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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Prevalence and multilocus genotypes of *Enterocytozoon bieneusi* in alpacas (*Vicugna pacos*) in Shanxi Province, northern China

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Abstract

Enterocytozoon bieneusi is a single-celled obligate pathogen that seriously threatens animal and public health. However, information on the prevalence and genotypes of *E. bieneusi* in alpacas in China is limited. In the present study, 366 fresh fecal samples from alpacas in Shanxi Province, northern China, were collected to detect *E. bieneusi* by nested PCR amplification of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA). The overall prevalence of *E. bieneusi* in alpacas was 4.4% (16/366), including 3.9% (12/305) in Yangqu County and 6.6% (4/61) in Dai county, respectively. Four known genotypes were identified, namely ALP1, ALP3, P, and SH11, all of which belong to the zoonotic group 1 by phylogenetic analysis. Moreover, ITS-positive samples were further characterized by PCR amplification of other four targets, including three microsatellites (MS1, MS3, and MS7) and one minisatellite (MS4). Multilocus sequence typing (MLST) showed that 5, 2, 3, and 3 types were identified at MS1, MS3, MS7, and MS4 loci, respectively, representing eight multilocus genotypes (MLGs). These findings contribute to the improved understanding of the prevalence and genotypes of *E. bieneusi* in alpacas in China and have important implications for controlling *E. bieneusi* infections in animals and humans.

Keywords *Enterocytozoon bieneusi* · Alpaca (*Vicugna pacos*) · Prevalence · Multilocus sequence · Genotypes · Shanxi Province

Introduction

The alpaca (*Vicugna pacos*) is a member of the family Camelidae and is prized for its wool fiber and meat (Franceschi et al. 2014). Over the last decades, alpacas have been introduced into several countries, such as Australia, Canada, New Zealand, and the USA, to establish a modern alpaca industry (McGregor 2006; Koehler et al. 2018). Alpacas were firstly introduced into China from Australia in 2002. Since then, a number of studies on the regulatory mechanisms of alpaca fiber growth and coat color have been conducted (Dong et al. 2011; Tian et al. 2015; Wang et al. 2015; Liu et al. 2018). However, there is limited information on parasite infection of alpacas in China (Zhang et al. 2019).

Enterocytozoon bieneusi, an opportunistic intestinal pathogen associated with microsporidiosis, is widespread in humans, domestic animals, and wildlife (Santín and Fayer 2011; Li et al. 2015; Li et al. 2016; Yue et al. 2017; Chen et al. 2018; Koehler et al. 2018; Zou et al. 2018). So far, more than 300 genotypes of *E. bieneusi* have been reported based on sequence variability within the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) (Henriques-Gil et al. 2010; Matos et al. 2012), of which, 4 genotypes (ALP1, ALP3, ALP7 and P) clustered to group 1 of zoonotic potential have been identified in alpacas, suggesting that alpacas are a potential source for human infection (Koehler et al. 2018; Zhang et al. 2019).

Though alpacas have been imported into China for about two decades, information on the prevalence and ITS

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genotypes of *E. bieneusi* in alpacas in China is limited (Zhang et al. 2019). In addition to ITS genotyping, multilocus sequence typing (MLST), which combines the ITS and four microsatellite and minisatellite markers (MS1, MS3, MS4, and MS7), has been effectively used to further infer population genetics and zoonotic potential of *E. bieneusi* (Feng et al. 2011; Tang et al. 2018). However, there have been no prior reports using MLST analysis to elucidate the genetic diversity of *E. bieneusi* in alpacas. Thus, the present study aimed to investigate the prevalence of *E. bieneusi* in alpacas in Shanxi Province, northern China, and then to determine the genotypes and multilocus genotypes using nested PCR amplification of the ITS sequences in conjunction with MLST tool.

Materials and methods

Specimen collection

Three hundred and sixty-six fecal samples were collected from alpacas in Dai County and Yangqu County, Shanxi Province. Fecal sample was collected individually from each alpaca, preserved in a sterile centrifuge tube (15 ml) with 2.5% potassium dichromate immediately and marked with sampling site and gender. Then, fecal samples were transported to the laboratory in a Styrofoam box with ice packs immediately. All of the samples were maintained frozen at − 20 °C before DNA extraction.

DNA extraction and PCR amplification

Each fecal sample was washed several times using sterilized distilled water by centrifugation at 13000*g* for 2 min to wash off potassium dichromate, followed by genomic DNA isolation utilizing the E.Z.N.A.® Stool DNA Kit (Omega, Bio-tek Inc., USA) according to the manufacturer's specifications. Each DNA extraction product was stored at − 20 °C until used for PCR amplification. The positivity of *E. bieneusi* in alpacas was determined by nested PCR amplification of an approximately 390 bp fragment of the ITS rRNA using primers F1 (5′-GGTCATAGGGATGAAGAG-3′) and R1 (5′-TTCG AGTTCTTTCGCGCTC-3′) for primary amplification, and F2 (5′-GCTCTGAATATCTATGGCT-3′) and R2 (5′-ATCG CCGACGGATCCAAGTG-3′) for secondary amplification. PCR reaction mixture (25 μl) contained 10× Ex*Taq* buffer (Mg^{2+} free), 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.625 U of Ex*Taq* DNA polymerase (Takara, Dalian), 2 μl of genomic DNA, and 0.4 μM primers. Each PCR reaction included negative and positive control. After an initial hot start (94 °C for 3 min), 35 cycles of denaturation (94 °C for 45 s), annealing (55 °C for 45 s), and extension (72 °C for 60 s) were performed, followed by a final extension at 72 °C for 10 min. To further reveal the genetic diversity of the *E. bieneusi* isolates, four additional markers (MS1, MS3, MS4, and MS7) were used in this study, and the primers and PCR conditions referred to previous descriptions (Feng et al. 2011; Li et al. 2012). The secondary PCR amplicons were examined by 2% agarose gel electrophoresis and visualized under UV light after staining in ethidium bromide.

Sequencing and phylogenetic analysis

All of the secondary positive amplicons were purified and sequenced by Sangon Biotech Co. Ltd. (Shanghai, China) in both directions. The software Clustal X 1.83 and the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to align the obtained sequences with reference sequences of *E. bieneusi* available in GenBank to determine the genotypes and multilocus genotypes based on the five markers (ITS, MS1, MS3, MS4, and MS7). Phylogenetic relationships were established by referring to the previous ITS sequences with obtained ITS sequences in the present study using the neighbor-joining (NJ) method and the Kimura 2-parameter model in Mega 7.0 (1000 replicates) (http://www.megasoftware.net/).

Statistical analysis

The differences in *E. bieneusi* prevalence in alpacas from different regions and different genders were analyzed by chi-square (χ^2) test using the software SPSS V20.0 (IBM, Chicago, IL, USA), and the P value < 0.05 was considered statistically significant.

Results and discussion

Of 366 fecal samples examined in this study, 16 (4.4%, 95% CI 2.28–6.46) were PCR-positive for *E. bieneusi* (Table 1). *E. bieneusi* was detected in the two investigated counties, and the prevalence of *E. bieneusi* in Dai County (6.6%, 95% CI 0.35–12.77) is slightly higher than that in Yangqu County (3.9%, 95% CI 1.75–6.11), but with no statistically significant difference ($P > 0.05$). In addition, no statistically significant difference in the prevalence of *E. bieneusi* was observed between male and female alpacas (Table 1).

Alpacas were first introduced into China from Australia in 2002. Since then, alpaca populations were rapidly growing in China. However, to our knowledge, no data have been published on the prevalence of *E. bieneusi* in alpacas from Shanxi Province, northern China. Our study showed that the prevalence of *E. bieneusi* in alpacas in Shanxi Province (4.4%) is lower than that in Xinjiang Province (15.1%) in China, Peru (51.6%), and Australia (9.9%) (Gómez-Puerta 2013; Koehler et al. 2018; Zhang et al. 2019). The difference may be attributed to geographical difference, but different management

Table 1 Factors associated with prevalence of *E. bieneusi* in alpacas in Shanxi Province, northern China

Factor	Category	No. tested	No. positive	Prevalence % (95% CI)	OR (95% CI)	*P* value
Region	Yangqu county	305	12	3.9 (1.75~6.11)	Reference	0.36
	Dai county	61	4	6.6 (0.35~12.77)	1.71 (0.53~5.50)	
Gender	Male	92	4	4.4 (0.18~8.52)	Reference	1.00
	Female	274	12	4.4 (1.96~6.80)	1.01 (0.32~3.20)	
Total		366	16	4.4 (2.28~6.46)		

95% CI 95% confidence interval, *OR* odds ratio

modes, climates, and sampling seasons could also affect *E. bieneusi* prevalence. Further epidemiological investigation of *E. bieneusi* in alpacas should be carried out to determine the reasons for the difference in *E. bieneusi* prevalence.

All ITS-positive samples were sequenced, and representative nucleotide sequences were deposited in GenBank under the following accession numbers: MK674973-MK674976 for the ITS sequences, MK674968-MK674972 for the MS1 locus, MK674963-MK674964 for the MS3 locus, MK674965-MK674967 for the MS4 locus, and MK674960-MK674962 for the MS7 locus. In the present study, 4 known genotypes were identified, namely ALP1, ALP3, P, and SH11, of which, only ALP3 was found in alpacas in Yangqu County, while other *E. bieneusi* genotypes (ALP1, P, and SH11) were detected in Dai County (Table 2). The three genotypes (ALP1, ALP3, and P) detected in alpacas in Australia were also found in the investigated alpacas in the present study. However, the ALP3 was the primary genotype of *E. bieneusi* in alpacas in China, which was different from the previous result reported in alpacas in Australia where the predominant genotype was ALP1 (Koehler et al. 2018), but in accordance with the report in Xinjiang, China (Zhang et al. 2019). Genotype SH11 was firstly found in children in a pediatric hospital in China (Wang et al. 2013). Our findings reveal that genotype SH11 infected alpacas. Genotype SH11 detected in the present study was not found in alpacas in Australia, which indicated that the alpacas got infected with this genotype during the last 17 years after importation into China. Moreover, several genotypes, such as ALP2, ALP4-6, IV, D, and Beb6, were detected in Peru but not found in the investigated alpacas in the present study. This could be explained by the fact that the alpacas were introduced into China from Australia, and a recent report showed that only three genotypes (ALP1, ALP3, and P) were detected in alpacas in Australia (Gómez-Puerta 2013; Koehler et al. 2018). Phylogenetic analysis showed that all the genotypes identified in the present study belonged to the zoonotic potential group 1, which suggested that the investigated alpacas could be a potential source for human infection with *E. bieneusi*.

A number of studies have revealed genetic diversity of *E. bieneusi* genotypes by using the MLST tool (Li et al. 2016; Wang et al. 2016; Zhang et al. 2016; Deng et al. 2017). To further analyze the genetic diversity of *E. bieneusi* in alpacas, a total of 16 ITS-positive samples were characterized by using the MLST tool (Feng et al. 2011). The results showed that 13, 14, 13, and 15 samples were positive for the MS1, MS3, MS4, and MS7 loci, respectively (Table 3). Sequence analysis identified five, two, three, and three types at each locus, and 11 samples were sequenced simultaneously successfully at four loci based on ITS genotypes, forming 8 distinct MLGs (named as MLG1-8) (Table 3), of which, seven MLGs were observed in the genotype ALP3 and one MLG in the genotype P (Table 3). The MLGs of *E. bieneusi* in alpacas showed complicated genetic diversity within the same ITS genotype. While MLG3, MLG5, and MLG6 were detected in two alpacas belonging to the genotype ALP3, respectively, the other four MLGs were detected in only one alpaca of the genotype ALP3, suggesting that the subgenotypes may differ from each other in their transmission potential. Further MLST analyses, employing a larger number of isolates from alpacas, are needed to better understand the population genetics, zoonotic potential, and transmission patterns of *E. bieneusi* in alpacas.

Table 2 Prevalence and genotypes of *E. bieneusi* in alpacas in Shanxi Province, northern China

Factor	Category	No. examined (*n*)	No. positive (%)	Genotype (*n*)
Region	Yangqu county	305	12 (3.9)	ALP3 (12)
	Dai county	61	4 (6.6)	ALP1 (1), P (1), SH11 (2)
Gender	Female	274	12 (4.4)	ALP1 (1), SH11 (2), ALP3 (9)
	Male	92	4 (4.4)	P (1), ALP3 (3)
Total		366	16 (4.4)	ALP1 (1), ALP3 (12), P (1), SH11 (2)

Table 3 Multilocus genotypes of *E. bieneusi* isolates in alpacas in Shanxi Province, northern China

Code	ITS genotype	Multilocus genotypes				
		MS1	MS3	MS4	MS7	MLGs
DG1	P	Type 5	Type 2	Type 1	Type 2	MLG1
G55	ALP3	Type 4	Type 2	Type 3	Type 1	MLG2
2-16/2-34	ALP3	Type 2	Type 2	Type 3	Type 1	MLG3
2-17	ALP3	Type 1	Type 2	Type 2	Type 1	MLG4
2-24/G62	ALP3	Type 3	Type 2	Type 3	Type 1	MLG5
5-21/7-23	ALP3	Type 2	Type 2	Type 2	Type 1	MLG6
7-3	ALP3	Type 3	Type 2	Type 3	Type 2	MLG7
7-28	ALP3	Type 3	Type 2	Type 2	Type 2	MLG8
G70	ALP3	Type 2	Type 2	–	Type 2	–
5-3	ALP3	Type 2	Type 2	Type 2	–	–
DM2	SH11	–	Type 1	–	Type 2	–
DM36	ALP1	–	–	Type 1	Type 3	–
DM40	SH11	–	–	–	Type 2	–

– unsuccessful PCR amplification

Conclusions

The present study revealed that the overall *E. bieneusi* prevalence in alpacas in Shanxi Province, northern China, was 4.4%. Four known ITS genotypes (ALP1, ALP3, P, and SH11) were identified, and the genotype ALP3 was the preponderant genotype in alpacas. All of the ITS genotypes identified in the present study belong to group 1, indicating that the alpaca is a potential source for human infection with *E. bieneusi*. Moreover, seven MLGs were identified based on ITS genotype ALP3, indicating high genetic diversity in alpacas. These findings extend the knowledge of the prevalence and genotypes of *E. bieneusi* in alpacas.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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