Infection, Genetics and Evolution 32 (2015) 265–270

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Prevalence of *Enterocytozoon bieneusi* and genetic diversity of ITS genotypes in sheep and goats in China

Wei Zhao [a], Weizhe Zhang [a], Dong Yang [a], Longxian Zhang [b], Rongjun Wang [b], Aiqin Liu [a,*]

[a] Department of Parasitology, Harbin Medical University, Harbin, Heilongjiang 150081, China
[b] College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou, Henan 450002, China

ARTICLE INFO

Article history:
Received 5 November 2014
Received in revised form 16 March 2015
Accepted 21 March 2015
Available online 27 March 2015

Keywords:
Enterocytozoon bieneusi
Genotyping
Sheep
Goats
Phylogeny
Gene diversity

ABSTRACT

Enterocytozoon bieneusi is the most common microsporidia species, recognized in more than 90% cases of human microsporidiosis and has been found in a variety of animal hosts. To explore the prevalence of *E. bieneusi* in sheep and goats in China, genetic diversity and zoonotic potential of *E. bieneusi*, 193 fecal specimens from 138 sheep and 55 goats from eight farms in Heilongjiang Province, China were analyzed for the occurrence of *E. bieneusi* by PCR and sequencing of the single internal transcribed spacer (ITS) of the rRNA gene. The average prevalence of *E. bieneusi* was 22.3% (43/193), with 22.5% (31/138) for sheep versus 21.8% (12/55) for goats. Altogether 14 genotypes of *E. bieneusi* were identified, including six known genotypes—BEB6 (n = 15), Peru6 (8), D (n = 6), O (n = 3), EbpC (n = 2), and EbpA (n = 1)—and eight novel genotypes named COS-I to COS-VII and COG-I (one each). Six of the genotypes were previously detected in humans. In phylogenetic analysis, the five novel genotypes COG-I and CCOS-IV to COS-VII were clustered into group 1 with zoonotic potential. These results indicate that these animals may play a potential role in the transmission of *E. bieneusi* to humans.

1. Introduction

Microsporidia, previously classified as protozoa and recently considered as fungi, are obligate intracellular eukaryotes and are emerging as opportunistic human pathogens. In fact, they are ubiquitous in nature and have an ability to infect almost all animal phyla (Didie and Weiss, 2006). The phylum Microsporidia contains over 150 genera and 1300 species (Keeling, 2009). To date, at least 14 microsporidian species, spread across eight genera, have been identified in humans (Mathis et al., 2005). *Enterocytozoon bieneusi* is the most common microsporidian species, causing more than 90% cases of human microsporidiosis (Didie and Weiss, 2006; Matos et al., 2012). This pathogen usually infects enterocytes of the small intestine, with clinical signs being characterized by chronic diarrhea, malabsorption and wasting in humans (Didie and Weiss, 2006). Most seriously, it can cause increased mortality through life-threatening diarrhea in immunocompromised individuals such as AIDS patients (Waywa et al., 2001; Matos et al., 2012). Since the first recognition of *E. bieneusi* in AIDS patients in 1985 (Desportes et al., 1985), the number of microsporidiosis caused by *E. bieneusi* has been increasing in HIV-infected patients with chronic diarrhea (Mathis et al., 2005; Matos et al., 2012). Epidemiological data also revealed the presence of *E. bieneusi* in immunocompetent persons with an asymptomatic infection (Mathis et al., 2005; Matos et al., 2012). In addition to humans, *E. bieneusi* has been detected in many species of animals, mainly including domestic and wild mammals as well as birds (Santin and Fayer, 2011).

Genotyping tools and phylogenetic analysis for *E. bieneusi* are very useful in understanding its host specificity, transmission routes, and clinical presentations of human microsporidiosis as well as evolution. Currently, sequencing of the single internal transcribed spacer (ITS) region of the rRNA gene is the standard method for genotyping *E. bieneusi* (Santin and Fayer, 2009) based on considerable genetic variations within *E. bieneusi* isolates of human and animal origins (Thellier and Breton, 2008). Molecular epidemiological surveys of *E. bieneusi* from different host species have resulted in a large number of ITS genotypes. So far, at last 204 genotypes have been described based on subtle differences within this 243-bp sequence (Karim et al., 2014a; Santin and Fayer, 2011). By phylogenetic analysis, currently, the published ITS genotypes of *E. bieneusi* are divided into eight different groups (Karim et al., 2014a). The first group, considered as a human-pathogenic group or group 1, is the largest and the most complicated since it includes almost all the *E. bieneusi* genotypes from humans and some *E. bieneusi* genotypes from domestic and wild

* Corresponding author. Tel.: +86 451 86674766; fax: +86 451 86673936.
E-mail address: liuaiqin1128@126.com (A. Liu).

http://dx.doi.org/10.1016/j.meegid.2015.03.026
1567-1348/

animals. In contrast, the remaining seven major clusters named as group 2 to group 8 are found mostly in specific hosts and wastewater (Thellier and Breton, 2008; Karim et al., 2014a). To date, at least 28 genotypes have been identified both in humans and animals (Matos et al., 2012; Santin et al., 2012). The findings of the same genotypes of *E. bieneusi* in humans and animals support presumption of zoonotic potentials (Matos et al., 2012).

Thus, it is important/necessary to carry out molecular epidemiological investigations of animal microsporidiosis caused by *E. bieneusi*, especially farm animals with large quantities and economic importance. The livestock health is not only a veterinary issue but also a public health issue because of the existence of zoonotic pathogens in them. Sheep and goats are common economic animals worldwide, however, only three studies reported *E. bieneusi* infection in sheep or goats (Stensvold et al., 2014; Lores et al., 2002; Li et al., 2014a). The aims of the present study were to examine the prevalence of *E. bieneusi* in sheep and goats in China, and genetic characterizations of *E. bieneusi* by PCR and sequence analysis of ITS gene, and to assess zoonotic potential by a neighboring-joining analysis of ITS sequences.

2. Materials and methods

2.1. Ethics statement

The present study protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Harbin Medical University (HMUIRB20130009). All work with animals strictly followed guidelines in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals. The farm animals involved in the present study were permitted by the farm owners or managers. During collection of the fecal specimens these animals were not hurt at all.

2.2. Specimen collection

193 fecal samples were randomly collected from 138 sheep and 55 goats on eight farms located in Heilongjiang Province, China in a one-year study from June 2013 to May 2014. Farms were selected only based on the owners' willingness to participate and the accessibility of animals for sampling. One fresh fecal specimen (approximately 10 g) was collected from each animal on the ground immediately after defecation by using a sterile disposal latex glove, and then was placed into individual 50 ml plastic containers. All the animals were healthy at the time of sampling. The ages of these animals ranged from two to ten months.

2.3. DNA extraction

After the fecal specimens were sieved, the filtrates were concentrated and washed three times with distilled water by centrifugation for 10 min at 1500 g. Genomic DNA was extracted from approximately 0.2 g washed fecal specimens using a QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany) according to manufacturer-recommended procedures. DNA was eluted in 200 μL of AE and stored at −20 °C prior to PCR analysis.

2.4. PCR amplification

All the DNA preparations were analyzed for the presence of *E. bieneusi* by nested PCR amplification of a 389 bp nucleotide fragment of the rRNA gene of *E. bieneusi* containing 76 bp of the 3′-end of SSU rRNA gene, 243 bp of the internal gene and 70 bp of 5′-region of LSU rRNA gene. Two pairs of primers, EBITS3 and EBITS4, and EBITS1 and EBITS2.4 were used in the first and the second PCR amplifications, respectively. The two cycling parameters were 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 40 s, and 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, with both of them having a final extension step at 72 °C for 10 min (Buckholt et al., 2002). TaKaRa Taq DNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all the PCR amplifications. A negative control with no DNA added was included in all PCR tests. All the secondary PCR products were subjected to electrophoresis in a 1.5% agarose gel and visualized by staining the gel with ethidium bromide.

2.5. Nucleotide sequencing

All the secondary PCR products of the expected size were directly sequenced with a set of primers used for the secondary PCR after being purified on an ABI PRISM 3730 XL DNA Analyzer by Sinogeno-max Biotechnology Co. Ltd. (Beijing, China), using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if necessary for some DNA preparations.

2.6. Sequence analysis

Nucleotide sequences obtained in the present study were aligned with each other and reference sequences downloaded from GenBank database by using the Basic Local Alignment Search Tool (BLAST) and Clustal X 1.83 (http://www.clustal.org/) to determine *E. bieneusi* genotypes. The genotypes were given the first published name if they were identical to known genotypes in GenBank. Meanwhile, the genotypes that produced ITS sequences with single nucleotide substitutions, deletions or insertions and were confirmed by the DNA sequencing of at least two PCR products were considered as novel genotypes. All the novel and known genotypes were only based on 243 base pairs of the ITS gene region of *E. bieneusi* according to the established nomenclature system (Santin and Fayer, 2009).

2.7. Phylogenetic analysis

To better present the diversity of all the genotypes obtained in the present study and the genetic relationship of novel ones in sheep and goats to known ones, a comparison of ITS region of all the sequences obtained here and reference sequences previously published in GenBank was performed using the software Mega 5 (http://www.megasoftware.net/) by constructing a neighbor-joining tree, based on the evolutionary distances calculated by a Kimura 2-parameter model. The reliability of these trees was assessed using bootstrap analysis with 1000 replicates.

2.8. Nucleotide sequence accession numbers

Nucleotide sequences of the ITS of the eight novel *E. bieneusi* genotypes obtained in the present study (COS-I to COS-VII for sheep and COG-I for a goat) were deposited in the GenBank database under accession numbers KJ850432 to KJ850434 and KP063053 to KP063057.

3. Results

3.1. Prevalence of E. bieneusi

193 fecal specimens were screened for the presence of *E. bieneusi* by nested PCR amplification of the ITS gene. 43 of which were positive for *E. bieneusi*, with overall infection rate of 22.3% (43/193)