Section 3.1

Multiple species

CHAPTER 3.1.6

ECHINOCOCCOSIS (INFECTION WITH *ECHINOCOCCUS GRANULOSUS* AND WITH *E. MULTILOCULARIS*)

SUMMARY

Human cystic echinococcosis, caused by Echinococcus granulosus sensu lato (s.l.)*, and alveolar echinococcosis, caused by* E. multilocularis*, are important public health threats in many parts of the world. Diagnosis of echinococcosis in dogs or other susceptible carnivores relies on the detection of adult cestodes of the* Echinococcus *genus or their eggs in the faeces or small intestine. Coproantigen and coproDNA assays have proven useful particularly for epidemiological screening programmes For the metacestode stage, diagnosis in humans is performed by imaging techniques supported by immunological tests, while in animals, diagnosis is based on post-mortem detection of the larval form that can infect almost any organ, particularly the liver and lungs, with subsequent species confirmation by polymerase chain reaction* (*PCR*) and DNA sequencing.

Detection of the agent: *It was previously accepted that there were five valid species of the genus* Echinococcus; *the current view however, informed by biology, epidemiology and particularly molecular genotyping, recommends the inclusion of at least nine species within the genus. All those species of* Echinococcus *known to cause cystic echinococcosis in the intermediate host may be referred to as* E. granulosus s.l., *whereas genotypes G1,3 which are closely related are now referred to as* E. granulosus sensu stricto (s.s.). *It is also widely believed that within* E. granulosus s.l., E. equinus (*G4*)*,* E. ortleppi (*G5*) *and* E. canadensis (*G6, G7, G8, G10*) *should be considered as distinct species although there is still some debate as to whether* E. canadensis *represents more than one species. Larval forms of* E. granulosus s.l. *and* E. multilocularis *in intermediate hosts can be detected by macroscopic and microscopic examination of visceral 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*. Genotyping via PCR/sequencing is the only method available to confirm the exact species of* Echinococcus *infecting animals. The small intestine is required at necropsy for the detection of adult* Echinococcus *spp. in definitive hosts* (*wild and domestic carnivores*)*. Handling infected material needs detailed safety precautions to avoid risk to the operator of contracting a potentially fatal disease.*

Coproantigen or CoproDNA tests: *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 only in some countries for surveys of* E. granulosus *in dogs and is currently used in surveys for* E. multilocularis *in populations of dogs and foxes in high endemic areas. Coproantigen detection is possible in faecal samples collected from dead or living animals or from the environment. However, as these tests were developed based on adult worm antigens, false positives may occur. PCR DNA methods for the detection of*

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E. multilocularis *and more recently* E. granulosus *in definitive hosts have now been validated 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. Moreover, analytical sensitivity and specificity can sometimes be poor as cross-reactivity between* Echinococcus *and* Taenia *species also may occur.*

Requirements for vaccines: *A vaccine for* E. granulosus *s.s. based on the EG95 recombinant antigen has proven to be safe and effective in livestock. Commercial EG95 vaccines are available and are manufactured in Argentina, Morocco and China* (*People's Rep. of*)*. The vaccines have gained regulatory approval in the countries of manufacture as well as a number of other countries. The EG95 vaccine was adopted in 2016 as a compulsory part of the national program for control of echinococcosis in China. No vaccine is available for Echinococcus infection in the parasites' definitive hosts.*

A. INTRODUCTION

The species within the genus *Echinococcus* are small (1–11 mm length) tapeworms of carnivores with a larval stage known as metacestode that proliferates asexually encysting in the internal organs of various mammals including humans. Until recently it was accepted that there were five morphologically distinct species in this genus: *Echinococcus granulosus s.l.*, *Echinococcus multilocularis*, *Echinococcus oligarthra*, *Echinococcus vogeli* and *Echinococcus shiquicus*. However, the current view, informed by biology, epidemiology and particularly molecular genotyping, recommends the inclusion of at least nine species within the genus. *Echinococcus granulosus s.l.*, formerly regarded as a single species with a high genotypic and phenotypic diversity, is now recognised as an assemblage of five cryptic species, which differ considerably in morphology, development, host specificity (including infectivity or pathogenicity for humans). This diversity is reflected in the mitochondrial and nuclear genomes. Based on phenotypic characters and gene sequences, *E. granulosus s.l.* has now been subdivided into *E. granulosus sensu stricto* (*s.s.*) (including the formerly identified genotypic variants G1, 3), *Echinococcus felidis* (the former 'lion strain'), *Echinococcus equinus* (the 'horse strain', genotype G4), *Echinococcus ortleppi* (the'cattle strain', genotype G5) and *Echinococcus canadensis*. The latter species, as recognised here, shows the highest diversity and is composed of the 'camel strain', genotype G6, the 'pig strain', genotype G7, and two 'cervid strains', genotypes G8 and G10 (Nakao *et al*., 2013; Romig *et al*., 2015). Studies performed on nearly complete mitochondrial genome and significantly long nuclear genetic DNA sequences suggest the previous G2 genotype be considered a microvariant of G3 (Kinkar *et al.,* 2017) and *E. canadensis* be considered a cluster composed of two different species (Laurimae *et al.,* 2018).

Echinococcus granulosus (s.l.) has a global distribution; *E. multilocularis* occurs in wide areas of the northern hemisphere, *E. shiquicus* is found in the Tibetan plateau and *E. oligarthra* and *E. vogeli* are confined to Central and South America. Nearly all the originally described species are infective to humans causing various echinococcal diseases, although in the most recent taxonomic classification there is no evidence of *E. shiquicus* and *E. felidis* infections in humans (Ma *et al*., 2015). Human cystic echinococcosis (CE), caused by *E. granulosus s.l.,* and alveolar echinococcosis (AE), caused by *E. multilocularis*, are important public health threats in many parts of the world (WHO/WOAH, 2001) caused by ingestion of eggs derived directly or indirectly from definitive hosts. The strong zoonotic potential of *E. granulosus s.l.* is mainly related to *E. granulosus s.s.* (Alvarez Rojas *et al.,* 2014). Clinical specimens and eggs of *Echinococcus* spp. should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Table 1. *Useful characteristics for identification of Echinococcus species* (*source: Xiao* et al.*, 2006*)

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	***E. granulosus* (*sensu lato*)**	***E. multilocularis***	***E. oligarthra***	***E. vogeli***	***E. shiquicus***
Distribution	Cosmopolitan	Holoarctic region	Neotropical region	Neotropical region	Tibet plateau
Definitive host	Wild and domestic carnivores	Foxes/dogs	Wild felids	Bush dog	Tibetan fox
Intermediate host	Ungulates	Microtine rodents	Neotropical rodents	Neotropical rodents	Plateau pika
Adult					
Body length (mm)	2.0–11.0	1.2–4.5	2.2–2.9	3.9–5.5	1.3–1.7
No. segments	2–7	2–6	3	3	2–3
Length of large hooks (µm)	25.0–49.0	24.9–34.0	43.0–60.0	49.0–57.0	20.0–23.0
Length of small hooks (µm)	17.0–31.0	20.4–31.0	28.0–45.0	30.0–47.0	16.0–17.0
No. testes	25–80	16–35	15–46	50–67	12–20
Position of genital pore					
a. Mature segment	Near to middle	Anterior to middle	Anterior to middle	Posterior to middle	Near to upper edge
b. Gravid segment	Posterior to middle	Anterior to middle	Near to middle	Posterior to middle	Anterior to middle
Gravid uterus	Branching laterally	Sac-like	Sac-like	Tubular	Sac-like
Metacestode	Unilocular cysts in viscera	Multilocular cysts in viscera	Unicystic cysts in muscles	Polycystic cysts in viscera	Unilocular cysts in viscera

1. *Echinococcus granulosus sensu lato* (*s.l.*)

The parasite is most frequently transmitted between the domestic dog and a number of domestic ungulate species. In countries where sheep farming plays an important role in the local economy, *E. granulosus s.s.* is maintained prevalently by a dog–sheep cycle. Sylvatic cycles involving different definitive and intermediate hosts (e.g. wolf or cervid) have been reported for *E. canadensis* (see Deplazes *et al.,* 2017 for further illustration) and for *E. felidis,* with lion or spotted hyena acting as definitive hosts. There is some intermediate host predilection in some strains – e.g. *E. equinus* in horses, *E. ortleppi* in cattle and *E. canadensis* in pigs, camels and cervids. The adult

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worm 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 and 49 µm in the first row, and between 17 and 31 µm in the second row. The gravid uterus has well-developed sacculations.
The metacestode, known as hydatid, is a fluid-filled bladder that is typically unilocular, although communicating chambers may also occur. Growth is expansive, and newly formed hydatids (daughter cysts) inside and, occasionally, outside the cyst may be produced. Individual CE cyst may reach up to 30 cm in diameter and occur mainly in liver and lungs. Other internal organs are affected less frequently. The infection with this stage is referred to as cystic echinococcosis

2. *Echinococcus multilocularis*

The parasite is transmitted primarily between wild definitive hosts (e.g. foxes, *Vulpes vulpes, V. corsac*, *Alopex lagopus*) 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.

This zoonotic parasite is found mainly in the Northern Hemisphere, and its life cycle is mainly maintained in wildlife (Deplazes *et al.,* 2017). Like *E. granulosus,* there are a number of genetic variants or haplotypes based on microsatellite EmsB and mitochondrial gene sequences. These are associated with different geographical regions and have been named the Asian, the Mongolian, the North American 1, the North American 2 and the European haplotypes. In Europe the prevalence of *E. multilocularis* in red foxes varied from zero to >10% in different countries, and over 50% in high endemic areas. *E. multilocularis* has also been detected in Arctic foxes (Deplazes *et al.,* 2017). Domestic dogs, raccoon dogs, golden jackals and wolves have also been shown to act as definitive hosts. Experimental studies indicate that domestic cats play an insignificant role in transmission (Kapel *et al.,* 2006). Rodents of the genus *Microtus, Arvicola, Myodes* and *Lemmus* are all known to be suitable intermediate hosts as are muskrats (*Ondatra zibethicus*), nutria/coypu (*Myocastor coypus*) and beaver (*Castor fiber*).

3. *Echinococcus oligarthra*

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 where they are also 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.

4. *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. oligarthra*. 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. oligarthra* 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 hooks, respectively. Those

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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. The hook-guard for *E. oligarthra* also divides the hook 50:50, compared with 30:70 for *E. vogeli.*
Echinococcus vogeli is a zoonotic agent with approximately 200 human cases in total reported in South America. The infection caused by the larval stage of this species may be referred to as neotropical echinococcosis.

5. *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 (Xiao *et al.,* 2005). 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 the anterior position of the genital pore in the gravid segment. The adult measures 1.3 to 1.7 mm.

The metacestode is found mainly in the lungs of pika and is essentially a unilocular minicyst containing fully developed brood capsules; however, oligovesicular forms have also been observed. It is differentiated from *E. granulosus* by the absence of daughter cysts within the fertile cyst (WHO/WOAH, 2001).

A detailed description of echinococcosis in humans and animals can be found in the WHO/WOAH Manual on echinococcosis (WHO/WOAH, 2001).

B. DIAGNOSTIC TECHNIQUES

Table 2. *Test methods available for the diagnosis of echinococcosis and their purpose*

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Method	Purpose (metacestode cysts in intermediate hosts)					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases[a]	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Parasite Identification /meat inspection	++	–	++	++	++	–
Antigen detection	–	–	–	–	–	–
PCR	++	–	–	+++	++	–
Detection of immune response						
ELISA	–	–	–	–	+	+
Purpose (adult worms in carnivorous definitive hosts)						
Detection of the agent						
Parasite isolation/ microscopy	+	+	+++	+++	++	–
Antigen detection	+	++	+++	+++	++	–
PCR	–	++	+++	+++	+++	–
Detection of immune response						
ELISA	–	–	–	–	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[a]Meat inspection may only identify cysts seen and not include subsequent tests to confirm they are due to Echinococcus granulosus infection.

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1. Detection of the agent

In the intermediate host, diagnosis depends on the meat inspection or post-mortem detection of the larval cyst form, which can occur in almost any organ, 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 the small intestine or the detection of specific coproantigens or coproDNA in faeces. Comprehensive reviews are available relating to diagnostic procedures for *E. granulosus s.l.* (Craig *et al.,* 2015) and *E. multilocularis* (Conraths & Deplazes, 2015).

Investigators carrying out these procedures are exposed to the risk of infection and severe disease, which must be minimised by appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4). Infective (egg/adult) material can be decontaminated by freezing at −80°C (core temperature) for 5 days, or by heating to 70°C for 1 hour. Face masks, disposable gloves and an apron must be worn. Chemical disinfection is not reliable, although sodium hypochlorite (10% bleach) can be used to destroy eggs. Contaminated material must be destroyed by incineration or autoclaving.

1.1. Diagnosis of larval echinococcosis in intermediate hosts

1.1.1. Necropsy

Whereas surveillance for *E. granulosus s.l.* in domestic animals may take place in licensed slaughter houses, that for *Echinococcus* spp. in wildlife must be done by field surveys. When undertaking surveillance work with *E. granulosus s.l.* 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 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.

CE cysts 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 also 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. Please refer to Chapter 3.10.3 *Cysticercosis* (*including infection with* Taenia solium) for information on other cestodes found at meat inspection.

i) Suspect parasite material should be removed from the organ by cutting with a scalpel to include the immediate host tissue, and kept in a cool location. (**NB**: parasite material in intact cysts will remain viable for more than 24 hours after death even at ambient temperatures. However viability will be prolonged by storage at 4°C for up to 72 hours. If material cannot be examined within this time, it should be stored either in 10% formol saline for subsequent microscopic examination or in 70–90% ethanol for subsequent DNA analysis. Ideally a sample of parasite material should be preserved in both media. Parasite tissues that are frozen will not be viable but can be examined morphologically on thawing and subjected to DNA analyses.

ii) For morphological analysis of cyst contents, fluid should be removed and retained using a syringe. The material inside the cyst should then be washed with saline and the contents examined under the microscope (×4 objective) for the presence of protoscoleces. Note that some CE cysts may be sterile and not contain protoscoleces. If no protoscoleces are present, the germinal layer on the inside of the cyst cavity may be observed as a gelatinous structure that can easily be pulled away. 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.

iii) In all cases exact species/genotype identification can only be made through extraction of DNA from ethanol-fixed or frozen material and subsequent genotyping by polymerase chain reaction (PCR) and, where needed, sequencing. This requires either protoscoleces or pieces of germinal layer to be present. Cysts removed from animals should be cut open after the fluid has been removed and pieces of cyst wall removed to 70% ethanol. It is important to remember that identification of the parasite genotype can give significant information on transmission cycles and that an individual animal may contain mixed infections of more than one genotype. Specific primers based on mitochondrial genes (cox 1, NAD1) and ribosomal genes (12s) have been identified for all *Echinococcus* species and related

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taeniids and are summarised by Roelfsema *et al.* (2016). These also include primers listed for the detection of adult worms in Table 3, Section B.2.2.1.

1.2. Diagnosis of adult parasites in carnivores

1.2.1. Necropsy

Necropsy is invariably employed in studies of echinococcosis in wildlife and is useful if domestic carnivores 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 PCR. It should also be emphasised that any possible contact with eggs is potentially very hazardous and requires risk management. Tissues should be deep frozen at between −70°C and −80°C for 3–7 days before necropsy to kill any eggs.

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 38±1°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 38±1°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 non-infectious. 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. *E. granulosus* is usually found in the first third of the small intestine of dogs and *E. multilocularis* in the mid/posterior sections. This approach has a greater than 95% sensitivity, except under low worm burdens where false negative results may occur.

Necropsy is considered to be the most reliable form of diagnosis for *E. multilocularis* in definitive hosts. It is a useful method for determining the prevalence in a population and the best way to determine worm burden. 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. *Echinococcus multilocularis* can survive in liquid nitrogen (around −200°C) for 35 years and still be infective.

1.2.2. Sedimentation and counting technique (SCT) (Eckert, 2003)

This well established technique has been widely used, but is less sensitive than the coproDNA (PCR) test.

i) The small intestine is incised longitudinally and cut into 20 cm long segments or into five 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.

1.2.3. 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. For all DNA

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characterisation, worms should be transferred to 70–90% ethanol. For morphological studies, the worms should be fixed in 5–10% formalin. Persons involved in such examinations should receive serological screening for anti-*Echinococcus* serum-antibodies at least once a year (WHO/WOAH, 2001).

Methods have been developed aimed at simplifying and improving epidemiological investigations in final host populations and allowing diagnosis in living animals. These methods include the detection of coproantigens and PCR DNA detection (see below).

1.3. Arecoline surveys and surveillance

Purgation with arecoline has been used to perform surveys of tapeworm infections in dog populations. Its use as a control agent has been superseded by praziquantel. Arecoline can cause discomfort to dogs and its use for diagnostics is not recommended.

2. Coprological tests

Adult *Echinococcus* worms inhabiting the intestine will release both surface or secretory molecules (antigens) and DNA (usually contained within eggs). Both types of molecules can be detected by assaying faecal samples. The sensitivity of the tests is strongly influenced by the worm burden and stage of maturity.

2.1. Coproantigen tests

Coproantigen ELISA (enzyme-linked immunosorbent assay) or coproELISA provides an alternative method for diagnosing canine echinococcosis, and both polyclonal and monoclonal antibodies have been used, directed against either somatic or excretory/secretory (ES) antigens. To date, only a few commercial coproELISAs have been reported. Wang *et al.* (2021) evaluated these tests and found that they have good sensitivity and specificity. However, the kits are not easily available. Moreover, several tests developed within individual research laboratories, have been described even if, a certain amount of variability between tests from different laboratories regarding sensitivity and specificity are reported.

CoproELISAs are usually genus-specific for *Echinococcus* spp. (Allan & Craig, 2006). For canine echinococcosis due to *E. granulosus* most authors report reasonable sensitivity (78–100%) and good genus specificity from 85% to greater than 95% as well as a degree of pre-patent detection (Deplazes *et al.*, 1992). Where cross-reactions occur these generally appear to be caused by infection with *Taenia hydatigena*, the most common taeniid of dogs, and attempts to improve specificity by using monoclonal antibodies in coproELISAs have not been able to eliminate this problem. CoproELISA sensitivity broadly correlates with worm burden of *E. granulosus*, however some low intensity infections (worm burdens <50–100) may give false negatives in coproELISA (Allan & Craig, 2006).

For detection of *E. multilocularis* infection of foxes, necropsy is time-consuming. Coproantigen testing by ELISA may offer 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 18–25°C for 1 week and in dog faeces frozen at –20°C. 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.

2.1.1. Typical coproantigen test procedure (*Echinococcus* genus specific) (Craig *et al.,* 1996)

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°C or lower. Supernatants that appear very dark or viscous are still acceptable for use.

ii) A 96-well ELISA microtitre plate is coated with optimal concentration (typically 5 µg per ml) of a protein A purified IgG fraction of rabbit anti-*E. granulosus s.l.* proglottid extract in 0.05 M bicarbonate/carbonate buffer, pH 9.6 (100 µl 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 µl 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 µl of neat fetal calf serum is added to all wells. This is followed by the addition of 50 µl per well of faecal sample supernatants is added (in duplicate wells). The plate is incubated at room temperature

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for 1 hour with plastic film to seal 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 µg/ml of an IgG rabbit anti-*E.-granulosus* proglottid extract peroxidase conjugate in PBST is prepared and 100 µl 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 µl per well of tetramethyl benzidene (TMB) or similar peroxidase substrate 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 650 nm. The enzyme-substrate reaction can be stopped by adding 100 µl of 1 M phosphoric acid (H_3PO_4) to each well. The colour turns from blue to yellow if positive and is read at 450 nm.

x) Laboratories should establish their own end-point criteria using standard positive and negative samples. Standards can also be obtained from the WOAH Reference Laboratory[1]. 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.

2.2. CoproDNA methods

2.2.1. Definitive hosts

While coproantigen ELISAs provide a better overall and practical alternative to arecoline purgation for pre-mortem detection of canine echinococcosis, their lack of species specificity is a disadvantage, especially for epidemiological studies. The amplification of small fragments of species-specific *Echinococcus* DNA in eggs or in faeces by PCR was first reported for *E. multilocularis* infections in foxes, with reduced inhibition and sensitivity subsequently increased by egg concentration through sieving and zinc chloride flotation of faecal samples (Mathis *et al*., 1996). Cabrera *et al.* (2002) applied this approach targeted to the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene of *E. granulosus* as proof of principle for PCR identification of eggs of *E. granulosus* (with an analytic sensitivity of four eggs) isolated from adult tapeworms and faecal samples from necropsied dogs in Argentina. The ability to perform PCR with faecal samples or extracts directly without first isolating taeniid eggs is an advantage, especially when relatively large numbers of samples require testing. However faecal material preserved in formol saline is not suitable for DNA amplification and 70% ethanol or freezing should be used. Commercial extraction kits designed for faecal specimens can be used to extract total DNA from canid faecal samples (1–2 g). This approach has been used with at least two coproPCRs based on the EgG1 Hae III repeat (Abbasi *et al.,* 2003) and the NADH dehydrogenase subunit 1 gene (ND1) (Boufana *et al.,* 2013).

In recent years there have been a number of key developments attempting to simplify DNA amplification (e.g. loop-mediated isothermal amplification [LAMP]) (Ni *et al*., 2014; Salant *et al*., 2012) and improve sensitivity and specificity (e.g. real-time PCR) (Dinkel *et al.,* 2011; Knapp *et al.,* 2014; Øines *et al.,* 2014). This is important in relation to differential diagnosis between *E. granulosus* genotypes, *E. multilocularis* and other taeniids that occur in the same geographical area. Multiplex PCR in particular are a useful approach to multispecies detection. (Dinkel *et al.,* 2011; Trachsel *et al.,* 2007). Currently there are several published PCRs for the *E. granulosus* complex and *E. multilocularis* (Table 3) and their great value is an extremely high specificity to the extent that a result can be taken as an alternative to the finding of worms at necropsy or purgation. A practical and cost-effective way to undertake testing of dogs or foxes on a large-scale is to adopt a serial testing strategy based on primary screening of all samples using the coproELISA, followed by testing of all positives using coproPCR ensuring that duplicate samples are taken from each animal and fixed appropriately for each technique.

Table 3. *PCR primers used for coproDNA detection* (*modified from Craig* et al., *2015*). *Tissue indicates that the technique is also compatible with DNA extraction from metacestode tissues*

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Gene (all sequences 5' → 3')	Species	Copro-sample	Tissue	Reference

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cox1 F: TCA-TAT-TTG-TTT-GAG-KAT-YAG-TKC R: GTA-AAT-AAM-ACT-ATA-AAA-GAA-AYM-AC	*E. granulosus s.l.*	Eggs	Yes	Cabrera *et al.*, 2002
EgG1HaeIII Eg1121a F: GAA-TGC-AAG-CAG-CAG-ATG Eg1122a R: GAG-ATG-AGT-GAG-AAG-GAG-TG	*E. granulosus* s.l	Faeces	Yes	Abbasi *et al.*, 2003
12sRNA Eg1f F: CAT-TAA-TGT-ATT-TTG-TAA-AGT-TG Eg1r, R: CAC-ATC-ATC-TTA-CAA-TAA-CAC-C	*E. granulosus* G1	Eggs/ Faeces	Yes	Stefanic *et al.*, 2004
12S rRNA G1: E.g.ss1 F: GTA-TTT-TGT-AAA-GTT-GTT-CTA E.g.ss1 R: CTA-AAT-CAC-ATC-ATC-TTA-CAA-T G5, G6, G7: E.g.cs1 F: (ATT-TTT-AAA-ATG-TTC-GTC-CTG) E.g.cs1 R: (CTA-AAT-AAT-ATC-ATA-TTA-CAA-C) To discriminate between *E. ortleppi* and *E. granulosus* G6/7, semi-nested PCRs specific for G6/7 (*g6/7 PCR*; e.g. camel. F: ATG-GTC-CAC-CTA-TTA-TTT-CA e.g.cs1 R) and for *E. ortleppi* (*g5 PCR*; e.g. cattle. F: ATG-GTC-CAC-CTA-TTA-TTT-TG E.g.cs1 R)	*E. granulosus* G1, G5, G6/7	No	Yes	Dinkel *et al.*, 2004
Cox1, NAD, rrnS Multiple sequences referred to	*E. multilocularis, E. granulosus, Taenia* spp.	Eggs	Yes	Trachsel *et al.*, 2007
Real-time multiplex-nested PCR Primer/probe sequence P60.short. F: TGG-TAC-AGG-ATT-AGA-TAC-CC P375.short. R: TGA-CGG-GCG-GTG-TGT-ACC CVF. F: TTA-ATG-ACC-AAC-ATT-CGA-AA CVF. R: AGG/T-ACA/G-TAG/C-CCC-ATA/G-AAA/T-GC Pnest. F: ACA-ATA-CCA-TAT-TAC-AAC-AAT-ATT-CCT-ATC Pnest. R: ATA-TTT-TGT-AAG-GTT-GTT-CTA CVF.light F: TCA/T-GCC/T-TGA-TGA/G-AAC-TTC-GGA/G-TCC CVF.light R: AC/TA/G-ATT-CCA-ATA/G-TTT-CAT-GTC/T-TCT emulti-fl: CTA-AAA-CTA-CAC-AAA-CTT-ACA-TTA-CTA--FL emulti-705; LC705-ACA-ATA-ATA-TCA-AAC-CAG-ACA-TAC-ACC-A-PH CaVuFe1-fl: ATA-CAC-TAT-ACA-TCT-GAC-AC-FL CaVuFe2-640: LC640-GCT-ACT-GCT-TTC-TCA-TCT-G-PH	*E. multilocularis, E. granulosus* (G1), *E. ortleppi, E. canadensis* (G6, G7), *other taeniids*	Faeces	Yes	Dinkel *et al.*, 2011
LAMP method Eg1121aGAA-TGC-AAG-CAG-CAG-ATGEg1122aGAG-ATG-AGT-GAG-AAG-GAG-TGFIP1echCTT-TTC-CGG-ATG-GGT-AGG-CAT-CTT-TTG-ATC-ACT-CCT-ATT-CTA-GCA-TGTBIP1echCGT-GCT-GTG-GAG-GTA-GTT-TCG-TTT-TCA-GTG-AGA-TGA-GTG-AGA-AGG	*E. granulosus* G1	Eggs	Yes	Salant *et al.*, 2012
ND1 Eg181, F: GTT-TTT-GGC-TGC-CGC-CAG-AAC Eg183, R: AAT-TAA-TGG-AAA-TAA-TAA-CAA-ACT-TAA-TCA-ACA-AT Em19/3, F: TAG-TTG-TTG-ATG-AAG-CTT-GTT-G Em6/1, R: ATC-AAC-CAT-GAA-AAC-ACA-TAT-ACA-AC	*E. granulosus* G1; *E. multilocularis; E shiquicus*	Faeces	Yes	Boufana *et al.*, 2013
Many mitochondrial and nuclear primers	*E. granulosus* complex G1–(G10)	(Eggs)	Yes	Boubaker *et al.*, 2013
Nad5 gene primers inc. (LAMP method) Primer name and sequence FIP: TTA-ACC-AAC-CAA-TAA-CAA-CCC-AGT-gaattc-GTG-GTG-TTA-GTT-ATT-TGG-TTA-GG BIP: ATG-TGA-CGT-TTG-GTG-TGG-TAG-TTA-gaattc-AAG-AAC-CAC-CAA-AAT-AAT-GTC-T F3: GTG-TGT-TGC-TAT-ATT-GCT-TGT B3: AAC-TTT-AAC-AAC-ATA-CAC-CTA-GT	*E. granulosus s.s* (G1)	Faeces	Yes	Ni *et al.*, 2014
Magnetic capture – PCR mt 12S rRNA gene **EMrtCO1 F**' (TGG-TAT-AAA-GGT-GTT-TAC-TTG-G), **EMrtCO1 R**' (ACG-TAA-ACA-ACA-CTA-TAA-AAG-A), and Zen probe: 56-FAM/-TCT-AGT-GTA/Zen/-AAT-AAG-AGT-GAT-CCT-ATT-TTG-TGG-TGG-GT/3IABkFq/	*E. multilocularis*	Faeces	Yes	Isaksson *et al.*, 2014

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Real-time PCR large ribosomal subunit gene (rrnL) Em-rrn primer: F CTG-TGA-TCT-TGG-TGT-AGT-AGT-TGA-GAT-TT Em-rrn R GGC-TTA-CGC-CGG-TCT-TAA-CTC Em-probe with reporter 6-carboxyfluorescein (FAM) and quencher tetramethylrhodamine (TAMRA): TGG-TCT-GTT-CGA-CCT-TTT-TAG-CCT-CCA-T	*E. multilocularis*	Faeces	Yes	Knapp *et al.,* 2014
Real-time PCR using a hydrolysis probe targeting part of the mitochondrial "large ribosomal subunit" gene rrnL-Em F CTG-TGA-TCT-TGG-TGT-AGT-AGT-TGA-GAT-TT rrnL-Em R GGC-TTA-CGC-CGG-TCT-TAA-CTC	*E. multilocularis*	Faeces	Yes	Knapp *et al.,* 2014; Knapp *et al.,* 2016
Multiphase approach described in Santolamazza *et al.,* 2020 Phase1: DNA extraction; Phases 2: PCR Cox1; Phases 3: RFLP AluI specific digestion for distinction of G1, G3,G4, G5, G6/7 and G8/G10; Phases 4: multiple PCR showing the specific banding patterns for the G4-G10 genotypes	*E. granulosus sensu lato* G1, G3,G4, G5, G6/7 and G8/G10	Not available	Yes	Cox1: Bowles *et al.,* 1992 modified by Bart *et al.,* 2006 RFLP AluI: Kim *et al.,* 2017 Multiple PCR: Boubaker *et al.,* 2017
Real-time PCR for multiple sequences referred to Cox1, Cox3, NAD5	*E. granulosus sensu stricto* (G1, G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6–8, G10)	Faeces	Yes	Maksimov *et al.,* 2020

3. Serological tests

3.1. Intermediate hosts

Serological diagnosis of ovine echinococcosis has long been considered a potentially important tool for epidemiological studies in endemic areas, as well as for surveillance of control programmes. It has been known for many years that sheep infected experimentally with *E. granulosus* can mount detectable specific IgG responses within weeks. However, serum antibody levels varied greatly in natural infections resulting in reduced sensitivity and cross-reactions with *Taenia hydatigena* or *T. ovis* infected animals. At present this approach cannot replace necropsy (Craig *et al.,* 2015; McManus, 2014).

3.2. Definitive hosts

Serodiagnostic tests for canine echinococcosis were considered to have good potential for practical testing of dogs for *E. granulosus* infection and, initially, as a potential substitute for arecoline purgation. Diagnostic specificity was good (>90%) but sensitivity was generally poor (35–40%) with natural infections, and was much lower when compared directly with coproantigen detection (Jenkins *et al.,* 1990). Further research to assess existing or develop better recombinant antigens may improve the sensitivity of serological tests for canine echinococcosis.

There is mounting evidence of cases where dogs developed alveolar echinococcosis (AE) caused by the larval stage. AE in dogs is fast-developing and life-threatening. Frey *et al.* (2017) evaluated the diagnostic performance of several antigens for serological detection of AE in dogs. Excellent performance of ELISA with recombinant EM95 antigen in combination with Western blot was demonstrated. The test can potentially detect AE in the early stages of development. Since AE in dogs may be concomitant with the intestinal adult stage, it was suggested that dogs from endemic areas should be tested for *E. multilocularis* using available methods, including the EM95 ELISA, before relocation into non-endemic regions.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Intermediate hosts

Application of an effective vaccine to reduce CE infection in livestock may have a substantial impact on the rate of transmission of the disease to humans (Lightowlers, 2006; Torgerson, 2003). A highly effective EG95 vaccine has been

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developed that can prevent infection with *E. granulosus s.s.* in the parasite's livestock intermediate hosts (Lightowlers *et al.,* 1996; Gauci *et al.,* 2011). Field trials of the vaccine have demonstrated that the vaccine reduces the level of cystic echinococcosis in sheep under natural conditions (Amarir *et al*., 2021; Larrieu *et al*., 2019). The ultimate aim of vaccination against *E. granulosus* is to reduce the parasite's transmission and reduce human exposure to cystic echinococcosis.

Two subcutaneous immunisations, approximately 1 month apart, induce protection against subsequent exposure to *E. granulosus* eggs. The vaccine is not believed to cure an infection existing prior to the animal being vaccinated. For that reason, young animals should receive their first vaccination around the time of weaning. The duration of immunity induced in young animals after a second vaccination is sufficient to protect them until 1 year of age, at which time a single booster vaccination elicits a strong protective response sufficient to induce long-lasting protection (Larrieu *et al*., 2019; Poggio *et al*., 2016). Hence, a vaccination programme involving two immunisations in recently weaned animals, followed by a single booster immunisation when animals are approximately 1 year of age, presents effective and practical programme.

The EG95 vaccine is manufactured in Argentina, China (People's Rep. of) and Morocco, and has gained regulatory approval in Morocco and a number of East and South Asian, and South American, countries.

1.2. Definitive hosts

Development of *E. granulosus* vaccines for dogs would ideally reduce worm fecundity and populations, and could be a valuable step towards the reduction (prevention) of the infection pressure on intermediate hosts, and thus reduce (prevent) infection in dogs. However, no clear evidence exists for immunologically-based protection against *Echinococcus* infection in definitive hosts. Attempts to actively immunise dogs against infection with *E. granulosus* have not produced consistent results.

2. Outline of production and minimum requirements for vaccines for intermediate hosts

The vaccine incorporates 50 µg of the EG95 antigen, expressed in *Escherichia coli*, together with adjuvant (Quil A or Montanide ISA 70 plus saponin) (Gauci *et al.,* 2011; Lightowlers *et al.,* 1996).

2.1. Characteristics of the seed

2.1.1. Quality criteria

Suitable *E. coli* strains (BB4 LE392.23 [F´ *lacI*^{q} ZΔM15 *proAB* Tn10 (Tet^r)] or BL21 (DE3) F$^-$*omp*T *hsd*S$_B$ (r$_B^-$, m$_B^-$) *gal dcm* (DE3) for expression of recombinant vaccine antigen should be acquired from a source that has been established as sterile and pure (free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate licensing authorities). The pGEX expression vector should be obtained from a source known to be free of extraneous agents. The EG95 DNA insert in the expression vector should be verified as having the sequence described (Gauci *et al.,* 2011; Lightowlers *et al.,* 1996).

2.2. Methods of manufacture

2.2.1. Procedure

Suitable *E. coli* strains are transformed with pGEX vector containing in-frame EG95 cDNA. Bacteria are cultured in a suitable medium such as Super Optimal Broth. Recombinant protein expression is induced by addition of isopropyl-ß-D-thiogalactosidase (IPTG) at a concentration of 0.2 mM with incubation for 3–5 hours, after which the culture supernatant is discarded and the bacterial pellets are resuspended in PBS, pH 7.4. After chilling on ice, *E. coli* are lysed by sonication, French Press or other suitable equipment. The extent of bacterial cell lysis is monitored by measuring an increase in the soluble proteins released from the ruptured cells by determination of protein concentration. Soluble and insoluble cellular proteins are separated by centrifugation. After a brief wash with PBS, insoluble proteins are solubilised in 8 M urea. Vaccine can be prepared from either the soluble fraction by glutathione agarose affinity purification as described by Lightowlers *et al.* (1996) or from the insoluble inclusion bodies as described by Gauci *et al.* (2011). Proteins are analysed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and quantified by scanning densitometry and determining protein concentration according to Bradford (1976).

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2.2.2. Requirements for ingredients

All ingredients used for vaccine production should comply with the requirements referred to in Chapter 1.1.8 *Principles of veterinary vaccine production*.

2.2.3. Final product batch tests

i) Sterility

Must comply with chapter 1.1.8.

ii) Safety

Batch safety testing is performed unless consistent safety of the product is demonstrated and approved in the regulatory approval dossier and the production process is approved for consistency with the standard requirements referred to in chapter 1.1.8.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions. Local and general reactions must be examined. The tests must be performed by administering the vaccine to the sheep in the recommended dose and recommended route of administration. Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes of vaccinated and control groups.

iii) Batch potency

Sheep are immunised according to the recommended protocol (two subcutaneous injections, 3–4 weeks apart). IgG antibody responses to the EG95 antigen are determined 2 weeks after the second immunisation as described by Heath & Koolaard (2012).

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NB: There is a WOAH Reference Laboratory for echinococcosis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for echinococcosis

NB: FIRST ADOPTED IN 1989 AS ECHINOCOCCOSIS/HYDATIDOSIS. MOST RECENT UPDATES ADOPTED IN 2022.

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