COPRO-DIAGNOSIS OF ECHINOCOCCUS GRANULOSUS INFECTION IN DOGS BY AMPLIFICATION OF A Newly IDENTIFIED REPEATED DNA SEQUENCE

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Abstract. Diagnosis of Echinococcus granulosus infection in dogs by detecting adult worms recovered post mortem or purged from the intestines after treatment with arecoline is not suitable for mass screening. Large-scale diagnosis by detection of copro-antigens is useful but only with relatively high intensity infections, and only by genus. To provide a more sensitive and specific diagnosis, a polymerase chain reaction (PCR) assay was developed, that amplified a target repeated sequence (EgG1 Hae III) newly identified in the genome of the common sheep strain of E. granulosus. This repeated sequence consists of approximately 6,900 copies, arranged in tandem, in groups of 2−6 repeats. The corresponding primers used in the PCR easily detected a single egg with no cross-amplification of DNA from closely related cestodes, including E. multilocularis and Taenia spp. Fecal samples from naturally infected dogs, with 2–10,000 E. granulosus worms at necropsy, were all PCR positive, while E. multilocularis or Taenia spp. positive controls as well as non-endemic controls were all PCR negative. This copro-PCR assay was demonstrated to be 100% specific and also detected all necropsy-positive E. granulosus-infected dogs. It is suggested that this copro-PCR assay has the potential for pre-mortem diagnosis of E. granulosus infection even in areas where E. granulosus and E. multilocularis are co-endemic.

INTRODUCTION

Cystic echinococcosis or cystic hydatidosis is a chronic helminthic zoonotic disease with a cosmopolitan distribution,1,2 and is especially prevalent in sheep-raising countries.3 The causative organism, the dog tapeworm Echinococcus granulosus, is transmitted cyclically between canines and numerous herbivorous livestock animals, which can serve as intermediate hosts. In herbivorous animals and in people who become infected by accidentally ingesting E. granulosus ova, the cystic larval form (hydatid cyst) develops, and can cause serious morbidity.1

Determining the infection rate in dogs is important for epidemiologic studies and surveillance of control programs, and is also useful for assessing the dynamics of transmission and the danger of infection. Traditionally, infection in dogs has been determined by identifying worms in intestinal washes post mortem, or following arecoline purgation.5 More recently, an enzyme immunoassay-based coproantigen test has been developed for this purpose.5 Coproantigen tests are genus specific with a specificity of approximately 97% (when worm burdens are more than 50−100 worms). However, sensitivity is relatively limited, resulting in an overall average test sensitivity of only approximately 60% for natural canine E. granulosus infection.5−6 While coproantigen tests have facilitated large-scale screening of definitive hosts,6,10 the need for improved detection sensitivity and for species-specific detection prompted the development of a copro-polymerase chain reaction (PCR) test, as has already been developed for identifying foxes infected with E. multilocularis, the causative agent of alveolar echinococcosis. In the latter case, amplification by PCR of species-specific sequences in the U1 small nuclear RNA gene10 or in the ribosomal RNA gene11 enabled identification of E. multilocularis-infected definitive hosts (foxes) with a high sensitivity and specificity.10,11 A PCR-based test for detecting E. granulosus eggs has recently been developed based on a previously identified mitochondrial cytochrome oxidase I (COI) gene.12 This test enabled specific identification of E. granulosus isolated ova, but with a sensitivity of only several hundreds of eggs. In the present study, we searched for tandem-repeated DNA sequences of E. granulosus for potential specific and highly sensitive diagnosis by PCR, as has been previously accomplished for detecting human schistosomes13,14 and Trichobilharzia.15 While tandem-repeated sequences were previously identified in the genome of the pig strain of E. granulosus,16 none has so far been used for diagnostic approaches. Since the sheep strain of E. granulosus (G1 genotype) is the most widespread and most likely to cause human infection,1,2 this strain was selected for the identification and characterization of tandem-repeated DNA sequences intended to serve as the amplification target for copro-PCR diagnosis of E. granulosus in dog feces. As far as we are aware, this is the first report of a PCR test for amplification of E. granulosus DNA from canine feces.

MATERIALS AND METHODS

Parasites. Fresh E. granulosus cysts in ovine liver tissue were obtained from the slaughter house in Nablus (the Palestinian Authority). Hydatid fluid was aspirated from the cysts and the cyst membranes were excised and scraped to remove protoscoleces. Echinococcus multilocularis protoscoleces were obtained from experimentally infected gerbils. Adult worms of Taenia hydatigena, T. pisiformis, T. ovis, Dipylidium caninum, and Mesocestoides sp. were recovered following necropsy from the intestines of dogs, then washed with phosphate-buffered saline and stored in 70% ethanol.

Isolation of DNA and preparation of genomic libraries. Materials for recombinant DNA technology including and enzymes for restriction, ligation, and dephosphorylation were obtained from New England Biolabs (Beverly, MA) when not indicated otherwise. Conditions for these procedures were used according to the recommendations of the manufacturer. High molecular weight DNA was prepared from protoscoleces of E. granulosus and E. multilocularis, and from the other helminths mentioned earlier in this report by using...
modifications of a procedure previously described. Briefly, frozen material was crushed in liquid nitrogen, then thawed into 2× extraction buffer (0.1 M EDTA, pH 8.0, 0.1 M Tris, pH 7.5, 0.2 M NaCl) containing 1% sodium dodecyl sulfate (SDS), protease K (1.5 mg/ml), and 1% β-mercaptoethanol. The solution was incubated for two hours at 37°C with intermittent gentle shaking, and nucleic acids extracted with phenol/chloroform, then precipitated in ethanol,10 dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and stored at −70°C.

Several genomic libraries were prepared from DNA of the sheep strain of E. granulosus. The one in which repeated sequences were identified was prepared as follows. DNA (0.5 μg) was partially digested with a mixture of Alu I and Rsa I restriction enzymes, and fragments with sizes ranging from 200 to 2,000 basepairs were cloned into the Sma I site of pBluescript plasmid DNA (Stratagene, La Jolla CA). Standard procedures were used for the various steps of cloning as previously described. Transformation was carried out with Escherichia coli XL1 Blue by established protocols. Transformed cells were plated out on LB agar plates containing ampicillin. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) were included in the final plating mixture for color selection of recombinant clones.

Selection of clones containing repetitive DNA from the Alu I Rsa I library. White colonies were plated on a grid, transferred to nylon filters, and initially screened with 32P-labeled total E. granulosus DNA for selecting clones exhibiting strong hybridization signals indicative of the presence of repeated sequences. Screening with total DNA from T. hydatigena and T. pisiformis was done similarly for excluding cross-hybridizing clones. Colony lysis and DNA denaturation and fixation onto the filters were carried out by a single step in a microwave oven as previously described.

Radiolabeling and filter hybridization. DNA was radiolabeled with 32P using the Ready-To-Go DNA Labeling Kit (Pharmacia Biotech, Piscataway, NJ) according to the instructions of the manufacturer. For colony hybridization and dot-blot and Southern-blot hybridizations, pre-hybridization was carried out for two hours at 60°C in the hybridization buffer containing 6× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5× Denhardt’s solution,18 100 μg/ml of salmon sperm DNA (Sigma, St Louis, MO), and 0.1% SDS. Hybridization was then carried out with 32P-labeled total DNA overnight in the same conditions, and the filters were washed once for 30 minutes at room temperature with 2× SSC, and twice for 15 minutes at 65°C with 2× SSC containing 0.1% SDS. The air-dried membranes were then exposed to x-ray film.

Dot-blot and Southern blot analyses. Comparative dot-blot hybridization was done for estimating the abundance of the repeated sequence. For this purpose, various concentrations of E. granulosus DNA and DNA of the repeated sequence amplified by PCR (see PCR assay) were dotted and comparatively probed with labeled total DNA and with the DNA of the repeated sequence amplification product. The DNA tested was dotted onto a nitrocellulose membrane by using a dot-blot apparatus (BioRad, Hercules, CA), denatured and neutralized by standard procedures,18 and finally hybridized with the radio-labeled probe as described earlier in this report. Analysis of the dot hybridization signals was performed using the public domain National Institutes of Health (Bethesda, MD) image program (developed at the National Institutes of Health and available on the internet at http://rsb.info.nih.gov/)

Southern blot analysis was done by standard procedures for determining the distribution of the repeated sequence. For this purpose, 500 ng of E. granulosus (G1) DNA was partially digested with Hae III, a restriction enzyme found to cut once within the new repeated sequence (see Results), and following Southern transfer to a nitrocellulose filter, hybridization was carried out as described earlier in this report.

Nucleotide sequencing. A Plasmid Isolation Kit (Qiagen, Chatsworth, CA) was used according to the instructions of the manufacturer for obtaining DNA from recombinant plasmids selected for sequence analysis. Nucleotide sequence analysis was carried out using Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Wallington, United Kingdom) according to the instructions of the manufacturer. An ABI PRISM 377 DNA Sequencer and ABI Sequencing Analysis software (Perkin Elmer Fostier City, CA) were used. The thermal profile was 10 seconds at 96°C, five seconds at 50°C, and four minutes at 60°C for 25 cycles. Consensus sequence was analyzed by using the Wisconsin Package (Genetic Computer Group, Inc, Madison, WI).

PCR assay. Primers were designed by using the PRIME software version 9.0, of the Wisconsin Package (Genetic Computer Group) based on the sequence information of the newly repeated sequence of E. granulosus, which is 269 basepairs long (see Results). A first set of primers was designed for amplifying the complete repeat, and the amplification product was used as a probe for analyzing the repeat abundance and distribution. These primers were as follows: Eg2691 5’-ACACCCAGCATGGAATTAC-3’ (upstream) and Eg2692 5’-ACGAGCATTGGAATTGC-3’ (downstream). The second pair of primers was designed for the diagnostic PCR. It amplifies a 133-basepair segment within the newly described repeat unit. The structure of these specific primers is as follows: Eg1121a 5’-GAAATGCAAGCAGCAGATG-3’ (downstream) and Eg1122a 5’-GAGATGAGTGAGAAGGAGTG-3’ (downstream).

Optimal PCR buffers were selected by using the OptiPrime PCR Optimization Kit (Stratagene). The PCR with both pairs of primers was carried out in a volume of 50 μl that contained 10 mM Tris-HCl, pH 9.2, 25 mM KCl, 1.5 mM MgCl2, 200 μM (each) dNTPs (Promega, Madison, WI), 0.4 μM of each of the amplification primers, 2.5 units of Tag DNA polymerase (Red Hot Taq DNA polymerase; Advanced Biotechnologies, Epsom, United Kingdom), and target DNA. For PCR with primers Eg1121a and Eg1122a, 1% formamide was added to the reaction mixture. The thermal profile used with the thermal cycler (MiniCycler; MJ Research, Watertown, MA) involved five minutes at 95°C, followed by 35 cycles, each of one minute at 95°C, one minute at 55°C, and one minute at 72°C, and a final elongation step at for 10 minutes at 72°C. The amplification products were subjected to electrophoresis on a 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) with a 100-basepair ladder size marker (Gibco-Life Technologies, Gaithersburg, MD).

Extraction of DNA from fecal samples of infected and control canines. Stray dogs collected in northern Jordan17 and Turkana, Kenya were humanely killed. Fecal samples were...
taken for DNA extraction and adult worms were recovered post mortem by intestinal washings and stored in ethanol. Fecal samples from 34 *E. granulosus*-infected dogs from Jordan (n = 27) and Kenya (n = 7) were included in the present study. Fecal samples from 18 control dogs, 8 caught in the same region in Jordan and 10 in Kenya, but with no adult *E. granulosus* worms found, served as endemic controls. The Kenyan dogs were found to be infected with other taeniids and *D. caninum*. Fecal samples from seven foxes collected in Franche-Comté, France and proven infected with *E. multilocularis* by recovery of adult worms at necropsy were also included in the present study as species-specificity controls. Fecal samples from uninfected dogs from the United Kingdom and Denmark served as non-endemic controls. Fecal samples from uninfected pets were used in experiments involving spiking with eggs and subsequent extraction of the DNA. The DNA for the PCR assay was extracted from samples of 0.3 mL of fecal sediment by using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany), or by the Invisorb Spin Stool DNA Kit (Invitek, Berlin, Germany) according to the instructions of the manufacturers.

RESULTS

Cloning and nucleotide sequence analysis of *E. granulosus* repeated sequences. A genomic library prepared by double restriction with *Alu I* and *Rsa I* yielded 204 recombinant clones, of which two containing repeated sequences were selected. The selected clones (AR5 and AR6) contained *E. granulosus* DNA segments of 770 and 792 basepairs, respectively, and each contained a pair of sequences arranged in tandem with unit lengths of 268 and 269 basepairs and with sequence identity of 98%. This sequence, shown in Figure 1, has not been previously reported. Restriction mapping of these sequences indicated that several restriction enzymes including *Hae III* cut once within each of the repeat units. We named the new sequence the EgG1 *Hae III* repeat (for species, strain, and the selected restriction enzyme cutting once within the sequence).

Arrangement in the genome of the EgG1 *Hae III* repeat. This arrangement was elucidated by Southern blot analysis using 32P-labeled EgG1 *Hae III* repeat (PCR product) to probe partially *Hae III*-digested *E. granulosus* genomic DNA. Six bands arranged in a ladder, with size increments of a unit repeat (268/9 basepairs) were obtained, as shown in Figure 2. This banding pattern indicates that the repeats are arranged in tandem with up to six repeat units in an array. Since the three first bands presented the strongest signals (> 90% of the signal intensity) it can be assumed that most of the repeats are arranged in groups of 2–3 repeats while those arranged in 4–6 repeats constitute a minority.

Abundance of the EgG1 *Hae III* repeat. The number of copies of the EgG1 *Hae III* repeat in the genome was calculated based on a comparative dot-blot analysis where varying concentrations of *E. granulosus* DNA (Figure 3A) and PCR products of the repeat (Figure 3B) were dot-blotted and probed with the 32P-labeled PCR product of EgG1 *Hae III*. The intensity of the hybridization dots obtained with the various concentrations of DNA, their corresponding densitometric peaks, and the curves representing the relationship between the quantity of DNA and hybridization signal intensity are shown in Figure 3. Signal intensity obtained with 10 ng of total *E. granulosus* DNA was roughly similar visually to that obtained with 100 pg of DNA of the EgG1 *Hae III* PCR product, thus indicating that the repeated sequence constitutes approximately 1% of the genome. A more precise evaluation of the repeat abundance was obtained by calculating the ratio between the integrals of the quantity of DNA to signal-intensity curves. The resulting ratio of 1.25% is the estimated abundance of the repeat. Given that the genome size of *E. granulosus* is estimated to be 1.5 × 10⁸ basepairs,
the approximate number of the repeat copies (a unit being 269 basepairs) is 6,900.

Detection specificity and sensitivity of the EgG1 Hae III-based PCR. The PCR results with DNA from *E. granulosus* and from related cestodes for specificity analysis, and results obtained with different quantities of DNA and with different numbers of *E. granulosus* ova for sensitivity analysis are shown in Figure 4. Ten-fold dilutions of *E. granulosus* DNA yielded a ladder of amplification bands with a detection sensitivity as low as 1 fg of DNA (Figure 4A). This degree of sensitivity should enable not only the detection of a single egg, but also the detection of infection in a pool of samples from several hosts, for improved cost efficiency. The ladder pattern of the amplification bands further demonstrates the tandem arrangement of this repeat. The size of the major bands obtained matches the theoretical size expected. Thus, the 133-basepair band constitutes the region amplified by the Eg1121a and Eg 1122a primers used (see Materials and Methods), and the other major bands (402, 671, and 940 basepairs) are larger by increments of 269 basepairs (the size of the unit repeat). Minor bands (approximately 300 and 600 basepairs), which sometime appear when larger amounts of *E. granulosus* DNA are amplified (Figure 4B), probably represent amplification products of a minor population of related repeats. As shown in Figure 4B, DNA from *E. granulosus* (1 ng) and DNA from feces spiked with *E. granulosus* ova (1,000, 100, 10, and 1) yielded a similar ladder of amplification products. By comparison, DNA from *T. ovis, T. pisiformis T. hydatidenae, D. caninum*, and *E. multilocularis* (10 ng each) yielded negative PCR signals, thus demonstrating the specificity of the test. DNA from *Mesocestoides, Schistosoma, Trichobilharzia, Echinostoma*, and *Toxoplasma* species also yielded negative PCR signals. In addition, DNA extracted from *E. granulosus* protoscoleces collected from hydatid cysts from goats and cattle from Jordan gave a similar PCR product banding pattern as those obtained from sheep, whereas those collected from horse and camel yielded different banding patterns. Since we did not examine DNA from well-characterized strains, the possible significance of these differences in banding patterns to strain differentiation is unclear, but is suggestive of the repeat sequence being more specific to the G1 genotype.

Identification of *E. granulosus*-infected dogs by the copro-PCR. Positive PCR signals are demonstrated in Figure 5 with DNA from fecal samples of dogs infected with low numbers of *E. granulosus* worms, while feces from foxes infected with nearly 2,000 *E. multilocularis* worms were PCR negative. These results further suggest that differentiation between these two species should be possible by the copro-PCR test described.

The results obtained with fecal samples from dogs naturally infected with *E. granulosus*, from endemic control dogs, and from non-endemic controls dogs are summarized in Table 1. Fecal samples from foxes infected with the related species *E. multilocularis* were also included in this study. All 34 fecal samples collected in Kenya (7) and Jordan (27) from dogs naturally infected with *E. granulosus* were PCR positive (sensitivity = 100%), including those from dogs with low numbers of worms recovered (2, 2, 7, 13, 15, 22, 27, 30, 30, 37, and 50 worms). By comparison, all 10 fecal samples collected in

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**Figure 3.** Estimation of the copy number of the EgG1 Hae III repeat in the genome of *Echinococcus granulosus* by comparative dot hybridization analysis. Signals obtained with various concentrations of genomic DNA (A) were compared with signals obtained with various concentrations of the polymerase chain reaction (PCR) product of the EgG1 Hae III repeat (B). The dots were probed with 32P-labeled PCR product of the repeat. 1 = dot blot; 2 = analysis of the dots by densitometry; 3 = a plot of signal intensity to DNA concentration.
the United Kingdom and in Denmark from uninfected dogs were PCR negative. Of the 18 samples from endemic control dogs (with no *E. granulosus* worms detected) collected in Jordan (8) and Kenya (10), four (14%) dogs were PCR positive, suggesting that either the test lacks specificity or that the PCR assay was more sensitive than *E. granulosus* worm recovery following necropsy. The second possibility seems more likely if one considers the high sensitivity and specificity of the assay. Examinations of fecal samples support the high specificity demonstrated by the lack of cross-amplification with DNA from other taeniids (Figure 4B). Thus, *E. multilocularis*-positive fecal samples from foxes (worm burdens ranging from 4 to 1,990) and fecal samples from endemic control dogs with *Taenia* spp. infections (from Kenya) were all PCR negative, suggesting a specificity of 100%.

**DISCUSSION**

Currently, the classic and most reliable methods for diagnosis of *E. granulosus* infection rely on parasitologic detection of adult worms at necropsy or after arecoline purgation. These methods are difficult to use in large-scale epidemiologic studies because they are laborious, bio-hazardous, and lack sensitivity. Copro-antigen detection assays developed relatively recently are generally more suitable and practical for this purpose, even though they have sensitivity limitations and are specific only for the genus *Echinococcus*. In areas where both *E. granulosus* and *E. multilocularis* are co-endemic, it is of utmost importance to identify the parasite to the species level, as well as to differentiate them from *Taenia* spp. Highly specific and sensitive copro-PCR assays have been developed for the detection of *E. multilocularis*, and have been used for screening of foxes in epidemiologic surveys. However, the development of a similar PCR-based test for diagnosis of *E. granulosus* infection in canids largely awaited the identification of target sequences suitable for sensitive and specific amplification. Primers based on the mito-
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<table>
<thead>
<tr>
<th>Source of fecal samples</th>
<th>Number of samples tested</th>
<th>PCR results</th>
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<tbody>
<tr>
<td>Non-endemic negative control dogs</td>
<td>10</td>
<td>10 (100%)</td>
</tr>
<tr>
<td><em>E. multilocularis</em>-infected foxes (from 4 to 1,990 worms detected)</td>
<td>18</td>
<td>14 (77%)</td>
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<tr>
<td><em>E. granulosus</em>-infected dogs (from 2 to 10,000 worms detected)</td>
<td>34</td>
<td>0</td>
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* Numbers of canines in each group are presented, and percentages of PCR-positive or -negative results in each group are indicated in parentheses.

The current assay amplified an *E. granulosus* 133-basepair fragment of the tandem repeat, and larger bands corresponding to size increments of 269 basepairs (the size of the unit repeat). All 34 fecal samples from dogs naturally infected with *E. granulosus* were found to be positive when tested by this PCR assay (Table 1), even when worm burdens were as low as two worms. The high sensitivity of the assay was demonstrated by showing that 1 fg of DNA and DNA extracted from feces spiked with a single egg gave clear PCR signals (Figure 4). The high specificity of the test was demonstrated by the lack of amplification signals with 10 ng of DNA from *E. multilocularis*, three *Taenia* species, and *D. caninum* (Figure 4B), as well as with DNA from *Mesocestoides* sp. and *Toxocara canis* and from other species of helminths. Also, although some of the endemic negative controls were found to be positive for other taeniid tapeworms at necropsy, no amplification could be observed, thus further suggesting the specificity of the current copro-PCR assay.

A similar conclusion was used to explain the occurrence of apparent false-positive results in necropsy-negative foxes from an endemic area of Germany when a highly sensitive and *E. multilocularis*-specific copro-PCR assay was used.11

Extraction of DNA is often hampered by the presence of inhibitory substances. All fecal samples that were found to be negative were verified for inhibition by spiking the samples with *E. granulosus* DNA, and none of them were found to be inhibited.

The absolute species specificity of the copro-PCR test for *E. granulosus* should enable more accurate diagnosis of infection, and data collection on the prevalence of dog infection, including dogs with low worm burdens. Furthermore, since the test is species-specific, it will be of potential great value in areas such as China and Russia where *E. granulosus* and *E. multilocularis* are co-endemic. Also, the high sensitivity of this test should enable testing of pools of samples for improved test cost efficiency, as previously done with other parasites.26,27

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