Chapter 2.1.4.

Echinococcosis/Hydatidosis

Summary

Diagnosis of echinococcosis in dogs or other susceptible carnivores relies on the demonstration of adult cestodes of the Echinococcus genus or their eggs in their faeces or small intestine. Recently, coproantigen and copro-DNA assays have proven useful for safe, fast and accurate diagnosis. In intermediate hosts, diagnosis depends on detection of the larval cyst form that can infect almost any organ, particularly the liver and lungs.

Identification of the agent: At present, five species of the genus Echinococcus are regarded as taxonomically valid. These are E. granulosus, E. multilocularis, E. oligarthrus, E. vogeli and E. shiquicus. Echinococcus oligarthrus and E. vogeli occur less frequently than the first two species. Until recently E. shiquicus had been discovered only in a specific region of the People’s Republic of China. These five species are morphologically distinct in both adult and larval stages. A number of intraspecific variants have been described for E. granulosus, which exhibit morphological and biological characteristics, and these can reliably be differentiated by DNA analysis. Some of the E. granulosa genotypes have been recommended for elevation to species status.

Larval forms of Echinococcus can usually be visually detected in organs. Special care has to be taken for a specific diagnosis of E. granulosus in instances where Taenia hydatigena in sheep is also a problem. Histological examination may confirm the diagnosis after formalin-fixed material is processed by conventional staining methods. The presence of a periodic-acid-Schiff positive, acellular laminated layer with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of metacestodes of Echinococcus. The identification of larval E. multilocularis in rodents and other hosts is possible by macroscopic or microscopic examination and by DNA detection using the polymerase chain reaction (PCR).

The small intestine is required at necropsy for the detection of adult Echinococcus spp. in wild or in domestic carnivores. The technique of carrying out surveys with the use of arecoline has been generally adopted for determining the prevalence of E. granulosus in dogs. Handling infected material presents a risk to the operator of contracting a potentially fatal disease. Significant progress is being made in the development of immunological tests for the diagnosis of intestinal Echinococcus infections by use of coproantigen detection. The technique has been used successfully in surveys of E. granulosus in dogs and is currently used in surveys for E. multilocularis in populations of dogs, foxes, and cats. Coproantigen detection is possible in faecal samples collected from dead or living animals or from the environment.

PCR/DNA methods for the detection of E. multilocularis and more recently E. granulosus in definitive hosts have now been established in specialised laboratories as diagnostic techniques.

Serological tests: Antibodies directed against oncosphere, cyst fluid and protoscolex antigens can be detected in the serum of infected dogs and sheep, but this approach is presently of limited practical use as it does not distinguish between current and previous infections. Cross-reactivity between Echinococcus and Taenia species also may occur.

Requirements for vaccines and diagnostic biologicals: Progress has been made in the development of an effective vaccine against infection with the larval stage of E. granulosus in sheep and cattle.
A. INTRODUCTION

Species under genus *Echinococcus* are small tapeworms of carnivores with larval (metacestode) stages known as hydatids proliferating asexually in various mammals including humans. There were four morphologically distinct species in this genus until recently when *Echinococcus shiquicus* was added to the previously known species: *E. granulosus*, *E. multilocularis*, *E. oligarthrus*, and *E. vogeli*. Discovered in the Shiqu County, the Qinghai-tibet plateau region of western Sichuan, the People’s Republic of China (57, 58), *E. shiquicus* is morphologically distinct both in adult and larval stages from other species.

A number of interspecific and intraspecific variants have been described for *E. granulosus*. Some genotypes of *E. granulosus* exhibit characteristic features that would justify the recognition as separate species according to some authors. Recently other species and genotypes of *Echinococcus* have been proposed (51). Further studies are needed to define the full range of genetic diversity (32, 37, 43, 50). *Echinococcus granulosus* has a global distribution; *E. multilocularis* occurs in wide areas of the Northern Hemisphere. *E. shiquicus* is found in the People’s Republic of China and *E. oligarthrus* and *E. vogeli* are confined to Central and South America. All five species are infective to humans causing various forms of echinococcosis. Human cystic echinococcosis, caused by *E. granulosus* and alveolar echinococcosis, caused by *E. multilocularis*, are important public health threats in many parts of the world (56).

Table 1. Useful characteristics for identification of *Echinococcus* species. Source: Xiao et al. (58)

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution</th>
<th>Definitive Host</th>
<th>Intermediate Host</th>
<th>Adult:</th>
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<tbody>
<tr>
<td><em>E. granulosus</em></td>
<td>Cosmopolitan</td>
<td>Dogs</td>
<td>Ungulates</td>
<td>Body length (mm) 2.0–11.0</td>
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<td>No. segments 2–7</td>
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<td>Length of large hooks (µm) 25.0–49.0</td>
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<td>No. testes 25–80</td>
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<td>Position of genital pore</td>
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<td>a. Mature segment Near to middle</td>
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<td>b. Gravid segment Posterior to middle</td>
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<td>Gravid uterus Branching laterally</td>
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<td>Metacestode Unilocular cysts in viscera</td>
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<tr>
<td><em>E. multilocularis</em></td>
<td>Holoarctic region</td>
<td>Foxes</td>
<td>Microtine rodents</td>
<td>Body length (mm) 1.2–4.5</td>
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<td>No. segments 2–6</td>
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<td>Length of large hooks (µm) 24.9–34.0</td>
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<td>b. Gravid segment Anterior to middle</td>
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<td>Gravid uterus Sac-like</td>
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<td>Metacestode Multilocular cysts in viscera</td>
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<tr>
<td><em>E. oligarthrus</em></td>
<td>Neotropical region</td>
<td>Wild felids</td>
<td>Neotropical rodents</td>
<td>Body length (mm) 2.2–2.9</td>
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<td>No. segments 3</td>
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<td>b. Gravid segment Anterior to middle</td>
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<td>Gravid uterus Sac-like</td>
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<td>Metacestode Polycystic cysts in muscles</td>
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<tr>
<td><em>E. vogeli</em></td>
<td>Neotropical region</td>
<td>Bush dog</td>
<td>Neotropical rodents</td>
<td>Body length (mm) 3.9–5.5</td>
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<td>No. segments 3</td>
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<td>Length of large hooks (µm) 49.0–57.0</td>
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<td>Length of small hooks (µm) 30.0–47.0</td>
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<td>No. testes 12–20</td>
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<td>Position of genital pore</td>
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<td>a. Mature segment Posterior to middle</td>
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<td>b. Gravid segment Anterior to middle</td>
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<td>Gravid uterus Tubular</td>
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<td>Metacestode Unilocular cysts in viscera</td>
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<tr>
<td><em>E. shiquicus</em></td>
<td>Tibet plateau</td>
<td>Tibetan fox</td>
<td>Plateau pika</td>
<td>Body length (mm) 1.3–1.7</td>
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<td>No. segments 2–3</td>
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<td>Length of large hooks (µm) 20.0–23.0</td>
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<td>Length of small hooks (µm) 16.0–17.0</td>
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<td>No. testes 12–20</td>
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</table>

**Echinococcus granulosus**

The parasite is transmitted between the domestic dog and a number of domestic ungulate species. The dog/sheep cycle is most important. Sylvatic definitive and intermediate hosts also occur, e.g. wolf/cervid. The adult varies between 2 and 11 mm in length and usually possesses from two to seven segments, averaging from...
three to four segments. The penultimate segment is mature, and the genital pore normally opens posterior to the middle in both mature and gravid segments. The last (gravid) segment is usually more than half the length of the entire worm. There are rostellar hooks of various sizes on the protoscolex in two rows. The size of the hooks varies between 25 to 49 µm in the first row, and between 17 and 31 µm in the second row. The gravid uterus has well-developed sacculations.

The larval stage is a fluid-filled bladder or hydatid cyst that is unilocular, although communicating chambers also occur. Growth is expansive, and endogenous daughter cysts may be produced. Individual bladders may reach up to 30 cm in diameter and occur most frequently in liver and lungs, but may develop in other internal organs. The infection with this stage is referred to as cystic echinococcosis.

The strain specificities of E. granulosus in domestic cycles include, dog/sheep in the Mediterranean region, South America (Argentina, Brazil, Chile, Peru and Uruguay), Africa (Ethiopia, Kenya and Sudan), the Middle East and Levant regions, Russia, Central Asia (Kazakhstan, Kyrgyzstan and Uzbekistan), Mongolia, the People’s Republic of China, Oceania and the United Kingdom; dog/horse in Belgium, Ireland and the United Kingdom; dog/cattle in Belgium, Germany, South Africa and Switzerland; dog/swine in Poland; and dog or wolf/reindeer in sub-Arctic regions of Norway, Finland and Alaska. The status of dog/camel strains requires further elucidation. This strain has recently been identified in human cases in Argentina, Nepal, the People’s Republic of China and Iran (3, 19, 44, 59). To date, all genotypes of E. granulosus except the dog/horse (G4) and the Finnish cervid (G10) strains have been found to infect humans.

**Echinococcus multilocularis**

The parasite is transmitted primarily between wild definitive hosts (e.g. Vulpes vulpes, V. ferrilata, V. corsac, Alopex lagopus, Canis latrans) and small arvicolid rodents (voles and lemmings). The adult varies between 1.2 and 4.5 mm in length and usually possesses from two to six segments, with an average of four to five. The penultimate segment is characteristically mature, and the genital pore is anterior to the midline in both mature and gravid segments. The gravid uterus is sac-like. On the rostellum, the larger hooks of the first row vary in size between 24.9 and 34.0 µm and the smaller hooks of the inner row between 20.4 and 31.0 µm.

The metacestode is a multivesicular structure consisting of conglomerates of small vesicles, usually not exceeding a few millimetres in diameter. Unlike E. granulosus, the larval mass often contains a semisolid rather than a fluid matrix. It proliferates by exogenous budding, which results in infiltration of tissues. Infection with this stage is commonly referred to as alveolar echinococcosis. There is no clear evidence for distinct strains or genotypes of E. multilocularis, though regional variations at the continental scale have been described (56).

This zoonotic parasite is found in the Northern Hemisphere, and its life cycle is mainly maintained in wildlife (25). The sylvatic cycle involves foxes and many species of wild rodents. Coyotes, raccoon dogs, wolves, wild cats, domestic dogs and cats (20, 27), however, may serve as definitive hosts while pigs, horses, primates and humans can be infected as intermediate hosts (25).

**Echinococcus oligarthrus**

The parasite typically uses neotropical wild felids as definitive hosts (e.g. Felis concolor, F. jaguarundi) and large rodents (e.g. Dasyprocta sp., Cuniculus paca) as intermediate hosts. The adult varies between 2.2 and 2.9 mm in length, and normally possesses three segments, the penultimate of which is mature. The genital pore is anterior to the middle in mature segments and approximately at the middle in gravid segments. The gravid uterus is sac-like.

The metacestode is polycystic and fluid-filled with a tendency to become septate and multichambered. The rostellar hooks of the protoscolex vary in length between 25.9 and 37.9 µm. The hooks are described in more detail in the next section and compared with those of E. vogeli. The single cyst may reach a diameter of approximately 5 cm. Predilection sites are internal organs and muscles. To date, there have only been a few reports of human disease. The parasite appears not to mature in dogs.

**Echinococcus vogeli**

The parasite typically uses the South American bush dog (Speothus venaticus) as a wild definitive host, but the domestic dog is susceptible, as are large rodents (e.g. Cuniculus paca) as intermediate hosts. The adult varies between 3.9 and 5.5 mm in length, and usually has three segments, the penultimate of which is mature. The genital pore is situated posterior to the middle in both the mature and gravid segments. The gravid uterus has no lateral sacculations and is characterised by being relatively long and tubular in form, compared with the other segments, which are sac-like.

The metacestode is similar to that of E. oligarthrus. It has been reported that the two species can be distinguished by comparing differences in the dimensions and proportions of the rostellar hooks on the protoscolex. The hooks of E. oligarthrus vary in length between 25.9 and 37.9 µm (average 33.4 µm) and between 22.6 and 29.5 µm (average 25.45 µm) for large and small hosts, respectively. Those of E. vogeli vary between 19.1 and 43.9 µm.
(average 41.64 µm) and between 30.4 and 36.5 µm (average 33.6 µm) for the large and small hooks, respectively. Also the hook-guard for *E. oligarthrus* divides the hook 50:50, compared with 30:70 for *E. vogeli*.

*Echinococcus vogeli* is a zoonotic agent with approximately 100 human cases reported in South America. The infection caused by the larval stage of this species is commonly referred to as polycystic echinococcosis.

**o Echinococcus shiquicus**

The parasite was found in the Tibetan fox (*Vulpes ferrilata*) its definitive host and the plateau pika (*Ochotona curzoniae*), the intermediate host. In most species of *Echinococcus*, the gravid segment is connected to a mature segment; however, a strobila consisting of only two segments (a gravid segment directly attaching to a premature segment) is unique to this species (56). The adult stage is morphologically similar to *E. multilocularis* but differs by its smaller hooks, fewer segments, upper position of genital pore in the premature segment and fewer eggs in the gravid segment. It is easily distinguishable from *E. granulosus* by its shorter length, branchless gravid uterus and anterior position of genital pore in the gravid segment. The adult measures 1.3 to 1.7 mm.

The metacestode is found in the liver and is essentially a unicellular minicyst containing fully developed brood capsules; however, oligovesicular forms have also been observed. It is differentiated from *E. granulosus* having no daughter cysts appearing within the fertile cyst (56).

A detailed description of echinococcosis in humans and animals can be found in the WHO/OIE Manual on echinococcosis (56).

**B. DIAGNOSTIC TECHNIQUES**

**1. Identification of the agent**

In the intermediate host, diagnosis depends on the detection of the larval cyst form, which can occur in almost any organ, but particularly in the liver and lungs. The diagnosis of echinococcosis in dogs or other carnivores requires the demonstration of the adult cestodes of *Echinococcus* spp. in their faeces or the small intestine or the detection of specific coproantigens or coproDNA.

Investigators carrying out these procedures are exposed to the risk of infection and severe disease, which must be minimised by appropriate procedures. Infective material can be decontaminated by freezing at –80°C (core temperature) for 48 hours, or –70°C for 4 days or by heating to 70°C for 12 hours (41, 45). Face masks, disposable gloves and an apron must be worn. Chemical disinfection is not reliable, although sodium hypochlorite may destroy a proportion of eggs (8). Contaminated material must be destroyed by heat; hot water, at temperature of 85°C or above, is very effective. The decontamination of laboratories can be achieved at reduced humidity (40%) combined with increased room temperature (30°C) for at least 48 hours.

**a) Diagnosis of *Echinococcus* eggs in environmental samples**

**o Faecal samples** (22, 49)

This is a concentration method in which a saturated solution is used to separate *Echinococcus* eggs from faeces. A faecal sample of 0.5–2 g is mixed with water or 0.3% Tween 20 in 1% formalin (42) in a 10–15-ml test tube and centrifuged (1000 g for 10 minutes) once or twice until the supernatant is clear. Sediment is mixed with either zinc sulphate 33% (1.18 sp. Gr.) or sucrose solution (1.27 sp. Gr.) and centrifuged at 1000 g for 5–10 minutes. The test tube is filled to the top and a cover-glass is placed on the tube. The cover-glass is examined microscopically 2–16 hours later.

**o Soil samples** (35)

A 20-g soil sample is placed in a 50-ml conical tube to which is added 40 ml of 0.05% Tween 80. The mixture is stirred vigorously and sieved through a 100-µm mesh. The suspension is centrifuged at 1000 g for 5 minutes and the supernatant is discarded. The remaining procedure follows the concentration method used for faecal sample examination.

**b) Diagnosis of larval echinococcosis**

**o Necropsy**

Whereas surveillance for *E. granulosus* in domestic animals may take place in licensed slaughter houses, that for *Echinococcus* sp. in wildlife must be done by field surveys. Specimens should be preserved by removal of tissue and fixation in 4% formal saline or kept cool at +4°C and deep-frozen at –20°C for subsequent examination. When undertaking surveillance work with *E. granulosus* in intermediate hosts, it is vitally important that data are stratified and reported according to the age of animals slaughtered. Prevalence
rates are strongly age dependent (53) and reports from abattoirs that may slaughter only young animals will substantially under-represent the true situation. This is because older animals may be heavily infected even when animals have very few larvae.

Larvae can be observed in many organs, but in large animals, such as sheep and cattle, palpation or incision should be done. Pigs, cattle, sheep and goats may be infected with larval *Taenia hydatigena*, and it is sometimes difficult to differentiate between these two parasites when they occur in the liver. In wild animals, such as ruminants and rodents, several other larval cestodes should be considered for differential diagnosis.

Formalin-fixed material can be stained by conventional histological techniques. The presence of a periodic-acid-Schiff (PAS) positive acellular laminated layer, underlying a connective tissue layer, and with or without an internal cellular, nucleated germinal membrane can be regarded as a specific characteristic of the metacestodes of *Echinococcus* spp. The presence of protoscolec differences within brood-capsules or in hydatid sand is also diagnostic for the genus. Genotyping of *E. granulosus* or *E. multilocularis* is usually done on DNA derived from protoscolec or larval tissue material that is frozen, refrigerated or preserved in 90% ethanol.

c) Diagnosis of adult parasites in carnivores

**o Necropsy**

Necropsy is invariably employed in studies of echinococcosis in wildlife and is useful if domestic dogs are humanely culled. It should be emphasised that it is necessary to isolate and identify the adult *Echinococcus*, because under normal conditions of faecal examination, the eggs of *Echinococcus* cannot be differentiated from those of *Taenia* spp. The eggs of *E. granulosus* and *E. multilocularis* can now be identified and differentiated from other taeniid eggs by polymerase chain reaction (PCR).

The small intestine is removed as soon as possible after death, and tied at both ends. If the material is not frozen or formalin fixed (4–10%), it should be examined quickly, as the parasite can be digested within 24 hours. Formalin does not kill eggs. The fresh intestine is divided into several sections and immersed in 0.9% saline at 37°C for examination. Worms adhering to the intestinal wall may be observed and counted by means of a hand lens (for *E. granulosus* and *E. vogeli*). For accurate counts, the unfixed intestine is best divided into four or six sections, opened up and immersed in 0.9% saline at 37°C for 30 minutes to release the parasites. The contents are washed into another container for detailed examination, and the intestinal wall is scraped with a spatula. All material is boiled and washed by sieving to eliminate most of the particulate material and to make it noninfectious. The washed intestinal contents and scrapings are placed on a black tray, and the worms are counted with the aid of a hand lens or stereoscopic microscope. *Echinococcus granulosus* is usually found in the first third of the small intestine of dogs and *E. multilocularis* in the mid/posterior sections.

Necropsy is considered to be the most reliable form of diagnosis for *E. multilocularis* in definitive hosts. It is an inexpensive method for determining the prevalence in a population and the best way to determine worm burden (15). Carcasses or intestines of definitive hosts for examination should be deep frozen at between −70°C and −80°C for 3–7 days before necropsy to kill any eggs. Eggs of *E. multilocularis* are resistant to freezing to −50°C.

Methods for quantitative determination of *E. multilocularis* in definitive host’s intestine.

**o Sedimentation and counting technique (SCT; 16, 21)**

This technique can be regarded as the ‘gold standard’ for assessing the sensitivity and specificity of other techniques.

i) The small intestine is incised longitudinally and cut into 20 cm long segments or into 5 pieces of approximately the same length. These pieces are transferred to a glass bottle containing 1 litre physiological saline (0.9% NaCl) solution.

ii) The glass bottle is shaken vigorously for a few seconds and the pieces of intestine are removed. The superficial mucosal layer is stripped by exerting pressure between thumb and forefinger to dislodge attached helminths.

iii) The glass bottle is left for 15 minutes for sedimentation to occur; the supernatant is then decanted. The glass bottle is refilled with physiological saline solution. This procedure is repeated 2–6 times until the supernatant is cleared of coloured particles.

iv) The sediment fraction is examined in small portions of about 5–10 ml in rectangular plastic or Petri dishes with a counting grid (9 × 9 cm) in transmission light under a stereomicroscope at a magnification of ×120.
v) If up to 100 worms are found, the entire sediment fraction is checked; if higher numbers are present, the total worm burden is calculated from the count of one subsample.

- **Intestinal scraping technique (IST; 12, 17)**
  i) Deep mucosal scrapings are taken at nearly equal distances from the small intestine using microscope slides (75 × 25 × 1 mm). Five mucosal scrapings from proximal, middle and posterior thirds of the small intestine (total 15) are recommended. Adherent materials are transferred to a square plastic Petri dish.
  
  ii) Scrapings are squashed between slides and examined under a stereoscopic light microscope (×120). Three slides are placed in one plastic dish and examined. *Echinococcus multilocularis* is usually found in the second half of the small intestine.

- **‘Shaking in a vessel’ technique (SVT; 15)**
  i) A plastic vessel (1 litre), which has a plastic screw-on lid with a central hole 6–7 cm in diameter is used. The hole is covered with a high-grade steel mesh (mesh size 500 µm) fixed into the remaining plastic ring with a hot soldering iron. Silicone is applied to seal the edges of the steel mesh.
  ii) The longitudinally opened small intestine is transferred to the vessel with all its contents; the vessel is closed with the lid and filled with water.
  iii) The vessel is inverted and shaken; the water is decanted. Vessel is refilled with water, and the process is repeated until the decanted water is clear.
  iv) The half-filled vessel is opened and the intestines are removed. The intestines are stripped between the thumb and forefinger to dislodge parasites stuck to the mucosa into the vessel.
  v) The vessel is closed again, refilled and shaken one last time draining as much water from it as possible.
  vi) The remaining sediment is filled into a 1 litre plastic jug and stored at 4°C. For prolonged storage, a 0.9% NaCl solution is added to the sediment to prevent the parasites from shrinking.
  vii) For analysis, the materials are placed into small glass Petri dishes and scanned along engraved lines using the stereomicroscope as above.

- **Preserving specimens**

  Intact worms are fragile and for morphological studies are best handled in normal saline with a Pasteur pipette. They are washed free of other material and left for approximately 30 minutes for all movement to cease. After removal of the fluid, cold 5–10% formalin (5°C) or FAA fixative (95% ethanol [80 ml], 37–40% formaldehyde [10 ml], and glacial acetic acid [5 ml]) is added and the worms are left for a further 12 hours. For staining, the worms are washed in water for 15 minutes and transferred to Mayer's paracarmine (carmine acid [1.0 g], aluminum chloride [0.5 g], calcium chloride [4.0 g], and 70% ethanol [100 ml]) for 12–24 hours. Excess stain is removed by immersion in 0.5–1.0% hydrochloric acid solution for a few seconds. Dehydration is accomplished by serial passage in ascending concentrations of alcohol (41, 50, 70, 85, 95, and 100%) for at least 15 minutes in each, with two changes in 100%. The alcohol is removed by xylo (10 minutes) and cleared with methyl salicylate or creosote. Prior to mounting in any suitable medium such as balsam, picolyte, etc., the specimens should be returned to the xylo for a few minutes. Persons involved in such examinations should receive serological screening for anti-*Echinococcus* serum-antibodies at least once a year (56).

Recently, some methods have been developed with the aim of simplifying and improving epidemiological investigations in final host populations and of allowing diagnosis in living animals. These methods include the detection of coproantigens and PCR DNA detection (see below).

d) **Arecoline surveys and surveillance**

Arecoline has been used to perform surveys of tapeworm infections in dog populations. Its use as a control agent has now been superseded by praziquantel. Arecoline is a parasympathomimetic agent. Its action results in sweating, and stimulation of salivary, lachrymal, gastric, pancreatic, and intestinal glands. It increases intestinal tonus and the mobility of smooth muscle, and this effect is responsible for purgation. The liver is the principal site of detoxification. Arecoline also has a direct action on the worm itself, by causing paralysis, but not death, and thereby making it relax its hold on the intestinal wall. Thus, it must be administered by the oral route. The accompanying purgation carries the worms out with the faeces. It is particularly suitable for baseline surveys of *E. granulosus*, however, 15–25% of dogs may not purge. Arecoline may also be used to purge dogs infected with *E. multilocularis*. In animals, arecoline purgation has been useful; again, the recovered tapeworms are identified morphologically. Products containing arecoline are no longer available as an anthelminthic, but can be obtained from chemical supply companies. As it has side-effects, old, infirm and pregnant animals should be excluded from treatment. A dose of 4 mg/kg should
result in purgation in under 30 minutes. Walking and abdominal massage of recalcitrant cases or enema for constipated dogs may avoid the use of a second dose (2 mg/kg), which should be given only sparingly.

Dogs that are purged successfully may produce at least two motions; the first will be formed faeces and can be ignored (or collected for laboratory tests as described later), but the mucus that follows may be productive. This can be divided into several samples and each examined separately, but this method is not recommended as the worms will be difficult to detect. Preferably, the mucus sample (about 4 ml) is diluted with 100 ml of tap water, covered with a thin layer of 1 ml of kerosene (paraffin) and boiled for 5 minutes. The kerosene prevents foaming and reduces the smell.

Investigators carrying out these procedures are exposed to risk of infection and severe disease. Personnel should wear whole body coveralls, boots, disposable gloves and a face mask. Coveralls should be boil washed after use, and boots disinfected in 10% sodium hypochlorite solution. The purge should be boiled as soon as possible after collection. Dogs may continue to pass eggs, proglottides and worms after the first purge, therefore, they should remain tethered for 2 hours after purgation and given access to drinking water. After arecoline testing, the area of ground used to tether dogs should be sprayed with kerosene and flamed.

e) Coproantigen tests

An alternative to arecoline testing, based on a faecal antigen-detection antibody sandwich enzyme-linked immunosorbent assay (ELISA), has been developed and has shown particular promise as coproantigens can be detected shortly after infection (10–14 days) and the level declines rapidly following expulsion of the worms. The sensitivity and specificity of the test have been estimated at 70% and 98%, respectively (2, 5, 8, 12, 13).

Both qualitative and quantitative results can be obtained from arecoline testing, which is most useful for base-line epidemiological studies on the comparative rates of infection with Taeniidae in dogs. Further studies may show that the coproantigen test may be more cost-effective than arecoline testing during routine surveillance of *E. granulosus* in the dog population (6).

ELISAs for specific coproantigen have now been developed that have sufficient specificity and sensitivity to replace arecoline testing for detecting *Echinococcus* in dogs and other definitive hosts (8). When testing for genus-specific *Echinococcus* coproantigens (against necropsy as a gold standard), specificity is around 98% and overall sensitivity approximately 70%; however, when mean worm burdens are >50–100, sensitivity approaches 100% (2, 8, 9, 13). Dogs, dingoes, foxes and wolves have been screened successfully for coproantigen ELISAs and, importantly, *E. multilocularis* worm infestations are also detectable in red foxes and domestic dogs (13, 46). When the capture ELISA uses either anti-ES or anti-somatic proglottid antibodies to *E. granulosus*, the sensitivity for *E. multilocularis* infection may be reduced, though genus specificity remains intact. Polyclonal- or monoclonal-antibody-based ELISAs for coproantigens exhibit high sensitivity and specificity to *E. granulosus* (~80%), even though they were developed for *E. multilocularis* (11, 44). However, for low worm burdens (<50), the sensitivity of the *E. multilocularis* coproantigen ELISA is below that of the mucosal smear method at necropsy (11).

The exact nature of *Echinococcus* antigens released in faeces for coproantigen detection has not been fully characterised. However, their stability in 5% formal saline after boiling and susceptibility to periodate treatment suggest involvement of large (>150 KDa) of carbohydrate antigen(s) with α-D-mannose, α-D-glucose, β-galactose and N-acetyl-β-glucosamine residues (8, 18).

Coproantigens can be detected prior to release of eggs by *Echinococcus* worms, and therefore are not related to egg antigen(s) (13, 44). This has the advantage of detection of prepatent infections. Furthermore, coproantigen levels return to the preinfection baseline within 5 days of anthelminthic treatment of infected dogs (13). More importantly, it reduces the biohazardous risk of exposure of personnel to potentially infective eggs during purgation or necropsy (26).

For detection of *E. multilocularis* infection of foxes, necropsy is time-consuming. Coproantigen testing by ELISA offers a specific practical alternative. Fox faecal samples should be taken at post-mortem from the rectum rather than from the small intestine. *Echinococcus* coproantigens are also stable in fox or dog faeces left at 20°C for 1 week and in frozen dog faeces. Coproantigen testing has also been successfully used to evaluate the efficacy of deworming wild foxes infected with *E. multilocularis* using praziquantel-laced bait, which proved to be a successful combination of eliminating the source of infection (24).

o Coproantigen test procedure (*Echinococcus granulosus*) (2, 9)

i) The faecal sample (collected per rectum or from the ground) is mixed with an equal volume of phosphate buffered saline (PBS), pH 7.2, containing 0.3% Tween 20 (PBST), in a capped 5 ml
disposable tube. This is shaken vigorously and centrifuged at 2000 \( g \) for 20 minutes at room temperature. Faecal supernatants can be tested immediately or stored at \(-20^\circ C\) or lower. Supernatants that appear very dark or viscous are still acceptable for use.

ii) A 96-well ELISA microtitre plate (Immulon #4, Thermo Electron Corporation) is coated with optimal concentration (typically 5 \( \mu g \) per ml) of a protein A purified IgG fraction of rabbit anti-E. granulosus proglottid extract (2) in 0.05 M bicarbonate/carbonate buffer, pH 9.6 (100 \( \mu l \) per well). The plate is covered and incubated overnight at 4°C.

iii) The wells are rinsed three times in PBST with 1 minute between washes; 100 \( \mu l \) of the same buffer is added to each well, and the plate is incubated for 1 hour at room temperature.

iv) The PBST is discarded and 50 \( \mu l \) of neat fetal calf serum is added to all wells. This is followed by the addition of 50 \( \mu l \) per well of faecal sample supernatants is added (in duplicate wells). The plate is incubated at room temperature for 1 hour with clingfilm seal covering the plate.

v) The wells are rinsed as in step iii, but the contents are discarded into a 10% bleach (hypochlorite) solution.

vi) An optimal dilution concentration of around 1 \( \mu g/ml \) of an IgG rabbit anti-E.-granulosus proglottid extract peroxidase conjugate (2) in PBST is prepared and 100 \( \mu l \) per well is added to all wells. The plate is incubated for 1 hour at room temperature (22–24°C).

vii) The wells are rinsed as in step iii.

viii) Next, 100 \( \mu l \) per well of tetramethyl benzidine substrate (TMB, KPL Labs) is added and the plate is left in the dark for 20 minutes at room temperature (22–24°C).

ix) Absorbance of wells is read at 630 nm. The enzyme-substrate reaction is stopped by adding 100 \( \mu l \) of 1 M phosphoric acid (\( H_3PO_4 \)) to each well. The colour turns from blue to yellow if positive.

x) Laboratories should establish their own end-point criteria using standard positive and negative samples. Standards can also be obtained from the OIE Reference Laboratories (see Table given in Part 3 of this Terrestrial Manual). Usually, the positive to negative threshold is taken as 3 standard deviations above the mean absorbance value of control negatives, or against a reference standard control positive using absorbance units equivalence.

\[ \text{Coproantigen test procedure} \ (Echinococcus multilocularis) \ (39, 42) \]

Sandwich ELISA using a monoclonal antibody EmA9 raised against adult \( E. \multilocularis \) somatic antigen (28).

i) 0.5 g of each faecal sample is placed in a centrifuge tube and a 1% formalin solution containing 0.3% Tween 20 is added to a total volume of 15 ml.

ii) After adequate mixing, the faecal solution is centrifuged at 1200 \( g \) for 10 minutes at room temperature. A supernatant fraction is used for the coproantigen detection assay.

iii) Flat-bottomed microtitre plates (Immulon 600, Greiner, Germany) are coated with 50 \( \mu l/well \) of 1 \( \mu g/ml \) rabbit IgG directed against adult \( E. \multilocularis \) excretory/secretory (ES) products in 0.05 M NaHCO\(_3\)/Na\(_2\)CO\(_3\) buffer (pH 9.6) and are left overnight at 4°C.

iv) The plates are washed three times with 250 \( \mu l/well \) PBS (pH 7.4) containing 0.05% Tween 20 (PBST), and blocked using 100 \( \mu l/well \) 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (22–24 C).

v) The plates are washed three times (with the wash disinfected with 10% bleach) and 50 \( \mu l \) of faecal supernatant is added to each well and the plates are incubated for 2 hours.

vi) The plates are again washed four times and 0.5 \( \mu g/ml \) of the biotinylated monoclonal antibody in 0.5% BSA/0.5% casein in PBST is added to each well and the plates are incubated for 1 hour.

vii) The plates are washed four times and streptavidine-biotinylated horseradish peroxidase complex (Amersham Life Science), diluted 1/1000 in 0.5% BSA/0.5% cCasein in PBST is added to each well and the plates are incubated for 1 hour.

viii) The plates are washed five times and 100 \( \mu l/well \) of substrate solution (20 mg of phenylenediamine (Wako) in 50 ml of 0.1 M citric phosphate buffer with 10 \( \mu l \) of \( H_2O_2 \)) is added.

ix) The plates are shaken immediately and placed in a 37°C incubator for 30 minutes. The reaction is stopped by adding 50 \( \mu l/well \) of 4 N \( H_2SO_4 \). The optical densities (OD) of the plates are read at 490 nm.

x) The cut-off value is calculated as the mean OD value plus 3 standard deviations of samples from uninfected animals.
This procedure was also used in a sandwich ELISA for *E. granulosus* coproantigen detection (45). In 2008, a latex agglutination test and immunochromatography in-house kit using EmA9 became available for coproantigen detection (23, 41).

**f) DNA recognition methods**

**Definitive hosts:** Copro-DNA has proven to be of value for the diagnosis of Echinococcosis in animal definitive hosts. DNA isolation from the faeces, however, is laborious.

PCR is a technically demanding and expensive technique. It is currently used mainly for confirmatory testing of coproantigen-positive samples or for identification of taenid eggs recovered from faeces. Table 2 presents the different PCR primers used for identification of copro-DNA from faeces in definitive hosts of genus *Echinococcus*.

Differential diagnosis of *E. granulosus* and *E. multilocularis* infections in definitive hosts may be achieved by specific detection of PCR-amplified DNA from *E. multilocularis* eggs present in faeces (4, 32). In practice, it is recommended to screen definitive hosts (e.g. foxes) using the coproantigen test and confirm with the PCR DNA test. In Europe, transmission of *E. multilocularis* generally occurs in regions where *E. granulosus* is not endemic or appears very infrequently. In other regions, including parts of the Near East (Turkey and Iran), Central Asia, Russia and the People’s Republic of China, these two species may occur together (10). Further evaluation of *E. multilocularis* infection is required to investigate intermittent shedding and duration of shedding of parasite DNA. Recently PCR has been developed for the detection of copro-DNA for *E. granulosus* and for genotypic differentiation. (36, 55)

As PCR is generally used as a confirmatory test, it is suggested to concentrate the taenid eggs by sequential sieving and an in-between concentration method step. DNA isolation from these eggs can be achieved using a simplified protocol of the alkaline lysis method combined with a commercial kit with no need for organosolvent extractions (30).

**Table 2. PCR primers used for copro-DNA detection (modified from Mathis & Deplazes [34])**

<table>
<thead>
<tr>
<th>Primer designation: primer sequences (5’–3’)</th>
<th>Ref.</th>
<th>Target, comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. multilocularis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTG-AGG-CGA-TGT-GTG-ATG-GAG-AGA-AGG</td>
<td>4</td>
<td>U1 sRNA gene: may yield non-specific products when used with metacestode material containing host DNA (unpublished observation)</td>
</tr>
<tr>
<td>CAA-GTG-GTC-AGG-GGC-AGT-AG</td>
<td></td>
<td>Mitochondrial 12S RNA gene; used in two-tube nested PCR</td>
</tr>
<tr>
<td>Outer primers:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P60 forward)</td>
<td>14</td>
<td>Mitochondrial 12S RNA gene; modified from ref. 14 for use in one-tube nested PCR</td>
</tr>
<tr>
<td>TTA-AGA-TAT-ATG-TGG-TAC-AGG-ATT-AGA-TAC-CC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P375 reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAC-CGA-GGG-TGA-CGG-GCG-GTG-TGT-ACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner primers:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pnest forward)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACA-ATA-CCA-TAT-ACC-ATT-CCT-ATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pnest reverse)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA-TTT-TGT-AAG-GTT-GTT-CTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 cont. PCR primers used for copro-DNA detection (modified from Mathis & Deplazes [34])**

<table>
<thead>
<tr>
<th>Primer designation: primer sequences (5’–3’)</th>
<th>Ref.</th>
<th>Target, comments</th>
</tr>
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<tr>
<td>Outer primers:</td>
<td>54</td>
<td>Mitochondrial 12S RNA gene; modified from ref. 14 for use in single PCR</td>
</tr>
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<td>(Em-1)</td>
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<td></td>
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</table>
TAA-GAT-ATA-TGT-GGT-ACA-GGA-TTA-GAT-ACC-C
(Em-2)
GGT-GAC-GGG-CGG-TGT-TGT-A
Inner primers:
(Em-3)
ATA-TTA-CAA-CAA-TAT-TCC-TAT-C
(Em-4)
ATA-TTT-TGT-AAG-GTT-GTT-CTA
(EM-H15)
CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC
(EM-H17)
GTG-AGT-GAT-TCT-TGT-TAG-GGG-AAG

48 NADH dehydrogenase subunit 1 (ND1) of mtDNA; cleavage with enzyme Cfo1 distinguish E. multilocularis from E. granulosus

E. multilocularis and E. granulosus

ND1
(NDfor2-)
AGT-TTC-GTA-AGG-GTC-CTA-ATA
(NDrev2-)
CCC-ACT-AAC-TAA-CTC-CCT-TTC

38 Repeated sequences from E. granulosus. ‘sheep strain’; yields banding pattern upon electrophoresis

Mitochondrial 12SRNA gene; specific for E. granulosus ‘sheep strain’

Amplify a fragment of the cox1 genespecific to E. granulosus

E. granulosus

(Eg1121a)
GAA-TGC-AAG-CAG-CAG-ATG
(Eg1122a)
GAG-ATG-AGT-GAG-AAG-GAG-TG

(Eg1f)
CATTAATGTATTTTGTAAAGTTG
(Eg1r)
CAC-ATC-ATC-TTA-CAA-TAA-CAC-C

(EgO/DNA-IM1)
forward
TCA-TAT-TTG-TTT-GAG-KAT-YAG-TKC
reverse
GTA-AAT-AAM-ACT-ATA-AAA-GAA-AYM-AC

47

Table 2 cont. PCR primers used for copro-DNA detection (modified from Mathis & Deplazes [34])

<table>
<thead>
<tr>
<th>Primer designation: primer sequences (5’–3’)</th>
<th>Ref.</th>
<th>Target, comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. multilocularis, E. granulosus and Taeniid spp.</td>
<td>52</td>
<td>Cestodes</td>
</tr>
<tr>
<td>(JB11)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Chapter 2.1.4. - Echinococcosis/Hydatidosis

AGA-TTC-GTA-AGG-GGC-CTA-ATA  
(JB12)  

AC-CAC-TAA-ATT-CAC-TTT-C  
(60.for.-mod)  

ATG-TGG-TAC-AGG-ATT-AGA-TAC-CC  
(375.rev.-mod)  

GGT-GAC-GGG-CGG-TGT-GTA-CC  

(Eg1f)  

CAT-TAA-TGT-ATT-TTG-TAA-AGT-TG  
(Eg1r)  

CAC-ATC-ATC-TTA-CAA-TAA-CAC-C  
(EM-H15)  

CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC  
(EM-H17)  

GTG-AGT-GAT-TCT-TGT-TAG-GGG-AAG  
(Cest1)  

TGC-TGA-TTT-GTT-AAA-GTT-AGT-GAT-C  
(Cest2)  

CAT-AAA-TCA-ATG-GAA-ACA-ACA-ACA-AG  
(Cest4)  

GTT-TTT-GTG-TGT-ATT-AAT-AAG-GGT-G  
(Cest5)  

GCG-GTG-TGT-ACM-TGA-GCT-AAA-C  
(Cest5seq)  

YGA-YTC-TTT-TTA-GGG-GAA-GGT-GTG  
(Cest5)  

GCG-GTG-TGT-ACM-TGA-GCT-AAA-C  
(Cest5seq)  

GAT-TCT-TTT-TAG-GGG-AAG-G  

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Organism/Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Eg1f)</td>
<td>52</td>
<td><em>Echinococcus granulosus</em> (sheep strain)</td>
</tr>
<tr>
<td>(Eg1r)</td>
<td>52</td>
<td><em>E. multilocularis</em></td>
</tr>
<tr>
<td>(EM-H15)</td>
<td>52</td>
<td><em>E. multilocularis</em></td>
</tr>
<tr>
<td>(EM-H17)</td>
<td>52</td>
<td><em>E. multilocularis</em></td>
</tr>
<tr>
<td>(Cest1)</td>
<td>52</td>
<td><em>E. multilocularis</em></td>
</tr>
<tr>
<td>(Cest2)</td>
<td>52</td>
<td><em>E. granulosus</em></td>
</tr>
<tr>
<td>(Cest4)</td>
<td>52</td>
<td><em>Taenia spp.</em></td>
</tr>
<tr>
<td>(Cest5)</td>
<td>52</td>
<td><em>Taenia spp.</em> (Sequencing primer for the 267 bp amplicon of the multiplex PCR)</td>
</tr>
</tbody>
</table>

**Intermediate hosts:** DNA hybridisation methods are not currently used for the detection of *E. granulosus* in livestock intermediate hosts. Molecular methods are, however, important in identification of isolates or strains of *E. granulosus* for epidemiological purposes (37).

For the identification of small, degenerated or calcified lesions of *E. multilocularis* in intermediate or aberrant hosts, PCR is of great value (33).

### 2. Serological tests

#### a) Intermediate hosts

Immunological tests, useful in humans, are less sensitive and specific in livestock and at present cannot replace necropsy (8, 30).

#### b) Definitive hosts

An extensive programme has been initiated to develop immunodiagnostic tests to control canine echinococcosis. Following ingestion of a cyst, dogs will be exposed at the intestinal level to various antigens during the establishment of the parasite and its development and oogenesis. Specific antibodies against oncosphere and protoscolex antigens can be readily detected in the serum of infected dogs. This methodology has not reached a practical stage as it does not differentiate between current and previous infections and false positives may occur with infections of *Taenia* species.
C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Intermediate hosts

Application of an effective vaccine to reduce hydatid infection in livestock would be likely to have a substantial impact on the rate of transmission of the disease to humans (29). As E. granulosus belongs to the Taeniid family, many aspects of its immunological relationship with its intermediate host are similar to that occurring in Taenia species. Moreover, it was considered that the vaccine development approach used in Taenia species such as the native host-protective antigens of T. ovis would also be successful for E. granulosus. Using recombinant DNA technology, an oncosphere antigen vaccine EG95 was shown to be capable of inducing a high level of protection against experimental challenge infection with E. granulosus eggs in sheep (31).

The EG95 vaccine has been licensed by the University of Melbourne and AgResearch New Zealand to a commercial group in the People's Republic of China (29).

2. Definitive hosts

While considerable research has been undertaken with crude antigens to protect dogs from echinococcosis, only limited evidence has been demonstrated so far. Recent studies using recombinant protoscolex antigens, however, look encouraging (7). Basic research on canine mucosal immunology and Echinococcus infection is required for progress.

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Echinococcosis/Hydatidosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).