ENTEROCYTOZOON BIENEUSI IN HUMAN AND ANIMALS, FOCUS ON LABORATORY IDENTIFICATION AND MOLECULAR EPIDEMIOLOGY

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Summary:

Human microsporidian infections have emerged following the onset of the AIDS pandemic. Microsporidia are unicellular eukaryotic parasites that form spores. They are an exceptionally diverse group of parasites that infect a wide range of eukaryotic cells in numerous invertebrate and vertebrate hosts. Of the 14 species newly described as pathogens in human, *Enterocytozoon bieneusi*, which causes gastrointestinal diseases, is the most common agent of human infections. In the past fifteen years, *E. bieneusi* was also identified in environmental sources, especially in surface water, as well as in wild, domestic and farm animals. These findings raised concerns for waterborne, foodborne and zoonotic transmission. Molecular analyses of the 243-bp internal Transcribed spacer-(ITS) of the rRNA gene have revealed a considerable genetic variation within *E. bieneusi* isolates of human and animal origins, supporting the potential for zoonotic transmission. The focus of this revue is to present and discuss recent advances in diagnosis and zoonotic potential of *E. bieneusi* infections.

KEY WORDS : Microsporidia, *Enterocytozoon bieneusi*, Internal Transcribed Spacer, diagnosis, molecular epidemiology, zoonotic.

The dramatic onset of the acquired immunodeficiency syndrome (AIDS) pandemic in the last 30 years, has completely changed the established knowledge of infectious diseases by favouring the emergence of numerous "new" pathogens in human, now qualified as opportunistic. This is particularly true concerning our perception of hosts-parasites interactions. Microsporidian infections, which are associated with a wide range of clinical symptoms in humans, illustrate well this new situation. While early investigation revealed that coccidian parasites were a leading cause of chronic diarrhoea in AIDS patients, the development of improved microscopic, immunological and molecular diagnostic methods has highlighted the importance of microsporidian infections in man (Didier & Weiss, 2006). Microsporidia are unicellular eukaryotic parasites that form spores. They are an exceptionally diverse group of parasites that infect a wide range of eukaryotic cells in numerous invertebrate and vertebrate hosts.

The phylum Microsporidia consists of more than 140 genera, with more than 1,200 species. Historically, these obligate intracellular parasites have long been recognized as pathogens in animal industries, first in silkworms in 1857, then in honeybees more than a century ago. They were later found in fish and more recently in mammals (reviewed in Mathis *et al.*, 2005). Prior to the middle of the 1980's only sporadic cases of microsporidia infections were well documented in humans (reviewed in Weber *et al.*, 1994). Today, 14 species in eight genera have been described as human pathogens (Didier, 2005). Two species, both responsible for gastrointestinal diseases, *Enterocytozoon bieneusi* Desportes *et al.*, 1985 and *Encephalitozoon intestinalis* (*Septata intestinalis* Cali *et al.*, 1993) Hartskeerl *et al.*, 1995, were the most frequently involved in cases of human infection (Desportes *et al.*, 1985; Hartskeerl, 1995). Both species have been found worldwide, but although hundreds of cases of *E. bieneusi* have been reported in the literature, only a tenfold lower rate of *E. intestinalis* cases are documented. It was further demonstrated that *E. bieneusi* can also infect immunocompetent patients, especially children, although the disease is less serious and the infection self-limited in the absence of immune abnormalities (Tumwine, 2002). Since mature spores are shed in the host's faeces, the transmission routes of this pathogen may involve person-to-person as well as waterborne or food-borne contaminations, especially in developing countries with low level of sanitation (Bern *et al.*, 2005). Together with the route of contamination, one of the first interrogations raised with the description of this new species was the origin of its reservoir.

Desportes *et al.*, first compared *E. bieneusi* with an enteric microsporidian *Microsporidium simiae* found in the Duski titi monkey *Callicebus moloch* (Desportes *et*

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al., 1985). The development of new tools for diagnosis and especially molecular methods have shown that *E. bieneusi* was present in a large number of domestic and wild mammals as well as birds (Haro *et al.*, 2006; Haro *et al.*, 2005; Kondova *et al.*, 1998; and reviewed in Mathis *et al.*, 2005). Thanks to molecular methods again, the potential of zoonotic transmission was quickly supported by phylogenetic studies showing that several genotypes can infect humans as well as animals (Curry, 1999; Dengjel *et al.*, 2001; Drosten *et al.*, 2005; Lobo *et al.*, 2006a; Santin *et al.*, 2006; Sulaiman *et al.*, 2003a; Sulaiman *et al.*, 2003b; Sulaiman *et al.*, 2004). The focus of this revue is to present and discuss the more recent advances on diagnosis and zoonotic potential of *Enterocytozoon bieneusi* infections.

THE ORGANISM

The life cycle of microsporidia includes, in a single cell, two consecutive stages, a proliferative merogonic stage, followed by a sporogonic stage, resulting in the production and emission in body fluids of numerous characteristic infective spores. The environmentally resistant spores (1 to 4 µm) have a typical three layers-thick wall and contain a remarkable long, coiled tubular extrusion apparatus, the polar tube. The polar tube has a crucial role in the invasion process, injecting the infective sporoplasm into the cytoplasm of the host cell. Microsporidia possess typical eukaryotic characteristics such as a true nucleus, an endomembrane system and a cytoskeleton. Other traits, including genome size and features of the transcriptional apparatus, are closer to that found in prokaryotes. Finally, some unique signatures (presence of chitin and trehalose, diplokaryotic stages in some species) and phylogenic analyses revealed a fungi and probably zygomycete origin of microsporidia (Canning *et al.*, 1998; Keeling, 2003; Mathis, 2000; Muller, 1997).

LABORATORY IDENTIFICATION OF THE MICROSPORIDIA

During the last twenty years, diagnostic methods to detect microsporidia infections have been remarkably improved. Several techniques for easy detection and species differentiation of microsporidia are now available.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

The TEM technique, which remains the gold standard for species identification, had first permitted the description and classification of Microsporidia genera and species. The examination of the intracellular prolife-rative forms, fine structure features of the spores, method of division and the nature of host-parasite interface are the main criteria used for diagnosis and species differentiation. TEM is very specific for diagnosis of infection but suffers from low sensitivity because of the small amount of sample that can be examined. Other drawbacks of the technique include high equipment cost, long sample preparation and considerable expertise. It is therefore not suitable for routine diagnosis (Field, 2002).

HISTOLOGICAL DIAGNOSIS

Histologic examination of tissue samples embedded in paraffin or after formalin fixation can be used in some clinical situations, both for diagnosis and to study physiopathological aspects of microsporidia infections inside infected cells. Various staining techniques have been described in the literature, and the more suitable are the fluorescent Uvitex 2B stain, Gram-derived stains, silver staining (Warthin-Starry), Giemsa stain and chromothrope 2R stains (Conteas *et al.*, 1996; Field *et al.*, 1993; franczen *et al.*, 1995; Kotler *et al.*, 1994; Simon, 1991).

NON SPECIFIC CYTOLOGIC DIAGNOSIS USING LIGHT MICROSCOPY

Microsporidia spores are the only developmental parasite stage that could be found in body fluids. They have been found in almost all human body fluids, but almost exclusively in stool or duodenal drainage for *E. bieneusi* species. In such a situation, the staining method must be able to stain the spores (content or/and wall) and to differentiate between Microsporidia spores and other elements present in samples, especially in stools. The main difficulties are that: *i)* spores are very small (from 1 µm for *E. bieneusi* to 3 µm for *E. intestinalis*); *ii)* identification of microsporidial spores in stool samples was very difficult since no staining technique existed when the importance of microsporidia intestinal infection in AIDS patients emerged in the 1980's. For clinical diagnosis, a concentration procedure appears to be helpful to increase the sensitivity (van Gool *et al.*, 1994). Specimens can be fresh or preserved (formalin or sodium acetate-acid solutions are most often used). Two main techniques have been developed successfully, and each one with various stains modifications. The modified trichrome stains (using chromotrope 2R) were first developed. In 1992, Weber *et al.* developed a technique based on the Wheatley's modification of the Gomori's trichrome stain with two important changes: a 10-fold higher concentration of the chromotrope 2R and a longer staining time (Weber *et al.*, 1992). Thereafter, several improvements to Weber's technique were proposed that allowed: *i)* to accelerate the process (in reducing the staining time by increasing the

staining temperature); and *ii)* to increase the contrast between spores and background (Kokoskin *et al.*, 1994; Moura *et al.*, 1997). In the same time, rapid and easy-to-perform staining methods were developed using chemo-fluorescent agents stains (Calcofluor White 2MR or Fungi-Fluor or Uvitex 2B) (van Gool *et al.*, 1993). The fluorescent agents bind chitin from the endospore. Globally, the sensitivity of both methods is good, but these types of stains are non specific and some bacteria, small fungi and artifactual material can interfere and give false positive results. Microscopists must be particularly experienced to perform these techniques with good performances (specificity), and the use of positive control material is highly recommended. Nevertheless, despite their quality of simplicity, moderate cost and good sensitivity, these techniques do not allow for species identification, which is absolutely necessary to start the relevant treatment. Infections caused by *E. intestinalis* were treated with albendazole (Molina *et al.*, 1998), while only fumagillin has been shown to be effective for eradicating *E. bieneusi* (Molina *et al.*, 2000).

SPECIFIC DIAGNOSIS

Polymerase chain reaction (PCR)-based methods are the most sensitive and specific tools available for diagnosis. A specificity and sensitivity of 100 % can be achieved under certain conditions. In addition they present the major advantage to allow further analysis to be achieved, such as genotypic identification at sub-species level. PCR diagnosis of *E. bieneusi* was first reported by Zhu *et al.*, 1993. Primers generally used for diagnosis target the small and large subunit rRNA genes (Franzen & Muller, 1995). The introduction of real time PCR, by reducing the risk of contamination, the labour time and the reagent cost, offer the possibility of a secure method suitable in routine diagnosis (Menotti *et al.*, 2003a, 2003b; Wolk *et al.*, 2002). Moreover, a multiplex real-time PCR method, which includes an internal control to detect inhibition of the amplification by components of the faecal matrix, was recently developed for the simultaneous detection of *E. bieneusi* and *Encephalitozoon* spp. in stool samples (Verweij *et al.*, 2007). However, despite the improvement described, these techniques remain costly, time consuming and need considerable expertise. In consequence, they are not readily available to many clinical laboratories, especially in non-industrialized countries.

IMMUNOFLUORESCENT REAGENTS

Immunofluorescent reagents using polyclonal or monoclonal antibodies (MAbs) directed against microsporidia spores present in body fluids seemed to be very helpful for diagnosis. Unfortunately, in faecal specimens, background staining, cross reactions with yeast and bacteria and poor sensitivity in comparison with chromotrope or chemofluorescent stains, prevented the use of polyclonal antibodies for routine diagnosis. More specific reagents using MAbs were then developed, but because of the lack of an *in vitro* culture system for *E. bieneusi*, these were limited to *Encephalitozoon* species spores (Aldras *et al.*, 1994; Beckers *et al.*, 1996; Enriquez *et al.*, 1997). Using spores from human faecal specimens obtained through an original method of purification, Accoceberry *et al.*, reported in 1999 the production and characterization of a species-specific MAb, 6E5-2D9, a mouse immunoglobulin G (IgG2a), raised against the exospore of *E. bieneusi* spore wall (Accoceberry *et al.*, 1999). Later, the same team produced a new MAb directed against *E. intestinalis* spore walls, usable under the same operational conditions (Thellier *et al.*, 2001). Antibody tests using these MAbs were further evaluated in comparison with species-specific PCR, and showed a similar level of performances (Alfa Cisse *et al.*, 2002; Breton *et al.*, 2007).

GENOTYPIC DIVERSITY AND HOST SPECIFICITY

A fundamental question raised with the discovery of new microsporidial species infecting immunocompromised patients was that of their natural origin. Is man a natural reservoir for the parasite or an accidental host in whom the infection becomes possible because of the deterioration of his immune system? For instance, after being identified in humans, spores of *E. bieneusi* were detected in numerous animal species, especially mammals but also birds, and in environmental source such as surface water, swimming pools or rivers (Coupe *et al.*, 2006; Dowd *et al.*, 1998; Fournier *et al.*, 2000; Haro *et al.*, 2006; Haro *et al.*, 2005; Santin *et al.*, 2004; Sparfel *et al.*, 1997). Analysis of the sequence of the 243-bp internal transcribed spacer rRNA gene has revealed a considerable genetic variation within *E. bieneusi* isolates of human and animal origins.

PHYLOGENETIC ANALYSIS

To better understand the genetic relationship among the *E. bieneusi* genotypes, alignment of the ITS region of 84 distinct sequences published to date was performed using the Muscle software. Then, a phylogram and a phylogenetic tree inferred by maximum-likelihood analysis were constructed using the PhyMl software. The most divergent sequence EntCanA isolated from a dog (GenBank accession number AF059610) was used as an outgroup in the phylogenetic analysis (similar methods to those used in Breton *et al.*, 2007), (Mathis *et al.*, 1999). The results are presented in Table I.

Genotypes*	Host/Country (Reference), *Human genotypes are in italics*	Accession number
Group 1		
CAF 3	*Human/Gabon* (Breton *et al.*, 2007)	DQ683748
Peru 8	*Human/Peru* (Sulaiman *et al.*, 2003a)	AY371283
Peru 10	***Human/Peru*** (Sulaiman *et al.*, 2003a), **Cat/Colombia** (Santin *et al.*, 2006)	**AY371285**
Peru 11 (Peru 12)	*Human/Peru* (Sulaiman *et al.*, 2003a), *Thailand* (Leelayoova *et al.*, 2006)	AY371286
Peru 13	*Human/Peru*&	EF014429
Peru 15	*Human/Peru*&	EF014431
Peru 17	*Human/Peru*&	EU140500
T	*Human/Thailand* (Leelayoova *et al.*, 2006)	AY945810
V	*Human/Thailand* (Leelayoova *et al.*, 2006)	AY945812
pigEBITS6	Swine/USA (Buckholt *et al.*, 2002)	AF348474
pigEBITS8	Swine/USA (Buckholt *et al.*, 2002)	AF348476
L	Cat/Germany (Dengjel *et al.*, 2001)	AF267142
EbfelA	Cat/Switzerland (Mathis *et al.*, 1999)	AF118144
4948-FL-2	Cattle/USA (Santin *et al.*, 2005)	DQ154136
WL7	Beaver/USA (Sulaiman *et al.*, 2003b)	AY237215
D (pigEBITS9, WL8, Peru 9, CEbC, PtEbVI)	***Human/Germany*** (Rinder *et al.*, 1998), **UK** (Sadler *et al.*, 2002), ***Peru*** (Sulaiman *et al.*, 2003a), ***Gabon, Cameroon*** (Breton *et al.*, 2007), ***Niger*** (Espern *et al.*, 2007), ***Thailand*** (Leelayoova *et al.*, 2006), ***Vietnam*** (Espern *et al.*, 2007), **Swine/USA** (Buckholt *et al.*, 2002), **Cattle/USA** (Santin *et al.*, 2005), **Korea** (Lee, 2007), **Macaque/USA** (Green *et al.*, 2004), **Germany** (Drosten *et al.*, 2005), **Muskrat, Racoon, Beaver, Fox/USA** (Sulaiman *et al.*, 2003b), **Falcon/UAE& (DQ793212, DQ793213), Dog/Portugal** (Lobo *et al.*, 2006a)	**AF101200**
Like-D	Cat/Colombia (Santin *et al.*, 2006)	DQ836345
pigEBITS5 (PEbA)	Swine/USA (Buckholt *et al.*, 2002), Korea (Jeong *et al.*, 2007)	AF348473
pigEBITS7	***Human/Thailand*** (Leelayoova *et al.*, 2006), **Swine/USA** (Buckholt *et al.*, 2002)	**AF348475**
Peru 6 (PtEbI, PtEbVII)	***Human/Peru*** (Sulaiman *et al.*, 2003a), **Pet Birds/Portugal** (Lobo *et al.*, 2006b), **Dog/Portugal**** (Lobo *et al.*, 2006a)	**AY371281**
PtEbII	Pet Birds/Portugal (Lobo *et al.*, 2006b)	DQ425108
Peru7	*Human/Peru* (Sulaiman *et al.*, 2003a)	AY371282
R	*Human/Thailand* (Leelayoova *et al.*, 2006)	AY945808
S	*Human/Thailand* (Leelayoova *et al.*, 2006)	AY945809
UG2145	*Human/Uganda* (Tumwine *et al.*, 2002)	AF502396
PtEbV	Kudu/Portugal (Lobo *et al.*, 2006a)	DQ885581
A (Peru 1)	*Human/Switzerland* (Breitenmoser *et al.*,1999), *Germany* (Rinder *et al.*, 1997), *Peru* (Sulaiman *et al.*, 2003a) *Thailand* (Leelayoova *et al.*, 2005), *Gabon, Cameroon* (Breton *et al.,* 2007), *Niger* (Espern *et al.*, 2007)	AF101197
B (Type I)	*Human/Switzerland* (Breitenmoser *et al.*,1999), *Germany* (Rinder *et al.*,1997), *France* (Liguory *et al.*, 2001; Liguory *et al.*, 1998), *UK* (Sadler *et al.*, 2002), *Cameroon* (Breton *et al.*, 2007)	AF101198
NIA1	*Human/Niger* (Espern *et al.*, 2007)	EF458628
Peru 3	*Human/Peru* (Sulaiman *et al.*, 2003a)	AY371278
CAF2	*Human/Gabon* (Breton *et al.*, 2007)	DQ683747
Type III	*Human/France* (Liguory *et al.*, 2001; Liguory *et al.*, 1998)	AF242477
Type V	*Human/France* (Liguory *et al.*, 1998)	AF242479
K (Type IV, Peru 2, BEB5, PtEbIII)	***Human/France*** (Liguory *et al.*, 2001; Liguory *et al.*, 1998), **UK** (Sadler *et al.*, 2002), ***Peru*** (Sulaiman *et al.*, 2003a), ***Uganda*** (Tumwine *et al.*, 2002), ***Gabon*** (Breton *et al.*, 2007), ***Cameroon*** (Sarfati *et al.*, 2006), ***Niger*** (Espern *et al.*, 2007), **Cat/Germany** (Dengjel *et al.*, 2001), **Colombia** (Santin *et al.*, 2006), **Portugal** (Lobo *et al.*, 2006a), **Cattle/USA** (Santin *et al.*, 2005; Sulaiman *et al.*, 2004), **Portugal** (Lobo *et al.*, 2006a)	**AF267141**
PtebIV	Dog/Portugal (Lobo *et al.*, 2006a)	DQ885580
P	Lama/Germany (Dengjel *et al.*, 2001)	AF267146
WL9	Beaver/USA (Sulaiman *et al.*, 2003b)	AY237217
WL10	Muskrat/USA (Sulaiman *et al.*, 2003b)	AY237218
WL11 (Peru 5)	***Human/Peru*** (Sulaiman *et al.*, 2003a), **Fox/USA** (Sulaiman *et al.*, 2003b), **Cat/Colombia** (Santin *et al.*, 2006)	**AY237219**
WL12	Beaver, Otter/USA (Sulaiman *et al.*, 2003b)	AY237220
E (EbpC, WL13, Peru 4)	***Human/Peru*** (Sulaiman *et al.*, 2003a), ***Thailand*** (Leelayoova et al., 2006), ***Vietnam*** (Espern *et al.*, 2007), **Swine/Switzerland** (Breitenmoser *et al.*, 1999), **Muskrat, Racoon, Beaver, Fox, Otter/USA** (Sulaiman *et al.*, 2003b)	**AF135832**
HAN1	*Human/Vietnam* (Espern *et al.*, 2007)	EF458627
WL14	Muskrat/USA (Sulaiman *et al.*, 2003b)	AY237222
WL15 (Peru 14)	***Human/Peru*& (EF014430)**, **Muskrat, Racoon, Beaver, Fox/USA** (Sulaiman *et al.*, 2003b)	**AY237223**
WL16	Muskrat, Racoon/USA (Sulaiman *et al.*, 2003b)	AY237224
WL17	Racoon/USA (Sulaiman *et al.*, 2003b)	AY237225

Groups (leftmost column): 1a, 1b, 1c, 1d

Table I. *(To be continued).*

	Genotypes*	Host/Country (Reference), *Human genotypes are in italics*	Accession number
1e	EbpB	Swine/Switzerland (Breitenmoser *et al.*, 1999)	AF076041
	EbpD	Swine/Switzerland (Breitenmoser *et al.*, 1999)	AF076043
	F (EbpA)	Swine/Switzerland (Breitenmoser *et al.*, 1999), Germany (Dengjel *et al.*, 2001; Rinder *et al.*, 2000), Cattle/Germany (Dengjel *et al.*, 2001)	AF135833
	G	Swine/Germany (Rinder *et al.*, 2000)	AF135834
	H (PebC)	Swine/Germany (Rinder *et al.*, 2000), Korea (Jeong *et al.*, 2007))	AF135835
	O	*Human/Thailand* (Leelayoova *et al.*, 2006), **Swine/Germany** (Dengjel *et al.*, 2001)	**AF267145**
	M	Cattle/Germany (Dengjel *et al.*, 2001)	AF267143
	pigEBITS1	Swine/USA (Buckholt *et al.*, 2002)	AF348469
	pigEBITS2	Swine/USA (Buckholt *et al.*, 2002)	AF348470
	pigEBITS3 (PebB)	Swine/USA (Buckholt *et al.*, 2002), Korea (Jeong *et al.*, 2007)	AF348471
	pigEBITS4 (PebD)	Swine/USA (Buckholt *et al.*, 2002), Korea (Jeong *et al.*, 2007)	AF348472
	U	*Human/Thailand* (Leelayoova *et al.*, 2006)	AY945811
	W	*Human/Thailand* (Leelayoova *et al.*, 2006)	AY945813
	CEbD	Cattle/Korea (Lee, 2007)	EF138198
1f	C (Type II)	*Human/France* (Liguory *et al.*, 2001; Liguory *et al.*, 1998), *Switzerland* (Breitenmoser *et al.*, 1999), *Germany* (Dengjel *et al.*, 2001)	AF101199
	Q	*Human/Germany* (Dengjel *et al.*, 2001)	AF267147
1g	**CAF1 (PEbE)**	*Human/Gabon* (Breton *et al.*, 2007), **Niger** (Espern *et al.*, 2007), **Swine/Korea** (Jeong *et al.*, 2007)	**DQ683746**
1h	**Peru 16**	*Human/Peru* (Sulaiman *et al.*, 2003a), **Guinea pig/Peru** (Cama *et al.*, 2007)	**EF014427**
	Group 2		
	I (CEbE)	Cattle/Germany (Dengjel *et al.*, 2001; Rinder *et al.*, 2000), Cattle/Korea (Lee, 2007)	AF135836
	J (BEB1, PtEbX, CEbB)	Cattle/Germany (Dengjel *et al.*, 2001; Rinder *et al.*, 2000), USA (Santin *et al.*, 2005; Sulaiman *et al.*, 2004), Korea (Lee, 2007), Portugal (Lobo *et al.*,2006a), Chicken/Germany (Reetz *et al.*, 2002)	AF135837
	N (BEB2)	Cattle/Germany (Dengjel *et al.*, 2001), USA (Santin *et al.*, 2005; Sulaiman *et al.*, 2004)	AF267144
	BEB3	Cattle/USA (Santin *et al.*, 2005; Sulaiman *et al.*, 2004)	AY331007
	BEB4	Cattle/USA (Santin *et al.*, 2005; Sulaiman *et al.*, 2004)	AY331008
	CEbA	Cattle/Korea (Lee, 2007)	EF138195
	CEbF	Cattle/Korea (Lee, 2007)	EF138194
	PtEbXI	Cattle/portugal (Lobo *et al.*,2006a)	DQ885587
	BEB6	Cattle/USA (Fayer *et al.*, 2007)	EU153584
	BEB7	Cattle/USA (Fayer *et al.*, 2007)	EU153585
	Group 3		
	WL4	Muskrat/USA (Sulaiman *et al.*, 2003b)	AY237212
	WL5	Muskrat/USA (Sulaiman *et al.*, 2003b)	AY237213
	WL6	Muskrat/USA (Sulaiman *et al.*, 2003b)	AY237214
	PtEbVIII	Cat/Portugal (Lobo *et al.*,2006a)	DQ885584
	Group 4		
	WL1	Racoon/USA (Sulaiman *et al.*, 2003b)	AY237209
	WL2	Racoon/USA (Sulaiman *et al.*, 2003b)	AY237210
	WL3	Racoon/USA (Sulaiman *et al.*, 2003b)	AY237211
	Group 5		
	CAF4	*Human/Gabon, Cameroon* (Breton *et al.*, 2007)	DQ683749
	PtEbXII	Marmoset/Portugal (Lobo *et al.*,2006a)	DQ885588
	Outliers		
	EntCanA (PtEbIX)	Dog/Switzerland (Mathis *et al.*, 1999), Portugal (Lobo *et al.*,2006a)	AF059610

* Abbreviations in brackets refer to the other genotype names assigned to identical sequences by various research teams; ** Reported as Peru6 and Peru6-var; & Unpublished.

Table I. – Epidemiological traits of the *Enterocytozoon bieneusi* genotypes. Genotypes found both in human and animals are listed in grey zones.

Using this method, five main groups (major clusters) numbered 1 to 5, were segregated from the most divergent sequence EntCanA. The first group, far the largest, included all except one of the previously reported *E. bieneusi* isolates from humans. The exception being one genotype, called CAF4, isolated recently from HIV-positive patients in Gabon and HIV-negative individuals in Cameroon (Breton *et al.*, 2007). This genotype is the most highly divergent genotype reported in human to date. In our analysis it formed a distinct major cluster, named Group 5, together with another genotype named PtEb XII isolated from a

marmoset in Portugal (Lobo *et al.*, 2006a). Marmosets originate from the primary forest in South America, so, it could indicate that similar hosts possibly exist in Central Africa. The other major clusters named Group 2, Group 3 and Group 4 are composed of genotypes only found in animals. Group 4 is to date host specific and found only in raccoon (Sulaiman *et al.*, 2003b). Group 3 is composed of three genotypes isolated from muskrat in USA and one isolated from a cat in Portugal (Lobo *et al.*, 2006a; Sulaiman *et al.*, 2003b). Group 2 consisted of genotypes isolated in farm animals, almost exclusively from cattle (10 genotypes), with one genotype also found in chicken (Dengjel *et al.*, 2001; Fayer *et al.*, 2007; Lee, 2007; Lobo *et al.*, 2006a; Reetz *et al.*, 2002; Rinder *et al.*, 2000; Santin *et al.*, 2005; Sulaiman *et al.*, 2004). Interestingly genotypes from cattle were found in very different countries and continents (Germany, Portugal, USA and Korea).

The analysis of the distribution of genotypes within the Group 1 is more complicated since it includes numerous genotypes from various origins: human, both HIV-positive and negative, but also domestic and wild animals. In our analysis this group is clearly subdivided in eight major clades named subgroup 1a to 1 h. Within subgroup 1a, eight genotypes were found only in HIV infected patients in Gabon, Peru and Thailand (Breton *et al.*, 2007; Leelayoova *et al.*, 2005; Sulaiman *et al.*, 2003a). Eight again were isolated from animals only, domestic such as swine, cattle and cat but also wild such as beaver (Buckholt *et al.*, 2002; Dendjel *et al.*, 2001; Jeong *et al.*, 2007; Mathis *et al.*, 1999; Santin *et al.*, 2005; Santin *et al.*, 2006; Sulaiman *et al.*, 2003b). More interestingly, three genotypes were found both in human and animals. Two of them were found in human, and to date in a single animal host: genotype pigEBITS7 was found in HIV-positive patients in Thailand and swine in USA while genotype Peru 10 was found in HIV-positive patients in Peru and cat in Colombia (Buckholt *et al.*, 2002; Leelayoova *et al.*, 2006; Sulaiman *et al.*, 2003a). The remaining genotype, named D, is very widespread: it was first found in human in Germany, then in other countries in Americas, Asia and Africa. It was also found in numerous and various animals (swine, cattle, macaque, muskrat, raccoon, beaver, fox, dog and falcon) (Breton *et al.*, 2007; Buckholt *et al.*, 2002; Drosten *et al.*, 2005; Espern *et al.*, 2007; Green *et al.*, 2004; Lee, 2007; Leelayoova *et al.*, 2006; Lobo *et al.*, 2006a; Rinder *et al.*, 1998; Sadler *et al.*, 2002; Santin *et al.*, 2006; Sulaiman *et al.*, 2003a, 2003b) (Table I). Type D was commonly reported in HIV-positive patients in Thailand (leelayoova *et al.*, 2006) and in Peru Sulaiman *et al.*, 2003a), and in two isolated cases in Europe (Rinder *et al.*, 1998; Sadler *et al.*, 2002). It represented 15 % of isolates from four species of wildlife animals in North America (Sulaiman *et al.*, 2003b) and 26 % of isolates found in cats in Colombia (Santin

et al., 2006), supporting a zoonotic route of transmission for this strain. Interestingly, type D, was recently isolated from 3 HIV-negative individuals in Cameroon (Breton *et al.*, 2007).

Within subgroup 1b, four genotypes were found only in HIV-positive patients, in Peru, Thailand and Uganda, two genotypes were found only in animal, both in pet birds in Portugal, and one genotype named Peru 6 was found both in human in Peru and in animals, pet birds and a dog, both in Portugal (Leelayoova *et al.*, 2006; Lobo *et al.*, 2006b; Sulaiman *et al.*, 2003a; Tumwine *et al.*, 2002). Subgroup 1c contains seven genotypes that were found only in human (Breitenmoser *et al.*, 1999; Breton *et al.*, 2007; Espern *et al.*, 2007; Leelayoola *et al.*, 2005; Liguory *et al.*, 2001; Liguory *et al.*, 1998; Rinder *et al.*, 1997; Sadler *et al.*, 2002; Sulaiman *et al.*, 2003a). One genotype named B appear to be prevalent in Europe while genotype A was found in four continents Europe, South America, Asia and Africa. Five genotypes were found only in animal (Dengel *et al.*, 2001; lobo *et al.*, 2006a; Sulaiman *et al.*, 2003b). Two genotypes named WL11 and K were found both in human and animal. To date WL11 was found in HIV-positive patients in Peru only, and in fox and cat, in USA and Columbia respectively (Santin *et al.*, 2004; Sulaiman *et al.*, 2003a, 2003b). Genotype K is most largely widespread since it was isolated in human in Europe, South America and Africa and in domestic animals (cat, and cattle) in the same continents (Breton *et al.*, 2007; Dengjel *et al.*, 2001; Espern *et al.*, 2007; Liguory *et al.*, 2001; Liguory *et al.*, 1998; Lobo *et al.*, 2006a; Sadler *et al.*, 2002; Santin *et al.*, 2006; Sarfati *et al.*, 2006; Sulaiman *et al.*, 2003a, 2003b; Tumwine *et al.*, 2002). It is to be noted that, on the contrary to types A and B, type K may be common in developing countries but rare in Europe (Liguory *et al.*, 2001; Sadler *et al.*, 2002). Within subgroup 1d, one genotype was found only in HIV-positive patients in Vietnam and three genotypes were found only in wild animals in USA (Espern *et al.*, 2007; Sulaiman *et al.*, 2003b). Here again, some genotypes, E and WL15, were found both in human and animals. Type E, has been reported from HIV-positive patients in Peru, Thailand and Vietnam (Espern *et al.*, 2007; Leelayoova *et al.*, 2006; Sulaiman *et al.*, 2003a) from swine in Switzerland (Breitenmoser *et al.*, 1999), and from wild mammals in the USA (Sulaiman *et al.*, 2003b). In subgroup 1e, all genotypes but two (n = 14) were isolated from animals. The animals involved were swine with ten genotypes isolated in Switzerland, Germany, Korea and Thailand and cattle with three genotypes from Germany and Korea (Breitenmoser *et al.*, 1999; Buckholt *et al.*, 2002; Dengjel *et al.*, 2001; Jeong *et al.*, 2007; Lee, 2007; Rinder *et al.*, 2000). The two exceptions were genotypes isolated in HIV-positive human in Thailand (Leelayoova *et al.*, 2006). Finally only one genotype named O was found

both in HIV-positive patients in Thailand and in swine in Germany (Dengjel *et al.*, 2001; leelayoova *et al.*, 2006). The subgroup 1f, 1g and 1h contained very few genotypes, 2, 1 and 1 respectively, isolated from very few studies (Breitenmoser *et al.*, 1999; Breton *et al.*, 2007; Cama *et al.*, 2007; Dengjel *et al.*, 2001; Espern *et al.*, 2007; Jeong *et al.*, 2007; Liguory *et al.*, 2001; Liguory *et al.*, 1998). Nevertheless subgroup 1h appear to be of a particular interest since genotype Peru 16 was to date the single isolate found in the same location in human and animals, a young child and its domestic guinea pig in Peru (Cama *et al.*, 2007).

To date, and considering the relatively low number of sequences analyzed, the number (n = 84) of different genotypes appear very high. However, at the moment very subtle differences (one base pair) between sequences are considered, which might not be relevant for genotype discrimination. Additional independent molecular markers are highly desirable in order to validate and clarify the *E. bieneusi* population's genetic structure.

Overall, the analysis of *E. bieneusi* ITS genotypes highlight the complexity of the species phylogeny and the particular ability of this parasite to adapt and develop in numerous and various hosts. Moreover, the parasitism of this species seems to be well tolerated since, in most of the cases reported in animal or human populations, it has minor effects on the health of the infected individuals. Even in AIDS patients, the pathogenicity of *E. bieneusi* was questioned since some authors have not found any association between intensity of microsporidia infection and clinical symptoms (Clarridge *et al.*, 1996; Rabeneck *et al.*, 1993). It is now clear that the parasite is most often symptomatic in the youngest individuals, human or animals, and in AIDS patients with a very low CD4 level (Gumbo *et al.*, 1999; Jeong *et al.*, 2007). In the light of actual knowledge, it seems that concerning AIDS patients, the clinical situation is linked with the genotypes of the *E. bieneusi* "subspecies" involved. According to Bern *et al.* in a risk factor study in Lima, some genotypes would be related to pathogenicity whereas some others are not (Bern *et al.*, 2005).

So, the epidemiology of *E. bieneusi* is still unclear. The available data stress the need for further molecular studies that will combine human, animal and environmental sources to better understand the relationships between genetic diversity, host specificity and pathogenicity of *E. bieneusi*.

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