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Original Article

High Prevalence of *Enterocytozoon bieneusi* Genotype BEB6 in Wild Boars in Lorestan Province, Iran: Potential Source of Zoonotic Transmission

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Abstract

Background: We aimed to investigate the prevalence of *Enterocytozoon bieneusi* and the circulating genotypes in wild boar in the western regions of Iran.

Methods: Fifty-two fecal samples were collected from wild boars in Lorestan province, Iran. After DNA extraction, the presence of *E. bieneusi* was evaluated by real-time PCR. A nested PCR targeting the internal transcribed region (ITS) was employed to characterize genotypes. The PCR products were sequenced, and the genetic diversity and relationships among the genotypes were identified using MEGA X and DnaSp (V5) software.

Results: Sixteen (30%) samples were positive for *E. bieneusi* using real-time PCR, and 11 (21%) were positive for nested PCR, which was sequenced. All 11 positive samples were identified as the BEB6 genotype (Group 2). The haplotype diversity was noted to be 0.182, and the nucleotide diversity, calculated using DnaSp, was 0. 00085.

Conclusion: The findings highlight the high prevalence of the genotype BEB6 in wild boars. The presence of this genotype suggests the circulation of *E. bieneusi* between domesticated animals and wild boars in Iran.

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Introduction

Microsporidia are a group of obligate intracellular, spore-forming eukaryotes that can infect a broad spectrum of vertebrate and non-vertebrate hosts (1-4). *Enterocytozoon bieneusi* is the most frequent species worldwide that can affect the small intestine of humans and animals (5). The parasite can cause persistent diarrhea and severe wasting syndrome in immunocompromised individuals (6, 7).

The transmission of *E. bieneusi* typically occurs through the fecal-oral route by ingestion of spores shed in feces (3, 8, 9). Domestic and wild animals are the main reservoirs of parasites, crucial in preserving *E. bieneusi* in the environment (10). Due to the increasing important of microsporidiosis as a veterinary concern, there is a growing need for effective diagnostic methods, management strategies, and a deeper understanding of the parasite's biology and epidemiology. *Enterocytozoon bieneusi* genotypes are typically classified into 1 to 11 groups based on their genetic characteristics, with each group comprising specific genotypes that share similar genetic traits (11, 12).

Wild boars (*Sus scrofa*), native to Europe, Asia, and North Africa, have increasingly drawn attention in ecological and epidemiological studies due to their adaptability to various habitats and their role as reservoirs for various pathogens (13, 14). In recent years, there has been growing concern regarding the potential transmission of microsporidia infections in wild boar populations, which can serve as reservoirs for these parasites (15). These animals can harbor different microsporidia species, some of which may be zoonotic, pose health risks, and can be transmitted to livestock and human populations (15). In wild boars, several *E. bieneusi* genotypes have been identified, including EbpA, D, EbpC, Pig-SpEb1, and KWB1–KWB4 (16, 17). Understanding the interactions between microsporidia and wild boars is crucial for assessing the dynamics of these infections, their ecological implications, and potential impact on wildlife management and public health.

Therefore, concerning the importance of zoonotic transmission of *E. bieneusi,* we aimed to investigate the molecular prevalence and genotypes of this parasite using sensitive, and rapid molecular methods.

Methods

Ethical considerations

All experimental protocols were in accordance with the ethical principles and the national norms and standards for conducting Medical Research in Iran. The study was performed in accordance with the relevant guidelines and declaration. The current study was approved by the Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1403.159).

Study design and sample collection

A cross-sectional study was conducted on wild boars in Iran to evaluate the prevalence of microsporidia using molecular methods, including nested PCR and real-time PCR. Samples were collected from 37 (71.15%) and 15 (28.85%) male and female wild boars, respectively. In addition, 40 (76.92%) and 12 (23.08%) of wild boars were adults and cubs, respectively. The selection of wild boars encompassed various genders (male and female) and age groups (adult and cub). Fifty two wild boars were randomly selected from Lorestan province, Iran. Fecal samples were collected from each selected wild boar following standard operating procedures to minimize contamination. Samples were stored in sterile containers and labeled with identification numbers corresponding to each wild boar. Samples were transported to the laboratory at 4°C and processed within 24 h.

DNA extraction

Total genomic DNA was extracted from fecal samples using the FavorPrep™ Stool

DNA Isolation Mini Kit (Favorgen Biotech, Ping Tung, Taiwan), as mentioned in the manufacturer's protocol. The quality and concentration of extracted DNA were assessed using a spectrophotometer.

Real-time PCR amplification

Real-time PCR was performed using a real-time Rotor-Gene Q (QIAGEN, Germany) instrument. Specific primers targeting the internal transcribed spacer (ITS) region of *E. bieneusi* were used, including EbITS-89F (5'-TGTGTAGGCGTGAGAGTGTATCTG-3') and EbITS-191R (5'-CAT CCAAC-CATCACGTACCAATC-3') (18). The real-time PCR reactions were conducted in a 15 µL total volume containing 7.5 µL of 2 × SYBR® Green master mix (BIOFACT, Korea), 0.5 ϱM of each primer (5 ϱM), 3.5 µL of distilled water, and 3 µL of template DNA (19). Amplification reactions were done as follows: 95 °C for 10 min followed by 40 cycles: 95 °C for 25 s, 60 °C for 30 s, 72 °C for 20 s, and ramping from 65 °C to 95 °C at 1° Cs^{-1}. DNA of *E. bieneusi* (Accession number: MW429405) as a positive control and sterile distilled water as a negative control were tested in each run. The real-time PCR assays were carried out in duplicate to check the reproducibility. The melting profiles were also analyzed using Rotor-Gene Q software to exclude non-specific amplifications and primer dimers. Real-time PCR results were considered negative when the Ct value was more than 38 or no amplification curve was obtained. All samples with Ct values above 35 were retested, and their melting curve was justified by the positive control to confirm the result.

Nested PCR amplification

Specific primers targeting *E. bieneusi* were utilized for nested PCR amplification (19). First-step PCR reactions were conducted in a final volume of 25 µL containing 12.5 µL of 2x Mastermix with 1.5 mM $MgCl_2$ (Ampliqon, Denmark), 10 ϱM of each primer and 1 µL of DNA. The outer primers were EbGeno-Fe (5'-TTCAGATGGTCATAGGGATG-3') and EbGeno-Re (5'-ATTAGAGCATTCCGTGAGG-3'), which amplified a 465-bp fragment of ITS.

In the second PCR, the product of the first step was used as a template, and inner primers EbGeno-Fi (5'-TCGGCTCTGAATATCTATGG-3') and EbGeno-Ri (5'-ATTCTTTCGCGCTCGTC-3') were utilized to amplify a 410-bp amplicon. Thermal cycling conditions for both steps were: initial denaturation at 94 °C for 5min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 55°C for 45 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min. DNA of *E. bieneusi* (Accession number: MW429405) as a positive control and sterile distilled water as a negative control was used in each run.

Gel electrophoresis

PCR products were subjected to gel electrophoresis on 1.3% agarose gels with TBE (0.09M Tris, 0.09M boric acid, 2mM EDTA), containing a safe stain for visualization. Bands were compared against a 100-bp ladder to confirm the presence of expected amplicons.

Sequencing and phylogenetic analysis

The PCR products were sequenced using an ABI 3130 sequencer (California, USA), and then sequences were analyzed using BLAST and BioEdit 7.2. Phylogenetic trees were constructed using the Maximum Likelihood method with MEGAX to elucidate the relationship among identified *E. bieneusi* genotypes (20). All generated sequences were submitted in the GenBank database with accession numbers PQ057787- PQ057797.

DnaSP analyses and Genetic diversity

The genetic correlation between our sequences and various *E. bieneusi* genotypes retrieved from the GenBank database was analyzed. The DnaSP (21) was employed to calculate haplotype diversity (Hd), segregating sites

(S), nucleotide diversity (π), and Tajima's D test (22).

Statistical analysis

Data analysis was performed using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA). Proportions were presented as percentages and mean ± standard deviation. Mann Whitney was used to compare demographic data with the significance level of $P<0.05$.

Results

Real-Time PCR Findings

The results indicated that the mean ± SD of Ct values for positive *E. bieneusi* samples in wild boars was 28.64 ± 4.26, and the mean ± SD of Tm values was 82.30 ± 0.77. The real-time PCR results showed high prevalence, with 16 (30.76%) wild boar samples testing positive for *E. bieneusi* (Table 1).

Table 1: Genotype distribution and accession numbers of *E. bieneusi*-positive samples.

NO.	Gender	Age	Real time-PCR	Nested-PCR	Genotype	Acc. no.
1	Female	Adult	+	+	BEB6	PQ057787
2	Male	Adult	+	+	BEB6	PQ057788
3	Female	Adult	+	+	BEB6	PQ057789
4	Female	Adult	+	+	BEB6	PQ057790
5	Female	Adult	+	+	BEB6	PQ057791
6	Female	Wild boar cub	+	+	BEB6	PQ057792
7	Male	Wild boar cub	+	+	BEB6	PQ057793
8	Male	Wild boar cub	+	+	BEB6	PQ057794
9	Male	Adult	+	+	BEB6	PQ057795
10	Female	Adult	+	+	BEB6	PQ057796
11	Male	Adult	+	+	BEB6	PQ057797
12	Male	Adult	+	Not Amplified	-	-
13	Female	Adult	+	Not Amplified	-	-
14	Female	Adult	+	Not Amplified	-	-
15	Male	Adult	+	Not Amplified	-	-
16	Male	Adult	+	Not Amplified	-	-

Nested PCR findings and genotyping of E. bieneusi

From 16 *E. bieneusi* positive samples, the identical fragment for genotyping was successfully amplified and sequenced in 11 (21.15%) samples. Nucleotide sequences of the ITS revealed the presence of BEB6 genotype in all 11 positive fecal samples of wild boars. Also, no significant association was seen between age (*P*: 0.4) and gender (*P:* 0.5) with the prevalence of *E. bieneusi*.

Phylogenetic analysis

A phylogenetic tree based on the *ITS* gene sequences confirmed the relationships between the detected *E. bieneusi* genotype and those previously reported in wild boars and domesticated animals (chickens, cattle, sheep, horses, and human). The phylogenetic tree

results indicated that the genotype BEB6 isolated from wild boars belongs to Group 2 and is placed in the same clade as other isolates derived from domestic animals and human samples in Iran (Fig. 1).

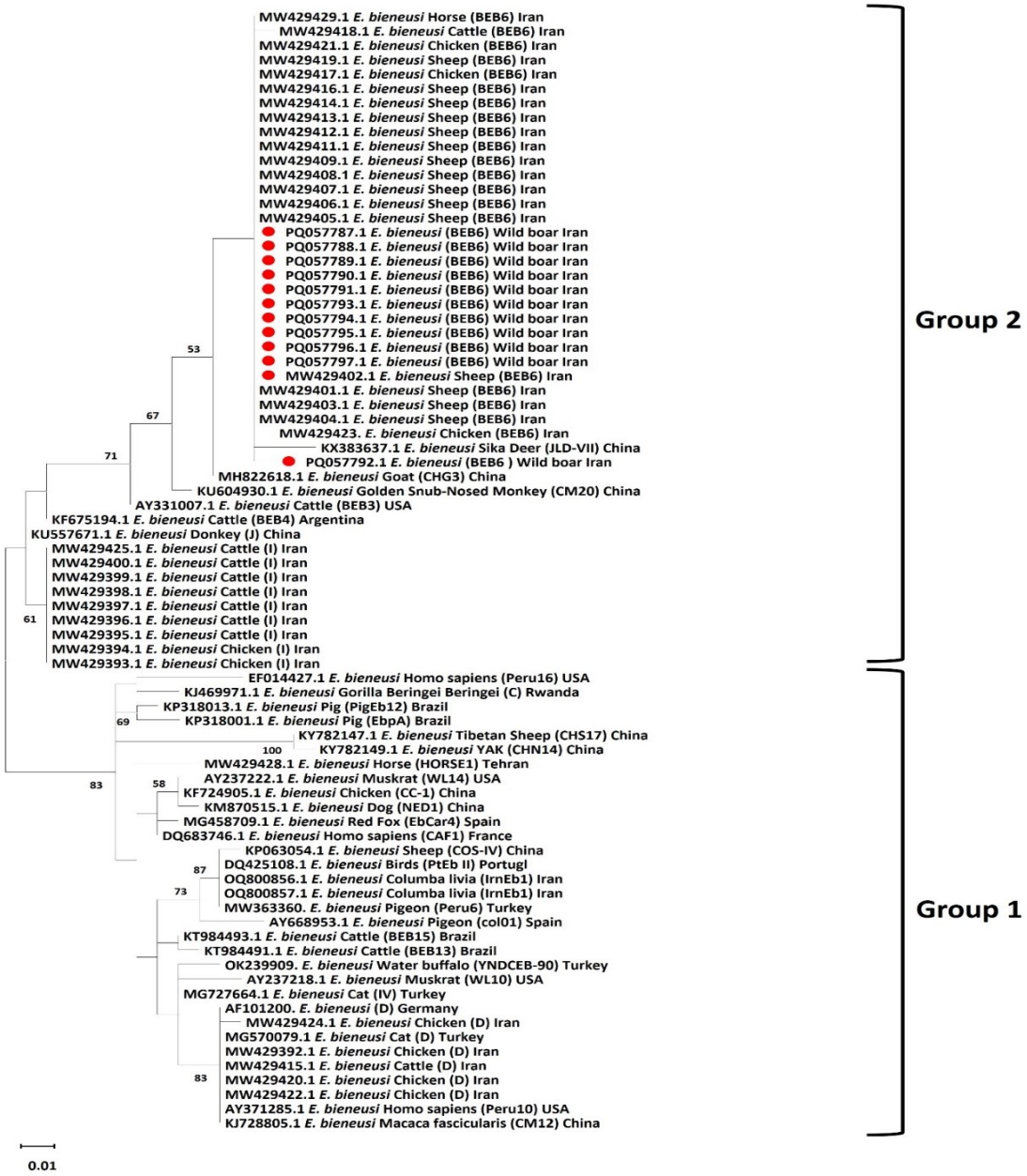

Fig. 1: Phylogenetic tree of the ITS gene of *E. bieneusi* BEB6 genotypes in zoonotic groups I and II based on ML and Hasegawa-Kishino-Yano algorithms. Sequence topologies were calculated using 1000 bootstrap replicates. The studied sequences are shown in red for the BEB6 genotype

DnaSP analyses and genetic diversity results

DnaSP analysis showed π and Hd indices among the BEB6 genotypes were 0.00085 and 0.182, respectively. In addition, two haplotypes were detected in the genotype BEB6, and the results of neutrality tests indicated negative (-1.12850) Tajima's D for the genotype BEB6.

Discussion

Enterocytozoon bieneusi is a microorganism recognized as a significant cause of intestinal microsporidiosis in various mammals, including humans. Recent studies have highlighted its prevalence in wildlife, raising concerns about zoonotic transmission (15, 23, 24).

In the current study, *E. bieneusi* was identified for the first time via real-time PCR as well as nested PCR in the wild boars from the mountainous regions of Lorestan, western Iran. Our findings revealed a high prevalence of *E. bieneusi* infection in the investigated wild boar population, highlighting the potential role of wild boars as reservoir hosts for *E. bieneusi*, which has important implications for the health of wild boars and public health.

In last decades, molecular techniques have been used for accurately detecting and identifying microsporidian species (25). In the present study, real-time PCR was employed to detect *E. bieneusi* in wildlife samples due to its high sensitivity. This technique can detect low levels of DNA, enabling to identify infections even in the early stages or in samples with low parasite loads (26). Specific primers ensure that only the target pathogen is amplified, reducing the chances of false positives from non-target organisms (27, 28). The real-time PCR analysis revealed a higher prevalence rate, with 16 out of 52 sampled wild boars (30.76%) testing positive for *E. bieneusi*. The higher detection rate suggests that real-time PCR may be a more sensitive method for detecting *E. bieneusi*. The high prevalence in this study, as shown in a study in Denmark, could be related to the detection method (29, 30). Overall, real-time PCR is a powerful tool in parasitology, enhancing our ability to detect, quantify, and understand wildlife parasitic pathogens (29).

Moreover, we used nested PCR as a highly sensitive method for detecting the ITS region of *E. bieneusi* to enhance the reliability of the results. Sequence analysis of the ITS gene of isolates enables the genetic characterization of *E. bieneusi,* which can provide new insights into their phylogenetic relationships, host specificity, and ecological prevalence (31). Furthermore, understanding the epidemiology of *E. bieneusi* in wild boars is crucial for developing effective control and prevention strategies to mitigate the risk of zoonotic transmission (32). In the present study, 11 out of 52 wild boars (21.15%) were found positive for *E. bieneusi* by nested PCR analyses. However, various studies in China have reported a high prevalence of *E. bieneusi* in domestic pigs. Wild pigs typically inhabit various ecosystems and have diverse diets, making them potentially less susceptible to certain *E. bieneusi* that thrive in domestic environments (33, 34). Studies in the Czech Republic and Germany reported that the prevalence of *E. bieneusi* in domestic pigs was up to 90% and 40%, respectively (35, 36). This is likely because pigs are raised in close quarters, facilitating rapid transmission of the parasite through direct contact and shared environments. Domestic pigs are often kept in confined conditions, which increases exposure to *E. bieneusi* due to overcrowding, poor hygiene, and restricted movement (35, 36). The prevalence of *E. bieneusi* reported by Feng et al. (16) in wild boars from Beijing was 42%. This difference could be attributed to varying ecological settings, climate, and habitat types in Iran and China, which may influence *E. bieneusi* life cycle and transmission dynamics. The prevalence in this study was 21.15%, which was higher than the study by Lee et al. (17) in South Korea, which reported a preva-

lence of 2.6%. The high prevalence in Iran could be related to differences in agricultural practices, wildlife management, and public health measures between the two countries.

Studies on *E. bieneusi* genotypes in wild boars have been conducted in various countries, providing insights into the diversity of genotypes across different geographical regions. For instance, genotype EbpA has been identified in wild boars in Spain (37). The genotypes PigEBITS5 and Type IV have been detected in wild boars in China (11), and the genotype EbpA has been found in wild boars in central Europe (38).

The genotyping of *E. bieneusi* from wild boar samples provides valuable insights into the genetic diversity and distribution of this parasite within the population (16). Interestingly, the sequencing results identified the BEB6 genotype in all positive samples identified by nested PCR. The genotypic diversity of *E. bieneusi* in Iran is high, and studies on its isolation from various hosts confirm this finding (8, 39, 40). So far, the *E. bieneusi* genotypes D, E, and J in Iran have been dominant in Iran (19, 41). The presence of the BEB6 genotype in a significant proportion of the positive samples suggests that this particular genotype is prevalent in the wild boar population under investigation (15). Genotyping data can help elucidate the genetic diversity of *E. bieneusi* strains and provide information on the transmission dynamics and potential sources of infection within the host population (42).

Research indicates shared genotypes between domesticated animals and their wild counterparts (43). These findings may be attributed to common environmental reservoirs. The presence of similar *E. bieneusi* genotypes in both populations suggests that there could be shared sources in the environment, such as water, as well as routes of fecal contamination that enable cross-transmission between wild and domestic pigs (38, 44). Recent reports indicate the presence of BEB6 genotype isolates in various sources across Iran, including water, vegetables, and humans (Acc no.: PP920042, ON746324).

The presence of BEB6 genotype in various sources indicates it is spreading in Iran. Furthermore, the identification of this genotype in wild boars raises questions about its zoonotic potential. Mohammad Rahimi et al. (45) identified BEB6 genotype of *E. bieneusi* in different domestic animals, including cattle and sheep, at the same location as the sampling for this study. This is evidence of genetic overlap between isolates from different reservoirs. The same genotype suggests that wild boars, cattle, and sheep may all be exposed to similar environmental reservoirs of the parasite, such as contaminated water sources, feed, or soil. This indicates a potential overlap in habitats and food sources and the possibility of cross-species transmission. The discovery of a common genotype implies that *E. bieneusi* can be transmitted between species, which raises concerns for disease management (11). Wild boar may act as reservoirs and vectors for the transmission of *E. bieneusi* to domestic livestock. The presence of a common *E. bieneusi* genotype in both domestic and wild animals highlights the zoonotic potential of this pathogen. This suggests that human populations, particularly farmers and ranchers living near these animals, may be at risk if *E. bieneusi* can transfer from animals to humans (45, 46).

Calculation of nucleotide diversity (0.00085) and haplotype diversity (0.182) in the isolates of this study showed that this diversity is less than that of *E. bieneusi* samples from previous studies on treated wastewater from southern Tehran, which had nucleotide diversity (0.00191) and haplotype diversity (0.529) (19). This difference may be related to the varying types of geographic habitats, which can support different levels of *E. bieneusi* diversity. Water bodies often host complex ecosystems with a broader range of hosts and parasites (47). The results of neutrality tests, particularly Tajima's D statistic, provide further insights into the evolutionary dynamics and demographic history of the BEB6 genotype; the

negative Tajima's D value (-1.12850) observed for the BEB6 genotypes suggests an excess of low-frequency polymorphisms, which could be indicative of population expansion, purifying selection, or a recent selective sweep in the *E. bieneusi* population.

Consistently, the negative Tajima's D value was also detected among *E. bieneusi* isolates derived from the water (-1.359) (19) and wild animals (-1.246) (47). Overall, the DnaSP analysis results shed light on the genetic composition, diversity, and evolutionary patterns of the BEB6 genotype of *E. bieneusi* (22). The combination of low nucleotide diversity, haplotype diversity, and negative Tajima's D values for the BEB6 genotypes suggests potential demographic changes, selective forces, or population dynamics affecting these genotypes (22). Understanding the genetic characteristics and evolutionary history of the BEB6 genotype is crucial for elucidating the transmission patterns, adaptation strategies, and pathogenic potential of *E. bieneusi* in different host populations and environments.

Conclusion

E. bieneusi was detected in 16 (30%) fecal samples collected from wild boars in the west of Iran. The molecular characterization showed the presence of the genotype BEB6 in all samples. Owing to the report of the genotype BEB6 in domesticated animals in the same region, the current findings suggest a probable circulation of *E. bieneusi* between domesticated animals and wildlife in the west of Iran.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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