Field and laboratory diagnostics of parasitic diseases of domestic animals: from sampling to diagnosis

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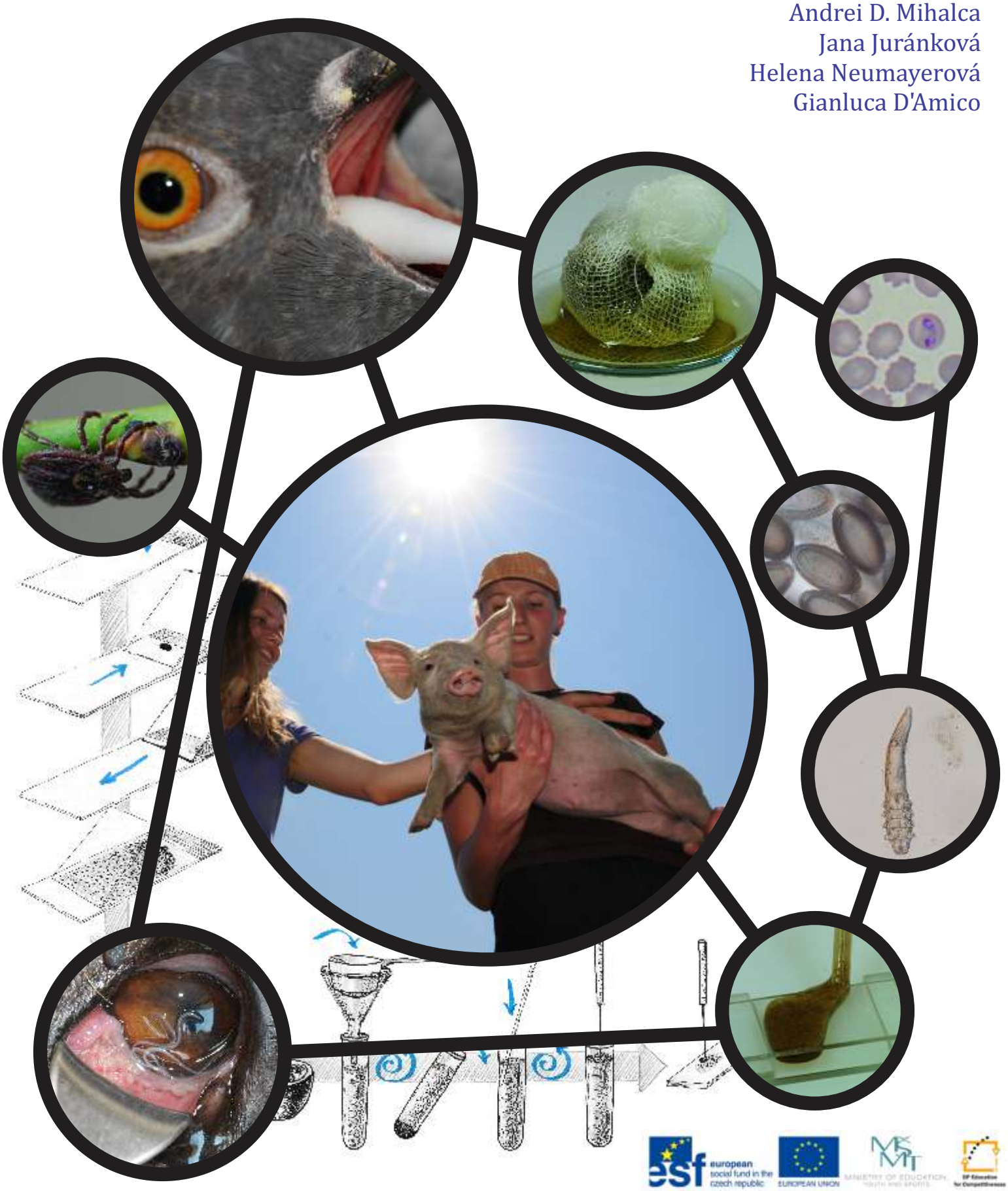

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FIELD AND LABORATORY DIAGNOSTICS OF PARASITIC DISEASES OF DOMESTIC ANIMALS: FROM SAMPLING TO DIAGNOSIS

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1 DETECTION OF PARASITES DURING THE NECROPSY

Necropsy is defined as the post-mortem examination of animal or human corpses with the specific aim to identify the cause of death or to assess the presence of lesions, toxins or infectious organisms. The term autopsy is rather used for human necropsy and is not considered for post-mortem examination of animals. Necropsy includes various post-mortem diagnosis techniques, from dissections and gross inspection to complex laboratory tests; its principles and methods will not be discussed here and readers should refer to a more general veterinary pathology textbook. The focus of this chapter will be on the specific steps to be followed for the identification of parasites during the necropsy of domestic animals. However, few more essential general indications will be also provided.

For most internal parasites, the necropsy is the most sensitive detection method, a kind of "gold standard" (figure 1). In many cases, although the coproscopy is negative, parasites can be found in the internal organs (digestive, respiratory). In other situations, the necropsy is the only diagnostic methods) for instance the presence of serosal parasites like *Gongylonema* in the esophagus wall).

1.1 General safety measures and preparatory steps

Necropsy of domestic animals poses various risks. We will refer here specifically to those related to the possibility of parasitic infections. However, readers must be aware on the existence of many other (non-parasitic) zoonotic risks posed by dead animals, some of them highly dangerous and potentially deadly (i.e. rabies, brucellosis, leptospirosis, etc.).

Domestic animals carry a great variety of zoonotic parasites, which can be accidentally transmitted to humans during necropsy, especially when the carcass is fresh. The routes of infection with zoonotic parasitic agents can be by direct contact, ingestion of infective parasitic stages, transcutaneous penetration, aerosol inhalation etc. The list of the most important parasitic zoonoses, the sources and routes of infection and host species are shown in table 1.

Table 1. List of most important parasitic zoonoses which can be acquired from domestic animals during necropsy

Hosts	Parasite	Route	Infective stage (source)
Protozoans			
All	*Giardia* spp.	Oral	cysts (faeces, intestine, fur)
All	*Cryptosporidium* spp.	Oral	oocysts (faeces, intestine, fur)
Cats	*Toxoplasma gondii*	Oral	oocysts (faeces, intestine, fur)
Helminths			
Dog	*Echinococcus* spp.	Oral	eggs (faeces, intestine, fur)
Dog	*Taenia* spp.	Oral	eggs (faeces, intestine, fur)
Dog, cat. cattle	*Toxocara* spp.	Oral	larvated eggs (faeces, intestine, fur)
Dog, cat, cattle	Hookworms (*Ancylostoma* spp., *Uncinaria* spp., *Bunostomum* spp.)	Transcutaneous	larvae (faeces, intestine, fur)
All	*Strongyloides* spp.	Oral, transcutaneous	larvae (faeces, intestine, fur)
Arthropods			
All	fleas (various species)	Direct contact	alive adult parasites (fur, skin)
All	ticks (various species)	Direct contact	all developmental stages (fur, skin)
All	*Sarcoptes* spp.	Direct contact	all developmental stages (fur, skin)
Birds	*Dermanyssus* spp.	Direct contact	all developmental stages (feathers, skin)

Figure 1

Team of scientists performing a parasitological necropsy on a jackal.

To reduce the risk of transmission of parasitic zoonoses during necropsy, general safety measures include: use of protection clothing (i.e. overalls, gloves, masks, rubber boots), thoroughly washing and disinfecting the hands after the necropsy and disposal of all potentially infective material according to the legal measures (i.e. incineration). If during necropsy the protection equipment is broken, it should be immediately replaced and the skin area in contact with infective material washed and disinfected.

To avoid most of the risks associated with necropsy, it is recommended to deeply freeze (-80°C) the carcasses prior to necropsy for minimum 3 days. However, this reduces the sensitivity of some laboratory diagnostic procedures (e.g. histology, morphologic identification of helminths, collection of living parasitic specimens, etc.).

In the specific case of parasitological necropsy, it is particularly important to avoid contamination of samples, mainly in the case of molecular diagnostic methods. Hence, if during necropsy tissues samples are collected for DNA detection, it is essential to initially neutralize any trace of nucleic acids from the dissection tools.

Before starting the necropsy, some general steps should be followed:

- preparation of the necropsy table: the table should be thoroughly cleaned before placing the dead animal in order to prevent external contamination (i.e. fleas, lice, ticks);
- preparation of the necropsy kit: except the general (standard, basic) necropsy kit, some specific materials and instruments are necessary for the parasitological necropsy. These include: fine tweezers for the collection of "delicate" parasites (thin helminths, immature ticks, other small arthropods etc.), microscopic slides for cytology (for intestinal mucosal touch imprints), Petri dishes, sedimentation cones, vials of various sizes for the collection of parasite specimens, ethanol, formalin, potassium dichromate (2.5% w/v aqueous solution), zip bags;
- preparation of the sample identification forms: printed tables, pencils, paint markers;
- if carcasses are frozen, they should be totally defrozen prior to necropsy. Depending on the size of the animal, this may take from few hours (small vertebrates) to 48 hours (dogs, cats).

1.2 The general steps of parasitological necropsy

To increase the sensitivity of the method (i.e. to detect as many parasites as possible), parasitological necropsy should follow certain steps, in a specific sequence. In general, these are the steps to be followed:

- identification of the animal: species, breed, sex, age, origin, ownership;

- examination of the body surface for external parasites (e.g. ticks, mites, insects);
- examination of the body surface for lesions which could be associated with parasites (crusts, nodules) and further investigations to detect them (skin scrapings, biopsies);
- skinning is recommended for certain host species to enhance the detection of subcutaneous parasites (i.e. *Dirofilaria repens* in carnivores);
- examination of the external mucosae and cavities for parasites (e.g. the conjunctival sac for ocular nematodes, the ear canal for mites);
- dissection and examination of body cavities for the detection of serosal parasites (e.g. *Setaria* in horses, cestode cysts) and examination of the surface of the organs (e.g. liver surface for larval cestodes);
- examination of internal organs, separately for each organ system (digestive system, respiratory system, circulatory system, urinary and reproductive tract, nervous system);
- gross and microscopic examination of muscles (for *Sarcocystis*, *Trichinella*), ligaments (*Onchocerca*) and bones (i.e. cytology of bone marrow);

Except these specific steps, when performing a parasitological necropsy, the general inspection of the carcass must be done. Some general signs of diseases must be checked. Anaemia is often an indication of parasitism with hematophagous parasites or the icterus (jaundice) may be one of the lesions associated with babesiosis.

1.3 Parasitological examination of the skin

The skin of dead animals hosts several types of parasites. Certainly, the most common ones are arthropod **external parasites** like mites, ticks, lice, fleas. Many external parasites leave the animal's body after the death of the host. So it is essential to examine the cutaneous surface as quickly as possible after the time of death. If this is not possible, the animal should be packed into closed, individual plastic bags before freezing/refrigeration. In such a case, after the body is removed from the bag, the content must be carefully examined for parasites. This can be easily done by placing all the debris from the bag on a white sheet of paper which must be examined very carefully, as many ectoparasites are very small and barely visible with the naked eye.

The diversity of external parasites in domestic animals is relatively high. Several groups can be commonly found during necropsy. Although our aim is not to prepare an exhaustive list of ectoparasitic species found on domestic animals, we will provide below an account of the most important groups commonly encountered on the skin's surface during necropsy. Readers should also refer to Chapter 5 for further details on the preservation and principle for identification of ectoparasites.

However, examination of skin and subcutaneous tissue is essential for diagnosing also **other parasitic diseases**. All of these will be presented in this chapter.

1.3.1 Mites

All domestic animal species can be parasitized by various species of mites. Some mites (i.e. *Dermanyssus* spp., *Cheyletiella* spp.) are located on the skins surface (fur, feather, skin). Finding and collecting these mites can be done after a **very detailed examination of the body surface** or after **rubbing or combing** it over a white sheet of paper. In very fresh, unfrozen carcasses, mites are usually mobile and can be easily observed. In carcasses which have been frozen prior to necropsy, identification of dead mites is difficult, and we recommend the examination of the debris resulting from skin rubbing or combing under a zoom stereomicroscope. To collect these mites, a fine tweezers should be used.

Mites located on the skin surface can be collected also using the **adhesive tape method**. For this method, after the firm application of the tape with its adhesive side onto the skin surface, this is transferred to a microscopic slide and examined. Submacroscopic mites will adhere to the tape.

In the case of mites located in the deeper layers of the skin (e.g. *Demodex* or mange mites of genera *Sarcoptes*, *Chorioptes*, *Psoroptes*) their finding requires the microscopic examination of **skin scrapings**. Skin scrapings are performed using a scalpel blade which is firmly rubbed against the skin.

Usually skin scrapings are collected from areas where the skin shows lesions like: crusts, loss of hair etc. After the scraping is perform, the content adhering to the scalpel blade is placed on a microscopic slide, cleared with lactophenol, covered with a cover slip and examined under the light microscope (figure 2). To enhance the adherence of debris and mites to the blade, this could be greased with lactophenol prior the scraping. For increasing the sensitivity, the skin scraping should be performed thoroughly enough to reach the deep layers of the skin. Moreover, repeated examinations are recommended, mainly from the marginal areas of the lesions. Skin scrapings can be used not only for the detection of mites but also for spores and hyphae of dermatophytes (*Microsporum*, *Trichophyton*).

Special attention should be paid in birds which can harbour mange-causing mites (*Knemidocoptes* spp.) not only on the body but also on their legs or beak.

The mites collected from the skin should be preserved in 70% ethanol for further analysis in properly labelled vials.

1.3.2 Ticks

Ticks can often be found on the body of animals during necropsy (figure 3). Usually they are located in areas of the body where the skin is thinner like the head, ears, neck, axilla, abdominal region, inguinal area etc. Hence, the thorough examination of these areas is essential for finding ticks. Moreover, the location of ticks on the body of animals is also dependant on the species and developmental stage. Ticks are present on animals in various developmental stages and status of engorgement; hence their size is heterogeneous, from less than 1 mm in larvae to more than 2 cm in fully engorged females. Finding the small larvae in the thick fur of the animals could be challenging and require a very careful examination. Ticks should be removed from the skin of dead animals using a very thin, sharp but strong tweezers. When examining dead animals, non-attached ticks can be often found crawling on the body surface or on the necropsy table. They must also be collected. All ticks should be placed in ethanol for further examination (species identification, pathogen detection).

1.3.3 Fleas and lice

Domestic animals carry a great variety of flea and lice species on their skin. Fleas usually leave the body after the death of animals and if the carcass was not packed in a plastic bag they will often escape the diagnosis. In animals frozen immediately after their death, the fleas usually survive for long periods of time and special attention must be paid as when removed from the bag, they will jump. The fleas are usually located in those skin areas which are hidden from direct light (thick fur, abdominal region) but in dead animals this feature is not noticed. However, there are certain flea species which are found attached to the host with predilection on the head, like for instance *Echidnophaga gallinacea* in birds. Fleas must be collected using a fine tweezers so their delicate morphologic features required for identification are not altered.

Unlike fleas, where only the adult stages are parasitic, lice spend their life exclusively on their hosts during all the developmental stages (eggs, immature stages and adults). Their collection from the fur of mammals or feathers of birds is done based on careful gross examination using fine tweezers. Alternatively, like for lice, they can be collected from a white sheet of paper (figure 4) placed under the animals, as described for mites. Lice must be preserved in ethanol for further laboratory analyses.

1.3.4 Other ectoparasites

Except mites, ticks, lice and fleas, also other parasites can be found (rarely) on the body surface of dead animals during necropsy. Most of the biting insects (mosquitoes, sandflies, biting midges, horse flies etc) are almost never found on carcasses as they are only feeding for very short periods of time and they will not attack only living hosts. However, certain groups like louse flies can remain in the deeper parts of the fur and careful examination must be performed to detect them.

Special attention must be given to insect larvae or eggs present of the skin surface, hair or feathers of dead animals or in various natural orifices (eye, ear, anus, genital, cloaca) of the carcasses. In the vast majority of cases, they are not true parasites, as many fly species will lay their eggs or larvae on the skin after the death of the host (figure 5).

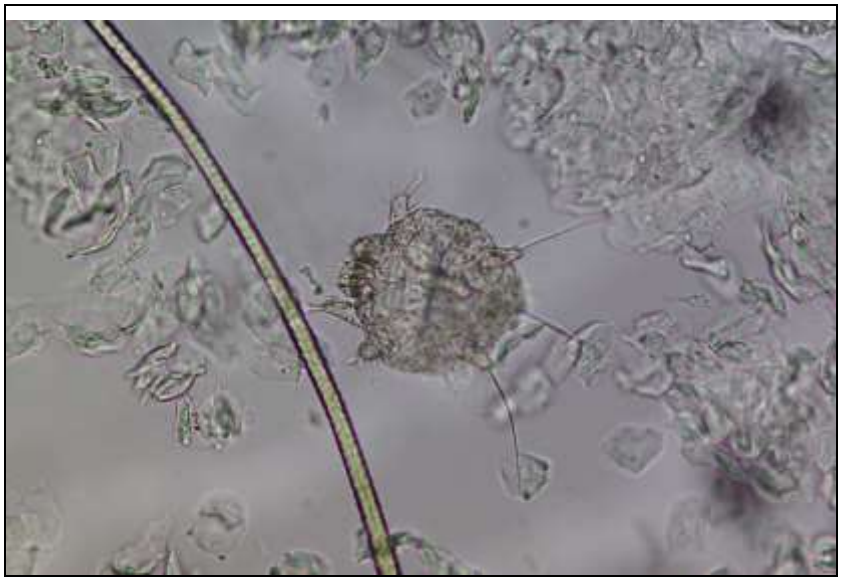

Figure 2

***Trixacarus caviae*, a parasite of guinea pigs as seen in direct examination of skin scraping cleared with lactophenol.**

Figure 3

***Ixodes vespertilionis*, a tick specific to bats pictured here while still attached to the body of its dead host.**

Figure 4

***Gliricola porcelli*, collected on a white sheet of paper from a dead domestic guinea pig.**

However, caution should be taken for differentiation from the true cutaneous or genital myiasis. Nevertheless, also the eggs or larvae of sarcophagous flies are important from a forensic perspective. Forensic entomology is a science dedicated to the study of these insects and readers should refer to such focused monographs.

Figure 5

Larval flies on decomposing carcass of cormorant.

Figure 6

Larval stages *Hypoderma diana* located in the subcutaneous tissues of European roe deer.

1.3.5 Subcutaneous parasites

Although not truly external parasites, many parasitic species are located under the skin of domestic animals (figure 6). These include various nematodes (*Dirofilaria repens* in carnivores; *Parafilaria* spp. of livestock), larval cestodes (subcutaneous coenurosis in rabbits) or larval insects (*Hypoderma* spp. larvae). Detection of these parasites during the necropsy involves various approaches. Some of the species (*Parafilaria*, *Hypoderma*) are producing subcutaneous nodules which are normally visible during the examination of the skin surface. Performing a small incision in these nodules may reveal the parasites. In other cases, the skin of the animal should be carefully removed and the exposed subcutaneous tissue must be examined carefully for the presence of nematodes (*Dirofilaria*, *Cercopithifilaria* - in dogs, *Stephanofilaria* - in cattle, *Parafilaria* and *Onchocerca* - in horses and cattle).

Larvae of *Taenia serialis* ("*Coenurus serialis*") are found as 3-5 cm vesicular, cyst-like structures under the skin or in the intermuscular connective tissue of rabbits or hares.

1.3.6 Unicellular parasites in the skin

There is a great variety of protozoan parasites responsible for systemic infection in animals. Sometimes, this systemic distribution involves the skin and tissues cysts of protozoans like *Toxoplasma* or *Neospora* could accidentally produce cutaneous lesions. These lesions should be differentiated from other medical conditions and the confirmation must follow a specialized histopathology diagnosis. However, certain severe protozoal diseases (e.g. leishmaniasis) are usually producing typical cutaneous lesions. These should be confirmed during necropsy and skin biopsies must be collected from the lesions. In the case of canine leishmaniosis, the typical skin lesions include mainly cutaneous necrosis. In the case of bovine besnoitiosis, the typical lesions are also cutaneous and include skin oedema and scleroderma.

1.4 Examination of the external mucosae and cavities

Several parasites have the typical habitat at the surface of external mucosae (i.e. conjunctival sac, buccal cavity, nasal cavity, genital mucosae, ear canal). Their careful examination is essential for the detection of such parasites. For instance, nematodes of genus *Thelazia* are located in the conjunctival sac of the eye in several domestic animal species (figure 7). In dogs, the still enigmatic *Onchocerca lupi* is located in the deep surface of the eye ball. For its demonstration, the eye should be removed from its orbit during necropsy. Molecular detection of *Leishmania* DNA is often successful in swabs collected from the conjunctival mucosa.

Dogs, cats and rabbits are often infected with ear mites (general *Otodectes* or *Psoroptes*). If crusts are present in the ear canal (figure 8) they should be examined after clearing with lactophenol under the microscope for observing such mites.

In certain groups of domestic animals (chicken, pigeons), flagellated protozoa of genus *Trichomonas* are responsible for severe buccal lesions. In very fresh and warm carcasses (hours old), these protozoans can be visualized under the microscope if fresh swabs are collected using physiological saline (figure 9).

It is very important to examine thoroughly the external openings of the genital system, both in females in males. The vulva/vagina but also the preputial cavity is often the site of severe infections with larval flies, responsible for genital myiasis. Larval flies can also be present in the external milk canal of the mammary glands in females. Their detection requires sometimes the longitudinal dissection of this canal.

The nasal cavities can also harbour parasites. In sheep, the nasal bot fly, *Oestrus ovis* are located here (figure 10). The pentastomids *Linguatula serrata* is located in the nasal cavities of dogs and other canids (figure 11). In dead animals these parasites can be removed by opening the nasal cavities or by washing with them with high pressure water.

1.5 Examination of body cavities

After the examination of the skin, external mucosae and cavities and subcutaneous tissues, the next step of the necropsy is the opening of the abdominal and thoracic cavities. Their examination for parasites is often omitted during standard necropsy. However, the serosal layers of the body cavities can also harbour parasites, free or encysted. Several nematode species are known to inhabit freely the peritoneal layers in domestic animals: *Setaria* in horses, *Acanthocheilonema* in carnivores or *Diplotriaena* in birds (figure 12). As most of these nematodes are whitish and very fine (thin), observing them is not easy. Hence, an experienced observer and a very vigilant examination is required. The serosal surface of the oesophagus can also be a site for parasitic infection. Adult nematodes of genus *Gongylonema* are located at this level in pigs or ruminants as a long, slender round worm, with very sinuous aspect (figure 13). The external surface of the oesophagus in cattle and buffaloes is often host to whitish, lens-like cysts of genus *Sarcocystis*.

The peritoneal cavity can also show signs of parasitic migration, visible mainly as lesions and often without the direct observation of parasites. Most of such migrating parasites are in their larval forms and

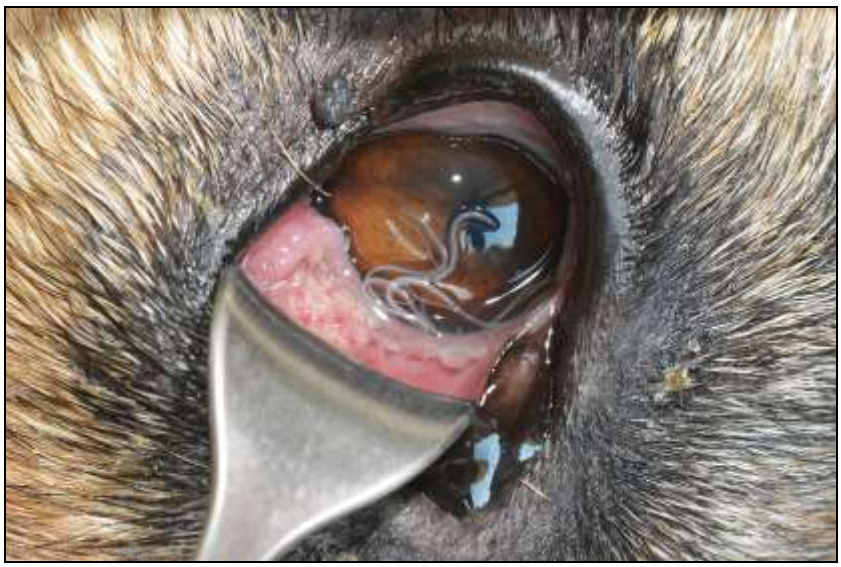

Figure 7

***Thelazia callipaeda*, parasitic in the conjunctival sac of domestic dog.**

Figure 8

Typical crusty lesions in rabbit infected with *Psoroptes* sp.

Figure 9

Collection of buccal swab for the direct diagnosis of trichomoniasis in pigeons.

Figure 10

Larva of *Oestrus ovis* in the nasal cavity of a sheep.

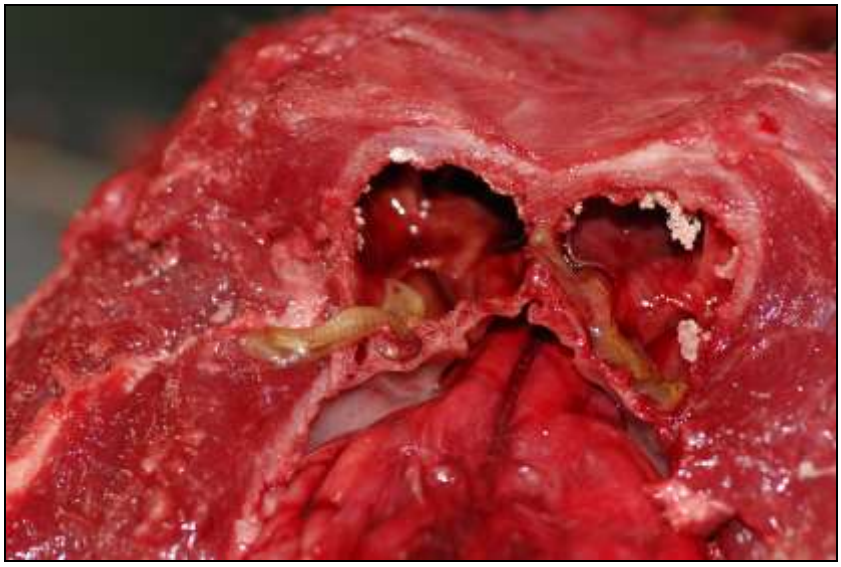

Figure 11

Adult *Linguatula serrata* in the nasal cavities of a dog.

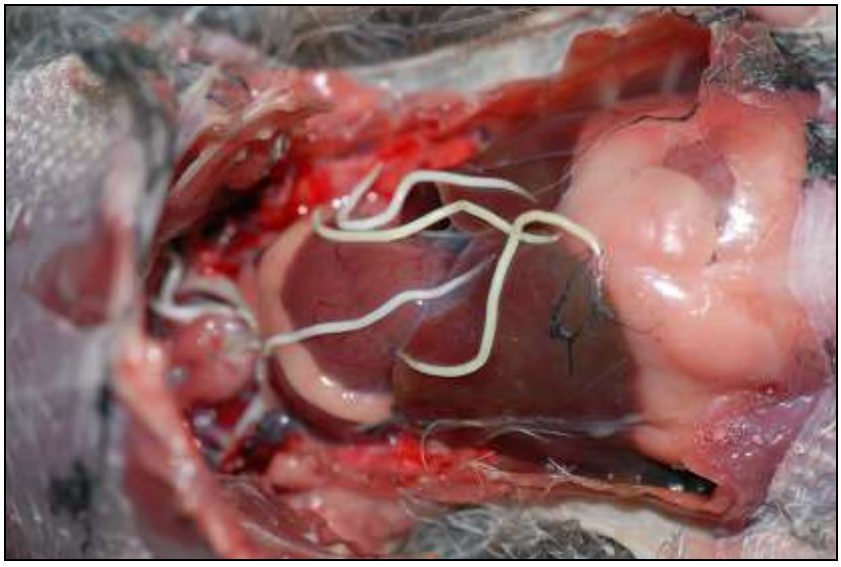

Figure 12

Nematodes of genus *Diplotriaena*, parasitic in the body cavity of a wild bird.

Figure 13

***Gongylonema* sp. in the oesophageal serosa of a wild boar**

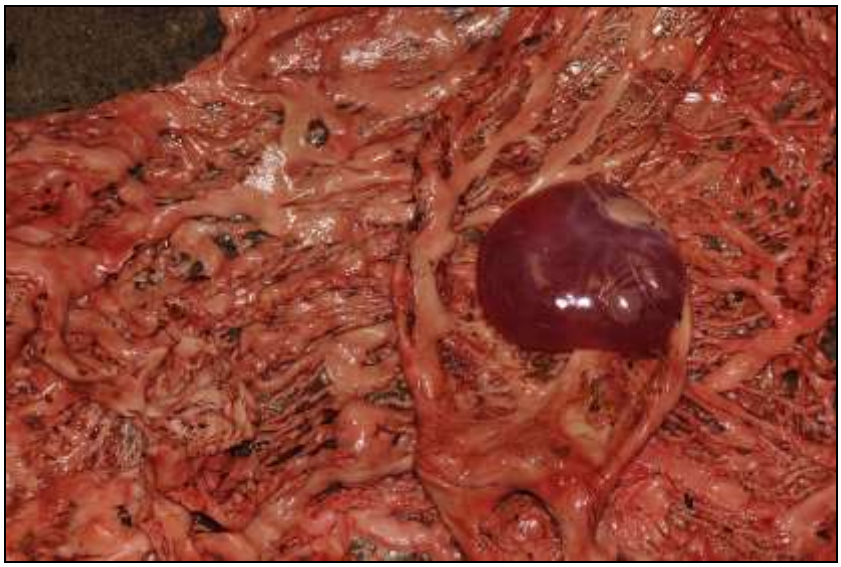

Figure 14

***Cysticercus tenuicollis* cyst on the abdominal serosa of a pig.**

Figure 15

Larval stages of *Linguatula serrata* in the mesenteric lymph node of a goat.

are too small for macroscopic detection. Such migration-associated lesions can be detected as fibrous path in the structure of the peritoneal layers covering the intestines or other abdominal organs. In rabbits for instance, the migration of the larval forms of *Taenia pisiformis* (known as *Cysticercus pisiformis*) are leaving migration traces on the surface of the liver. Fully developed larvae will also be visible as clusters of fluid-filled vesicles (cysts) on the liver surface. The surface of the other abdominal organs (spleen, kidneys, pancreas) must be also examined during this step, mainly for the presence of larval *Echinococcus*. Similarly, the larval forms of *Taenia hydatigena*, known as *Cysticercus tenuicollis* (figure 14) are attached to the peritoneal tissue as large cystic vesicles, often in large numbers.

When examining the body cavities, the parasitological necropsy must include the examination of lymph nodes. Lymph nodes are usually the first to respond to any infectious invasion and in such cases are significantly enlarged. System parasitic invasions like those with *Toxoplasma gondii*, *Sarcocystis* spp., *Hepatozoon canis*, *Leishmania* spp., *Babesia* spp., *Theileria* spp. will be often associated with lymph node hypertrophy. Collection of aspirates or biopsies from lymph nodes followed by cytology examination can reveal parasitic stages. Moreover, the lymph nodes can be the site of infection with metazoan parasites. The larval stages of *Linguatula serrata* are located in the lymph nodes (figure 15) of herbivore intermediate hosts (usually sheep and goats). To visualize them, cross sections should be performed in the lymph nodes.

1.6 Examination of internal organs

This is probably the step of the necropsy which will reveal the highest number of parasites (figure 16). After the body cavities are opened, all the organs must be removed and clearly separated by organ systems. Each of the following systems will be examined separately: digestive system, respiratory system, circulatory system, urinary system and reproductive system.

1.6.1 Examination of the digestive system

The digestive system is probably the host to most of the internal parasites of domestic animals. Virtually all domestic animal species carry parasites in their digestive tract, mostly in the intestine. However, also the other segments are equally important. During the necropsy, the digestive system must be examined entirely, from the pharynx and oesophagus (the examination of the buccal cavity has been described above) to the rectum.

The ***oesophagus*** can harbour various parasites in domestic animals. Probably the most prominent example is *Spirocerca lupi*, the cancer-causing nematode of dogs. In birds, in relation to the oesophagus is the crop. In domestic poultry, the crop and the oesophagus can host several types of parasites, the most common being nematodes of genus *Capillaria*. As they are very fine nematodes, the examination of the content should be done carefully. In pigeons, the crop can be the site of infection with flagellated of genus *Trichomonas*. However, they can be found by direct microscopic examination using warm physiological saline only in very fresh carcasses.

The ***forestomachs*** of ruminants are embryological related also to the oesophagus. Several parasites can be located here, but the most prominent are trematodes of genus *Paramphistomum* (figure 17). They are large enough and reddish in colour, so they are relatively easily detectable, fixed to the ruminal papillae.

The ***stomach*** is also a very important site for parasites. Its content must be examined in detail, as many parasites which are not attached can be mixed in the gastric content. Serial washings may be required (the method is described below). Various groups of parasites can be found in the stomach, depending on the species. Some of them are free in the stomach content, others are fixed to the mucosa while others can be located deeply in the stomach wall.

Many domestic species carry nematodes (i.e. spirurids in pigs (figure 18), strongyles in ruminants or rabbits, etc). Particular attention must be paid in ruminants where the nematodes located here are usually very thin and they can escape detection by a non-trained eye. In horses, the stomach can be the host of larval insects of genus *Gasterophilus*, which are usually strongly attached to the mucosa, in clusters. Several species of parasitic nematodes are producing nodular lesions in the stomach. For instance *Physaloptera* or *Cylicospirura* are producing gastric nodules in domestic carnivores. In waterfowl,

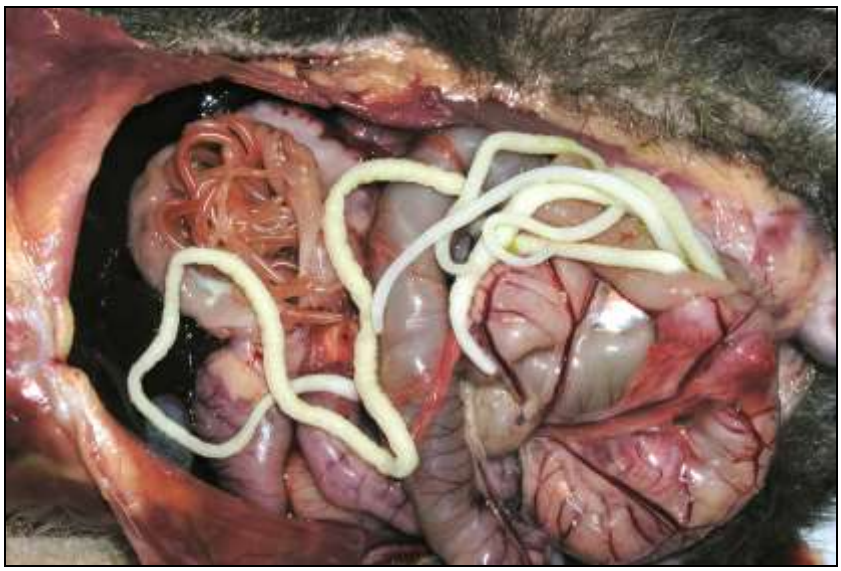

Figure 16

Poliparasitism in hedgehogs as revealed during necropsy.

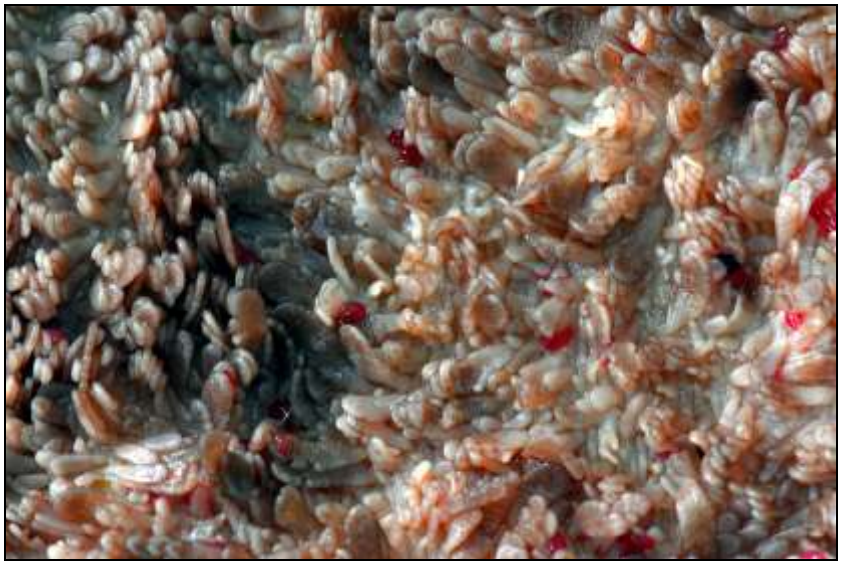

Figure 17

The red colour of *Paramphistomum* sp. is evident between the ruminal papillae.

Figure 18

Gastric spirurid nematodes in the content of the gastric mucosa of pigs.

nematodes of genus *Eustrongylides* can be found in the deeper layers of the stomach wall, producing tumour-like lesions (figure 19). Birds are also particular, as they usually have two stomachs. The gizzard can for instance harbour strongyles of genus *Amidostomum* in waterfowl or spirurids of genus *Acuaria* in gallinaceous birds. Nematodes of genus *Tetrameres* in various species of domestic and wild birds are difficult to be detected as they are deeply embedded in the proventricular glands.

The ***intestine*** is the site for a extremely large diversity of parasites. It is beyond the scope of this guide to provide a list of intestinal parasites in domestic animals. However, certain representative examples will be used throughout this section, but it should not be treated as an exhaustive list. First of all, it is important to understand that during the parasitological necropsy, the intestine must be examined along its entire length. The intestine is placed in a straight position, uncoiled on the necropsy table. It should be always opened using a scissor, along all its length, including the cecum (or both ceca in birds). As macroscopic, sub-macroscopic but also microscopic parasites can be found in the intestine, it must be examined using various approaches.

After the intestine is completely open along its entire length, the first step is to perform a gross, visual inspection for macroscopic parasites (trematodes, tapeworms, nematodes, acanthocephalans, insect larvae) (figures 20-21). They should be collected with fine tweezers and conserved according to the objectives of the subsequent laboratory methods to be employed. Initially, all macroparasites are placed in Petri dishes, in physiological saline (figure 22).

In general, formalin is indicated for morphological examination while ethanol is the choice if molecular methods are to be considered. Usually, we recommend a combination of the two approaches. In the case of tapeworms, their collection should be done with caution, as usually the fine scolex is attached to the intestinal mucosa and it can be broken and remained attached.

After the collection of macroparasites, the next step is collection of intestinal biopsies or samples for intestinal cytology. Usually these should be done according to the host species for unicellular parasites (coccidia, other Apicomplexa). For such purposes, it is recommended that the specimens are collected from the intestinal areas with visible lesions. Other intestinal protozoa like ciliates or flagellates can be found only in very fresh carcasses using a direct wet mount in (warm) physiological saline from the intestinal content. Biopsies must be collected by the standard pathology protocols in formalin. For the cytological diagnosis of protozoan, clean microscopic slides must be gently applied to the mucosal surface of the intestine and stained.

A very important step of the parasitological necropsy during the examination of the intestine is the collection of faecal samples. We strongly recommend this, followed by immediate examination using coprological methods (see chapter 3). The results of the intra-necropsy coproscopy can be very useful as an indication for the presence of certain more difficult to detect parasites.

One of the most important parasitological techniques during the examination of the intestine is the successive washing method. This method is recommended for finding small and/or thin intestinal parasites. Often, the small nematodes (i.e. *Strongyloides*, trichostrongyles), larval nematodes, small intestinal trematodes (i.e. *Alaria* or other trematodes in dogs or intestinal flukes in birds) are very difficult or almost impossible to be detected during the visual inspection of the intestinal mucosa. Moreover, one of the most important zoonotic parasites, cestodes of genus *Echinococcus* are so small, that their detection in the intestinal content is extremely unlikely macroscopically. The successive washing method (repeated sedimentation) is significantly increasing the sensibility of these findings. The method is simple but time-consuming. However, its use is essential for a complete parasitological necropsy. The steps to be followed are:

- the longitudinally opened intestine is placed between the handle of a large surgical scissor over a large metal or plastic tray with water;
- by applying a moderate pressure on the closed scissor, the intestine is slowly pulled through the handles while its content together with the superficial layer of the mucosa are collected in the tray; this step can be repeated several times until all the intestinal content is collected; preferably,
- after all the content is collected in the tray, all the content is placed in sedimentation cones;

Figure 19

Eustrongylides excisus **adults deeply buried in the wall of the stomach in a cormorant.**

Figure 20

Heavy intestinal infestation with adult *Macracanthorhynchus hirudinaceus*.

Figure 21

Anoplocephala perfoliata **adults found during horse necropsy in large intestine.**

Figure 22

Adult tapeworm *Moniesia expansa* after necropsy of lamb small intestine.

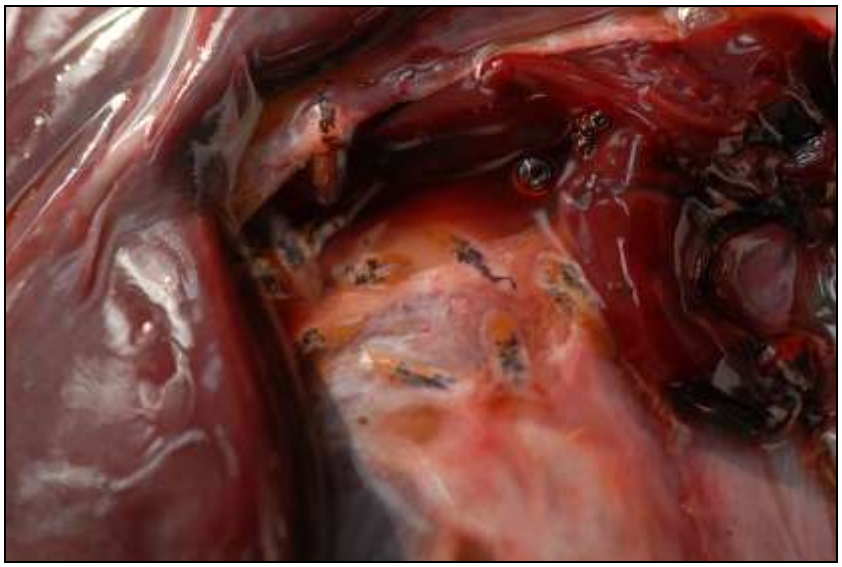

Figure 23

Adult flukes *Dicrocoelium dendriticum* in liver.

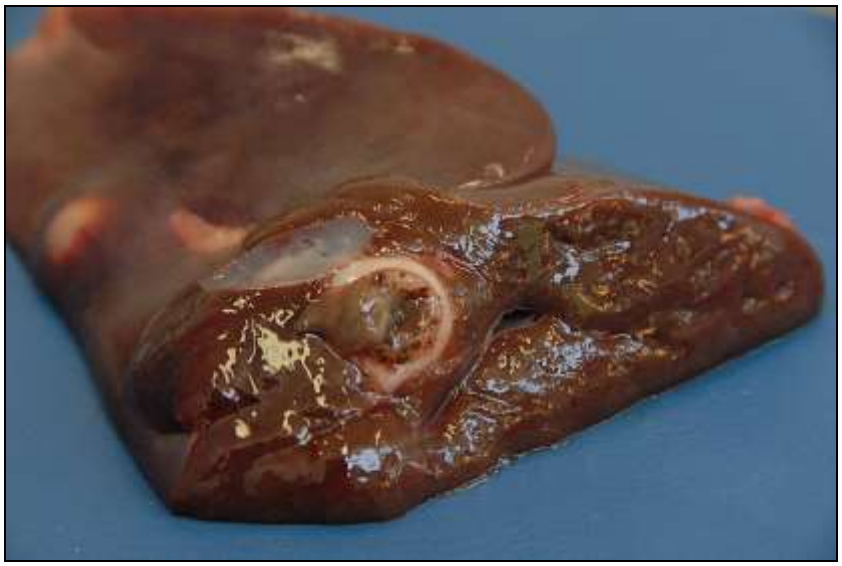

Figure 24

A cross-section of liver with liver fluke *Fasciola hepatica* in a bile duct.

- after several hours, the supernatant is eliminated and new clean water is added over the sediment;
- this step is repeated several times until the supernatant will remain clear;
- the sediment is placed in small volumes in Petri dishes and the content is examined under the dissection microscope;
- the helminths should be collected as mentioned above;
- all the above steps should be performed separately for each intestinal segment (duodenum, jejunum etc.) to ensure a correct evaluation for each.

The ***liver*** is an important organ for parasites. In various host species it can be infected with a great diversity of parasites. The most common examples include: *Fasciola* in herbivores, *Dicrocoelium* in ruminants, larval *Echinococcus* in herbivores etc. Liver macroparasites can be located on the surface of the liver (*Cysticercus*), in the liver parenchyma (*Echinococcus* larvae) or in the bile ducts and gall bladder (*Fasciola*, *Dicrocoelium*) (figure 23). Finding these parasites requires different methods:

- parasites located on the liver surface can be observed by visual inspection;
- parenchyma parasites like larval *Echinococcus* can be observed because of evident organ deformation or, smaller ones, after sections through the organ;
- helminths in the bile ducts are found following carefully dissecting the ducts with a fine scissors.
- a more sensitive method for detecting especially the young forms of *Fasciola hepatica* is to squeeze or tease the liver tissue through the cut sections (figure 24).

An interesting situation can be found in pigs heavily infected with *Ascaris suum*. In this case, due to high parasite loads, individual nematodes can migrate erratically to the common bile duct or even to the liver producing ruptures.

In rabbits, the liver is the host of the coccidia *Eimeria stiedai*. As this is a unicellular parasite located in the epithelial cells of the bile ducts, its presence can be demonstrated only microscopically. Normally, in the clinical disease, the infection with these hepatic coccidia is associated with severe necrotic hepatitis. Sections through the necrotic lesions will reveal a whitish fluid. This must be placed on a slide and examined under the microscope for the presence of various developmental coccidial stages.

1.6.2 Examination of the respiratory system

The respiratory consist of tubular and parenchymatous structures which can all carry parasites. The examination of nasal cavities has been described above.

The larynx is rarely a site for parasitic infection. In tropical areas, on the laryngeal mucosa, nematodes of genus *Mammomonogamus* can be present.

The ***trachea*** and the ***bronchi*** should be examined for the presence of nematodes in birds (for *Syngamus*), ruminants and horses (*Dictyocaulus*), pigs (*Metastrongylus*) and carnivores (*Oslerus*, *Eucoleus*, *Crenosoma*). To find all these respiratory nematodes, the **trachea and the bronchi must be longitudinally opened** and the mucosa surface examined in detail. Some of the nematodes are very fine (i.e. *Eucoleus*) and their very thin body is difficult to see, mainly in the level of infestation is low. In other cases, the nematodes form large balls which obstruct the airways (figure 25).

Other typical lungworms are located deeper in the air tract. Performing **serial sections to the lungs** is required for their identification. Protostrongylids of small ruminants and *Aelurostrongylus* of cats are such examples. The serial sections through the lungs are also indicated as a method for finding otherwise not-visible cysts of *Echinococcus*.

The lungs are often a migration site for other parasitic nematodes (ascarids, hookworms) and their larvae are located deep in the pulmonary tissue. Despite their small size, they can be found if small pieces of lung tissues are examined using the Baermann method (see chapter 3).

1.6.3 Examination of the circulatory system

The cardiovascular parasites can be detected during the necropsy mainly in carnivores. The right ventricle and the initial segment of the pulmonary

arteries should be inspected for the presence of *Dirofilaria immitis* (figure 26) and *Angiostrongylus vasorum* in dogs and cats.

When performing necropsy in horses, the cranial mesenteric artery and its branches must be examined for the presence of lesions (thrombosis, arteritis, aneurysms). At a closers inspection under the dissecting microscopes, these lesions might reveal the presence of the larval stages of *Strongylus vulgaris*.

During the necropsy, blood (or blood clot) should be collected for parasitological laboratory tests. Hemoparasites (located in the erythrocytes, leukocytes or free in the plasma) can be eventually detected also after the death of the animals. The reader must refer to the dedicated section (chapter 2). The microscopic examination of the typical, stained, blood smears is not feasible due to very rapid haemolysis after the death of animals. However, erythrocyte parasites as *Babesia* or *Theileria* could be detected in smears from the spleen or the brain cortex capillaries.

1.6.4 Examination of the urinary system

The urinary system rarely hosts parasites in domestic animals. Notable exceptions are dogs and cats. The surface of the urinary bladder must be examined in carnivores under a dissection microscope, for the detection of the very fine, hair-like *Capillaria plica* nematodes on its surface. *Dioctophyma renale*, one of the largest nematode of domestic animals can be detected in the kidney pelvis.

Urine can be also collected during necropsy and after its centrifugation, the microscopic examination of the sediment can reveal the presence of parasitic stages (eggs).

1.6.5 Examination of the reproductive system

Although few parasites are located here in domestic animals, the examination of the reproductive system can yield important results. Few parasites are located at this level in domestic animals. One notable example is *Prosthogonimus*, a genus of flukes parasitic in the oviducts, cloaca or bursa of Fabricius during the necropsy of infected chicken, turkeys, ducks or geese.

However, probably the most important part of the post-mortem examination of the genital system is the collection of samples from foetuses or the necropsy of aborted specimens. Many parasites in domestic animals are responsible for abortions and often, these are associated with severe and characteristic lesions in the foetal tissues. The heteroxenous coccidia *Toxoplasma gondii* and *Neospora caninum* can be detected in fresh specimens using various laboratory techniques: histopathology, immunohistochemistry, molecular biology. Proper samples should be collected for employing these techniques from the brain, myocardium, spleen or liver. Any body fluids present are not normal and using a syringe they should collected as well for further analysis (figure 27). An important tissue is the placenta, which could serve the same purposes as the tissues mentioned above.

1.6.6 Examination of the nervous system

Many system parasites can be located in the tissues of the central nervous system. Typically, *Toxoplasma gondii* produce necrotic lesions in the brain. Biopsies should be collected during necropsy for further histopathology or other more specific techniques (i.e. immunohistochemistry).

In sheep, it is recommended that the skull should be opened and the brain examined, as this is the typical location of the *Coenurus cerebrals*, the larval stages of *Taenia multiceps*, a tapeworm of dogs. The cysts of *Coenurus* are vesicle-like structure, with variable size, usually several cm, filled with a clear liquid.

In cattle, early larval stages of *Hypoderma bovis* can be found in the spinal canal, in the fat between the dura mater and the vertebral body.

1.6.7 Examination of the musculo-skeletal system

The parasitological necropsy should include also the examination of muscle and ligaments. The examination for muscular parasites is also a compulsory technique during the slaughterhouse exam. Although the majority of the parasites present

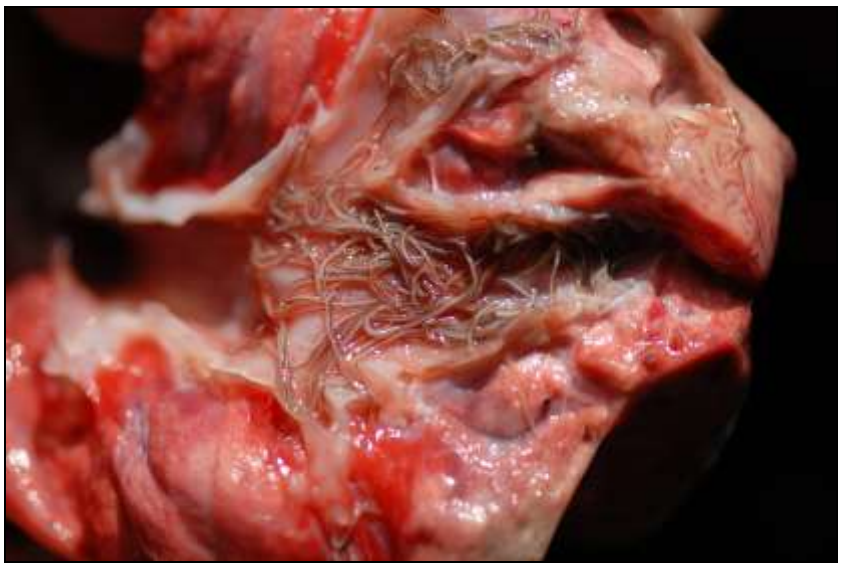

Figure 25

***Metastrongylus* sp nematodes are obstructing the large bronchi in a pig.**

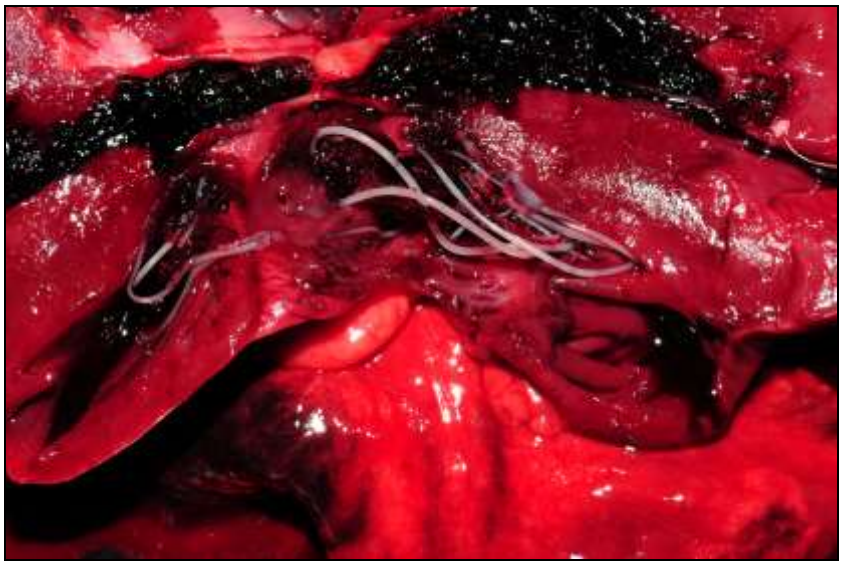

Figure 26

Adults of *Dirofilaria immitis* in the heart of a golden jackal.

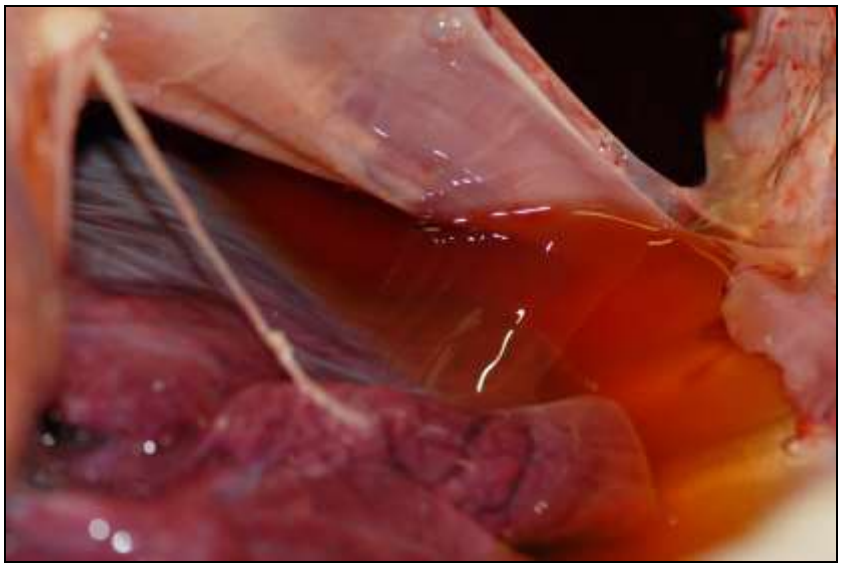

Figure 27

Peritoneal sanguinolent fluid in a abortion caused by *Neospora caninum*.

in the muscles are generally visible during gross examination, some of them require special techniques followed by microscopy. Pigs and cows can harbour larval stage of *Taenia solium* and *T. saginata*, a condition known as muscular cysticercosis. The *Cysticercus* cysts are bladder worms and have a vesicle-like appearance, filled with a clear liquid. They are located in the skeletal muscle, predominantly in the masticatory muscles but also in other areas. Heteroxenous coccidia of genus *Sarcocystis* are also located in the skeletal muscle of their intermediate hosts (horses, ruminants, pigs). Sometimes, the cysts of *Sarcocystis* are visible macroscopically, but other times their presence can be detected only during microscopic examination. The gross appearance of the macrocysts of is usually lens shape, 1 mm - 1 cm size, with a whitish colour and fat-like aspect. Microcysts are visible only microscopically and they are usually accidentally found during the trichinelloscopy in pigs. Other systemic tissue parasites can produce muscular lesions and their presence can be detected in histological section from the skeletal muscles. Muscular biopsies should be collected during necropsy for the diagnosis of the *Hepatozoon americanum* infection in dogs.

Typical muscular parasites are various species of *Trichinella*. They cannot be seen macroscopically and they do not produce gross muscular lesions. The detection of the larvae is done either by trichinelloscopy or by artificial digestion. Reader can refer to these methods in general veterinary parasitological textbooks or food hygiene guides.

Rarely, the ligaments can also be infected with parasites. *Onchocerca cervicalis* and *O. gutturosa*, are nematode located in the *ligamentum nuchae* of horses and cattle, respectively.

Further details on the specific methods to detect tissue parasites are given in Chapter 3.

2 BLOOD AND BLOOD SAMPLES

Blood is a key-material, used during examination of alive animals. Easy *intra vitam* collection from the animals makes the blood essential for detection and identification of various parasitic organisms, ranging from protists to helminths. The presence of circulating stages of parasites in peripheral blood often replects the life cycle of the parasit, showing seasonal and also diurnal fluctuation. Microfilariae are notoriously known for their periodicity in the peripheral blood of the host. The different periodicities of microfilariae correspond with the biting habits of their principal vector.

Detection methods include various techniques such as simple direct examination of blood under a light microscope, blood smear staining methods, concentration and staining techniques, fluorescence microscopy, and molecular detection. Moreover, the collected blood and separated blood serum represent an essential material for a range of assays detecting the antibodies as part of indirect diagnostics.

2.1 Blood samples collection

Collection of the blood in the field is certainly specific and rather different compared to blood collection at the veterinary clinic (figures 28-29). It is invasive technique which requires particular skills (figure 30) of collector and manipulators/assistants. The procedure should be as fast as possible to reduce the stress of the animal and to avoid coagulation of the blood. Therefore, it is necessary to prepare all the equipment before the sampling. From practical point of view, it's better to collect the blood with syringe and immediately transfer to collection tubes.

2.1.1 Equipment

Equipment for blood collection differs according to sampled animal species. There is a range of more or less sophisticated modification, including several commential systems (S-Monovette, BD-Vacutainer...). The final preference for particular way of blood collection usually involves personal preferences, price, availability, animal species, purpose for the blood collection, volume need etc etc. Basic equipment for blood samples collection includes:

- restraining devices
- needles, syringes
- vacuum collection tubes (e.g. Hemos,) - for large animals
- tourniquet – for small animals
- disinfection, (shaver)
- cotton pads, paper towels
- collection tubes (according to intended diagnostic tests)
- slides, slide boxes, markers, notebook

2.1.2 Preparation of animal for sampling

Sampled animal is immobilized to avoid injury of itself and manipulators (figure 31). Manipulation should be calm to avoid stress of the animal (figure 32). It is recommended to let the animal owner/keeper to immobilize the animal. Collection site is prepared by shaving of the hair (in mammals, when possible/needed) and disinfection with solution containing alcohol. Sterile conditions should be kept in all blood collection methods. An animal's blood volume is about 10% of its body weight. In general the volume of blood removed at any bleeding should not exceed 1.5% of body weight (assuming that 1 ml of blood weighs 1 gram). If blood is collected at repeated intervals, much less volume should be collected per each sampling to avoid serious haematological problems.

Figure 28

Collection of blood from *vena jugularis externa* in a feral horse from Danube Delta, Romania.

Figure 29

Collection of blood from *vena caudalis mediana* in a cow from Danube Delta, Romania.

Figure 30

Collection of the blood in the field requires particular skills.

Figure 31

The manipulator checks if the animal is properly immobilized to avoid injuries.

Figure 32

Manipulation should be calm to avoid stress of the animal.

Figure 33

Blood samples for the more advanced laboratory techniques are prepared and preserved in the field.

2.1.3 Collection sites

- Horse: *Vena jugularis externa*
- Cattle: *V. jugularis externa, v. caudalis mediana (v. coccygea)*
- Small ruminants: *V. jugularis externa*
- Swine: *V. jugularis, v. cava cranialis, vv. auriculares*
- Dog, cat*: V. cephalica accesoria, v. saphena lateralis, v. jugularis v. femoralis*
- Rabbit: *Vv. auriculares*, (cardiocentesis – anesthetized only)
- Chicken: *V. brachialis*, right *v. jugularis*, (cardiocentesis – anesthetized only)
- Snake: cartiocentesis, *v. coccygea* (larger snakes)
- Lizard: *v. coccygea, v. jugularis*
- Turtle, tortoise: coccygeal veins, *v. jugularis*

After sampling keep short compression of the used vein to stop bleeding. Process the sample(s) immediately.

2.2 Preparation of blood samples for examination and preservation

Some diagnostic methods can be used directly in the field laboratory with basic equipment, whereas other techniques require special labs and instruments. The methods which can be easily performed in the field lab include fresh blood microscopy, microscopy of stained thin blood smear and Knott's test. Other methods such as immunological tests and molecular detection are dependent on particular laboratory equipment. Samples for these advanced laboratory techniques are prepared and preserved in the field lab for further examination (figure 33).

2.2.1 Preparation of blood sample for serology

Serological tests are used for detection of antibodies (or antigens) from the blood sera. Serum is taken after the blood coagulation into the sterile tube. The full blood can coagulate itself or can be centrifuged (10 minutes at 800 g). Serum can be taken after blood coagulation by Pasteur pipette, by automatic pipette or by needle and syringe. For fast examination a range of quick serological tests is available. For further examination in specialized laboratory, serum samples are frozen at -20 °C. As every defreazing decreases the activity of immunoglobins, it should be avoided as much as possible and samples should be transported frozen.

In case freezer is not available, sera can be stored using electrophoretic paper (Whatman) discs, dried and stored at +4 °C. When stored at room temperature (18 °C) significant decrease in titre can occur. Some studies describe glycerol preservation for certain serology tests.

2.2.2 Preservation of blood sample for consequent molecular examination

Currently, range of blood parasites can be diagnosed based on the DNA detection. There are several ways how to preserve the blood for consequent DNA isolation. Probably the easiest and most commonly used is collection to the EDTA tubes. EDTA tubes contain a certain amount of anticoagulant that needs to be mixed in an exact proportion to the blood. Gently invert the EDTA tubes with collected blood several times to reach a proper mix of additive and blood; do not shake. Tubes are then marked and stored in a freezer at least at -20 °C. Alternatively, blood can be stored in tubes with 70% ethanol or frozen. Preferably, even the EDTA or EtOH preserved samples houdl be frozen for longer storage.

In large scale epidemiological studies, Flinders Technology Associates (FTA) cards are becoming increasingly popular for the rapid collection and archiving of a large number of blood samples for molecular analysis. FTA® paper is a commercial product (Whatman) consisting of filter paper impregnated with a mix of chemicals which serve to lyse cells, to prevent growth of bacteria, and to protect the DNA in the sample. Blood samples are applied to the FTA® paper and air-dried, then put in the tube. Tubes are stored at room temperature until processed in the specialized laboratory. Relatively low volume of preserved blood (and resulting low volume of isolated DNA) can complicate the repeated examination and/or detection of various pathogens from FTA papers.

If other mean of preservation is accidentally not available, the small volumes of blood for molecular examination can be dried on normal filtration paper, soft tisse paper, toilet paper etc.

2.3 Microscopy of native sample

Principle

For quick detection of extracellular blood parasites (microfilariae, trypanosomes), preparation and immediate examination of native sample is possible. It is performed immediately after blood collection from fresh blood or alternatively from fresh uncoagulated blood (in EDTA or heparin). Moving parasites are visible under light microscope. As this is not a concentration techniques, parasites occurring in low parasitaemia are difficult to detect.

Equipment

- Glass slides, cover slips
- Pipettes
- Microscope

Procedure

1. Put a small drop of uncoagulated blood on the slide and cover with cover slip
2. Examine by microscope immediately

Note: fresh blood is coagulating quickly, especially at higher temperatures (more than 20 °C).

2.4 Preparation of thin blood smears

Principle

Permanent stained thin blood smears are used for detection of intracellular and extracellular parasitic stages present in peripheral blood of the hosts. It is fast and easy technique which is performed immediately after blood collection. Blood films are made on glass microscopic slides. After drying, the slides are fixed, stained and examined using light microscope.

Equipment

- Clean standard size glass slides
- Stable flat mat
- Markers
- Slide box

Procedure (figure 34)

1. To do a blood smear you need two slides. On one slide the blood sample is placed - this is the "sample" slide. The second slide is used to smear the drop of blood (can be called the "spreader"). You can use regular microscopic glass, cover slip or „special“ thin glass spreader available on market.
2. Clean the sample slide by wiping it with alcohol, if it is not perfectly clean. Handle slides by edges only.
3. Place a small (!) drop of blood close to the end of the slide.
4. Take the spreader, and holding at an angle of about 45 deg, touch the blood with one end of the slide so the blood runs along the edge of the slide by capillary action. Push carefully along the length of the first slide to produce a thin smear of blood. Note that the blood is being dragged (not pushed!) by the spreader.
5. Completed smear must be uniform, homogeneous and moderately thin. The slide must have its long edges straight, should end before the end of the sample slide and has to pass into the end in a "feathered edge" - a region where the blood cells are well separated.
6. Air dry and fix the sample slide.
7. It is always recomendable to make 2 smears; label smears clearly with alcohol proof pencil. Once dried place in the slide transport containers for further use (e.g. staining).

The most common errors (figure 35) in the preparation of blood smears are:

- too much blood - thick smear
- too little blood - short smear
- too long time after the blood collection - blood clotting and longitudinal stripes

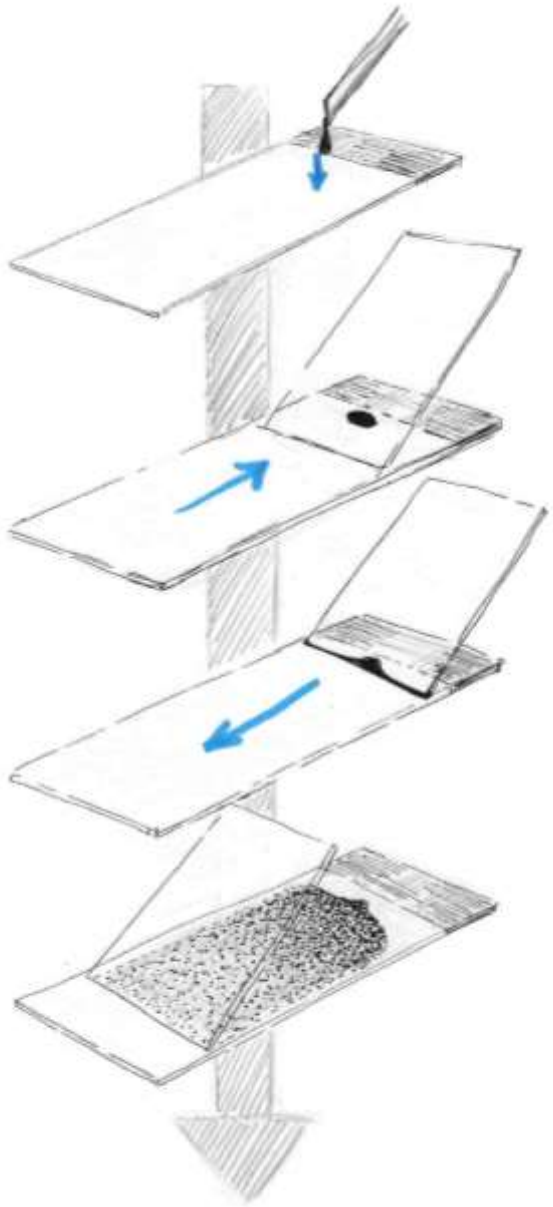

Figure 34

Preparation of thin blood smears. Small drop of fresh uncoagulated blood is smeared on the slide using another microscopic glass.

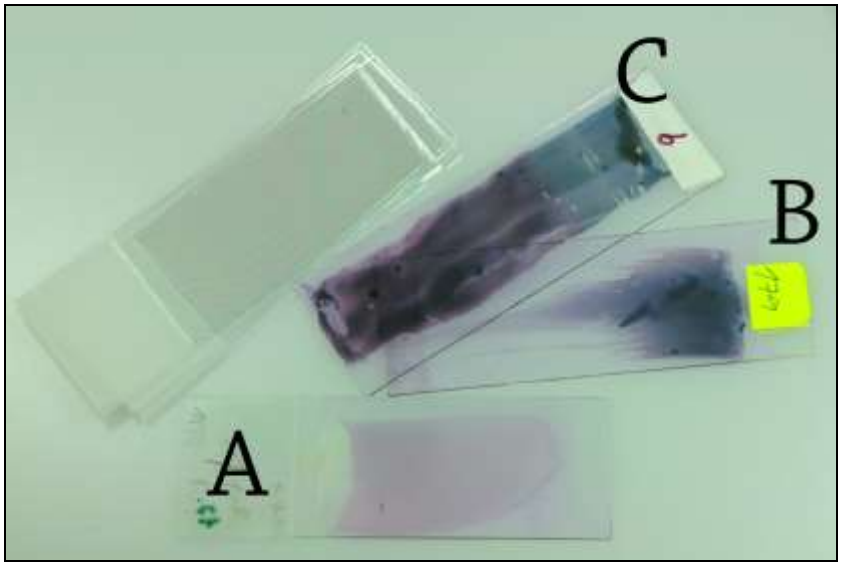

Figure 35

Permanent stained thin blood smears must be uniform, homogeneous and moderately thin (A). Short smear due to little amount of blood or too long time after the blood collection causing blood clotting and longitudinal stripes (B). Long thick smear due to too much blood, scalloped coat is caused by shaking hand (C).

- poorly degreased glass - speckled smear
- shaking hand - scalloped coat

Fresh blood smears made in the field must be protected against dust which sticks at the slide and after staining appear as artefacts during microscopy.

2.5 Thick blood smear

Principle

A thick blood smear is a drop of blood on a glass slide. Thick films allow screening a larger volume of blood and are more sensitive than the thin film. They are used mainly in humans for detection of malaria stages in the blood. Thick smears consist of a thick layer of lysed red blood cells. The thick smear is, in fact, a concentration method as the blood components (incl. parasites) are more concentrated in comparison to equal area of thin blood smear. Thick films are not suitable for evaluation of parasite morphology; therefore the thin smear should be usually used for species identification.

Equipment

- Glass slides
- Tray for putting the slides

Procedure

1. Put a small drop of blood in the centre of the slide
2. Spread the drop in a circular pattern until it is the size of 1.5 cm^2 (use stick or corner of another slide); the density of smear should hardly allow read the words, if placed over newsprint
3. Let the smear to dry thoroughly in horizontal position at least 30 minutes to several hours at room temperature
4. Do not fix thick smears with methanol or heat
5. If there will be a delay in staining smears, dip the thick smear briefly in water to haemolyse the erythrocytes
6. Stain the smear with Giemsa

Note: Insufficiently dried smears and too thick smears can detach from the slides during staining. The risk is increased in smears made with non-coagulated blood. Thick smears should be protected from dust and insect and also from hot environments to prevent heat-fixing.

2.6 Staining of blood smears

2.6.1 Giemsa staining

Principle

Giemsa stain is a classic blood film stain for peripheral blood smears. Red blood cells stain pink, platelets are light pale pink, lymfocyte cytoplasm stains sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin has dark purple colour. This staining technique is considered gold standard for detection of intracellular and extracellular protozoan blood parasites (e.g. detection of malaria or trypanosomiasis).

Equipment

- Tray for putting the slides
- Coplin jar with methanol
- Coplin jars with Giemsa stain
- Water

Procedure

1. Fix air-dried film in absolute methanol by dipping the film briefly (two dips) in a Coplin jar containing absolute methanol.
2. Remove and let air dry.
3. Stain with diluted Giemsa stain (1:20, vol/vol, 5%) for 20 min. For a 1:20 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water in a Coplin jar.
4. Wash by briefly doping/washing the slide in and out of a Coplin jar of tap water (one or two dips). Note: Excessive washing will decolorize the film.
5. Let air dry in a vertical position

2.6.2 Diff Quick staining

Principle

It is a commercial variant (figures 36-37) of Romanowsky dye, commonly used in histology because of the speed and resolution. It is time better than Giemsa, since it shortens the original 4 minute process to 15-second, allowing a selective increase in eosinophils and basophils, depending on the length of dip slides in staining solution. This staining is suitable for quick detection of blood parasites in the field (figures 38-40).

Equipment

- Diff Quick components:
- Fixing agent - Triarylmethane dye and methanol (5x1 sec.)
- Diff Quick solution I - xanthan, pH buffer, sodium azide (5x1 sec.)
- Diff Quick solution II - Thiazin, pH buffer (5x1 sec.)
- Water

Procedure

1. Fixing of the blood smear in fixing agent - 5x1 sec.
2. Staining with Diff Quick solution I - 5x1 sec.
3. Staining with Diff Quick solution II - 5x1 sec.
4. Rinsing under running water for several seconds, than drying in a vertical position

2.7 Modified Knott's test

Principle

This concentration and staining technique is used for detection of microfilariae in peripheral blood (figure 41). The test is more sensitive in comparison with permanent stained blood smear because it allows examination of much larger volume of blood. Principle of the method is based on hemolysis and further staining of white blood cells and extracellular microfilariae. Microfilariae stain in blue color.

Equipment

- Formaldehyde
- Methylene blue or green
- Tubes, Pasteur pipettes
- Centrifuge
- Glass slides, cover slides

Procedure

1. Mix 1 ml of uncoagulated blood (in heparin or EDTA) with 10 ml of 2% formaldehyde
2. Centrifuge 3–5 min, 1500 rpm
3. Pour off supernatant and mix sediment with few drops of 1% methylene blue (or green)
4. Put a drop on glass slide, cover and microscope - examination of a whole sediment is necessary

2.8 Polycarbonate membrane filtration

This technique is widely used for separating microfilariae from blood. It is very sensitive, enabling very low parasitemia to be detected. Nucleopore polycarbonate membranes, 25µm diameter, 5µm pore size, are held in a Millipore Swinnex filter holder, using a rubber gasket to secure the membrane.

Procedure

1. Place the membrane on the holder with a drop of water
2. Draw up 10–20 ml of 1:1 saline diluted blood into a 20ml syringe
3. Connect the syringe to the filter and gently push the blood through the filter membrane
4. Repeat until all of the blood has been filtered
5. Draw up 20 ml of saline into the syringe, flush through the filter, repeat using air
6. Unscrew the top of the filter and discard the gasket into chloros; use forceps to transfer the membrane to a slide.

Figure 36

Staining of blood smears in the field laboratory. Stained blood smears should be protected against dust which sticks at the slide and appears as artefacts during microscopy.

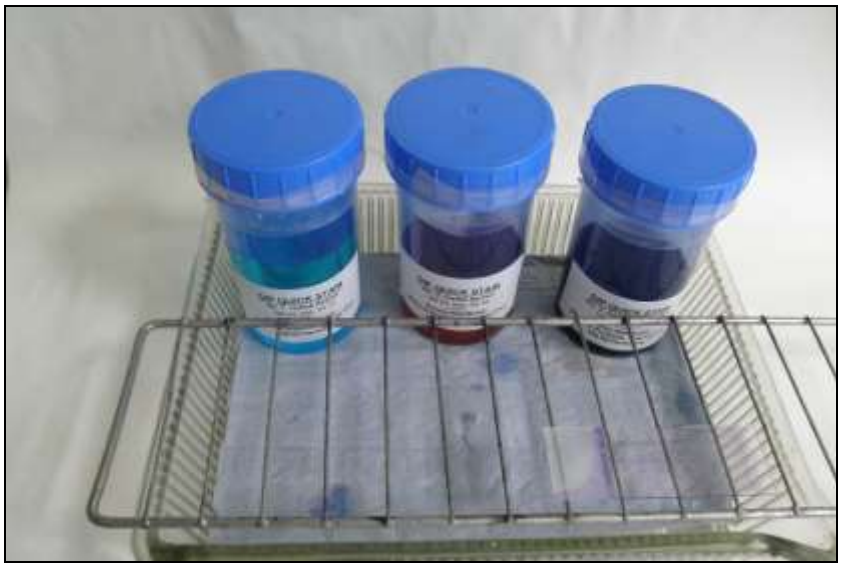

Figure 37

Diff Quick staining consists of three components: fixing agent, solution I and solution II. This technique allows fast staining of thin blood smears.

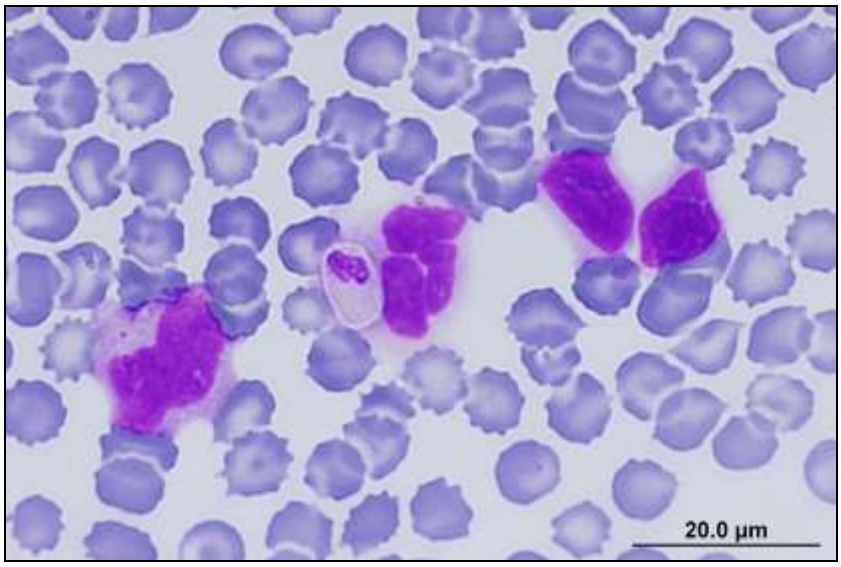

Figure 38 (Photo: Bára Mitková)

A gamont of Hepatozoon canis in a blood smear stained with Diff Quick from a naturally infected dog.

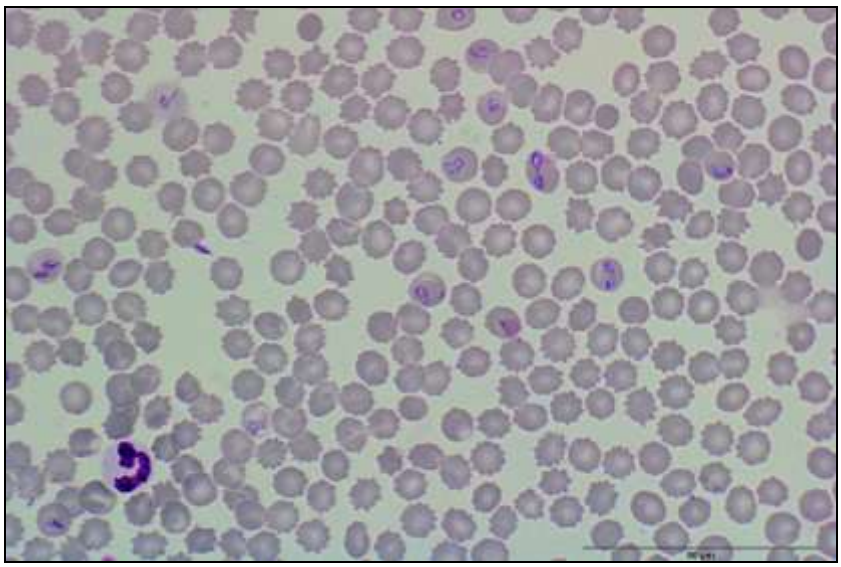

Figure 39

A blood smear stained with Giemsa from a dog. Merozoites of *Babesia canis* in red blood cells have typical drop shape.

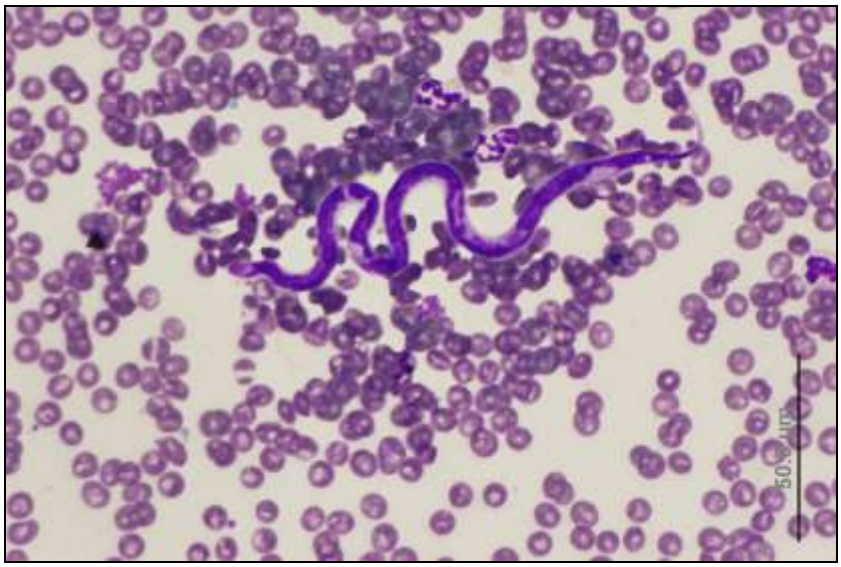

Figure 40

A microfilaria surrounded by blood cells in blood smear stained by Giemsa.

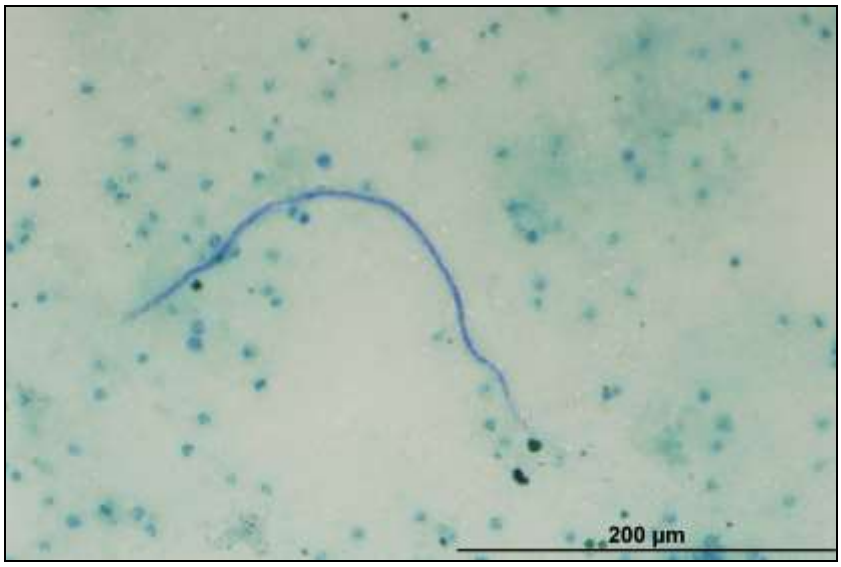

Figure 41 (Photo: Bára Mitková)

Microfilaria of dog's parasite Dirofilaria repens detected by Knott's test. Microfilaria and leukocytes are stained in blue colour, background is greenish and erythrocytes are haemolysed.

7. Add a drop of saline to the membrane and cover with a cover slip
8. Examine the membrane under the microscope, using a 100× magnification; examine any microfilariae found using a 400× magnification (40× objective)

2.9 Buffy coat examination

Principle

A buffy coat is a leukocyte and platelet concentrate, which appears as the upper, lighter portion of the blood clot occurring when coagulation is delayed or when blood has been centrifuged. It contains 10–20 times concentrated leukocytes. A layer of clear fluid (plasma), a layer of red fluid containing most of the erythrocytes, and a thin layer in between (buffy coat) is visible after centrifugation of the blood. The colour of buffy coat is usually whitish, sometimes green if the blood sample contains large amounts of neutrophils. This method is recommended mainly for malaria detection and detection of *Leishmania* amastigotes, but it can be used also for detection of other blood parasites that do occur in leucocytes (like *Hepatozoon* in mammals). Buffy coat stained smear can be used for microscopic detection of parasites. The buffy coat layer can be also used for fluorescence detection of parasitic stages, for DNA isolation and molecular analysis or for inoculation of cultures for *Leishmania* diagnosis.

Equipment

- Centrifuge
- Pipettes
- Collection tubes/slides - according to intended examination method

Procedure

More techniques including commercial procedures are available for preparing of buffy coat. The simplest is centrifugation of anticoagulated blood:

1. Whole blood in anticoagulant (EDTA, sodium heparin) is centrifuged at 200 g for 10 min at room temperature
2. The concentrated white blood cells at the plasma and erythrocyte interface is carefully removed using a pipette

2.10 Microscopy of blood smears

Using light microscopy, imaging is done with a 10× ocular and 10× objective at first. Increasing in progression, it is best to view smears up to a total magnification of 1000× using the 10× ocular mainly while searching for intracellular protozoan parasites. Extracellular blood parasites (trypomastigotes, microfilariae) are visible with lower magnification.

Drying related artefacts or a stain related artefacts may be seen on the slide. Internal reflections or contamination in the sample (or in the optics) can cause refractions which can complicate microscopic examination.

3 DETECTION OF PARASITES LOCALIZED IN TISSUES

This chapter presents way of collection and preservation of tissue samples and also mentions special techniques for detection of some parasites located in various tissues. In tissues we can find mainly protozoan parasites and helminths, but sometimes we can even discover larvae of warble flies, thus we can say that there is wide parasitical spectrum.

In case of life animals we don't have many options how to detect parasites in tissues. Commonly we are able to detect antibodies using indirect methods or to use some developed methods, but usually we don't have such equipment in field conditions, so we can only collect samples for further analysis. In the field we can directly carry out classical clinical examination using palpation, take blood etc. In case of suspicious lesions we can even perform needle biopsy. For example in case of dogs suspicious from *Leishmania* infection or even in those without clinical signs we are not able to detect the parasite in blood and serological testing is tricky, so in the field we commonly use skin scrapings in dogs with skin lesions, and also in asymptomatic ones we can easily perform conjunctival swabs with rapid slide diagnostics without need of problematic spleen biopsy.

In carcasses or slaughtered animals the spectrum of possible diagnostic procedures is wider (for detailed procedures see chapter necropsy). Of course in larger parasitical stages we commonly use just naked eye assessment, we can see cysts of various tapeworms in organs (cysticercus, echinococcus etc.), tissue cysts of some *Sarcocystis* spp. etc. On the other hand for most of the other parasites microscopy examination or other special techniques are required (*Trichinella*, *Toxoplasma*, majority of *Sarcocystis* spp., *Encephalitozoon* spp.).

Techniques for tissue parasites detection are sometimes problematic due to irregular distribution of parasitical stages in various tissues. In some species like *Trichinella* spp., *Sarcocystis* spp., *Toxoplasma* etc. we should consider typical predilection sites of these parasites. For example *Trichinella* ssp. is generally mostly concentrated in diaphragm pillars (pigs, wildboras), in tongue (horses) or in muscles of legs (carnivores). Cysts of *Toxoplasma gondii* are in large numbers usually in brain. Typically, the cysts of *Sarcocystis* spp. Are localized in muscles, however, the location of cysts may vary between species.

3.1 Collection of samples

Before sampling, it is good to know the exact purpose of a study. The size of the tissue can vary from mg (in case of molecular detection) to kg, so we can pick suitable amount of sample. Mainly in animals with unknown cause of death we usually collect as many samples as possible. To maximize the quality of scientific or diagnostics results it is important to collect samples as fresh as possible. In live animals, even in the terrain conditions, we can perform biopsy, scrapings, eye swabs etc. But also often we collect tissues from slaughtered animals or from dead ones. In case of carcasses the use of tissues is limited according to a level of decomposition. During sampling we should ensure sterile or at least clean instruments. All the samples should be visibly labeled with important information about the material with permanent markers which are resistant to commonly used chemicals. Samples should be transported and examined as soon as possible or directly preserved for subsequent analysis.

3.1.1 Equipment

- Various bags, zip bags
- Various tubes
- Knife
- Scissors
- Tweezers
- Permanent markers

3.1.2 Preservation of tissue samples

In general, there are many ways how to preserve the tissue samples, depending of course mainly on the diagnostic methods that follow. In case of rapid processing of samples it is possible to cool the samples in the box with cooling bags or in the fridge. In case of longer storage, we can freeze tissues to avoid process of decomposition. In the field also dry ice can be used for a short term freezing. For most of the follow-up analyses freezing at minus 20°C is suitable, usually we perform freezing using travel freezers which are supplied with electricity or work on the basis of generator.

BUT: the freezing always destroys fine structures of the host tissues and parasites. Keep in mind, that histopathological examination, helminth staining, examining the fine structures using the elctron microscopy etc is almost impossible from frozen tissues! The material for these analyses should be preserved separately before freezing. On the other hand, the isolation of the DNA (but not RNA) from frozen material gives usually very good results. Alternatively, the tissue for consequent DNA-based analyses can be preserved in abolute EtOH (96 %). For the detection of RNA, deep frezing (liquid nitrogen or -80) or commercial buffers (like RNAlater®). Whole parasites or some bigger parts of tissues can be also preserved in 70% EtOH. Lower concentrations of alcohol are not suitable for proper preservation.

Formalin fixation is widely used mainly because of histopathology (figure 42) and also for the purpose of whole parasites fixation etc. Its advantage is, it is not dissolving fats and samples can be kept for a longer period (years). Commonly we work with 4–10% formalin solution. The amount of formalin should be in ideal conditions 100x bigger than the amount of sample. In the field conditions we usually work at least with 10x bigger amount of the fixation solution. In case of histological examination is planned, the perfect size of a tissue block is about 0.5×0.5×0.5 cm. Before the fixation process, perfused tissues should be washed in saline solution to remove blood. Changing of the fixation solution after few hours is appropriate, esp. in case of larger samples.

3.2 Macroscopical identification of parasites in tissues

Determination of some parasites is possible just through detailed naked eye assessment usually after slaughtering or during necropsy, because some parasitical stages are so visible and typical, therefore cannot be substitute. For example the macroscopic cysts of *Sarcocystis* spp. can appear as tiny white threads (figure 43) or grain-shape objects. Cysts of tapeworms are forming transparent vesicles of various size with various type of content, depending on tapeworm species (see below the examination of cysticerci and echinococcus). Some parasites are also forming typical lesions (for example milk spots in the liver after migration of *Ascaris suum* larvae, nodules in lungs containing the lungworms). In organs, we can commonly find migratory or adult stages of various helminths (for more information see chapter necropsy).

3.3 Squashed samples

Principle

Some parasites are forming cysts and other structures in tissues and in case of wisely selected sample are detectable by squashing a piece of tissue between two slides followed by microscopy (i.e. cysts of *Sarcocystis* spp. in muscles (figure 44), *Toxoplasma gondii* in brain, *Alaria alata* in muscles or organs).

Equipment

- Microscopic slidey
- Cover slips
- Scissors
- Tweezers
- Microscope

Procedure

1. Prepare small pieces of tissue by cutting (max 2x2 mm)
2. Put the tissue in to the central part of the glass and put cover glass on it
3. Take another glass and make pressure equally on whole slide
4. If the sample is squashed enough(the tissue is transparent), microscopy can be performed

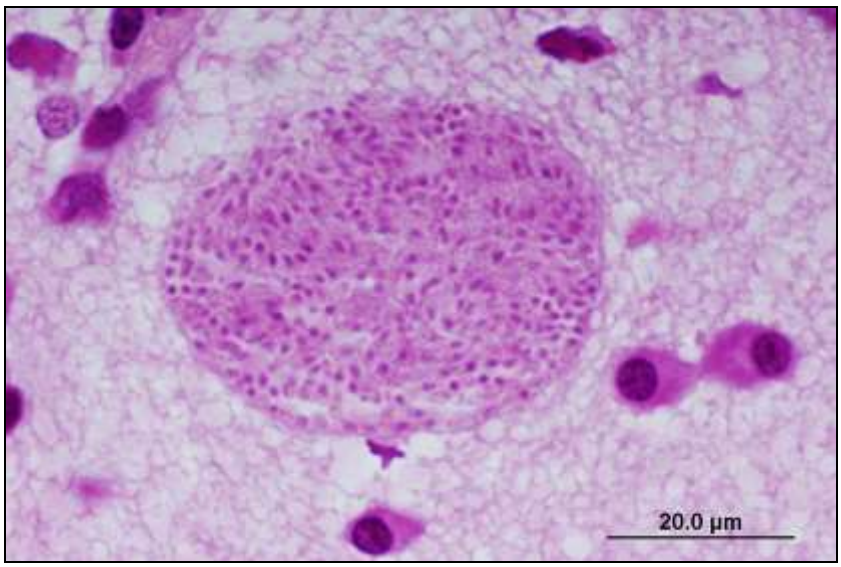

Figure 42

Example of a histopathology result of a sample from brain of mice with content of *Toxoplasma gondii* cyst containing bradyzoites. Sample was prepared classically using 4% formalin fixation followed by cutting and hematoxylin-eosin staining.

Figure 43

White sarcocysts of *Sarcocystis* sp. visible by naked eye in muscles of abdominal wall of dissected rodent.

Figure 44

***Sarcocystis* sp. originating from a wild boar muscle, using classical squashed sample of diaphragm pillar.**

3.4 *Trichinella* detection: trichinoscopic examination

Trichinellosis is a dangerous zoonotic helminthiasis. Detection of infective stages in meat is important part of the food hygiene measures minimizing the risk of the human infections. The prevalence and spectrum of dominant hosts differ geographically, with importance of wild (sometimes also domestic) omnivores and carnivores. Presence of *Trichinella* L1 larvae is typically diagnosed in muscles well irrigated with blood. The predilection sites can differ in various host species. Larvae of *Trichinella* species are usually encapsulated, with size around 350–600 µm, oval in shape after visualization using trichinoscopic examination (figure 45). Although the trichinoscopic examination is not acceptable in EU countries for detection of *Trichinella* in commercially bred and hunted animals any more (see below), it is interesting and rather useful way for detection of tissue larvae for research purposes. The threshold sensitivity of this method is limited (3 larvae/1g of sample).

Principle

Trichinoscopic examination is just a modification of a squashed sample. Detection is done visually by microscopy identification of encapsulated cysts with L1 larvae of *Trichinella* in squashed pieces of muscles. Non-encapsulated species are not easily detectable by this method. In case of positive finding we can see L1 larvae in the cysts (0.4–0.6×0.2–0.3 mm). The placement of numerous samples and higher pressure used for squashing the meat is the main advantage.

Equipment

- Incandescent-lamp trichinoscope with 30–40× and 80–100× magnification or a stereomicroscope with a substage transmitted light source of adjustable intensity.
- Compressorium = a simple device consisting of two glass plates (one of which is divided into fields), connected by screws.
- Small curved scissors.
- Tweezers.
- Knife for cutting specimens
- Dropping pipette
- Acetic acid and a potassium hydroxide solution for brightening any calcifications and softening dried meat

Procedures

In general one plate of compressorium is filled with 1.0 ± 0.1 g of meat, normally corresponding with 28 oat-kernel size pieces (figure 46).

If the flesh of the specimens to be examined is dry and old, the preparations must be softened for 10–20 minutes before pressing with a mixture of one part of potassium hydroxide solution to about two parts of water.

1. Fill the compressorium with pieces of meat of appropriate size.
2. Push firmly both plates of compressorium together using screws on both sides of plates to get a transparent view through squashed pieces of meat.
3. Check the filled compressorium using the 100× magnification.

Note: The threshold sensitivity of this method is 3 larvae/1g of sample. Non-encapsulated species are not easily detectable by this method. In case of positive finding we can see larvae in the cysts (0.4–0.6×0.2–0.3 mm).

3.5 Digestion technique for *Trichinella* detection

Trichinella detection could be also done by artificial digestion using pepsin and HCl digestion solution to release larvae from muscles. Moving larvae are detected in the solution after digestion followed by sedimentation (figure 47). Digestion technique according to "COMMISSION REGULATION (EC) No 2075/2005 for laying down specific rules on official controls for Trichinella in meat" is mostly not a suitable method for a field laboratory. The principle of this method is artificial digestion using water, pepsin and HCl at 46 to 48°C followed by sedimentation.

Figure 45

Larvae of *Trichinella spiralis* visible microscopically in muscle examined by the compression method.

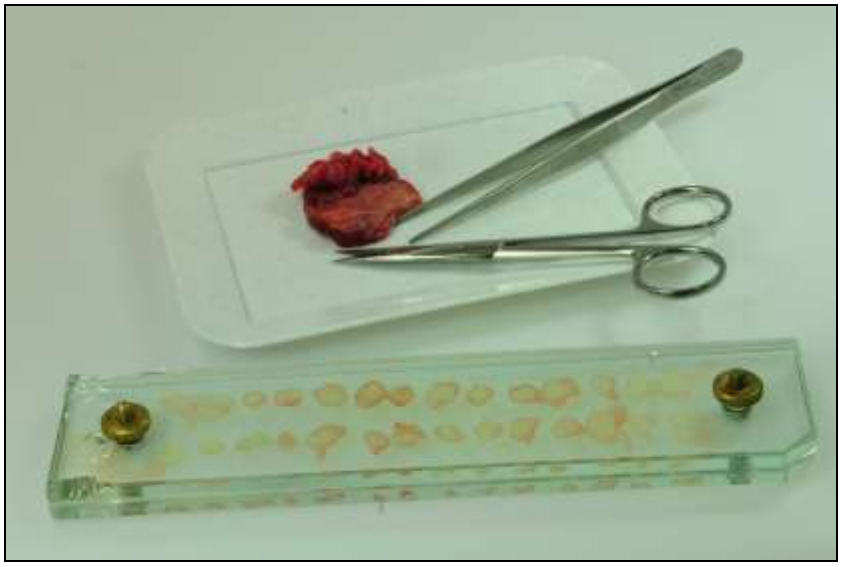

Figure 46

Compressorium filled with pieces of diaphragm pillars after squashing. Trichinoscopic examination in field conditions ready to be checked by microscopy.

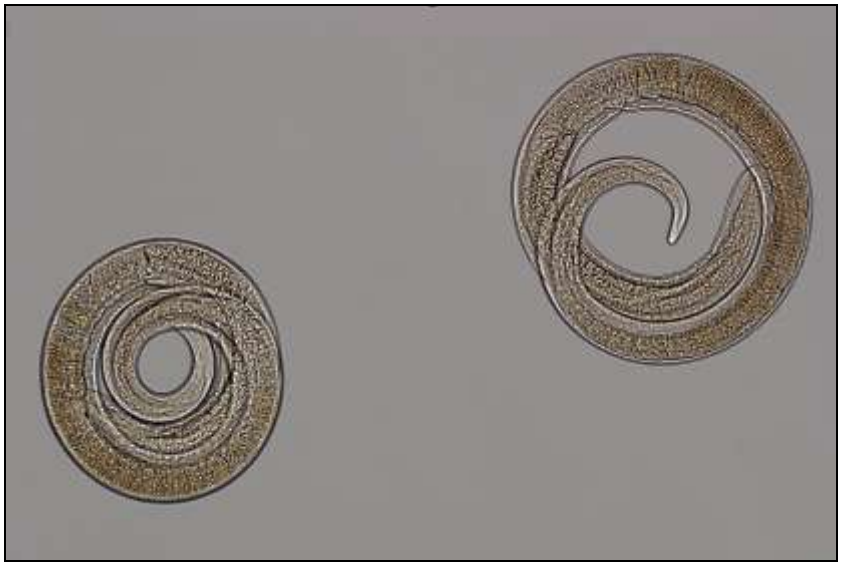

Figure 47

Free L1 larvae of *Trichinella spiralis* visible microscopically after digestion technique processing of muscle tissue.

Various modifications of this technique are also used for *Toxoplasma gondii*, where we use lower temperature and addition of NaCl, at the end sample should be neutralized. For *Sarcocystis* spp. detection we mainly use trypsin solution. In case of *Alaria alata* detection in adipose tissues the process should be done without any digestion, mesocercariae are just left to migrate in warm water after chopping but the equipment and procedure is similar.

3.6 Detection of cysticerci

Principle

Cysticerci of some tapeworms are commonly present in muscles, tongue, heart, oesophagus, diaphragm, and also on the surface of organs. In case of slaughtered cows or carcasses of cows we can perform palpation on the tongue. Small nodular changes in the size of 0.5–1 cm palpable in the muscle of tongue may indicate the presence of developmental stages of *Cysticercus bovis* (figure 48). In these cases, the incision can be done on the tongue and masseters. Positive finding is a detection of *Cysticercus* scolex with a typical position and shape of hooks and suckers. Similarly we can test pigs for presence of *Cysticercus cellulosae*, main predilection site is tongue. In case of rabbits and hares we able to detect Cysticercus pisiformis typically located around abdominal organs etc.

Equipment

- Tweezers
- Knife
- Scalpel
- Scissors
- Slides
- Microscope

Procedure

1. Palp suspected predilection sites, find the cyst
2. Cysts with scissors or scalpel and tweezers are dissected from the muscle
3. Cysticerci are inserted between two large slides and are slightly compressed and inspected under a microscope at 40 × magnification.

3.7 Microscopic confirmation of *Echinococcus*

Echinococcus cysts can be found in domestic animals mainly in liver, lungs and in other organs. *Echinococcus* cysts are usually localized in the parenchyma of organs in contrast to cysticerci of other morphologically similar cysts of various tapeworms (figure 49). The cyst (or secondary cysts) may contain many protoscolices which are free or they are forming capsules.

Equipment

- Tweezers
- Knife
- Scalpel
- Scissors
- Glasses
- Needle
- Syringe
- Microscope

Procedure

1. Dissect the cyst with scissors and tweezers from organs
2. Check the morphology
3. Check the presence of protoscolices by needle puncture followed by microscopy

When necessary, collect the samples for molecular typing (ethanol or frozen) and/or histopathology (formalin).

3.8 Imprint of lymph node/ aspirate from lymph node

Enlargement of the lymph nodes could be one of clinical signs during leishmaniosis in dogs. *Leishmania* amastigotes are mainly concentrated in lymphatic system (lymph nodes, spleen) (figure 50). In case of negative conjunctival swab, or even without it, we can use the needle biopsy from suspicious lymph nodes or an imprint of lymph node (mainly in case of carcasses) to check the presence of *Leishmania* amastigotes.

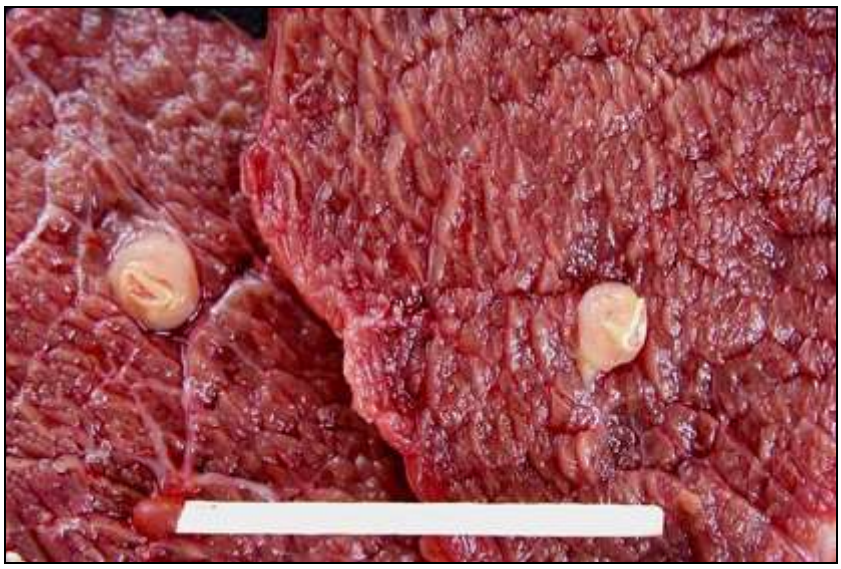

Figure 48 (Photo Břetislav Koudela)

Cysticercus bovis **in the muscle of infected cow, gross assessment.**

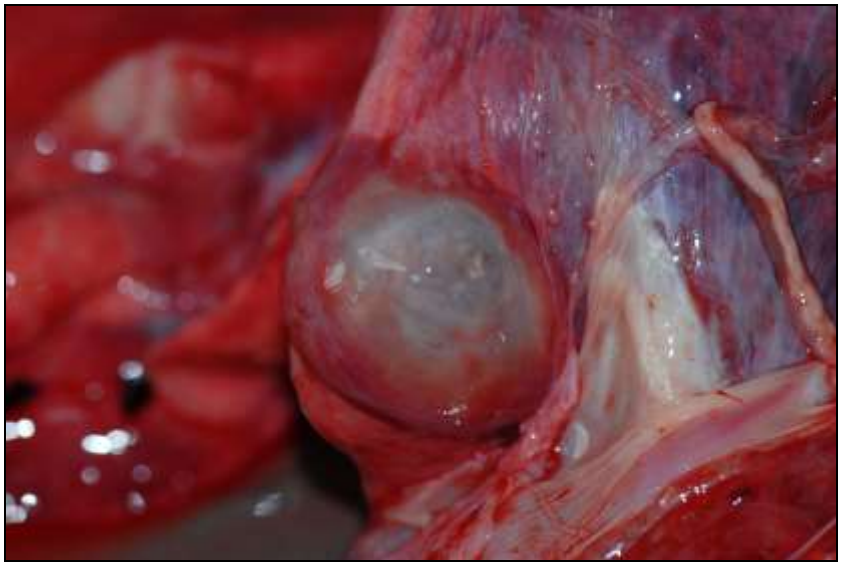

Figure 49

Echinococcus granulosus **(hydatid) cyst in the lung of goat.**

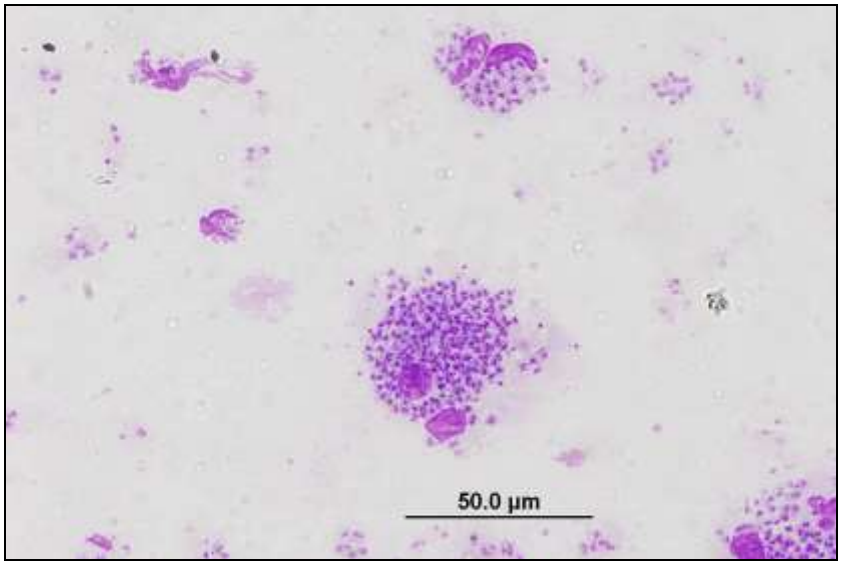

Figure 50

Imprint of lymph node with ***Leishmania major*** **amastigotes Giemsa staining.**

Figure 51

Punch needle biopsy in a dog for the detection of *Cercopithifilaria* spp.

Procedure

- Fixation of animal/necropsy of the animal
- Using sterile needle aspirate the content of lymph node/preparation of a lymph node using blade and tweezers from dead animals
- Coat the slide with content of aspirate/imprint the lymph node on the slide
- Use methanol fixation and Giemsa stain

Check the slide using microscopy using large magnification (amastigotes are really small, mid of each amastigote is darkly purple, the size is just around 3μm)

3.9 Conjunctival swab

Principle

Conjunctival swab is a novel, easy and promising way of *Leishmania* spp. diagnostics without complicated invasive sampling, thus feasible in field conditions. Amastigotes are present in the conjunctiva, even in asymptomatic dogs with *Leishmania* infection. Result can by visualized immediately after the staining by microscopy, or can be detected later using more advanced methods like PCR.

Equipment

- Cotton swab
- Glass
- Giemsa staining
- DNase-free tubes

Procedure

1. Fixation of animal
2. Make a swab from both lower conjunctivas using sterile cotton swabs manufactured for bacteriological isolation
3. Brake the swab tips off, transfer to DNase-free tubes and stored at −20°C until next use (in case of following PCR).
4. Swabs can be also directly transfer on the microscopic slide, followed by Giemsa stain (see chapter method for blood parasite detection) and examined by direct microscopy

3.10 Skin biopsy

Principle

Several filaria species parasitic in domestic animals produce microfilariae which are not detectable in the blood, as they do not produce microfilaremia. The microfilariae of the canine parasites of genus *Cercopithifilaria* are located in the skin and their

detection is based on the identification of larvae with specific morphology in skin biopsies.

Equipment

- scissors
- punch biopsy needle
- physiological saline solution
- microscope
- tubes
- microscope slides and cover slips

Procedure

1. The hair is cut on a small area of skin in the dorsal region of the withers.
2. With the aid of a small biopsy punch needle, a circular piece of skin is collected (figure 51) and placed in physiological saline at 37°C for 10 minutes.
3. After 20 minutes, 20 µl of the sediment are examined under the microscope for the detection of typical microfilaria.

4 COPROSCOPIC METHODS AND TECHNIQUES

This chapter presents diagnostic techniques to identify and quantify stages of unicellular and metazoan endoparasites (cysts/oocysts/eggs/larvae) using the examination of faecal material. Generally, the faecal material represent one of the most suitable media for parasitological diagnostics, in general because of two closely interconnected reasons: (i) it is the most common way through which the parasites leave the host and enter the external environment, and (ii) it is the material easily obtainable by non-invasive means. Alternatively, the same spectrum of methods can be used to examine the content of digestive tract obtained during the necropsy.

Faecal specimens are examined for the presence of protists (trophozoites, cysts, oocysts) and helminths (eggs, larvae). The stages that are found in faeces are usually those that naturally serve for the parasite dispersal, and, as such are usually well adapted for survival outside the host. This fact provides a great advantage, as the examination of the faecal samples can be postpone after the collection. Moreover, the faecal samples are almost the only sample that can be collected and brought for examination by inexperienced owners of animals.

The adult nematodes, larvae of stomach botflies and segments of tapeworms are usually visible to naked eye, but the stages of protozoan organisms (as trophozoites, cysts, oocysts), and helminths (eggs and larvae) can be seen only with microscope. The major obstacle of faecal examination is the fact, that the stages of parasites are distributed in relatively large volume of material that is not really suitable for direct microscopic examination. In order to see the objects we are looking for, the faecal material must be properly prepared and examined.

It is important to understand also the limitations of the coproscopic diagnostics. The demonstration of parasite stages in the faeces provides positive evidence that the animal is infected, but only roughly indicates the degree of infection, because various factors can limit the accuracy and significance of amount of cysts/oocysts/eggs/larvae shed/detected in particular time. Generally, the numbers of eggs/larvae per gram of faeces can show some correlation with the number of mature adult worms present in the host organisms. However, our failure to demonstrate the stages in particular faecal sample does not necessarily mean that the parasites are absent; they may be present in an immature stage or in low numbers or the test used may not be sufficiently sensitive. Furthermore, cysts/oocysts/ eggs/larvae are not evenly distributed throughout the faeces, so the quantity of faeces passed can affect the number of parasite stages per gram. To make the interpretation even more complicated, it is necessary to note, that the morphology of stages of parasites diagnosed in faeces is commonly insufficient for detailed (up to genus or species level) determination of the parasites. Morphologically uniform oocysts of intestinal Cryptosporidium spp. or undistinguishable eggs of strongyles can be mentioned as typical examples. Altogether, the coproscopic examination is a powerful part of intravital diagnostics of the parasites; however, one should always interpret the results with extreme caution, being aware of limits of combination of particular technique with particular parasites.

4.1 Faecal sample collection and preservation

To maximize the value and credibility of the results, the faecal samples for parasitological examination should be collected fresh. The survival of stages of various parasites greatly differs. As faeces age, diagnosis is complicated as many nematode eggs develop and hatch into the larvae within 24 hrs after the defecation. Contaminants such as free-living soil nematodes, fly larvae, mites, and other arthropods often invade the faeces and complicate our diagnosis.

Number and volume of faecal samples depends on several factors, including health status of the animal, intended methods of examination and also financial resources. A volume of the sample can depend on animal size, optimal amount is 5-10g (e.g. 5-10

pellets of faeces of small ruminants or 1–2 faecal ball of horse). For routine screening of asymptomatic animal and for post therapy control, a single sample should be sufficient. For symptomatic or newly arrived animals sequential collection of faeces for 3 days is recommended to increase diagnostic power. To assess the parasite status of a group of animals we should examine min. 30 % of small groups to 30 animals or 10 % of larger groups. If individual sampling is not possible, we can collect adequate number of faecal samples from the animal box, pen, barn or pasture.

The samples should be dispatched as soon as possible to a laboratory in suitable containers such as screw cap bottles, plastic containers with lids, disposable plastic sleeves/gloves used for collecting the samples or plastic bags. Each sample should be clearly labeled with animal identification, date and place of collection.

Samples should be packed and dispatched in a cool box to avoid the stages developing and hatching, if prolonged transport time to a laboratory is expected. If coolants are not available, samples can be stored in 10% formalin (one part faeces, nine parts 10% formalin) to preserve parasite eggs. However, one should always keep in mind, that formalin kills most of the organisms on faeces. It is appreciated in case of zoonotic parasites, however, formalin-fixed faeces cannot be used for faecal cultures and sporulation of oocysts. Samples expected for oocysts sporulation should be keep in 2.5% potassium dichromate solution ($K_2Cr_2O_7$) or 1% sulfuric acid solution (H2SO4). Moreover, such samples are not suitable for any diagnostic methods based on the DNA detection. For molecular methods, we can fix the faecal samples in pure 96 % ethanol, RNAlater or in some cases (oocysts) in 2.5% potassium dichromate.

When the samples are received in the laboratory, they should immediately be stored in the refrigerator (4–8°C) until they are processed, unless cultivations or direct smears are to be prepared for the detection of live and motile parasites from fresh un-cooled samples. Samples can be kept in the refrigerator for up to 1 week without significant changes in majority of parasite stages counts. E.g. strongyle eggs develop to larvae within few days. Samples should not be kept in the freezer.

4.2 Faecal examination techniques

4.2.1 Gross examination of faeces

Principle

The examination of faeces should always start with gross examination by naked eye to determine the consistency (hard, formed, loose, watery), color, distinctive odor and presence of mucus, pus, blood and large parasites as nematodes, proglottids of tapeworms or larvae of bot flies. The presence of macroscopic parasites (or structures resembling them) is commonly a reason why owners of the animals ask for the parasitological checkup.

Equipment

- Tweezers
- Petri dishes
- Magnifying glass
- Microscope slides and cover slips
- Microscope

Procedure

The specimen is visually observed. Suspect structures should be removed to Petri dish under magnifying glass. Smaller structures (e.g. proglottids of tapeworms) should be placed on a microscopic slide to a drop of saline, gently press with cover slip and examine with the microscope using the 40-100x magnification.

4.2.2 Direct smear method

Principle

The direct smear is used, when motile trophozoites (trichomonadids, amoebas, giardia) are suspected in the samples or in a situation, why the first results is required immediately (e.g. during the necropsy). Usually, the diarrheic or loose stool samples or intestinal content are processed this way, as the trophozoites become distorted or form cysts in formed faeces during normal intestinal transit. Specimens must be examined immediately, before low external temperature decreases trophozoite motility. Therefore, the refrigeration should not be

Figure 52

Macroscopic view of parasites in dog faeces: adults of roundworm *Toxocara canis.*

Figure 53

***Dipylidium caninum* proglottids - macroscopic findings on fresh dog faeces.**

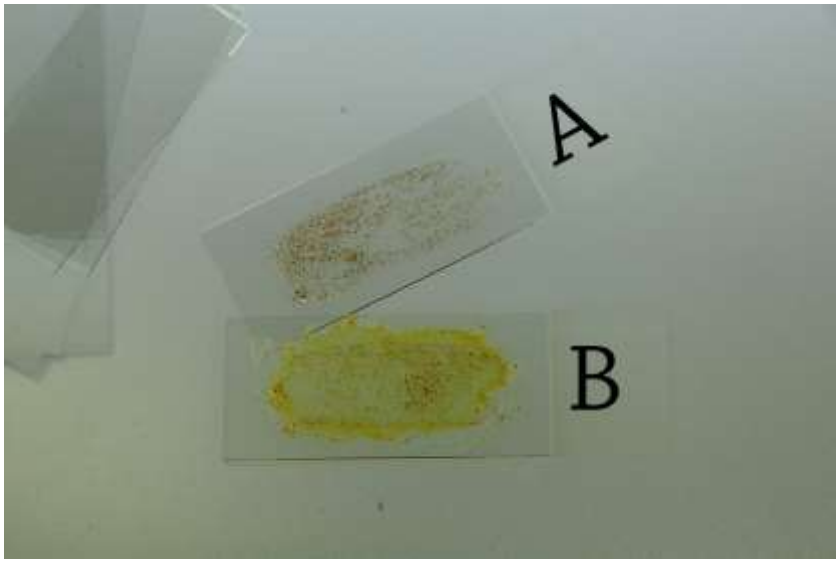

Figure 54

A macroscopic view of slides with a native faecal smear (A), and the faecal smear stained with Lugol solution (B).

used prior to preparing direct smears of faecal samples.

Logically, more frequent the stages in sample are, better chance to detect them. In severe infections (which are usually those manifested clinically), you can detect virtually stages of any protist or metazoan parasites by this simple method; especially clinical coccidioses or severe infections by strongylids can be easily diagnosed this way.

Equipment

- Microscope slides and cover slips
- Basic saline
- Faecal loop or applicator stick
- Microscope

Procedure

1. Place a drop of liquid faecal sample or the sample mixed with drop of saline on a microscope slide
2. Place a cover slip over the specimen
3. Examine the sample with the microscope adjusted for ample contrast using the 100–400x magnification

4.2.3 Faecal stains

The direct smear can be stained for better visibility of parasite stages or their structures (figure 54). The fresh specimens must be examined immediately before undesirable changes in morphology of parasites.

4.2.3.1 Lugol's iodine stain

Principle

Lugol's iodine staining is making the cell nuclei more visible. It can be used as an indicator of starches in organic compounds.

Equipment

- Microscope slides and cover slips
- Saline
- Faecal loop or applicator stick
- Lugol's iodine
- Microscope

Procedure

1. Place a drop faecal sample or the sample mixed with drop of basic saline on a microscope slide.
2. Add drop of Lugol's iodine.
3. Place a cover slip over the specimen.
4. Examine the sample with the microscope adjusted for ample contrast using the 100–400x magnification.

4.2.3.2 Trichrome stain

Principle

The Trichrome stain is a rapid staining procedure for differentiation of parasites from debris in faecal samples. It can be used for staining of cysts and trophozoites of intestinal protists in fresh specimens. Cysts and trophozoites will appear blue-green, background material usually stain green. Karyosomal material of *Entamoeba* is well defined and stains a deeper blue-green to purple in contrast to the cytoplasm. Helminth eggs and larvae usually stain red to purple.

Equipment

- Microscope slides and cover slips
- Faecal loop or applicator stick
- Iodine alcohol solution
- 70%, 95%, 100% ethanol
- Acid-alcohol solution
- Trichrome stain
- Xylene
- Mounting medium
- Microscope

Procedure

1. Put a small drop of suspension of diarrheic calf faeces on a microscopic slide and make a smear (similar to a blood smear).
2. Air dry the smear.

3. Stain the smear with iodine alcohol solution for 10 min.
4. Wash with 70% ethanol for 5 min 2x.
5. Stain the smear with Trichrome stain for 6-8 min.
6. Wash with acid-alcohol solution (two quick dips) cca 5-10 sec.
7. Wash with 95% ethanol for 5 min
8. Wash with 95% ethanol for 5 min
9. Wash with100% ethanol for 3 min.
10. Fix the smear in xylene for 3 min.
11. Mount with mounting medium and cover slip. Slide should be allowed to dry overnight before examination is attempted.

4.2.3.3 *Cryptosporidium* specific acid stains

Principle

Acid staining (figure 55) is making the oocysts of cryptosporidium more visible. It is based on the oocysts acid-resistance, when we initially stain everything in the faecal smear and then unstained with acid or acid-alcohol. As a result, only the acid-resistant structures remain colored.

Miláček-Vítovec modification of acid stain

Equipment

- Microscope slides
- Applicator stick
- Coplin jars
- Methanol
- Anilin-carbol-methylviolet
- 1% sulfuric acid
- 1% tartrazin
- Tap water
- Immersion oil
- Microscope

Procedure

1. Put a small drop of suspension of diarrheic calf faeces on a microscope slide and make a smear.
2. Fix air-dried slide in absolute methanol by dipping the slide briefly (two dips) in a Coplin jar.
3. Remove and let air dry it.
4. Stain the smear with aniline-carbol-methyl violet stain for 30 min.
5. Wash it briefly in tap water.
6. Differentiate with 1% water solution of sulfuric acid (H2SO4) for 1 min.
7. Wash the slide with tap water.
8. Stain the smear with tartrazine for 2 min.
9. Wash the slide in tap water and air dry it in a vertical position.
10. Examine the sample with the microscope using the 100-1000x magnification. Stained cryptosporidian oocysts are blue-violet on a yellow to yellow-green background (figure 56).

Ziehl-Nielsen modification of acid stain

Equipment

- Microscope slides
- Applicator stick
- Coplin jars
- Methanol
- Carbol fuchsin stain
- 3% acid alcohol
- Methylene blue solution
- Tap water
- Immersion oil
- Microscope

Procedure

1. Put a small drop of suspension of diarrheic calf faeces on a microscope slide and make a smear.
2. Fix air-dried slide in absolute methanol by dipping the slide briefly (two dips) in a Coplin jar.
3. Remove and let air dry it.
4. Stain the smear with carbol fuchsin stain for 15-20 min.
5. Wash off the stain with tap water.
6. Differentiate with acid alcohol for 1 min.

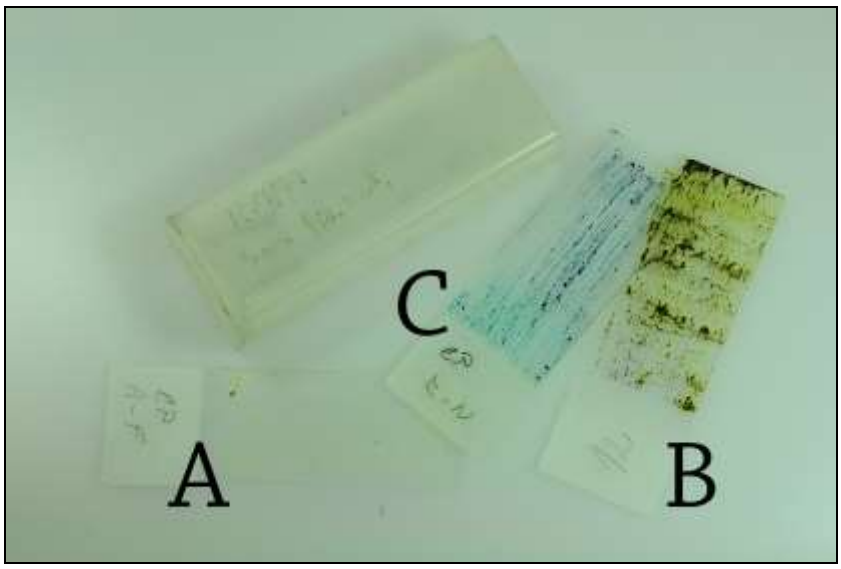

Figure 55

A macroscopic view of slides with an unstained faecal smear (A), faecal smear stained by Miláček-Vítovec method (B) and Ziehl-Nielsen method (C).

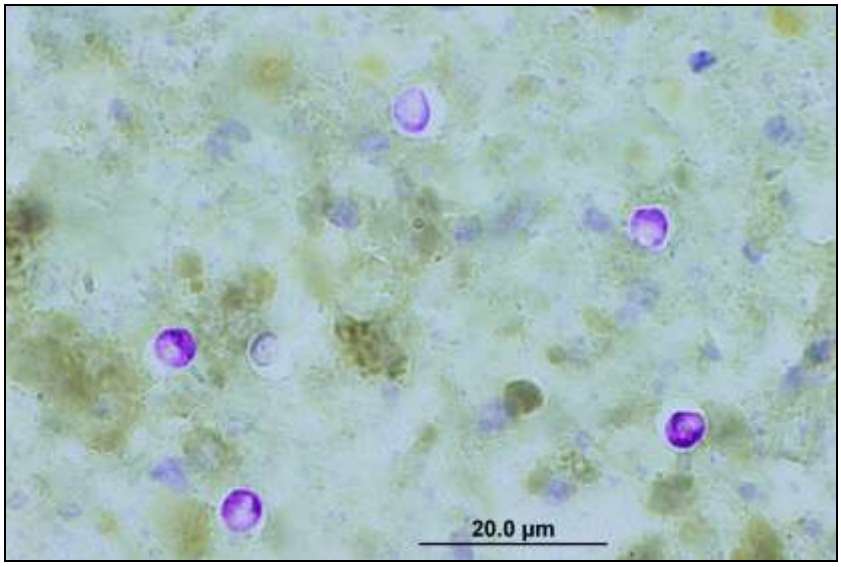

Figure 56

Oocysts of *Cryptosporidium parvum* in Miláček-Vítovec stained faecal smear appear as violet on the yellow background.

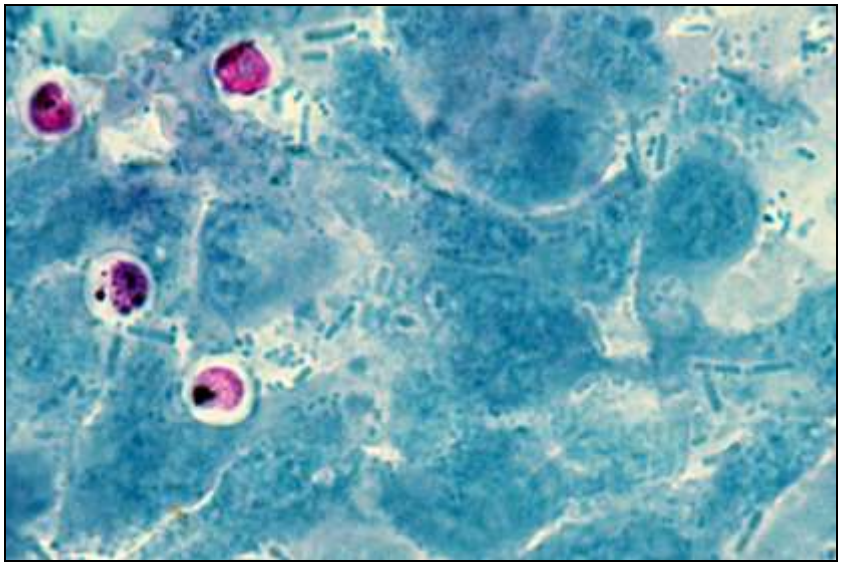

Figure 57

Oocysts of *Cryptosporidium parvum* in Ziehl-Nielsen modification of acid stain appear as bright red on the blue-green background.

7. Wash the slide with tap water.
8. Cover the stain with methylene blue solution for 1-2 minutes
9. Wash the slide with tap water and air dry it in a vertical position.
10. Examine the sample with the microscope using the 100-1000x magnification. Stained cryptosporidian oocysts are pink-red on a blue-green background (figure 57).

4.2.4 Faecal concentration methods based on flotation

As mentioned above, it is the low density of diagnostically important stages of parasites that makes the major obstacle for their detection and correct diagnosis. Then, the most commonly used diagnostic tests in parasitology are the concentration methods based either on the sedimentation or flotation of parasite stages. The flotation techniques separate the cysts/oocysts/eggs presented in the faeces from debris, and concentrates them into a minimum amount of solution for easy identification and enumeration under the microscope. As the basis for the flotation methods is the Archimedes principle, the specific gravity of the solutions used for flotation is important (table 2). When the specific gravity is too low, many stages does not float. Whereas the solution specific gravity is too high, it can cause deformations or rupture of the stages and also results of presence of large amount of debris on the surface. Most stages of parasites float efficiently at solutions of specific gravity 1.2–1.3.

Table 2. Common flotation solutions

Solution	Specific gravity	Ingredients/1l H2O
Sodium nitrate	1.20	338 g sodium nitrate
Sodium nitrate	1.30	616 g sodium nitrate
Sugar	1.20	1170 g sugar + 9 ml phenol as preservative
Sheather's sugar	1.30	1563 g sugar + 9 ml phenol as preservative
Zinc sulfate	1.20	493 g zinc sulfate
Saturated salt	1.20	400 g sodium chloride

4.2.4.1 Passive flotation

Principle

Passive flotation relies strictly on gravity to separate parasite stages and debris. It is less sensitive than centrifugal flotation (see below).

Equipment

- Mortar with pestle
- Wash bottle with water
- Sieve or double layer cheesecloth
- Funnel
- Test tube
- Test tube rack
- Flotation solutions
- Transferring loop
- Microscope slides and cover slips
- Microscope

Procedure

1. Place ~3g of faeces (nut-size volume) into a mortar, add ~30ml of tap water and stir the content thoroughly.
2. Pass the suspension through a sieve or double layer cheesecloth and a funnel into 10 ml test tube.
3. After 30 min of sedimentation, remove carefully supernatant and mix the sediment with the flotation solution.
4. Wait 15 min for passive flotation and then remove upper layer on a microscopic slide using transferring loop and the drop cover with cover slip. Respectively, when you fill the tube with flotation solution to maximum to have a cap, you can transfer the upper layer exactly with a cover slip, which you put directly on microscopic slide.

Examine the sample with the microscope using the 100–400x magnification.

4.2.4.2 Centrifugal flotation

The centrifugal flotation is basic process of preparing a faecal sample for parasitological examination. The sample should first be centrifuged with water to remove water-soluble pigments, free lipids, and other debris. Then the centrifugation-flotation concentrates cysts/oocysts/eggs from the faecal material into an upper layer of flotation solution.

The method of centrifugal flotation is more sensitive than the passive flotation, because it magnifies gravitational forces, thereby accelerating the downward movement of more dense debris and the upward movement of less dense parasite stages. Centrifuges with swinging bucket rotors are preferred because they allow each tube to be filled more than is possible with fixed-head rotors. Some laboratories prefer to place the cover slip on the sample tube during the centrifugation steps. This is not possible with fixed-head rotors, because small vibrations cause lost of cover slip during centrifugation. Some laboratories perform the centrifugations with the fluid level in the tube at the maximum possible, and then transfer the tube into a stationary rack before placing the cover slip on the sample to allow parasite stages to adhere to the cover slip. Sensitivity is equivalent for both variations.

Sheather's sugar flotation method:

Equipment

- Mortar with pestle
- Wash bottle with water
- Sieve or double layer cheesecloth
- Funnel
- Test tube
- Test tube rack
- Sheather's sugar solution (1.3 sg)
- Transferring loop
- Microscope slides and cover slips
- Microscope

Procedure (figure 58)

1. Place ~3g of faeces (nut-size volume) into a mortar, add ~30ml of tap water and stir the content thoroughly.
2. Pass the suspension through a sieve or double layer cheesecloth and a funnel into 10 ml test tube.
3. Centrifuge the tube for 2 min at 2000 rpm for sedimentation.
4. Remove carefully supernatant and mix the sediment with the with Sheather's sugar solution (1.3 sg).
5. Centrifuge the tube for 2 min at 2,000 rpm for flotation.
6. Remove upper layer on a microscopic slide using transferring loop, when the drop cover with cover slip or when you pour under the upper layer the tube with Sheather's sugar solution to maximum to have a cap, you can transfer the upper layer exactly with a cover slip.
7. Examine the sample with the microscope using the 100–400x magnification (figure 59).

Commercial flotation kits

Principle

Commercial flotation kits were developed for easy and quick preparation of faecal sample for parasitological examination without centrifugation. Benefit is also no possibility of cross contamination. The kits compose mainly of a mixing tube, a filter, a filtration tube and flotation solution. Disadvantage can be a higher price.

Equipment

- *Commercial* kit
- Microscope slides and cover slips
- Microscope

Procedure

The procedure should be make exactly according manufacturer´s instructions. Examples include:

OVASSAY PLUS®

https://www.zoetis.com.au/products/453/ovassay-plusreg.aspx

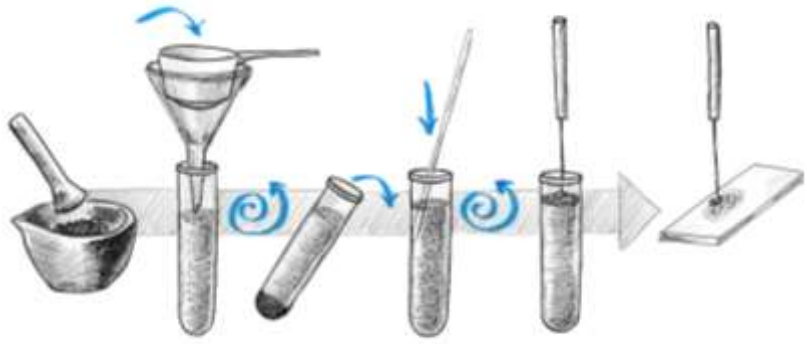

Figure 58

A schema of centrifugation-flotation process for most of parasite stages detection.

Figure 59

A microscopic view of many parasites as the result of centrifugation flotation processing of sheep faeces.

Veterinary Parasep Faecal Filters:

http://www.labtechnik.cz/Parasep-Faecal-Filter-d498.htm?tab=description or

http://www.woodleyequipment.com/veterinary-diagnostics/general-products/veterinary-parasep-faecal-filters-288-112-.php

FECA FLOTATION DEVICE

http://www.vedco.com/index.php/product-listing/108-VINV-FECL-FLOT

4.2.4.3 Quantitative techniques

All diagnostic methods can be semi-quantitative for scoring numbers of parasite stages on microscopic slides with 5 levels: negative, + (1–10 parasite stages on a slide), ++ (1–2 parasite stages in most of fields on a slide), +++ (2–10 parasite stages in most of fields on a slide), ++++ (more than 10 parasite stages in most of fields on a slide).

Quantification of parasite stages in specimen can help to indicate clinical impact of the infection and necessity of therapy. The simplest and most effective method for determining the number of eggs or oocysts per gram of faeces is the McMaster counting technique, we can use also other counting chambers

as Bürker´s chamber, or commercial system as Flotac® can be used.

McMaster counting technique:

Principle

The McMaster counting technique is a quantitative technique to determine the number of eggs or oocysts present per gram of faeces (EPG or OPG). A flotation fluid is used to separate eggs or oocysts from faecal material in a counting chamber. Sensitivity of this method is 50 and more eggs/oocysts per gram of faeces.

Equipment

- Balance
- Wash bottle with water
- Measuring cylinder
- Mortar with pestle
- Sieve or double layer cheesecloth
- Funnel
- Beakers and 15 ml conical centrifuge tube
- Flotation fluid (e.g. saturated salt)
- Pasteur pipettes
- McMaster counting chamber (figure 60)
- Microscope

Procedure

1. Weigh 3 g of faeces and place into mortar.
2. Add 42 ml of water and mix (stir) the contents thoroughly.
3. Filter the suspension through a sieve into a beaker and poured into 15 ml conical centrifuge tube.
4. Centrifuge the tubes at 1500 rev/min for 3 min
5. Remove the supernatant and then mix the sediment with a saturated salt solution to 15 ml.
6. Fill both sides of the McMaster counting chamber with the solution (figure 61).
7. Wait 3-5 minutes until eggs float to the top layer (figure 62).
8. Examine the prepared sample under a microscope at 100x magnification.
9. Count all eggs or oocysts within the engraved area of both chambers.

The number of eggs/oocysts per gram of faeces is calculated:

y × 50 = EPG/OPG

y - number of eggs (in the both chambers)

50 - coefficient to calculate EPG/OPG

Example: We found 12 eggs in chamber 1 and 15 eggs in chamber 2 = (12 + 15) x 50 = 1350 EPG.

Flotac® quantification:

Recently, various attempts to find standard, universal and more sophisticated method of parasite detection and quantification resulted into development and spread of FLOTAC technique. Originally, this technique was designed for studies on the prevalence and intensity of soil-transmitted helminth infections in humans based on the egg quantification. However, in past decade, the method was applied by several teams for various applications, covering also the detection of parasites (including protists) in wild and domestic animals. The method is based on the principals identical to Mc Master technique described above - on flotation of the eggs/larvae/cysts in solution with high specific gravity. However, instead of passive flotation (as used in Mc Master chamber), the FLOTAC device is centrifuged and examined after (figures 63-65).

Procedure

1. Preparation of the samples starts with homogenization and filtration. The samples of weight 10 g have to be homogenized in 90 ml of water (the dilution 1:10) and filtered. 15 ml conical tubes are used, being filled with the filtered suspension up to 11 ml and then centrifuged for 3 min at 1500 rpm (about 170 g).
2. After centrifugation the supernatant have to be discarded and the two pellets (sediments) weighted if the primary weight of sample was unknown.

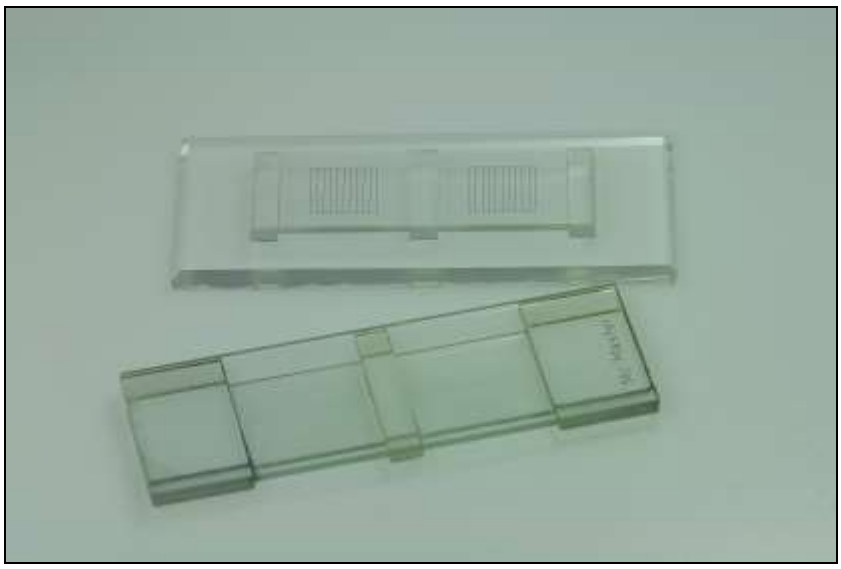

Figure 60

McMaster chamber - different types.

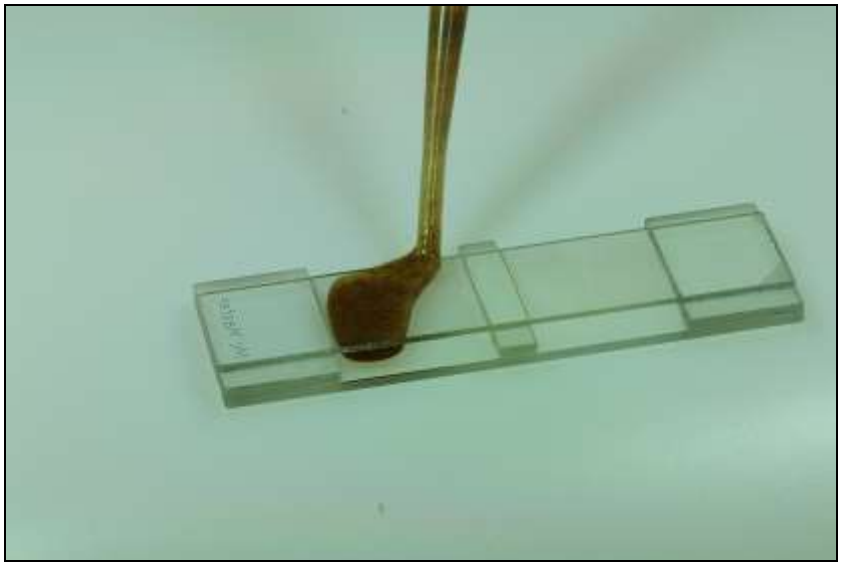

Figure 61

Process of filling McMaster chamber with prepared sample in salt solution.

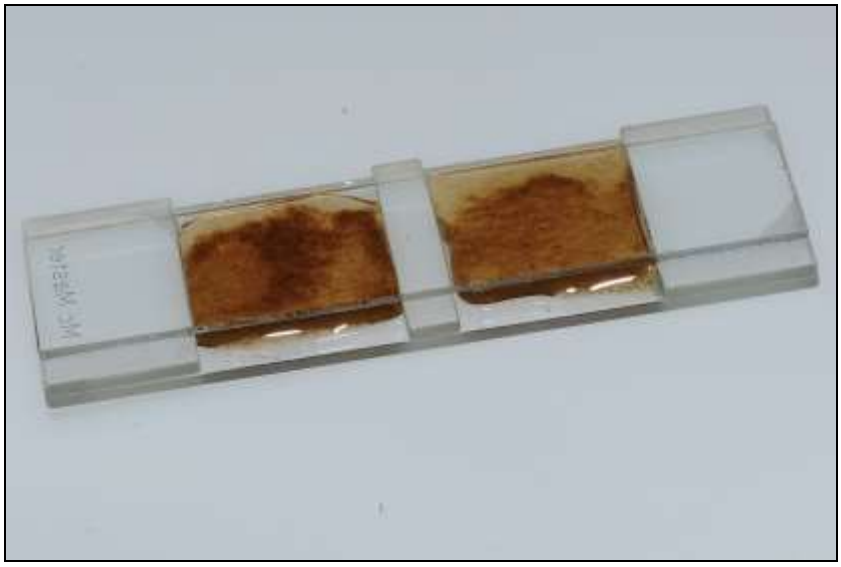

Figure 62

McMaster chambers prepared for examination under microscope.

Figure 63

Flotac set.

Figure 64

Flotac gadget with flotation chambers prepared to use.

Figure 65

Flotac gadget is fixed in a special extension in the centrifuge.

3. Based on the type of host examined and parasites expected, different flotation solutions are recommended to re-suspend the pellets, e.g. sodium chloride solution, zinc sulfate solution or Sheather´s sugar solution of different density from 1.2 till 1.45 (Cringoli et al., 2010). Samples have to be homogenized again and suspension should be filled into the two flotation chambers of the FLOTAC apparatus.
4. Closed FLOTAC than centrifuged for 5 min at 1000 rpm (about 120 g); after centrifugation, the top parts of the flotation chambers have to be translated and each chamber should be read under the microscope.
5. Different magnifications can be used, 100x and 400x, respectively for the research of egg/larvae of helminths and cysts/oocysts of protozoa. The results are than expressed in arithmetic mean of eggs/oocysts per gram (EPG/OPG) of faeces.

Notes: Although the method gained a lot of attention, its usage for routine diagnostics has two major drawbacks: first, the special type of centrifuge is needed, which is large, difficult to carry and expensive; second, it is rather difficult to find the universal flotation solution for the samples with unknown spectrum of the parasites. Considering these fact, FLOTAC represent a valuable alternative mainly for larger studies in well-equipped labs that requires the standardization of the quantification approach and deal with larges sets of samples of the same origin. For details, the readers should refer to the FLOTAC instructions and related studies. The extremely valuable part of the FLOTAC testing procedures is the exact data about the usability of various flotation solutions for detecting various parasites, which can be applied to modify standard flotation procedures as described above.

4.2.5 Sedimentation techniques

4.2.5.1 Simple gravity sedimentation

Principle

Most acanthocephalan eggs, trematode eggs or ciliate cysts are relatively large and heavy. The sedimentation technique is a qualitative method for detecting these heavy eggs or cysts in the faeces.

Equipment

- Balance or teaspoon
- Mortar with pestle
- Wash bottle with tap water
- Sieve or double layer cheesecloth
- Funnel
- Beakers or plastic containers
- Watch glass or small Petri dish
- Microscope

Procedure (figure 66)

1. Put ~3g of faeces (nut-size volume) into a mortar, add ~30ml of tap water and stir the content thoroughly.
2. Pass the suspension through a sieve or double layer cheesecloth and a funnel into 50 ml beaker.
3. Let stand for 5 min for sedimentation.
4. Remove carefully supernatant and mix the sediment with the tap water.
5. Let stand for 5 min for sedimentation.
6. Repeat this procedure until the water above the sediment is clear (figure 67).
7. Pour of the water, let only 1–2 ml of the sediment, and transfer it to a watch glass or small Petri dish.
8. Examine the sediment with the microscope using the 40–100x magnification (figures 68-70).

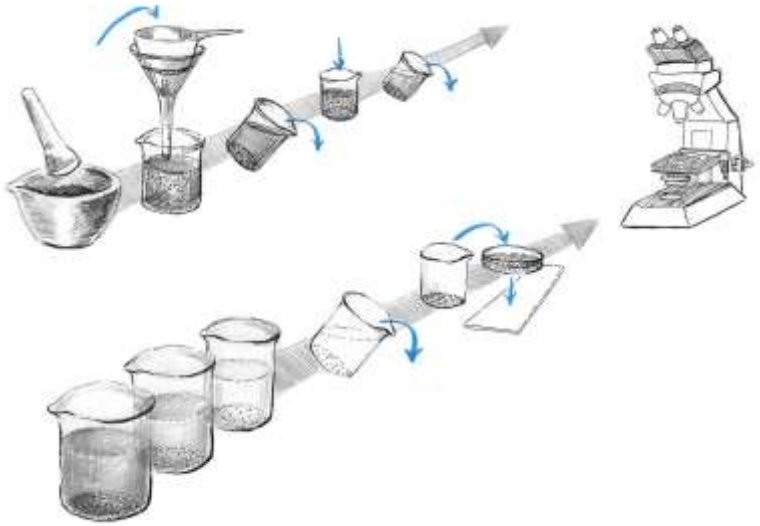

Figure 66

A schema of sedimentation process for heavy eggs/cysts detection.

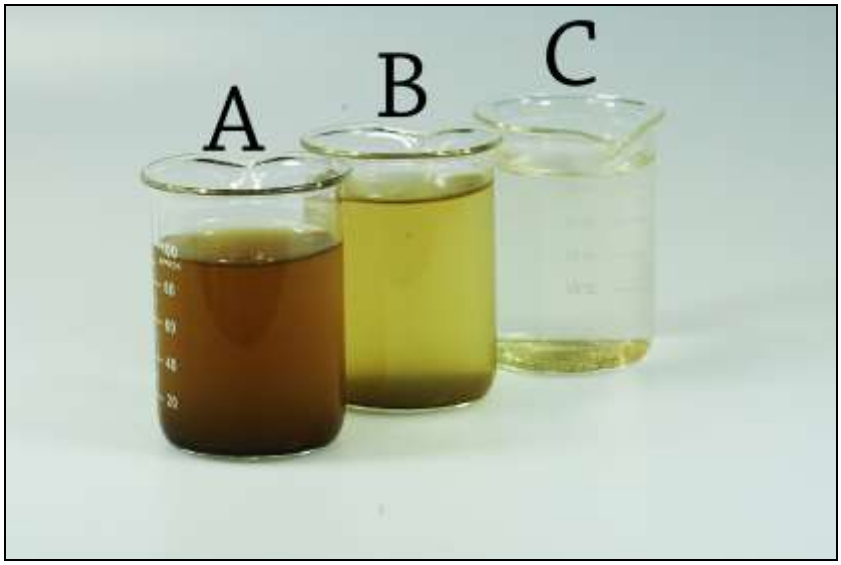

Figure 67

Process of sedimentation after the first (A), the second (B) and the final (C) washing of the sediment of faecal sample.

Figure 68

Acanthocephalid eggs visible under microscope after sedimentation of wild pig (*Sus scrofa*) faecal sample.

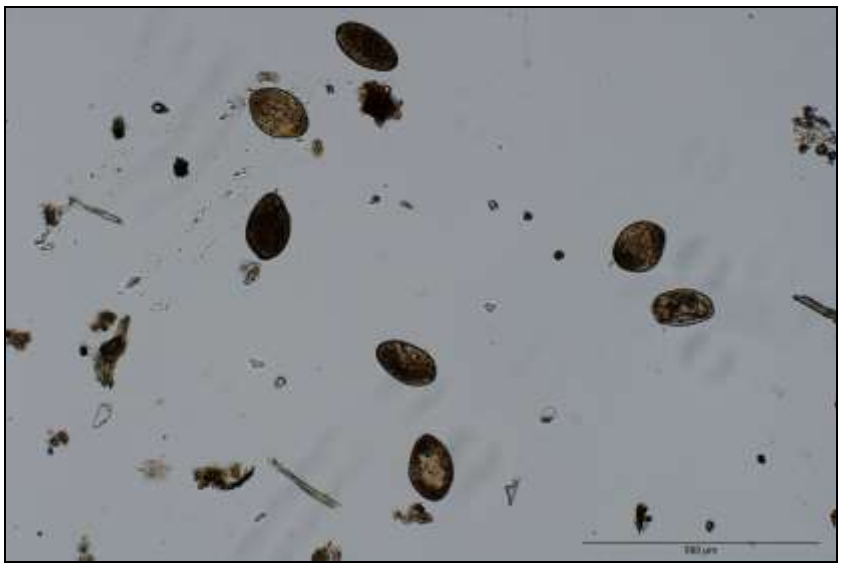

Figure 69

Heavy eggs of fluke (*Fascioloides magna*) visible under microscope after sedimentation of faeces of ruminant.

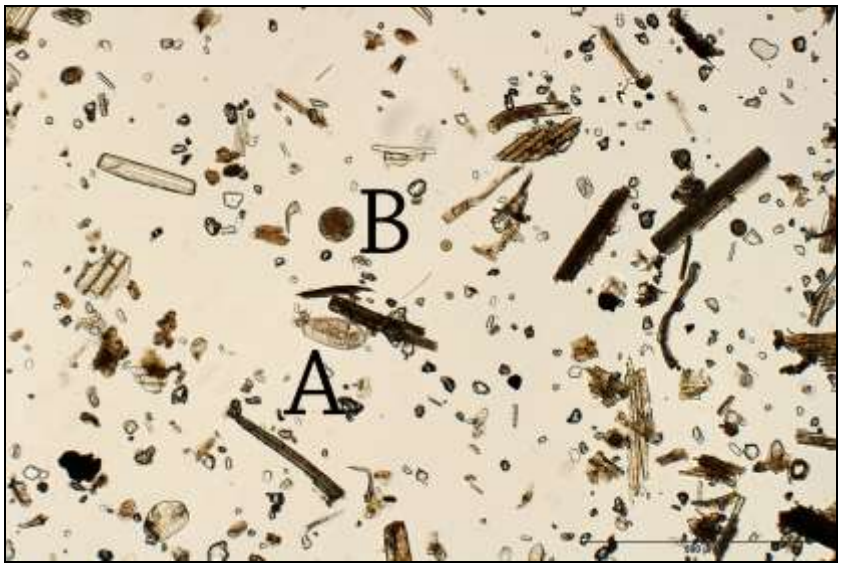

Figure 70

A microscopic view on a result of sedimentation: *Paramphistomum* sp. egg (A) and *Buxtonella* sp. cyst (B).

4.2.5.2 MIF (Merthiolate-Iodine-Formaldehyde) sedimentation

Principle

The sedimentation technique is established for large and heavy parasite stages, while ether which is part of MIF solution helps remove fat and mucus from the faecal sample. Lugol's iodine is added for increasing contrast of parasite stages and their internal structures.

Equipment

- Ether
- Glycerin
- Merthiolate (Thiomersal)
- Distilled water and tap water
- 40% formaldehyd
- Mortar with pestle
- Sieve and funnel
- Sedimentation 15ml conical tubes
- Pasteur pipettes
- Microscope slides and cover slips
- Lugol's iodine
- Microscope

Procedure

1. Mix solution of 500 ml of distilled water, 400 ml of 1% merthiolate solution, 50 ml of 40% formaldehyde and 10 ml of glycerin.
2. Put faecal sample into a mortar, add tap water and stir the content thoroughly.
3. Pass the suspension through a sieve and a funnel into a tube.
4. Mix a mixture with 5 ml MIF solution and 6ml of ether in 15 ml tube.
5. Centrifuge 2 min at 1500 rpm.
6. Discard a supernatant.
7. Transfer the sediment with Pasteur pipette on microscope slide, add drop of Lugol's iodine and cover with cover slip.
8. Examine the sample with the microscope using the 40-100x magnification.

4.2.6 Larvoscopic methods

4.2.6.1 Baermann technique

Principle

The Baermann technique is used to isolate lungworm larvae from faecal samples. The method is based on the active migration of larvae (attracted with wet) from faeces to water and their subsequent collection and identification.

Equipment

- Scale or teaspoon
- Funnel (size ~ 8 cm diameter)
- Funnel stand
- Rubber or plastic tubing
- Rubber bands
- Clamp or clip
- Double layer cheesecloth
- Simple thin stick
- Petri dishes or watch glass
- Pasteur pipette
- Microscope slides and cover slips
- Microscope

Procedure (figure 71)

1. Prepare the apparatus of funnel with rubber tubing closed with the clip in funnel stand (figure 72).
2. Weigh or measure ~ 3-10 g of faeces and place it on a piece of double-layer cheesecloth.
3. Form the cheesecloth around the faeces as a "pouch".
4. Place the pouch containing faeces in the funnel.
5. Fill carefully the funnel with lukewarm water, covering the faecal material.
6. Leave the apparatus in place for 24 hours, during which time larvae actively move out of faeces and ultimately collect by gravitation in the stem of the funnel.
7. Drop a few ml of fluid from the stem of the funnel into a small Petri dish.
8. Examine under microscope for live lungworm larvae (L1) using magnification 40-100x (figure 73).
9. Use Pasteur pipette for a transfer of larvae from positive samples on a microscopic slide for identification at 200-400x magnification. It is important to differentiate between lungworm larvae (figures 74-75) and larvae of gastrointestinal or free-living nematodes.

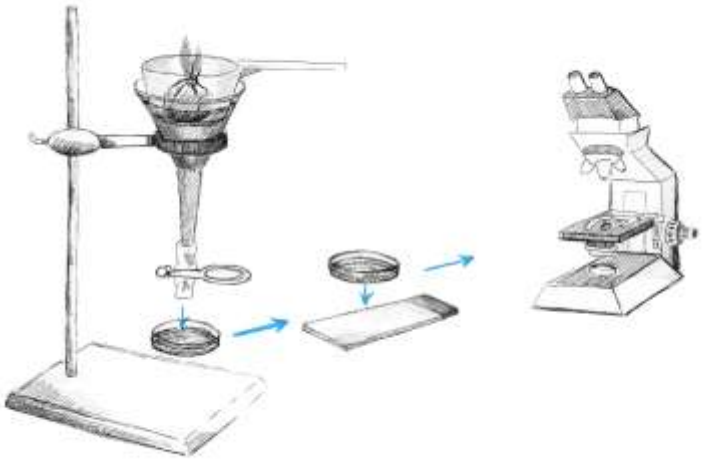

Figure 71

A schema of Baermann apparatus for larvae detection.

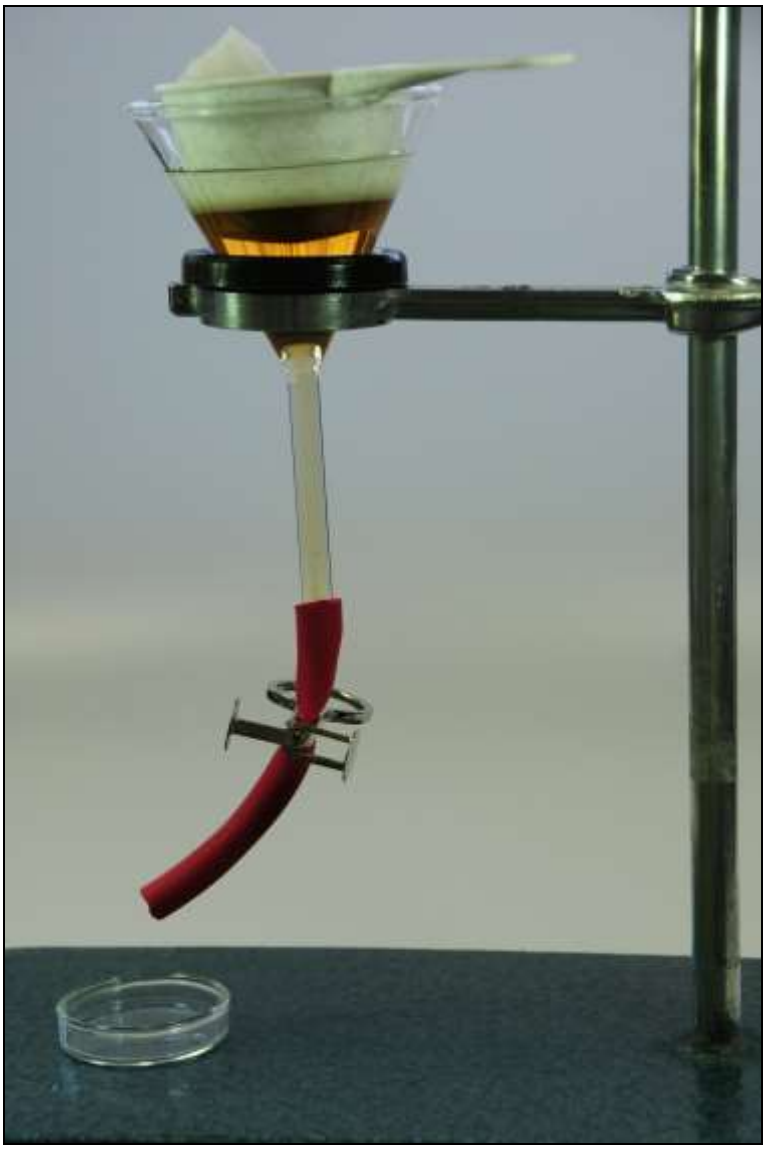

Figure 72

Baermann apparatus of funnel with rubber tubing closed with the clip in funnel stand. The faeces are places in a bundle of double-layer cheesecloth in a sieve in funnel filled with water.

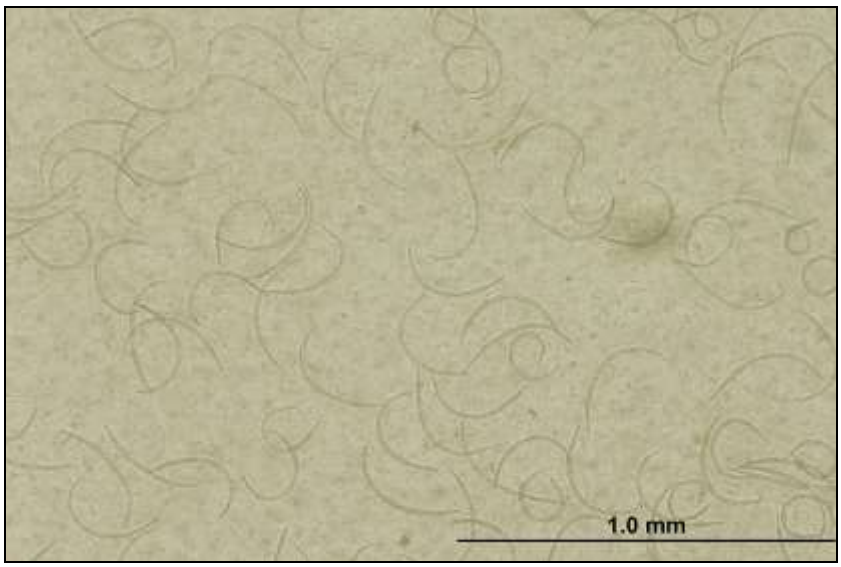

Figure 73 (Photo T. Šimonovská)

Numerous larvae of *Crenosoma striatum* obtained from Baerman larvoscopic method of faeces from strongly invaded hedgehog.

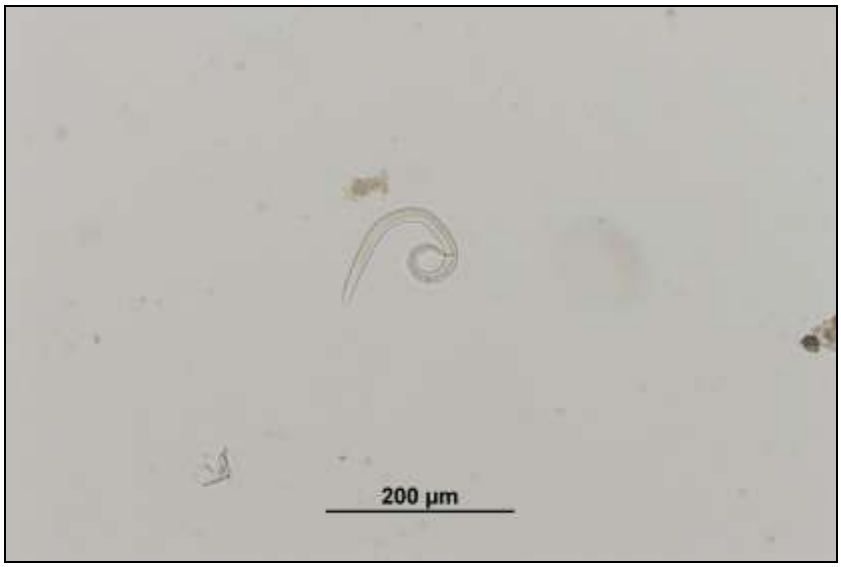

Figure 74

A single larva of *Crenosoma striatum* as a result of Vajda larvoscopic method of hedgehog faeces.

Figure 75

Larva L1 of *Angiostrongylus vasorum* obtained from Baermann larvoscopy of wild wolf (*Canis lupus*) faecal sample. Note typically curved tail with a dorsal spine.

4.2.6.2 Vajda method

Principle

The Vajda technique is simple method for larvoscopic examination based on the same principle as Baermann technique - the active migration of lungworm to water, their collection and identification. The Vajda method is less time-consuming, suitable for formed faeces.

Equipment

- Balance or teaspoon
- Tweezers
- Double layer cheesecloth
- Petri dishes or watch glass
- Pasteur pipette
- Microscope slides and cover slips
- Microscope

Procedure (figure 76)

1. Weigh or measure ~ 3–5 g of faeces and place it on a piece of double-layer cheesecloth.
2. Form the cheesecloth around the faeces as a "pouch".
3. Place the pouch containing faeces on a small Petri dish or watch glass.
4. Fill the Petri dish with lukewarm water.
5. Leave the sample for 1 hour, during which time larvae actively move out of faeces to the Petri dish (figures 77-78).
6. Remove the pouch from the Petri dish.
7. Examine the content under microscope for live lungworm larvae (L1) using magnification 40–100x.
8. Use Pasteur pipette for a transfer of larvae from positive samples on a microscopic slide for identification at 200–400x magnification.

4.3 Faecal cultures

4.3.1 Coprocultures of nematode larvae

Principle

Many nematode eggs are morphologically similar and cannot be clearly differentiated in faecal samples. In these cases, coprocultures can be used for developing nematode eggs into the infective larvae (L3). These L3 larvae can be morphologically distinguished into the genus.

Equipment

- Water
- Mortar with pestle
- Jars or containers
- Petri dishes or watch glass
- Pasteur pipette
- Microscope slides and cover slips
- Microscope

Procedure

1. Put faecal sample into a mortar and stir the content thoroughly.
2. Faeces should be moist and crumbly. If faeces are too dry, add water.
3. Transfer the mixture to jars or other containers.
4. Leave the culture at room temperature for 14–21 days, by which time all larvae should have reached the infective stage. If an incubator is available, the culture can be placed at 27 °C for 7–10 days. Add water to cultures regularly every 1-2 days.
5. Larvae are recovered using the Baermann technique (see above).
6. Use Pasteur pipette for a transfer of larvae from the samples on a microscope slide for their identification at 200–400x magnification. The counts for each species provide an estimate of the composition (%) of the parasite population of the host. Identification of infective larvae is based on keys.

Figure 76

A schema of preparing a Vajda larvoscopic method.

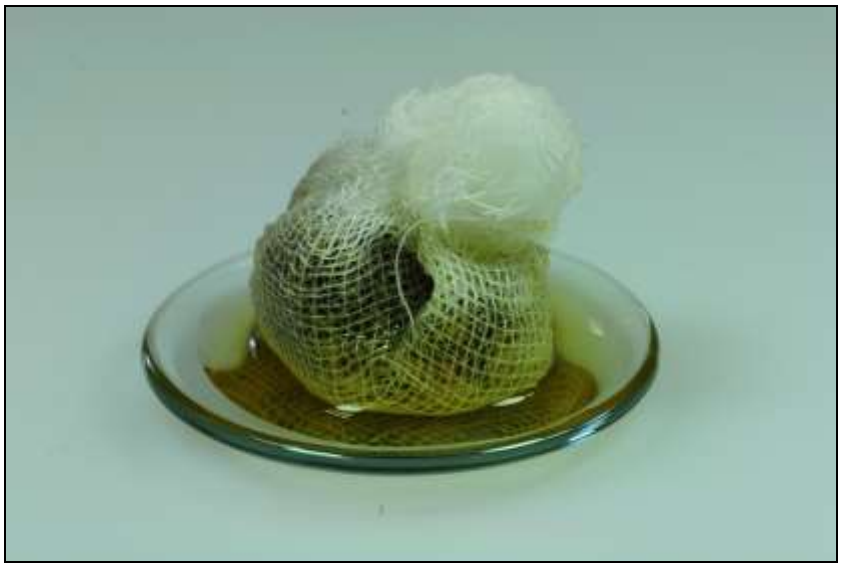

Figure 77

A sample prepared by Vajda method waiting for larvae migration to water on Petri dish.

Figure 78

Modification of Vajda larvoscopic method using bigger volume of water in a beaker and fixation of a bundle with faeces.

4.3.2 Sporulation of coccidia

Principle

Exogenous stages of coccidia - the oocysts sporulate under propitious conditions of oxygen saturation, humidity and temperature to become infective in several days. Sporulation of unsporulated oocysts is necessary for their morphological determination to genus and in some oocysts to species also (figures 79-80).

Equipment

Mortar with pestle

- Wash bottle with tap water
- Sieve and funnel
- 2.5% potassium dichromate solution ($K_2Cr_2O_7$) 1% sulfuric acid solution (H_2SO_4).
- Petri dishes with lid
- Pasteur pipette
- Microscope slides and cover slips
- Immersion oil
- Microscope

Procedure

1. Put the faecal sample into a mortar, add tap water and stir the content thoroughly.
2. Pass the suspension through a sieve and a funnel into a Petri dish to a low layer, add potassium dichromate.
3. Leave the suspension at room temperature for 3–14 days, mix each day for oxygenation, add potassium dichromate if solution evaporate.
4. Use Pasteur pipette for a transfer of suspension on a microscopic slide for oocysts identification at 1000x magnification. Identification of oocysts is based on keys.

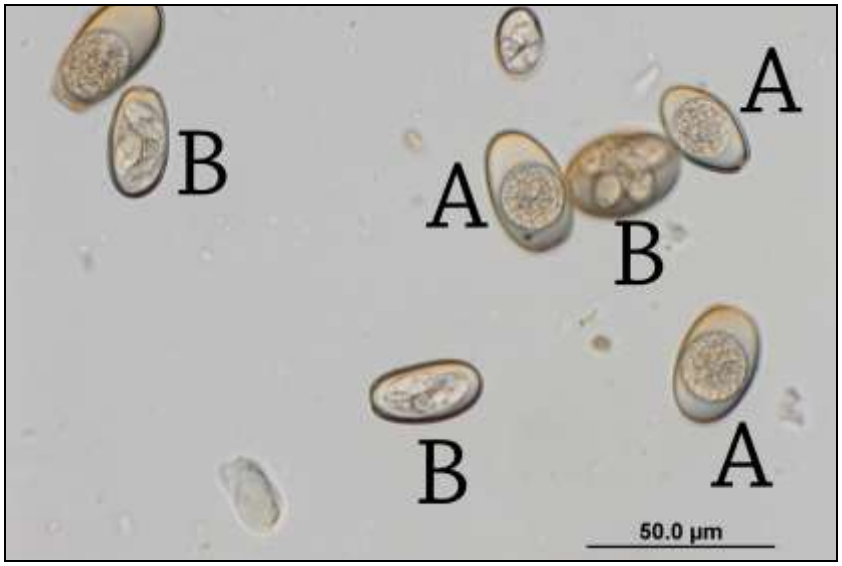

Figure 79

A sporulation of oocysts. Unsporulated (A) and sporulated (B) oocysts of rabbit *Eimeria* spp.

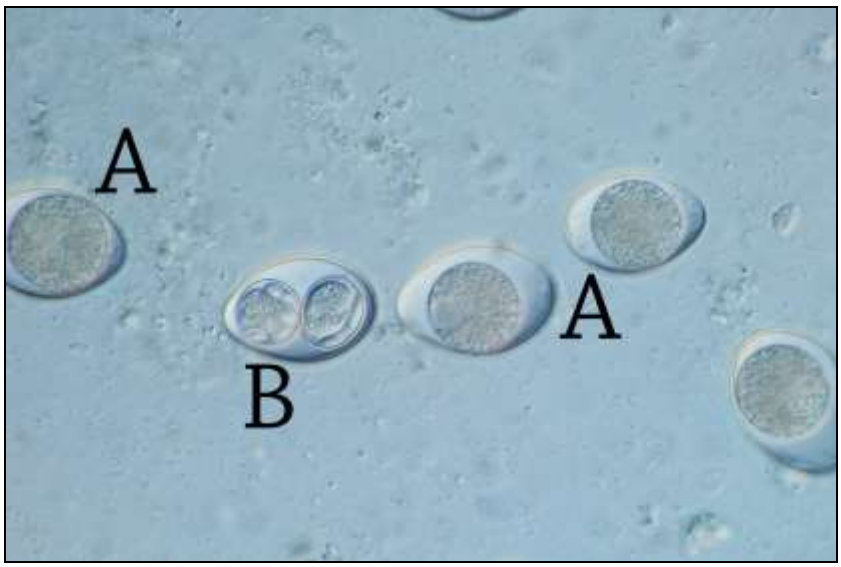

Figure 80

Unsporulated (A) and sporulated (B) oocysts of *Isospora felis*.

5 ECTOPARASITES AND VECTORS

5.1 Introduction

The ectoparasites are invertebrate organisms included in the phylum Arthropoda. The word arthropod has Greek origin, "*árthron*" standing for "joint" and "*pous*" for "leg", giving them the main morphologic characteristic: "jointed leg". The body of the arthropods is segmented and they have an exoskeleton and jointed appendages.

This phylum included the Arachnida, the Insecta, the Myriapoda and the Crustacea classes, on which the first two contains species with significant veterinary importance.

The ectoparasites of the class Arachnida have the body divided in two parts: the abdomen and the cephalothorax. The cephalotorax is covered by a solid shield on the dorsal side, and it is unsegmented. The first pair of the appendages used for feeding (chelicerae) can be seen in front of the mouth, and behind of it are positioned the second pair of appendages (pedipalps), of which function differs from order to order. A characteristic of the mouth is the absence of jaws. They have simple eyes and no antennae or wings.

The sub-class of the class Arachnida that has a major importance in veterinary practice is the Acari sub-class, which includes the mites and ticks.

The ectoparasites are living in or on the skin of other organisms - the host, but not within their body. Between the large number of the existing arthropod species, only a reduce number developed the capacity to inhabit directly on the host's expense.

However, depending on the species, they spend different periods of time on the hosts and this could be harmful for the latter, but usually, they do not kill the hosts. This type of relationship is defined as parasitism.

There are two type of parasitism with regards to the association between the arthropod ectoparasite and vertebrate host:

- obligatory parasitism: when the ectoparasite is totally dependent on the host, by inhabiting in association with the latter during all its life (continuous association) (e.g. many lice species);
- facultative parasitism: when the ectoparasite is not totally dependent on the host, he only feeds or only lives occasionally on this (intermittent contact) (e.g. ticks).

The host represents the main resources for the ectoparasites, including:

- food: blood, lymph, tears, sweat, skin debris, hair, etc.;
- the body of the hosts constitutes the environment for living and the hair/fur offer protection against the external environment;
- the host can also provide transportation for the parasite.

The infestation with ectoparasites can occur in all animals, including: farm animals, pets, poultry, laboratory animals, etc. Some of them are parasitising a wide range of hosts (e.g. ticks), while other ectoparasites are host-specific (e.g. lice). Other ectoparasites are not only highly host-specific, but they can parasite the body of the host in only one defined area (e.g. some mite species).

In addition to the parasitic role, the ectoparasites are vectors of many pathogens that they typically transmit to the hosts during the time of feeding or defecating: vector role.

5.2 Field diagnostics

5.2.1 Ectoparasites collection from hosts

Depending on the size of the ectoparasite, they are collected directly off the hosts using a properly sized **tweezers** in case of the large ectoparasites (e.g. fleas, myiasis associated larvae and ticks) (figures 81-83); the small specimens can be collected with a **moistened paintbrush**. The unattached ticks, fleas

Figure 81

Heavy flea infestation with *Ctenocephalides* in a dog

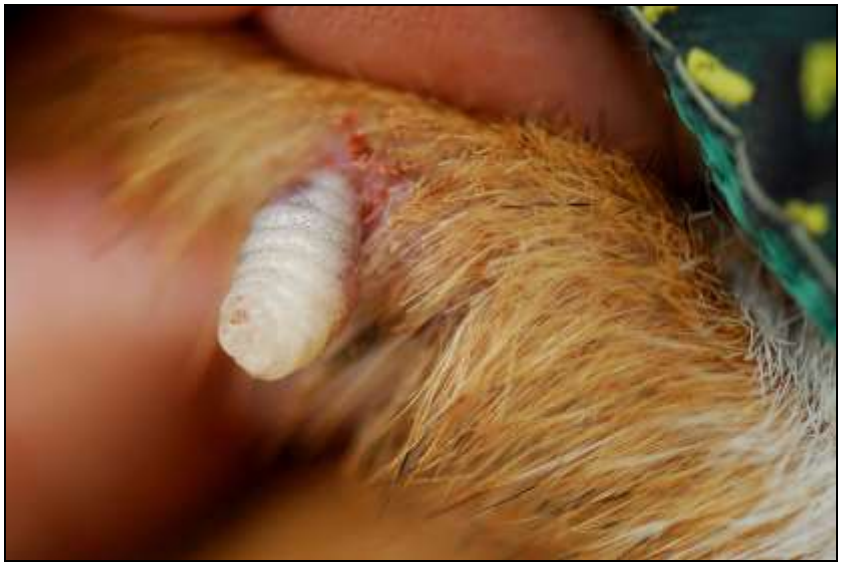

Figure 82

Manual removal of a larva of *Cordylobia anthropophaga* from the subcutaneous tissues of a dog.

Figure 83

Fully engorged nymphs of *Rhipicephalus sanguineus*, the brown dog tick on the ear pinna of a dog.

or mites can be taken off by **combing or brushing** the host on the top of a white surface or sheet of paper.

For example, flea infestation can be diagnosed by brushing the hair or coat of the host over a moistened white paper or towel because the flea faeces stain in red when they dissolve on the wheat surface. As fleas are adapted to hang on and crawl in then fur in places that are hard to be reached by the hosts (e.g. ventral side of the neck, tail) it is also recommended the brushing to be performed in these areas.

After collection, the fleas should be identified by using a dissecting microscope. If the sample examination is done immediately, a dish of alcohol should be placed in the bottom of a container and a sharpened stick should be used to pick up the fleas. After immersing the stick into the alcohol, the fleas will adhere to the stick. In this way, each flea will be passed in the dish of alcohol. If fleas preservation is needed, ethanol 70–80% should be used. It is not recommended to use higher concentrations of ethanol, or isopropyl and methyl alcohol, the samples will be destroyed.

To identify microscopically some species of ectoparasites, the hair of the host is collected by **brushing or plucking** and then immersed in mineral oil (e.g. liquid paraffin) and examined under the microscope. By using this technique, there are identified the eggs of some attached to the hair shaft (e.g. lice and *Cheyletiella* spp.) and even some adult ectoparasites (e.g. lice and different species of mites). When examining the plucked hair, especially from feet lesions, it is really important to closely check the bulb and the lower third part of the shaft as this is a useful technique to diagnose the demodicosis, by identifying the follicular mite *Demodex* spp. If one of the symptoms of the host is alopecia, it is really important to closely examine the upper part of the hair shaft: if this part of the hair is fractured, it can be concluded that this type of alopecia it is self-induced by pruritus.

A useful technique to identify the ectoparasites and material present in the coat or on the skin surface is the "**acetate strip examination**" (e.g. scotch tape or sellotape). The strips of the acetate tape are repeatedly applied on the hair or on the skin surface, and then mounted on the glass slides and examined under the microscope (figure 84). This method is usually used to identify the surface mites. In case of severe infestations, *Otodectes* spp. (body infestation) and even *Demodex* spp. could be identified.

Another routine method used to diagnose the ectoparasitic infestation, especially for the definitive diagnoses of the surface mites (e.g. *Cheyletiella* spp., *Otodectes* spp. and *Trombicula* spp.) is "**superficial skin scraping**" (called also epidermal surface examination). The coat hair should be removed by gentle clipping (in addition, the hair can be also examined by using the methods mentioned above) and then the skin surface is scraped by using a scalpel blade. The collected material should be mounted in liquid paraffin, mineral oil or potassium hydroxide (KOH) 10% on a glass slide. Alternatively, the liquid paraffin could be spread over the skin surface and then scraped by using a blunt scalpel blade. The obtained sample of superficial epidermis is then spread over a glass slide, covered by a glass cover slip and then microscopically examined (figure 85). If this technique is used, is important to note that the 10% KOH it is forbidden to be applied directly on the skin. If the sample is harvested by dry skin scraping, it could be digested for a period of in 20 minutes in 10% KOH and then centrifuged. The concentrate of mites can be harvested from the surface of the sample.

No matter what scraping technique is used, it is highly recommended to take multiple scrapings in order to increase the chance of ectoparasite identification. When performing this test, it is also important to select the area preferred by the suspected parasite and to take in consideration the nature of the dermatosis (for example, *Cheyletiella* spp. are usually found on the dorsal part of the body).

An extension of the technique described above is the "**deep skin examination**" (deep epidermal examination). It is recommended to use this method during or following skin squeezing between the thumb and forefinger. After removing the epidermal layer as suggested above, the operation is repeated until capillary ooze or slight bleeding appear. This method is used to diagnose the burrowing and deep follicular mites (e.g. *Sarcoptes scabiei, Notoedres* and *Demodex*). To increase the chances of ectoparasite detection, it is recommended to scrape multiple sites. If more than one scraping specimens are taken from the same place they can be examined on the same slide, but excessive amount of sample should be avoid. After mixing the skin scrapings with liquid

Figure 84

Chirodiscoides caviae **revealed on the fur of a guinea pig using the scotch tape method**

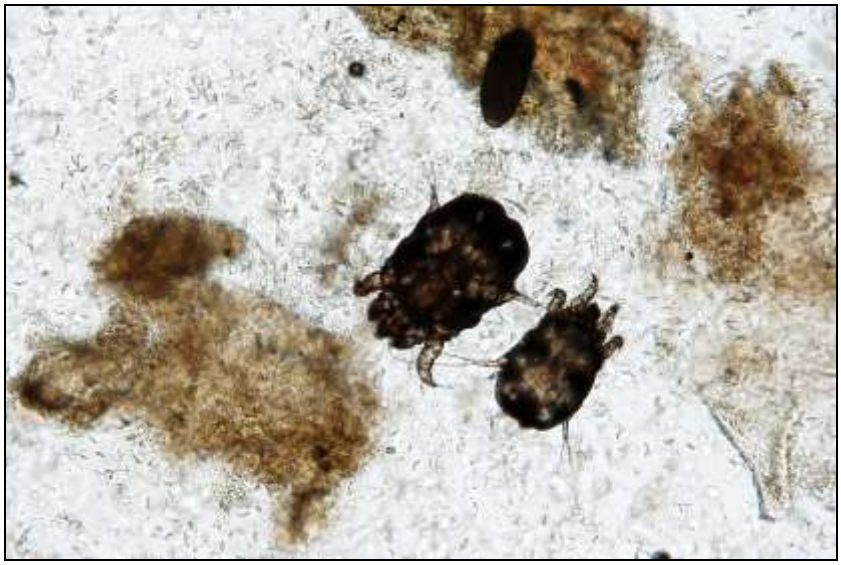

Figure 85

Psoroptes **sp. mites identified in the ear od a rabbit using superficial skin scrapping.**

Figure 86

Demodex canis **from the dermis of a dog, identified using the deep skin scrapping..**

Figure 87

Ear mite *Otodectes cynotis* stages on swab from kitten ear under microscope.

Figure 88

***Dermanyssus gallinae* mites in the cage of a pet budgerigar.**

Figure 89

***Dermacentor marginatus* adult female questing on vegetation.**

Figure 90

Researcher performing a dragging on vegetation for the collection of questing ticks.

Figure 91

A styrene foam box containing dry ice is placed on a plastic board near a dog den for the collection of *Rhipicephalus* spp. ticks.

Figure 92

Horse-flies are commonly seen feeding on animals during the warm season. They can be easily collected using hand nets.

Figure 93

***Hippobosca longipennis* feeding on the injured skin of a dog.**

paraffin and applying the glass cover slip, the sample should be examined under a binocular microscope (figure 86).

The definitive diagnosis is done based on the findings. For example, if a mite, egg or faeces of sarcoptic mange mites are identified, it is enough evidence to diagnose a sarcoptic infestation. In case of *Demodex* spp., the finding of one or two parasite is not sufficient to diagnose a parasitic infestation as these ectoparasites are normal commensals; additional samples should be taken and closely examined, and then corroborate the findings with the clinical history of the patient.

An important note is that in some cases, the absence of the parasite in the examined sample does not exclude the parasitic infestation. For example, in case of sarcoptic mange and cheyletiellosis, the presence of the parasite confirms the diagnosis, but the absence of it does not infirm the diagnosis.

If the animal has skin lesions characterized by epidermal hyperplasia and exfoliation, or other lesions caused by different type of ectoparasites that are leading to epidermal debris (e.g. *Chorioptes* spp., *Cheyletiella* spp., *Sarcoptes* spp., *Psoroptes* spp., *Otodectes* spp. , and *Demodex* mites, fleas or lice), the dried exudate or debris can be collected either using a small jar or a vacuum cleaner adapted with a filter. The collected sample should be further examined under the microscope.

If is this suspected that the animal could have ear mites (e.g. *Otodectes* spp.), a closely examination with the otoscope could evidence the parasite. However, it is recommended to take a sample from the external year by using a cotton swab and then examine under the microscope (figure 87).

5.2.2 Ectoparasites collection from environment

The free-living fleas, ticks or mites are also find in the environment of the hosts (e.g. bedding, nests or faeces). They can be identified in bedding, nests and faeces. If they are not find after a though search, the material can be passed by sieves with decreasing mesh size.

For example, the poultry mites (e.g. *Dermanyssus* spp.) are staying on the birds only during the night, while in the daylight it is mandatory to examine the environment of the birds (figure 88). This should be performed by searching in the nests or other housing structures, and then, the samples should be examined in laboratory.

The ectoparasites can also be collected from the surrounding vegetation by using a hand net.

Free-living tick collection (trapping)

The most common method is the “blanket dragging or flagging” (figure 89-90). A piece of white cotton

towel or woolen blank of approximately 1 m^2 is attached at one side to a bar. The dragging method is performed by passing the material across the low-lying vegetation; the ticks should attach to the piece of white material.

The flagging method is done by waving the material through and over a higher vegetation, and the questing ticks will be trapped in its fibers because they respond to the body movement and heat, and to the carbon dioxide (CO_2) released during expiration.

Both of these methods are effective, but it is recommended to be used depending on the season, tick species and developmental stage of the tick. Usually, flagging is more effective to trap adult ticks in spring or winter months. In order to achieve the best results, the combination of these methods can be used.

The dry ice baited tick trap

It is a method used to collect the ticks that are not responsive to dragging techniques. The principle of this method is based on the fact that humans and animals, by CO_2 emission during exhalation process, attract the ticks and stimulate them to go questing for a blood meal, playing this way an essential role in the host seeking process. As the dry ice is the solid form of CO_2, this agent is used by placing it in a container, where the ticks will be attracted (figure 91). To easily identify the trapped ticks is recommended to use a white surface or a sticky tape. This method is usually used to collect the ticks from environments with high humidity (e.g. forest and rivers areas), but sampling should be done in warm seasons. An advantage is that the thick capturing is performed without using humans or other animals as a source of CO_2.

Free-living adult flies collection

The hand nets method can be efficiently used. These nets are usually deep bags of fine mesh that have a circular, wire-stiffened opened on the top. The flies can be taken off when they are on the hosts (figures 92-93), faeces or feeding on the death animals. In addition to the hand net, it can also be used a glass tube that should be inverted over the fly and different types of baited traps to draw in and catch the adult flies.