Identification and genotyping of Enterocytozoon bieneusi in China

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In this study, the prevalence of *E. bieneusi* in China was investigated. Twelve genotypes of *E. bieneusi* were identified, of which, 10 were novel genotypes. Further, 41.6% of the genotypes were found in both human and animals. This is the first report of *E. bieneusi* in China.

Microsporidia are a diverse group of obligate intracellular pathogens consisting of approximately 1,300 formally described species in 160 genera that infect a wide range of invertebrate and vertebrate hosts, including human (1, 2). *Enterocytozoon bieneusi*, the most frequently diagnosed microsporidial species in humans, was first reported in an AIDS patient in 1985 (3). Over the last two decades, *E. bieneusi* has been detected in human, other mammals and birds in more than 30 countries (4, 5, 6, 7). However, the prevalence of this parasite in China has been unclear.

Currently, sequencing of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene has been regarded as the standard method for species identification and genotyping of *E. bieneusi* (8). More than 90 genotypes or variants of *E. bieneusi* in human and animals have been identified (2). Recent molecular epidemiological studies indicated that some genotypes are host-specific (8). Further, 8 genotypes (WL15, D, Peru6, EbpC, Peru10, Peru16, WL11, Type IV) have been found in both human and animals, indicating the potential for zoonotic transmission and the importance of surveillance of the epidemiology of *E. bieneusi* (8).

We investigated the prevalence of *E. bieneusi* infection in human and animals in China. A total of 220 fecal samples were collected. Among them, 40 fecal samples were from
diarrheal children in the First Hospital of Jilin University in Changchun city, northeast China; 61 pig fecal samples were from a livestock production facility; 26 fecal samples were collected from dogs in a pet market; 93 fecal samples were from cows. Samples of both human and animals were collected in the same area around Changchun city; however, the human samples were not from the same farm as animal ones. The collection of human and animal stool samples was approved by the ethical committee of the Institute of Zoonosis, Jilin University. DNA was extracted from each fecal sample with a modified protocol as described (9).

A nested PCR with primers based on the specific ITS sequences of *E. bieneusi* was applied for pathogen identification as previously reported (10,11). *E. bieneusi* ITS sequences were determined and a multiple alignment was performed using the ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) program. To assess the extent of genetic diversity and evolutionary relationships between the previously known *Enterocytozoon* spp. genotypes and novelty, a neighbor-joined tree was constructed using the MEGA 4.0 program (12) based on the evolutionary distances calculated by the Kimura two-parameter model (13).

*Enterocytozoon bieneusi* specific sequences were amplified from 56 samples (9 from humans, 2 from dogs, 35 from cow, and 10 from pigs) of the 220 fecal samples (Table 1). The 56 sequences were classified into 12 genotypes, which included the previously reported genotypes I and J (14, 15, 16) and 10 novel types (named CHN1 to CHN10) (Table 1). Genotypes I and J, originally detected in cattle (16), were surprisingly identified in both cow and human samples in this study. Genotype I was found in 3 children and 8...
cow samples; and Genotype J was found in 3 children and 9 cow samples. Interestingly, the two genotypes co-infected with other genotypes in all positive samples. Of the novel genotypes, CHN1 was the most common genotype found in this study, which was detected in 5 children, 9 cow, and 4 pigs. Genotype CHN2 was found only in human samples. Genotypes CHN3 and CHN4 were also found in both human and cow samples. Genotypes CHN5 and CHN6 were only found in dogs in this study. Similarly, genotypes CHN7 to CHN10 were likely pig-specific. CHN7 and CHN8 were found in mono-infections, while CHN9 and CHN10 co-infected with other genotypes.

The ITS sequences of the 10 new genotypes were highly homologous to those of genotypes I, J, K, and G published earlier (14, 15, 16, 17, 18) (Fig. 1). Types CHN1, CHN2, CHN3, CHN5, and CHN6 differed from J by one to four positions, while type CHN4 is 2 bp shorter than type K. Types CHN7 and CHN8 differed from G by one and two nucleotides, respectively. Similarly, types CHN9 and CHN10 differed from type I by one and two nucleotides. A neighbor-joining tree was constructed by aligning the ITS sequences of 10 new genotypes and that of genotypes I, J, K, and G (Fig. 2). Due to the high similarity of the sequences, no obvious clusters related to host preference were observed. Thus E. bieneusi is a mammalian parasite with the ability to infect multiple species.

In this study, we conducted the first investigation on the prevalence of E. bieneusi in China. Though E. bieneusi was first identified in a patient with clinical HIV infection (3), there is still no direct correlation between E. bieneusi infection and clinical disease. Of the 40 samples of the diarrheal children, only 22.5% was E. bieneusi positive and all these...
children were infected with two or more genotypes of the parasite, which indicated that the
diseases may not be directly caused by *E. bieneusi*. Further studies in this area are
obviously necessary.

In an earlier study, it was reported that an unusual *E. bieneusi* genotype was found in
seven guinea pigs and a 2-year-old child in the same household in the USA in 2007, which
strongly suggested the possibility of zoonotic transmission (19). In this study, among the 12
genotypes identified, 6 genotypes were found in children, and 5 of them were also detected
in cow and pigs. Further, we also found human infections of genotypes I and J, which were
only found in cattle in other countries. Due to the fact that all samples were collected from
the same location, the data provide a direct evidence that *E. bieneusi* of animal origin is
infective to human. Interestingly, only two genotypes (CHN5 and CHN6) were found in the
samples collected from dogs and no human infection by these two genotypes were found.
Taking together, these results provide further evidence that *E. bieneusi* of animal origin
may be infective for humans. The data argued for the importance of epidemiological
control and prevention from both agricultural and medical sections.

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Figure legends

Figure 1. Sequence variation in the ITS region of the rRNA gene of E. bieneusi isolates. The ITS sequences of the 10 distinct new genotypes (CHN1 to CHN10) identified in this study were aligned with that of the 4 known genotypes (I, J, K and G). Dots indicate sequence identity to J, while dashes indicate deletions. The GenBank accession numbers for CHN1 to CHN6 are HM992509 to HM992514 and CHN7 to CHN10 are HM992516 to HM992519, respectively.

Figure 2. Phylogenetic relationship of the 10 distinct new genotypes (CHN1 to CHN10) identified in this study with four known genotypes (I, J, K and G) of E. bieneusi inferred by a neighbour-joining analysis of internal transcribed spacer sequences, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1,000 replicates.
Table. Prevalence of *E. bieneusi* in 220 samples.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number of samples examined</th>
<th>Number of samples positive by PCR (%)</th>
<th>Genotypes (number of positive hosts)</th>
<th>Co-infected genotypes (number of positive hosts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>40</td>
<td>9 (22.5)</td>
<td>I (3), J (3), CHN1 (5), CHN2 (2), CHN3 (4), CHN4 (3).</td>
<td>I &amp; CHN2 &amp; CHN3 (1); I &amp; CHN3 (1); J &amp; CHN1 (1); J &amp; CHN1 &amp; CHN2 &amp; CHN4 (1); CHN1 &amp; CHN4 (1); I &amp; CHN1 &amp; CHN4 (1).</td>
</tr>
<tr>
<td>Dogs</td>
<td>26</td>
<td>2 (7.8)</td>
<td>CHN5 (1), CHN6 (1).</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>93</td>
<td>35 (37.6)</td>
<td>I (8), J (9), CHN1 (10), CHN3 (14), CHN4 (2).</td>
<td>I &amp; J (5), CHN1 &amp; CHN3 (1), I &amp; J &amp; CHN1 (1), CHN1 &amp; CHN4 (1), J &amp; CHN1 (1), CHN3 &amp; CHN4 (1).</td>
</tr>
<tr>
<td>Pigs</td>
<td>61</td>
<td>10 (16.4)</td>
<td>CHN1 (4), CHN7 (4), CHN8 (1), CHN9 (1), CHN10 (2).</td>
<td>CHN1 &amp; CHN10 (1), CHN1 &amp; CHN9 (1).</td>
</tr>
<tr>
<td>Total</td>
<td>220</td>
<td>56 (25.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Genotypes

- CHN2 - human
- CHN5 - dogs
- CHN1 - human, cow, pigs
dogs
- CHN6 - human, cattle
- I - pigs
- CHN9 - pigs
- CHN10 - human, cow
- CHN3 - human, cattle
- J - human, cow
- CHN4 - human, cattle and dogs
- K - pigs
- G - pigs
- CHN7 - pigs
- CHN8 - pigs