ESCCAP

EUROPEAN SCIENTIFIC COUNSEL COMPANION ANIMAL PARASITES®

4 Parasitological Diagnosis in Cats, Dogs and Equines

ESCCAP Guideline 04 First Edition – November 2022

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INTRODUCTION

Correct diagnosis of infection and/or disease is a prerequisite for appropriate treatment and effective control, and the diagnosis of parasitic diseases is no exception. A tentative diagnosis based on clinical signs, haematology, chemistry, pathology or histopathology can be helpful in choosing specific diagnostic analyses for the direct or indirect detection of parasites (or a combination of both).

The development of standard procedures and good laboratory practices are of fundamental importance in assisting the veterinary health professional. Whether confirming the diagnosis of infection in pets presenting clinical signs compatible with parasitic disease or screening companion animals for infection as part of a wider programme of prevention and control, there is a need for accurate and reliable methods.

Direct detection

Parasitological laboratory methods frequently allow the direct morphological detection of parasites or the direct detection of parasite molecules such as parasite antigens or DNA.

Direct detection of parasites is possible through:

- morphological identification (macroscopic, microscopic, directly or after concentration, after staining or labelling with specific antibodies, after cultivation for fungal pathogens);
- immune diagnostic detection of parasite antigens;
- biomolecular detection of parasitic DNA;
- detection of parasite macromolecules through mass spectrometry; and
- in vitro cultivation for multiplication or development of parasitic stages.

Limiting factors include:

- irregular parasite distribution and excretion;
- incorrect sampling, storage and transportation;
- limited available genetic/proteomic information; and
- low sensitivity or specificity in some cases.

Caveat: Biomolecular detection of DNA or antigen may occasionally lead to false-positive results as they may persist for some time after the infection has cleared.

Indirect detection

For certain parasitic infections, direct detection is not always possible. This can either be due to their localisation in the body, because the parasite is present in very low densities and therefore not reliably detected by direct methods, or because the method is too technically laborious or invasive for the patient. In such cases, indirect detection, through the diagnosis of immune reactions to the parasites, can be very helpful. Indirect detection methods involve the detection of specific antibodies from different substrates.

Limiting factors include:

- variable and delayed immune reactions (depending on the point in time after infection, intensity, localisation and type of reaction);
- persistency and cross-reactivity of antibodies; and
- low sensitivity or specificity in some cases.

Antibodies can persist for varying time spans after the infection has cleared, so their ability to detect current infections may be limited.

This guideline is intended for use by veterinary professionals who carry out routine diagnostic procedures in their practice for the detection of parasitic infections, and for those who submit samples regularly to external laboratories.

It has been divided into chapters, based on the material being tested and the methods available. It also includes general information on sample collection, handling and preservation.

SAMPLING, SHIPPING AND STORAGE OF DIAGNOSTIC MATERIAL

General considerations

The accuracy of diagnostic tests largely depends upon the correct sampling, storage and shipping of test materials. Each sample must be labelled appropriately (including patient and owner identification along with the date of sampling), shipped in tightly sealed, unbreakable containers (labelled according to national and international legislation for the transport of potentially infectious material), quickly transferred to the laboratory and accompanied by correctly completed forms to enable laboratory staff to assign the sample and to carry out the requested test. Blood/serum samples must be sent in a leak-proof plastic tube with a screw cap in a cool pack with an overnight courier. The tube should be placed in a zip-lock bag/specimen bag in case the tube cracks or breaks, wrapped in protective material and placed in a sturdy shipping box.

Many laboratories provide specific guidelines for sampling and shipping. Furthermore, general and special safety precautions must be considered and toxic substances or infectious materials properly disposed of.

Sampling and storage

1. Faecal material

Faecal samples should be collected rectally or immediately after deposition in order to prevent contamination with material from the environment which may contain free-living nematodes. Analysis should be performed with fresh samples or after conservation at 4°C because the eggs of some helminths and coccidian oocysts may continue to develop and change their diagnostic characteristics. In older faecal samples, lungworm first stage larvae (L1) may not be sufficiently viable for migration using the Baermann technique. The larvae of *Strongyloides* spp. and strongylids may hatch from their eggs after hours or days respectively and the samples may return a false-negative result for egg detection. To prevent further development of nematode eggs, samples and containers should contain as little oxygen as possible and be tightly closed.

In veterinary parasitology, faecal samples are not usually conserved for a long time before examination. Longer-term storage may require preservation e.g. with chemicals (see specific literature for media) or by freezing at -20°C. In such cases, it should be noted that some parasite stages, especially nematode larvae, strongylid and other thin-shelled helminth eggs, cannot be reliably detected in preserved or previously frozen material.

2. Serum

In most cases, serum samples for the identification and quantification of antigens or antibodies, are obtained from peripheral blood. Ideally, the blood should be allowed to flow freely into the tube; this helps to prevent haemolysis. Whenever possible, at least 5 ml of blood should be taken to ensure a serum volume of 2 ml for a whole test panel. After coagulation of at least 20 minutes, the serum is separated from the coagulum by centrifugation, transferred to a fresh tube and preferably shipped straight away. If necessary, serum can be stored at 4°C for a maximum of 24 hours before shipping. Longer storage, if required, should take place at -20°C. Please note that centrifugation before complete coagulation may cause haemolysis which can interfere with the test; leaving the blood for too long may lead to microbial growth and subsequent degradation of the sample. If serum is not available, plasma can be used for some tests; this must be confirmed with the laboratory prior to testing.

3. Whole blood

For the microscopic or molecular detection of parasites or parasite DNA in whole blood (directly or after concentration, e.g. in the Knott's test), sample tubes with an anticoagulant are used (EDTA, Li-heparin). Whole blood samples can be stored at 4°C for a maximum of 4 days. Freezing samples at -20°C to facilitate longer storage, will still allow DNA detection but limit the isolation and identification of parasitic stages.

4. Isolated parasites

Living arthropods (and skin scrapings) should be kept in a tightly closed container to prevent their escape. Some moist paper tissue may be added to prevent dehydration. For further examination they should be fixed in 70% ethanol. Parasites from faeces, vomit, skin etc. should be collected separately and conserved in physiological saline solution (short-term) or in 70% ethanol.

STANDARD EQUIPMENT OF A PARASITOLOGICAL LABORATORY

Since microscopy is a standard technique for the evaluation of samples, a well-maintained light microscope is indispensable for parasitological diagnosis. Microscopic measurements are often necessary for identification, therefore equipment for accurate standardised measurements should be available. For objects visible with the naked eye, a stereomicroscope is usually the best option for detailed examination (see Table 1).

Table 1: Recommended magnification ranges and criteria for identification of parasite stages

Stages	Magnification range*
Eggs and larvae of helminths, small arthropods	Low magnification for screening: 40–100x For details: 400x
Protozoa	Medium to high magnification: 400–650x Blood smears, very small objects in faeces: 1000x (oil immersion)
Large arthropods, adult helminths (or parts of them, e.g. proglottids)	Low magnification (stereomicroscope)

* Please note that magnification is calculated as the ocular magnification (usually 8–10x) x the objective magnification for a light microscope whereas a stereomicroscope uses only one set of magnifying lenses.

Different features such as size, shape, colour and morphology of the surface, of the shell itself and the contents must be considered when diagnosing parasite stages under a microscope. For the identification of nematode larvae (lungworms, strongylids of horses, hookworms of dogs and cats or *Strongyloides* spp.), mobility can be considered when they are living. Furthermore, specific structures such as the morphology of the anterior part, the oesophagus and tail end are helpful for identification.

Further equipment for a parasitological diagnostic laboratory usually includes:

(A) for coproscopy:

- weighing equipment
- pestle and mortar
- plastic container and spatula, tongue depressor, plastic spoon or similar
- plastic stick or slim spatula
- cotton swab
- plastic squeeze bottle
- centrifuge tubes (12–15 ml; 50 ml)
- table centrifuge with swing-out rotor
- beaker (250 ml) or other container suitable for sedimentation
- funnel
- tea strainers with a sieve size of approximately 0.5–1 mm (for flotation or sedimentation–flotation) and 0.3 mm (for sedimentation)
- wire loop (bent to a right angle, approximately 6 mm in diameter), or other devices to remove droplets of flotation solution from the surface
- microscope slides
- coverslips
- immersion oil
- Petri dish (glass or plastic, with a gritted bottom for easier microscopic examination)
- methylene blue solution (ink)
- water jet pump (or 10–20 ml pipettes)
- cotton gauze (20 x 20 cm)
- plastic or glass Pasteur pipettes
- worm egg or oocyst counting chamber (e.g. McMaster, FLOTAC)

In addition, for the Baermann technique:

- funnel fixation apparatus
- rubber tube and tubing clamp
- calibrated measuring cylinders (100 ml and larger)
- magnetic stirrer and stirring magnets

For coprocultures also:

- jam jar 500 ml (or similar)
- Petri dish slightly larger in diameter than the glass jar
- culture chamber at 25°C, in darkness
- autoclaved sawdust, charcoal or vermiculite

(B) for haematology and serology

- Different equipment is necessary depending on the format and read-out method e.g. a fluorescence equipped microscope for IFAT or a plate reader for ELISA etc., as well as laboratory pipettes and staining equipment.

(C) for skin scrapings:

- 10% KOH solution or lactophenol
- mineral oil or glycerine

MEDIA AND SOLUTIONS

- 0.9% NaCl (physiological saline solution)
- flotation media (see Table 2):
- Lugol's solution (10 g potassium iodide, 5 g iodine, ad 200 ml aqua dest.)
- diethyl ether[1]
- SAF (sodium acetate-acetic acid-formalin): 4.5 g sodium acetate, 6.0 ml concentrated acetic acid, 12.0 ml formalin[1]
- Ziehl-Neelsen stain: carbol fuchsin, malachite green, acidic alcohol (15 ml HCl (37%) in 485 ml EtOH), methanol (use staining set)

[1] ether, formalin or merthiolate-iodine-formaldehyde (containing mercury) are used for the SAF concentration technique (see 1.2.7.), the Telemann-Rivas method (see 1.2.8.) and/or the MIFC (merthiolate-iodine-formaldehyde concentration) technique (not described in this guideline). The choice, among others, of the adopted method may be determined by the different laboratory standard safety measures regarding these solutions.

Table 2: Ingredients and properties of flotation solutions for coproscopic parasitological analyses (selection)

Flotation solution	Quantity	Volume of water	Specific gravity*
Saturated salt solution (NaCl)	340–360 g NaCl	1000 ml	~1.18–1.2
Zinc chloride salt solution ($ZnCl_2$ + NaCl)	275 g $ZnCl_2$ + 262 g NaCl	1000 ml	1.3
Zinc chloride solution ($ZnCl_2$)	440 g $ZnCl_2$	1000 ml	1.3
Zinc chloride solution ($ZnCl_2$)	660 g $ZnCl_2$	1000 ml	1.45
Zinc sulfate solution ($ZnSO_4$)	760 g $ZnSO_4$	1000 ml	1.3
Magnesium sulfate solution ($MgSO_4$)	350 g $MgSO_4$	1000 ml	1.28
Sucrose solution**	550 g sucrose	440 ml	1.28
Saturated salt solution with 50% glucose	375 g glucose + 250 g NaCl	Up to 1000 ml	1.27
Sucrose salt solution**	50 g sucrose + 100 ml saturated salt solution		1.33

* at room temperature, to be measured with a **hydrometer**.

** add 0.7 ml formalin (37%) or 1 g crystalline phenol for every 100 ml to prevent bacterial and fungal growth or store at 4°C.

1. FAECAL EXAMINATIONS

1.1. General information

Macroscopic examination

Prior to processing, each faecal sample should be examined macroscopically for excreted helminths (e.g. roundworms, tapeworm proglottids, pinworms) or blood (for the preservation of isolated parasites, see Introduction).

Microscopic examination

Allows the detection of excreted eggs, egg packets, larvae, oocysts, trophozoites (only in fresh faeces, if not mixed with a preservation solution) and cysts.

Helminth eggs and protozoan (oo)cysts should be differentiated based on their:

- shape: round, oval, polygonal, lemon-shaped;
- size: large = approximately 80–150 (–300) µm (e.g. *Fasciola hepatica* eggs, *Dipylidium caninum* egg packets), medium = approximately 60–80 (–120) µm (Ancylostomatidae eggs, Strongylidae eggs), small = approximately 40–60 µm (e.g. *Strongyloides* eggs, Taeniidae eggs) and very small = approximately <40 µm (e.g. coccidia and *Cryptosporidium* oocysts, *Giardia* cysts);
- shell/envelope: thickness, surface (e.g. smooth, rough, dented), colour, distinctive features such as pole caps or micropyles; and
- content (in relation to the time after faecal deposition): unsegmented cells, blastomeres, embryo or larva in helminth eggs, number of sporocysts and sporozoites and other structures in coccidia and other protozoa.

Nematode larvae are differentiated according to their total length, the morphology of the cuticular sheath, the digestive tract and of their anterior and posterior ends. They can be distinguished from free nematode larvae based on the absence of additional sheaths. Free-living nematode larvae have a distinct mouth opening and are a darker colour than infective larval stages; they may have oesophageal bulbi and several other particular features.

Depending on the operative principle, distinction is made between different coproscopic methods: direct procedures without concentration (i.e. faecal smears without staining, detection of motile stages or stained smears) as opposed to methods for concentration of parasitic stages:

- sedimentation
- flotation (with or without centrifugation)
- combined sedimentation–flotation
- Baermann technique
- specific concentration methods, such as Telemann-Rivas method, SAFC, etc.

Conclusiveness of coproscopic examinations

The listed methods can frequently detect a range of different parasites; however, no single method is equally suitable for all stages and parasites present. The preferred method should be selected according to the expected parasite stages and several methods will be required to cover the complete spectrum of possible infections.

In general, the greater the quantity of faeces, the greater the probability of detecting parasite stages (higher sensitivity, i.e. fewer false-negatives). However, the amount of examined faeces will be limited by the degree of contamination in the processed sample: too much debris decreases sensitivity (i.e. more false-negatives), and also specificity.

Since a larger quantity of faeces can be processed using a **combined sedimentation–flotation method**, the level of sensitivity is higher, particularly in the case of low levels of excretion. Therefore, this is the **preferred method** for routine examinations. Similar to flotation and sedimentation, this method is classified as semi-quantitative when performed with consistent methodology and defined quantities of faecal samples. Active flotation supported by centrifugation is preferred over passive flotation. Also, the choice of flotation solution, and therefore the specific gravity of that solution, is fundamental because some parasites are not detected at high specific gravity and others not at low specific gravity.

The sensitivity of coproscopical methods is limited by the following factors:

- Infections must be in the patent phase, i.e. stages (eggs, larvae, (oo)cysts) must be present in the substrate indicating an ongoing infection with multiplication/generation of offspring. Pre- or postpatent infections cannot be detected by copromicroscopy.
- Not all stages/parasites are excreted continuously; several samples from individual animals (e.g. for 3 consecutive days) or re-sampling may be necessary.
- Frequently, low-level infections only produce a small number of detectable stages. Increasing the amount of faeces or performing multiple analyses per animal can be advantageous as outlined above.

Diarrheoa can dilute samples by increasing faecal volume and thus decreasing sensitivity. This can be avoided by using the combined sedimentation–flotation method which concentrates liquid faeces by sedimentation prior to parasite concentration by flotation.

As a consequence of the low sensitivity of copromicroscopy, **negative results** must be interpreted with care. The fact that no parasite stages are detected in the faeces does not preclude a parasitic infection when there are typical clinical signs (e.g. infections that are still prepatent). Such cases warrant further examination. In some cases, alternative methods can be adopted (i.e. antigen or antibody detection).

On the other hand, direct detection of parasitic stages has, in general, a high specificity and false-positive results are rare when the analysis is performed correctly. False-positive results can arise from coprophagy in dogs (i.e. observed intestinal passage of the cat parasites *Toxocara cati* or *Toxoplasma gondii* in faecal samples of canids). Therefore, owners should be advised to try and prevent this behaviour, particularly for the days leading up to a coproscopic sample being taken, to avoid false-positive detection of passing parasite stages. Antigen detection, which is commercially available for some canine nematode species, can circumvent this problem.

For the interpretation of results in a clinical context, parasitological findings must be evaluated together with other diagnostic findings (e.g. clinical examination, haematology etc.) and in the case of zoonotic stages, their implications for human health.

1.2. Coproscopic methods

1.2.1. Sedimentation in water

Principle:
With this method, parasite stages with a relatively high specific gravity (e.g. trematode eggs, especially those of *Fasciola hepatica* and *Opisthorchis felineus* but also oocysts of *Eimeria leuckarti*) and heavy faecal particles quickly sediment in water. By repeated sedimentation and decanting of the supernatant, lighter faecal particles are removed. This method is usually applied to detect liver fluke eggs from horse faeces, but also from cats.

Faecal material required:
5–10 g of faeces (sensitivity increased by repeated sampling).

Procedure:

1. Mix the faecal sample with water into a homogenous solution with a spatula. If the consistency is very firm, mix it up with water beforehand using a pestle and mortar.
2. Pour the faeces/water suspension through a tea strainer (mesh size 0.3 mm) into a suitable container (e.g. a beaker or similar) and rinse the sieve with a strong jet of water from a plastic squeeze bottle until the beaker is full.
3. Leave it to stand for 3 minutes.
4. Pour off the supernatant in a single motion without interruption (or siphon it off with a water suction pump).
5. Refill the beaker with water.
6. Repeat sedimentation three times in total. The final supernatant should be almost translucent.
7. Add 1–3 drops of methylene blue (ink) to the last sedimentation and mix it thoroughly by swirling.
8. Pour the whole sediment into a (lined) Petri dish.
9. Examine the sediment under a microscope or stereomicroscope (20–50x magnification).

For standardised detection of *Fasciola* eggs in horse samples, a commercial device, FlukeFinder®, is also available.

1.2.2. Flotation with centrifugation

Principle:
Flotation is performed for the detection of helminth eggs, protozoa cysts and oocysts which have a specific gravity lower than that of the flotation solution. In a suspension of a faecal sample mixed with a flotation solution with a known specific density, lighter parasitic stages accumulate on the surface, whereas heavier particles sink or remain in suspension.

A number of different flotation solutions can be used (see Table 2). The chosen solution should be prepared at least one day before analysis and the specific gravity checked with a hydrometer.

Using saturated sodium chloride solution, eggs of *Strongyloides* (fresh faeces), strongylids, ascarids, oxyurids, coccidian oocysts and other protozoan cysts can be detected. With this method, parasite stages float as a result of their low specific gravity in a saline solution with higher specific gravity. The detection of *Trichuris*, *Capillaria*, taeniid, spirurid or oxyurid eggs and *Eimeria leuckarti* oocysts may be uncertain as they require flotation solutions of higher specific gravity. *Giardia* cysts are deformed, while trematode eggs are not detected. Prolonged storage of helminth ova or protozoa stages in hypertonic solutions can lead to the deformation of stages, complicating diagnosis. Nematode larvae can occasionally be found in flotation preparations; however, they usually shrink quickly and morphological diagnosis is severely compromised.

In addition to the above-mentioned stages, *Trichuris*, *Capillaria*, taeniid and spirurid eggs and *Giardia* cysts (deformed) can be detected using zinc sulfate, zinc chloride, magnesium sulfate or sucrose/sucrose salt solutions. Used solutions containing zinc (heavy metal) need to be collected and disposed of as special waste.

Several adaptations to the standard flotation technique are possible, some of which are described below.

Faecal material required:
Small animals: 4–5 g (approximately the size of a walnut), possibly collected on each of 3 successive days, analysed as a subsample of the pooled sample; horses: up to 20 g of faeces.

Procedure:

1. Prepare the flotation solution at room temperature and verify the specific gravity with a hydrometer (if not available, until the salt is almost completely dissolved).
2. Mix the faecal sample with a 10-fold volume of flotation solution to a homogenous solution. If the consistency is firm, pre-soak the sample in water.
3. Pour the faecal suspension through a tea strainer and by means of a funnel into two centrifuge tubes per sample. Leave a 1 cm margin from the top of each tube to avoid spillage within the centrifuge.
4. Centrifuge the tubes for 3–5 minutes at 300 x *g*.
5. With a wire loop (or similar device), remove 3–5 drops from the surface of the suspension and place on a microscope slide.
6. Cover the slide with a coverslip and examine microscopically starting with a 40x magnification. Focus to the correct optical level using small air bubbles or particles as a reference. The entire area of the coverslip can then be examined at a higher magnification (100–400x). In cases of only moderately dirty samples, examination without a coverslip at a low magnification can increase sensitivity since eggs and cysts are concentrated on the convex surface of the drops.

There are several options for the microscopic examination of parasite stages that float on the surface of the samples:

- several drops from the surface of the faecal suspension can be transferred to a slide using a wire loop bent into a right angle and then examined (with or without a coverslip) under the microscope. Take care not to breach the surface of the suspension when removing the droplets and ensure that the eyelet is cleaned between samples (washing followed by flaming).
- position a coverslip on the brim of a filled centrifuge tube (the coverslip should be in contact with the surface of the flotation solution) and centrifuge with the coverslip in place. The coverslip can then be removed, positioned on a microscope slide and examined under a microscope. The floating parasite stages are transferred to the slide along with the fluid adhering to the underside of the coverslip. Centrifugation with a coverslip works best with a sucrose solution.
- position a coverslip on the tube after centrifuging (to do this, the tube must be filled to the top so that the suspension forms a meniscus) then wait for a few minutes to allow the parasitic stages to float.

1.2.3. Combined sedimentation–flotation method

With this method, parasite stages accumulate first, forming a sediment in water, and then float as a result of their low specific gravity in a flotation solution with a higher specific gravity. The advantage of this combination method is the higher sensitivity because a greater quantity of faeces (up to 20 g) can be examined.

The method can be used to accurately identify all classes of helminth eggs and most protozoan oocysts and cysts. Detection of nematode larvae and trematode eggs requires expertise because they become deformed. It is not possible to detect protozoan trophozoites and amoeba cysts using this method.

Faecal material required:
For dogs and cats, a minimum of 4–5 g of faeces (approximately the size of a walnut) is collected. In some cases, to increase the likelihood of detection, it can be useful to collect faecal samples on each of 3 successive days and analyse a subsample of the pooled sample. For horses: up to 20 g of faeces.

Procedure:

1. Place the sample in a mortar and mix it thoroughly with water using the pestle.
2. Pour the faeces/water suspension through a tea strainer by means of a funnel into two centrifugation tubes. Rinse the faeces through the strainer using a heavy stream of water from a spray bottle (no material should be lost in the process).
3. Centrifuge for 8 minutes, at approximately 500–700 x *g*, for sedimentation.
4. Pour off the supernatant in one motion without interruption (or siphon it off with a water suction pump) until a maximum of 5 mm sediment remains.
5. Fill the tubes with flotation solution.
6. Centrifuge the tubes for 3–5 minutes at 300 x *g*.
7. Proceed as for the flotation technique (see 1.2.2.).

Alternatively, if no centrifuge is available, mix the faecal sample with water and leave the tubes at room temperature for 30 minutes to one hour, pour off the supernatant and mix the sediment with the flotation solution until a meniscus can be observed. Add a coverslip, wait for 10–20 minutes (depending on the flotation solution), put the coverslip on a glass slide and then examine under a microscope. This method is less sensitive than those based on centrifugation and usually only detects parasite stages present in higher numbers.

1.2.4. Modified McMaster egg counting technique

Principle:
This technique is adopted for **quantitative** faecal examination of helminth eggs or coccidian oocysts. A defined amount of faeces is floated in a chamber and counted microscopically under a marked field. From the counted number of stages, the amount in 1 g of faeces is calculated and expressed as epg (egg count per g) or opg (oocyst count per g). Egg counting is frequently adopted for quantitative determination of strongylid eggs in horses, but it is also applicable to other parasite stages that float. Usually, saturated NaCl flotation solution is used; by using flotation solutions with higher specific density, heavier eggs can be detected.

For field studies on drug resistance, the technique is applied before and after anthelmintic treatment (faecal egg count reduction test, FECRT). The FECRT is a screening test for treatment efficacy.

In control programmes with targeted selected treatments, epg counts provide information on the role of individual animals for pasture contamination and can be used to support decision making in selective treatment procedures for horses (see ESCCAP's Guideline 8: A guide to the treatment and control of equine gastrointestinal parasite infections).

Due to its detection limits (see below), the modified McMaster egg counting technique is not recommended for the screening of parasites in animals, especially for highly relevant endoparasites (i.e. *Echinococcus* spp. in carnivores or *Parascaris* spp. in horses).

Faecal material required:
At least 4 g of faeces.

Procedure:

1. Mix exactly 4 g of faeces with approximately 30 ml of flotation solution in a mortar and pestle.
2. Pour the faeces suspension through the sieve and funnel into the cylinder; squeeze the sieve residue with a pestle; flush the mortar and pestle with flotation solution.
3. Remove the sieve and funnel and fill up the flotation solution to 60 ml.
4. Transfer this suspension into a beaker and mix well by continuously stirring, e.g. with a magnetic stirrer.
5. Quickly remove a sample with a pipette and fill the first compartment of the counting slide with a volume of at least 0.15 ml. The counting grid must be filled completely; no air bubbles must be introduced.
6. Mix the suspension thoroughly again and take a second sample to fill the second compartment of the slide.
7. Leave the slide for 5–10 minutes in order to allow the eggs/oocysts to float.
8. Place the counting slide on the microscope and count all the parasite stages within the counting grid (40–100x magnification).

Calculation of epg/opg:
epg/opg = (numbers of eggs/oocysts from all counted grids (N)/amount of faeces (g) x counting grid area (cm^2)) x volume of faecal suspension (ml)/height of counting chamber (cm) x number of counted grids (n),

- with N being the average number of eggs/oocysts counted per grid;
- with n being the number of grids counted;
- volume under the grid = counting grid area (cm^2) x height of the counting chamber (cm).

As an example:
When the amount of faeces = 4 g and the volume of the suspension = 60 ml and the volume under the grid = 0.15 ml, the technical lower detection limit is 100 epg/opg. With slides having two chambers and when the total number of eggs counted **under both grids** are added, the technical lower detection limit per slide is 50 epg/opg.

$$\text{epg/opg} = \frac{\text{counted eggs}}{\text{amount of faeces (g) x size of grid (cm}^2\text{)}} \times \frac{\text{volume of faecal suspension (ml)}}{\text{height of chamber (cm) x number of grids counted}}$$

epg/opg = eggs/oocysts per gram of faeces
size of the grid: 1 x 1 cm = 1 cm^2
height of chamber = 0.15 cm
volume under the grid = 0.15 ml (calculated from the height of the chamber and the size of the grid)
number of grids counted: can vary, therefore this is included in the formula

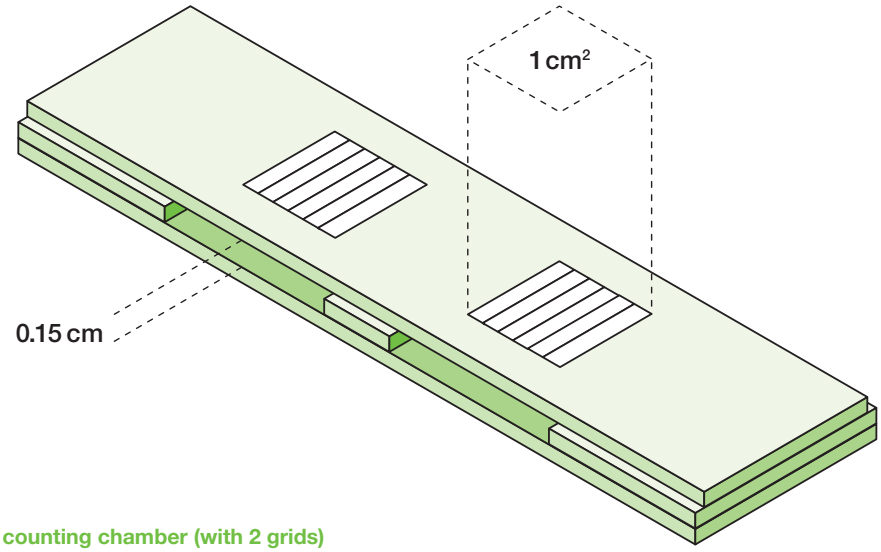

Figure 1: McMaster counting chamber (with 2 grids)

1.2.5. Mini-FLOTAC

Principle:
Mini-FLOTAC allows detection and quantification of gastrointestinal parasites, including coccidia oocysts, helminth eggs and nematode larvae. It is derived from the original FLOTAC apparatus but has the added advantage of requiring no centrifugation step. Mini-FLOTAC can be performed on either fresh or fixed faecal samples, offering an opportunity to process samples days or weeks after transfer to the laboratory. The sensitivity of Mini-FLOTAC is 5 oocysts/eggs/larvae/coccidia per gram of faeces.

Mini-FLOTAC comprises two components: the base and the reading disc. There are two 1 ml flotation chambers which are designed for the examination of faecal sample suspensions allowing a maximum magnification of 400x.

Fill-FLOTAC 2 and 5 are disposable sampling devices, which are part of the FLOTAC and Mini-FLOTAC kits. They consist of a container, a collector (2 g or 5 g) and a filter. These kits facilitate the performance of the first four consecutive steps of the Mini-FLOTAC techniques, i.e. sample collection (including weighing), homogenisation, filtration and filling. The kit and process of the Mini-FLOTAC is described below.

For dogs and cats

Faecal material required:
At least 2 g of faeces.

Procedure:

1. Add 18 ml of flotation solution to the Fill-FLOTAC 2 container.
2. Using a spatula, fill the conical collector of the Fill-FLOTAC device with 2 g of faeces and level the surface.
3. Homogenise the sample by moving the conical collector up and down.
4. Put a tip onto the Fill-FLOTAC, homogenise briefly and then fill the two chambers of the Mini-FLOTAC device until a small meniscus is formed.
5. After 10 minutes, translate the reading disc by rotating clockwise and place the device under a microscope using the adapter supplied with the kit. The Mini-FLOTAC can be examined at a maximum of 400x magnification.
6. Examine the sample by reading both chambers of the Mini-FLOTAC. Multiply each counted egg by 5 to obtain the egg/larva/oocyst per gram count.

Note: for the reliable detection of taeniid eggs, flotation solutions with a higher density must be used (see Table 2).

For horses

Faecal material required:
At least 5 g of faeces.

Procedure:

1. Add 45 ml of flotation solution (i.e. saturated NaCl) to the Fill-FLOTAC 5 container.
2. Using a spatula, fill the conical collector of the Fill-FLOTAC device with 5 g of faeces and level the surface.
3. Homogenise the sample by moving the conical collector up and down.
4. Put a tip onto the Fill-FLOTAC, homogenise briefly and then fill the two chambers of the Mini-FLOTAC device until a small meniscus is formed.
5. After 10 minutes, translate the reading disc by rotating clockwise and place the device under a microscope using the adapter supplied with the kit. The Mini-FLOTAC can be examined at a maximum of 400x magnification.
6. Examine the sample by reading both chambers of the Mini-FLOTAC. Multiply each counted egg by 5 to obtain the egg per gram count.

1.2.6. Double centrifugation technique for the detection of *Anoplocephala* eggs in horse faeces

The detection of tapeworm eggs in horse faeces using the standard flotation method with 20 g of faeces provides only limited sensitivity. Using a combined sedimentation–flotation procedure, during which two centrifugation steps are employed with 15 g of faeces, provides a higher detection rate.

Faecal material required:
15 g

Procedure:

1. Thoroughly mix the faecal sample (minimum 15 g) with at least 40 ml of water using a spatula.
2. Place the strainer on top of the 250 ml beaker and transfer the faecal sample to the strainer. Pass all the liquid through the strainer by pressing the faecal sample using the spatula.
3. Transfer the filtrate to 50 ml tubes.
4. Centrifuge for 10 minutes at 400 x *g*.
5. Remove the supernatant using a suction pump or pipette.
6. Dissolve the remaining pellet in 1–2 ml of sucrose solution and transfer suspension to a 15 ml tube.
7. Fill up with sucrose solution.
8. Centrifuge for 10 minutes at 200 x *g*.
9. Using a wire loop, transfer drops from the (complete) surface of the centrifuged faecal suspension to a glass slide and cover with a coverslip.
10. Microscopically examine the complete area under the coverslip as described in section 1.2.2.

1.2.7. SAFC technique

Principle:
The **SAFC** (**S**odium Acetate Acetic **A**cid **F**ormalin **C**oncentration) technique is suitable for detecting encysted and vegetative stages of protozoa (e.g. *Giardia*) and small eggs of some trematodes like *Opisthorchis* and *Metorchis*. Previous preservation of fresh faecal samples in SAF solution is used. By processing the sample, fat components are removed by ether and parasitic stages are concentrated. Variants of the SAFC technique are the Telemann-Rivas method (see 1.2.8.) and the MIFC technique (not described in this guideline).

SAF solution:
20 ml concentrated acetic acid
40 ml formaldehyde (37%)
15 g sodium acetate
ad 1 L aqua dest.

Faecal material required:
Minimum of 1 g fresh faeces transferred into sample tubes with 10 ml SAF solution.

Procedure:

1. Agitate the sample tube containing faeces and SAF solution.
2. Pour half of the suspension through gauze into a centrifuge tube and centrifuge for 2 minutes (500 x *g*).
3. Decant the supernatant, add 8 ml of physiological saline solution and stir the sediment with a plastic spatula or stick.
4. Add 3 ml diethyl ether, close the tube and mix the contents by gently shaking.
5. Remove the lid and centrifuge for 5 minutes (500 x *g*) which allows the formation of several layers.
6. Detach detritus from the tube wall with a stick and decant the supernatant.
7. Transfer two or more drops of the sediment to a slide. Optionally add Lugol's solution, then cover the drops with a coverslip.
8. Microscopically examine the complete area under the coverslips as described in section 1.2.2.

1.2.8. Telemann-Rivas method

Principle:
This method is a variant of the SAFC technique. This method concentrates eggs, cysts and larvae in faecal samples with high fat concentrations (like those from carnivores).

Faecal material required:
1 g, fresh.

Procedure:

1. Weigh 1 g of fresh faeces.
2. Place the faeces in a glass jar and add 5 ml of acetic acid (at least 5% concentration).
3. Homogenise thoroughly with a stick and filter through a double layer of gauze.
4. Transfer the faecal solution into a centrifuge tube (12 ml).
5. Add an equal quantity of diethyl ether and mix by shaking.
6. Centrifuge for 5 minutes (300 x *g*). The mixture separates into 4 layers (from top to bottom): diethyl ether/detritus (including fat components)/acetic acid/sediment. The sediment is often very small.
7. Draw off the sediment using a pipette and transfer it to a slide. Optionally add Lugol's solution for detection of some parasitic stages (i.e. *Giardia* cysts), cover the drops with a coverslip.
8. Microscopically examine the complete area under the coverslips as described in section 1.2.2.

1.2.9. Baermann technique

Principle:
This method is usually used for the isolation of motile nematode larvae (L1 of lungworms, *Strongyloides*). The Baermann technique can also be used to recover third stage larvae (L3) of strongylids from coproculture. The principle is based on larval migration (positive hydrotaxis) and gravity, therefore can only be performed with living larvae from fresh faeces. By weighing the faeces and counting all present larvae (or representative aliquots), a quantitative analysis is possible.

Faecal material required:
At least 4–10 g of faeces; sampling for three consecutive days is helpful to overcome the intermediate shedding of L1 of lungworms.

Procedure:

1. Prepare the Baermann apparatus by placing the funnel into the funnel fixation apparatus and the rubber tubing onto the funnel end, with the clamp parallel to the angular cut of the rubber and the first opening side of the clamp closer to the bottom. Pour some tap water into the funnel. Make sure that the clamp is not leaking by e.g. filling the apparatus the day before use.
2. Mix the faeces properly (sawdust can be used to loosen the faeces if the sample is firm and compacted) and place the homogenous mixture into the gauze.
3. Place the gauze containing the faeces into the funnel and add water until almost entirely covered.
4. Stand the sample for at least 12 hours at room temperature. The larvae migrate out of the faeces into the tube and sink (gravity) to the funnel end.
5. By opening the rubber clamp, the first drops can be analysed under a microscope (40–400x magnification). If larvae are moving, 1 drop of Lugol's solution can be added, which kills the larvae making it easier to identify their morphological characteristics. Alternatively, collect 10–14 ml of the fluid in a tube, centrifuge for 2 minutes (500 x *g*), discard the supernatant and examine 0.5–1 ml of the sediment.

For the quantitative determination of the number of larvae per gram of faeces (LPG), previously weigh the faeces, count all the larvae (or aliquots) in the sediment and extrapolate the LPG.

1.2.10. Faecal larval culture

Principle:
Coprocultures are performed with **horse** faeces for recovering third stage larvae (L3) of gastrointestinal nematodes: eggs of trichostrongylids and strongylids are cultivated to L3 allowing differentiation at genus level.

Faecal material required:
At least 50 g of faeces, taken directly from the rectum or immediately after defaecation to avoid contamination with soil nematodes.

Procedure:

1. Mix faeces with sterilised sawdust to a moist, crumbly mass (if needed add tap water, but ensure that the water remains absorbed; when the mixture is too moist the larvae will not develop).
2. Transfer the mixture to a screw-top glass jar, filled halfway, and loosely close the lid (air must still be able to get into the jar).
3. Incubate the jar for 7–10 days in the dark at 25°C, allowing the developed larvae to migrate up the glass walls of the jar.
4. Open the jar and press the faecal culture down with a pestle or a spoon.
5. Fill the jar carefully to the brim with tap water without stirring the faecal culture.
6. Press the lid of a glass Petri dish firmly onto the neck of the glass jar, then turn the culture upside down.
7. Half fill the Petri dish with water and let the jar and dish stand for 12 hours, allowing the larvae to pass from the culture glass into the fluid of the Petri dish.
8. Collect the fluid in the Petri dish with a pipette and differentiate the larvae under the microscope (see 1.2.9.).

Alternatively, after incubation and development of third-stage larvae (point 3), these can be concentrated by the Baermann method (see 1.2.9.).

1.2.11. Faecal smears, staining

Principle:
Direct faecal smears on microscope slides may be a faster option for i.e. the detection of vast numbers of lungworm larvae or protozoa trophozoites. However, without concentration, this procedure has low sensitivity and is in most cases insufficient for reliable parasite detection. The only exception is the staining for detection of *Cryptosporidium* described here.

Oocysts of *Cryptosporidium* can be detected in thin faecal smears after staining. After modified Ziehl-Neelsen staining (acid-fast staining) the oocysts appear as pink-coloured roundish objects (diameter 4–6 µm) on a turquoise background. After carbolfuchsin staining according to Heine, oocysts are visible as colourless structures on a reddish background. Other staining, such as Kinyoun staining, is also suitable to detect such oocysts.

Faecal material required:
1 g of faeces, semi-liquid or liquid (mixed with water when too firm).

Procedure (Ziehl-Neelsen) stain:

1. Distribute faecal material as a thin layer on a microscope slide using a cotton swab.
2. Air dry for approximately 30 minutes at room temperature.
3. Fix in methanol for 5 minutes in a staining station.
4. Air dry.
5. Colour with carbolfuchsin for 4 minutes in a staining station.
6. Rinse with cold running tap water.
7. Decolour with acid alcohol in a staining station until there is no more colour flowing.
8. Rinse with cold running tap water.
9. Counterstain with malachite green for 4 minutes in a staining station.
10. Rinse with cold running tap water.
11. Air dry.
12. Carry out a microscopic examination with 400–1000x magnification (oil immersion), searching for pink-coloured roundish objects (diameter 4–6 µm).

Procedure (Heine stain):

1. Mix a drop of faeces with carbolfuchsin and spread thinly on a microscope slide.
2. Leave to air dry until the surface appears dull and immediately examine microscopically with oil immersion as described above.

1.2.12. Adhesive tape method

A direct impression technique uses transparent sticky tape (approximately 1 cm wide and 4 cm long) to collect material from around the anus of horses for the detection of *Oxyuris equi* eggs. The same method can be applied to obtain *Echinococcus* and other taeniid eggs from the perianal area of dogs. The sticky side of the tape is pressed several times onto the skin. Next, it is pressed (also sticky side down) onto a slide. The tape serves as a coverslip and the sample can be evaluated microscopically.

1.2.13. Commercial examination kits for faecal analysis/ InPouch™ TF Feline/coproantigen tests

Various kits, for performing the flotation procedure, are commercially available to facilitate faecal examination (e.g. Parasite Diagnosis System®, ParaTest®, Fecalyzer®, Ovatec® Plus); these kits work with a ready-to-use flotation solution (specific gravity 1.2) and analysis vessels.

In general, these kits are best suited for faecal analysis in dogs and cats. They are appealing because they are user-friendly and are a hygienic way of handling and examining faecal samples.

However, it should be taken into consideration that the kits are only able to process small amounts of faeces because the vessel is small, and the procedure may involve mounting the faecal sample on the underside of the strainer insert. As a result, kits are only suitable for samples collected from carnivores and, in the case of light infestations, have a lower detection sensitivity (smaller quantity of faeces examined, lower specific gravity of the flotation solutions, no concentration by centrifugation) compared to the combined sedimentation–flotation method described above. Moreover, it is not usually possible to macroscopically inspect the sample (e.g. for the presence of proglottids) if the animal's owner has already closed the container holding the faecal sample before bringing it in for analysis. Compared to conventional methods, analysis using a kit incurs a higher cost and produces more plastic waste because disposable components are used.

InPouch™ TF Feline

This procedure is used for the cultivation and detection of *Tritrichomonas foetus* trophozoites in cat faeces. Faecal matter (0.03 g, corresponding to the size of a pin head) is introduced by means of a sterile cotton swab into a commercially available pouch (InPouch™ TF Feline) containing cultivation medium. The pouch is labelled and dated and then closed for cultivation in a vertical position at 37°C for 24 hours, then kept at room temperature in darkness. The pouch is examined for trophozoites under a microscope after 24 hours, 48 hours and then every second day up to 12 days.

Although the test is considered specific for *T. foetus*, non-pathogenic flagellates such as *Pentatrichomonas* can appear in culture, necessitating molecular differentiation.

Coproantigen tests and other antibody-guided parasite detection assays

Coproantigen tests qualitatively detect antigens of parasites in faecal matter and are commonly used as point-of-care assays in practice, but also in specialised laboratories, since the sensitivity is high when used for examination of the intended host species. The formats for coproantigen detection can be direct immunofluorescence assays (DFA) on slides, ELISA or enzyme immunoassay (EIA)-formatted antigen detection tests in 96-well plates, or immunochromatographic dipstick tests (ICT) (see also Table 3). Because tests designed for use in dogs and cats are now available, these are preferred over those developed for human stool samples due to enhanced quality assurance, especially for specificity.

In the case of *Giardia*, it should be noted that coproantigen detection does not always correlate with sickness, and test results must be interpreted in conjunction with clinical signs.

Table 3: Examples of commercially available coproantigen tests (without claim for completeness)

Assay	Manufacturer	Parasite detected	Format	Host species*
Anigen Rapid CPV/CCV/Giardia Ag 2.0	BioNote, Korea	*Giardia*	immunochromatography	dog, cat
*FAST*est® GIARDIA Strip	Megacor, Austria	*Giardia*	immunochromatography	dog, cat
*FAST*est® CRYPTO-GIARDIA Strip	Megacor, Austria	*Giardia*/ *Cryptosporidium*	immunochromatography	dog, cat
SNAP® *Giardia*	IDEXX, USA	*Giardia*	ELISA	dog, cat
Merifluor® *Cryptosporidium*/*Giardia*	Meridian Bioscience, UK	*Giardia*/ *Cryptosporidium*	direct immunofluorescent detection	dog
PetChek™ IP Fecal Dx™**	IDEXX, USA	*Giardia* ascarids Trichuridae hookworms	ELISA	dog, cat***
Speed Giardia™	BVT-Virbac, France	*Giardia*	immunochromatography	dog, cat
Uranotest® Giardia	Uranovet, Spain	*Giardia*	immunochromatography	dog, cat
WITNESS® GIARDIA	Operon S.A., Spain****	*Giardia*	immunochromatography	dog, cat

* according to the manufacturer's instructions; some assays can be extended to other species as described in the literature.

** PetChek™ IP collection kit sold by veterinary practices, samples to be submitted to manufacturer for testing. Fecal Dx™ refers to the same tests and the same company.

*** described for use in dogs.

**** distributed by Zoetis.

2. SEROLOGY

2.1. General information

Serological tests are preferred over direct detection in chronic infections, where low parasite densities in substrate for direct detection reduce the success rate, e.g. in babesiosis or neosporosis, or in cases where the infected tissue cannot be sampled and stages are not directly accessible in body fluids. Examples of this are tissue-dwelling protozoa that cause chronic infections in tissues, e.g. brain or ocular tissue for *Toxoplasma gondii* or *Encephalitozoon cuniculi*. In the diagnosis of canine leishmaniosis, serological assays are used alongside direct detection to support the correct interpretation of the infection and immunological status of the animals, to monitor the response to treatment, and to anticipate a potential relapse.

Antigen detection

Similar to coproantigen tests, which detect antigen in faeces (see 1.2.13.), the serological detection of antigens means that parts of the parasite (somatic antigens) or excretory/secretory antigens produced by the parasite are directly detected in blood or serum. Similar to DNA detection, this provides direct evidence of the presence of the parasite in the blood circulation and therefore information on the current status of infection. *Dirofilaria immitis* and *Angiostrongylus vasorum* are blood parasites of which circulatory antigens can be serologically detected (see Table 4).

Table 4: Selection of serological tests commercially available for the diagnosis of parasites or vector-borne pathogens of dogs, cats and horses.

(Sources: www.megacor.at; www.laboklin.de; www.idexx.ch; uranovet.com and others; without claim for completeness)

Assay	Manufacturer	Parasites or vector-borne bacteria detected	Format	Host species
Antigen detection tests				
Anigen Rapid CHW Ag 2.0	BioNote, Korea	*Dirofilaria immitis*	Lateral flow immunochromatography	dog
DiroCHEK®	Zoetis, USA	*Dirofilaria immitis*	ELISA	dog
*FAST*est® HW Antigen	Megacor, Austria	*Dirofilaria immitis*	ELISA	dog
Angio Detect™ Test	IDEXX, USA	*Angiostrongylus vasorum*	Lateral flow immunochromatography	dog
Speed Diro™	BVT-Virbac, France	*Dirofilaria immitis*	Lateral flow immunochromatography	dog
Uranotest® Dirofilaria	Uranovet, Spain	*Dirofilaria immitis*	Lateral flow immunochromatography	dog, cat
WITNESS® Dirofilaria	Zoetis, USA	*Dirofilaria immitis*	Lateral flow immunochromatography	dog
Antibody detection tests				
ANAPLASMA-ELISA DOG	Afosa, Germany	*Anaplasma phagocytophilum*	ELISA	dog
Anigen Rapid Leishmania Ab	BioNote, Korea	*Leishmania infantum*	Lateral flow immunochromatography	dog
BABESIA-ELISA DOG	Afosa, Germany	*Babesia canis*	ELISA	dog
EHRLICHIA-ELISA DOG	Afosa, Germany	*Ehrlichia canis*	ELISA	dog
Diagnosteq	University of Liverpool, UK	*Anoplocephala perfoliata*	ELISA	horse
EquiSal	Austin Davis Biologics, UK	*Anoplocephala perfoliata*	ELISA (using saliva)	horse
LEISHMANIA-IgG ELISA DOG	Afosa, Germany	*Leishmania infantum*	ELISA	dog
SARCOPTES-ELISA 2001 DOG	Afosa, Germany	*Sarcoptes scabiei* var. *canis*	ELISA	dog
SNAP® *Leishmania*	IDEXX, USA	*Leishmania infantum*	ELISA	dog
Speed Leish K™	BVT-Virbac, France	*Leishmania infantum*	Lateral flow immunochromatography	dog
Uranotest® Ehrlichia-Anaplasma	Uranovet, Spain	*Ehrlichia canis*, *Anaplasma platys*	Lateral flow immunochromatography	dog
Uranotest® Ehrlichia	Uranovet, Spain	*Ehrlichia canis*	Lateral flow immunochromatography	dog
Uranotest® Leishmania	Uranovet, Spain	*Leishmania infantum*	Lateral flow immunochromatography	dog
Mixed antigen (Ag) -antibody (Ab) detection tests				
SNAP® 4Dx® Plus	IDEXX, USA	*Dirofilaria immitis* (Ag) *Borrelia burgdorferi* (Ab) *Ehrlichia canis* (Ab) (*E. ewingii*, *E. chaffeensis*) *Anaplasma phagocytophilum* (Ab), *A. platys* (Ab)	ELISA	dog
Uranotest® Quattro	Uranovet, Spain	*Dirofilaria immitis* (Ag) *Leishmania infantum* (Ab) *Ehrlichia canis* (Ab), *A. platys* (Ab)	Immunochromatography	dog

Antibody detection

The detection of specific antibodies against parasites in a single sample indicates whether the animal has been previously exposed to this antigen/parasite. As the production of antibodies is delayed, they can usually only be detected 2–3 weeks after the primary infection. Antibodies also persist after elimination of the pathogen, in many cases for weeks to months/years with great individual and pathogen-specific variations. It is important to consider antibodies that are derived from sources other than infection, such as a previous vaccination (e.g. against *Leishmania* or *Babesia*) or maternal antibodies in very young animals. Cross-reactivity with other organisms or non-specific reactions may limit the specificity of serological tests.

To reduce false results, serological tests can be repeated after a certain time period (usually 2–3 weeks) to see if values have changed. If an animal with previously negative or low values has an increasing or positive value, this can be interpreted as an active/ongoing infection; if the value is waning or converted to negative, the infection is terminated. There are, however, exceptions to this rule.

To test for fluctuations in antibodies, either quantitative ELISAs (with unit, score or concentration values) or semiquantitative IFA assays (titres) can be used. In-clinic rapid assays are qualitative in nature and only allow a decision as to whether an animal is serologically positive or negative.

The formats of antibody detection resemble those of antigen detection, except that the substrate for detection is reversed. Since most formats are restricted to specialised laboratories, because specific equipment is necessary in order to carry out the tests, the methods are not described in detail in this document and only the different formats and available in-practice tests are listed.

Typically, antibody detection is applied to evaluate the infection status for extraintestinal protozoa that cause chronic infections, such as *T. gondii*, *E. cuniculi* (cats and dogs), *Neospora caninum* (dogs), *Sarcocystis neurona* (horses) as well as many vector-borne pathogens, including *Leishmania infantum*, *Babesia canis*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Dirofilaria immitis* (cats), *Angiostrongylus vasorum* (dogs), *Babesia caballi* and *Theileria equi* (horses). For dogs, antibody detection against metacestodes in alveolar echinococcosis can be employed in specialised laboratories.

In addition, infection with *Anoplocephala perfoliata* in horses can be diagnosed by the detection of serum antibodies or antibodies in saliva. For dogs, direct detection of sarcoptic mange may be supported by using a serological antibody test.

Some laboratories offer “travel packages” for dogs, combining different tests (serology, PCR, antigen detection, Knott’s test and others) for a variety of “exotic” pathogens. However, one must consider the differences in incubation times and the onset of clinical signs of disease. Retesting can be advisable in some cases.

Some countries impose national regulations regarding testing for equine piroplasmosis.

Quality assurance in serological test systems

As with all diagnostic tests, serological assays have varying levels of sensitivity and specificity. It is good clinical practice to always include positive and negative controls in a serological test. Many laboratories offer background information on the technical quality of their tests and interpretation aids, especially regarding interference with e.g. vaccination titres, cross-reactivity with closely related pathogens and other factors that must be considered for test interpretation.

Depending on the clinical presentation and the requirements of the test, it might be advisable to combine a serological test with other direct detection methods or, if this is not possible, with another indirect test system for antibody detection. Western blotting is often used as a reference test for human infections, but due to cost and time restrictions, its application in veterinary diagnostics is usually limited to research, with a few exceptions.

Where applicable, immunoglobulins produced during active, subacute infections (IgM) are differentiated from those dominating chronic infections (IgG).

To evaluate the success of treatment in the case of canine leishmaniosis, serology may not be sufficient as antibodies can persist for several months or years following clinical cure because the parasite cannot be eliminated. Therefore, further laboratory analyses evaluating the patient's immunological and health status are recommended. Quantitative PCR allows the parasitic load to be estimated in comparable tissues, which could be useful for follow-up during the treatment of canine leishmaniosis although this approach needs to be thoroughly evaluated.

2.2. Quantitative methods for antibody detection

2.2.1. Indirect fluorescent antibody test (IFAT)

The indirect fluorescent antibody test is based upon the detection of specific antibodies, which bind to antigens (i.e. inactivated whole parasites) immobilised on glass slides and are subsequently detected by a secondary antibody. This antibody binds to the invariable region of the primary (serum) antibody that is covalently linked to a fluorophore. With fluorescent light microscopy, the fluorophore of the bound antibody is detected at the appropriate wavelength. The technique is very accurate when carried out properly but is time consuming and requires a certain level of training and expertise. However, for small animals it is still widely used because it is less prone to non-specific reactions. The disadvantage of IFATs is that not all antigens are equally accessible to the antibodies, so sensitivity may be low compared to other tests. Since the results are expressed in titres, they give a semiquantitative result which can be useful for follow-up studies.

2.2.2. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) detects specific antigens or antibodies that bind to a processed antigen (i.e. whole lysate of native antigens or recombinant antigens) or antibodies, absorbed to a plastic surface (indirect ELISA). The secondary antibody is, in this case, labelled with an enzyme that induces a colour change of a chromophore substrate that can be measured and quantified by measuring the optical density. A more elaborate form is the sandwich ELISA which does not bind the antigen directly to the plastic surface but uses antibodies linked to this surface to "catch" specific antigens. This method is of use when the direct linking of antigen mixtures to the solid phase leads to a decrease in the specificity of the test. Other ELISA formats are possible.

ELISA is a more standardised test format as it comes in 96 well-plates and is frequently customised with controls included, so pathogen-specific expertise is not necessary to carry out the test. Test results are usually expressed as absorbance values, test scores, concentrations or antibody units and in most cases only one dilution of diagnostic material is used for specific antibody detection, and low dilutions for antigen detection. Therefore, the intensity of the reaction is not always correlated with the quantity of the antibodies present (in strong positive reactions the system may be saturated).

Since ELISA formats are more standardised than IFATs and the optical readout is less subjective than microscopy, quantitative ELISAs are preferable over IFAT for the detection of changes in antibody titres over time.

In particular cases, substrates other than blood can be tested, such as in the case of *Anoplocephala perfoliata* in saliva of horses (EquiSal®).

2.3. Qualitative methods

2.3.1. Rapid test systems

Enzyme-based rapid tests (using antibodies covalently bound to an enzyme and employing a washing step) or immunochromatographic tests (using antibodies bound to colloidal gold and no washing step) are available for the quick point-of-care diagnosis of various infectious diseases. They are supplied by specialised companies who maintain the quality assurance of their tests and supply all materials including the positive and negative controls which are included in the test. The tests are easy and quick to perform and do not require specific training. However, they should not be used for cases and hosts other than those specified in the test documents. The assessed level of antibodies or antigen is usually not accurate enough to determine an increase or decrease in titres.

3. SKIN EXAMINATION

3.1. General information

This section is dedicated to the identification of external parasites that cause disease directly due to their presence on the skin of dogs, cats, horses and small pet mammals.

Fleas, lice, mites and ticks all cause parasitic disease, either through direct action and/or through the transmission of pathogens.

The diagnosis of skin infestation with smaller ectoparasites such as mites, and the differentiation of isolated specimens, usually require microscopic identification of one or more life stages of the parasite. Diagnostic success depends upon the correct collection of material and, after any necessary processing, examination under a light microscope, preferably with the condenser down and the light source dimmed.

The following section provides descriptions of the most commonly used isolation methods for ectoparasites.

3.2. Flea combing

A “flea comb” is a fine-toothed comb used to collect debris from an animal’s coat.

In the apparent absence of the highly mobile fleas, flea faeces may be detected by combing the animal and placing the collected material onto damp white paper, tissue or cotton wool: the black spots of flea faeces become surrounded by a red ring of undigested blood.

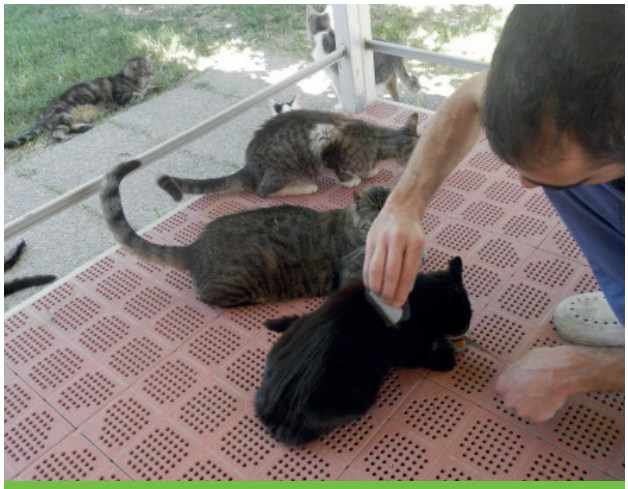

Figure 2: Flea combing

Figure 3: Flea faeces containing blood evidenced by the addition of water

Combing may also be useful for diagnosing *Cheyletiella* and louse infestation. The collected debris can be placed in a Petri dish and examined using a stereomicroscope: mites may be seen walking amongst the debris ("walking dandruff"). Another way of identifying lice or *Cheyletiella* mites is by collecting the dandruff from the comb with sticky tape (see 1.2.12). Native microscopy allows the identification of these parasites or their eggs.

3.3. Skin scrapings

Skin scrapings allow the sampling of the first layers (superficial) or all layers (deep) of the epidermis in order to detect ectoparasites that either remain on the surface of the skin or burrow or reside into deeper structures, like hair follicles. Sampling is performed at the edge of cutaneous lesions or in ectoparasite predilection sites.

Scrapings are performed with a scalpel blade or a curette. Mineral oil should be used on the scalpel blade and skin to allow debris and parasites to adhere to the blade. If clipping is necessary, only scissors should be used, and it is preferable to scrape skin that has not been damaged by excessive scratching.

The sample should be transferred into a transparent plastic tube with a large opening that can be tightly sealed.

Initially, the skin material and the walls of the plastic container can be examined using a stereomicroscope or a magnifying glass, as mites and other arthropods tend to migrate out of the skin material. The collected material can then be smeared on a slide, covered with a coverslip and examined microscopically at 40–100x magnification. Mineral oil (or a drop of glycerol) should also be placed on the slide.

Both superficial and deep skin scrapings should ideally be taken from relatively large parts of the body, bordering the suspected areas.

For **dogs and cats**, scrapings are recommended in order to establish the diagnosis of mange (e.g. *Sarcoptes scabiei*, *Notoedres cati*), demodicosis (*Demodex* spp.), cheyletiellosis (*Cheyletiella* spp.) or chigger infestation (larvae of *Trombicula autumnalis*). In particular:

- In cases of suspected *Demodex canis* infection, it is sensible to begin with a trichogram (see 3.5.): if *Demodex* mites are present and attached to the hairs, it is no longer necessary to perform a skin scraping.
- For *S. scabiei* in dogs, deep skin scrapings should be taken from the elbows, ear margins and lateral hocks. It has been reported that over 50% of scrapings in dogs with sarcoptic mange are negative; therefore, to enhance sensitivity, several samples (6–10) should be taken from various suspected spots on the animal. Alternatively, some laboratories offer ELISAs detecting antibodies against *S. scabiei*, helping to improve diagnosis (see Table 4).
- For *Demodex gatoi* in cats, the same procedure as for *S. scabiei* scrapings in dogs can be adopted.
- For *Cheyletiella* mite detection, the dorsum and ear flaps can be scraped; however, the recommended technique is the adhesive tape method (see 3.4.).
- Skin scrapings will also detect endoparasites in skin, i.e. nematode larvae of *Pelodera*, *Uncinaria*, *Ancylostoma* or promastigote stages of *Leishmania*. Occasionally, *Dirofilaria* microfilariae can also be found. Further biomolecular differentiation should be carried out for species confirmation.

Skin scrapings can also be adopted for the detection of mites occurring on **small pet mammals**.

Deep skin scrapings are recommended for detecting follicular *Demodex* mites which live deep in the hair follicles. For detection of such *Demodex* mites, areas of alopecia should be scraped with a blade or curette covered with mineral oil in the direction of hair growth until capillary blood appears. The skin should be squeezed before or during scraping to promote extrusion of *Demodex* mites from the hair follicles.

Evaluating the numbers and vitality of different life stages is useful for assessing treatment response. While healthy dogs are frequently reported to harbour low numbers of *Demodex*, clinically affected animals usually have a high mite burden and direct diagnosis has a high sensitivity in the latter cases. However, it has been reported that deep scrapings can give false-negatives for *Demodex*, in particular from hard-to-scrape areas (eyes, interdigital spaces) and from some breeds (old English sheepdog, Scottish terrier, Shar-pei). In these cases, trichograms (see 3.5.) may be more useful in yielding enough diagnostic material. Recently, it has been shown that adopting the adhesive tape method (see 3.4.) and squeezing the skin has a higher diagnostic success rate than deep scraping for *Demodex*; this procedure is also less painful for dogs and cats.

In **equines**, deep skin scrapings can be used to detect *Demodex* as well as stages of nematodes (*Pelodera*, *Strongyloides* and *Habronema*).

Superficial skin scrapings

In **dogs**, for the more superficial, short-bodied Demodex mites, superficial skin scrapings can be performed, as an alternative to the adhesive tape method (see 3.4.) and trichogram (see 3.5.).

In **equines**, superficial skin scrapings can be used for the identification of *Chorioptes*, *Psoroptes*, *Sarcoptes* mites, *Trombicula* larvae and forage mites (*Pediculoides ventricosus*, *Pyemotes tritici*, *Acarus farinae*).

Superficial skin scrapings (as well as the adhesive tape method) are also helpful to detect lice.

Processing skin material with potassium hydroxide solution (KOH) or lactophenol

The scraped material can be processed by adding a 10% KOH solution or lactophenol for at least 2–3 hours, frequently overnight, at room temperature. Alternatively, heating up the KOH solution in a suitable container shortens the procedure. The KOH solution and lactophenol macerate the keratin substances of the skin leaving the chitinous exoskeletons of arthropods intact. The suspension can be examined in a Petri dish under a stereomicroscope or on a glass slide under a microscope (40–100x magnification). For accurate identification, the arthropods need to be transferred to a slide and differentiated. Potential arthropods to be identified: biting and sucking lice, mites (*Cheyletiella*, *Trombicula*, *Demodex*, *Sarcoptes*, *Notoedres*).

3.4. Adhesive tape method

A direct impression technique (therefore also called the acetate tape impression method) uses transparent sticky tape (approximately 1 cm wide and 4 cm long) to collect debris from the surface of the skin and hair coat, as described in 1.2.12. The sticky side of the tape is pressed several times onto the skin. Next, it is pressed (also sticky side down) onto a slide. The tape serves as a coverslip (magnification: 40–100x).

Usually, several samples are taken from different areas of the skin. This technique is especially useful for *Cheyletiella* mites and short-bodied *Demodex* mites as a **larger** surface area can be sampled very quickly.

In **equines**, the acetate tape impression method can be used to detect *Chorioptes equi*, *Dermanyssus gallinae* and lice. For follicle-dwelling *Demodex*, the tape can be placed on the selected lesion as an alternative to deep skin scrapings. The skin should then be squeezed under the acetate tape and then the tape affixed to a glass slide.

3.5. Trichogram

A trichogram is the microscopic examination of plucked hair. Positive hair plucks can replace skin scrapings in areas that are difficult to scrape such as the eyelids, peri-ocular area, muzzle or feet, or when lesions are very painful. Forceps are used to forcefully pluck hairs in the direction of hair growth. If possible, skin is squeezed prior to and during sampling. At least 40 (ideally 50–100) hairs are then placed onto a slide with mineral oil and a coverslip and evaluated under low power magnification. In cases of suspected *Demodex* infection, it is sensible to begin with a trichogram: if *Demodex* mites are present and attached to the hairs, it is no longer necessary to perform a skin scraping.

3.6. Mite detection in ear wax

The collection of ear wax from **dogs and cats** often yields excellent results in animals affected by *Otodectes cynotis*. Ear mites can frequently be observed with otoscopic examination and the abundant, dark brown background debris is typical. This debris can be collected either with a cotton swab (when waxy) or with a curette (when dried and crusty) and evaluated under a microscope after mincing with a scalpel blade and being mixed with mineral oil.

The same approach can be used to detect *Psoroptes cuniculi* in **rabbits**' ears, although the amount of debris produced in such an infestation may require digestion with potassium hydroxide (KOH) or lactophenol to release the mites.

3.7. Biomolecular techniques

As for endoparasites, in cases where the morphology of arthropods does not allow their identification, DNA-based techniques may be adopted (see 4.5.). For this procedure, live arthropods can be preserved in 70% alcohol or frozen.

4. MISCELLANEOUS

4.1. Blood smears

4.1.1. General information

Freshly prepared smears made from blood samples collected in an anticoagulant can be used to diagnose a number of parasitic infections. Even though the presence of parasites in the blood can be rare, especially in chronic forms of disease, the ease and rapidity of a blood smear can offer a quick and inexpensive diagnostic approach.

Materials required:

- two slides wiped with alcohol
- blood sample in anticoagulant

Procedure:

1. Place a very small drop of blood near the end of one slide.
2. Place the end of the other slide on the first so that the short-sided edge of the slide is just below the drop of blood.
3. Hold the second slide at an angle of 30°.
4. Push the second slide so that the edge barely touches the drop of blood; by capillary action, a thin line of blood will spread along the edge of the slide.
5. Quickly drag the spreader slide along the entire length of the sample slide in one fluid motion (the smear should end in a "feathered edge").
6. Air dry and stain (Diff Quick).

Air dried slides are fixed in methanol for 5–10 minutes and stained with Giemsa, Diff-Quick, Hemocolor or other commercially available pre-made solutions to enable recognition of the characteristics of the protozoa. It should be noted that stain artefacts from poorly kept stains can lead to false-positive results.

4.1.2. Pathogens detectable in blood smears

Piroplasmids

The protozoa are located within red blood cells. Peripheral capillary blood, taken from the ear pinna or the tip of the tail or a buffy coat preparation (see 4.2.), may yield higher numbers of parasitised cells and a rapid diagnosis of the acute disease is therefore possible when a sick animal is first presented. In chronic cases, parasitaemia is normally low. Based on the size of the merozoites within the erythrocytes, large (>3 µm) and small (<3 µm), *Babesia* or *Theileria* species may be distinguished (see Table 5), but a molecular approach is needed for reliable differentiation as large *Babesia* can present as atypical small forms. Different *Babesia* species can mostly be morphologically differentiated between each other based upon the number of merozoites, the angle between them and their position within the erythrocyte (see Table 5). Case history (time spent in endemic areas, tick infestation), clinical signs and other diagnostic procedures such as seroconversion and DNA detection (see 4.5.) may support the diagnosis.

Some countries impose national regulations for the importation of horses and testing for equine piroplasmosis.

Table 5: Diagnostic features for the morphometric identification of piroplasmid agents in dogs, cats and horses.

For details about pathogenicity, vectors and occurrence see ESCCAP's Guideline 5: Control of Vector-Borne Diseases in Dogs and Cats.

Piroplasmids in dogs			
	Size (merozoites)	**Position in erythrocyte**	**Angle/other**
Babesia canis	large	central	acute
B. vogeli	large	central	acute
B. rossi	large	central	acute
B. gibsoni and *gibsoni*-like	small	central	-
B. conradae	small	central	-
*B. vulpes**	small	central	-
Piroplasmids in cats			
B. felis	small	central	Maltese cross
Babesia spp.	large	central	-
Cytauxzoon spp.	small	variable	rod- or comma-shaped
Piroplasmids in horses			
B. caballi	large	central	acute
Theileria equi	small	variable	pleomorph (1–4 merozoites, including Maltese cross)

* *Babesia vulpes* includes the previously used terms *Theileria*/*Babesia annae* and *Babesia microti*-like.

Hepatozoon spp.

Hepatozoon spp. are vector-transmitted (by oral ingestion of infected ticks) protozoal agents. In regions of Europe with a warm climate, *Hepatozoon canis* is a common parasite in canids, while *Hepatozoon americanum* occurs in canids in the USA. *Hepatozoon felis* and *H. silvestris* are rare cat parasites. On rare occasions, *H. canis* can also be found in cats. Gamonts can be found mainly in neutrophil granulocytes as typical brick-shaped structures in blood smears.

Larvae (microfilariae) of *Dirofilaria* and other filarioid nematodes

Microfilariae of pathogenic (*D. immitis, D. repens*) and apathogenic filarioid nematodes can be found in blood smears and therefore need to be differentiated (see 4.4.).

Rickettsiae (Anaplasmataceae)

Ehrlichia spp. are vector-borne, Gram-negative, obligate intracellular bacteria. In Europe, *Ehrlichia canis* is the aetiological agent of canine monocytic ehrlichiosis (CME). This pathogen infects mainly monocytes wherein the typical, but rarely observed, microcolonies (morulae) develop. *Candidatus Neoehrlichia mikurensis* is the aetiological agent of neoehrlichiosis in dogs. Further *Ehrlichia* spp. (*E. chaffeensis, E. ewingii*) are endemic in the neotropics and rarely identified in Europe. *Ehrlichia canis* or a closely related species has been described in cats but has no veterinary relevance.

Anaplasma spp. are vector-transmitted, Gram-negative, obligate intracellular bacteria. In Europe, *A. platys* have been reported in domestic dogs and *A. phagocytophilum* in dogs, cats, horses and others. They infect platelets (*A. platys*) or predominantly neutrophil granulocytes (*A. phagocytophilum*) respectively and develop into typical microcolonies (morulae) which can be observed by light microscopy in the acute stage.

Mycoplasma spp. (syn. *Haemobartonella* spp.) are small Gram-negative bacteria that attach to the surface of red blood cells, e.g. *Mycoplasma haemocanis* and *M. haemofelis*, in dogs and cats, respectively. Other less pathogenic species have been described mainly in cats: *Candidatus Mycoplasma haemominutum* and *Candidatus Mycoplasma turicensis* but also in dogs: *Candidatus Mycoplasma haematoparvum*. DNA detection methods support detection and enable differentiation.

4.2. Buffy coat method

For this procedure, a microhaematocrit tube is filled with EDTA blood, sealed at one end and centrifuged in a haematocrit centrifuge for 5 minutes. Trypanosomes and microfilariae accumulate in the zone between the buffy coat layer and plasma. Leucocytes are concentrated for *Anaplasma phagocytophilum*, *Ehrlichia* spp. or *Hepatozoon* spp. As *Babesia* spp. infect reticulocytes rather than mature erythrocytes, their detection in the buffy coat is more sensitive than in whole blood. For microscopic examination, the haematocrit tube is scratched with a small glass cutter at the level of the buffy coat and broken so that the buffy coat layer and plasma can be transferred to a slide. A drop of physiologic saline is added, or a stained smear can be performed to visualise the parasites.

4.3. Fine needle aspirates

4.3.1. General information

Some protozoa and vector-borne pathogens are intracellular obligates of lymphocytes/monocytes/macrophages. Thus, aspiration of haematopoietic tissues (e.g. lymph nodes, bone marrow, spleen) can be important for both their morphological identification through smears and/or collection of material for biomolecular analysis (see 4.5.). Fine needle aspirates from inner organs, e.g. liver, can also be used for the diagnosis of infections with metacestodes of *Echinococcus* spp. (alveolar and cystic echinococcosis). If the spleen is used for this purpose, the aspirate should be performed without negative pressure in order to avoid bleeding.

Materials required:

- 15 ml syringe
- 22–20 gauge needle
- Glass slides

Procedure:

1. Insert the needle into the tissue and create negative pressure within the syringe by pulling back the plunger to 5–10 ml.
2. Maintain this negative pressure, redirect the needle in several directions without removing the tip of the needle from the tissue.
3. Release the syringe plunger and remove the needle from the tissue (do not remove the needle from the tissue without releasing the plunger as all collected material will be sucked into the syringe).
4. Disconnect the syringe from the needle and fill it with air.
5. Reconnect the syringe to the needle and use the air to blow the sample out of the needle onto one or more glass slides.
6. Smear, dry and stain as for blood smear (see 4.1.).

4.3.2. Pathogens detectable in fine needle aspirates

Leishmania infantum

Lymph node aspirates, especially from animals with lymphadenopathy, are most convenient while bone marrow and spleen sampling is more invasive but may be indicated for infected clinically healthy dogs.

Amastigotes, with the typical "T" shaped nucleus/kinetoplast structure, can be seen within the cytoplasm of monocytes/macrophages and also free within the smeared material.

In cases where limping is present, amastigotes can be observed in fine needle aspirates of synovial fluid.

Aspirates from cutaneous lesions is also a feasible way to detect amastigotes infection in macrophages of *Leishmania* by cytology.

Ehrlichia canis

Sensitivity of microscopic detection can be higher in fine needle aspirates of lymph nodes, bone marrow or spleen or in buffy coat preparations compared to blood smear, but currently DNA detection by PCR is the recommended method for diagnosis.

4.4. Detection and identification of microfilariae

4.4.1. General information

The diagnosis of infection by filarioid nematodes of companion animals can be carried out through the isolation and morphological identification of circulating microfilariae. Blood samples should be examined after concentration by the Knott's test or with a filter method (commercial test kit: Difil-Test®). Wet blood smears do not allow species identification and have a very low sensitivity.

It is important to point out that the number of circulating microfilariae does not necessarily correlate with the adult worm burden, i.e. highly microfilaraemic dogs may harbour few adult worms. Also, the time of blood sampling may influence the sensitivity for detecting microfilariae: for both *D. repens* and *D. immitis*, a trend for higher microfilaraemia during night hours is described.

Microfilariae can be differentiated by morphometric measurements, by acid phosphatase staining or, especially in doubtful cases, by biomolecular methods allowing species diagnosis through DNA isolation from whole blood and concentration of microfilariae by centrifugation. There are some limitations in the morphological identification of circulating microfilariae. This includes the influence of different fixation techniques on the size of the larvae, poor staining with the acid-fast technique in an aliquot of larvae in a sample, and co-infections with a disproportionate number of larvae from different species. Species-specific (real-time) PCR is the method of choice for species differentiation.

Finally, even though heartworm infection (*D. immitis*) can be reliably diagnosed by serology, it is always recommended to test for circulating microfilariae.

In cats that are amicrofilaraemic or negative in antigen detection but show a clinical picture compatible with dirofilariosis, antibody detection is an alternative to demonstrate infection.

4.4.2. Knott's test

This method concentrates all of the microfilaria present in 1 ml of EDTA blood by centrifugation. These are then stained with methylene blue.

Briefly, 1 ml of EDTA-blood is diluted in 9 ml of 2% formalin and the solution agitated for several minutes. It is then centrifuged at 390 x *g* for 5 minutes. The supernatant is removed and one drop of 1% methylene blue is added to the sediment for morphometric analyses or examined for acid phosphatase activity, although the common procedure for microfilariae identification is currently biomolecular (see 4.4.1.). Slides are examined at 40x magnification for the presence of larvae, and at 100x magnification for morphological features. For maximum sensitivity, the whole sediment should be analysed.

4.4.3. Modified Knott's test

Alternatively, living microfilariae can be detected more rapidly by their movement when a modified Knott's test is used. For this, four drops of 2% saponin solution and 5 ml of distilled water are added to 1 ml EDTA blood to destroy the erythrocytes. The suspension can be examined under a microscope, as above. Living larvae are motile and can easily be detected microscopically (40x magnification).

4.4.4. Filtration method (Difil-Test®)

This commercial kit concentrates microfilariae from 1 ml of EDTA blood onto a filter that has an area about the size of a small coverslip. With this technique, the microfilariae tend to be straight rather than coiled or wavy in silhouette.

The kit includes lysing solution, stain, filters and a cartridge. Briefly, the procedure is as follows:

- The filter is placed on the base of the filter cartridge and the cartridge is assembled.
- 1 ml of blood is added to 9 ml of lysing solution in a large syringe and mixed together. The haemolysed blood solution is forced through the filter by attaching it to the port on the filter cartridge.
- The filter is rinsed by forcing an equal volume of water through the port on the filter cartridge.
- The filter cartridge is opened, the filter removed and is placed on a microscope slide.
- A drop of stain, provided in the kit, is placed on the filter.
- A coverslip is placed over the filter to disperse the stain and the slide is ready for viewing.

4.4.5. Acid phosphatase staining

There are several distinguishing features that can differentiate the microfilariae of *D. immitis* from those of *D. repens* (and from apathogenic filarioid nematodes). This is particularly important in areas where both *D. immitis* and *D. repens* are present (see ESCCAP's Guideline 5: Control of Vector-Borne Diseases in Dogs and Cats).

The microfilariae of different filarioid nematodes can be distinguished by their acid phosphatase staining patterns (see Table 6):

- *D. immitis* microfilaria displays two spots (excretory and anal pores);
- *D. repens* microfilaria has only one spot (anal pore);
- *Acanthocheilonema dracunculoides* microfilaria shows three areas of enzymatic activity (anal pore, internal body, excretory pore); and
- *A. reconditum* microfilaria shows a diffuse light red pattern.

Commercial kits are available that can be carried out in a laboratory. However, staining methods are gradually being superseded by DNA isolation from concentrated microfilariae.

4.4.6. Microfilariae measurements

Alternatively, microfilariae measurements can help to differentiate them: for this, mean length should be based on the size of 10 larvae (see also ESCCAP's Guideline 5: Control of Vector-Borne Diseases in Dogs and Cats). It should be noted that the preparation (especially the fixation technique) can considerably alter the length and features of the microfilariae.

Table 6: Morphological differentiation of microfilariae[1] according to length, width and shape

Species	Length (µ)	Width (µ)	Features
Dirofilaria immitis	301.77 ± 6.29 290–330	5–7	No sheath, cephalic end pointed, tail straight with the end pointed. APh-S: two activity spots located around the anal and the excretory pores.
D. repens	369.44 ± 10.76 300–370	6–8	No sheath, cephalic end obtuse, tail sharp and filiform often ending like an umbrella handle. APh-S: one spot around the anal pore.
Acanthocheilonema reconditum	264.83 ± 5.47 260–283	4	No sheath, cephalic end obtuse with a prominent cephalic hook, tail button hooked and curved. APh-S: activity throughout the body.
A. dracunculoides	259.43 ± 6.69 190–247	4–6.5	Sheath, cephalic end obtuse, caudal end sharp and extended. APh-S: three spots which include an additional spot in the medium body.

[1] Microfilariae (n=10) measured after concentration by the Knott's test; when using the Difil® test, lengths are shorter. APh-S: acid phosphatase stain.

4.5. Biomolecular detection and differentiation methods: polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP)

4.5.1. General information

The polymerase chain reaction (PCR) is an increasingly popular and powerful laboratory method for the diagnosis of many conditions. It is specific and, depending on the test substrate and stage of infection, it is also highly sensitive and can assist the practitioner in diagnosing active infections when clinical signs are suspect. It is indicated for vector-borne pathogens and parasites that require sensitive detection and/or differentiation beyond the range of morphology.

If PCRs are used for diagnostic and not differentiation purposes, sensitivity will, among other factors, depend upon the copy number of the target gene.

PCR is only carried out in specialised laboratories. Accurate results therefore depend upon correct collection, preservation and posting of samples. Using two different PCR protocols can increase sensitivity and specificity.

General guidance includes:

- As PCR is very sensitive, cross-contamination of samples must be avoided. Samples must be taken with, and stored in, single-use equipment wherever possible.
- Samples should be kept refrigerated (4°C) and posted with ice packs or continuously frozen at -20°C or colder.
- Samples should arrive in the laboratory no later than 3 days after collection.

Loop mediated isothermal amplification (LAMP) is a single tube technique for the amplification of DNA. Due to its simplicity and low costs, LAMP is already used by clinicians to detect certain pathogens, either as a simple screening assay in the field or at the point of care. Reagents/kits are commercially available from some companies. In contrast to PCR technology, in which the reaction is carried out with a series of alternating temperature steps or cycles, isothermal amplification is carried out at a constant temperature and does not require a thermal cycler. LAMP has been observed to be more resistant than PCR to inhibitors in complex samples such as blood or faeces, possibly due to the use of a different DNA polymerase (typically Bst – *Bacillus stearothermophilus* – DNA polymerase rather than Taq polymerase as in PCR).

DNA extraction can be carried out on virtually any substrate, but blood and tissue aspirates are the most frequently analysed samples.

Blood:
Several vector-borne pathogens such as *Ehrlichia*, *Anaplasma*, *Bartonella* and *Babesia* can be diagnosed by identifying their DNA in peripheral blood. PCR has also been used to detect and differentiate the filarioid species that release microfilariae into the bloodstream.

There are many PCR protocols for blood and each laboratory has its own, including the best anticoagulant to use. Most prefer EDTA, but it is best to check with the laboratory of choice. A maximum of 0.5–1 ml of blood will usually suffice.

Tissue aspirates:
Leishmania infantum and *Ehrlichia* spp. are present in lymphoid organs and fine needle aspirates can be used for PCR testing. Skin biopsies can also be taken for PCR for *Leishmania*. Following aspiration, the aspirate can be squirted onto a filter paper or cotton swab that can then be preserved in a sterile test tube at 4°C. Smearing material onto glass slides has been shown to have lower sensitivity.

Parasites:
There may be occasions where molecular identification of individual parasites is necessary (helminths, arthropods). These should be preserved in 70% ethanol and shipped in air-tight containers.

Faeces:
Faecal samples are also directly used for detection of DNA parasitic stages. As an example, *Tritrichomonas foetus* detection in faeces from cats is actually recommended over accumulation by faecal culture (see 1.2.13.). Currently copro-PCR is used for *E. multilocularis*, different coccidia (i.e. *Toxoplasma*/*Hammondia*), *Cryptosporidium* and *Giardia*.

For parasites that require differentiation of species or genotypes that cannot be achieved morphologically, e.g. for *Giardia duodenalis*, *Cryptosporidium* spp., oocysts of *Toxoplasma*/*Hammondia*, ascarid or taeniid eggs, genotyping (on faeces or isolated parasites) can be helpful to determine zoonotic species or genotypes etc. In certain cases with low numbers of parasitic stages, or where DNA extraction is challenging (i.e. for taeniid or ascarid eggs, see below), parasitic stages in faeces should ideally be previously concentrated. As an example, the flotation and sequential sieving method can be adopted (see below).

Flotation and sequential sieving:
Helminth eggs can be accumulated from faecal samples by combining flotation in zinc chloride solution followed by sequential sieving using sieves with decreasing mesh sizes (21–200 µm). Therefore, using the appropriate mesh size of the last sieve, eggs (in particular taeniid eggs and *Toxocara* eggs for differentiation) can be concentrated allowing i.e. DNA isolation.

4.5.2. Specific information

In principle, all relevant parasites can be detected or confirmed by PCR or other genetic methods. Several machines offering contemporaneous automated detection of infectious agents are already on the market but are not validated in veterinary medicine.

In literature, depending on the parasite, the following options may be available:

- specific primers for distinct parasite species replicating only the targeted genus and/or species;
- multiplex PCR combines different (specific) primers;
- traditional PCR vs. real-time PCR; and
- LAMP.

When a PCR product is obtained, the product can be identified based on its size by gel electrophoresis (with or without previous fragmentation by restriction enzymes), or by sequencing the nucleotide sequences and comparing them with available gene sequences.

Interpretation:
All PCR results should be interpreted as parts of a combination of clinical criteria and other diagnostic tests.

Some examples, without claim for completeness:

Protozoa

Leishmania infantum

PCR can be used for both the diagnosis of infection and the monitoring of dogs undergoing treatment for canine leishmaniosis. Several laboratories also offer quantitative PCRs (real-time, etc.) which can indicate the amount of parasite and the increase/decrease in parasite load. These are also useful for monitoring response to treatment.

Even though tissue aspirates (lymph nodes, spleen, bone marrow) or skin samples (in cases of skin lesions) are the samples of choice for *L. infantum*, laboratories will also carry out analyses on blood. There are some concerns over false-negatives (no white cells in the blood harbouring the parasite) due to low sensitivity, although sensitivity of real-time PCRs targeting multicopy genes seems to be satisfactory. Non-invasive samples like conjunctival swabs are now also recommended. For this purpose, both eyes should be sampled and the swabs should contain a sufficient number of cells.

Babesia

The sensitivity of PCR has proven higher than blood smear examination, especially for the diagnosis of chronically infected dogs. However, false-negative results cannot be completely excluded. Identification of species and subspecies can be important in terms of treatment options (large vs. small *Babesia*) and prognosis.

Toxoplasma gondii

Toxoplasma gondii oocysts excreted by cats can barely be morphologically differentiated from those of the apathogenic *Hammondia hammondi* in routine diagnostics. Together with serological procedures differentiating specific antibodies in sera of cats and intermediate hosts, PCR can be performed from oocysts or for identification of *T. gondii* bradyzoite cysts in meat. In dogs and/or cats with neurological disorders compatible with toxoplasmosis, PCR from cerebrospinal fluid or aqueous humour can be carried out.

Neospora caninum

Oocysts of *N. caninum* are excreted for very short periods and in low density in dog faeces. As with *T. gondii*/*H. hammondi*, the oocysts of *N. caninum* can hardly be distinguished from *Hammondia heydorni* (occurring in faeces from dogs or foxes) in routine diagnostics and *T. gondii*/*H. hammondi* (in case of coprophagia of cat faeces). Therefore, a PCR analysis is indicated for their identification.

In clinically suspect dogs, a serological analysis together with muscle biopsies, liquor and/or cerebrospinal fluid by PCR are diagnostic options.

Giardia

Classical tests for *Giardia* infections are normally not species-specific. If, for clarification of the zoonotic potential, the assemblage needs to be determined, multilocus PCR and sequencing can be performed.

Tritrichomonas

A PCR, distinguishing *T. foetus* in cat faeces from other trichomonads, overcomes parasite detection in culture (InPouch®, see 1.2.13.) and its drawbacks (temperature sensitive, time).

Bacteria transmitted by arthropod vectors

Bartonella

Even though the gold standard for the diagnosis of bartonellosis is blood culture, this can be time consuming and expensive. It is also possible to detect *Bartonella* DNA in samples of blood, tissue, cerebrospinal fluid or aqueous humour. A combination of enrichment culture followed by PCR amplification results in increased sensitivity.

Ehrlichia/*Anaplasma*

A positive PCR result generally confirms the presence of an infection by these tick-borne bacteria. However, a negative PCR result does not exclude the presence of an infection.

As with blood cytology, sensitivity appears to be greater for *Ehrlichia* PCR when performed on lymph nodes, spleen, bone marrow aspirates or buffy coats.

Helminths

Taeniid eggs

In *Echinococcus* endemic areas it is recommended to differentiate *Echinococcus* spp. from other taeniid eggs in order to clarify the potential for zoonotic transmission. Eggs can be isolated by flotation, the adhesive tape technique of the perianal area or by flotation and sequential sieving of faeces, assuming biosafety rules (equipment in specialised labs). PCR is performed with specific primers, by multiplex PCR targeting different taeniid species, or by broad helminth PCR followed by sequencing.

Toxocara

It has been shown that approximately one third of dogs positive for *Toxocara* are excreting *Toxocara cati* eggs (following coprophagia). These eggs can be differentiated from *T. canis* eggs by egg measurements or more reliably by PCR.

Lungworms

Aelurostrongylus abstrusus is the most frequently occurring cat lungworm. In some countries, however, other lungworms such as *Troglostrongylus* spp. and *Oslerus rostratus* may be present and expertise is required for morphological differentiation of L1. Alternatively, PCR can be performed from L1 isolated from faeces, or by analysis of tracheal swabs or bronchoalveolar lavage (lower sensitivity).

The L1 of the most frequent lungworms found in dogs, *A. vasorum* and *C. vulpis*, can be easily differentiated morphologically. However, on single occasions it may be helpful to use biomolecular methods for confirmation.

Capillaria

Dogs, cats and other mammals may harbour different *Capillaria* species, which have different life cycles. Not all are taxonomically definitively attributed. PCR may help to identify the species.

Filarioid nematodes

Species-specific (real-time) PCR from EDTA blood is the method of choice for species differentiation of microfilariae (see 4.4.1.), although morphological analysis and acid phosphatase staining may still be in use.

Aberrant helminths

When aberrant helminths are morphologically indistinguishable or need to be extracted from histologically fixed samples, PCR can be performed, also from histological staining or skin scrapings.

Arthropods

In cases where the morphology of arthropods precludes their identification, DNA-based techniques may be adopted.

Dermatophytes

A commercially available panel includes *Microsporum* spp., *M. canis* and *Trichophyton* spp. real-time PCR tests and it performs with high sensitivity and specificity. Skin scrapings and/or hairs should be collected and sent to the laboratory. The results are available in a few days.

4.6. DERMATOPHYTE DETECTION

4.6.1. General information

Dermatophytosis is diagnosed by a combination of different and complementary tests, including Wood's lamp (for dogs and cats only) and direct examination to document active hair infection, mycological culture to identify the fungal species involved and monitor response to therapy, and sometimes skin biopsy for nodular or atypical presentations. The detection of dermatophytes by PCR is now possible for dogs and cats in Europe (see 4.5.2.).

4.6.2. Direct examination of hairs

Dermatophytoses may be detected by the microscopic examination of hairs. However, the sampling technique and expertise of the examiner strongly influence the sensitivity of this procedure. In addition, despite optimal sampling and examination, false-negative trichograms (see 3.5.) cannot be excluded. Therefore, only a positive trichogram is meaningful. Potassium hydroxide (KOH) or mineral oil with or without the addition of stains (lactophenol cotton blue for example) can be used to better detect fungal elements. Hairs infected by dermatophyte fungi usually present as enlarged and swollen structures with a rough and irregular surface. The hair surface typically demonstrates clusters or chains of fungal spores (arthroconidia) (2–4 µm for *Microsporum canis*).

4.6.3. Wood's lamp examination

Examination of the hair coat with an ultraviolet lamp (Wood's lamp) is a good screening method for dermatophytosis in dogs and cats. When exposed to the light, hairs invaded by some dermatophyte species, including *M. canis*, glow green. Hairs infected by other dermatophyte species never fluoresce and some topical medications may mask fluorescence. Thus, negative results following Wood's lamp examination do not rule out dermatophytosis. The observation of fluorescence should be confirmed by microscopic examination of hairs (even though the recognition of infected hairs is not always easy and may require an experienced eye) (see 4.6.2.).

4.6.4. Mycological culture

Mycological culture remains the most reliable technique for confirming dermatophytosis in dogs and cats. Sample collection may be obtained through skin scrapings, plucking hairs (under Wood's lamp) or brushing the hair coat with a sterile toothbrush, a little piece of sterile carpet or a dust-catching cloth. Several media (like Sabouraud dextrose agar) are suitable for mycological cultures. Colonies of dermatophyte species such as *M. canis* may develop in a few days. Dermatophyte test media (DTM) are regularly used in veterinary medicine. However, only very few attempts have been made to evaluate the performance of such media with samples collected from animals and the use of DTM alone without microscopic identification of macroconidia is not recommended for the diagnosis of animal dermatophytoses. Ideally, the material collected from the animals should be sent to a laboratory with an expertise in veterinary mycology. In the laboratory, specific identification is made by microscopic examination of the fungal colonies. The number of colonies may help to distinguish between mechanical carriers and infected animals. Mechanical carriage is due to the contamination of/from the environment and is usually associated with a limited number of dermatophyte colonies in culture. Infection leads to a massive production of fungal spores (arthroconidia) and is usually associated with a very high number of dermatophyte colonies in culture.

APPENDIX 1 – BACKGROUND

ESCCAP (European Scientific Counsel Companion Animal Parasites) is an independent, not-for-profit organisation that develops guidelines and promotes good practice for the control and treatment of parasites in companion animals. With the proper advice, the risk of diseases and parasitic transmission between animals and humans can be minimised. ESCCAP aspires to see a Europe where companion animal parasites no longer threaten the health and wellbeing of animals and humans.

There is a great diversity in the range of parasites and their relative importance across Europe and the ESCCAP guidelines summarise and highlight important differences which exist in different parts of Europe and, where necessary, specific control measures are recommended.

ESCCAP believes that:

- Veterinarians and pet owners must take measures to protect their pets from parasitic infections.
- Veterinarians and pet owners must take measures to protect the pet population from risks associated with travel and its consequent potential to change local parasite epidemiological situations through the export or import of non-endemic parasite species.
- Veterinarians, pet owners and physicians should work together to reduce the risks associated with zoonotic transmission of parasitic diseases.
- Veterinarians should be able to give guidance to pet owners regarding risks of parasite infection and diseases and measures which can be taken to minimise these risks.
- Veterinarians should attempt to educate pet owners about parasites to enable them to act responsibly not only for their own pet's health but for the health of other pet animals and people in their communities.
- Veterinarians should, wherever appropriate, undertake diagnostic tests to establish parasite infection status in order to provide the best possible advice.

To achieve these objectives, ESCCAP produces:

- Detailed guidelines for veterinary surgeons and veterinary parasitologists
- Translations, extracts, adaptations and summarised versions of guidelines which address the varied requirements of European countries and regions

Versions of each guideline can be found at www.esccap.org

Disclaimer:

Every effort has been taken to ensure that the information in the guideline, which is based on the authors' experience, is accurate. However, the authors and publishers take no responsibility for any consequence arising from the misinterpretation of the information herein nor is any condition or warranty implied. ESCCAP emphasises that national, regional and local regulations must be borne in mind at all times before following ESCCAP advice.

APPENDIX 2 – GLOSSARY, ABBREVIATIONS AND USEFUL LINKS

GLOSSARY

Sensitivity	The rate of true-positive samples (as judged by a "Gold Standard" test) or the likelihood that an infected animal can be detected as positive by the test.
Sensitivity [%]	True-positive samples/(True-positive samples + False-negative samples)*100.
Specificity	The rate of true-negative samples (as judged by a "Gold Standard" test) or the likelihood that an uninfected animal can be detected as negative by the test.
Specificity [%]	True-negative samples/(True-negative samples + False-positive samples samples)*100.
Direct detection methods	Detect the presence pathogen itself, either by visualisation (e.g. by microscopy), by detecting genetic material (e.g. PCR or LAMP) or by detecting molecules specific to the parasite in question (e.g. antigen, usually composed of proteins in different variations).
Indirect detection methods	Serological methods which determine previous contact to parasites by detection of specific antibodies induced by infection. As antibodies are usually produced some time after initial infection and prevail after parasite clearance, they do not necessarily indicate the presence of active infections. Antibody detection can be performed in different formats, e.g. Western blotting, direct or indirect immunofluorescence assays, ELISA, lateral flow assays and others. Detection is accomplished by the use of labelled secondary antibodies. Many, but not all, of these tests allow for a quantification of antibody levels (titres) e.g. for follow-up studies during the course of infection or treatment.

ABBREVIATIONS

CME	Canine monocytic ehrlichiosis
DFA	Direct immunofluorescence assay
DTM	Dermatophyte test medium
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPG	Egg count per gram
FECRT	Faecal egg count reduction test
ICT	Immunochromatographic dipstick test
IFAT	Indirect immunofluorescence antibody test
KOH	Potassium hydroxide solution
L1	First stage larvae
L3	Third stage larvae
LAMP	Loop mediated isothermal amplification
LPG	Larvae per gram
MIFC	Merthiolate-iodine-formaldehyde concentration
OPG	Oocyst count per gram
PCR	Polymerase chain reaction
SAF	Sodium acetate-acetic acid-formalin
SAFC	Sodium acetate-acetic acid-formalin concentration

USEFUL LINKS

How to measure with a microscope:
www.youtube.com/watch?v=_CkcYrns-6I
www.youtube.com/watch?v=_eu9OrNM_wY
www.youtube.com/watch?v=GdRLPAo_j1Y
parasitology.cvm.ncsu.edu/vmp930/m_keys.html

The modified McMaster egg counting technique:
youtu.be/rkSGe-L4Sec

Images of parasite stages can be found on the following pages:
www.parasite.org.au/para-site/contents/toc.html
www.ksvdl.org/resources/news/diagnostic_insights_for_technicians/august2019/common-intestinal-parasites-pt1.html
www.ncvetp.org/parasite-image-database.html
quizlet.com/64375340/parasites-in-veterinary-medicine-flash-cards/

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