MOLECULAR EPIDEMIOLOGY OF HUMAN MICROSPORIDIOSIS CAUSED BY *ENTEROCYTOZOON BIENEUSI*

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Abstract. *Enterocytozoon bieneusi* is a common human pathogen, responsible for more than 90% of overall human microsporidian infections. The epidemiology of *E. bieneusi* infections, however, is not well studied, and little is known about its transmission routes. *Enterocytozoon bieneusi*-like spores have been reported in surface and ground water in the United States and France, and a possible outbreak of microsporidiosis involving 200 persons was associated with drinking water in France. Recently, genotyping tools based on DNA sequencing of the internal transcribed spacer of the rRNA gene have been used in elucidating *E. bieneusi* transmission. Studies conducted in Europe (Switzerland, France, Germany, and UK) and Uganda identified nine *E. bieneusi* genotypes in humans. A more recent study in Peru with a larger sample size confirmed the presence of three of the nine genotypes in humans, but also found eight other genotypes that have not been found in humans before. As expected, geographic differences are present in the distribution of *E. bieneusi* genotypes in humans, with genotype B being the predominant genotype in Europe and genotype A the most common genotype in Peru. Four of the human-pathogenic genotypes have been found in domestic animals and wild mammals, and many of the 40 or so *Enterocytozoon* genotypes identified in animals are genetically related to the human-pathogenic genotypes, indicating that zoonotic transmission of *E. bieneusi* is possible. Nevertheless, host-adapted *Enterocytozoon* spp have been found in dogs, cattle, and some wild mammals (muskrats and raccoons), which do not have apparent public health significance. Further studies of *E. bieneusi* transmission in humans and animals in other regions should be helpful to elucidate the epidemiology of microsporidiosis.

INTRODUCTION

Among the 14 or so human-pathogenic microsporidia species, *Enterocytozoon bieneusi* is the most common. It is one of the common causes of chronic diarrhea in AIDS patients (van Gool *et al*, 1995), and has recently been detected in HIV-negative persons (Lores *et al*, 2000a). In addition to causing human disease, it has also been frequently found in many animals, especially mammals (del Aguila *et al*, 1999; Lores *et al*, 2002a).

Little is known about the transmission routes of human microsporidiosis. Nevertheless, human-pathogenic microsporidia have been reported in surface and ground water in the United States (Dowd *et al*, 1998). In France, a municipal water supply was suspected as the source of a possible outbreak of microsporidiosis involving 200 persons (Cotte *et al*, 1999), and spores of *E. bieneusi* were detected in river water (Sparfel *et al*, 1997; Fournier *et al*, 2000). Recently, several DNA sequencing tools based on the internal transcribed spacer of the rRNA gene have been developed to genotype *E. bieneusi* isolates from humans and animals, which has helped to clarify some issues related to the transmission of *E. bieneusi* in humans (Rinder *et al*, 1997; Liguory *et al*, 1998; Breitenmoser *et al*, 1999; Buckholt *et al*, 2002; Sulaiman *et al*, 2003b).

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E. BIENEUSI INFECTION IN HUMANS

Enterocytozoon bieneusi was first identified as a human pathogen at the beginning of the AIDS epidemic (Desportes *et al*, 1985). Since then, it has been shown to be a major cause of chronic diarrhea in HIV-positive persons, with infection rates varying widely among different studies, even though most studies have reported rates between 10 and 30% in AIDS patients with chronic diarrhea. Low CD4+ cell count is a major risk factor for *E. bieneusi* infection in HIV-positive persons, with most infected patients having CD4 cell counts lower than 100 cells/μl (Navin *et al*, 1999). Other risk factors for *E. bieneusi* infection are not clear, but a study conducted in Zimbabwe showed an association of *E. bieneusi* infection in AIDS patients with living in rural areas, consumption of nonpiped water, contact with cow dung, and household contact with an individual with diarrhea (Gumbo *et al*, 1999). No significant seasonal variation in the prevalence of

intestinal microsporidiosis was seen in AIDS patients in the US in a multi-year study (Conteas *et al*, 1998).

Even though sporadic cases of *E. bieneusi* infection have been known for some time, especially in association with travel to Africa (Fournier *et al*, 1998), it has been only recently that *E. bieneusi* was considered a pathogen in immunocompetent persons (Table 1). An early study conducted in Niger showed the presence of *E. bieneusi* in 8 of 990 HIV-negative children (Bretagne *et al*, 1993). Two recent studies in Thailand and Zimbabwe, however, have reported much higher infection rates (13/87 and 2/6, respectively) in HIV-negative children or adults with diarrhea (Gumbo *et al*, 2000; Wanachiwanawin *et al*, 2002). Whether *E. bieneusi* causes diarrhea in children is not yet clear, as one large-scale study in Uganda recently has shown similar *E. bieneusi* infection rates in children with or without diarrhea (310/1,779 or 17.4% in children with diarrhea, *versus* 112/667 or 16.8% in children without diarrhea) (Tumwine *et al*, 2002). Nevertheless, children with *E. bieneusi* infection had significantly longer diarrhea than those without infection. A study conducted in Spain showed a similar high prevalence rate of *E. bieneusi* in elderly patients (8 of 60; 17.0%) (Lores *et al*, 2002b). In Uganda, *E. bieneusi* infection in children was more common in rainy seasons and was strongly associated with younger age (Tumwine *et al*, 2002).

E. BIENEUSI INFECTIONS IN ANIMALS

Enterocytozoon bieneusi or *E. bieneusi*-like organisms have also been found in many mammals, including dogs, cats, cattle, pigs, rhesus monkeys, and a llama (Rinder *et al*, 1998, 2000; Breitenmoser *et al*, 1999; del Aguila *et al*, 1999; Mathis *et al*, 1999; Chalifoux *et al*, 2000; Dengjel *et al*, 2001; Buckholt *et al*, 2002; Lores *et al*, 2002a; Fayer *et al*, 2003; Sulaiman *et al*, 2003b). More recently, *E. bieneusi* was detected for the first time in a non-mammalian host, chickens (Reetz *et al*, 2002). Even though most of the reports represent individual case reports or descriptions, recent surveys in pigs, cattle, and wild mammals indicate that *E. bieneusi* infection is common in animals. A slaughterhouse study in Massachusetts, USA showed a 32% prevalence rate of *E. bieneusi* in adult pigs (Buckholt *et al*, 2002). A survey of farms in the eastern United States reported an *E. bieneusi* infection rate of 3.1% in calves (Fayer *et al*, 2003). We have also shown a 12.7% overall infection rate of *Enterocytozoon* spp in muskrats, raccoons, beavers, otters, and foxes in four counties in Maryland, USA (Sulaiman *et al*, 2003b). In pigs, the prevalence of *E. bieneusi* infection is highest during summer in the United States (Buckholt *et al*, 2002).

The species nature of *Enterocytozoon* in animals is not entirely clear. Even though the term *E. bieneusi* is frequently used in describing *Enterocytozoon* from animals, recent molecular studies have suggested that some isolates from certain animals are genetically distinct from *E. bieneusi* in humans. For example, a genotype found in dogs (AF059610) showed significant sequence differences from *E. bieneusi*, not only in the internal transcribed spacer (ITS) region, but also in the small and large subunit regions of the rRNA gene (Mathis *et al*, 1999). Similarly, some isolates in muskrats, raccoons, and cattle are genetically so different from *E. bieneusi* that they may represent individual species (Sulaiman *et al*, 2003b, 2004).

Table 1
Prevalence of *Enterocytozoon bieneusi* in immunocompetent persons in various studies.

Study location	Age group	Nature of diarrhea	Prevalence of microsporidiosis	Species	Reference
Niger	Children	Unknown	8/990 (0.8%)	*E. bieneusi*	Bretagne *et al*, 1993
Thailand	Children	Yes	13/87 (14.9%)	*E. bieneusi*	Wanachiwanawin *et al*, 2002
Uganda	Children	Yes/no	With diarrhea: 310/1,779 (17.4%) Without diarrhea: 112/667 (16.8%)	*E. bieneusi*	Tumwine *et al*, 2002
Cuba	Adults	Yes/no	0/136	-	Escobedo and Nunez, 1999
Zimbabwe	Adults	Yes	2/6 (33.3%)	*E. bieneusi*	Gumbo *et al*, 2000
Spain	Elderly	Yes (47/60)	8/60 (17.0%)	*E. bieneusi*	Lores *et al*, 2002

Table 2
Summary of the ITS genotypes of *Enterocytozoon* spp.

Genotype	Host	GenBank accession no.	Reference
A, Peru1	Human	AF101197, AY371276	Rinder *et al*, 1997; Breitenmoser *et al*, 1999; Sulaiman *et al*, 2003a
B, Type I	Human	AF101198, AF242475	Rinder *et al*, 1997; Liguory *et al*, 1998
C, Type II	Human	AF101199, AF242476	Rinder *et al*, 1997; Liguory *et al*, 1998; Breitenmoser *et al*, 1999; Dengjel *et al*, 2001
Q	Human	AF267147	Rinder *et al*, 2000
Type III	Human	AF242477	Liguory *et al*, 1998
Type V	Human	AF242479	Liguory *et al*, 1998
UG2145	Human	AF502396	Tumwine *et al*, 2002
Peru3	Human	AY371278	Sulaiman *et al*, 2003a
Peru6	Human	AY371281	Sulaiman *et al*, 2003a
Peru7	Human	AY371282	Sulaiman *et al*, 2003a
Peru8	Human	AY371283	Sulaiman *et al*, 2003a
Peru10	Human	AY371285	Sulaiman *et al*, 2003a
Peru11	Human	AY371286	Sulaiman *et al*, 2003a
D, PigEBITS9, WL8, Peru9	Human, macaque, pig, fox, beaver, muskrat, raccoon	AF101200, AF023245, AF348477, AY237216, AY371284	Rinder *et al*, 1999, Chalifoux *et al*, 2000, Buckholt *et al*, 2001, Sulaiman *et al*, 2003b, 2003b
Type IV, K, BEB5, Peru2	Human, cat, cattle	AF242478, AF267141, AY331009, AY371277	Liguory *et al*, 1998, Dengjel *et al*, 2001; Tumwine *et al*, 2002; Sulaiman *et al*, 2003a, 2004
EbpC, E, WL13, Peru4	Human, pig, beaver, muskrat, raccoon, otter, fox	AF076042, AF135832, AY237221, AY371279	Deplazes *et al*, 1996, Breitenmoser *et al*, 1999, Rinder *et al*, 2000, Sulaiman *et al*, 2003b
WL11, Peru5	Human, fox	AY237219, AY371280	Sulaiman *et al*, 2003a, 2003a
M	Cattle	AF267143	Dengjel *et al*, 2001
I, BEB2	Cattle	AF135836, AY331006	Rinder *et al*, 2000, Sulaiman *et al*, 2004
J, BEB1	Cattle, chicken	AF135837, AY331005	Rinder *et al*, 2000, Dengjel *et al*, 2001, Reetz *et al*, 2002, Sulaiman *et al*, 2004
N	Cattle	AF267144	Dengjel *et al*, 2001
BEB3	Cattle	AY331007	Sulaiman *et al*, 2004
BEB4	Cattle	AY331008	Sulaiman *et al*, 2004
L	Cat	AF267142	Dengjel *et al*, 2001
EbfelA	Cat	AF118144	Mathis *et al*, 1999
Enterocytozoon sp	Dog	AF059610	Mathis *et al*, 1999
P	Llama	AF267146	Dengjel *et al*, 2001

Table 2 (continued)

Genotype	Host	GenBank accession no.	Reference
EbpA, F	Pig, cattle	AF076040, AF135833	Breitenmoser *et al*, 1999, Rinder *et al*, 2000, Dengjel *et al*, 2001
EbpB	Pig	AF076041	Breitenmoser et al. 1999
EbpD	Pig	AF076043	Breitenmoser *et al*, 1999
G	Pig	AF135834	Rinder *et al*, 2000
H	Pig	AF135835	Rinder *et al*, 2000
O	Pig	AF267145	Dengjel *et al*, 2001
PigEBITS1	Pig	AF348469	Buckholt *et al*, 2001
PigEBITS2	Pig	AF348470	Buckholt *et al*, 2001
PigEBITS3	Pig	AF348471	Buckholt *et al*, 2001
PigEBITS4	Pig	AF348472	Buckholt *et al*, 2001
PigEBITS5	Pig	AF348473	Buckholt *et al*, 2001
PigEBITS6	Pig	AF348474	Buckholt *et al*, 2001
PigEBITS7	Pig	AF348475	Buckholt *et al*, 2001
PigEBITS8	Pig	AF348476	Buckholt *et al*, 2001
WL1	Raccoon	AY237209	Sulaiman *et al*, 2003b
WL2	Raccoon	AY237210	Sulaiman *et al*, 2003b
WL3	Raccoon	AY237211	Sulaiman *et al*, 2003b
WL4	Muskrat	AY237212	Sulaiman *et al*, 2003b
WL5	Muskrat	AY237213	Sulaiman *et al*, 2003b
WL6	Muskrat	AY237214	Sulaiman *et al*, 2003b
WL7	Beaver	AY237215	Sulaiman *et al*, 2003b
WL9	Beaver	AY237217	Sulaiman *et al*, 2003b
WL10	Muskrat	AY237218	Sulaiman *et al*, 2003b
WL12	Beaver, otter	AY237220	Sulaiman et al. 2003b
WL14	Muskrat	AY237222	Sulaiman *et al*, 2003b
WL15	Beaver, muskrat, raccoon, fox	AY237223	Sulaiman *et al*, 2003b
WL16	Muskrat, raccoon	AY237224	Sulaiman *et al*, 2003b
WL17	Raccoon	AY237225	Sulaiman *et al*, 2003b

MOLECULAR EPIDEMIOLOGY OF *E. BIENEUSI* INFECTION

Several genotyping tools have been developed to characterize *Enterocytozoon* spp from humans and animals (Rinder *et al*, 1997; Liguory *et al*, 1998; Breitenmoser *et al*, 1999; Buckholt *et al*, 2002; Sulaiman *et al*, 2003b). All these tools are based on PCR analysis of the ITS of the rRNA gene. Most tools employ DNA sequencing of the PCR products and comparison of obtained ITS sequences with those in the databases. One tool, however, uses restriction fragment length polymorphism (RFLP) analysis to differentiate genotypes (Liguory *et al*, 1998). Thus far, more than 50 genotypes of *E. bieneusi* and *E. bieneusi*-like organisms have been described in humans, pigs, cattle, dogs, cats, muskrats, raccoons, beavers, otters, foxes, etc (Table 2). Because there are only a few such studies of small numbers of persons and animals, it is likely that genotypes described so far are only the tip of the iceberg of total genotypes present in humans and animals. Thus far, most of the described genotypes are from humans, pigs, cattle, and five species of wild mammals. There is no standard nomenclature for *Enterocytozoon* genotypes, and the same genotype frequently has different names by different investigators (Table 2).

Seventeen *E. bieneusi* genotypes have been found in humans, in Germany, Switzerland, France, UK, Uganda, and Peru (Table 2). Usually several *E. bieneusi* genotypes circulate in humans in a given area, even though it is unclear whether this is an indication of

multiple transmission routes (Table 3). For example, a recent study of samples from 89 HIV-positive patients in Lima, Peru showed the presence of 11 *E. bieneusi* genotypes (Sulaiman *et al*, 2003a). Fewer genotypes were seen in earlier studies in other areas, but the number of isolates examined was mostly small (Table 3). The only other studies characterizing a large number of samples used PCR-RFLP in genotyping, which probably underestimated the genetic diversity of the *E. bieneusi* presented (Liguory *et al*, 2001).

It seems that different *E. bieneusi* genotypes predominate in different human populations (Table 3). In France, genotype B (Type I) was the most abundant (75%) genotype in the 88 HIV-positive patients characterized (Liguory *et al*, 2001). Genotype B was also the most prevalent *E. bieneusi* genotype in 25 patients analyzed in Germany and Switzerland (in 12 patients), and 13 patients in northwest England (in 11 patients) (Dengjel *et al*, 2001; Sadler *et al*, 2002). In contrast, in a recent study of HIV-positive persons in Peru, genotype A (Peru1) was the most prevalent *E. bieneusi* genotype (39%), and six of the 11 genotypes identified in Peru have never been seen in other areas before (Sulaiman *et al*, 2003a). Nevertheless, some *E. bieneusi* genotypes have wide geographic distributions. For example, genotype K has so far been found in humans in all three continents studied: Europe (France and England), Africa (Uganda), and South America (Peru) (Table 3). It has also been found in a cat in Germany and in cattle in Portugal and the United States (Dengjel *et al*, 2001; Sulaiman *et al*, 2004).

There are also multiple *E. bieneusi* genotypes in animals. At least eight *E. bieneusi* genotypes have been described in pigs in Germany and Switzerland in several reports (Breitenmoser *et al*, 1999; Rinder *et al*, 2000; Dengjel *et al*, 2001). A more recent study in Massachusetts showed the presence of 11 *E. bieneusi* genotypes in adult pigs, two of which were previously reported (Buckholt *et al*, 2002). Thus, there are at least 17 *E. bieneusi* genotypes in pigs. Likewise, there are also at least seven *Enterocytozoon* genotypes in cattle (Rinder *et al*, 2000; Dengjel *et al*, 2001; Sulaiman *et*

Table 3
Enterocytozoon bieneusi ITS genotypes in humans in different geographic areas.

Location	Patient type (n)	Genotype (n)	Reference
France	HIV+ (88) HIV- (12)	Type I/B (66) Type II/C (18) Type III (3) Type IV/K (12) Type V (1)	Liguory *et al*, 1998, 2001
Germany Switzerland	HIV+ (25)	A (6) B (12) C (5) D (1) Q (1)	Rinder *et al*, 1997; Dengjel *et al*, 2001
UK	HIV+ (13)	B (11) D (1) K (1)	Sadler *et al*, 2002
Uganda	HIV- children (10)	K (6) UG2145 (4)	Tumwine *et al*, 2002
Peru	HIV+ (89)	Peru1/A (35) Peru2/Type IV/K/BEB5 (18) Peru3 (1) Peru4/E/EbpC/WL13 (1) Peru5/WL11 (3) Peru6 (1) Peru7 (8) Peru8 (4) Peru9/D/*Pig*EBITS9/WL8 (9) Peru10 (3) Peru11 (6)	Sulaiman *et al*, 2003a

al, 2004). A recent survey of 465 wildlife specimens in Maryland showed the presence of 17 *Enterocytozoon* genotypes in beavers, foxes, muskrats, otters, and raccoons, 14 of which had never been seen in other animals (Sulaiman *et al*, 2003b).

The high occurrence of *E. bieneusi* and *E. bieneusi*-like organisms in animals has raised the question of the public health significance of *Enterocytozoon* spp in animals. Even though there were some earlier debates about the zoonotic potentials of *E. bieneusi* from animals (Breitenmoser *et al*, 1999), recent phylogenetic analyses of ITS sequences from *E. bieneusi* isolates of humans and animals have clearly shown a genetic relatedness of human *E. bieneusi* genotypes with many animal isolates (Rinder *et al*, 2000; Dengjel *et al*, 2001; Sulaiman *et al*, 2003b). In all phylogenetic trees constructed with the neighbor-joining or maximum likelihood method, human *E. bieneusi* isolates clustered together with all isolates from pigs, many isolates from wild mammals (muskrats, raccoons, beavers, otters, and foxes) and a few isolates from cattle, dogs, cats, rhesus monkeys, and a llama (Fig 1). Thus, these *E. bieneusi* genotypes could infect both humans and a broad range of mammals.

Indeed, four *E. bieneusi* genotypes have been found in both humans and animals. (1) Genotype D (PigEBITS9, WL8, or Peru9) has been found in humans in Germany, England, and Peru, and rhesus monkeys, pigs, beavers, raccoons, muskrats, and foxes in the United States. (2) Genotype E (EbpC, WL13, Peru4) has been seen in HIV-positive persons in Peru and has an ITS sequence identical to one genotype reported in pigs in Switzerland, and beavers, muskrats, raccoons, otters, and foxes in the United States. (3) Genotype K (genotype IV, Peru2, and BEB5) has been found in humans in France, England, Uganda, and Peru, a cat in Germany, and cattle in the US and Portugal. (4) Genotype Peru5 (WL11) has been found in humans in Peru and foxes in the US (Table 2).

Phylogenetic analyses have also shown that some isolates from animals are genetically distinct, and thus may represent *Enterocytozoon* species other than *E. bieneusi* (Sulaiman *et al*, 2003b, 2004). In particular,

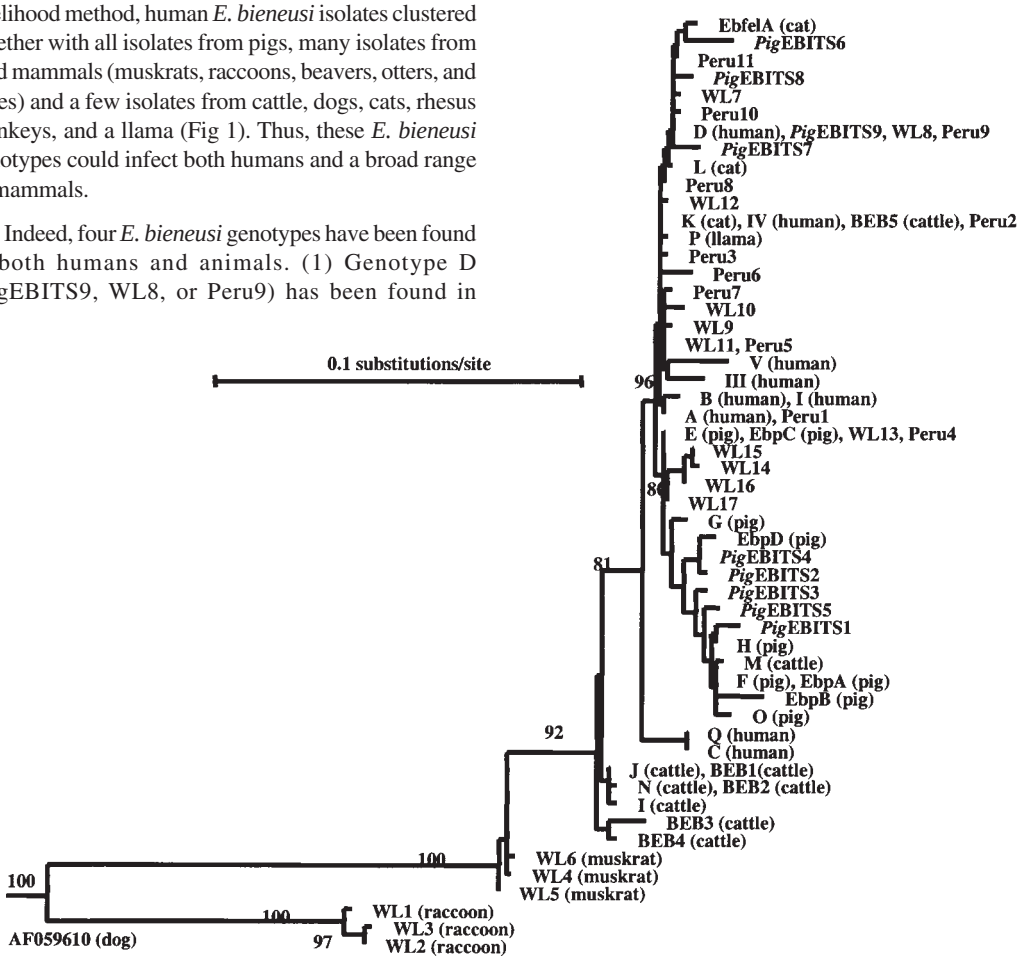

Fig 1- Phylogenetic relationship among known *Enterocytozoon* genotypes as revealed by a neighbor-joining analysis of ITS sequences, based on genetic distances calculated by the Kimura 2-parameter model. Numbers on branches are percent bootstrapping values from 1,000 replicates.

some genotypes from muskrats and raccoons form clusters near the base of phylogenetic trees. Likewise, most bovine genotypes cluster together in a group that is separated from the genotypes of humans and other animals (Fig 1). The most divergent *Enterocytozoon* sp is a genotype from dogs (GenBank accession No. AF059610). These parasites do not likely have significant public health importance. Two human *E. bieneusi* genotypes (Q and C) are also very divergent from other *E. bieneusi* genotypes from humans and mammals. It is not yet clear whether these two are also host-adapted genotypes with strict host specificity.

Conclusion

The molecular epidemiology of *E. bieneusi* is still in infancy. So far, only a few studies have been conducted by several groups of investigators. Most previous genetic characterizations of *E. bieneusi* were done in a few species of animals, such as pigs, cattle, and several wild mammals. The lack of human studies in diverse areas has severely hampered our understanding of *E. bieneusi* transmission. This is further exacerbated by the absence of well-designed case-control and longitudinal studies to identify risk factors and their association with the results of genetic characterizations. There is also an urgent need to develop diagnostic tools to analyze environmental samples and use alternative genetic loci as genotyping targets. Nevertheless, the results of studies conducted so far have already significantly improved our understanding of the complexity of *E. bieneusi* transmission in humans and the zoonotic potentials of parasites of animal origin. The challenge is to use an integrated approach to comprehensively characterize *E. bieneusi* transmission in humans and animals, which in turn would lead to better identification of infection patterns, disease spectrum and risk factors involved in the acquisition of infection, and better understanding of the clinical and public health significance of genetic polymorphism and the development of immunity.

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