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Genetic polymorphisms of *Echinococcus granulosus* sensu stricto in the Middle East[☆]

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ABSTRACT

Echinococcus granulosus sensu stricto is a cosmopolitan parasite causing cystic echinococcosis in humans and livestock. Recent molecular phylogeographic studies suggested the rapid dispersal of the parasite by the anthropogenic movement of domestic animal hosts. In the present study, genetic polymorphism of *E. granulosus* s. s. in the Middle East, where the domestication started, was investigated to validate the dispersal history of the parasite. Thirty-five and 26 hydatid cysts were collected from Iran and Jordan, respectively, and mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene was sequenced. Chinese and Peruvian specimens were also analyzed for comparison. Haplotype network analysis demonstrated the existence of a common haplotype EG01 in all populations. Although EG01 and its one-step neighbors were the majority in all regions, most of the neighboring haplotypes were unique in each locality. Haplotype diversity was high but nucleotide diversity was low in Iran, Jordan and China. Both diversities were lowest and only a few haplotypes were found in Peru. Neutrality indices were significantly negative in Iran, Jordan and China, and positive but not significant in Peru. Pairwise fixation index was significant for all pairwise comparisons, indicating genetic differentiation among populations. These results suggest a evolutionary history of *E. granulosus* s. s. in which a genetic subgroup including EG01 was selected at the dawn of domestication, and then it was rapidly dispersed worldwide through the diffusion of stock raising. To approach the origin of the ancestral strain, extensive sampling is needed in many endemic regions. To evaluate the hypothetical evolutionary scenario, further study is needed to analyze specimens from diverse host species in wider regions.

1. Introduction

Echinococcus granulosus sensu lato is a complex of species of cestode causing cystic echinococcosis (CE), one of the most important and widespread parasitic zoonoses [1]. The larval stage of *E. granulosus* s. l. utilizes ungulate livestock (sheep, cattle, goat, horse, etc.) as the intermediate hosts, and the predatory canids harbor adult tapeworms in the intestine. Recent phylogenetic studies based on both mitochondrial and nuclear DNA genes revealed that *E. granulosus* s. l. consists of at least 4 valid species, *E. granulosus* sensu stricto (genotype G1–G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5) and *Echinococcus canadensis* (G6–G10) [2–5], although the species status of *E. canadensis* is still controversial [3,6]. Among them, *E. granulosus* s. s. is known to have a broad geographical distribution and a wide host range [7]. The three known genotypes of *E. granulosus* s. s. are called the sheep (G1), the Tasmanian sheep (G2) and the buffalo (G3) strains. A latest phylogeographic study revealed the existence of a common and predominant mitochondrial DNA (mtDNA) haplotype of *E. granulosus* s. s. in far distant countries, China and Peru, suggesting the recent and rapid dispersal of the parasite by the anthropogenic movement of host animals [8]. The study provides us with good insight into the evolutionary history and the origin of the cosmopolitan parasite. As the domestication of ungulate livestock is considered to have started in the Middle East around 10,000–12,000 years B.P. [9,10], we hypothesized that the common haplotype in China and Peru originated in the Middle East and has spread following the anthropogenic diffusion of domestic animals. Recently, the similar hypothesis was proposed based on the

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[☆] Nucleotide sequences determined in this report were deposited into DDBJ/EMBL/GenBank databases under the accession numbers of JQ250806–JQ250817 and AB688590–AB688621. We all dedicate this joint paper to the late Mrs. Hikari Ito who continuously encouraged our collaborative projects from the beginning but passed away on 30 May 2012.

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investigation of the mtDNA genetic variability of *E. granulosus* s. s. in Europe [11] To evaluate this hypothesis, investigation of the genetic variations of *E. granulosus* s. s. in the Middle East is essential.

CE is one of the major zoonotic parasitic diseases in the Middle East and has been reported from all countries [12]. Molecular identification of the etiological agents of CE in both humans and livestock revealed the presence of *E. granulosus* s. s. (G1–G3) and *E. canadensis* (G6–G7) in Iran [13–16] and Turkey [17–21], and *E. canadensis* (G6) in Egypt [22]. However, to date extensive studies on the intraspecific genetic variations of *E. granulosus* s. s. have not been reported. The present study aims to clarify the mtDNA genetic polymorphisms of *E. granulosus* s. s. in Iran and Jordan, and to estimate the dispersal history of the cosmopolitan parasite by comparing other geographical populations.

2. Materials and methods

2.1. Geographic isolates and DNA sequencing

A total of 35 cysts were collected from sheep (N = 23), cattle (3), camel (1), goat (2) and humans (6) in Shiraz county in Iran. Twenty-six cysts were obtained from sheep in the Northern governorates of Irbid (7), Zarqa (6), Balqa (5) and Karak (8) in Jordan. For comparison, 29 and 30 isolates of *E. granulosus* s. s. in China and Peru, respectively, were randomly chosen from the specimens which were used in a previous study [8].

The genomic DNA of each specimen was extracted from ethanol-preserved larval cysts using DNeasy blood and tissue kit (Qiagen), and subsequently used as template for polymerase chain reaction (PCR). The mtDNA fragments of cytochrome *c* oxidase subunit I (*cox1*) were amplified by PCR using previously reported primer pairs which were designed to amplify the complete *cox1* gene of *Echinococcus felidis* [23]. Obtained PCR products were treated with ExoSAP-IT (GE Healthcare) to remove excess dNTPs and primers, and directly sequenced with a BigDye^{TM} Terminator v3.1 and a 3500 DNA sequencer (Applied Biosystems). As a result, the sequences of *cox1* (1609 bp) gene were determined for all specimens.

2.2. Data analysis

Obtained sequences were aligned by Clustal W 2.0 [24] and the amino acid sequences were inferred from the nucleotide sequences by flatworm mitochondrial genetic code [25]. The population diversity indices (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated for each four sub-populations (Iran, Jordan, China and Peru) using DnaSP 5.1 [26]. The population genetics package Arlequin 3.1 [27] was used to conduct neutrality tests to calculate the neutrality indices of Tajima's D [28] and Fu's Fs [29] in each population. The negative value of both indices suggests either some forms of negative selection or population expansion, while the positive value suggests either some forms of balancing selection or population bottleneck. The identification of haplotypes and the drawing of their networks were computed by TCS 1.2 software [30] using statistical parsimony [31]. The network estimation was run at 95% connection limit. The degree of gene flow among the four sub-populations was estimated using a pairwise fixation index (Fst) as determined by the Arlequin 3.1.

3. Results

In total, 44 haplotypes were found in 120 isolates. Nucleotide sequences of all the haplotypes were deposited into DDBJ/EMBL/GenBank databases under the accession nos. JQ250806–JQ250817 and AB688590–AB688621. Among the point mutations observed in 54 variable sites, 36 (66.7%) were synonymous and 18 (33.3%) were non-synonymous substitutions. The maximum values of the pairwise divergence of the sequences was 0.8% in Iran, 0.6% in Jordan, 0.4% in China, 0.1% in Peru and 0.8% within total population groups. In the public databases, only 9 sequences of *E. granulosus* s. s. (accession numbers = AB522646–AB522647, AB551110, AB622277, AF297617, DQ856466, GQ168811–GQ168812, GQ856692–GQ856693) had similar length (1608–1611 bp) and covered the same region of *cox1* gene, and none of them was 100% identical to any of the haplotypes obtained in the present study. The pairwise divergence between the obtained haplotypes and the complete *cox1* gene sequence of G1 genotype (AF297617) ranged from 0.1% to 0.8%. Although there were some *cox1* gene sequences of G2 and G3 genotypes in the database, all of them were short, less than 400 bp. Moreover, some of the sequences of G2, DQ131582 from Italy and AY686559 from Romania, are identical to EG3 from Europe, which was defined as G3 genotype [11].

As shown in Fig. 1, the existence of a common haplotype EG01 was confirmed. EG01 was a major haplotype in all the geographic populations and was constantly placed in the center of the haplotype networks. The nucleotide sequence of EG01 was 100% identical to the haplotypes previously reported as dominant in China and Peru (G01: accession number = AB491414) [8], and in Europe (EG1: JF513058) [11], although the sequence lengths of these known haplotypes are less than half (789 bp and 351 bp, respectively) of EG01. Chinese haplotypes exhibited a typical star-like expansion from a main founder haplotype EG01, as previously demonstrated using shorter (789 bp) *cox1* gene sequences [8]. The haplotypes in Iran and Jordan also exhibited a star-like expansion from EG01, but the haplotype networks were represented by rather complex structures compared to the one from the Chinese population. The Peruvian haplotypes showed the simplest structure, with only 3 haplotypes. Although EG01 alone was not predominant in any regions, more than half of each population was occupied by EG01 and its one-step neighbor haplotypes (Fig. 1). On the other hand, the majority of the haplotypes other than EG01 were unique in each population and only few were shared between Iran and Jordan (EG03 and EG04) or among Iran, Jordan and China (EG02). In Iran, EG01 was obtained from humans (N = 1), sheep (3) and cattle (3). Eight haplotypes (EG01, 03–06, 08, 11, 12) were found from sheep, and 5 haplotypes (EG01, 02, 05, 07, 10) were obtained from humans. Other hosts, cattle, goat and camel showed only one haplotype each (EG01, 02 and 09 respectively).

The haplotype diversity (Hd) was high in Iran, Jordan and China, but low in Peru where only 3 haplotypes were found in 30 isolates (Table 1). The nucleotide diversity (π) was low in all populations, and the lowest value was obtained in the Peruvian population. In Iran, Hd was also high in sheep (0.933 ± 0.122) and humans (0.846 ± 0.046), and π was low in sheep (0.0017) and humans (0.0015). Both diversities were zero in other hosts, because only one haplotype was obtained from each animal. Neutrality indices obtained by Tajima's D and Fu's Fs test were both negative in Iran, Jordan and China, and the value was highest in the Chinese population. Although Fs values were significantly negative ($P < 0.02$) in all the three populations, the D value was significantly negative only in Jordan and Chinese populations. By contrast, both neutrality indices were positive in the Peruvian population, but those values were not significantly deviated from zero. To estimate the degree of gene flow among four geographic populations, the pairwise Fst was computed. The Fst values among the populations ranged from 0.037 to 0.229, and all of them were statistically significant (Table 2). Fst value was generally high between Peruvian and other populations. These statistically significant Fst values that indicate all geographical populations are genetically differentiated.

4. Discussion

The existence of a common haplotype EG01 in far distant geographic regions was confirmed, as previously reported in China and Peru [8]. EG01 has the identical sequence with the dominant