

Zoonotic potential of *Enterocytozoon bieneusi* among children in rural communities in Thailand

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Abstract – *Enterocytozoon bieneusi* is a common opportunistic intestinal pathogen worldwide. Genotype distribution of *E. bieneusi* differs by geography and host immunity. In order to investigate the prevalence, genotype characteristics, and host specificity of *E. bieneusi* in the community, we conducted a preliminary cross-sectional study among children in Western and Northern Thailand. Seventy-eight (78) and 102 stool samples were collected; the prevalence of *E. bieneusi* was 3.8% and 2.9% by nested PCR in Western and Northern Thailand, respectively. Three genotypes were identified: Genotype D predominated, followed by EbpC, and then novel genotype ETMK1. The first two genotypes have zoonotic potential. Analysis of the genetic proximity of the *E. bieneusi* ITS sequences from our study, compared with those published in genetic databases, showed that all positive samples were classified into Group 1, the largest group consisting of various host specificity. The present study demonstrates the possible zoonotic transmission of *E. bieneusi* in rural communities in Thailand. A large-scale investigation of both human and animal samples, as well as improvements in the available phylogenetic tools, will be required to elucidate transmission routes of *E. bieneusi* in this area.

Key words: microsporidia, zoonosis, *Enterocytozoon bieneusi*, children, Thailand.

Résumé – **Potentiel zoonotique d'*Enterocytozoon bieneusi* chez les enfants de communautés rurales en Thaïlande.** *Enterocytozoon bieneusi* est un pathogène intestinal opportuniste commun et mondial. La distribution des génotypes d'*E. bieneusi* change selon la géographie et l'immunité des hôtes. Pour étudier la prévalence, les caractéristiques des génotypes et la spécificité aux hôtes d'*E. bieneusi* dans la communauté, nous avons effectué une étude transversale préliminaire chez des enfants de l'ouest et du nord de la Thaïlande. Soixante-dix-huit (78) et 102 échantillons de selles ont été récoltés. La prévalence d'*E. bieneusi*, étudiée par PCR, était de 3,8 % et 2,9 %, respectivement, dans l'ouest et le nord de la Thaïlande. Trois génotypes ont été identifiés : le génotype D prédominait, suivi par EbpC, et par le génotype nouveau ETMK1. Les deux premiers génotypes ont un potentiel zoonotique. L'analyse de la proximité génétique des séquences ITS des *E. bieneusi* de notre étude, comparées avec celles publiées dans les bases de données, montre que tous les échantillons positifs sont classés dans le Groupe 1, le plus grand groupe, qui inclut des spécificités d'hôtes variées. Cette étude démontre la possible transmission zoonotique d'*E. bieneusi* dans les communautés rurales de Thaïlande. Une étude à grande échelle d'échantillons à la fois humains et animaux et des améliorations dans les outils phylogénétiques disponibles seront nécessaires pour élucider les voies de transmission d'*E. bieneusi* dans cette zone.

Introduction

Enterocytozoon bieneusi Desportes et al., 1985 [8] is the most common cause of intestinal microsporidiosis. *E. bieneusi* infections in immunocompetent hosts are usually self-limiting,

while infections in immunocompromised hosts can be life-threatening, especially in patients with AIDS [8]. Recently, awareness of microsporidiosis in non-HIV-infected populations has increased, and infections among organ-transplant recipients, children, the elderly, and patients with malignant disease and diabetes, have been reported [10]. Microscopic diagnosis of *E. bieneusi* is difficult because the organism is small and similar in size to bacteria. Molecular techniques, such as PCR, are more

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sensitive than microscopy and have been more widely used in recent times [14–16]. The numbers of *E. bieneusi* genotypes, based on the internal transcribed spacer (ITS) nucleotide sequence of the ribosomal RNA gene, have increased rapidly; currently, over 100 genotypes have been published in GenBank [29, 30].

The genotype distribution of *E. bieneusi* differs by geography. Anthroponotic host-specific genotypes are frequently observed in developed countries, while in developing areas, both host-specific and non-host-specific genotypes have been identified [4, 7, 33]. In addition, recent molecular epidemiological studies have demonstrated that genotype distribution differs between HIV and non-HIV patients [23, 36]. In Thailand, zoonotic genotype D is most commonly identified in HIV patients, however, only anthroponotic genotype A has been identified in the community and in non-HIV individuals [20–22]. Factors influencing this difference in genotype distribution are not clearly understood. There may be an important association in the host/organism relationship, such as host immune status, virulence, or host specificity of the organism itself [23]. So far, molecular epidemiological studies of *E. bieneusi* have been mainly conducted in HIV and non-HIV patients, while only a few studies have been conducted in the community. For a better understanding of the basic epidemiological characteristics of the organism, such as infection sources and zoonotic potential, surveillance in rural communities is required.

Therefore, we conducted a cross-sectional study among children in rural communities in Western and Northern Thailand. We investigated the prevalence of *E. bieneusi* by nested PCR, genotype characteristics, and host specificity. A phylogenetic tree was constructed for further evaluation of zoonotic potential.

Materials and methods

Study design

In Thailand, in June and December 2011, 79 and 102 stool samples were collected from children in Kanchanaburi (age 4–12 years) and Nan (age 4–6 years) Provinces respectively. The community in Kanchanaburi Province is located on the Thai-Myanmar border, in Western Thailand. The village in Nan Province is located on the Thai-Lao border, in Northern Thailand. Both communities are known endemic areas for parasitic infections, due to low socio-economic status and poor hygiene standards. The parents of the children received instructions for stool collection and provided consent for the investigation. Stool samples were kept in cool conditions during transportation and preserved at -80°C until DNA extraction.

DNA extraction, PCR amplification, and nucleotide sequencing

DNA was extracted from the samples using a commercially available DNA extraction kit (PSP Spin Stool DNA Kit, STRATEC Inc., Germany) in accordance with the manufacturer's instructions. Acquired DNA was stored at -20°C . A nested PCR was performed to amplify a fragment of the large and

small subunit of the rRNA gene, including the entire ITS region. The outer primer pair was EBITS3 (5'-GGT CAT AGG GAT GAA GAG-3') and EBITS4 (5'-TTC GAG TTC TTT CGC GCT C-3'). The inner primer pair was EBITS1 (5'-GCT CTG AAT ATC TAT GGC T-3') and EBITS2.4 (5'-ATC GCC GAC GGA TCA AGT G-3') [5]. Each 25 μl PCR mixture contained 1 \times PCR buffer, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 2.5 U Taq polymerase (Fermentas, USA), and 0.25 μM of each primer. For primary and secondary PCR, reaction conditions were designed as follows: 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Two microliters of the initial PCR products was used as the template for secondary PCR. Secondary PCR produced fragments of 390 bp. The PCR products were subjected to electrophoresis in a 2% agarose gel and visualized by staining the gel with ethidium bromide. All amplified products were sequenced in both directions using the secondary PCR primers EBITS1 and EBITS2.4 on an ABI 3730xl DNA analyzer (Applied Biosystems). The genotypes of *E. bieneusi* from each specimen were confirmed by the homology of the sequenced PCR products to the published sequence in GenBank.

Genetic proximity of the *E. bieneusi* ITS sequences

Analysis of the genetic proximity of the *E. bieneusi* ITS sequences from different origins was performed using MEGA Software Version 4 [35]. The evolutionary distance between the different isolates was calculated using the Kimura 2-parameter method, and phylogenetic trees were constructed using the neighbor-joining algorithm. Branch reliability was assessed using bootstrap analyses (1000 replicates).

Ethical approval

This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2012-064-01).

Nucleotide sequence accession numbers

The sequence of *E. bieneusi* novel genotype ETMK1 in the present study was submitted and deposited in GenBank with Accession No. [JX914568](https://www.ncbi.nlm.nih.gov/nuclot/JX914568).

Results

Prevalence and genotypes of *E. bieneusi* positive samples are shown in Table 1. The prevalence of *E. bieneusi* was 3.8% and 2.9% in Kanchanaburi and Nan Provinces, respectively. In Kanchanaburi province, three samples (3.8%) were positive; two samples were genotype D and one was novel genotype ETMK1. ETMK1 was one base different from genotype EbfelA, L and V as shown in Table 2 (EbfelA position 93 [A→C]; L position 118 [A→G]; V position 130 [G→A]). In Nan Province, three samples (2.9%) were positive. Two samples were genotype D and one was genotype EbpC.

Table 1. Prevalence and genotypes of *E. bieneusi* among children in Kanchanaburi and Nan provinces, Thailand.

Source (Province)	Number of samples examined	Number of positive samples (Prevalence %)	Genotype (number of samples)	Subtype
Kanchanaburi	78	3 (3.8%)	D (2) ETMK1 (1)	1a 1a
Nan	102	3 (2.9%)	D (2) EbpC (1)	1a 1d
Total	180	6 (3.3%)		

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

Position No.	31	77	93	113	117	118	130	137	141
D	G	G	C	C	T	G	G	C	T
V	–	–	A	–	–	A	A	–	–
ETMK1	–	–	A	–	–	A	–	–	–
L	–	–	A	–	–	–	–	–	–
EbfelA	–	–	–	–	–	A	–	–	–
A	–	–	T	–	G	–	–	T	–
B	A	A	T	–	G	–	–	T	–
K	–	–	T	–	G	–	A	–	–
EbpC	–	–	T	A	G	–	–	–	C

The result of the phylogenetic tree was poorly reliable due to low bootstrap values (<20) in the internal branches. However, the majority of the classification in the phylogenetic tree matched with the study by Thellier and Breten [37]; therefore, we followed their classification. Group 1, the largest clade, is further subdivided into eight clades (subgroups 1a–1h). All of the positive samples in the present study were classified into Group 1; genotype D and ETMK1 were classified into subgroup 1a and genotype EbpC into subgroup 1d.

Animal hosts of genotype D, EbpC, EbfelA, and L are shown in Table 3. Except for the novel genotype ETMK1, all positive genotypes in the present study have zoonotic potential. A variety of domestic and wild animal hosts have been reported in genotype D and EbpC, while only felines have been reported as an animal host in genotype EbfelA and L.

Discussion

The zoonotic potential of the *E. bieneusi* positive samples in the community and predominance of *E. bieneusi* genotype D are key distinguishing features in this study. In Thailand, the most prevalent genotype in HIV patients was reported to be zoonotic genotype D [22]. However, only anthroponotic genotype A has been identified in the community or non-HIV individuals [20, 21]. This is the first report in Thailand to identify zoonotic genotype D in the community.

Prevalence of intestinal microsporidiosis in HIV patients varies widely from 1.5% to 50%, depending on differences in geographic region and diagnostic method [2]. In developed countries, prevalence of intestinal microsporidiosis has decreased after the propagation of highly active antiretroviral therapy (HAART) [12, 39]. However in developing areas, intestinal microsporidiosis is still highly prevalent among HIV

patients due to the limited availability of HAART [13]. In addition, microsporidia have been recently identified in non-HIV immunosuppressed individuals, such as organ-transplant recipients, children, the elderly, and patients with malignancy and diabetes [10].

In this study, the prevalence of *E. bieneusi* infection was 3.8% and 2.9% in Western and Northern Thailand, respectively. Evaluation and comparison of the prevalence with previous studies are difficult due to a paucity of investigations in the community as well as differences in conditions. In Thailand, *E. bieneusi* has been detected in young children in orphanages; the prevalence was 4.1% by microscopy [21]. In communities around pig farms, the prevalence was 1.4% by microscopy [20]. Taking into consideration these previous studies in Thailand, the infection rate in our investigation is within expectations.

E. bieneusi genotype is influenced by geography. In European countries, genotype B has been most frequently detected followed by genotypes A and C [3, 7]. These genotypes have been reported in humans only. In Africa, zoonotic genotype K has been frequently identified in Uganda and Gabon [4, 38], while anthroponotic genotype A was the most prevalent in Cameroon and Niger [4, 13]. In Latin America, anthroponotic genotype A was most commonly found, followed by zoonotic genotype Type IV and D in Peru [34]. In Australia, only genotype B has been reported as a causative genotype [33]. In China, CHN1, 3, and 4 were all reported, each of which has potential for zoonotic transmission [40].

Overall, anthroponotic genotypes are commonly seen in developed countries, while both anthroponotic and zoonotic genotypes are observed in developing areas. Opportunities for zoonotic transmission are assumed to be higher in developing countries, especially in rural parts due to frequent animal contact. The transmission routes of *E. bieneusi* are still not

Table 3. Animal hosts in *E. bieneusi* genotype D, EbpC, EbfelA, and L in published records.

Genotype	Subtype	Animal hosts	Country	References	
D	1a	Domestic animals			
		Cattle	Korea	[18] [19]	
			USA	[31]	
		Dog	Portugal	[24]	
		Horse	Colombia	[32]	
		Pig	Czech Republic	[28]	
			Japan	[1]	
			USA	[5]	
		Wild animals			
		Beaver	USA	[34]	
		Falcon	Abu Dhabi	[26]	
		Macaque	USA	[34]	
			Germany	[11]	
		Muskrat	USA	[34]	
Raccoon	USA	[34]			
EbpC	1d	Domestic animals			
		Pig	Thailand	[22]	
			Japan	[1]	
			Germany	[27]	
		Wild animals			
		Beaver	USA	[34]	
		Fox	USA	[34]	
Muskrat	USA	[34]			
	Otter	USA	[34]		
EbfelA	1a	Feline	Switzerland	[25]	
L	1a	Feline	Germany	[7]	

completely understood. Several transmission routes, including direct person-to-person, zoonotic, and food and waterborne, have been reported [9]. With regard to zoonotic transmission, several genotypes have been identified from both humans and animals; genotypes with broad host specificity may be responsible for the zoonotic transmission [30]. Additionally, Cama *et al.* [6] reported possible zoonotic transmission from domestic guinea pigs to a child with no evidence of immunosuppression. In the present study, involvement of zoonotic transmission routes has been observed. Food and waterborne transmission, however, cannot be ruled out since both serve as vehicles for the organism.

Distribution of genotype is reported to be different between immunocompromised and immunocompetent hosts. According to Liguory *et al.* [23], in France, genotype B was most frequently observed in HIV patients, whereas genotype C in non-HIV-infected patients. Both genotypes have been reported in humans only. Similar results were observed in the Netherlands; genotype C was identified in non-HIV patients only. Genotype D, the most prevalent genotype in this study, is widely distributed, and is often reported in humans in many countries in Europe, Africa, Latin America, and Asia. However, genotype D has always been identified in HIV patients, except for three HIV-negative individuals identified in a rural community in Cameroon [37]. Although the factors influencing these differences in genotype distribution are still undetermined, the involvement of host immunity, pathogenicity of the organism, and routes of transmission have been hypothesized [23].

Genotype D and EbpC have less host specificity. Genotype D has been reported in a wide range of animals: domestic animals such as dogs, horses, and swine, and in wild animals such as beavers, falcons, fox macaques, muskrats, and raccoons [30]. Genotype EbpC also has been identified in a broad range of wild animal hosts, but not in domestic animals; only in pigs has it been reported. It is assumed that transmission between humans, domestic and wild animals occurs in these genotypes. In Thailand, genotype EbpC has frequently been identified in pigs, and they may be the source of transmission in the area [20]. ETMK1, a novel genotype in this study, was one base different from genotype L, V, and EbfelA. Genotype L and EbfelA are as yet reported in felines only [30], however, genotype V has been identified in humans. Considering the similarity, ETMK1 may have zoonotic potential especially related with felines.

Analysis of the genetic proximity of the *E. bieneusi* ITS sequences from our study with those previously published in genetic databases demonstrated that genotype D and ETMK1 were classified into subgroup 1a and genotype EbpC into subgroup 1d. According to Thellier and Breton [37], the largest groups (Group 1) consist of both anthroponotic and zoonotic strains, whereas the other groups consist of host-adapted zoonotic strains with low public health priority. Group 1 is divided into eight major subgroups: subgroup 1a and 1d are large, both human or animal-specific and human-animal common genotypes are classified into the clades. There is a problem in constructing a phylogenetic tree in *E. bieneusi* ITS sequence

– it is the only available polymorphic marker in *E. bienersi* and is not reliable enough for statistical support. Detailed subtype classification therefore differs among researchers, resulting in confusion in the classification itself, and hampering evaluation of host specificity of the organism [4, 11, 17, 37]. New sets of markers will be required for further analysis [29].

This study had some limitations. First, host immunity such as HIV status was not investigated in the present study; immunocompromised patients may have a higher infection rate of *E. bienersi* zoonotic strains. Second, the sample size was not large enough to fully analyze genotype characteristics in the community. Third, only human samples were collected in this study; animal samples are required to evaluate further zoonotic transmission routes. The present study is a preliminary study and we are planning a large-scale longitudinal study in which animal and environmental samples, in addition to human samples, will be collected and investigated comprehensively.

In conclusion, this investigation demonstrated zoonotic strains of *E. bienersi* with a predominance of genotype D in rural communities in Thailand. Our findings show possible zoonotic transmission of *E. bienersi* in rural communities in Western and Northern Thailand. A future large-scale study to investigate humans and animals, as well as the improvement of available phylogenetic tools, will be required to elucidate epidemiological characteristics of *E. bienersi*.

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