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SHORT REPORT: THE USE OF A POLYMERASE CHAIN REACTION TO DETECT *ECHINOCOCCUS GRANULOSUS* (G1 STRAIN) EGGS IN SOIL SAMPLES

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Abstract. Cystic echinococcosis is a re-emerging disease in central Asia. A total of 120 soil samples taken from 30 gardens of rural homesteads in southern Kazakhstan were analyzed for the presence of taeniid eggs using a concentration technique. Of these, 21 (17.5%) were shown to be contaminated with taeniid eggs. These isolated taeniid eggs were further analyzed using a polymerase chain reaction specific for the G1 (sheep) strain of the tapeworm *Echinococcus granulosus*, and five samples were shown to be positive. This study demonstrates the widespread contamination of the environment with *E. granulosus* eggs in an *Echinococcus*-endemic area and thus the potential for indirect transmission of *E. granulosus* to humans from such sources.

The larval stages of the tapeworm *Echinococcus granulosus* are the causative agent of cystic echinococcosis (CE), one of the most important cestode infections causing morbidity and mortality in humans and significant economic losses in livestock. Cystic echinococcosis occurs on all continents with the highest prevalences being found in the Mediterranean basin, eastern Europe, central Asian Republics, China, north Africa, and South America.[1] Control programs have been implemented with variable success in many regions with high incidence of CE (e.g., Cyprus, China, Chile, Spain), and the disease has been eradicated on some island countries (Iceland, New Zealand, Tasmania, Falkland Islands, and part of Cyprus).[2] However, there is strong evidence that this disease is an emerging problem in many countries worldwide.[3] For example, the incidence of human CE has increased 3–4-fold in the last 10 years in Kazakhstan and Kyrgystan.[4,5]

A prerequisite for initiating control programs is the understanding of the transmission biology of *E. granulosus*, and the epidemiologic characteristics in a given area include the assessment of baseline data of infections of definitive and intermediate hosts and risk factors for transmission to humans. Dog ownership, particularly length of dog ownership, and contact with dogs is an established risk factor for human echinococcosis.[6] However, a number of studies have suggested that indirect transmission of both *E. granulosous* and *E. multilocularis* may occur.[5,7–11] An association of human infection with the type of water supply, the use of non-enclosed vegetable gardens, or the consumption of food that is not protected from flies has been suggested. In addition, several studies have failed to find an association of human CE with dog ownership or the keeping of dogs.[5,7–11] Consequently, contamination of the environment with *Echinococcus* eggs must represent a possible source of infection for humans. *Echinococcus* eggs have indeed been identified in the environment in a single study using a monoclonal antibody directed against *Echinococcus* oncospheres,[12] but this method has not been used in further epidemiologic studies. The purpose of this study was to evaluate the use of the highly specific polymerase chain reaction (PCR) that can detect *E. granulosus* eggs in dog feces[13] to detect environmental contamination of soil samples with this parasite.

To investigate this, 120 soil samples were collected from 30 gardens of a village in Almaty Oblast in southern Kazakhstan, an area highly endemic for *E. granulosus*.[4] Four samples were taken from defined sampling sites. These were directly in front of the house, from the area where dogs were tied, from the area where livestock were kept, and from the "summer kitchen", a recreational area where residents often relax and eat, especially during the summer months. Each sample consisted of approximately five grams of soil. The recovery of helminth ova from the soil was done using a flotation method. This is a technique modified from one previously described,[14] which has been reported as being efficient in isolating helminth eggs from soil samples with recovery rates, for example, of 70% for *Toxocara* ova. Briefly, five grams of soil was first passed through a 4-mm^2 mesh to remove course debris. The remaining soil was suspended in a 5% solution of KOH at a ratio of 1:2 using a high speed vortex. The suspension was then centrifuged in 50-mL tubes at 500 × *g* for three minutes. The supernatant was discarded, the pellet was resuspended in KOH, and the process was repeated. The pellet was then suspended in the flotation media, a saturated solution of $NaNO_3$ (specific gravity = 1.35), and centrifuged at 4,500 × *g* for 15 minutes. The flotation media in each centrifuge tube was then topped up to form a positive meniscus on which a cover slip was superimposed and left to stand for at least five minutes. The cover slips were then examined microscopically. Any eggs detected by microscopic examination were carefully washed into a tube and identification at the genus level was confirmed using an inverted microscope. Eggs were then stored at −80°C for further analysis.

The DNA was isolated from samples containing taeniid eggs. Samples were centrifuged, and alkaline lysis of the eggs in the sediments and neutralization was performed as previously described.[15] Twenty microliters of proteinase K and 200 μL of lysis buffer of a commercial kit (Qiamp DNA mini kit; Qiagen, Hilden, Germany) were added to the samples, and the samples were digested for 10 minutes at 56°C. Samples were then centrifuged (13,000 × *g* for one minute), and supernatants transferred into new tubes. Fifty microliters of Chelex beads (50% [w/v] in distilled water; Bio-Rad Laboratories, Hercules, CA) was added to each tube, and the tubes were kept rotating for 30 minutes at room temperature. After centrifugation as described earlier, supernatants were transferred into new tubes into which 250 μL of 100% ethanol was added. After vortexing for 15 seconds, the samples were loaded onto the columns of the kit, washed according to the kit protocol, and the DNA was eluted in 100 μL of 10 mM Tris-HCl, pH 8.3, and stored at −20°C until use.

Echinococcus granulosus–specific DNA was detected with a modified PCR[13] with primer sequences Eg1f 5′-CAT TAA TGT ATT TTG TAA AGT TG-3′ and Eg1r 5′-CAC ATC ATC TTA CAA TAA CAC C-3′, which amplify a sequence of the mitochondrial 12S ribosomal RNA and is specific for the *E. granulosus* G1 (sheep) strain. Each sample was tested in triplicate, one with 25 μL of sample solution, the second with 2 μL, and the third using 2 μL spiked with 1 μL (10^5 copies) of a cloned, size-modified control target. This control target, which detects amplification inhibition, yields an amplicon of 292 basepairs upon amplification, which is easily discriminated from the *E. granulosus*-specific product (255 basepairs) after gel electrophoresis.[13] The samples were also examined using *E. multilocularis*-specific primers.[15]

Of the 120 soil samples examined, 21 samples from 18 different households contained taeniid eggs, 4 contained *Toxocara* spp., 6 contained *Toxascaris leonina*, and 5 contained *Trichuris* spp. Of these 21 samples positive for taeniid eggs, six were from samples taken directly in front of the house, five from the area of the "summer kitchen", six from the area where the dogs were tied, and four from where domestic stock were kept. Five of the 21 samples positive for taeniid eggs were PCR positive for *E. granulosus* G1 strain. Each of these five samples were from different households. Two of them were from samples taken in front of the house, one from the area around the "summer kitchen," and the other two from areas where the dogs were tied. The PCR used had already been shown to be highly specific for the G1 strain of *E. granulosus*.[13] Thus, the positive PCR results on eggs isolated from several soil samples confirm that the ova of zoonotic strains of *E. granulosus* can be recovered from environmental samples. All samples containing taeniid eggs were negative for *E. multilocularis*. Therefore, the remaining taeniid eggs were almost certainly *Taenia* spp. originating from dogs (e.g., *Taenia hydatigena* and *T. multiceps*) or from cats infected with *T. taeniaeformis*. However, as yet there are no specific techniques to confirm the species identity of these eggs.

Of the five soil samples positive for *E. granulosus* in the PCR, three contained less than 10 detected taeniid eggs, with one containing only two eggs. This illustrates that the method is feasible for identifying *Echinococcus* eggs in environmental samples such as soil. Therefore, this technique will be useful in documenting the extent of environmental pollution with *E. granulosus* eggs in future epidemiologic studies.

In both Kazakhstan and neighboring Kyrgystan, a disproportionate number of cases of CE have been recorded in children.[4,5] The finding of *Echinococcus* eggs in more than 4% of the soil samples from a village community illustrates the possibility of transmission to such individuals while playing in the garden, even if they have no direct contact with an infected dog. Such transmission from environmental sources may also help to explain some of the anomalous epidemiologic studies that have failed to detect an association with dog ownership or contact as a risk factor for developing CE. Furthermore, aerosol transmission, resulting in lung cysts, is a theoretical possibility: eggs introduced experimentally into the lungs of lambs were capable of hatching and developing into hydatid cysts.[16] A study in nearby Bishkek[5] in northern Kyrgystan suggested that approximately 50% of human cases of CE have lung cysts, which is far higher than in most other studies. Thus, the cold continental weather conditions that predominate for a large part of the year in the study area may allow the survival of eggs in dust samples and thus promote transmission to humans and explain such anomalously high numbers of pulmonary CE. An assessment of oncosphere viability of samples recovered from soil would be helpful in exploring this hypothesis.

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