First Molecular Characterization of *Echinococcus multilocularis* in Turkey

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Abstract

This study aimed to find out the occurrence of *Echinococcus multilocularis* in foxes in Erzurum province, the highest endemic region for human alveolar echinococcosis in Turkey. The sedimentation and counting technique was used to reveal adult *Echinococcus* spp. in the intestines of foxes. One out of the 10 foxes was infected with *E. multilocularis*. The adult worms were analyzed morphologically and molecularly and were confirmed to be *E. multilocularis* by species-specific PCR. Pairwise comparisons between the *12S rRNA* sequences of the *E. multilocularis* isolate from Erzurum and other *E. multilocularis* isolates showed 100% similarity of the Erzurum isolate with European isolates. With this study, the presence of *E. multilocularis* in a fox in Erzurum was confirmed by PCR, and molecular identification of *E. multilocularis* is reported for the first time in Turkey.

Keywords: *Echinococcus multilocularis*, fox, molecular characterization, Turkey

Introduction

ALVEOLAR ECHINOCOCCOSIS (AE), caused by the tapeworm *Echinococcus multilocularis*, is one of the most severe parasitic diseases in humans and represents one of the 17 neglected diseases prioritized by the World Health Organization (WHO 2012).

E. multilocularis is characterized predominantly by its sylvatic life cycle, which contains two mammalian hosts: rodents as intermediate hosts and wild canids as definitive hosts (Knapp et al. 2015).

E. multilocularis distribution is restricted to the northern hemisphere, including Central Europe, Russia, Central Asian republics, northern Japan, parts of North America, and some countries of the Middle East (Eckert et al. 2001, Ito et al. 2003, Torgerson et al. 2010). Investigation of definitive and intermediate hosts of *E. multilocularis* has been neglected in Turkey. No detailed published data are available on *E. multilocularis* in animals in Turkey except for one earlier report (Merdivenci 1963) of *E. multilocularis* in a fox in the northwest of Turkey. There have been case series reports that describe AE in humans from Erzurum province (Miman and Yazar 2012). In contrast, to date, there are no data on the abundance of *E. multilocularis* in definitive hosts in this region.

From earlier mitochondrial studies, two geographical genotypes of *E. multilocularis* were described among European

isolates (M1) and among Chinese, Japanese, Alaskan, and North American isolates (M2) (Bowles et al. 1992, Bowles and McManus 1993, Okamoto et al. 1995). Recent classification schemes using multiple mitochondrial loci and microsatellites suggested greater diversity in *E. multilocularis* than previously thought (Nakao et al. 2009, Knapp et al. 2012, Gesy et al. 2014).

Although Turkey is known as an endemic area for AE, molecular investigations and characterizations of the isolates have not been studied in this region so far. With this aim, a project named “Molecular Epidemiology of Cystic and Alveolar Echinococcosis in Erzurum Region” was planned to present molecular characterization of cystic echinococcosis (CE) and AE in humans and animals in this region. Based on this progressing project, we wish to present our preliminary findings of this study, which describes the occurrence of *E. multilocularis* in foxes from a highest endemic region for human AE and represents its first molecular characterization in Turkey.

Materials and Methods

Study area

The study was conducted from December 2015 to February 2016 in Erzurum (39°54'31" N, 41°16'37" E) province. The province is located in the eastern part of Turkey, at an

elevation of 1853 meters above sea level. It receives an annual rainfall of 453 mm. The temperature range is from -35°C to 35°C .

Sample collection

The intestines of 10 foxes (Red fox, *Vulpes vulpes*) that died on roads by car crashes were collected. A standard form including the place and date was filled out. The intestines, which were good conserved due to freezing temperatures, were placed in labeled ziploc bags, stored at -86°C for at least 7 days (Deplazes and Eckert 1996) to reduce the risk of laboratory infection by inactivating any *Echinococcus* oncospheres, and subsequently stored at -20°C until further examination. Ethical approval was obtained from the Ataturk University Animal Research Local Ethics Committee (Approval No. 2015/27) for the study.

The sedimentation and counting technique

The sedimentation and counting technique (SCT) was done as previously described by Hofer et al. (2000). Because worms were numerous, an aliquot was examined and the total parasite count was estimated from the proportion of the aliquot to the total sediment. The intensity of infection was classified as low (1–100), medium (101–1000), or high (>1000) worm burden (Duscher et al. 2005). All isolated adult worms were stored in 70% ethanol and 10% formalin for molecular and microscopic identification, respectively.

Morphological examination

Adult worms of *E. multilocularis* were differentiated using morphological characteristics, including size, length of gravid proglottids, shape of uterus, and position of genital pore by light microscopy and stereomicroscopy.

Isolation of genomic DNA and PCR amplification

Isolation of DNA from adult worms was executed by using Qiaamp DNA mini kit (DNeasy Tissue kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were then processed according to the kit's protocol and the obtained DNA was stored at -20°C until use.

The mitochondrial 12S ribosomal RNA (rRNA) gene was subjected to analysis using classic PCR with species-specific primers Em.nest/for and Em.nest/rev (Dyachenko et al. 2008). *E. multilocularis* positive DNA provided by the Laboratory of Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, as positive control and distilled water as negative control were included in all of the PCR runs. Amplification products were analyzed by 1.5% agarose gel electrophoresis, stained by ethidium bromide, and photographed using UV transillumination (Vilber Lourmat, Quantum ST4 1100).

Sequencing and phylogenetic analysis

Bidirectional sequencing was performed commercially with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) using the ABI PRISM[®] BigDye terminator cycle sequencing kit. Sequence data were then subjected to BLASTN (RefSeq mRNA) searches of the

FIG. 1. *Echinococcus multilocularis* adults isolated from a fox in Erzurum province ($\times 40$, stereomicroscopy). Color images available online at www.liebertpub.com/vbz

E. multilocularis genome database at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/).

All sequenced data were edited and aligned using Bioedit 7.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html) following naked eye checking. Phylogenetic tree and pairwise calculations were obtained by using MEGA 5.1 software (Tamura et al. 2011).

Results

SCT and morphology

The entire intestines of 10 foxes were examined by SCT and 1 was found to be infected with *E. multilocularis* with high (~ 4110 worms) worm burden. The characteristics and morphology of *E. multilocularis* were seen in all mature worms (Fig. 1).

Molecular characterization and phylogenetic analysis

The adult worms from the infected fox were molecularly analyzed with species-specific PCR and were confirmed to be *E. multilocularis* by 12S rRNA gene sequence analysis. The sequence was deposited to GenBank with the accession number KU711929. Erzurum isolate was aligned and compared with some previously published sequences of related *Echinococcus* species available from GenBank.

Pairwise comparison between the 12S rRNA sequences of the Erzurum *E. multilocularis* isolate and European *E. multilocularis* isolates showed 100% identity, whereas it was 99.5% with the Japanese isolates.

Discussion

Echinococcosis remains a serious public veterinary health concern in many areas of the world. Torgerson et al. (2010) suggested that Turkey is a highly endemic area for human AE, and the estimated annual incidence is 100 cases. Altintas (2003) reported that AE predominantly occurs in the eastern Anatolian region (especially Erzurum province) of the country. Reports of ~ 207 cases of AE in Turkey were published over the 1987–1994 period. Between 2000 and 2010, a total of

162 new AE cases were reported. Of these 162 cases, 83 belong to Erzurum province (Miman and Yazar 2012). To date, there is no data available on the abundance of *E. multilocularis* in definitive hosts including foxes in this region. In this study, adult *E. multilocularis* tapeworms were found in the intestine of 1 out of the 10 red foxes. This is the first report describing the presence of *E. multilocularis* in foxes in Erzurum and the second in Turkey.

The intraspecific diversity of *E. multilocularis* was first described by sequencing coding or noncoding fragments of mitochondrial DNA. These analyses revealed very low diversity among geographically distinct isolates: nucleotide diversity in *E. multilocularis* was estimated to be about 10 times lower than in *E. granulosus* s.l. (Haag et al. 1997). *E. multilocularis* adults from a fox were molecularly characterized for the first time with this study in Turkey. The isolate was deposited to GenBank and compared with some other published *Echinococcus* isolates from GenBank. The Erzurum isolate showed maximum identity with *E. multilocularis* isolates from Europe (GenBank acc. nos.: KR229983, KR229987, KF171965, KF171966, JN175268, EU043372, L49455) with a ratio of 100%, and this ratio was followed by isolates from Japan (GenBank acc. nos.: AB243207, AB244598) with 99.5%.

In conclusion, the presence of *E. multilocularis* in a fox in Erzurum, a highest endemic region of AE confirmed by PCR, and molecular characterization of *E. multilocularis* are presented for the first time in Turkey. Large scale studies will be conducted to investigate the prevalence of *E. multilocularis* in definitive and intermediate hosts in the region.

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Author Disclosure Statement

No competing financial interests exist.

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