First report and multilocus genotyping of *Enterocytozoon bieneusi* from Tibetan pigs in southwestern China

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Abstract

Enterocytozoon bieneusi is a common intestinal pathogen and a major cause of diarrhea and enteric diseases in a variety of animals. While the *E. bieneusi* genotype has become better-known, there are few reports on its prevalence in the Tibetan pig. This study investigated the prevalence, genetic diversity, and zoonotic potential of *E. bieneusi* in the Tibetan pig in southwestern China. Tibetan pig feces (266 samples) were collected from three sites in the southwest of China. Feces were subjected to PCR amplification of the internal transcribed spacer (ITS) region. *E. bieneusi* was detected in 83 (31.2%) of Tibetan pigs from the three different sites, with 25.4% in Kangding, 56% in Yaan and 26.7% in Qionglai. Age group demonstrated the prevalence of *E. bieneus*i range from 24.4%(aged 0 to 1 years) to 44.4%(aged 1 to 2 years). Four genotypes of *E. bieneusi* were identified: two known genotypes EbpC (n=58), Henan-IV (n=24) and two novel genotypes, SCT01 and SCT02 (one of each). Phylogenetic analysis showed these four genotypes clustered to group 1 with zoonotic potential. Multilocus sequence typing (MLST) analysis three microsatellites (MS1, MS3, MS7) and one minisatellite (MS4) revealed 47, 48, 23 and 47 positive specimens were successfully sequenced, and identified ten, ten, five and five genotypes at four loci, respectively. This study indicates the potential danger of *E. bieneusi* to Tibetan pigs in southwestern China, and offers basic data for preventing and controlling infections.

Introduction

Microsporidia are obligate intracellular eukaryotic pathogens, classified as fungi, which are composed of approximately 1300 species in 160 genera[1]. To date, 17 microsporidia species are known to infect humans, and of these, *E. bieneusi* is the most prevalent, accounting for over 90% of cases of human microsporidiosis[2]. Since its first detection in an HIV/AIDS patient in 1985, a growing literature attests to *E. bieneusi* expanding range of hosts [3-5]. In humans, infection by microsporidia results in self-limiting diarrhea and malabsorption, most seriously, immunocompromised and immunocompetent patients more susceptible to *E. bieneusi* infection[6]. Normally, fecal-oral routes serve as the main infection pathways in humans and animals, while human inhalation of *E. bieneusi* spores has also been documented[7, 8].

PCR-based molecular techniques may be used to analyze the *E. bieneusi* genome, and for diagnosis. Based on the nested PCR amplification of internal transcribed spacers (ITS) of small subunits of ribosomal rRNA (SSU rRNA), over 240 *E. bieneusi* genotypes have been identified globally[9-11]. Phylogenetic analysis reveals that these genotypes clustered into nine groups. Group 1 is considered zoonotic, and is composed of genotypes from humans and a few animals, while groups 2-9 have particular host associations or are found in wastewater[5, 11]. To better comprehend *E. bieneusi* genetic diversity and molecular characteristics, high-resolution multi-locus sequence typing (MLST) using three microsatellites (MS1, MS3 and MS7) and one minisatellite (MS4) as markers was used to explore genotype taxonomy and transmission routes [9, 12, 13].

In the southwest of China, Tibetan pigs are widely kept for livelihood and are economically important for farmers, especially on the plateau. Tibetan pigs have firm black hair which differs from that of the common pig, and are sturdy, outdoor foragers. They may act as reservoirs for *E. bieneusi* spores and zoonotic transmission of disease. Although much research has been carried out on *E. bieneusi*[14-16], few studies have examined its epidemiology or Tibetan pig-associated genomes in China[17, 18], and Tibetan pigs in southwestern China have been entirely unstudied. Therefore, this study aimed to establish the incidence and molecular characteristics of *E. bieneusi* in Tibetan pigs, to use ITS and MLST to evaluate its genetic diversity, and to assess the potential for zoonotic transmission of microsporidiosis between Tibetan pigs and humans.

Materials and methods

Ethics statement

The study was conducted in accordance with the Research Ethics Committee and the Animal Ethics Committee of Sichuan Agricultural University. Prior to fecal specimen collection, permission was obtained from the keepers of the animals whenever possible.

Collection of Tibetan pig fecal specimens

Fresh fecal specimens were collected from 266 Tibetan pigs during June–October 2017. Samples were obtained mainly from three cities in Sichuan province, southwestern of China, including Yaan (n=50), Kangding (n=201) and Qionglai

(n=15) (Table 1). The ages of Tibetan pigs sampled ranged from 1 to 2 years. Specimens were collected using sterile disposable latex gloves immediately after being defecated on to the ground, and transferred into 50 ml plastic containers. All experimental Tibetan pigs were not any diarrheic or gastrointestinal conditions. Samples were stored at 4 °C in 2.5% (w/v) potassium dichromate.

DNA extraction

Before conducting DNA extraction, potassium dichromate was removed from the fecal samples with distilled water by centrifugation for 10 minutes at 1500 × g, three times. Genomic DNA was extracted from 200 mg of washed fecal matter using the EZNA1 Stool DNA kit (Omega Biotek, Norcross, GA, USA). Prior to use in PCR analysis, DNA was stored and frozen at −20 °C.

PCR amplification

E. bieneusi species/genotypes were determined using a nested PCR amplification of the entire ITS region, and positive specimens were further detected by MLST analyses using the MS1, MS3, MS4, and MS7 loci. The primers and cycling parameters implemented for these reactions were as previously described[12, 19]. Negative controls were included in all PCR analyses. The secondary PCR products were subjected to electrophoresis in a 1.5% agarose gel and visualized under UV light by staining the gel with GoldView (Solarbio, China).

Nucleotide sequencing and phylogenetic analysis

Secondary PCR amplicons of anticipated size were sequenced in both directions by Life Technologies (Guangzhou, China) with an ABI 3730DNA Analyzer (Applied

Biosystems, Foster City, CA, USA) using the BigDye® Terminator v3.1 cycle sequencing kit. Sequence accuracy was confirmed by bidirectional sequencing, and new PCR secondary products were re-sequenced, if necessary. To identify the *E. bieneusi* genotype, the sequences generated were respectively aligned with known reference sequences using BLAST and ClustalX 1.83. Mega 7.0 was used to construct the phylogenetic tree using the neighbor-joining (NJ) method (the Kimura two parameter model) with 1000 bootstrap replicates[20]. Novel genotype(s) of *E. bieneusi* were named according to the established system of nomenclature[21].

Statistical analysis

The variation in *E. bieneusi* infection rates in Tibetan pigs between different areas, gender, and ages were compared using the Chi-square test. All tests were two-sided, with $P < 0.05$ indicating statistical significance. SPSS version 22.0 was used on all data. 95% confidence intervals (95% CIs) were calculated to explore the strength of the association between *E. bieneusi* occurrence and each factor.

Nucleotide sequence accession numbers

Representative nucleotide sequences of *E. bieneusi* isolates were deposited in GenBank under accession numbers from MG581429–MG581432 for ITS sequences and MH142189–MH142213 for the microsatellite (MS1, MS3, and MS7) and minisatellite (MS4) loci.

Results

Occurrence of *E. bieneusi* in Tibetan pigs

133 Of the 266 Tibetan pigs sampled 83 (31.3%) were PCR-positive for *E. bieneusi*.
134 The epidemiology and genotypes of *E. bieneusi* in different areas are given in Table 2.
135 Infection rates detected in Tibetan pigs were 25.4%, 56% and 26.6% in Kang ding, Ya
136 an and Qiong lai, respectively. Infection rates by age and gender are given in Table 1.
137 Differences between the three areas were significant ($\chi2=17.648$, df =2, $p<0.01$). In
138 addition, the female Tibetan pig groups (17.7%, 47/266,) had a higher *E. bieneusi*
139 prevalence than the male groups (13.5%, 36/266,). The difference in infection rate
140 was also significant ($\chi2=8.906$, df =1,P=0.003); however, in the present study, high
141 infection rates were observed in 1–2 year-old pigs (41.51%, 22/53) and 0–1 year-olds
142 (33.33%, 61/183); however, these rates were not significantly different ($\chi2 = 1.240$, df
143 =1, P>0.05).
144 Table1 .Factors associated with prevalence of *Enterocytozoon bieneusi* in Tibetan
145 pigs in southwestern China

Factor	Category	No. tested	No. positive	(%)(95%CI)	P-Value
Region	Kangding	201	51	25.37(0.193-0.314)	
	Qionglai	15	4	26.67(0.013-0.520)	<0.01
	Yaan	50	28	56.00(0.417-0.703)	
Age(years)	0-1	183	61	33.33(0.264-0.402)	0.318
	1-2	53	22	41.51(0.278-0.552)	
Gender	Male	82	47	57.32(0.464-0.683)	0.003
	Female	184	36	19.57(0.138-0.254)	
Total		266	83	31.20(0.256-0.368)	

146

147 **Genotype distribution of *E. bieneusi* in Tibetan pigs**

Nucleotide sequences from ITS-PCR were obtained from the 83 *E. bieneusi*-positive specimens. Four genotypes were detected, including two known genotypes (EbpC, Henan-IV) and two novel genotypes, which were named SCT01 and SCT02 (Table2). Genotype EbpC was the most prevalent (21.8%, 58/266), and was detected in samples from all three cities. Genotype Henan-IV was only found in Kang ding (8.6%, 23/266). The novel genotypes SCT01 (0.3%, 1/266) and SCT02 (0.3%, 1/266) were only found in single specimens, both of which came from Ya an, and are the first newly-detected *E. bieneusi* genotypes from Tibetan pigs.

Table 2. Occurrence and genotypes of *E. bieneusi* in Tibetan pigs from different cities in southwest China.

Region	Farm ID	Prevalence (%)	Genotypes (n)
Kangding	Farm 1	31/102(30.40)	EbpC(18),Henan-IV(n=13)
	Farm 2	20/99(20.20)	EbpC(12),Henan-IV(n=8)
Yaan	Farm 3	14/28(50.00)	EbpC(n=14)
	Farm 4	10/22(45.45)	EbpC(n=12),SCT01(n=1),SCT02(n=1)
Qionglai	Farm 5	4/15(26.67)	EbpC(n=4)
Total		83/266(31.20)	EbpC(58),Henan-IV(n=23),SCT01(n=1),SCT02(n=1)

Phylogenetic relationships of *E. bieneusi* ITS genotypes

Phylogenetic analysis based on ITS gene sequences, the four *E. bieneusi* genotypes obtained from the present study (two known and two novel genotypes) were classed as a single group (group 1) and further clustered into subgroup 1d, indicating zoonotic potential (Fig 1).

Fig 1. Phylogenetic relationship of *Enterocytozoon bieneusi* groups, the relationship between *E. bieneusi* genotypes identified in this study and other known genotypes deposited in the GenBank was inferred by a neighbor-joining analysis of ITS sequences based on genetic distance by the Kimura-2-parameter model. The numbers on the branches represent percent bootstrapping values from 1,000 replicates, with more than 50% shown in tree. Each sequence is identified by its accession number, genotype designation, and host origin. Genotypes with black triangles and open triangle are novel and known genotypes identified in this study, respectively.

Multilocus genotyping of *E. bieneusi*

Positive specimens were further characterized by PCR analyses of MS4, MS1, MS3 and MS7 to improve taxonomy and population genotypes of *E. bieneusi.* 47, 48, 23 and 47 *E. bieneusi* isolates were amplified at the MS1, MS3, MS4, and MS7 loci, respectively, but only 12 samples were PCR-positive simultaneously at all four loci. Four distinct MLGs were observed in Henan-IV and six distinct MLGs in EbpC, named MLG1-4 and MLG5-10, respectively (Table 3). These results reveal high genetic diversity in the Henan-IV and EbpC genotypes of *E. bieneusi* in Tibetan pigs.

Table3 Multilocus characterization of *Enterocytozoon bieneusi* isolates in Tibetan pigs in Southwestern China

Discussion

In the present study, infection rates of varied between 25.4%-56% in different districts, with an overall infection rate of 31.2%. This rate is lower than the documented prevalence of *E. bieneusi* for: wild boars in Sichuan province, China (41.2%), domestic pigs in Jilin province, China (45.1%), wild boars in central Europe (33.3%) and pigs in the State of Rio de Janeiro, Brazil (59.3%) [14, 15, 22, 23]. However, infection rates recorded in this study were higher than those for pigs in Jilin, China (16.4%), central Thailand (28.1%) and Japan (30%)[14, 16, 22]. Differences in infection rates between these studies may be largely attributable to climate and farming mode. Prevalences also varied across sample sites. Kang ding, the only site on the Western Sichuan Plateau, had a prevalence of 25.4%, possibly reflecting the

ITS genotype	Multilocus genotype						No. of MLGs
	MS1	MS3	MS4	MS7	GenBank accession Nos.	MLGs	
Henan-IV	Type II*	Type I*	Type III	Type II	MH142190,MH142204,MH142200,MH142212	MLG1	1
Henan-IV	Type I	Type III*	Type III	Type II	MH142193,MH142205,MH142200,MH142212	MLG2	1
Henan-IV	Type I	Type I*	Type II*	Type I*	MH142193,MH142204,MH142199,MH142210	MLG3	1
Henan-IV	Type II*	Type II*	Type II*	Type II	MH142195,MH142206,MH142199,MH142212	MLG4	2
Ebpc	Type II*	Type I*	Type II*	Type II	MH142196,MH142204,MH142199,MH142209	MLG5	2
Ebpc	Type X*	Type I*	Type IV*	Type I*	MH142189,MH142204,MH142203,MH142213	MLG6	1
Ebpc	Type I	Type I*	Type II*	Type II	MH142193,MH142204,MH142199,MH142212	MLG7	1
Ebpc	Type II*	Type I*	Type I	Type IV*	MH142196,MH142204,MH142201,MH142213	MLG8	1
Ebpc	Type III*	Type IV*	Type I	Type III*	MH142194,MH142207,MH142201,MH142209	MLG9	1
Ebpc	Type I	Type IV*	Type I	Type II	MH142193,MH142207,MH142201,MH142209	MLG10	1

195 area's high temperatures, and UV radiation, which may limit survival of *E. bieneusi*
196 *s*pores and reduce transmission. Other factors influencing infection levels may include
197 geo-ecological conditions, feeding/herd densities, herd management, sample size, and
198 the condition of host animals. Differences in prevalence in Tibetan pigs between Ya
199 an and Kang ding are thought to reflect differences between traditional and modern
200 herd management and breeding technologies.

201 Of the four genotypes identified in this study EbpC was the most prevalent (21.8%,
202 58/266), and has been found in a number of animals, including cattle, dogs, cats,
203 birds, non-human primates, bears, squirrels, sheep, foxes, deer, and humans[3, 5, 13,
204 23-28]. EbpC is the prevalent *E. bieneusi* genotype associated with pig infection in
205 China, reflecting *E. bieneusi*'s dominance as a porcine parasite. In addition, we also
206 detected 26 records of Henan-IV (solely in Ya an), a zoonotic genotype associated
207 with human infections in Henan province in China, and thus far only recorded from
208 China, where it demonstrates strict host specificity[29], occurring only in pigs and
209 humans. To the best our knowledge, the two genotypes EbpC and Henan-IV, which
210 were examined for the first time in Tibetan pigs in the present study, which may be a
211 key reservoir host of these genotypes (Table 4).

212 ITS gene sequence analysis revealed two novel genotypes, SCT01 (n=1) and
213 SCT02 (n=1), both of which were detected in Ya an and clustered into group 1
214 zoonotic genotypes with public health significance. Other genotypes in this group
215 include Henan-III in humans and EbpC from humans or wild boars [13, 29, 30].
216 Modes of transmission and zoonotic potential of *E. bieneusi* genotypes remain poorly

217 known, and further molecular epidemiology studies are required. MLST holds
218 promise for ongoing investigation of *E. bieneusi* taxonomy and genetic diversity[12].
219 Nine, five, three and four novel genotypes were detected at MS1, MS3, MS4 and MS7
220 loci, respectively. Analysis of 12 samples at four gene loci identified eight novel

Genotype (Synonym)	Host	Location	Isolate	Reference
EbpC (E, Peru4,	Pig	Shanghai	3	[12]
WL13, WL17)	Pig	Heilongjiang	10	[12]
	Pig	Heilongjiang	3	[31]
	Pig	Heilongjiang	3	[32]
	Pig	Jilin	1	[33]
	Pig	Mongolia	1	[33]
	Tibetan pig	Sichuan	58	This study
	Red panda	Shanxi	5	[34]
	Human	Shanghai	1	[35]
	Human	Henan	39	[29]
	Human	Heilongjiang	11	[36]
	Human,pig,Monkey	Guangxi	4	[37]
	Squirrel	Sichuan	3	[25]
	Wild boar	Sichuan	85	[30]
	nonhuman primates	Hebei	1	[38]
	nonhuman primates	Hubei	3	[38]
	nonhuman primates	Hunan	4	[38]
	nonhuman primates	Being	2	[38]

221 MLGs, including three genotype EbpC MLGs and five genotype Henan-IV MLGs
222 (Table 3).
223
224
225
226 Table4. Host ranges and geographical distribution of *Enterocytozoon bieneusi*
227 genotype in this study in China

	nonhuman primates	Henan	5	[39]
	Water	Shanghai	37	[40]
	Wastewater	Shanghai	2	[41]
	Wastewater	Shanghai	2	[42]
	Wastewater	Shandong	1	[41]
	Wastewater	Hubei	5	[41]
	Camel	Xinjiang	23	[43]
	Fox	Heilongjiang	5	[44]
	Chicken	Heilongjiang	2	[31]
	Dog	Heilongjiang	2	[45]
	Dog	Shanxi	1	[46]
	Cattle	Henan, Ningxia	6	[47]
	Cattle	Hubei, Tianjin	1	[48]
	Calve	Xinjiang	2	[49]
	Deer	Henan	4	[50]
	Deer	Henan	3	[51]
	Deer	Jilin	1	[51]
	Human	Henan	1	[29]
	Human	Heilongjiang	3	[36]
	Chicken	Jilin	2	[31]
	Camel	Xinjiang	1	[43]
	Horse	Xinjiang	21	[52]
	Cattle	Xinjiang	2	[49]
Henan-IV	nonhuman primates	Hebei	2	[38]
	nonhuman primates	Shanxi	1	[38]
	nonhuman primates	Shanghai	1	[38]
	Pig	Heilongjiang	5	[32]
	Tibetan pig	Sichuan	23	This study
SCT01	Tibetan pig	Sichuan	1	This study
SCT02	Tibetan pig	Sichuan	1	This study

Conclusions

This study revealed an average *E. bieneusi* infection rate of 31.2% in three cities in Sichuan province, and is the first report of EbpC and Henan-IV in Tibetan pigs in

China. Genetic diversity was characterized using MLST, and ten MLGs were identified. These results identify Tibetan pigs as possible vectors for zoonotic transmission of human microsporidiosis; Tibetan pigs widespread use and frequency of human contact make them a significant public health risk in southwest China. Thus, measures are needed to control the transmission of *E. bieneusi* and to develop effective vaccines and drugs for use in the event of widespread human microsporidiosis.

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