Comparative diagnostic value of PCR and ELISA methods in the diagnosis of *Echinococcus granulosus* infection

Valoarea comparativă metodelor de diagnostic PCR și ELISA în infestația cu *Echinococcus granulosus*

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**ABSTRACT**

Echinococcosis/hydatidosis is a parasitic zoonosis, caused by *Echinococcus* genus. According to the WHO classification, it is a zoonosis because the causative parasites pass through more than one vertebrate hosts (e.g. dogs to humans) in order to finish their biological cycle. The disease raises numerous health problems regarding both humans and animals. The complexity of the epidemiology and pathology of this disease results from the transmission cycle and from the physiological features that link the parasite with its host species. Cystic echinococcosis has a cosmopolite spreading and a general receptivity. Human echinococcosis presents a polymorphism, ranging from asymptomatic forms to highly complicated ones, extremely severe. This parasitosis continues to represent an important public health problem in most parts of the world, in spite of the progress in the domains of surveillance and control.

**Methods:** In order to compare the diagnosis value of copro-ELISA method and PCR technique in *Echinococcus granulosus* infection of dogs, 37 samples were selected randomly, from dogs that had positive copro-ELISA results for *Echinococcus* spp. The surveys were conducted during October-November 2008. 37 samples of faeces were examined, by copro-ELISA method for detection of *Echinococcus* spp. coproantigens (Chekit Echinotest, Idexx Laboratories, Switzerland) and by nested-PCR (using the primers P60.for., P375.rev. and Eg1.for. Eg1.rev.) for identifying DNA of *Echinococcus granulosus*, G1 sheep strain.

**Results:** Of the 37 faecal samples examined, 35 (94.6%) were positive for both methods, as these dogs were infected with *Echinococcus granulosus*. Only 2 samples (5.4%) were PCR negative but positive for copro-ELISA test.

**Conclusion:** The identification of DNA by PCR technique is a certainty in the diagnosis of *Echinococcus granulosus* infection in dogs and can be used to confirm positive samples at copro-ELISA test.

**Keywords:** *Echinococcus granulosus*, dog, copro-ELISA, copro-PCR

**Introduction**

To establish accurately the infection with the species of the genus *Echinococcus* in the dog, as definitive host, it requires the use of efficient diagnostic methods in order to reflect a real level of infection (Eckert et al., 2000). Over time, in the researches carried out on intestinal echinococcosis in dogs, many methods have been applied for identification of positive cases. Due to inconveniences in terms of the usual methods of diagnosis (purigation with arecoline hydrobromide - high risk of dissemination of oncospheres in the environment; necropsy - an animal requires euthanasia; the coproparasitological techniques have low sensitivity), there were many diagnostic methods developed for achieving better sensitivity and specificity and without threatening the life of the animal. Thanks to recent findings focusing on molecular biology and immunology, it has been perfected and introduced in laboratories, techniques with high sensitivity and specificity, including enzyme
immunoassay tests - ELISA (Enzyme-Linked-Immunosorbent Assay), and methods of identification of parasite's DNA structure - PCR (Polymerase Chain Reaction) which are considered for the moment as reference methods (Eckert and Deplazes, 1999, Mathis and Deplazes, 2006)

Materials and methods

Aim of study: The aim of the study was to compare the diagnostic value of copro-ELISA and copro-PCR techniques in natural Echinococcus granulosus infection of dogs.

Period: The survey was conducted during October-November 2008, at the Discipline of Parasitic Diseases, Faculty of Veterinary Medicine, Cluj-Napoca, Romania.

Experimental design: 37 samples were collected, from dogs that had positive copro-ELISA results for Echinococcus spp. The dogs belonged to counties from northwestern Romania. The faecal samples were examined by two modern diagnostic methods: copro-ELISA for detection of Echinococcus spp. coproantigens and by PCR technique for identifying DNA of Echinococcus granulosus, G1 sheep strain.

For the ELISA technique we used the commercially available Chekit Echinotest (IDEXX Laboratoires, Switzerland). The Chekit Echinotest is designed for the detection of Echinococcus granulosus and Echinococcus multilocularis coproantigens in dogs, foxes and cats, thus is only genus specific. The test was performed according to the manufacturers instructions, using 1g of faecal material diluted 1:4 in the kit’s sample dilution buffer. The samples were run in duplicates. After centrifugation of the sample suspension (3,000 g at room temperature for 10 min), the supernatants were used for ELISA. Estimations of coproantigens level were carried out. For the ELISA technique we used the commercially available Chekit Echinotest (IDEXX Laboratoires, Switzerland). The Chekit Echinotest is designed for the detection of Echinococcus granulosus and Echinococcus multilocularis coproantigens in dogs, foxes and cats, thus is only genus specific. The test was performed according to the manufacturers instructions, using 1g of faecal material diluted 1:4 in the kit’s sample dilution buffer. The samples were run in duplicates. After centrifugation of the sample suspension (3,000 g at room temperature for 10 min), the supernatants were used for ELISA. Estimations of coproantigens level were carried out. The interpretation of results was realized following the attached protocol from the above producer. Results were expressed in value % and values >40% were considered as positive.

For the copro-PCR, the DNA extraction was carried out after the protocol described by Mathis et al. (1996). It is based on a method for concentration of oncospheres from faeces because their number and dispersion in the faecal mass is not constant, and in case of few oncospheres, we have to increased their chances of isolation. Of the concentrate obtained, we selected 10 oncospheres and release them into a solution of 0.02N NaOH. This was heated to 95°C for 10 minutes and 1 ml of lysate was used directly for PCR.

The PCR was performed in two steps (nested PCR). For the first PCR the primers used were P60.for. (5'-TTAGATATATGGTACAGGATTAGATA CCC-3') and P375.rev. (AACCGAGGGTGACGGGCTGTTACC-3'-5') which amplified a product of 373 base pairs specific for cestodes and for the second PCR we used the primers Eg1.for. (5'-CATTAATGTATTTTGTAACGGTGG-3') and Eg1.rev. (3'-CACATCATCTTACAATAACACC 5') which amplified a product of 255 base pairs of the species Echinococcus granulosus, the G1 sheep strain.

The PCR mix had the following composition: 12.5 ml Master Mix, primers (50 pmol each), 2.5 ml DNA obtained after extraction and 9 ml bidistilled sterile water, with a final volume of 25 ml. The first amplification was performed after the following program: initial denaturation (95°C for 5 min.), denaturation (94°C for 60 sec.), hybridization (55°C for 60 sec. - 40 cycles), elongation (73°C for 30 sec), final elongation (72°C for 7 min.).

In the second PCR, the mixture of amplification (PCR mix) had the following composition: 12.5 ml Master Mix, primers (50 pmol each), 2μl DNA amplification product and 9.5 ml sterile bidistilled water, with a final volume of 25 ml. The second amplification was performed after the following program: initial denaturation (98°C for 5 min.), denaturation (94°C for 30 sec.), hybridization (55°C for 45 sec. - 30 cycles), elongation (72°C for 45 sec.), final elongation (72°C for 7 min.). For the visualization of amplicons 4 μl of amplification product was used and we added 2 ml of staining solution. Then the migration was performed in 1.5% agarose gel, TBE-buffer (Triss, boric acid, EDTA) at 80V, 72 mA, for 60 minutes (Dinkel et al., 1998). After the solidification of the agarose, the electrophoretic liquid was poured into the tank for migration (buffer), then the PCR products of amplification, were filled in the agar wells, along with a molecular weight marker.

Results and discussion

The prevalence values obtained in dog intestinal echinococcosis differed according to the diagnostic test applied.

The ELISA method

Of the 37 faecal samples examined using the enzyme immunoassay test (ELISA), all the
samples were positive for *Echinococcus* spp. coproantigens. The optical density (OD) values of positive samples ranged between 40 and 146. Distribution of optical density values (OD) in ELISA, of the faecal samples from the randomly selected dogs in northwestern Romania, is given in figure 1.

![Figure 1. Distribution of ELISA OD values for faecal samples of dogs (n=37) tested for *Echinococcus* coproantigens](image)

**PCR for Echinococcus granulosus, sheep strain**

To determine the real prevalence of *Echinococcus granulosus* in dogs, after performing the copro-ELISA test, the 37 samples of faeces were subjected to DNA extraction and nested-PCR. Following the studies performed for identification of *E. granulosus* DNA, G1 sheep strain by PCR technique, using the primers pairs *(P60.for.)*, *(P375.rev.)* and *(Eg1.for.)* *(Eg1.rev.)*, we obtained an amplicon of 255 pb at 35/37 of dogs examined (fig. 2).

![Figure 2. Identification of *E. granulosus* DNA, by copro-PCR, 255 pb amplicon](image)

Comparing the diagnostic value of copro-ELISA and copro-PCR in intestinal echinococcosis of dogs, of the 37 faecal samples examined, 35 were positive for both methods used (94.6%), as these dogs were infected with *Echinococcus granulosus*. Only 2 samples (5.4%) were PCR negative but positive for copro-ELISA test (table 1). These were false positive samples.
Tabell1. Comparative results of copro-ELISA and copro-PCR exam in the diagnosis of *Echinococcus granulosus* infection in dogs (n=37).

<table>
<thead>
<tr>
<th>Test</th>
<th>PCR +</th>
<th>PCR –</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpAgELISA +</td>
<td>35 (94,6%)</td>
<td>2 (5,4%)</td>
<td>37 (100%)</td>
</tr>
<tr>
<td>CpAgELISA –</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Our preliminary results, show that the identification of DNA by PCR technique is a certainty in the diagnosis of *Echinococcus granulosus* infection in dogs and should be used to confirm positive samples at copro-ELISA test.

The ELISA test for *Echinococcus* coproantigens, available in our country, is only genus specific, so although the 37 samples positive for coproantigen at ELISA, 2 samples were negative for *Echinococcus granulosus* in DNA identification by PCR, but it cannot be excluded the possible presence of *Echinococcus multilocularis* or the existence of cross-reaction with *Taenia* spp.

Absence of *Echinococcus granulosus* in PCR may be due to elimination of oncospheres intermittently or during prepatent infections.

*Intra-vitam* diagnosis of infection with various species of cestodes from carnivores, has always been difficult because oncospheres (eggs) from all species of the family *Taeniidae* cannot be identified morphologically. Lately, two diagnostic alternatives had been shown to solve this problem, the coproantigen detection by ELISA (Craig, 1997; Hoyle et al., 2001) and the DNA identification by PCR (Mathis and Deplazes, 2006).

Discovering the ELISA technique to detect parasite antigens in stool samples has made important improvements in diagnosis of echinococcosis in dogs: positive results were obtained in ELISA even during prepatency (Ahmad and Nizami, 1998), ELISA values become negative in 2 -4 days after suppression (Jenkins et al., 2000) and ELISA results were correlated well with the load of parasites in the intestine of dogs (Craig et al., 1995).

In Romania, due to the evolution of endemic echinococcosis in humans, a key goal is to determine the prevalence of infection with *Echinococcus granulosus* in dogs from areas of the country, where the flock of sheep is numerous, knowing that the presence and persistence of the parasite takes place mainly through the participation of the dog as definitive host and sheep as intermediate host. With the implementation of ELISA enzyme immunoassay tests, that goal has become feasible in our country, too.

In studies conducted in northwestern counties from Romania, it was observed that the prevalence of *Echinococcus* spp. coproantigens in dogs was high (28% in Cluj county; 12.5% in Satu-Mare county) (Stefania Seres et al., 2006; Stefania Seres et al., 2008), which is a signal for the need to implement efficient control programs in echinococcosis, starting with the detection of positive dogs. Also, in 2005, Iacobiciu et al., are describing a prevalence of 86.74% to 100.000 inhabitants of cystic echinococcosi in humans.

It was difficult to detect the *Echinococcus granulosus* infection in dogs and to obtain real data on its’ prevalence in our country, because the use of diagnostic techniques (necropsy of the small intestine, purgation with arecoline, coproparasitologic techniques) had unsatisfactory specificity and sensitivity. These shortcomings have been overcome by introducing the ELISA technique and the molecular tools.

Numerous studies from abroad emphasize the applicability of the ELISA technique in epidemiological screenings of intestinal echinococcosis in carnivores. Thus, Lahmar et al. (2007), in a comparative study using different diagnostic methods in screening of *Echinococcus granulosus* in dogs, recommended the use of ELISA method, as this test confirms the best copro-positive samples, compared to the purgation with arecoline or necropsy.

The PCR technique is used on a larger scale in copro-diagnosis of echinococcosis in the definitive hosts, and is considered for the moment the most specific diagnostic method (Mathis and Deplazes, 2006). By using specific primers, the *Echinococcus* infection can be diagnosed up to species level, with a specificity of 100% (Stefanic et al., 2004; Dinkel et al., 2004).

Our results, are similar with other studies. Thus, an interesting study was conducted on 65 dogs experimentally infected with *E. granulosus.*
The authors compared the diagnostic methods: purgation with arecoline, copro-ELISA, copro-PCR and necropsy. Infection rate by necropsy examination was 89.2%. Purgation with arecoline had only 43% efficiency in dogs, after a single administration, this value increasing to 76.9% after administration of two doses. Copro-ELISA test detected the *E. granulosus* infection in 82.8%, with positive and negative predictive values of 96 and 44.4%. *E. granulosus* DNA was detected in 25.9% of faecal samples by copro-PCR even in the prepatent period. The final results showed that copro-ELISA is more sensitive than purgation with arecoline. Also, copro-PCR technique detected DNA of *E. granulosus* in dog feces by 21 days before the elimination of oncospheres (Lahmar et al., 2007).

Other studies have revealed similar results for copro-diagnosis by PCR in *E. granulosus* infection. Abbasi et al., 2003, used for diagnostic in dogs, a repetitive sequence (EgG1 Hae III), identified in the genome subtype with *E. granulosus* sheep strain, and obtained a specificity of 100%, identifying any positive necropsy examination.

In a study performed in dogs from northern Spain, the coproparasitologic examination revealed the presence of *Taeniidae* oncospheres in 10.4% of dogs, while the copro-ELISA test was positive in 87% of cases, concluding that the coproparasitologic method underestimates the real prevalence of *Echinococcus granulosus* infection (Benito et al., 2006). Similar results were obtained by Craig et al. (1995), the copro-ELISA test had a very high negative predictive value (98.8%), making it suitable for the screening of dog populations with low prevalence of *E. granulosus*.

Thus, in areas with low prevalence of intestinal echinococcosis, the copro-ELISA test with its ability to assess the current status and intensity of infection, is a method of choice. However our study shows that further investigation is desirable, because of possible false positive results, using the identification of DNA by PCR, conclusion which is underlined also by Stefanic et al., 2004. In our study the real prevalence of intestinal echinococcosis in dogs was obtained by PCR (94.6%).

Conclusions

1. Prevalence of *Echinococcus* spp. coproantigens in the studied dogs, revealed by ELISA was 100%, but besides that the used kit was only genus specific, the technique can give false positive results (5.4%).

2. In our study the real prevalence of intestinal echinococcosis in dogs was obtained by PCR (94.6%).

3. Identification of DNA by PCR technique is a method of certainty in the diagnosis of *Echinococcus granulosus* in dogs and should be used to confirm ELISA positive cases.

**REZUMAT**

diagnosticul infestației cu *Echinococcus granulosus* la câini și poate fi folosită pentru a confirma probele pozitive la copro-testul ELISA.

References


