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Patent and pre-patent detection of *Echinococcus granulosus* genotypes in the definitive host

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Abstract

The detection of *Echinococcus granulosus* in dogs is important for epidemiological surveillance and evaluation of cystic hydatic disease control programs. We report the efficacy of two PCR-based methods to detect patent and pre-patent infection in dogs experimentally infected with *E. granulosus*. The detection is based on amplification of a fragment of a mitochondrial gene (Mit-PCR) and a DNA repetitive element (Rep-PCR) of *E. granulosus*. We tested the ability of both methods to detect several genotypes of the parasite. Both PCR methods could detect *E. granulosus* in pre-patent and patent periods, even when microscopical observation of eggs resulted negative in fecal samples. The Mit-PCR produced the same amplification pattern for all the parasite genotypes tested while the amplification patterns with the Rep-PCR differed among groups of strains. Fecal samples collected from dogs of an endemic area were diagnosed with more sensitivity than arecoline hydrobromide purgation. These molecular methods could be applied in the confirmation of coproantigen-positive fecal samples and to verify the success of control programs.

Keywords: *Echinococcus granulosus*; PCR; Fecal; Genotypes; Experimental infection; Patency

1. Introduction

Echinococcus granulosus is the causative agent of cystic hydatid disease (CHD), a zoonosis that affects the public health and economy in many areas worldwide. Domestic livestock, wild life animals and humans infected with the larval stage of the parasite develop fluid-filled cysts, most often in the liver and lungs. Dogs are the usual definitive hosts and the parasite eggs shed in their feces become the source of infection for sheep, pigs, cows and other intermediate hosts including humans.

Detecting *E. granulosus* infection in dogs is important for epidemiological surveillance and evaluation of control programs. Traditionally, the methods used for detecting dog infection were dog necropsy and further intestinal examination, and arecoline hydrobromide purgation [1]. The latter method is inconvenient due to environmental contamination, low sensitivity and reproducibility; and detrimental effects on animals [2]. Assays for detection of parasite antigens in fecal samples from definitive hosts have been developed [3], but this procedure is not species-specific [4,5] and its sensitivity appears to be affected by parasite burdens [4,6,7].

Molecular methods, especially those based on PCR should provide the required specificity and sensitivity for *E. granulosus* detection in dogs. Although a PCR-based method for detecting a patent infection in the definitive host has been recently developed [8], it was not reported whether the assay could detect a pre-patent infection, i.e before eggs are released in the feces.

Ten distinct genotypes (G1–G10) of *E. granulosus*, differing in biological characters such as intermediate host specificity and developmental rates have been described [9–14]. Although the sheep–dog strain (G1 genotype), is the predominant strain infective to humans, there is increasing evidence that other strains can also cause significant human hydatid disease. In Argentina, for example, Tasmanian sheep (G2 genotype), cattle (G5 genotype) and camel (G6 genotype) strains produced 42% of cases of human CHD [15]. Also, the camel strain was

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reported to cause CHD in humans from Iran [16] and East Africa [17]. This genetic variation makes it necessary to assess the performance of a PCR-based assay to detect a range of *E. granulosus* strains for reliable diagnosis in definitive hosts.

Cabrera et al. [18] showed that a primer pair designed for amplification of a fragment of the mitochondrial cytochrome oxidase subunit 1 (*coxI*) gene was able to specifically identify *E. granulosus* DNA extracted from oncospheres. In addition, a primer set and a copro-PCR method were described for the detection of *E. granulosus* infection in dogs with high sensitivity and specificity [19]. The amplification target is a repetitive DNA element present in the genome of the sheep strain of *E. granulosus* but the efficacy of this latter method for detection of other *E. granulosus* strains was not documented. In the present study, the performances of both copro-PCR methods have been evaluated for the detection of distinct strains of *E. granulosus* and for diagnosis of pre-patent infections.

2. Materials and methods

2.1. DNA extraction and purification

Total *E. granulosus* genomic DNA was prepared from fresh, frozen in liquid nitrogen, or 70% ethanol preserved protoscoleces or germinal layer from individual *E. granulosus* cysts by conventional techniques [20]. In brief, protoscoleces from an individual cyst or adult worm were washed three times in phosphate-buffered saline, pH 7.2, crushed in liquid nitrogen, and digested in 200 mg proteinase K/ml and 0.5% SDS during 3 h at 56 °C. After proteinase K treatment, phenol/chloroform extractions were made and DNA was precipitated with ethanol [20]. The germinal layers were processed as described by Kamenetzky et al. [21]. *E. granulosus* genotypes were determined by sequencing a fragment of the mitochondrial *coxI* gene as described [10]. All faecal samples from dogs were examined microscopically in triplicate to determine the presence of taenid eggs. DNA extraction and purification, from 200 mg of each fecal sample, was performed using the QIAmp DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Eluates of purified DNA samples were strored at −20 °C for 24–48 h after extraction until their use in PCR reactions. Ten microliters of eluate were employed as template in PCRs.

2.2. Echinococcus granulosus eggs

Isolated *E. granulosus* eggs were obtained from a gravid proglottid of an adult worm (previously genotyped as the G1 genotype) that was mechanically disrupted. The released eggs were washed three times with phosphate saline buffer and counted under the microscope.

2.3. Experimental infection

E. granulosus protoscoleces were aspirated from sheep liver hydatid cysts collected from a slaughterhouse in Urumqi, Xinjiang, PR China. Five dogs, previously treated with 10 mg/body weight of albendazole to remove all tapeworms, were each fed 0.8 ml packed and sedimented protoscoleces (approximately 100,000) together with a small amount of cyst fluid. Animals were maintained under humane conditions, according to the Guide for the Care and Use of Laboratory Animals [22]. Stool samples were collected at 25, 33, 35, 39, 43, 46 and 50 days post-infection (p.i.). In addition, two samples were collected at 55 days p.i. from two dogs. All samples were maintained in 70% ethanol at −70 °C for at least two weeks to inactivate oncospheres. After the collection of the last sample, all dogs were humanely killed and autopsied to determine parasite burdens.

2.4. Samples from endemic areas

Stool samples were collected from nine domestic dogs from Carmen de Patagones, Buenos Aires province and Ingeniero Jacobacci, Río Negro province, Argentina, after arecoline hydrobromide purging. The samples were kept in 70% ethanol at room temperature until their shipment to the laboratory, where they were stored at −70 °C for at least two weeks to inactivate oncospheres.

2.5. Polymerase chain reactions (PCR)

Three PCR reactions were performed: Mit-PCR for mitochondrial DNA amplificaction, Rep-PCR for repetitive DNA amplification and one control Cnt-PCR. For Mit-PCR, the primer set EgO/DNA-IM1 (forward primer 5′-TCA-TATTTGTTTGAGKATYAGTKC-3′, and reverse primer 5′-GTAAATAAMACTATAAAAGAAAYMAC-3′), designed to amplify a fragment of the *coxI* gene specific for *E. granulosus* [18], was used. The thermal profile used with this set of primers involved 15 min at 95 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C followed by a final elongation step of 10 min at 72 °C. This PCR is specific for *E. granulosus*, as was demonstrated by Cabrera et al. [18].

For Rep-PCR the primer set used was Eg1121a/22a (forward primer Eg1121a 5′-GAATGCAAGCAGCAGATG-3′ and reverse primer Eg1122a 5′-GAGATGAGTGAGAAG-GAGTG-3′) that amplifies a DNA repetitive element from *E. granulosus* genome [19]. The thermal profile involved 15 min at 94 °C, followed by 35 cycles, each of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and a final elongation step for 10 min at 72 °C. This PCR method has proved to be specific for *E. granulosus* [19].

As bacterial proteases and nucleases in feces, as well as cell debris, bile acids, and other factors, may prevent amplification by physicochemical and enzymic effects [23], a control was required to be sure that no false negatives by defective extraction and/or PCR inhibition occurred. The control used involved a polymerase chain reaction (Cnt-PCR) performed with eluates of each sample analyzed to detect DNA from bacteria that are always present in the intestine of dogs. The primer set used, DG74 5′-AGGAGGTGATCCAACCGCA-3′ and RW01 5′-AACTGGAGGAAGGTGGGGAT-3′, amplifies