

## DIRECT DETECTION OF PARASITES

Indisputable evidence of infection is obtained when the parasites themselves are recovered from clinical samples. A variety of techniques have been developed to find, recover and identify parasites from host material, some involving little more than visual examination of hosts or host material, but most involving microscopic examination of host tissues or concentrates and comparative morphometry.

### Parasite size

Parasites range greatly in size - covering seven orders of magnitude. Some adult tapeworms are several metres in length ( $10^1$  m) while some protozoa are only several micrometres (= microns) in size ( $10^{-6}$  m = 1  $\mu$ m). As a rough guide to the sizes of parasites, protozoan (oo)cysts are measured in  $\mu$ m, helminth eggs (nematodes, cestodes, trematodes) in tens of  $\mu$ m, and arthropod eggs in hundreds of  $\mu$ m. The adult stages of metazoan parasites grow to larger sizes, adult arthropods ranging from millimetres to centimetres, and adult helminths from millimetres to metres. In comparison, bacteria are an order of magnitude smaller than protists (measuring only microns) while viruses are another two orders of magnitude smaller still (measuring only nanometres =  $10^{-9}$  m).

Comparative sizes of parasites

Parasite assemblage	Approximate size range	
	(oo)cysts/eggs	trophozoites/adults
protozoa	4-50 $\mu$ m	5-100 $\mu$ m
cestodes	40-80 $\mu$ m	1 cm – 10 m
nematodes	50-100 $\mu$ m	1 mm – 1 m
trematodes	20-150 $\mu$ m	1 mm – 5 cm
mites	150-250 $\mu$ m	0.3-0.6 mm
lice	300-600 $\mu$ m	2-4 mm
fleas	400-600 $\mu$ m	1-2 mm
ticks	400-600 $\mu$ m	0.2-2 cm
flies	800-1,200 $\mu$ m	0.5-2 cm

### Systems of magnification

Despite considerable size variation between parasites, most developmental stages used for diagnostic purposes are microscopic in size (comprising cellular and subcellular features), thus parasitologists need to develop good microscopy skills. Low power magnification of specimens can be made using hand-held magnifying lenses giving up to 10 times magnification, medium power magnification is achieved using dissecting microscopes whereby eyepiece and objective lenses give from 8-40 times magnification, and high-power magnification is provided by compound (dual-lens imaging) light microscopes where eyepiece and objective lenses give from 40-1,000 times magnification. Most eyepiece lenses provide 10 times magnification and the most commonly used objective lenses provide a further 4, 10-, 20-, 40- or 100-times magnification. All magnifications can be achieved using dry objectives (air between specimen and objective lens), except 1,000 times which can only be achieved using oil immersion (high refractive index of viscous oil between specimen and objective lens prevents light scatter and facilitates image capture). However, the higher the magnification of the objective lens, the smaller the working distance (WD) between the lens and the specimen (e.g. 10 times lens WD ~ 6.8 mm; 40 times lens WD ~ 0.73 mm; 100 times lens WD ~ 0.12 mm). This means that focussed images at high power magnifications can only be achieved with small flat specimens that can be positioned close to the objective lens (usually under thin coverslips). Optical resolution is defined as the smallest distance between two points on a specimen that can still be distinguished by the observer as separate entities. The human eye has a resolution limit of around  $10^{-4}$  m (= 0.1 mm). In light microscopy, the resolving power of each objective lens (i.e. the size of the smallest object that can be seen clearly) can be calculated using the formula  $RP = \lambda/(2NA)$ : where  $\lambda$  = light wavelength (usually set at 550 nm (halfway point between limits of visible light)); and NA = the numerical aperture of each lens (given by the manufacturer on the lens barrel). For the 10 times lens, the NA is usually 0.25 so the  $RP = 550/(2 \times 0.25) = 1,100$  nm = 1.1  $\mu$ m. For the 40 times lens, the NA is usually 0.65 so the  $RP = 550/(2 \times 0.65) = 423$  nm = 0.42  $\mu$ m. For the 100 times lens, the NA is usually 1.25 so the  $RP = 550/(2 \times 1.25) = 220$  nm = 0.22  $\mu$ m. In comparison, electron microscopes have a resolution limit of around  $10^{-10}$  m (= 0.1 nm = 0.0001  $\mu$ m).

## **Parasite appearance**

Most endoparasite developmental stages have little in the way of natural colour, most appearing opaque or translucent. Some appear coloured, however, by virtue of ingested host material (e.g. blood-sucking parasites often have a red gut) or because they contain pigmented internal elements. While the adult stages of many ectoparasites can be pigmented (sometimes elaborately), their immature developmental stages can often be quite inconspicuous. Parasites are therefore difficult to see in host material because they blend in with their surroundings. The visibility of parasites can be improved considerably either by staining the parasites or by introducing optical contrast into the specimen. Many histochemical staining techniques have been used to differentially stain parasite developmental stages in smears or sections, usually by darkly staining parasite cell nuclei and lightly staining their cytoplasmic contents (e.g. Giemsa, haematoxylin, Feulgen, fuchsin, Ziehl-Neelsen, acid-fast, methyl-green, Gentian/methyl-violet, iodine, Indian ink, nigrosin, carmine, Masson's trichrome, silver impregnation, etc.). When examined under a light microscope, optimal bright-field white-light illumination systems are used to provide visual acuity. More recently, a range of fluorochrome stains have been employed to detect parasites by their fluorescence when viewed under ultra-violet illumination (e.g. fluorescein isothiocyanate, Texas red, diamidino-phenylindole, acridine orange, SYBR green, ethidium bromide, SYTO blue/red fluorescent dyes, etc.). If unstained specimens are examined under the light microscope, suboptimal illumination (condenser down, diaphragm partially closed) or special illumination (dark-field, phase-contrast or differential interference-contrast) may be used to provide contrast by light diffraction or refraction. The developmental stages of many parasites often have refractile properties, especially in their external coverings (membranes, cuticle, tegument, etc.) and in any internal globular contents.

## **Parasite identification**

When parasite developmental stages are found, they are usually identified by comparing their morphological characteristics to documented records. This is the conventional taxonomic approach to specific or generic identification. The level of identification required to implement an appropriate course of treatment for parasitic disease varies greatly, sometimes requiring the causative species (or even strain) to be identified, but often only requiring the genus or family to be identified (e.g. the diagnosis of gastrointestinal nematodes is often sufficient to commence a course of anthelmintic treatment). Many morphotypic characters have been described for parasites, some being qualitative (presence/absence), some quantitative (size) but most being combinations of features (shape, appearance). Many generic characters relate to whole organismal features, such as body plan (tissues/organs/cells), motility, feeding, attachment, appendages, encystment, etc. Most species-specific characters involve progressively smaller features (internal structures, organs/organelles, adornments, etc.). After several centuries of conventional morphometric taxonomy, numerous taxonomic keys have been developed for the identification of parasite species. Most have been published as dichotomous taxonomic keys requiring the user to make a series of binary choices about different character states. Unfortunately, many keys require comprehensive knowledge of specialist terminology and they are often based on well-preserved and well-prepared specimens (both of which may be lacking). More recently, moves have been made to produce computerized taxonomic keys which are random-access (no fixed starting point), polychotomous (multiple choice), annotated (glossaries and illustrations provided) and multi-media (combining text, tables, figures, photos, videos, etc.). Irrespective of the resources used, diagnosticians still rely heavily on their learning and experiences with many different parasite groups. Conventional parasitological courses must therefore be preserved in undergraduate and postgraduate university programs to provide the necessary theoretical and practical expertise required by practitioners.

Diagnostic laboratories are quick to adopt new tests based on contemporary technologies. Over the last decade, numerous immunological and molecular biological techniques have been applied to the detection of specific host proteins (notably enzymes and immunoglobulins) as well as parasite antigens and nucleic acids. In particular, enzyme immunoassays and polymerase chain reactions have revolutionized diagnostic technologies, but many tests are still only available with research institutions and have yet to be adapted for use in diagnostic laboratories, medical or veterinary clinics or under field conditions. Naturally, the diagnostic accuracy, sensitivity and specificity of many of these tests remain to be determined before their commercial application. Modern biotechnology, nanotechnology and translational research endeavours are constantly inventing some very clever technologies which hopefully will make these new tests more available in the future (dot/spot technologies, new generation immuno-diffusion, immuno-magnetic separation, immuno-electrophoresis, novel substrates, etc.). Nonetheless, despite the rhetoric and promise of new technological advances, diagnostic parasitology should remain an integrated multidisciplinary exercise, where clinicians make decisions based on holistic evidence (including presentation, history, physical examination and laboratory findings).

## MACROSCOPIC EXAMINATION

Visual examination of the patient is a key part of the physical examination process and should be conducted on all patients irrespective of presentation. While it may only reveal parasites that are large enough to be seen by the naked eye, it also serves to provide information about the possible site, extent and severity of infection through observation of clinical signs, such as body condition, coat condition, suspect lesions, etc. Indeed, signs of inflammation have long been known to assist in disease diagnosis (the four cardinal signs being *rubor* = redness, *calor* = heat, *tumor* = swollen, *dolor* = pain). The detection of visible parasites depends to a large extent on the sites of infection that can be examined. Ecto-parasitic arthropods (fleas, lice, flies, ticks, but not mites) may be found on external body surfaces hidden in hair, plumage or orifices, and sometimes in clothing or bedding. Looking for attached or embedded stages can be time-consuming, but cursory examination can have severe consequences (e.g. missing paralysis ticks on dogs can prove fatal). Often, finding ecto-parasites can be fortuitous, but it should not preclude a more detailed and systematic examination, particularly of predilection sites if specific parasites are suspected. Attention should also be paid to host products, such as excreta. Tapeworm segments or whole nematodes may occasionally be detected in or on fresh faecal samples, and whole worms may rarely be found in vomitus or sputum samples. Most endo-parasitic helminths detected by visual examination are adult life-cycle stages, as their eggs and larval stages are usually microscopic. Protistan parasites are unicellular and therefore microscopic in size, but occasionally feeding trophozoites or encysted stages may be macroscopic in size (e.g. white spot disease of fish caused by the ciliate *Ichthyophthirus*). The majority of parasites detected by visual examination may appear to be robust, but they degrade quite quickly when removed from the host and they can assume unnatural appearances unless properly preserved and processed.

### Specimen preparation

In order to avoid preparative artefacts, macroscopic parasites should be recovered and processed according to standard protocols which have been developed for different parasitic groups. Larger rotund parasites (flies, bugs, ticks) may be prepared using entomological techniques designed to preserve and present whole organisms for macroscopic examination (often aided by magnifying glasses) of specimens pinned on boards, while smaller and/or flatter parasites (small arthropods, tapeworms, flukes, some nematodes) may be processed using histological/histochemical techniques designed to show internal features by microscopic examination (dissecting or compound microscopy) of specimens mounted on slides.

**Removal/recovery:** Individual ticks can be carefully removed with fine forceps (using slow lever action to avoid ripping out mouthparts), fleas can be pursued and picked out of the coat, lice can be picked out or combed out, adult flies can be trapped, fly larvae can be teased or excised from lesions, suspect skin lesions can be scraped with a scalpel blade, and whole or worm fragments worms can be picked out of excreta.

**Fixation:** Large insects or arachnids can be prepared as whole mount entomological specimens after they have been killed (usually using ethyl acetate fumes (nail polish remover) or injected with acetone), relaxed in a steam chamber (restoring some pliability for manipulation) and set by pinning on mounting boards and air-dried (in presence of antifungal compounds such as Lysol or camphor). Smaller macroscopic parasites may be preserved by hot or cold fixation for subsequent transport, storage, processing and mounting. Arthropods are best fixed in 70% alcohol, usually containing 5% glycerine to provide viscous support for delicate stages and appendages (they remain pliable for years). Larval insects can be relaxed during fixation by immersing in 30% alcohol, heating to almost boiling point, cooling to ambient temperature and transferring to 70% alcohol. Cestodes can be relaxed in tap water, wound onto a flat card to hold their shape and then fixed in hot 70% alcohol. Trematodes can be purged of gut content by transferring them several times between warm saline and warm water before sandwiching them between two slides and fixing in hot 70% alcohol. Alternatively, if parasites are required for DNA extraction, they can be snap-frozen in dry-ice/ethanol or liquid nitrogen (although such freezing is impractical in many situations) or fixed in absolute ethanol.

**Cleaning:** Arthropods can be cleansed of organic debris and decolourized either by alkaline digestion (10% potassium hydroxide digests soft tissues but not chitinized elements) or clearing in lactophenol. Larger specimens can be punctured with a small pin to allow reagents to enter and act on soft internal tissues. Specimens can be partially dissected at this stage if required (e.g. the posterior ends of fly larvae can be excised for separate mounting).

**Staining:** Prior to mounting, cestodes and trematodes may be stained with Mayer's haemalum or Mayer's paracarmine or lactocarmine (acid-alcohol is used to differentiate colour development).

**Dehydration/clearing:** Specimens are dehydrated (water interferes with mounting and observation) by placing in absolute alcohol (several changes if required) and then cleared (replacing alcohol) in methyl salicylate, xylol, cedar wood, clove or wintergreen oil (miscible with mounting media).

**Mounting:** Temporary and permanent mounts are usually made on glass slides by placing/covering specimens in liquid mounting media (mostly Canada balsam although synthetic analogues are available), manipulating them with fine probes (if required) to lie in an appropriate orientation (dorso-ventral or lateral), covering with a coverslip ensuring all air-bubbles are expressed and then curing (hardening) mounts either at room temperature or in an oven for several days.

## MICROSCOPIC EXAMINATION

Most parasite developmental stages are too small to be seen unaided by the human eye, but they can be found and viewed in all their glory when magnified by microscopy. A range of techniques have been developed to prepare and process samples of host materials (predominantly faeces, blood and tissues) for microscopic examination: involving smears, suspensions, swabs, sediments, floats, concentrates, filtrates, cultures and sections. All vary in the amount of host material that can be conveniently examined so their thresholds of detection vary considerably. Those techniques which concentrate parasites from host materials have a higher level of detection than those which examine host materials raw or even diluted. In all cases, it is assumed that the intensity of infection (parasite abundance) is high enough for infections to be detected. However, it is best to remember that the lack of detection does not always mean absence of infection. Parasites are also not homogeneously distributed throughout host materials and many exhibit intermittent excretion or release of developmental stages during the course of infection. It is therefore hoped that samples were collected from the host during the patent (infectious) period of infection (when parasite developmental stages are being produced and released) rather than the pre-patent (incubation) period beforehand or the latent period thereafter. Some diagnostic protocols request that multiple consecutive samples be collected, some require multiple samples from different sites and most involve thoroughly mixing bulk samples before aliquots are taken (to ensure the aliquots are representative of the whole). International/national standard procedures have been developed for many diagnostic tests to ensure their integrity (reproducibility and reliability), particularly when import/export or quarantine certification is required. Considerable practice is required to confidently identify parasite stages in host materials by light microscopy. Novices frequently have problems with improper size perspective, wrong magnification, loss of plane of focus, lack of contrast, and the presence of air bubbles, artefacts and organic contaminants.

### Sample collection

Biological samples may be collected from hosts ante-mortem (whilst alive) or post-mortem (when deceased). Obviously, less invasive techniques are used to collect samples from live hosts (excreta, exudates, venepuncture, scrapings, tissue biopsy) and the objective is to keep the patient alive through timely diagnosis and treatment. Nonetheless, considerable information may be obtained from post-mortem examinations of deceased patients (particularly relevant in outbreak situations), as more invasive procedures may be used to sample internal tissues and organs.

**Blood:** Samples of peripheral blood may be collected from vertebrate hosts by skin prick or venepuncture. The fresh blood is usually collected into sterile tubes containing chemicals designed to inhibit or enhance blood coagulation and to preserve blood elements. When whole blood or plasma is required when still fluid, most blood samples are collected into tubes with added EDTA, citrate or heparin anticoagulant (commercial tubes with pink, blue or green tops). When serum samples are required, blood is collected into tubes without additives (white tops) or with added coagulants/clot-activators (gold or red tops). Haemolymph may also be collected from some larger invertebrate hosts by needle aspiration of vascular sinuses.

**Faeces:** Stool samples may be collected into range of containers, some with spoons fitted into the lids. Container size should be appropriate for the host, and the stool sample should be as fresh as possible and collected per rectum rather than off the ground. Veterinarians often use plastic gloves to collect samples direct from domestic animals (using digital stimulation to relax the anal sphincter). Human patients usually collect their own stool samples in their toilets, but often with great reluctance (thus compliance rates can be very low). Doctors should carefully explain to patients not only the importance of collecting samples, but also how to go about it (defaecate into a large bucket-like container and then transferring a subsample into the small container provided). If the patient is experiencing diarrhoea, collecting a stool sample can be quite a messy enterprise.

**Vomitus:** Stomach content is usually expelled spontaneously rather than on demand. Nonetheless, vomitus may be salvaged from various receptacles (sinks, trays, buckets, clean floors, etc.) and collected into appropriate airtight containers (often faecal containers).

**Sputum:** Mucus and phlegm can be collected from the respiratory tract by getting the patient to cough up sputum into containers. In cases involving heavy congestion, patients may require percussion drumming over the chest/back (with cupped hands) and postural drainage (head below level of lungs). Normal saline may sometimes be used for tracheal washes or bronchial lavages.

**Swabs:** Bacteriological swabs (sticks with cotton tips) may be used to collect fluids and/or cells from natural orifices (mouth, throat, nasal passages, ears, eyes, umbilicus, vagina, rectum) and surface lesions into sterile tubular containers.

**Scrapings:** Broad-bladed scalpels may be used to scrape suspect skin lesions for ecto-parasites. The epidermis is usually scraped until the subdermis is reached and begins to ooze serum. The scalpel blade and skin scrapings (plus any attached hair/feathers) are placed into a sterile container for transport to the laboratory. An unusual skin-sampling technique used to diagnose pinworm infections is to use transparent adhesive (sticky) tape to harvest any eggs attached to peri-anal skin.

**Biopsies:** Small pieces of host tissues (mainly surface lesions but occasionally internal organs) may be collected from living hosts by needle aspiration, punch biopsy or scalpel excision (lumpectomy). The tissues are usually fixed or frozen for histological examination but they can also be examined as wet mounts or dried smears.

**Necropsies:** Blocks of any tissue can be taken from deceased hosts during post-mortem examination. Pathologists may sample a range of vital organs but they often only collect suspect tissues on the basis of their unusual appearance (inflammation, haemorrhage, lesions, etc.). The tissues are fixed or frozen for histological examination but they can be examined as wet or dry mounts or used in digestion, concentration or culture techniques. Tissues and any contained parasites may degrade quite quickly after death of the host so samples should be collected reasonably fresh to avoid post-mortem autolysis and artefactual appearances.

### Wet suspensions/mounts

Host fluids can be examined under the light microscope simply by mounting drops on glass slides under coverslips. These wet mounts can be examined undiluted or diluted in warm saline (sometimes containing mucolysin to improve sample viscosity). The mounts may be examined for live motile organisms (protozoan trophozoites, worm larvae, small arthropods) or encysted stages (cysts, oocysts, eggs). Preservatives, stains or lytic agents can also be introduced under the coverslip to improve visibility. Drops of blood can be examined for motile trypomastigotes or microfilariae. Erythrocytes obscuring vision can be lysed by adding distilled water, dilute formalin, saponin or ammonium hydroxide. Faecal samples can be mixed with saline and examined for motile or encysted stages. Dilute iodine or methylene blue can be added to highlight protozoan nuclei (flagellates, ciliates, amoebae) and helminths (eggs, larvae). Tracheal washes can be examined for lungworm larvae. Skin, gill and fin scrapings can be diluted in appropriate media (saline for terrestrial hosts and source water for aquatic hosts) and examined for protozoan trophozoites, worm larvae or ecto-parasitic arthropods. Wet mounts can also be prepared of concentrates, filtrates or cultures to facilitate diagnosis.

### Dried smears

Permanent mounts can be prepared by making smears on slides, air-drying them, fixing them by heat (flaming) or alcohol (transient immersion), and staining with a variety of histochemical stains. These procedures are routinely used in microbiology laboratories for Gram staining bacteria. In parasitology, they are used less often as many parasite developmental stages are destroyed by air-drying or have tough resistant walls and are relatively impervious to stains. Streak smears can be made of faecal material and mucosal swabs, impression smears can be made from the cut surfaces of solid organs, and thin or thick smears can be made of blood or other fluid samples. Dried smears can also be prepared of concentrates, filtrates or cultures to facilitate diagnosis. Faecal smears can be stained with iron haematoxylin, acid-fast, trichrome or nigrosin stains to highlight protozoan trophozoites and/or cysts. Blood smears are most often stained with Giemsa (Romanowsky) stains to highlight the nuclei and cytoplasm of blood cells (for differential counts) and parasitic cells (extracellular microfilariae and trypanosomes and intracellular haemogregarines, haemosporidia and piroplasms). The preparation of good thin blood smears requires considerable practice – trained haematologists just seem to know exactly how much blood to use, what angle to hold the spreader, how much pressure to exert during smearing, etc. The objective is to make a cell monolayer that tapers/feathers out along the slide. Most smears are examined by bright-field light microscopy using high-power oil-immersion lenses (1,000 times magnification).

### Tissue sections

Pieces of host tissue collected by biopsy or autopsy can be processed immediately as squash/teased preparations or processed later after being preserved by chemical fixation or freezing. Small pieces of fresh tissue can be placed in saline on a glass slide and then squashed, macerated or teased apart to disrupt tissue structure and reveal (or release) any contained parasites (encysted worm larvae, protozoan cysts/spores, mites). Special tissue compression devices consisting of two glass plates (trichinoscopes) have even been developed for the examination of animal muscles for encysted *Trichinella* larvae (trichinoscopic examination). It is more usual, however, for blocks of tissue to be fixed in 10% phosphate-buffered formalin for subsequent histological processing. Formalin fixes proteins and prevents enzymatic degradation. It perfuses well into tissues so block sizes can be around 1 cm<sup>3</sup> (= 1 cc) and tissues can be held in fixative for long periods (2% glutaraldehyde is often used to fix tissues for electron microscopy - it better preserves membranous structures, but it does not perfuse far into tissues and long-term fixation causes tissues to become brittle). Once fixed, tissue blocks are dehydrated in a graded series of alcohol solutions, cleared in solvent (xylene, toluene or less volatile 'histo-clear'), and then infiltrated and embedded in paraffin wax. Tissue sections are cut at ~5 µm thickness on a microtome and enrobed onto glass slides. The sections are then stained (usually with haematoxylin and eosin, but many specialized stains may be employed), dehydrated in alcohol, cleared in solvent and mounted under coverslips in media (Canada balsam or synthetic media such as Deepex) prior to examination by bright-field light microscopy. Modern pathology laboratories have automated systems for bulk processing tissues into wax and staining sections. More recently, cryo-techniques have been developed to cut frozen sections on a cryomicrotome from pieces of tissues that have been snap-frozen (either in a mixture of dry ice/ethanol or in liquid nitrogen). Freezing preserves the tissues without degrading proteins or antigenic epitopes so enzymatic-labelling or immuno-labelling techniques can be used to screen for parasitic material in the sections. DNA can also be better extracted from frozen tissues for molecular biological techniques.

## Parasite concentration techniques

Various concentration techniques have been developed to recover parasites from larger sample volumes in order to improve the threshold of detection. Samples can be processed by sedimentation, floatation, filtration, centrifugation, digestion or culture. Concentrates can be examined qualitatively (presence/absence of parasites) or quantitatively (parasite count per volume/weight of sample, or percentage parasitaemia).

### Concentration from faeces

Infectious micro-organisms voided with host faeces contaminate the environment and are transmitted to new hosts via the ingestion of contaminated food and/or water. Many of the stages passed into the external environment are well protected for their transient free-living sojourn by being encysted within membranous casings which are relatively impervious to external environmental influences. Most exogenous stages are non-feeding and live on their own food reserves but they can survive for long periods of time under the right conditions by being dormant with low metabolic requirements. Faecal samples may contain protozoan cysts (amoebae, flagellates, ciliates), protozoan oocysts (apicomplexans), worm eggs (nematodes, cestodes, trematodes) or worm larvae (some nematodes). As excrement, faecal samples also contain large amounts of waste material (including fibrous and particulate material) and huge numbers of bacteria - the presence of both masking the detection of parasites. Several techniques are used to remove confounding materials and to concentrate parasites on the basis of their differential buoyancy in various solutions.

**Sedimentation:** Most parasite developmental stages sediment in water so faecal samples placed in at least 10 volumes of water and thoroughly mixed will allow fibrous and fatty material to float to the top while parasites settle to the bottom. Sedimentation by gravity will take several hours so the process can be accelerated by mild centrifugation over several minutes. Long thin centrifuge tubes (usually 10 mL volume) are used to provide good separation between sedimented parasites and floating detritus. Faecal samples from herbivores frequently contain abundant fibrous plant material which can be removed after centrifugation by vacuum-trap aspiration. Faecal samples from some carnivores frequently contain abundant fatty material which can be removed by centrifuging the samples in 10% ether to dissolve the fat. Where significant biohazards are indicated, faecal samples can be fixed in dilute formalin (or formol-ether or merthiolate-iodine-formalin) prior to centrifugation. Stains can also be introduced during sedimentation - the most commonly used one being iodine-trichrome stain to stain protozoan nuclei or methylene blue to stain worm eggs. An aliquot of the sediment is recovered and examined for parasite ova/cysts by light microscopy. Sedimentation techniques are best used to detect trematode ova (especially fluke eggs) which sediment readily (presumably due to their high iron-content). The problem with simple sedimentation is that heavy particulate material and most bacteria also sediment thereby obscuring parasites.

**Floatation:** Various floatation techniques are therefore used to separate parasites from faecal samples and sediments. When placed in high specific-gravity (SG) solutions, most parasite ova/cysts float to the meniscus due to their high starch content in the form of food reserves. Solutions commonly used include saturated sugar (sucrose, Sheather's solution) or saturated heavy metal salt solutions (sodium chloride, sodium nitrate, sodium iodide, zinc sulphate, magnesium sulphate, potassium iodide) which have SG ranging from 1.2-1.3. Most coccidial oocysts and nematode eggs float in solutions with a SG ~1.2 (e.g. saturated sodium chloride), but larger oocysts and the eggs of most cestodes and trematodes are heavier and require solutions with a SG  $\geq$  1.3 (e.g. saturated sucrose or magnesium sulphate). Faecal samples or sediments are placed in at least 10 volumes of floatation medium and centrifuged for several minutes (~300 xg for 2-5 minutes) so that parasites float through the viscous media while particulate material (and most bacteria) continue to sediment. Platinum (bacteriological) loops are usually used to collect drops of the meniscus for mounting on glass slides. Alternatively, some techniques require the tubes to be slightly overfilled producing a convex meniscus projecting above the tube rim so that coverslips can be placed in direct contact for several minutes and then lifted off (not slid) and transferred to glass slides (the viscous meniscus sticks to the coverslip). Several test kits are commercially available to sediment and/or float faecal samples all in one tube. Mounts of faecal floats are examined by light microscopy using phase- or interference-contrast illumination if unstained or bright-field illumination if stained. Samples should be examined as soon as practicable because prolonged exposure of parasite ova/cysts to many of the floatation media results in artefactual appearances due to osmotic distortion. Most direct floatation or consecutive sedimentation-floatation techniques are used as qualitative screens for the presence/absence of parasites, or at best to semi-quantitate the numbers present according to arbitrary subjective ranks (e.g. +, ++, +++ and ++++ corresponding to <5, 5-20, 20-100 and >100).

**Worm egg counts:** A modification of a floatation technique is used with special glass slide chambers to quantitate the number of gastrointestinal nematode eggs present in faecal samples from domestic animals, especially ruminants. A set weight of faeces is diluted in a set volume of floatation medium and a thoroughly mixed aliquot is then added to graduated chambers of known volume (McMaster, Whitlock, Universal and paracytometer slides have chambers ranging from 0.3-0.6 mL). The top glass plates of these chambers have been etched with grids so that a known area (and thus volume) can be examined. The number of nematode eggs present is counted by medium-power light microscopy and the number of eggs per gram of faeces (epg) is calculated as follows:

$$\text{epg} = (\text{number of eggs counted in chamber} / \text{chamber volume in mL}) \times (\text{faecal dilution factor})$$

where the faecal dilution factor = (total volume of diluted faeces in mL / weight of faeces added in g).

Most counts are conducted using 2 g of faeces mixed into a total of 60 mL of magnesium sulphate and then counting the number of eggs in one chamber, thus  $\text{epg} = (\text{number}/0.5) \times (60/2) = \text{number} \times 60$ . Variations of this basic protocol include using more faeces (4 g for horses, 5 g for cattle) and counting less than one whole chamber if eggs are numerous but then proportionally adjusting the formula. The relationship between faecal epg and the actual worm burden in the gut of the host varies considerably depending on the time course of infection and the worm species present. Infections may still be in the pre-patent period (before egg laying commences), host immune responses may suppress nematode reproduction, and worm fecundity has been shown to be reduced when the intensity of infection increases. Generally, there is a poor correlation between epg and worm burden for nematodes with low fecundity, such as many *Trichostrongylus* species; but a good correlation for nematodes which are prolific egg layers, such as *Haemonchus*, *Oesophagostomum* and *Chabertia* species. It is also important to remember that certain worms, such as *Haemonchus contortus*, *Trichostrongylus axei* and most *Teladorsagia/Ostertagia* species, can also undergo arrested larval development (hypobiosis) in the host when external environment conditions are unfavourable for parasite development and survival, thus animals are infected but not excreting worm eggs. Worm egg counts provide an indication, but not an exact measure, of what is going on inside the animal. Still, lots of eggs always means lots of worms. As a general rule, farmers are recommended to drench their animals if worm egg counts are greater than 1,000 epg.

**Drench resistance testing:** Nematode populations may be managed by a variety of anthelmintic drugs. At the turn of the century, various metal compounds (lead, tin, copper) were used to reduce worm burdens but they exhibited high toxicity to their hosts. They have now given way to synthetic compounds manufactured by the pharmaceutical industry. A very successful group of compounds called the benzimidazoles (or white drenches) were developed in the 1960's followed by another group called the levamisoles (or clear drenches). The first group acted upon microtubules causing worm paralysis and expulsion while the second group blocked cholinergic neurotransmission causing paralysis and expulsion. Regrettably, worm populations have now developed resistance against both groups of drugs. A third group of compounds called the avermectins (ivermectin in particular) was developed in the 1970's and they inhibited neurotransmission in worms. There are numerous reports of drench resistance against avermectins overseas and several in Australia. Producers are therefore faced with difficult choices – which drench to use and will it be effective? A subsidiary industry has now emerged which screens flocks for drench resistance and recommends the most appropriate drug to use to control worm populations. Basically, they involve drenching test groups of sheep with white, clear, combination and/or avermectin drenches (plus an untreated control group) and then testing 10-14 days later to see if faecal egg counts have been reduced by at least 95% for the drench to be declared effective.

**Filtration:** Several faecal filtration techniques have been developed to diagnose lungworm infections by facilitating the migration of lungworm larvae out of faecal samples. Adult lungworms lay eggs but they usually hatch while passing through the host intestines so that first stage larvae (L1) are found in faeces. The larvae will migrate out of faecal samples placed on filter paper or wrapped in gauze or muslin and placed in warm water in a funnel or Petri dish. Aliquots of the crude filtrate may be collected after several hours and examined for larvae by light microscopy.

### **Concentration from blood**

Many parasites infect the blood of vertebrate hosts and rely on blood-sucking invertebrate vectors for their transmission between hosts. Vector-borne blood parasites include kinetoplastid flagellates (trypanosomes), eucoccidia (haemogregarines, haemococcidia), haematozoa (haemosporidia, piroplasms) and filarial nematodes (microfilarial stages). The blood stream of vertebrate hosts is a lovely place to live (rich in nutrients and oxygen) provided the parasite can survive living in pressurized vessels and avoiding host homeostatic mechanisms (e.g. clotting) and immune defences (e.g. innate phagocytic and inflammatory responses and adaptive humoral and cell-mediated responses). Blood contains many different cell types to infect: erythrocytes or red blood cells (anucleate in mammals, nucleate in birds, reptiles, fish); leucocytes or white blood cells (granulocytic neutrophils, basophils, eosinophils and agranulocytic lymphocytes and monocytes); as well as a viscous fluid medium (plasma) rich in sugar, proteins, etc. Many protozoan parasites specialize in infecting red blood cells in which they grow and divide, but with the consequence of lysing the cell when mature, thus contributing to host anaemia. Other parasites occur extracellularly in the plasma deriving nutrients at the expense of the host. Haemoparasites may be few in number and easily obscured by the masses of cells in blood samples so a range of filtration and centrifugation techniques have been developed to concentrate parasites from blood samples.

**Knott's technique:** Microfilariae can be concentrated from unclotted blood samples firstly by lysing red blood cells through the addition of a lytic agent (commercial 0.8% ammonium chloride buffers or distilled water or 2% formalin or 0.5% Triton or Saponin detergent) and then pelleting the worm larvae by mild centrifugation (~800 xg for 5 mins). Aliquots of the sediment may be mounted on slides, stained with methylene blue (0.1%) and examined by low-power light microscopy.

**Membrane filtration:** Microfilariae may be recovered from unclotted blood samples firstly by lysing red blood cells through the addition of a lytic agent (commercial 0.8% ammonium chloride buffers or distilled water or 2% formalin or 0.5% Triton or Saponin detergent) and then capturing worm larvae by syringe-chamber filtration through polycarbonate membranes (Nucleopore, 8 µm pore size). The membrane may be washed and stained with methylene blue (0.1%) and then drained and mounted on a glass slide for low-power light microscopy.

**Microhaematocrit (capillary tube) centrifugation:** When unclotted blood samples are centrifuged, erythrocytes sediment (due to the heavy iron content in haemoglobin) while leucocytes settle on top of the red cell sediment as a white buffy coat. Extracellular haemo-parasites such as trypanosomes and microfilariae accumulate in the buffy coat layer and intra-erythrocytic parasites such as haemosporidia and piroplasms are often found in the upper portion of the red cell layer (infected cells are less heavy). Microhaematocrit centrifugation is often used to examine the buffy coat for parasites or to enrich parasitized cells in the red cell layer. Blood is taken up by capillary action into thin capillary glass tubes which are then sealed at one end with plasticine. The tubes are placed radially on a flat plate which is subject to mild centrifugation (~1,000 xg for 5 mins). A calibrated card can be used to read the haematocrit (percentage packed cell volume) and the tube can then be examined directly under a low-power light microscope (for parasites wriggling in the buffy coat) or the tube can be scored and snapped using a glass cutter or diamond pencil so the buffy coat can be expressed onto a slide for light microscopy.

**Density gradient centrifugation:** Unclotted blood samples can be centrifuged through solutions of increasing density so that sample components will band at different levels (technique known as density-gradient or isopycnic centrifugation or equilibrium sedimentation). The most commonly used media for blood cell separations are 20-70% sucrose solutions which can be prepared manually in 10% increments or obtained commercially as Ficoll-Paque/Hypaque solutions of high molecular weight sucrose polymers and sodium diatrizoate (note: caesium chloride solutions are inappropriate as they are used to harvest DNA by ultracentrifugation). The blood samples are loaded onto sucrose gradients and subject to low-speed centrifugation to avoid cell lysis (~60-100 xg for 10 mins). Such centrifugation is frequently used in cell biology and immunology laboratories to harvest lymphocytes for cell culture or hybridoma technologies. However, density-gradient techniques have been used to recover extracellular microfilariae and trypanosomes from whole blood samples, although the technique is more laborious and fastidious than filtration or microhaematocrit centrifugation.

### Concentration from tissues

Many parasites are not content to dwell in the luminal spaces of tubular organs or the blood stream of their hosts – instead, some actively invade the deeper tissues and organs of their hosts either transiently (as migrating stages) or for prolonged intervals (as encysted stages). Many protozoa form multiplicative stages (trophozoites, tachyzoites, bradyzoites, cystozoites, amastigotes, meronts, schizonts, cysts, pseudocysts) in host tissues; many being temporary and linked to acute disease syndromes although some can persist and cause chronic lesions and/or disease. Many helminth parasites have juvenile (larval) or adult (immature and mature) developmental stages which actively penetrate tissues and lodge in internal organs, some even becoming dormant (hypobiotic). Some geohelminths infect their hosts by transdermal penetration of the skin resulting in larval migrans. Other nematodes exhibit an obligatory pulmonary migration through the lungs before maturing as adults in their final predilection sites. All cestodes form encysted larval stages (metacestodes) in the tissues of intermediate hosts, and most trematodes infect their hosts when cercariae penetrate host tissues. All ectoparasites (fleas, lice, flies, ticks, mites) feed on host fluids/tissues by penetrating the skin, and some even invade subdermal tissues for part or all of their life-cycles. A range of techniques have been developed to recover parasites from host tissues, including washes, perfusions, smears and digests.

**Gut content (worm counts):** At post-mortem, the entire gastro-intestinal tract may be removed from herbivorous animals (mainly domestic animals such as sheep, goats and cattle) and examined for parasitic nematodes (mostly in the abomasa and small intestines). The numbers of worms present can be quantitated by extrapolating counts made on aliquots of gut content. Different portions of the gut are ligated (tied off) and processed separately. Abomasa are opened by cutting along the greater curvature and expelling the content into a sizeable container (bucket). Intestines are stripped from mesentery and the contents expressed into a separate container. The organs are washed several times with small quantities of water and the washings collected into the appropriate containers. The final volume in each container is made up to 1 litre, the contents are thoroughly mixed and a 100 mL aliquot taken. The aliquot is passed through a 400 µm mesh sieve to remove fine particulate material and the filtrate containing worms is back-flushed from the sieve and made up to 300 mL in water. A 30 mL aliquot is taken, added to a petri-dish, stained with a few drops of iodine (decolourized with sodium thiosulphate after a few minutes) and examined under a dissecting microscope. The number of worms present is counted and the total number of worms calculated by accounting for the two dilution factors; i.e. total worms = count x 1000/100 x 300/30 = count x 10 x 10 = count x 100. In many instances, different worm genera (and sometimes species) can be identified on the basis of their size and appearance; e.g. abomasal contents may contain *Haemonchus* sp. (large barbers-pole worms up to 25 mm long, females with white ovaries wound spirally around red intestine), *Teladorsagia circumcincta* (slender brown worms up to 12 mm long, uniform in thickness) and *Trichostrongylus axei* (small worms up to 5 mm long, tapering markedly at anterior end).

**Gut digests (developing worms):** Hypobiotic larvae (L4) and immature adult worms that burrow into the gut of ruminants can be recovered by enzyme-acid digestion of host tissues. Whole abomasa or stripped intestinal mucosa are incubated in at least 2 volumes of 1% pepsin in 3% hydrochloric acid (pH ~1.0) at 42°C for at least 2-3 hours. The digest is washed through a 400 µm mesh sieve and the filtrate containing worms back-flushed and retained. Aliquots are then examined microscopically for nematode developmental stages.

**Skin digests (burrowing mites):** Ecto-parasitic mites that burrow in the skin of animals may be recovered by alkaline digestion of skin scrapings taken from suspect lesions. Hair/fleece/feathers from the site may be removed by clipping/shaving and paraffin or white oil may be used to coat the skin before a broad-bladed scalpel is used to scrape the skin to the required depth (at least to stratum granulosum). The skin scraping is added to at least 10 volumes of 5-10% potassium hydroxide (which digests skin, keratin and hair, but not mites or fungi) in a centrifuge tube and the tube placed in a hot water (slow rolling boil) for 10-20 minutes. It is then centrifuged at low speed (~400 xg) for 1-2 minutes. The supernatant is discarded and a drop of the slurry/sediment is mounted on a slide for microscopic examination for mites (medium-high power with contrast).

**Muscle digests (encysted coccidia, worm larvae):** Parasite developmental stages that encyst within host musculature may be recovered by enzyme-acid digestion of post-mortem meat samples to reveal cystozoites/bradyzoites of tissue cyst-forming coccidia (such as *Sarcocystis* and *Toxoplasma*) and encysted/hypobiotic nematode larvae (such as *Trichinella*). Muscle samples are blended in at least 2 volumes of 1% pepsin in 3% hydrochloric acid (pH ~1.0) and incubated at 42°C for at least 2-3 hours. The resultant slurry is centrifuged at medium speed (~1,000 xg) for 10 minutes to pellet particulate material, including freed parasites. Aliquots of the sediment are examined microscopically for protozoan zoites and nematode larvae.

**Lung perfusions (lungworms):** Larval and adult lungworms may be recovered from domestic animals by perfusing intact lungs or rinsing cut lungs (bronchi and bronchioles) with warm 10% sodium bicarbonate (to remove mucus). The resultant washings are then rinsed through nylon-/wire-mesh sieve (aperture 45 µm) with warm saline. The filtrate is examined by low power microscopy for lungworm developmental stages.

**Tissue smears (piroplasms):** Intraerythrocytic stages of piroplasms (*Babesia/Theileria*) concentrated/sequestered in capillary blood may be better observed in impression smears prepared from biopsy/necropsy material of brain, spleen, kidney or heart than in thin smears prepared from blood collected by peripheral venepuncture. Impression smears are air-dried, fixed by flooding with methanol, mordanted in May-Grünwald for 2 minutes and then stained with Giemsa prior to microscopic examination.

## **Parasite cultivation**

The diagnosis of many infectious diseases involves the isolation of microbes from host samples and their cultivation in liquid media, on nutrient substrates or in tissue cell cultures for subsequent identification. Bacteria can be selectively cultivated in defined media and viruses amplified in susceptible cell lines. Conventional methods involving Petri dishes, test tubes and flask cultures are lengthy and laborious and have now been largely superseded by rapid mini-system assays (using strips of chambers containing defined reagents), immune-assays (for host antibodies or microbial antigens) or molecular biological assays (for microbial proteins or nucleic acids). By comparison, the cultivation of parasites for diagnostic purposes is uncommon and complicated by their complex life-cycles often involving multiple hosts, multiple developmental stages, slow development, long generation times and, for most species, unknown culture conditions. *In vitro* cultivation can therefore be tedious and time-consuming, often taking days before results become apparent. While specific parasite developmental stages may be cultivated *in vitro* under maintenance or growth conditions, to complete their life-cycles most parasites require cultivation *in vivo* in living hosts. Despite the inherent difficulties, suitable techniques have been developed to cultivate a small but growing number of parasite species for the:

- differential diagnosis of clinical infections;
- amplification of parasite numbers for phenotypic and/or genotypic characterization;
- assessment of parasite virulence/pathogenesis;
- isolation and maintenance of unique cultivars;
- comparison of clinical and/or environmental isolates for phylogenetic analyses;
- screening of potential therapeutic agents;
- testing for drug resistance/susceptibility;
- parasite attenuation and vaccine development; and
- producing defined reagents for immunological and/or biochemical assays.

## ***In vitro* culture**

A number of culture techniques have been developed for protozoan parasites but few are available for helminth parasites and even fewer for arthropod parasites (due to their more complex life-cycles and fastidious culture requirements). Parasites can be amplified in number from diagnostic samples (faeces, blood or tissues) by *in vitro* culture on semi-solid substrates (e.g. amoebae on agar plates/slopes), in liquid nutrient broths/media (e.g. haemoflagellates, helminth larvae) or in tissue cultures of host cells or surrogates (e.g. malaria in erythrocytes). Success depends on many factors, including the preparation of suitable inocula and recipient media/cells and their culture under appropriate conditions (time, temperature, atmosphere). In microbiology, there are three basic types of pre-culture isolation methods: enrichment (inoculation of sample into selective media); dilution (sequential dilution of sample in defined media); and physical methods (selection and transfer of individual cells by micropipetting, differential centrifugation, sieving, silicone oil plating, flow cytometry, agar plating or electromigration into defined media). Then, there are three basic types of culture systems: xenic (parasite grown in presence of undefined flora); monoxenic (parasite grown in presence of single additional species, usually provided as a feeder organism); and axenic (parasite grown alone in absence of any other metabolizing cells). The culture of parasites in tissue cell cultures involves their intra- or epi-cellular development in association with host cells (or surrogates) maintained as monolayers in chambers or as suspensions in flasks. Cultivars may be maintained by subculturing at suitable intervals, cryopreserved at ultra-low temperatures using cryoprotectants, and sometimes by desiccation of encysted resistant forms. There are a growing number of living or cryopreserved type cultures available from various governmental or institutional agencies (such as the ATCC = American Type Culture Collection, CDC = Centers for Disease Control), most being documented in registers maintained by parasitological societies or museums.

Parasitic protozoa have been isolated and grown in a variety of media and tissue cell cultures. Several free-living amoebae cause opportunistic tissue infections in humans known as PAM, SAM or GAM (= primary, secondary or granulomatous amoebic meningoencephalitis caused by *Naegleria*, *Entamoeba* and *Balamuthia* species respectively). Clinical isolates of these amoebae grow best in tissue cell cultures while environmental (water/soil) isolates grow best in diphasic xenic or monoxenic cultures. Enteric protozoa causing diarrhoeal diseases (including *Entamoeba*, *Giardia*, *Dientamoeba* and *Balantidium*) grow best in monophasic (liquid) or diphasic (liquid overlay on substrate) media and an increasing number of cultures have subsequently been axenized. The urogenital flagellate *Trichomonas* grows well in defined media and easy-to-use InPouch cultures have found widespread use in screening programmes. Enteric spore-forming protozoa, including the coccidian *Cryptosporidium* (known now to be a gregarine) and various microsporidian genera (known now to be fungi), grow well in tissue cell cultures, as does the tissue cyst-forming coccidian *Toxoplasma*. Two different methods are used to culture haemoprotozoa: monophasic or diphasic media culture for kinetoplastid haemoflagellates (blood or tissue stages of *Trypanosoma* and *Leishmania* species) and erythrocyte cell cultures for apicomplexan parasites (blood stages of haemosporidian *Plasmodium* and piroplasm *Babesia* species).

Cultivation of protozoan parasites

Protozoan parasite	Genus/species	Developmental stages	Cultivation*
tissue amoeba	opportunistic <i>Naegleria</i> spp.	trophozoites	xenic/monoxenic (non-nutrient agar); axenic (PYG, DGM 21A media); tissue culture (monkey kidney, rat glioma cells)
tissue amoeba	opportunistic <i>Acanthamoeba</i> spp.	trophozoites	xenic/monoxenic (non-nutrient agar); axenic (PYG, DGM 21A media); tissue culture (monkey kidney, rat glioma cells)
tissue amoeba	opportunistic <i>Balamuthia</i> spp.	trophozoites	xenic/monoxenic (non-nutrient agar); axenic (PYG, DGM 21A, BMS media); tissue culture ( monkey kidney, rat glioma, human brain microvascular endothelial cells (HBMEC))
urogenital flagellate	<i>Trichomonas vaginalis</i>	trophozoites	xenic (InPouch TV, Trussell & Johnson's media); monoxenic (Cleveland's medium), axenic (Linstead's, Diamonds TYI-S-33, YI-S media)
enteric amoeba	<i>Entamoeba histolytica</i>	trophozoites	xenic (Locke-egg, Robinson's, Boeck & Drbohlav's, Balamuth's, Diamond's TYSGM-9 media); monoxenic (Diamond's TYI-S-33, YI-S, LYI-S-2, Cleveland's media); axenic (Diamond's TYI-S-33, YI-S, LYI-S-2 media)
enteric flagellate	<i>Giardia intestinalis</i>	trophozoites	axenic (modified Diamond's TYI-S, YI-S media; Keister's modified TYI-S-33 medium (= ATCC medium 2695))
enteric flagellate	<i>Dientamoeba fragilis</i>	trophozoites	xenic (Locke-egg, Robinson's, Loeffler's media)
enteric ciliate	<i>Balantidium coli</i>	trophozoites	xenic/monoxenic (Locke-egg, Robinson's, Barret & Yarbrough's, Glasser & Coria's, Boeck & Drbohlav's media)
enteric microsporan	various	meronts, sporonts, spores	tissue culture (Madin-Darby canine kidney (MDCK), monkey kidney (E6), human lung fibroblasts (HLF), foetal bovine lung fibroblasts (FBF), lung fibroblasts (MRC-5), rabbit kidney (RK-13) cells)
enteric coccidia	<i>Cryptosporidium</i> spp.	meronts (schizonts)	tissue culture (human rectal tumour (HRT), baby hamster kidney (BHK), chicken chorioallantoic membrane (CAM), HFL, primary chicken kidney (PCK), MDCK, human colonic adenocarcinoma (Caco-2) cells)
tissue coccidian	<i>Toxoplasma</i>	tachyzoites	tissue culture (African green monkey kidney cells (Vero), lung fibroblasts (MRC-5), Henrietta Lacks' "immortal" cells (HeLa) cells)
haemosporidian	<i>Plasmodium</i> spp.	erythrocytic stages	tissue culture (human 'O' erythrocytes in Rosewell Park Memorial Institute (RPMI) 1640 medium with human 'AB' serum in HEPES buffer)
haemosporidian	<i>Plasmodium falciparum</i>	exoerythrocytic stages	tissue culture (hepatoma, chimpanzee primary hepatocytes, human hepatocytes, rat hepatocytes in RPMI 1640/minimal essential medium (MEM))
haemosporidian	<i>Plasmodium falciparum</i>	gametocytogenesis	tissue culture (young erythrocytes in RPMI 1640 medium with hypoxanthine)
haemosporidian	<i>Plasmodium falciparum</i>	sporogonic stages	tissue culture ( <i>Drosophila</i> cells on Matrigel substrate in RPMI 1640 medium)
blood piroplasm	<i>Babesia</i> spp.	merozoites	tissue culture (bovine erythrocytes in agitated suspension culture or microaerophilous stationary phase (MASP) culture; Ham's F10/12medium)
haemoflagellate	<i>Trypanosoma</i> spp.	trypomastigotes	axenic (Novy, McNeal & Nicolle (NNN) diphasic medium, salt-agar with rabbit blood)
tissue flagellate	<i>Leishmania</i> spp.	amastigotes	axenic (Schneider's, JH-31 with 25% foetal calf serum (FCS); tissue culture (mouse macrophage cells)
tissue flagellate	<i>Leishmania</i> spp.	promastigotes	axenic (Schneider's medium with inactivated 30% FCS; Trager's, Steiger's, Beren's media)

cf. Ahmed NH 2014 Cultivation of parasites. Trop Parasitol 4:80

Clark CG, Diamond LS 2002 Methods for cultivation of luminal parasitic protists of clinical importance. Clin Microbiol Rev 15:329

Parasitic helminths may be maintained in various crude and defined media, and a small number of culture techniques have been developed to support their continued development to the next life-cycle stage (thus allowing additional diagnostic characters to be examined). Xenic culture of faeces from humans and animals may facilitate the recovery of nematode larvae, notably threadworms (*Strongyloides*), hookworms (*Ancylostoma*, *Necator*, *Bunostomum*), lungworms (*Angiostrongylus*, *Metastrongylus*), strongyles (*Oesophagostomum*, *Chabertia*) and trichostrongyles (*Trichostrongylus*, *Ostertagia*, *Haemonchus*, *Nematodirus*, *Cooperia*); the latter two groups being most prevalent in ruminants. Faecal cultures involve incubating moist faeces at 29°C for several (7-10) days and then collecting any emergent nematode larvae (from condensate on container walls or by inverting the container into a Petri dish to allow larvae to migrate out). Several modifications include the following: Baermann technique (culture moist faeces in filter funnel and collect emergent larvae in filtrate); Harada-Mori technique (culture faeces in tube with filter paper insert onto which emergent larvae migrate); and agar plate technique (culture faecal sample on agar plate onto which emergent larvae migrate creating visible tracks). Emergent larvae are collected onto slides and then straightened by the application of gentle heat and fixed/stained with Lugol's iodine. Axenic or tissue cell cultures also support the maintenance or further larval development of microfilariae of the filarial nematodes (*Dirofilaria*, *Brugia*, *Wuchereria*, *Onchocerca*, *Stephanofilaria*, *Parafilaria*, *Setaria*) harvested from host blood or tissues. Axenic cultures have also allowed larvae of the tissue nematodes *Trichinella* and *Anisakis* to undergo further development.

The absence of a digestive tract in cestodes has provided challenges for their culture. Studies have been confined to hatching processes, metacestode (larval) development and maturation to adults (involving segmentation and organogenesis), mainly of aquatic (pseudophyllidean) species. However, various axenic media have been used to maintain specific developmental stages of cyclophyllidean *Taenia* and *Echinococcus* species to harvest oncosphere, cysticercus or adult worm excretory/secretory (ES) products for biotechnological purposes (esp. vaccine development). Similarly, trematodes with blind digestive tracts (sac-like) have proven difficult to culture, but a variety of axenic media have been found to support the partial development of digenean trematodes (*Schistosoma*, *Parvatrema*, *Metagonimus*, *Microphalloides* and *Paragonimus* species) from pre- or post-molluscan stages (ova to miracidia, or cercariae/metacercariae to adults).

Cultivation of helminth parasites

Helminth parasite	Genera	Developmental stages	Cultivation*
enteric nematode (rhabditid)	<i>Strongyloides</i> (threadworms)	ova to larvae	xenic (Harada-Mori, Baermann, agar plate cultures)
enteric nematode (ancylostomatoid)	<i>Ancylostoma</i> , <i>Necator</i> , <i>Bunostomum</i> (hookworms)	ova to larvae	xenic (Harada-Mori moisture culture); axenic (Waymouth's MB752/1 (WYM) medium)
enteric nematode (trichostrongyloid)	<i>Trichostrongylus</i> , <i>Ostertagia</i> , <i>Haemonchus</i> , <i>Nematodirus</i> , <i>Cooperia</i>	ova to larvae	xenic (Baermann, Harada-Mori, agar plate cultures)
enteric nematode (strongyloid)	<i>Oesophagostomum</i> , <i>Chabertia</i> (nodular worms)	ova to larvae	xenic (Baermann, Harada-Mori, agar plate cultures)
enteric/tissue nematodes (metastrongyloid)	<i>Angiostrongylus</i> spp. <i>Metastrongylus</i> sp. (lungworms)	ova to larvae to adults	axenic (Ham's F-12, modified White's (WMB), National Cancer Institute (NC) tissue culture (TC) 109, RPMI 1640, WYM media, Chernin's balanced saline solution (CBSS) with horse or calf serum)
enteric nematode (ascaridid)	<i>Anisakis</i> sp.	larvae to adults	axenic (TC 199 medium with bovine pepsin digestive fluid and bovine blood)
tissue nematode (enoplid)	<i>Trichinella</i> spp.	larvae to adults	axenic (NCTC 135 with bovine serum); tissue culture (human colonic adenocarcinoma (Caco-2) cells)
blood nematodes (filarial)	<i>Dirofilaria</i> sp. (heartworm)	microfilariae	tissue culture (mosquito cells in M41 or serum-free Ham's F-12 media)
blood/tissue nematodes (filarial)	<i>Onchocerca</i> , <i>Mansonella</i> , <i>Stephanofilaria</i> , <i>Parafilaria</i> , <i>Setaria</i>	microfilariae	axenic (various tissue culture media); tissue culture (monkey kidney feeder cells)
blood/tissue nematodes (filarial)	<i>Brugia</i> sp. <i>Wuchereria</i> sp.	microfilariae to adults	tissue culture (monkey kidney/mosquito cell lines)
tissue cestode (cyclophyllidean)	<i>Taenia</i> spp.	oncosphere to cysticercus	axenic (RPMI 1640 with 10% FCS, Dulbecco's MEM); tissue culture (calf kidney monolayer in Leibovitz's medium)
tissue cestode (cyclophyllidean)	<i>Echinococcus</i> sp.	protoscoleces	axenic (RPMI 1640, diphasic S.10E.H, Dulbecco's MEM media); tissue culture (murine Hepa 1-6 feeder cells)
blood trematode (digenean)	<i>Schistosoma</i> spp.	eggs to miracidia	axenic (RPMI/MEM with 10% FCS; Earle's buffered solution with glutamine)
blood trematode (digenean)	<i>Schistosoma</i> spp.	cercariae to schistosomula to adults	axenic (human serum; NCTC 109 medium with 50% human or rabbit serum)
enteric/liver trematodes (digenean)	<i>Parvatrema</i> sp. <i>Metagonimus</i> sp. <i>Microphalloides</i> sp. <i>Paragonimus</i> spp.	metacercariae to adults	axenic (NCTC 109 medium with 20% chicken, bovine or horse serum; or chicken embryo extract (CEE), Eagle's MEM with 20% bovine serum)

\*cf. Ahmed NH 2014 Cultivation of parasites. *Trop Parasitol* 4:80

Yasuraoka K, Hata H 2003 In vitro cultivation of parasitic helminths. *Prog Med Parasitol in Japan* 7:211.

## ***In vivo* culture**

Laboratory animals may be used as surrogate hosts to isolate and/or amplify parasites from diagnostic samples as their inoculation into natural hosts is often precluded by ethical and/or logistical considerations. However, most parasites are host-specific and their cross-transmission to surrogate (non-natural) hosts fails. Nonetheless, *in vivo* culture has been established as a successful diagnostic aid for several parasites, but unfortunately the process is time-consuming (taking up to weeks). Ideally, laboratory animals should be derived from specific-pathogen-free (SPF) colonies to rule out the possibility of natural infections. Mice have proven to be very susceptible to infections by the tissue cyst-forming coccidian *Toxoplasma gondii*, with tachyzoites becoming evident in peritoneal fluids 7-10 days after the intraperitoneal inoculation of test samples. Hamsters are susceptible to tissue-dwelling kinetoplastid *Leishmania* species, with amastigotes developing in their spleens 4-6 weeks after inoculation. Guinea pigs, rats and mice are susceptible to various blood-dwelling kinetoplastid *Trypanosoma* species, with trypomastigotes becoming evident in peripheral blood samples two weeks after inoculation. Triatomid bugs are susceptible to the kinetoplastid *Trypanosoma cruzi*, with epimastigotes developing in their intestines 10-30 days after being allowed to feed on patient's blood (procedure known as xeno-diagnosis).

A range of animal models of infection have been developed for various protozoan and helminth parasites, primarily to facilitate research on pharmacology (drug screening), immunology (vaccine development) and pathology (pathogenesis). Well-defined animal models\* exist for amoebic dysentery (in mice, gerbils, hamsters), trichomoniasis (mice, rats, hamsters, guinea-pigs, squirrel monkeys), giardiasis (mice, rats, gerbils, rabbits, dogs, cats, sheep), sleeping sickness (mice, rats, monkeys), Chagas' disease (mice, hamsters, rabbits, dogs), leishmaniasis (hamsters, mice, rats), coccidia (mice, chickens), cryptosporidiosis (neonatal or immunocompromised mice, rats, ruminants), toxoplasmosis (mice, cats), malaria (mice), echinococcosis (mice, gerbils, voles, cotton rats), schistosomiasis (mice, snails), and various gastrointestinal nematodes, including hookworms (hamsters), ascarids (chickens) and trichostrongyles (jirds, rabbits). Other parasites, particularly those of veterinary significance, have been propagated in their natural hosts (domestic and wild captive animals) for a few (sometimes many) generations as part of applied research programmes conducted by public or private institutions around the world. Information on living parasite collections, and their availability to other workers, has been documented electronically by various agencies; such as ASPIC (Australian Society for Parasitology Information on Collections), ALA (Atlas of Living Australia) and GBIF (Global Biodiversity Information Facility).

\*Zak O, Sande MA (eds) 1999 *Handbook of Animal Models of Infection. IV. Parasitic Infection Models. Chapters 92-106.* Academic Press, Elsevier.

## **Medical imaging**

Medical imaging may be used to detect certain parasites, or their effects, in the internal tissues and organs of hosts, notably those parasites that reside or migrate within tissues often causing inflammatory or space-occupying lesions. Various specialized techniques have been developed whereby light, X-rays, sound waves, electromagnetic waves or charged particles are used to create images of internal tissues. Imaging techniques are generally described as non-invasive, but radiographic (X-ray) techniques often involve the introduction of radio-opaque contrast media (barium, iodine, air) to help visualize internal structures, and nuclear medicine functional imaging techniques involve the use of short-lived radio-isotopes or energetic particles to create images; e.g.

- endoscopy (EN): uses flexible fiber-optic endoscopes inserted into tubular organs to take images.
- ultrasound (US): uses high frequency sound waves that are reflected by tissues to produce images.
- radiography: uses X-rays (XR) to differentially penetrate soft and hard tissues to produce negative images.
- fluoroscopy: uses a constant flow of XR at low-doses to provide real-time images.
- computed (axial) tomography (CT scans): uses synchronized movements of XR sources and radiation detectors to create images of internal tissues.
- magnetic resonance imaging (MRI): uses powerful magnets and radio frequency pulses to excite and polarize hydrogen nuclei (protons) of water molecules in tissues producing detectable signals.
- positron emission tomography (PET, SPECT): uses short-lived isotopes that are metabolically active and/or preferentially absorbed by active tissues to identify abnormalities using gamma-cameras.

Imaging techniques may be used to detect specific macroscopic parasites, or collections of microparasites, within tissues, but they are often used to detect particular lesions (often inflammatory and/or space-occupying) previously shown to be associated with certain parasites. Definitive diagnosis using alternative techniques is often required. According to conventional radiological texts, there are four active parasitic infections where diagnosis can reliably be made using imaging alone: ascariasis, trichuriasis, cerebral cysticercosis and urinary schistosomiasis. Other parasites may be recognized by their images, but they are often dead (degenerating and/or calcified). Lesions typically associated with parasites have been found in the central nervous system (commonly brain, rarely spinal cord), thorax (often lungs, sometimes heart), abdomen (tubular and solid organs, hepatobiliary tree, spleen, urogenital system), muscles (striated musculature) and skin (subcutaneous tissues). Radiologic manifestations include calcifications, erosions, ulcerations, inflammation, oedema, organ distention (sometimes mega-organ syndrome), mucosal thickening, fibrosis, nodularities, strictures, filling defects, hypertension, ascites, varices and ischaemia.

Medical imaging of parasitic infections

Type of parasite	Disease	Site of infection	Imaging technique*
protist (archamoeba)	amoebiasis	brain, thorax, abdomen	XR, CT, MRI, EN
protist (diplomonad)	giardiasis	abdomen	XR, CT, MRI
protist (kinetoplastid)	leishmaniasis	abdomen	CT, MRI, PET
protist (kinetoplastid)	Afr. trypanosomiasis	brain	CT, MRI
protist (kinetoplastid)	Am. trypanosomiasis	brain, thorax, abdomen	XR, CT, MRI
protist (gregarine)	cryptosporidiosis	abdomen	US, CT
protist (coccidian)	toxoplasmosis	brain, thorax, abdomen	XR, CT, MRI, PET, SPECT
protist (piroplasm)	babesiosis	thorax	US, CT
protist (haemosporidian)	malaria	brain, abdomen	CT, MRI
nematode (metastrongyloid)	angiostrongyliasis	brain, abdomen	XR, CT
nematode (ascaridid)	ascariasis	thorax, abdomen	XR, CT, MRI, US, EN
nematode (rhabditid)	strongyloidiasis	thorax, abdomen	XR, CT
nematode (ascaridid)	toxocariasis	brain, thorax, abdomen	XR, CT, MRI
nematode (ascaridid)	baylisascariasis	brain	MRI
nematode (ancylostomatoid)	hookworm disease	abdomen, muscles	XR, EN
nematode (camallanid)	dracunculiasis	subcutaneous tissues	XR
nematode (filaroid)	filariasis	thorax, abdomen, subcutaneous	XR, US, CT, MRI, PET
nematode (spiruroid)	gnathostomiasis	subcutaneous tissues	CT
nematode (enoplid)	trichinosis	brain, muscle	CT
nematode (enoplid)	trichuriasis	abdomen	XR, CT, MRI
nematode (enoplid)	capillariasis	abdomen	XR, CT, MRI
cestode (cyclophyllidean)	cysticercosis	brain, muscle, thorax, abdomen	XR, CT, MRI, US
cestode (cyclophyllidean)	echinococcosis	brain, thorax, abdomen, bladder	XR, CT, MRI, US
cestode (cyclophyllidean)	coenurosis	brain	CT, MRI
cestode (pseudophyllidean)	sparganosis	brain, abdomen, muscles	XR, CT, MRI
trematode (digenean)	schistosomiasis	brain, thorax, abdomen, bladder	XR, CT, MRI, US, EN
trematode (digenean)	paragonimiasis	brain, thorax, abdomen, muscle	XR, CT, MRI
arthropod (pentastome)	pentastomiasis	muscle, thorax, abdomen	XR

\*cf. Haddad MC, Abd El Bagi ME, Tamraz JC (Eds) 2008 *Imaging of Parasitic Diseases*. Springer.  
Palmer PE 1985 *Diagnostic imaging in parasitic infections*. *Pediatr Clin Nth Amer* 32:1019