

The diagnosis of *Echinococcus granulosus* in dogs

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Abstract. The problem of diagnosing *Echinococcus granulosus* in dogs has still only been partially resolved, even after the advent of biotechnology. The eggs of taeniid Cestoda are extremely similar, and thus identification by microscopic examination of the faeces is risky and non-specific. For this reason, *Echinococcus granulosus* was traditionally diagnosed in dogs *ante mortem* after an arecoline hydrobromate purge. The faeces were examined macro and microscopically to establish if the adult tapeworm or its proglottids were present. Although this method is 100% specific, it is bio-hazardous and time-consuming, requires trained personnel, and its sensitivity varies. In the 1990s copro-antigens were discovered and characterised. These are released by the adult worm in the faeces. This made it possible to use enzyme-linked immune-adsorbent assay (ELISA) for *in vitam* diagnosis of *Echinococcus granulosus*. In recent years several PCR protocols have been published on the identification of *Echinococcus granulosus* DNA from eggs or from adult parasites and new ways of diagnosing this cestode have been developed.

Key words: diagnosis, dogs, echinococcosis, copro-antigens, PCR.

The Gold Standard technique of *Echinococcus* infection in the definitive host is the recovery of adult parasites in the intestine after necropsy. The best known method is described by Eckert *et al.* (2001) as the "sedimentation and counting technique" (STC). This is based on examining the faeces in the small intestine of the dogs with a microscope and counting the number of adult parasites. Although this method is 100% sensitive and specific it is also time consuming and bio-hazardous and can only be used on dead animals.

This paper reviews other alternative methods for diagnosing echinococcosis in dogs. These may be used for routine *in vitam* diagnosis, epidemiological studies and disease control plans.

Before starting, it is important to remember that these experiments involve a potential risk of infection for humans, so safety precautions should be taken when handling this material. Eggs can be deactivated by freezing at -80°C for at least 4-7 days or by heating to $\geq 60^{\circ}\text{C}$ for 5 min (Eckert *et al.*, 2001).

Classical methods

Arecoline purging

Ante mortem diagnosis of canine echinococcosis has traditionally been performed by purging with arecoline hydrobromate (Eckert *et al.*, 2001). Arecoline is a parasymphathomimetic drug that, when given to dogs in tablet or liquid form at doses between 1.75-3.5 mg/kg body weight, purges the entire intestinal contents, increases intestinal peristaltic movements,

and paralyses the tapeworms. These can then be collected and identified.

This technique has been used in many control programs all over the world in recent decades. It has got 100% of specificity, however it has certain limitations. For example, its sensitivity is limited since not all dogs respond to the purge, and not all infected dogs eliminate *Echinococcus granulosus*, it is also bio-hazardous and time-consuming and must be administered by trained personnel (Eckert *et al.*, 2001). In situations where endemic rate of *Echinococcus granulosus* in the dog population is low, the predictive value of the test diminishes as the percentage of infected dogs decreases (Schantz, 1973).

This is an unpleasant technique but is the only quantitative technique that can be used on living dogs and it continues to play an important role in epidemiological studies. Most epidemiological data, and the models developed from them, come from the results of this method (Torgerson *et al.*, 2003).

Macroscopic and microscopic examination

Adult parasites or proglottids can be recovered and identified by macroscopic exam of faeces, but unless purging is used the chances of finding the adult Cestoda are extremely inconsistent. In addition copro-microscopic examination (sedimentation and flotation technique) to detect *Echinococcus granulosus* eggs is, unfortunately, not a useful method of diagnosing for this parasite.

Echinococcus granulosus eggs are morphologically indistinguishable to those of other taeniid Cestoda, emission of eggs is variable and inconstant (and naturally not present in the prepatent period). Copro-microscopic examination may be used successfully if it is combined with other more specific techniques, such as PCR examination of DNA from the isolated eggs.

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Detection of serum antibodies

Specific antibodies against oncosphere and protoscolex antigens can be readily detected in the serum of infected dogs (Heath *et al.*, 1985; Jenkins and Rickard, 1985). This is not yet a practical method, as it does not differentiate between current and previous infections and there are some problems related to cross-reactivity or specificity of the test (Gasser *et al.*, 1994).

The detection of serum antibodies using parasite antigens in ELISA has been considered unsuitable for reliable diagnosis of intestinal *Echinococcus* spp. infections because there is a poor co-relationship between the presence of specific antibodies and the worms (Eckert *et al.*, 2001). The development of diagnostic methods based on the detection of faecal copro-antigens has superseded this diagnostic method. Indeed a comparison of copro-diagnosis and serology detection found that identifying copro-antigens was 2.5 times more sensitive than antibody detection (Walters and Craig, 1992; Craig *et al.*, 1995).

New techniques

Copro-antigens detection

An alternative to arecoline testing, based on a faecal antigen-detection antibody sandwich enzyme-linked immunosorbent assay (ELISA), has been developed recently. This has shown particular promise, as copro-antigens can be detected shortly after infection (10-14 days) and their level declines rapidly following expulsion of the worms (within 3-4 days) (Malgor *et al.*, 1997).

The test is based on a parasite-specific layer of captured IgG antibodies which retains antigens from faecal supernatants. Copro-antigen detection ELISA tests have been developed that uses polyclonal antibodies to *Echinococcus granulosus* excretory/secretory (ES) antigens. *Post mortem* examination of naturally infected dogs showed 56% sensitivity and 96% specificity (Deplazes *et al.*, 1992).

Allan *et al.* (1992), using antiserum to somatic antigens in copro-antigen detection, found this was 88% sensitive in naturally infected dogs. False negative results in these studies have been attributed to low worm burden. Indeed, when the results were compared with those of *post mortem* examination, overall sensitivity was 63% but this increased to 92% in dogs with more than 100 worms (Deplazes *et al.*, 1992).

Copro-antigens can be detected prior to the release of the eggs by *Echinococcus* worms, and therefore are not related to egg antigens (Deplazes *et al.*, 1992; Sakashita *et al.*, 1995). Positive ELISA results were obtained during the prepatent period in dogs as early as 5 days post infection (Deplazes *et al.*, 1992; Sakashita *et al.*, 1995; Nonaka *et al.*, 1996). This has the advantage of being able to detect prepatent infections.

ELISA copro-tests can also detect heat-stable anti-

gens. They have been used in a number of studies in the Middle East, Wales, Southern and Eastern Europe, and South America (Deplazes *et al.*, 1992; Sakashita *et al.*, 1995; Eckert *et al.*, 2001). The high sensitivity of monoclonal antibodies (MAb) to parasite specific antigens could increase the reliability of copro-antigen detection. Some sandwich ELISA systems have been evaluated for their ability to detect *E. granulosus* copro-antigens. These used a monoclonal antibody produced against somatic extract of *E. multilocularis* (Sakai *et al.*, 1995; Malgor *et al.*, 1997). Although the test was very sensitive (100%) in naturally and experimentally infected animals, there were also cases of cross-reactivity with *Taenia hydatigena* (Malgor *et al.*, 1997).

Recently the first MAbs for *E. granulosus* copro-antigen detection were produced: two IgM murine monoclonal antibodies (MAbs), EgC1 and EgC3, against the excretory/secretory (E/S) products of *E. granulosus* adult worms (Casaravilla *et al.*, 2004). A copro-antigen capture ELISA was developed using a rabbit polyclonal antibody against E/S products from adult tapeworms as catching antibodies, and each of the MAbs as detecting antibody. The assays detected 7 out of 8 (EgC1), and 8 out of 8 (EgC3) experimentally infected dogs (worm numbers ranging from 61 to 57,500), and none (n=8) of the negative control samples. Faecal samples from 2 dogs experimentally infected with *E. multilocularis* were not recognised by the EgC1 assay. This suggests that this is a potential species-specific diagnostic tool for discriminating *E. granulosus* and *E. multilocularis* infections (Casaravilla *et al.*, 2004). These advances made in applying MAbs in ELISA tests for copro-antigen detection suggest that this may be an interesting research line in order to develop new and more sensitive kits for diagnosing echinococcosis in dogs.

PCR protocols

It would be useful to develop more specific techniques in cases where the presence of the parasite in the dog population is relatively low (Christofi *et al.*, 2002), as well as for discriminating between dogs with *Echinococcus* and those with other taeniid infections. Several PCR tests were developed for detecting *E. granulosus*-specific DNA (Cabrera *et al.*, 2002; Abbasi *et al.*, 2003; Dinkel *et al.*, 2004; Stefanic *et al.*, 2004).

Two different protocols could be used for isolating the DNA: one extracts DNA from the total amount of faeces, and the second isolates and concentrates first taeniid eggs by combining sequential sieving with flotation solutions (Mathis *et al.*, 1996). This second protocol seems to be most useful because: faeces could contain substances that inhibit DNA amplification, only a limited amount of material can be processed in DNA extraction, and laborious purification of the DNA is often indispensable (Stefanic *et al.*, 2004).

DNA is obtained by alkaline treatment (to lyse the

eggs), neutralisation, proteinase K digestion and DNA purification using organo-solvent extractions and/or DNA adsorbing matrices or, more recently, by the use of commercially available kits designed for DNA isolation from faeces (Abbasi *et al.*, 2003; Štefanic *et al.*, 2004). The available protocols mentioned above for detecting *E. granulosus* are all designed to detect G1 *E. granulosus* (Sheep strain). The protocol evaluated by Dinkel *et al.* (2004) describe primers that detect strains G5 (Cattle strain), G6 (Camel strain) and G7 (Pig strain), with the additional possibility of strain typing by means of a second PCR.

Only the protocols of Abbasi *et al.* (2003) and Štefanic *et al.* (2004) were tested on faecal or environmental material, while Cabrera's *et al.* (2002) had a sensitivity limit of at least 100 eggs per gram of faeces when applied to infected dogs (Abbasi *et al.*, 2003).

The PCR test used by Abbasi *et al.* (2003) found 100% sensitivity and specificity using DNA samples extracted from 0.3 ml of faeces from 34 infected and 18 non-infected dogs, and found positive results even when the sample contained only two *E. granulosus* eggs.

Conclusions

Although PCR is a very sensitive technique for detecting parasite-specific DNA from a very small number of eggs, it is not quantitative and is not suitable for large scale screening of samples. So, at the moment the method of choice for *in vitam* diagnosis of *E. granulosus* in large populations of dogs should be the detection of copro-antigens. This is highly sensitive, fast and cheap.

Detection of *E. granulosus* by ELISA has a very high negative predictive value. However positive prediction using this method becomes poor when the prevalence of the parasite in dogs is very low (Christofi *et al.*, 2002). In this epidemiological situation, copro-antigen positive dogs could be screened with a PCR-based technique to distinguish between true and false positive results. In the future, it may be possible to diagnosis copro-antigens using highly specific MAbs for the through ELISA test. This could be used for large scale screening in control programs and epidemiological studies.

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