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Strain Differentiation in Microsporidia

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Microsporidia are obligate intracellular, spore-forming protozoa and are regarded as newly emerging pathogens . *Enterocytozoon* spp. as well as *Encephalitozoon* spp. are recognized as major aetiological agents in chronic diarrhoea of immunocompromized patients . The detection and differentiation of strains within microsporidial species is a prerequisite for the elucidation of their hitherto unknown reservoirs and their mode of transmission . In *Enterocytozoon bieneusi*, the most prevalent human-pathogenic microsporidium, 6 different genotypes of the internal transcribed spacer (ITS) of the rRNA gene are known so far, with 12 polymorphic sites . This pathogen has infrequently been detected in 2 animal hosts only, pigs and rhesus macaques, and only the genotype of the latter has also been found in a human patient, too . *Encephalitozoon cuniculi* has a wider confirmed spectrum of animal hosts, but only one polymorphic site is known in the ITS, differing in 3 different numbers of a tetranucleotide repeat. Therefore, further genomic targets may have to be characterized, too . Few data are available on strain differentiation in *Encephalitozoon intestinalis* and *E. hellem.*

Keywords : Microsporidia, *Enterocytozoon*, *Encephalitozoon*, Strains, genotypes

INTRODUCTION

Why do we need to differentiate between strains of microsporidia? — One good reason might be to find the reservoirs of these pathogens and understand the ways that humans get infected. Indeed, both aspects are still unknown for any of the microsporidial species causing disease in humans.

Microsporidia are newly emerging pathogens. The most frequent species in human infections, *Enterocytozoon bieneusi*, has only been known since 1985 [3], and is found almost exclusively in immunocompromized patients. But recently, this species has also been detected in pigs [2] and rhesus macaques [8]. However, does this mean that we get infected from pigs or monkeys? How could this be found out?

Obvious possibilities seem to be traditional epidemiolgical studies on correlations and risk factors. However, there simply are not that many pig farmers, or researchers who work with rhesus monkeys and also suffer from AIDS and microsporidiosis to obtain a large enough numer of cases for a case control study, leave alone prospective cohort studies.

An elegant way to solve this dilemma is offered by the differentiation of individual strains of these parasites. If enough discriminating features can be found, it would be possible to compare the diversity of strains within the potential animal reservoirs with the diversity of strains found in human infections. By describing this diversity and the population structure within the individual microsporidial species, it may then also be possible to address, for example, the question if there are certain strains that may be characteristic for infections of HIV negative patients with microsporidia, as some early data already suggest [7]. Or more generally speaking, to find out if some strains might be more infective to humans than others, if different strains lead to different clinical symptoms, and also what their geographic distributions are.

Unfortunately, practical problems hinder

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the application of traditional morphological as well as other phenotypical approaches. One reason is the small size of microsporidia, making them difficult to detect by light microscopy. *E. bieneusi*, for example, measures only 1-1.5 µm. Furthermore, even ultrastructurally, some species, for example *Encephalitozoon* spp., are morphologically indistinguishable. Another reason is that *E. bieneusi* cannot be cultured effectively, thus making biochemical and immunological characterizations difficult, too.

For these reasons, molecular genotypical characterizations suggest themselves as alternatives to phenotypic techniques. In particular, non-coding regions of the genome, such as the internal transcribed spacer (ITS) of the rRNA gene (rDNA) have already been demonstrated to possess informative polymorphisms that allow the differentiation between individual strains within a species. Furthermore, knowledge of the primary DNA sequences of the target gene will generate quantifiable data to elucidate phylogenetic relationships between strains.

ENTEROCYTOZOON BIENEUSI

In 1997, the first characterization of strains within this species was described [11]. Nine polymorphic sites were found within the ITS of the rRNA gene which separated 3 genotypes between 8 patients with AIDS. Samples of 2 patients taken over a period of 9 and 11 weeks demonstrated strain stability during the course of infection, a result that was later confirmed in 9 other patients with AIDS [7].

The initial report stressed that a minimal requirement for the application of the term "strain" should be that more than one isolate exists with the same genotype, as opposed to the term "individual". Furthermore, as the rDNA is a multi-copy gene whose individual copies need not all be identical, it was cautioned that different "classes" of rDNA sequences might exist and should be looked for, in order to compare orthologous rather than paralogous genes.

Until now, there exists evidence for the existence of at least 6 different strains of *E.*

Table 1 *E. bieneusi* genotypes

	Position in the ITS													
Genotype	17	31	77	81	93	113	117	124	137	141	149	178	Source	References
A	G	G	G	C	T	C	G	G	T	T	G	G	human (n=3)	[11]
B	G	A	A	C	T	C	G	G	T	T	G	G	human (n=1) human (n=3)	[15][1] [11]
A or B?[2]	-	-	-	-	-	-	-	-	-	-	-	-	human (n=51)	[7][2]
C	T	A	G	T	T	T	G	A	C	T	T	A	human (n=2)	[11]
C?[2]	-	-	-	-	-	-	-	-	C	-	-	A	human (n=8)	[7][2]
D	G	G	G	C	C	C	T	G	C	T	G	G	human (n=1) monkey (n=3)	unpublished [8][3]
E	G	G	G	C	T	T	G	G	C	C	G	G	pig (n=1)	[2][4]
D or E?[2]	-	-	-	-	-	-	-	-	C	-	-	-	human (n=3)	[7][2]
F?[2]	-	-	-	-	-	-	-	-	-	-	-	A	human (n=3)	[7][2]

[1]94 % identical with genotype "B" in the ITS, similar to GeneBank accession number L20290
[2]a total of 6 products were sequenced, 4 of which "matched" L20290[1], while the other 2 showed "cleavage sites" which are implied to correspond to 2 of 3 further genotypes presented; the other 59 products were characterized by RFLP patterns only and the specific nucleotide at the polymorphic sites could only be deduced if it overlapped with a restriction site. The genotype designation is therefore based on compatibility, but cannot be proven without the primary DNA sequences
[3]GeneBank accession number AF023245
[4]GeneBank accession number U61180

bieneusi, and the number of polymorphic sites has increased to 12 (Table 1). These are conservative figures and taking into account that different rDNA classes may have been described by some researchers, for example Zhu et al. [15], whose ITS sequence differs by 12 deletions, mostly near multiple repetitions of the same nucleotide, and 3 substitutions from the consensus sequence found in subsequent reports [1, 8, 11], although one report [7] claims to have "matched" the GeneBank submission no. L20290 by Zhu et al. [15]. Possibly though, *Taq* polymerase as well as sequencing errors might account for some of these discrepancies, as there are already 3 differences between the Genebank submission L20290 from January 11, 1995 (positions 273, 311, and 324) compared to the originally published sequence [15]. In this context it cannot be stressed enough that the identity of each nucleotide in the DNA sequence of any particular genotype must be confirmed by at least one other, independently generated clone from DNA amplified in a separate PCR reaction. Cloning is indispensable because with "direct" sequencing of PCR products, mutations generated during the first cycles may be over-represented in the final product instead of being "averaged out".

In the largest survey to date, 65 individual infections with *E. bieneusi* were analyzed by PCR-RFLP with two restriction enzymes, *Nla*III and *Fnu*4HI, on a 210 bp fragment of the rDNA's ITS [7]. Most unfortunately, only 6 of the isolates were also sequenced. PCR-RFLP has the major drawback that all mutations between the restriction enzymes' recognition sites are invisible to this method, and even their exact position within the recognition site and the specific nature of the mutations that cause the loss of the site are inaccessible. In this particular case, 3 recognition sites were available that covered only 2 of the already known 12 polymorphic sites within the ITS (positions 137 and 178 in Table 1). Four genotypes could be differentiated, of which 2 are already known to consist of at least 2 distinguishable genotypes each themselves (A or B, and D or E in Table 1). One genotype ("F" in Table 1) can already be determined to be new by this method. Obviously, it would be highly desirabl to sequence all isolates.

What conclusions can be drawn for *E. bieneusi* ? Certainly different strains exist within this species and the ITS of the rRNA gene appears to be useful to differentiate between them. However, detections of this parasite outside humans have only recently been reported. The ITS genotype "D" was demonstrated in a monkey [8], and has also been found in one human patient (unpublished data). Another genotype ("E") found in a pig [2] has not yet been confirmed in humans except for a PCR-RFLP match of 3 patients by Liguory et al. [7]. Another preliminary finding is that the only two immunocompromized, but HIV negative patients, also from that study, were both compatible with a single genotype, namely "C".

Most interesting in this context is a recent environmental detection of *E. bieneusi* in surface water [12], but unfortunately the strain was not differentiated. Certainly, much more work on animal hosts and environmental occurrence of this protozoan parasite is needed.

ENCEPHALITOZOON CUNICULI

In *E. cuniculi*, the ITS is much shorter than in *E. bieneusi*, only between 33 and 41 bp, compared to 243 bp in *E. bieneusi*. There is only one polymorphic site detected to date, and the polymorphism is in the number of direct repeats of a GTTT motif. Until now, 3 genotypes with 2, 3, and 4 repeats have been characterized from 5 different host species, including humans (Table 2). It was concluded that the organism is a zoonotic parasite [1].

From the distribution of genotypes and hosts there appears to be a correlation because either only 1 genotype, or 2 genotypes differing by only 1 GTTT repeat have been found for each host so far. However, the absolute numbers of isolates for each host are still small and the significance of any correlation has yet to be verified.

In this context, and in contrast to the situation in *E. bieneusi*, the question must be addressed if the relatively short ITS in *E. cuniculi* with its single polymorphic site is sufficient for strain differentiation in that species. For a better resolution it may be necessary to characterize other target genes, too. Mathis et al. [10] have already described 4 distinct genotypes of the 16S rRNA gene, including 2 genotypes for the triple GTTT

Table 2 *E. cuniculi* genotypes and hosts

No. of GTTT repeats	Host	Origin	Reference
2	mouse (n=2)	Czech. Rep., UK	[4]
	blue fox (n=4)	Norway	[9]
3	rabbit (n=3)	USA	[4]
	rabbit (n=l)	USA	[6]
	rabbit (n=9)	Switzerland	[9]
	mouse (n=1)	USA	[4]
	human (n=5)	Switzerland	[10]
4	dog (n=2)	USA	[4]
	human (n=1)	USA	[6]
	human (n=1)	USA	[4]
	human (n=1)	Switzerland	[10]

repeat in the ITS, and a further genotype for which the ITS DNA sequence was not determined. Taken together, 5 different genotypes can already be distinguished in *E. cuniculi* if both target sequences are considered.

ENCEPHALITOZOON INTESTINALIS AND *ENCEPHALITOZOON HELLEM*

Few data are available on possible strains in these 2 species. For *E. intestinalis*, differences in genotypes could not yet be reliably differentiated. A report claiming 3.1% differences in a 394 bp portion of the 16S rDNA [5] compared to an earlier 16S rDNA sequence [14] still awaits reproduction for either of the two isolates to confirm the absence of polymerase errors and sequencing artifacts. In a comparison of a 237 bp, ITS containing portion of the rRNA gene from *E. intestinalis* specimens from 5 different patients with AIDS, we did not detect any polymorphic sites (unpublished data).

In 2 reports on *E. hellem* rDNA sequences from 3 and 5 isolates from humans, respectively [6,13], identical sequences were found. However, the 305 bp portion common to both reports differed by 1 substitution and 1 insertion of 1 bp each near the 3' end of the 16S rDNA. Again, both sequences should be closely checked. There is only one report so far [10] where 2 genotypes could be differentiated within a single study. Together with another GeneBank submission (L19070), a total of 3 genotypes were described in the 16S coding region by detection of 9 polymorphic sites.

CONCLUSION

In order to find the reservoirs of microsporidia and understand how humans become infected it will be necessary to differentiate and characterize microsporidial strains. Molecular techniques offer the advantage of producing quantifiable, informative data that are comparably easy to obtain and independent of environmental influences. However, there is an urgent need to agree on the genomic targets to be investigated in order to make the data comparable between different laboratories. For *E. bieneusi*, the ITS of the rRNA gene is very promising, while for *Encephalitozoon* spp. alternative targets, for example the 16S rDNA must be considered because of the short length of their ITS (28 - 46 bp). Furthermore, suitable techniques must be employed to not only *minimize* polymerase and sequencing artifacts, but also to allow their subsequent detection, should they occur anyway. To simply trust, for example, proofreading polymerases or sequencing techniques with "very low" error rates does not suffice, if the identity of every nucleotide position is crucial in products of several 100's bp in length. The identity of at least 2 independently amplified and cloned DNA

sequences of each isolate is thus a minimal requirement. If differences are detected, more independentiy generated clones need to be characterized in order to detect polymerase errors and rare genetic polymorphisms. If multi-copy genes are considered, even more independent clones per isolate are needed to exclude the possibility of comparing paralogous gene copies. By consideration of these aspects, unnecessary work on uninformative target genes or methods that do not elucidate reliable primary DNA sequences can be avoided for the urgently needed generation of further, reliable and comparable data on strain differentiations in microsporidia as a prerequisite to find their reservoirs and the mode of their transmission.

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