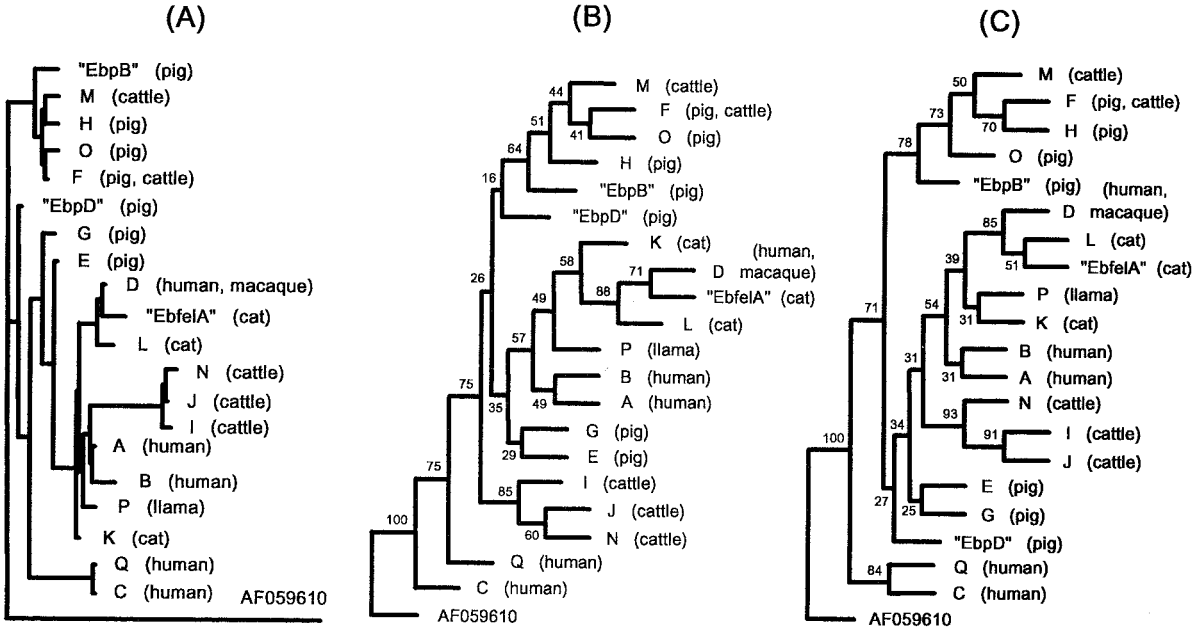

FIG. 1. Phylogenetic trees based on comparisons of the *E. bieneusi* ITS sequences from the genotypes given in Table 1. The dendrograms were constructed using the maximum-likelihood method, choosing the tree with the lowest natural logarithm likelihood score (ln likelihood = −822) (A); distance matrix (neighbor-joining) analysis (B); and maximum-parsimony algorithms employing bootstrapping of more than 1,000 replicates each (C). Bootstrap values given are percentages, and each value indicates how often the group of genotypes indicated to the right of the respective fork occurred among the 1,000 replicates. The ITS sequence of a taxonomically unresolved species related to *E. bieneusi* (GenBank accession number AF059610) was used as the outgroup.

TABLE 3. Genotypic comparison of 41-bp sections from the *E. bieneusi* ITS from humans and animal hosts

ITS positions considered	Species with identical ITS sections[a]
1–41	Human (A, D), pig (E, H), cattle (J, N), cat (K, L, EbfelA); human (B), pig (F, G, O, EbpD, EbpB), cattle (F, M)
42–82	Human (A, D), pig (E, G), cat (K, L, EbfelA), llama (P); human (C, Q), pig (F, H, O, EbpD, EbpB), cattle (F, I, J, M, N)
83–123	Human (A, B, Q), cat (K), llama (P); human (C), pig (E, G), cattle (I, J)
124–164	Human (D), cat (K, L), llama (P)
165–205	Human (A, B, D), pig (E, EbpD), cattle (I, J, N), cat (K, L, EbfelA), llama (P)
206–243	All genotypes are identical

[a] Genotype(s) is shown in parentheses.

only genotype found in two different host species and that we report here the detection of genotype F, previously known from pigs only, in the feces of a calf, too. It can easily be anticipated that with an increase in the number of genotyped *E. bieneusi* samples from different hosts, more genotypes shared by different host species will be detected, and that these will eventually also include those from humans as well.

Another, earlier line of argument doubted the zoonotic potential of *E. bieneusi* by referring to an analysis of the first 13 available *E. bieneusi* genotypes in which all of the 6 genotypes from pigs grouped together in two clusters and both of the genotypes from cattle fell into another, separate branch (12). It was concluded that specific genotype clusters were associated with specific host species and that *E. bieneusi* in humans was not dependent on an animal reservoir (12). However, this assumption was based on a simple distance matrix plot done without bootstrap analysis. In contrast, a rather different conclusion emerged after inclusion of the additional genotypes described in this report in a more stringent phylogenetic analysis employing all three of the major methodologies (maximum-likelihood, neighbor-joining [representing a distance matrix method], and maximum-parsimony analyses), especially in conjunction with the determination of the robustness of the inferred phylogenies by bootstrap analysis (Fig. 1). As described in Results, each of the phylogenetic analyses failed to demonstrate the existence of monophyletic groups consisting only of *E. bieneusi* genotypes from humans. Instead, with the exception of genotypes C and Q, which appear to be paraphyletic in relation to the other *E. bieneusi* genotypes, genotypes A, B, and D from humans grouped with those from pigs (genotypes E and G), cats (genotypes K, L, and EbfelA), and llama (genotype P) by any of the three methods. Similarly, monophyly of a cattle cluster (genotypes I, J, and N) is only moderately supported by bootstrap values of 85 and 93% in neighbor-joining and maximum-parsimony analyses and moreover is located in the human-pig-cat-llama cluster, described above, in maximum-likelihood and maximum-parsimony analysis (Fig. 1). Similarly, the seemingly monophyletic group consisting of genotypes F, H, M, O, and EbpB from pigs and cattle is supported by bootstrap values of only 64 and 78% in neighbor-joining and maximum-parsimony analyses, respectively.

In conclusion, it now appears to be only a matter of time until identical (ITS) genotypes from *E. bieneusi* will be found in humans and animals, although it should be emphasized that this must not be mistaken as a prerequisite for demonstrating the zoonotic potential of *E. bieneusi*. The now-available spectrum of *E. bieneusi* genotypes and their thorough phylogenetic analysis no longer support a transmission barrier between animals and humans. Further evidence is provided by the successful transmission of *E. bieneusi* spores from humans and rhesus macaques to gnotobiotic pigs (10). Notwithstanding, the absence of a transmission barrier for *E. bieneusi* between animals and humans does not preclude the possibility that this parasite may also be transmitted, possibly even as the prevalent mode, from person to person. Nevertheless, the zoonotic potential of *E. bieneusi* can no longer be denied.

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ADDENDUM

Ray Borrow, Public Health Laboratory Service, Withington Hospital, Manchester, United Kingdom, has recently detected the *E. bieneusi* genotype K in the stool of a patient with AIDS (F. Sadler, N. Peake, R. Borrow, T. Rowland, and A Curry, unpublished data).

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