INDICATION OF THE PRESENCE OF TWO DISTINCT STRAINS OF ECHINOCOCCUS GRANULOSUS IN IRAN BY MITOCHONDRIAL DNA MARKERS

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Abstract. Sixteen isolates of Echinococcus granulosus, collected from Iranian patients at surgery, and from domestic animals, including sheep, goats, cattle, and camels at slaughterhouses in Tehran and central and southern Iran were analyzed for DNA nucleotide and predicted amino acid sequence variation within regions of the mitochondrial cytochrome c oxidase I (COI) and NADH dehydrogenase subunit I (NDI) genes. A polymerase chain reaction–restriction fragment length polymorphism method, based on the DNA sequence variation in the NDI gene, was also used to rapidly survey the E. granulosus isolates. The isolates were categorized into two distinct and uniform genotype groupings. The analysis clearly indicated that the camel/dog strain (G6 genotype) of E. granulosus as well as the cosmopolitan, common sheep strain (G1 genotype) occur in Iran. The G1 genotype was found present in all four human isolates examined and it was more prevalent in domestic animals than the camel-restricted G6 genotype. In E. granulosus-endemic areas of Iran it is evident, therefore, that the majority of E. granulosus-infected livestock animals can potentially act as reservoirs of human infection, and this has important implications for hydatid control and public health.

Cystic hydatid disease (CHD), caused by Echinococcus granulosus, is prevalent in most parts of Iran, especially in rural areas where offal from slaughterhouses is incorrectly disposed of or where slaughtering is practiced on farms. Various surveys throughout the country have indicated that hydatid cysts are commonly found in sheep, cattle, goats, donkeys, camels and wild boar (Sus scrofa). Furthermore, human cases are regularly observed in the major cities of Tehran, Isfahan, and Shiraz and widespread recovery of adult worms has been reported from dogs, jackals, and wolves throughout Iran.

A phenomenon demanding close attention in the biology of hydatid disease in endemic areas is the extensive genetic variation that is characteristic of E. granulosus. Numerous studies have provided evidence that E. granulosus exists as a complex of different strains that differ in a wide variety of criteria that impact on the epidemiology, pathology, and control of CHD. Furthermore, there is evidence to suggest that some strains are more infective to humans than others. Molecular techniques, especially polymerase chain reaction (PCR)-based methods and DNA sequence analysis, have now been used extensively to characterize strain groupings within E. granulosus and, to date, nine distinct genotypes (G1-G9) have been identified. Strain typing surveys, using DNA approaches, have been undertaken on E. granulosus materials collected from a number of countries including the United Kingdom, Australia, Kenya, and China, Switzerland, and Poland. In terms of the Middle East, E. granulosus isolates have been collected and characterized using molecular techniques from Lebanon and Jordan. We have now undertaken the molecular examination of a group of isolates collected from Iran, and report the presence of different strains in Iran and other Middle Eastern countries.

MATERIALS AND METHODS

Parasites. Echinococcus granulosus hydatid cysts were collected from a range of hosts, including humans undergoing surgery, from various areas of Iran (Table 1). Cysts were processed separately and in this study an isolate represents protoscoleces collected from an individual hydatid cyst. Protoscoleces were aspirated from cysts, rinsed in saline, fixed in 95% (v/v) ethanol, and transported to the Brisbane laboratory for analysis. The protoscoleces were rinsed several times with distilled water to remove the ethanol prior to DNA extraction.

Molecular analysis. Total genomic DNA was prepared from individual isolates (Table 1) using a commercial DNA extraction kit (Qiagen, Diagen, Hilden, Germany) following the manufacturer’s instructions. Details of primers for PCR and automatic sequencing of partial mitochondrial cytochrome c oxidase I gene were amplified using standard PCR methods and the COI and NDI sequences were obtained automatically using the Applied Biosystems Terminator Cycle sequencing kit and Perkin-Elmer Cetus (Norwalk, CT) DNA Thermal Cycler 480. The DNA sequences were analyzed using the Genetics Computer Group Sequence Analysis Software Package.

The NDI PCR products obtained with DNA extracted from representative E. granulosus isolates were purified using a QIA Quick PCR purification kit (Qiagen) and then digested with the restriction endonuclease Bfa I (New England Biolabs, Inc., Beverly, MA). Routinely, 20 μl of PCR product was digested with 5 units of Bfa I for 3.5 hr. After digestion, the fragments were separated by electrophoresis on a 2.8% (w/v) Tris-borate (pH 7.4)/EDTA agarose gel containing 2% (w/v) low-temperature gelling agarose and 0.8% (w/v) agarose, and then stained with ethidium bromide.

RESULTS

Cytochrome c oxidase I gene. Partial nucleotide sequences obtained for the COI gene (391 bp) of 16 E. granulosus isolates obtained from different hosts (Table 1) were aligned with published sequences (366 or 391 bp) for the
G1-G8 genotypes. Fourteen isolates, including the four human isolates examined, produced identical sequence to that of the common domestic sheep strain (G1 genotype) with two isolates (both of camel origin) sharing identical sequence to the well characterized camel strain (G6 genotype). An alignment of the published, predicted partial amino acid G1 COI sequence to the camel strain (G6 genotype) is presented in Figure 1.

**NDH dehydrogenase subunit I gene.** Partial nucleotide sequences (471 bp) were also obtained for the NDI gene of five selected *E. granulosus* isolates obtained from different hosts (Table 1). The sequences were aligned with published sequences for the G1-G9 genotypes. The two isolates shown earlier to correspond to the G1 genotype by COI sequencing again shared absolute identity with the published G1 sequence. Fourteen isolates analyzed, four human, one sheep, one goat, and one cattle are shown as examples, shared complete identity with the G1 sequence, whereas the two camel isolates were identical to the published G6 sequence. A dot indicates an amino acid that is conserved relative to the published G1 sequence.

**Analysis of the NDI gene by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis.** The NDI DNA nucleotide sequences were obtained for the NDI gene of nine representative (of 16 examined) Iranian isolates of *Echinococcus granulosus* analyzed in the study aligned with the published (GenBank/EBI Data Bank accession number U50464). The G1 COI sequence (top line) served as a reference. Fourteen isolates analyzed, four human, one sheep, one goat, and one cattle are shown as examples, shared complete identity with the G1 sequence, whereas the two camel isolates were identical to the published G6 sequence. A dot indicates an amino acid that is conserved relative to the published G1 sequence.

**TABLE 1**

<table>
<thead>
<tr>
<th>Host</th>
<th>Isolate no</th>
<th>Organ</th>
<th>Geographic origin</th>
<th>COI genotype</th>
<th>NDI PCR-RFLP genotype</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>1</td>
<td>Liver</td>
<td>North Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human</td>
<td>2</td>
<td>Liver</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human</td>
<td>3</td>
<td>Lungs</td>
<td>North Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human</td>
<td>4</td>
<td>Lungs</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>5</td>
<td>Liver</td>
<td>Tehran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>6</td>
<td>Liver</td>
<td>South Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>11</td>
<td>Liver</td>
<td>Central Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
<td>7</td>
<td>Liver</td>
<td>South Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
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<td>Liver</td>
<td>South Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
<td>9</td>
<td>Liver</td>
<td>South Iran</td>
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<td>+</td>
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<tr>
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<td>South Iran</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Goat</td>
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<td>Heart</td>
<td>Tehran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
<td>13</td>
<td>Heart</td>
<td>Tehran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Camel</td>
<td>15</td>
<td>Lungs</td>
<td>Central Iran</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Camel</td>
<td>17</td>
<td>Lungs</td>
<td>Central Iran</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ + = analysis performed; = = analysis not performed.

**FIGURE 1.** Inferred partial amino acid sequences of mitochondrial cytochrome c oxidase I (COI) for nine representative (of 16 examined) Iranian isolates of *Echinococcus granulosus* analyzed in the study aligned with the published (GenBank/EBI Data Bank accession number U50464). The G1 COI sequence (top line) served as a reference. Fourteen isolates analyzed, four human, one sheep, one goat, and one cattle are shown as examples, shared complete identity with the G1 sequence, whereas the two camel isolates were identical to the published G6 sequence. A dot indicates an amino acid that is conserved relative to the published G1 sequence.

**FIGURE 2.** Inferred partial amino acid sequences of mitochondrial NADH dehydrogenase subunit I (NDI) for five Iranian isolates of *Echinococcus granulosus* analyzed in the study aligned with the published (GenBank/EBI Data Bank accession number S63066) G1 NDI sequence as a reference. The human, sheep, and goat isolates shared complete identity with the G1 sequence, whereas the two camel isolates were identical to the published G6 sequence. A dot indicates an amino acid that is conserved relative to the published G1 sequence.
The DNA sequence variation within regions of the mitochondrial COI and NDI genes of 16 isolates of *E. granulosus* has indicated the transmission of two strains in Iran, the common sheep/dog (G1 genotype) and the camel/dog (G6 genotype) forms. The variation in the NDI sequences of the G1 and G6 genotypes was confirmed by a PCR-RFLP assay that results in characteristically different and thus discriminatory banding patterns being generated for the two genotypes.

In areas where camels occur together with other livestock animals, humans are likely to be exposed to the camel strain as well as the sheep strain through contact with carnivorous definitive hosts, especially dogs. Nevertheless, there have been no direct reports of infection of humans by the camel strain of *E. granulosus*, which suggests strongly that it has low or no infectivity to humans. Indeed, the four human isolates examined here were each categorized as being the G1 genotype. This is consistent with results from earlier isoenzyme25 and DNA studies26,27 on a significant number of human isolates of *E. granulosus* collected from the Turkana region of Kenya, all of which were genotyped as the sheep strain. Both the sheep and camel strains are present in Turkana and their life cycle patterns overlap in intermediate as well as definitive hosts.27 It is puzzling that there is long-standing epidemiologic evidence from several areas in the Middle East, which has suggested that camels are an important reservoir for human infection.13,28 Clearly this apparent paradox will only be fully explained by extensive molecular examination of human isolates of *E. granulosus* from this and adjoining areas. In this regard, however, it is worth recounting the fact that in Somalia, where almost one-third of the world’s camel population is concentrated and where camels and dogs are frequently infected with *E. granulosus*, human cases of hydatid disease, apart from a single arbitrary cyst in a woman, have not been recorded.29

Although the number of Iranian isolates examined was not extensive, the G1 genotype was far more prevalent in domestic animals than the G6 genotype being identified in sheep, goats, and cattle. In *E. granulosus*-endemic areas of Iran it is evident, therefore, that the majority of *E. granulosus*-infected livestock animals can potentially act as reservoirs of human infection, and this has important implications for hydatid control and public health.

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