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Phylogenetic analysis of genetically distinct *Enterocytozoon bieneusi* infecting renal transplant recipients

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Abstract

Enterocytozoon bieneusi (*E. bieneusi*), infecting renal transplant (RT) recipients may be transmitted anthropologically or zoologically. Accordingly, we aimed to a) evaluate genotypes of *E. bieneusi* infecting RT recipients, and b) infer phylogenetic interpretation on transmission of different genotypes among infected hosts. Stool samples of 22 RT recipients infected with microsporidia (identified using modified trichrome staining) were subjected to species identification. All *E. bieneusi* positive samples were subjected to genotyping. The phylogenetic tree was constructed using Mega 5 software. Of 22 microsporidia infected RT recipients, 21/22 (95.5%) had *E. bieneusi*. ITS sequences of 21 *E. bieneusi* were classified into eight genotypes (Ind1 to Ind8). Among them, 4 (Ind5 to Ind8) were novel. Genotypes Ind2, Ind3, Ind4, Ind7 and Ind8 showed close sequence similarity to genotypes reported exclusively from humans. Phylogenetic analysis further supported their anthroponotic transmission. Genotypes Ind1, Ind5 and Ind6 showed close sequence similarity to genotypes reported from both animals and humans. Phylogenetic analysis further supported their zoonotic transmission. Anthroponotic transmission of *E. bieneusi* was more common among males (11, 100% vs. 7/10, 70%; P = 0.05), presenting with diarrhea (11, 100% vs. 6/10, 60%; P = 0.02) and watery stool (10/11, 91% vs. 5/10, 50%; P = 0.03).

Keywords

Microsporidia, intestinal microsporidiosis, immune-compromised patients, internal transcribed spacer, genotyping

Introduction

E. bieneusi is the most prevalent species infecting humans. (Leelayoova *et al.* 2005; Akinbo *et al.* 2012; Ghoshal *et al.* 2015) It has also been detected in faeces of various animals and birds including dogs, pigs, raccoon, wild boar, pigeons, aquatic birds, respectively. (Mathis *et al.* 2005; Sulaiman *et al.* 2003b; Slodkowicz-Kowalska *et al.* 2006) Previously, the spores of *E. bieneusi* have been detected in faeces of infected hosts, air and various water bodies. (Mathis *et al.* 2005) Therefore, transmission of *E. bieneusi* among infected humans may involve fecal-oral route, ingestion of contaminated water, or contact with faeces of infected animals. (Rinder *et al.* 2000) Thus, *E. bieneusi* infecting immunocompromised hosts may be transmitted either from human (anthroponotic spread) or animal to human (zoonotic spread). Genotyping and phylogenetic analysis of *E. bieneusi* would be very useful in understanding its route of transmission among humans in different geographical areas. Genotyping of *E. bieneusi* is based upon the sequencing of its internal transcribed spacer (ITS) region of rRNA gene. (Santin and Fayer 2009) Studying molecular epidemiology through genotyping of *E. bieneusi* would reveal its route of transmission among infected hosts. (Thellier and Breton 2008) It is known that *E. bieneusi* does not respond well to the available drug of choice, Albendazole. (Conteas *et al.* 2000) Therefore, preventing transmission of *E. bieneusi* among susceptible hosts like HIV-infected and renal transplant patients could help reduce its infection among them.

Data on genetic diversity of *E. bieneusi* infecting renal transplant (RT) recipients is scanty. (Pomares *et al.* 2012) Therefore, we aimed to a) evaluate genotypes of *E. bieneusi* among RT recipients and b) infer phylogenetic interpretation on transmission of different genotypes among infected hosts.

Materials and Methods

Study population

Twenty two RT recipients positive for microsporidia on light microscopy using modified trichrome staining (MT staining) were included during April 2012 to November 2015. Data on

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demographic, clinical and laboratory parameters were recorded in a standard questionnaire for each patient. The study protocol was approved by the Institutional Ethics Committee (2014-85-PhD-77).

Sample Collection

Three consecutive stool samples from each renal transplant recipient were collected. Samples were stored at –40°C in normal saline for deoxyribose nucleic acid (DNA) extraction.

Species identification

DNA was extracted from stool samples using QIAamp Qiagen mini stool kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instruction with some modifications. The extracted fecal DNA was subjected to amplify the conserved region of small subunit rRNA (SSU rRNA) gene of microsporidia using previously published forward C1 (5'CACCAGGTTGATTCTGCC-3') and reverse C2 (5'GTGACGGGCGGTGTGTAC-3') primers. (Raynaud *et al.* 1998) The amplified fragments were subjected to digestion using restriction endonucleases HinfI and HindIII to differentiate among four human microsporidia species causing intestinal microsporidiosis, *E. bieneusi*, *E. intestinalis*, *E. cuniculi* and *E. hellem* (Raynaud *et al.* 1998).

Genotyping of *E. bieneusi*

Genotyping of *E. bieneusi* was performed through nucleotide sequence analysis of the ITS region of rRNA gene. To amplify the ITS region of rRNA gene, a two-step nested PCR was performed. For the primary PCR, a PCR product of 410 bp was amplified using primers AL4037 5' GATGGTCATAGGGATGAAGAGCTT 3' and AL4039 5' ACGGATCCAAGTGATCCTGTATT 3'. The PCR mixture consisted of 1 or 2 µl of DNA, 200 µM (each) deoxynucleoside triphosphates, 1X PCR buffer (Perkin-Elmer, Foster City, Cal-if.), 3.0 mM $MgCl_2$, 5.0 U of Taq polymerase (GIBCO BRL, Frederick, Md.) and 200 nM (each) primers in a total of 100 µl of reaction mixture. The reactions were performed for 35 cycles (94°C for 45 s, 55°C for 45 s and 72°C for 60 s), with an initial hot start (94°C for 3 min) and a final extension (72°C for 10 min). For the secondary PCR, a fragment of 392bp were amplified using 2.5 µl of primary PCR, primers AL4038 5' AGGGATGAAGAGCTTCGGCTCTG 3' and AL4040 5' AGTGATCCTGTATTAGGGATATT 3'. The conditions for the secondary PCR were identical to the primary PCR. (Sulaiman *et al.* 2003b).

Sequencing

The amplified products were sequenced by commercially available sequencing services, Bangalore Genei, Bangalore, India. Electropherograms were analyzed. Sequences were aligned with previously reported ITS sequences of *E. bieneusi* using standard software (Chromas program, Technelysium Pvt. Ltd, Sydney, Australia and Bio Edit v 7.0.5, Ibis Biosciences, Carlsbad, California, respectively). Identified sequences were submitted to national centre for biotechnology information (NCBI).

Phylogenetic analysis

E. bieneusi ITS sequences were determined and a multiple alignment was performed using the ClustalW program (http://www.clustal.org/). Phylogenetic analysis was done using MEGA 5.0 program employing distance matrix and maximum-likelihood parameter. A neighbour joining tree was generated using Kimura two-parameter model.

Statistical analysis

Data was checked for normal distribution using Shapiro-Wilk test. Categorical, parametric and non-parametric continuous data were presented as proportion, mean, standard deviation and median, inter quartile range (IQR), respectively. Chi-square, independent t and Mann-Whitney U tests were used to compare between categorical, parametric and non-parametric continuous data, respectively. P values below 0.05 were considered significant for all statistical analysis. Statistical analysis was done using SPSS version 15 (SPSS, Inc., Chicago, IL, USA).

Results

Light microscopy detection

A total of 22 RT recipients had spores of microsporidia using MT staining, 16 of which have already been reported in our previous study (Ghoshal *et al.* 2015).

Table I. Distribution of Genotypes of *E. bieneusi* identified among 21 renal transplant recipients

S.no.	Genotype identified	No. of patients (%age)
1.	Ind 1	4 (19%)
2.	Ind 2	1 (4.8%)
3.	Ind 3	5 (23.8%)
4.	Ind 4	3 (14.3%)
5.	Ind 5	4 (19%)
6.	Ind 6	2 (9.5%)
7.	Ind 7	1 (4.8%)
8.	Ind 8	1 (4.8%)

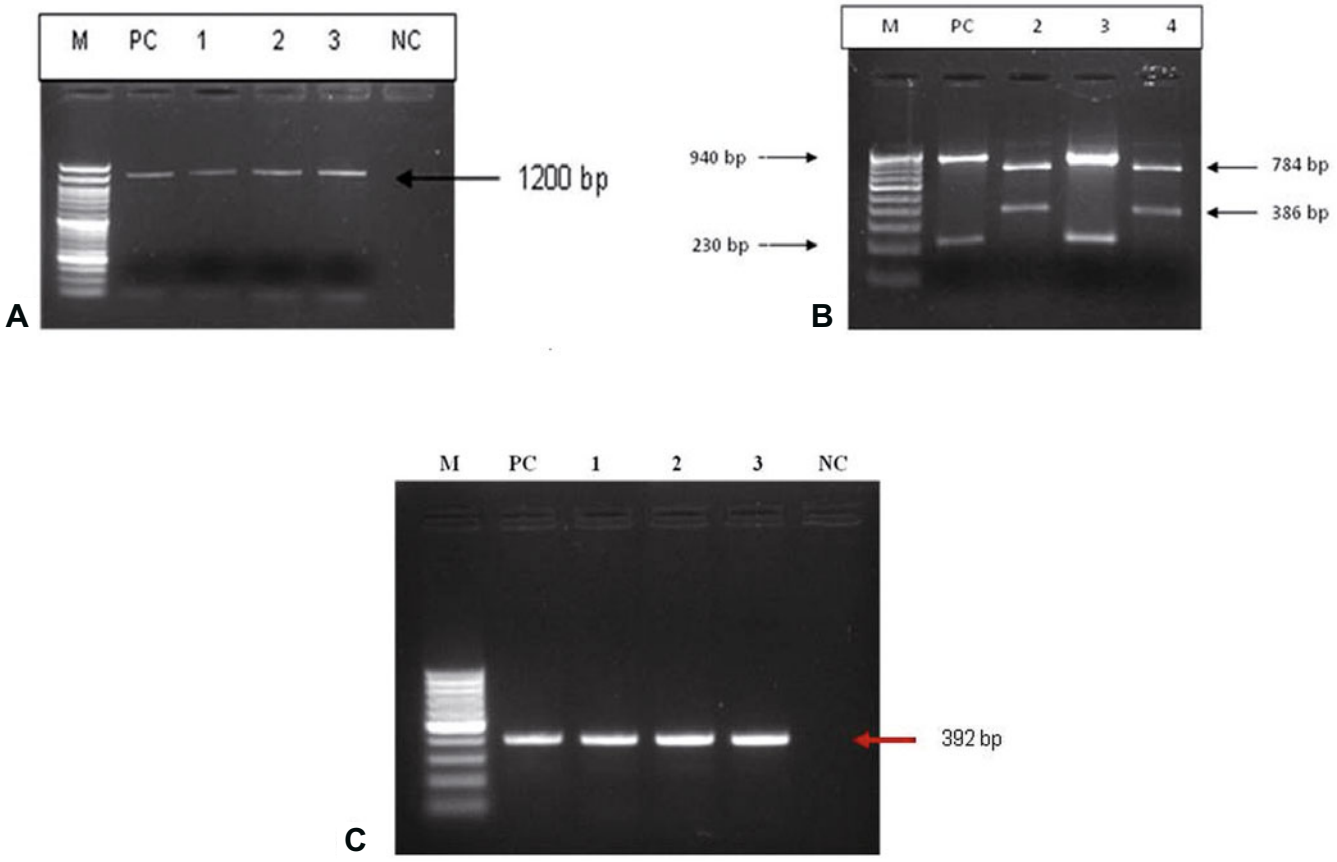

Fig. 1. A – An agarose gel showing PCR products of human fecal samples positive for Microsporidia. Lane M, 50 bp ladder; Lane PC, positive control for Microsporidia; Lane 1–3, Microsporidia positive samples; Lane NC, negative control **B –** Digestion pattern of PCR products using restriction enzyme HinfI and Hind III. Lane M, 100 bp ladder; Lane PC, positive control for *E. bieneusi* using HinfI; Lane 2&4, samples positive for *E.bieneusi* using HindIII; Lane 3, samples positive for *E. bieneusi* using HinfI. **C –** An agarose gel showing secondary PCR amplified products of ITS of *E. bieneusi*. Lane M, 100 bp ladder; PC, positive control of ITS of *E. bieneusi*; Lane 1–3, samples amplified for ITS of *E. bieneusi*; NC, negative control

Species identification

PCR picked up 21/22 (95.5%) samples positive for microsporidia on stool microscopy. Fig. 1A shows 1200 bp amplified product of microsporidia using specified primers. *E. bieneusi* was identified as the causative species among all 21 samples using restriction enzymes, which was further confirmed by sequencing. Fig. 1B shows the pattern of bands obtained using restriction enzymes.

Genotypes of *E. bieneusi*

ITS sequences of 392 bp in size were amplified among all 21 stool samples positive by PCR (Figure 1C). These 21 sequences were classified into 8 genotypes namely Ind1, Ind2, Ind3, Ind4, Ind5, Ind6, Ind7 and Ind8. Four genotypes (Ind1 to Ind4) have already been detected among HIV-infected patients at our centre, while other 4 (Ind5 to Ind8) are novel. Table I shows the number of patients belonging to each genotype identified. Genotypes Ind1, Ind5 and Ind6 showed 98–99% similarity with genotype D and L reported in animals (cats, horse) and humans with 4 polymorphic sites (Table II a). Genotypes Ind2, Ind3, Ind4, Ind7 and Ind8 showed 94–99% similarity to host specific genotype A reported exclusively in humans with 9 polymorphic sites (Table II b).

Table II. Polymorhic sites in the ITS of *E. bieneusi* (a) Genotypes D (consensus), Ind1, Ind5, Ind6; (b) Genotypes A, Ind2, Ind3, Ind4, Ind7, Ind8

(a)

Genotypes	Nucleotide positions in ITS of *E. bieneusi*			
	94th	**98th**	**167th**	**193rd**
D (Consensus)	G	T	G	G
Ind1	**A**	**.**	**.**	**.**
Ind5	.	.	A	A
Ind6	A	C	.	.

Represents same base pair as that of consensus sequence

(b)

Genotypes	Nucleotide positions in ITS of *E. bieneusi*								
	37th	61st	73rd	80th	100th	142nd	167th	179th	228th
A (Consensus)	T	G	G	G	G	G	A	T	T
Ind2	_	.	.	.	.	.	**T**	.	.
Ind3	.	.	.	.	.	.	.	.	_
Ind4	_	.	.	.	.	.	.	.	.
Ind7	.	.	A	A	A	A	.	C	.
Ind8	.	A	.	.	.	.	.	.	.

.Represents same base pair as that of consensus sequence;
Represents deletion as that of consensus sequence

Phylogenetic analysis

In the phylogenetic tree, genotypes Ind1, Ind5 and Ind6 were clustered with zoonotic genotypes D and L (Fig. 2). Therefore, these genotypes could have been transmitted via zoonotic spread (from animals to humans) supporting presumption of zoonotic potential of *E. bieneusi*. Genotypes Ind2, Ind3, Ind4, Ind7 and Ind8 were clustered with host specific genotype A (Fig. 2). Therefore, these genotypes could have been transmitted via anthroponotic route (human to human).

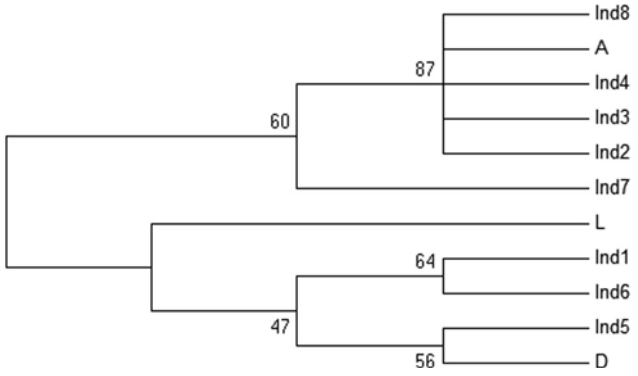

Fig. 2. Phylogenetic relationship of genotypes Ind1 to Ind8 with known genotypes D and A of *E. bieneusi* inferred by neighbour joining analysis of the ITS region based on genetic distances calculated by the Kimura two-parameter model. Numbers on the branches are percent bootstrapping values from 1000 replicates

Demography and clinical symptoms of *E. bieneusi* infected RT recipients

Anthroponotic transmission of *E. bieneusi* was more common among males (11, 100% vs. 6, 60%; P = 0.02), presenting with diarrhoea (11, 100% vs. 7, 70%; P = 0.05) and watery stool (10, 91% vs. 5, 50%; P = 0.03). Other features like age, stool frequency, fever, nausea, vomiting, abdominal pain and presence of pets were comparable among both anthroponotic and zoonotic route of transmission (Table III)

Nucleotide accession number assigned to novel genotypes

The gene bank accession numbers for sequences of genotypes of *E. bieneusi* are KP325473-KP325476 and KU360238-KU360241.

Table III. Association of demographic and clinical symptoms of *E. bieneusi* infected renal transplant recipients with anthroponotic and zoonotic route of transmission

Variable	Transmission route		P value
	Anthroponoti (n = 11)	Zoonotic (n = 10)	
Age (mean, SD)	33.4 (11.2)	40.6 (12.9)	0.193
Gender (Male)	11 (100%)	7 (70%)	0.05*
Diarrhoea	11 (100%)	6 (60%)	0.02*
Duration of diarrhoea in days (median, IQR)	60 (32–90)	47.5 (2–90)	0.214
Stool frequency (median, IQR)	4 (3–5)	2 (2–4.5)	0.194
Stool character (watery)	10 (90.9%)	5 (50%)	0.03*
Fever	3 (27.3%)	2 (20%)	0.69
Nausea, vomiting	2 (18.2%)	4 (40%)	0.26
Abdominal pain	3 (27.3%)	4 (40%)	0.53
Presence of Pets	2 (18.2%)	5 (50%)	0.12

Chi square, independent t and Mann-Whitney U tests were used for categorical, parametric and non-parametric continuous data. [*= P≤0.05]

Discussion

E. bieneusi is the most common microsporidia causing intestinal microsporidiosis among immunocompromised hosts especially HIV-infected patients. (Didier and Weiss 2011) Moreover, renal transplant recipients on immunosuppressive therapy presenting with cellular immunodeficiency are at an increased risk of infection with *E. bieneusi*. This is the first study to report identification of *E. bieneusi* genotypes among renal transplant recipients in India.

E. bieneusi is a complex species with varied hosts ranging from humans to domestic and wild animals. Genotyping of *E. bieneusi* is a valuable tool for molecular epidemiology investigations (Breton *et al.* 2007). Genetic diversity of *E. bieneusi* depends upon analysis of ITS sequences, which depicts its route of transmission among humans. In the present study, three genotypes (Ind1, Ind5, Ind6) showed close sequence similarity with zoonotic genotypes L and D reported in cats, horses and were clustered with them in phylogenetic tree. Zoonotic *E. bieneusi* has been reported in cats ranging from 5–31.3% (Mori *et al.* 2013; Mathis *et al*. 1999; Dengjel *et al.* 2001; Santin *et al.* 2006). Genotype L has often been identified among cats infected with *E. bieneusi* (Santin *et al.* 2006). Similarly, Genotype D has been identified in a variety of hosts including humans as well as domestic (cats, cattle, dogs) and wild animals (raccoons, fox) supporting zoonotic route of transmission (Santin and Fayer 2011). In our study, RT recipients infected with zoonotically transmitted *E. bieneusi* were in contact with pets. However, it was not statistically significant possibly due to small number of cases studied. Therefore, possible transmission of *E. bieneusi* from infected animals to humans through faecal-oral route is being suspected strongly. However, we did not look for *E. bieneusi* spores in faeces of domestic pets like cats, dogs in our geographical region. Spores of *E. bieneusi* have been detected among various domestic and wild animals, birds and humans worldwide. (Mathis *et al.* 2005) However, transmission route of *E. bieneusi* among RT recipients in this geographical region is still enigmatic. Therefore, the current study throws light on presumptive zoonotic transmission of *E. bieneusi* among RT recipients.

Anthropological transmission of *E. bieneusi* among HIV infected patients has been reported worldwide. In our study, five genotypes (Ind2, Ind3, Ind4, Ind7 and Ind8) showed close sequence similarity with host specific genotype A exclusively identified among humans. Phylogenetic tree depicted grouping of these genotypes with host specific genotype A. This shows genetic relatedness among them. Genotype A has been identified among HIV-infected patients from Peru, children and pig farming community from Thailand (Leelayoova *et al.* 2005; Sulaiman *et al.* 2003a; Leelayoova *et al.* 2009). Our sequences were closely related to genotype A, therefore; we can presume that they were transmitted anthropologically through enteric carriage. Another genotype C has been reported to infect exclusively transplant recipients from France and Netherlands (Liguory *et al.* 2001; ten Hove *et al.* 2009). However, we did not find genotype C in our study group. This could be due to geographical variation and lack of common source of infection. In our study, anthroponotic transmission of *E. bieneusi* among RT recipients was associated with diarrhoea and watery stool. Similarly, in a study from Peru, Genotype A was predominant among HIV infected patients with diarrhoea (Sulaiman *et al.* 2003b). However, in another study from Thailand, no gastrointestinal symptoms were associated with infected hosts (Leelayoova *et al.* 2009). There could be possibility of association of severity of disease with anthropologically transmitted genotypes infecting patients. However, to establish this, large number of samples infected with various genotypes of *E. bieneusi* should be studied. Therefore, the present study support that in our geographical region, *E. bieneusi* could be transmitted anthropologically among renal transplant recipients.

In our study, patients with controlled state of immune-suppression showed higher susceptibility to both host specific and non-specific genotypes of *E. bieneusi*. This could be attributed to the fact that microorganisms tend to be less specific in relation to hosts with a disturbed immunological barrier (Karp and Auwaerter 2007). Therefore, preventive measures may help immunocompromised patients to avoid *E. bieneusi* infection among them.

In conclusion, we identified eight genotypes (Ind1 to Ind8) of *E. bieneusi* infecting renal transplant recipients. Among them, four genotypes (Ind5 to Ind8) are novel. *E. bieneusi* is presumed to be transmitted anthropologically as well as zoonotically among infected RT recipients. Therefore, preventive measures may help immunocompromised patients to avoid infection of *E. bieneusi* among them. However, further studies including large number of *E. bieneusi* spores detected in both humans and animals should be looked for confirming its route of transmission among infected hosts.

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