

Chapter 2

Techniques of Sampling, Preparation and Staining of Cytological Specimens

2.1 Introduction

There are many skin lesions and they differ from one another morphologically. Papules, pustules, epidermal collarettes, scales, erosions, ulcers, plaques, nodules and swellings are clinically easy to recognise. Some of these lesions may be present simultaneously in the same patient, representing different stages of the same disease or clinical signs of different coexisting diseases.

Cytology is one of the fastest, most inexpensive and easy to perform of the diagnostic techniques available. As with all diagnostic tests, the quality of the specimens is basic for correct interpretation; even if more than one sampling technique can be performed from the same lesion, there is always one that is most suitable, offering more guarantees of obtaining a representative sample. This chapter discusses the sampling methods in canine and feline skin cytology. Starting from clinical lesions, the reader is provided with fundamental information for a correct preparation and staining of slides.

Based on the above, the execution of a correct cytological sampling cannot disregard three basic steps:

- (a) *The choice of the lesion to be sampled*
- (b) *The choice of the sampling method most suitable for that particular lesion*
- (c) *Proper preparation of the slide*

2.2 Sampling Techniques

In practice, the most commonly used sampling techniques include the *impression smear*, the *fine needle biopsy*, with or without aspiration, and the *scraping* (Raskin and Meyer 2015; Valenciano and Cowell 2014).

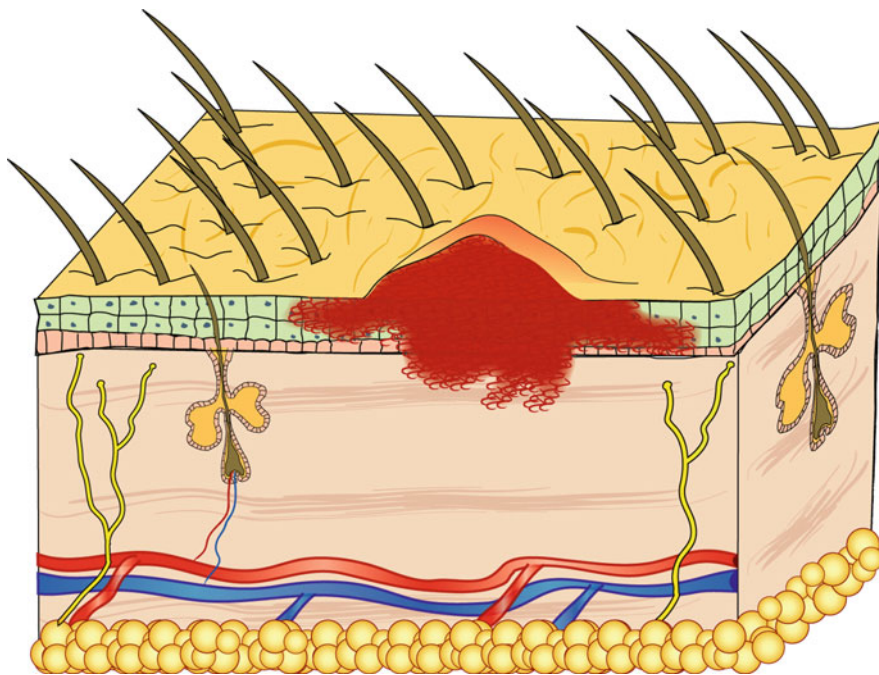


Fig. 2.1 Schematic representation of a papule

2.2.1 *Impression Smears*

The *impression smear* is the only executable method we can perform when cells must be sampled from non-raised, superficial “wet” (exudative) lesions. This technique is frequently used because with this method, it is possible to collect cells simply by placing a slide on the lesion. As the cells easily adhere to the slide, it is sufficient to gently place it on the lesion without exerting excessive pressure, which could alter the cell morphology. Nevertheless, there are some basic precautions that differ according to the lesion that must be investigated. The skin lesions from which cells can be obtained using the impression smears technique are numerous and are covered below.

2.2.1.1 Cytological Sampling from *Papules*

Papules are small, solid, raised and palpable primary lesions, usually erythematous, smaller than 1 cm (Figs. 2.1 and 2.2). Histopathologically, papules are composed of inflammatory or, exceptionally, neoplastic cells, located in the superficial dermis and usually cause a slight hyperplasia of the overlying epidermis. Intra-epidermal exocytosis of inflammatory cells or small ulceration of the epidermis, the latter



Fig. 2.2 Multiple erythematous papules on the abdomen of a dog with superficial pyoderma

often located on the top of the papule, cause discharge of a small amount of exudate that, once dehydrated, gives rise to a small crust (Fig. 2.3). These types of lesions are called *crusted papules* and are frequently observed in cats with miliary dermatitis and in dogs with scabies.

Papules with an intact surface are too small for FNB and cells cannot be collected using the imprint technique; therefore, only the papular crusted lesions permit the collection of a few cells using this method.

Some dermatologists use the term *nodular papule* to describe a lesion that is raised and round in shape, which is of intermediate size between a papule and a nodule. Nodular papules are less than 1 cm and even if they can sometimes slightly exceed this size, they do not appear large enough to justify the definition of nodules (Fig. 2.4). These lesions, compared with papules, provide more diagnostic specimens, because through FNB they can supply sufficient cells.

The author prefers to treat the cytological findings of papular nodular lesions separately from those of papules, as he believes that the former are characteristic of some skin diseases and for this reason, deserve a more detailed cytological description.

Sampling Technique

In general, *papules* do not yield many cells, but, in some cases, even the few cells collected may be useful in the interpretation of the lesions.

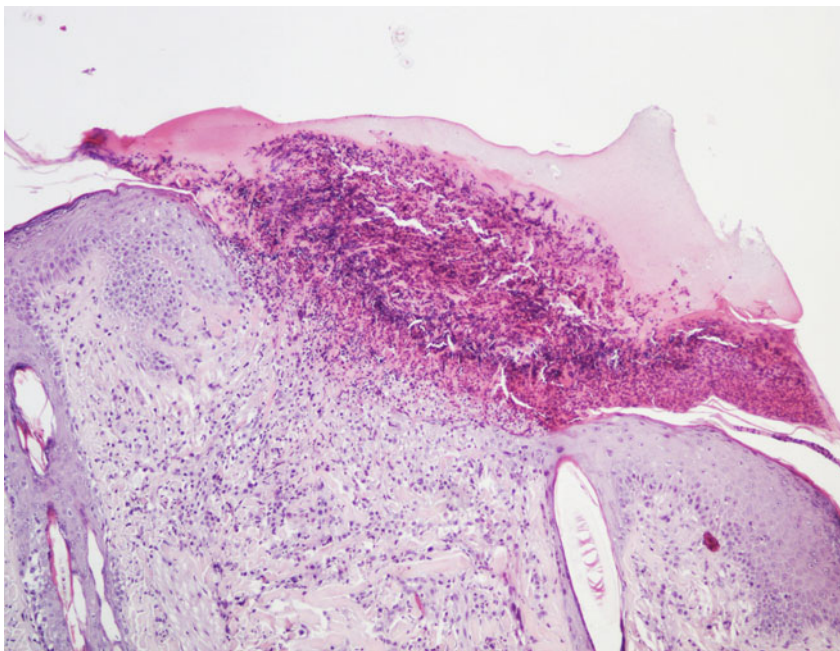


Fig. 2.3 Histology of a crusted papule: note the crust covering the ulcer



Fig. 2.4 Papular nodules in a dog with the papular form of leishmaniasis

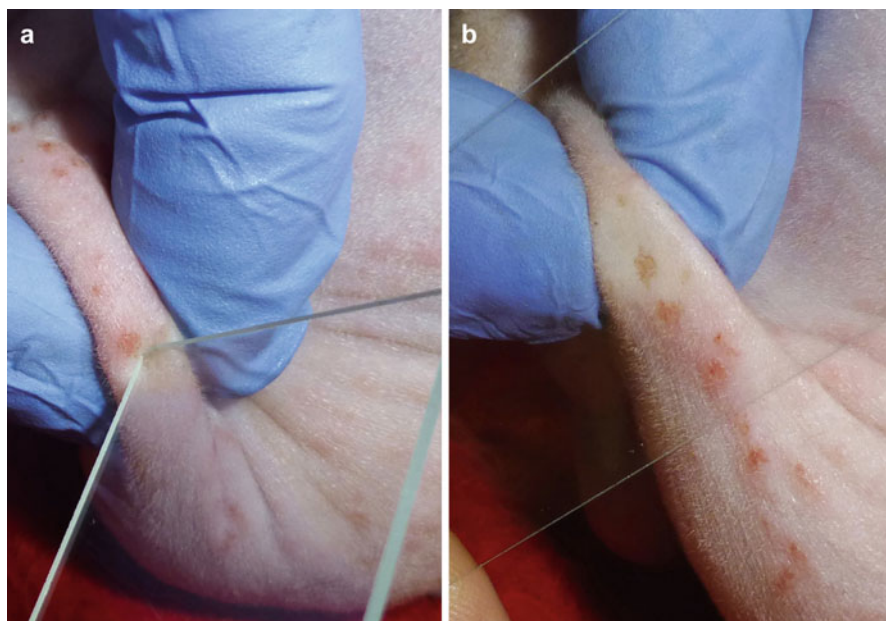


Fig. 2.5 (a) Removal of a crust with a slide, (b) placing the slide onto the exposed exudate

In the case of papules with an intact epidermis, the collection of cells is not possible using an impression smear. When the papules ulcerate or when spongiosis promotes the leukocytic exocytosis through the epidermis, it is possible to collect some cells by gently placing a slide on top. To collect cells from *crusted papules*, the superficial crust must be removed before placing the slide on the exposed exudate (Fig. 2.5). In the case of *nodular papules*, it is possible try collecting cells via FNB using a small needle (23 G), a technique that will be discussed later.

2.2.1.2 Cytological Sampling from *Pustules*

Pustules are raised, soft, yellowish, intra-epidermal or intra-follicular skin lesions. The yellowish colour of the pustules is closely related to their content of granulocytes, both neutrophils and eosinophils, which are the main cells that comprise the purulent exudate (Figs. 2.6 and 2.7).

By definition, pus is an inflammatory exudate composed of karyolytic neutrophils, necrotic debris and bacteria; according to this definition, the term pus should only be used to describe the content of pustules from pyoderma. As there are many diseases that produce a *sterile* purulent exudate, the term *pus* should refer only to its granulocytic composition, and should not imply a bacterial infection.

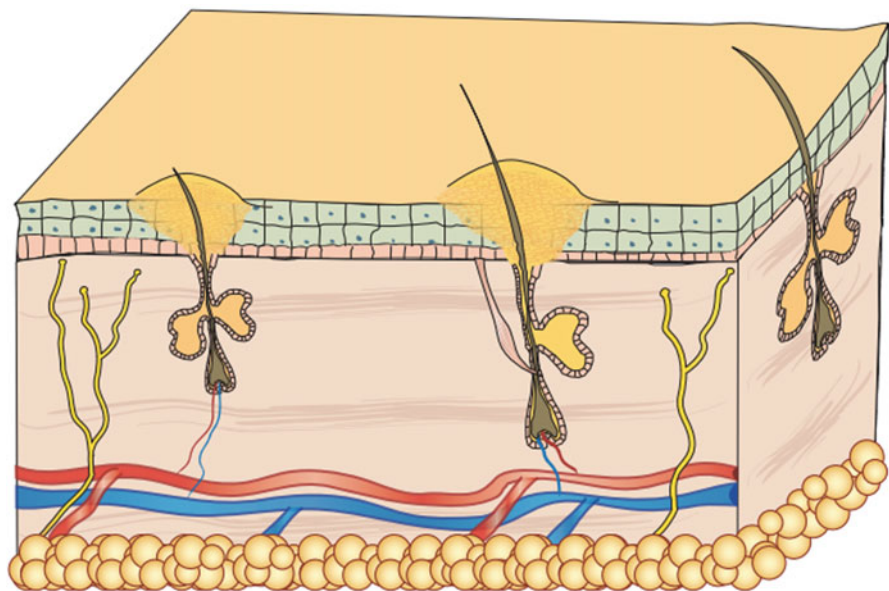


Fig. 2.6 Schematic representation of a follicular pustule

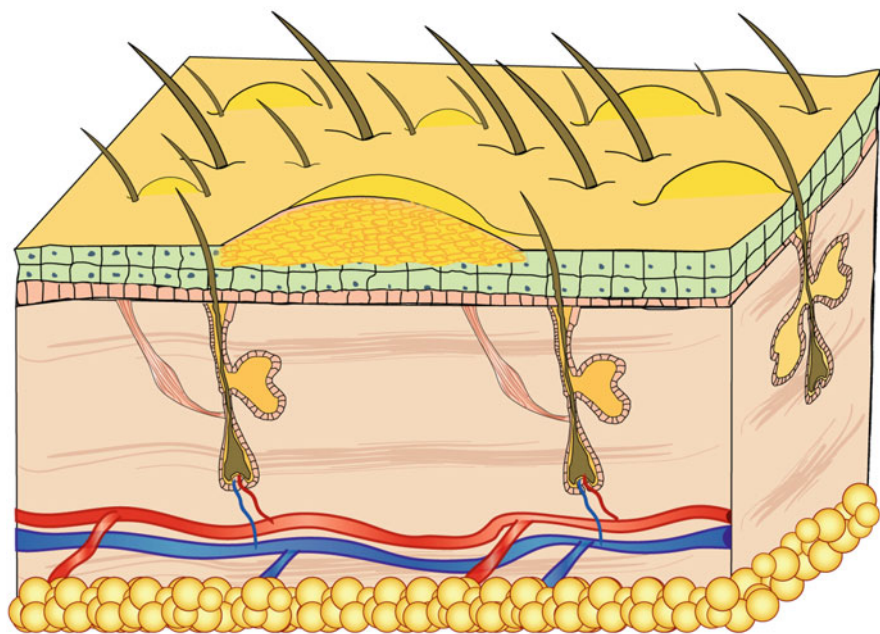


Fig. 2.7 Schematic representation of a non-follicular pustule



Fig. 2.8 (a) Histology of a follicular pustule; note how the pustule is located on the centre of the follicle; (b) clinical aspect of a follicular pustule: a hair shaft emerges from the centre of the pustule

In addition to granulocytes, pustules may contain other cell types (keratinocytes), microorganisms (bacteria), parasites (*Demodex* spp.) etc.. The detection of these components in cytological samples provides important diagnostic clues.

Clinically, pustules can be divided into *follicular* and *non-follicular*. *Follicular pustules* are characterised by pus located in the follicular lumen and are clinically recognisable by their small size and by a hair shaft protruding from its centre. As follicular pustules are very small, they are always round and less than 1 cm in size (Fig. 2.8). The term *follicular pustule* is also used in histopathology to describe an intramural follicular sterile pustule, sometimes observed in pemphigus foliaceus. In these cases, the pustules are so small that they are not clinically detectable; thus, when a hair emerges from a pustule, infectious folliculitis should be suspected (Fig. 2.9).

In *non-follicular pustules*, pus is not present in the follicular lumen, but it is intra-epidermal and more frequently intra-corneal (Fig. 2.10). Depending on their extent, non-follicular pustules can be of variable size, round or irregular in shape. They may be small and localised between follicles or so large that they inevitably encompass many hairs, ranging from a few millimetres to over 2 cm (Fig. 2.11). The clinical aspect and localisation of pustules not only has a descriptive meaning, it may also help the clinician to better interpret the cytological results.

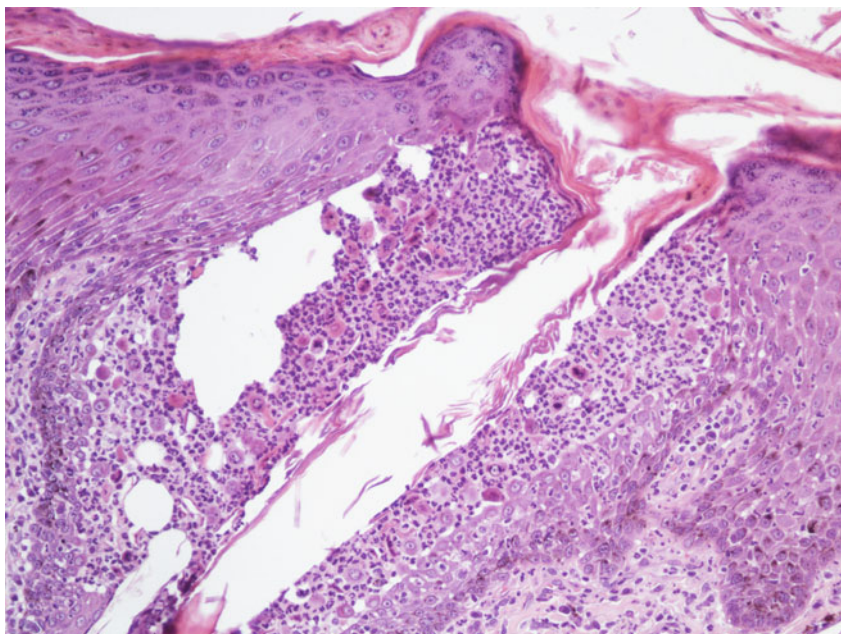


Fig. 2.9 Histology of two intramural acantholytic follicular pustules in course of pemphigus foliaceus. Note how the pustules are confined in the follicular wall and, as they do not emerge from the center of the follicle, they are not macroscopically evident

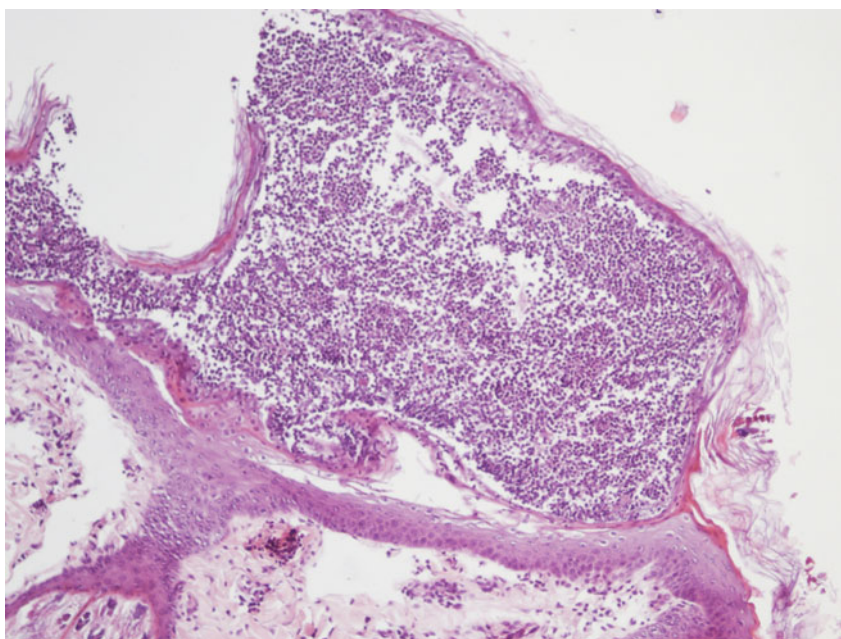


Fig. 2.10 Histology of a non-follicular pustule: note how the pustule does not involve the follicle



Fig. 2.11 Large and non-follicular pustules of irregular shapes in a dog with juvenile impetigo

Sampling Technique

Pustules may be the most important lesions in canine skin cytology, as they usually represent the direct action of a disease or a microorganism. In the case of large pustules, cells can be collected using the impression smear technique: breaking the base of the pustule with a fine needle and lifting the keratinocytes that form its “roof”. The pus is exposed and a sample can be taken by gently placing a slide. This method is a variant of the technique conceived by Dr. Arnault Tzanck, a French dermatologist who was a pioneer of diagnostic cytology in the early 20th century and for this reason, the technique is known as the *Tzanck test* (Fig. 2.12).

When pustules are very small, like follicular pustules, this method is very difficult to perform and the risk of penetrating the dermis with the needle and causing bleeding is very high. In these cases, the shorter part of the slide must be slightly and laterally pushed against the base of the pustule, allowing the pus to be transferred onto it (Fig. 2.13).

Once collected, the material must be gently smeared onto another slide and left to dry. Pustules tend to rapidly dehydrate and give rise to yellowish crusts or epidermal collarettes. In these cases, as described below, it is possible to obtain cytological specimens by collecting the exudate present below the crust at the edges of the collarettes.



Fig. 2.12 The Tzanck test: rupture of a large pustule with a needle

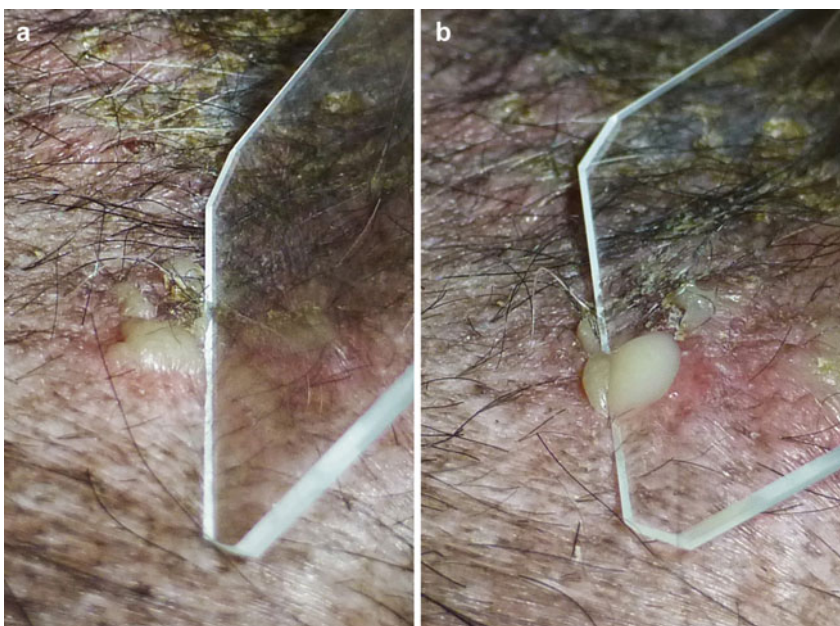


Fig. 2.13 (a) Lateral pressure placed on a small pustule with a slide; (b) the pus is transferred onto the slide

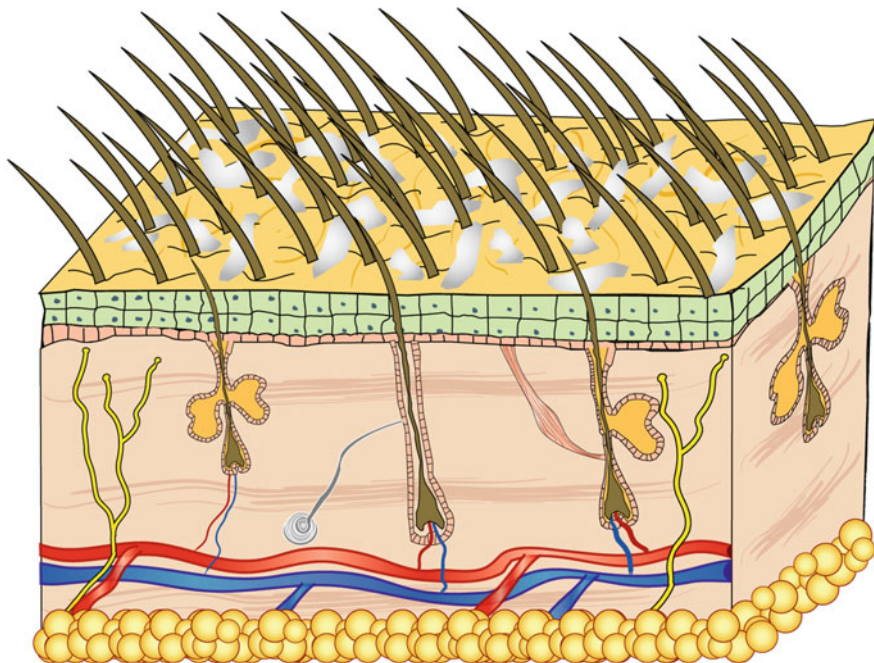


Fig. 2.14 Schematic representation of scales

2.2.1.3 Cytological Sampling from Intact Skin Covered with “Dry” or “Oily/Waxy” Scales

Scales are aggregates of corneocytes that represent the final product of the physiological keratinisation process of the epidermis and are continuously released into the environment, both singly and in small clusters (Fig. 2.14). When scales are visible to the naked eye, the clinical finding is described as *exfoliative* or *desquamative dermatitis*, which is observed in many canine and feline skin diseases. Scaling diseases recognise both *primary* and *secondary* causes; primary causes are usually linked to genetic or idiopathic disorders and in these cases, cytology has no diagnostic interest. Scales are also very common in secondary keratinisation defects, usually caused by infectious (leishmaniasis, dermatophytosis, pyoderma, *Malassezia* spp.), parasitic (demodicosis), and autoimmune diseases (pemphigus foliaceus), in addition to neoplasia (epitheliotropic lymphoma), whose cytology provides diagnostic information or can direct clinicians in selecting additional diagnostic tests.

Sampling Technique

Placing a slide onto an oily/waxy skin allows keratinocytes and different microorganisms present on their surface to be collected. In patients with dry scaly dermatitis, the impression smear technique does not permit the cells to adhere to the slide; in these cases, sampling requires the use of a piece of transparent acetate tape (Fig. 2.15). This method is very useful when searching for yeasts and dermatophytes,



Fig. 2.15 Many scales remain stuck to the adhesive acetate tape

but it can be used to collect any type of cell or parasite present on the skin surface, as will be discussed in the Chap. 3.

2.2.1.4 Cytological Sampling from *Erosions*

Erosions are superficial lesions characterised by the loss of the outer layers of the epidermis and do not involve the dermis (Fig. 2.16). Determining the integrity of the basement membrane is not always clinically possible, particularly when the erosion is deep and its base is composed only of the basal layer of the epidermis. Cytological examination confirms or rule outs an ulcer, based on the presence or absence of red blood cells respectively.

Epidermal collarettes are the most frequent erosive lesions observed in canine dermatology and are also more valuable for cytological examination. Collarettes are round erosion, with an erythematous or hyperpigmented central area and peripherally bordered by scales, in case of vesicles, or crusts in case of pustules (secondary lesions). As vesiculo-bullous lesions are extremely rare in pets and are mostly located at the dermal–epidermal junctions, rupturing them produces an ulcer; for this reason, almost all epidermal collarettes are secondary to dehydration and breakage of a pustule. Collarettes are very common in dogs because of the high frequency of superficial pyoderma in this species; indeed, these lesions are

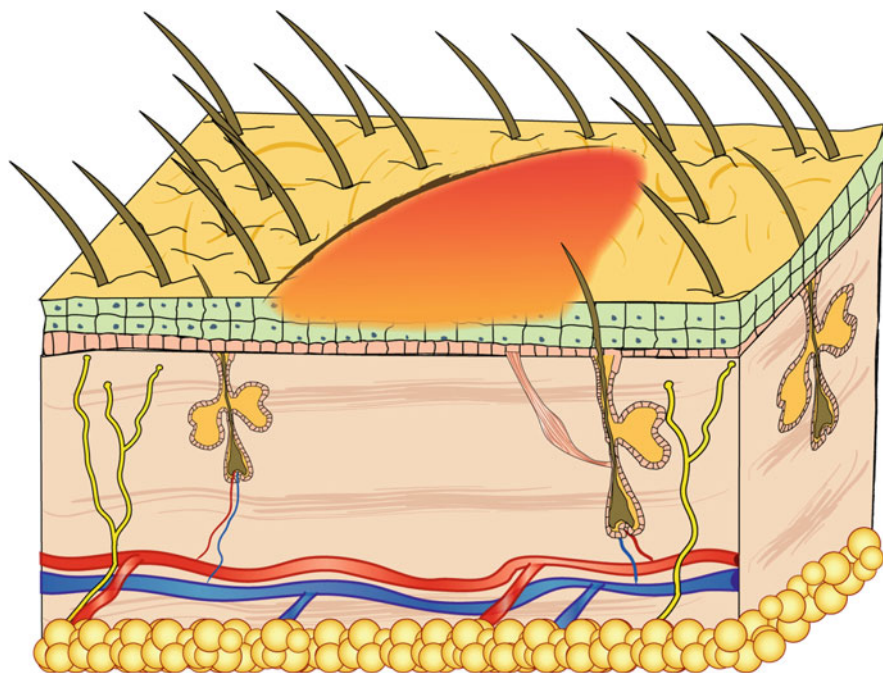


Fig. 2.16 Schematic representation of an erosion. Note how the lesion is located in the epidermis with no dermal involvement

frequently found in combination with papules and pustules. Rarely, epidermal collarettes are the only type of detectable lesion and, thus, the only lesion that can be sampled (Fig. 2.17).

In some dogs affected by so-called spreading pyoderma, the intra-corneal diffusion of the infection leads to the development of large and confluent, erosive, collarette-like lesions, which originate from a clinical presentation similar to exfoliative dermatitis. Less frequently, collarettes are observed in other pustular sterile diseases such as pemphigus foliaceus, in which cytology from collarettes could be very useful.

Sampling Technique

Because of their superficial location, erosions are flat and exudative lesions; therefore, placing a slide on their surface can provide the clinician with useful information. As for all lesions exposed to the external environment, cytological specimens obtained from collarettes using the impression smear technique must always be interpreted with caution. Clinicians must be aware of the ever-present possibility of the secondary contamination of specimens from both environmental bacteria and, in the case of excessive licking, the oral cavity (e.g. *Simonsiella* spp., rod-shaped and filamentous bacteria).

The collection of cells from epidermal collarettes is performed by lifting the crusts that delimit the lesion and placing a slide on the exposed fresh exudate.

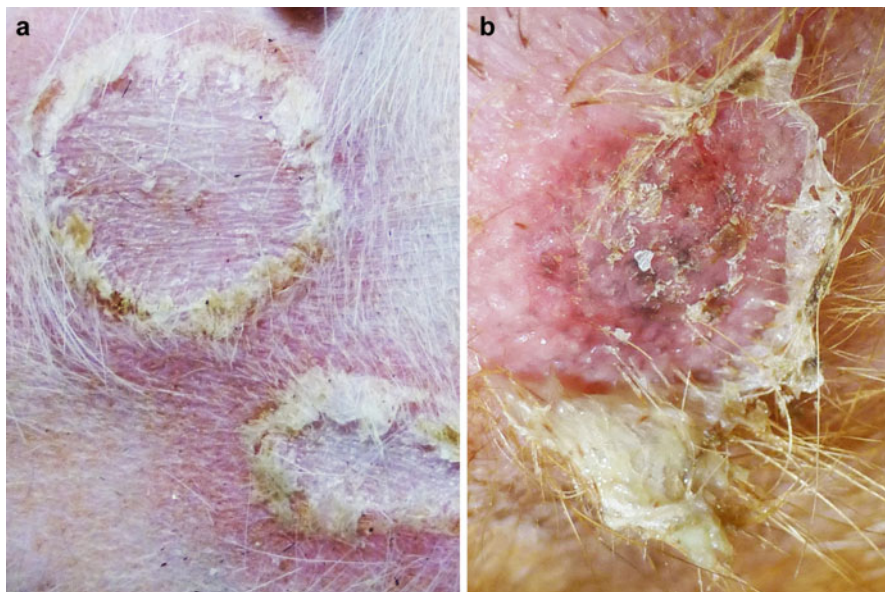


Fig. 2.17 (a) Epidermal collarettes with a dry central area; (b) purulent exudate is present under the peripheral crusts

2.2.1.5 Cytological Sampling from *Ulcers*

Ulcers are deep lesions that represent an excavation in the skin with a loss of all epidermal layers and part of the dermis (Fig. 2.18). Based on this definition, it is clear why it is common to find red blood cells in cytological specimens from ulcers (Fig. 2.19). Samples from ulcers are usually of poor quality and highly haemocontaminated; thus, only in rare cases do ulcerative diseases provide useful specimens. Most ulcerative diseases, such as cutaneous lupus, vesiculo-bullous, sub-epidermal diseases, erythema multiforme etc., can be diagnosed only by histopathology. Nevertheless, there are some ulcerative diseases for which cytology may provide an immediate diagnosis.

Sampling Technique

The method of sampling differs according to the depth of the ulcer. In deep ulcers with raised edges, the impression smear technique is not feasible because the slide cannot reach the bottom of the ulcer; in these cases, cells can be sampled by scraping (Fig. 2.20). In shallower ulcers with loss of only small portions of the dermis, the cells may be collected by placing the slide.

2.2.1.6 Cytological Sampling from *Crusts*

Crusts are secondary lesions, composed of inflammatory cells, keratinocytes, debris and, possibly, of hair shaft fragments, blood and microorganisms (Fig. 2.21).

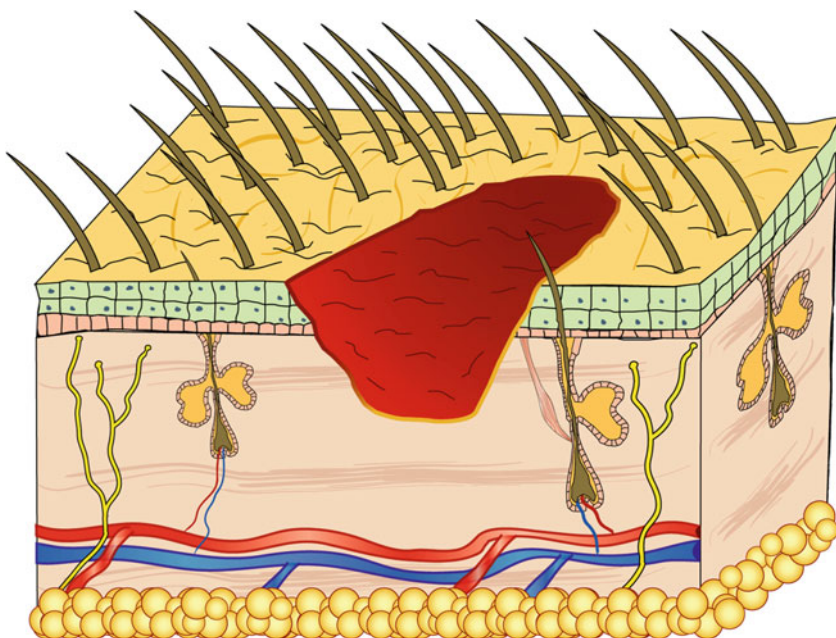


Fig. 2.18 Schematic representation of an ulcer. Note how the lesion is deep and involves the dermis



Fig. 2.19 Round ulcer covered with an haematic crust



Fig. 2.20 In the case of deep ulcers, placing a slide does not permit the cells to be collected

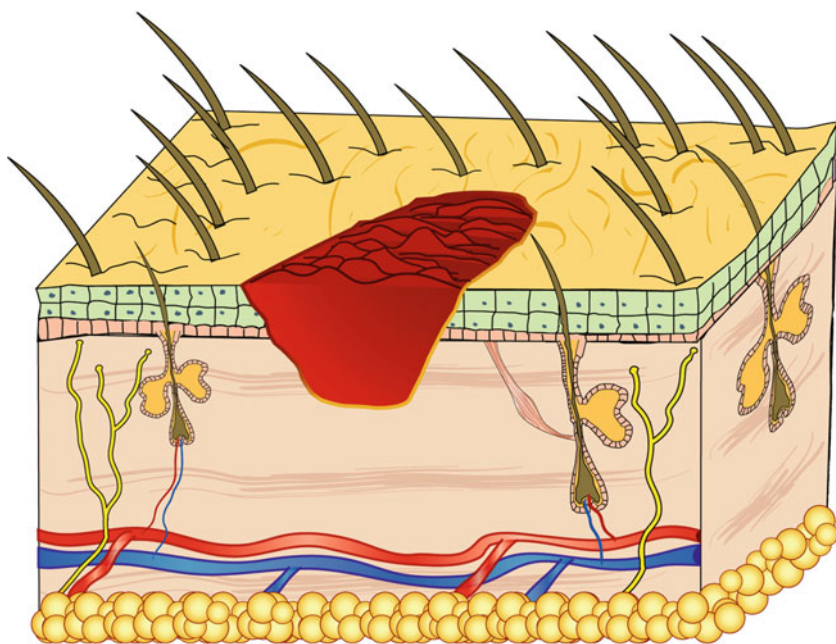


Fig. 2.21 Schematic representation of a haematic crust covering an ulcer

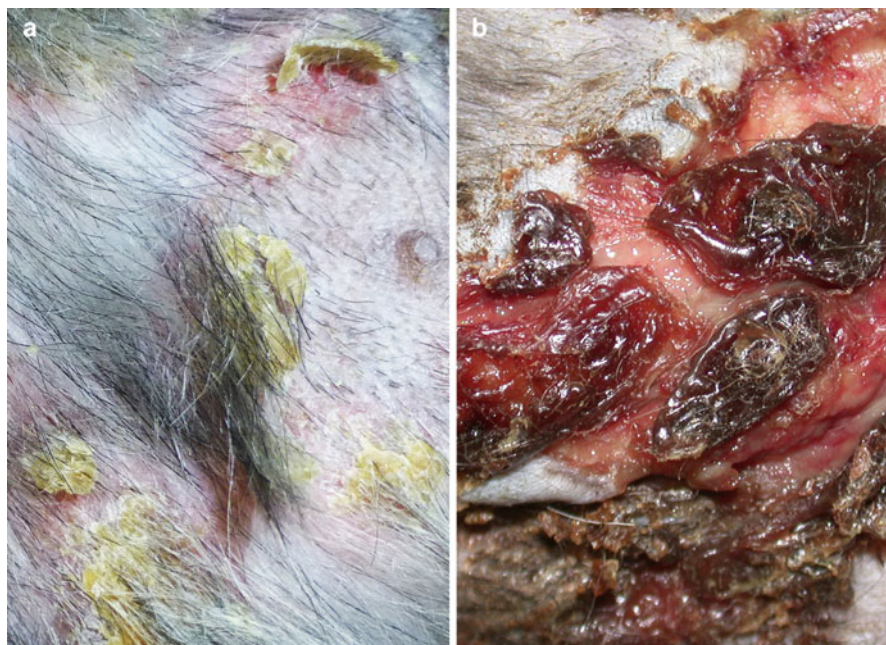


Fig. 2.22 (a) Yellowish superficial crust secondary to pustule dehydration; (b) dark red haematic crusts secondary to a deep lesion

Clinically, the crusts are classified as *superficial* and *deep*. The former are confined to the epidermis and therefore, as this portion of skin is not vascularised, they are not haematic, but usually characterised by a yellowish-brown colour; deep crusts are instead secondary to an ulcer and therefore, as they contain blood, appear dark, red-black in colour (Fig. 2.22). This clinically different appearance is easily recognised by visual examination and allows the clinician to obtain useful information regarding their aetiology. Cytologically, the superficial crusts are the most interesting, as they represent dehydrated pustules, whereas, in the case of haematic crusts, the specimens are more haemocontaminated and provide less useful results.

Sampling Technique

Sampling of cells from the crust is performed following its removal and by placing a slide on its inner surface or on the exposed pus (Fig. 2.23).

2.3 Fine Needle Biopsy (With or Without Aspiration)

2.3.1 Cytological Sampling from Nodules and Plaques

By definition, *nodules* are raised lesions, dermal or subcutaneous in location, greater than 1 cm in size and with a tendency to grow in height rather than in width (Figs. 2.24 and 2.25). The *plaques* are also raised lesions, but they tend to extend further in width



Fig. 2.23 Purulent exudate exposed after the crust has been removed

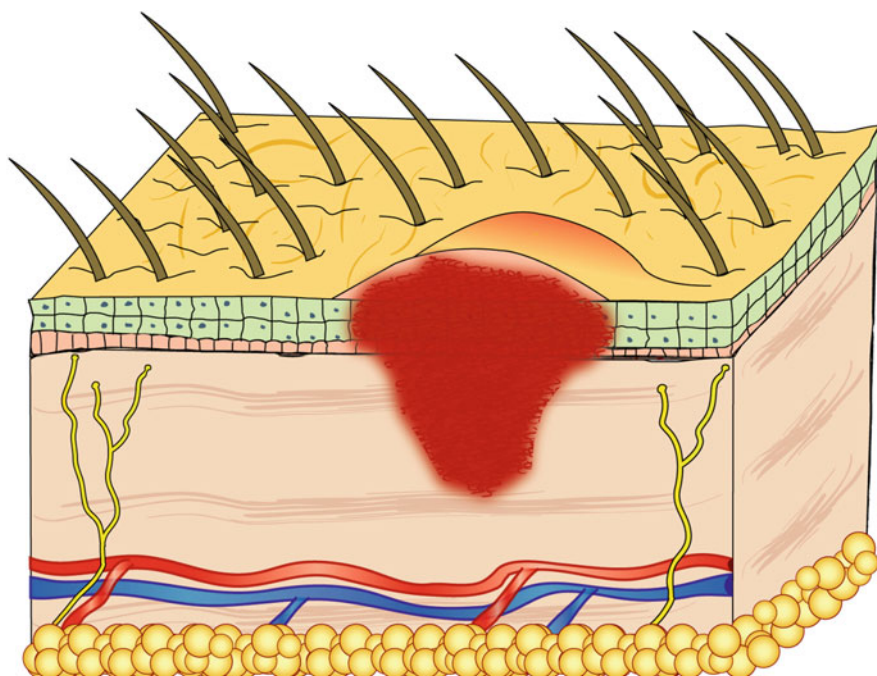


Fig. 2.24 Schematic representation of a nodule



Fig. 2.25 Round, alopecic, nodular, benign, cutaneous histiocytoma in a dog

than in height (Fig. 2.26). Large nodules are defined *masses*. Nodules and plaques may be composed of both inflammatory and neoplastic cells, or can be caused by the accumulation of fat (xanthomatosis) or mineral salts (calcinosis) in the dermis.

All nodules and plaques can be investigated using FNB, which is the best method when very small nodules must be sampled, especially those located in delicate areas such as the eyelids or mucocutaneous junctions. When cells are few and not representative of the lesion, or if it is necessary to sample a large cutaneous mass, it is better to perform a forced aspiration with needles of higher gauges (fine needle aspiration biopsy [FNAB]).

Sampling Technique

In cases of plaques or nodules, the fine-needle biopsy (FNB) or the FNAB is the most appropriate technique (Fig. 2.27).

The FNB technique is based on the simple insertion of a needle into a nodule. In most cases, this is adequate for collecting a high number of cells. A needle with a small gauge (23–25 G) is usually adequate for sampling nodules and plaques. The author routinely uses small needles as they reduce the possibility of vessel rupture and secondary haemocontamination. The needle must be inserted into different areas of the lesion, taking care to perform continuous and repeated *rotational* and *back and forth* movements. These measures are mandatory because many nodules and plaques are not uniform and may have a mixed composition, be necrotic or have central or multifocal cystic areas. As many nodules and plaques are ulcerated, the impression smear technique can provide contaminated samples. In these cases, FNB must be always pre-



Fig. 2.26 Plaque in a dog affected by *calcinosis cutis*

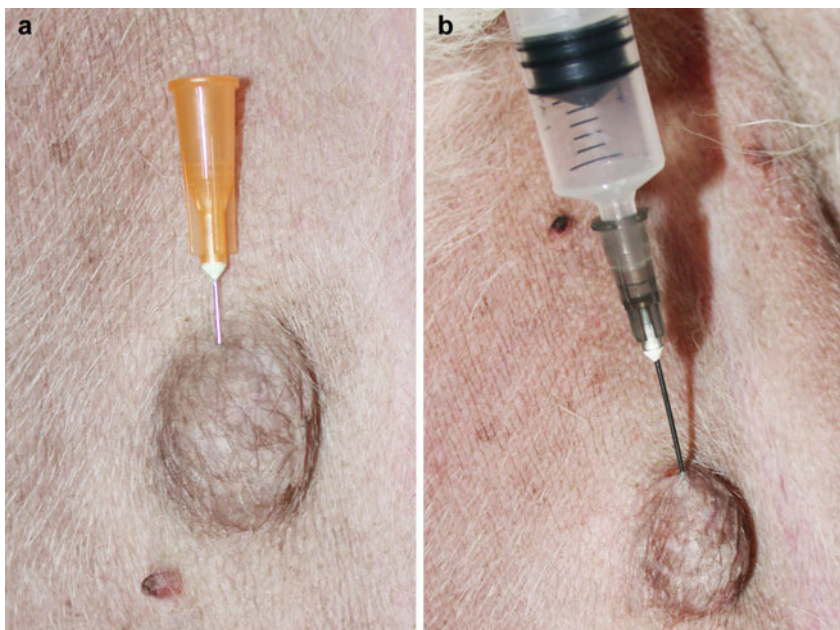


Fig. 2.27 (a) Fine needle biopsy; (b) fine needle aspiration biopsy



Fig. 2.28 Intradermal fine needle aspiration biopsy in a dog with leishmaniasis

ferred as it permits cells to be collected from deeper and uncontaminated areas of the same lesion, reducing the possibility of sampling unrepresentative inflammatory cells.

Cells from superficial dermis can also be obtained, in flattened lesions, by FNB or FNAB. In some non-nodular lesions, as in the course of desquamative lesions, where it is not possible to obtain the diagnosis by sampling superficial cells, it is possible to try to collect cells located just below the epidermis. A 23-gauge needle connected to a 5-ml syringe must be inserted into the sub-epidermal area and aspiration movements made. In this way, it is possible to collect cells that are indicative of the origin of the lesion.

In cases of exfoliative leishmaniasis, amastigotes are frequently localised in the subepidermal dermis; therefore in more lucky cases, they can be collected with this technique (Fig. 2.28).

2.4 Scraping

2.4.1 Cytological Sampling from Ulcers

As mentioned, samples collected from ulcers using the impression smear technique can produce non-diagnostic specimens, often comprising only contaminated specimens (Fig. 2.29).

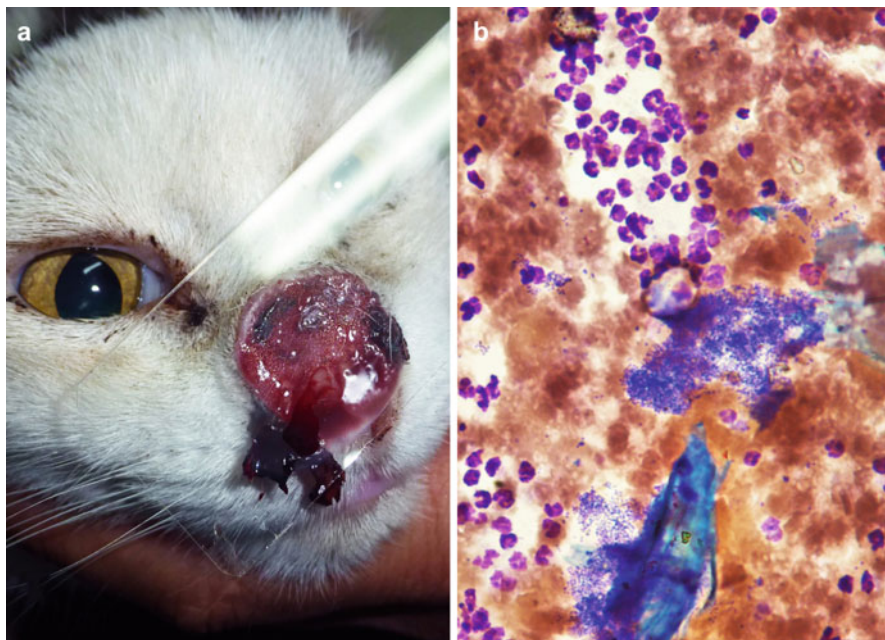


Fig. 2.29 (a) Impression smear technique used on the ulcerated nose of a cat with squamous cell carcinoma; (b) blood and many contaminant bacteria dispersed with arranged in large groups

Scraping is a traumatic method that allows numerous cells to be collected. The traumatic detachment of cells from the original tissue has the disadvantage of causing the vessels to break and of constantly providing haemocontaminated specimens. *Scraping* is therefore the most suitable technique for deep ulcers, because it enables the collection of cells from areas unreachable with other less invasive methods. A typical example is the *squamous cell carcinoma* in cats where the neoplastic keratinocytes deeply infiltrate the dermis, causing ulcerative lesions. In these cases, FNB is not a good method of sampling, whereas scraping provides more possibilities for obtaining diagnostic cells.

Sampling Technique

As ulcers are typically covered with a haematic crust containing inflammatory–necrotic cells or debris, sampling by scraping the upper material would be useless. According to the above, the first scrapes must be carried out to remove the superficial debris and after these, other scrapes must be gently performed to collect more representative specimens.

It is possible to use any sharp instrument, such as a scalpel blade or the edge of a glass slide; whichever tool is used, it is critical that multiple delicate scrapings must be repeated to gradually reach the bottom of the ulcer without excessive bleeding (Fig. 2.30).



Fig. 2.30 Cells collected via scraping of an ulcerative lesion

2.5 Preparation of Slides

The reading of a cytological specimen requires the slides to be correctly prepared. As already mentioned, once the lesion and the sampling technique have been chosen, the success of good preparation also depends on the manual skill of the operator who, with experience, is able to prepare slides of high quality. When the *impression smear* technique is performed on wet and exudative skin, it is sufficient to place the slide onto the surface to determine the direct transfer of cells (Fig. 2.31). The clinician must take care to place the slide gently to minimise or avoid cell damage.

In the case of sampling by *needle*, either by *FNB* or by *FNAB*, the material collected must be transferred onto a slide. This step is very delicate and fundamental to obtaining good-quality samples. The collected material should not be sprayed too energetically onto a slide, but must be gently deposited to approximately 1 cm from the frosted area of the slide. In this way, all the cells are contained in the central part of the slide. To avoid exerting too much pressure on the cells, the use of a 1-ml syringe is usually sufficient, and only if a tiny amount of material escapes from the needle should a larger syringe, either 2.5 or 5 ml be used. When collecting a large amount of material, it is mandatory not to deposit all of it on a single slide. Distributing only a small amounts of material onto more



Fig. 2.31 Impression smear technique: a slide is placed on an exudative lesion

slides has two advantages: (a) obtaining a specimen distributed in a single layer that permits the rapid dyes (e.g. Romanowsky) to penetrate and stain the cells, (b) obtaining more slides, some of which can be used for special or immunocytochemical staining.

Once sufficient material has been deposited on the slide; this must be gently smeared using a second slide. The two slides, maintaining a constant pressure and speed, are gently slid in opposite directions (Figs. 2.32 and 2.33). It will be down to the experience of the operator to evaluate, case by case, the pressure to be exerted on the two slides to obtain a high-quality sample. On good-quality slides, all the material must be contained in the central area without reaching the edges, forming a sort of ellipse (Fig. 2.34).

In the case of fluids, such as that collected from apocrine cysts, the slides may stick together, making it difficult for one slide to slip onto the other. This phenomenon can cause marked cell damage, which can be avoided by preparing slides of liquid consistency with the same method as that used for blood smears.

Material collected by *scraping* must be transferred onto a slide. In general, because with this method a large number of cells is collected and because rapid stains fail to penetrate specimens that are too thick, it is necessary to exert a higher pressure on the slides. With experience, clinicians acquire the skill of producing excellent slides.



Fig. 2.32 Cytological material sprayed too energetically on a slide and not smeared

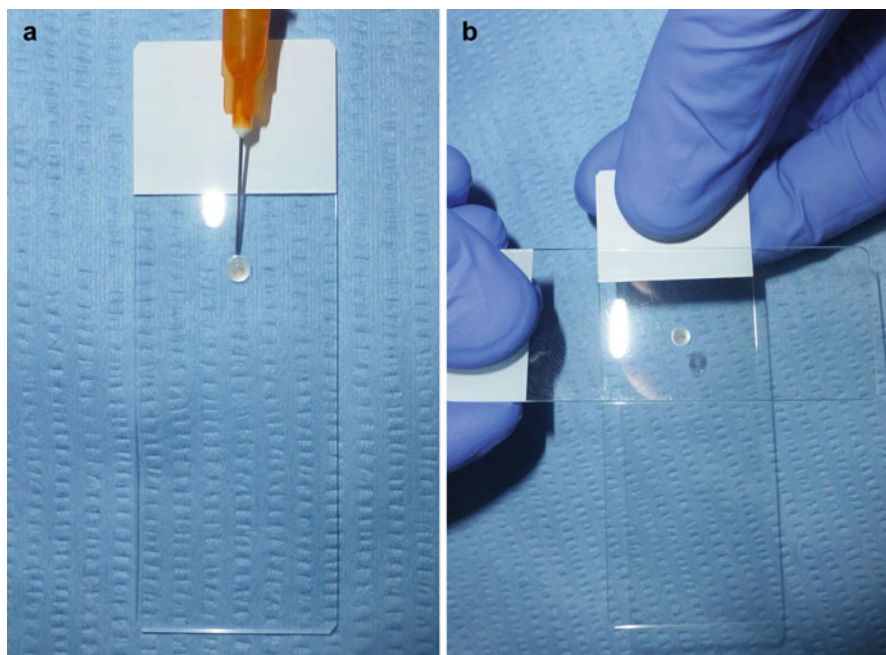


Fig. 2.33 (a) The collected material is gently transferred onto the slide; (b) smear technique: another slide is placed on the collected material and slipped onto it



Fig. 2.34 Ellipsoidal shape of the smeared material characterises a well-made slide

2.6 Staining of Slides

The last step before observation under the microscope is *staining*. In recent years, cytology has become a very common diagnostic method in veterinary practice; thus, many vets carry out staining of blood smears or cytological samples from skin lesions or internal organs on a daily basis.

The first rule that clinicians must respect before staining slides for microscopic evaluation is to avoid contact between unstained slides and the formalin vapours; indeed, formalin fumes do not permit the stains to penetrate the cells, which remain faintly coloured and have a *faded appearance* (Fig. 2.35).

2.6.1 Romanowsky Stains (Wright's, Giemsa)

Most veterinarians use rapid Romanowsky dye, a polychromatic stain originally composed of methylene blue and eosin. The staining method comprises a first dip in an alcoholic fixative (methyl alcohol) and two consecutive passages in two stains: the first is Azure B (N,N,N, -Trimethylamine), a basic dye that is a metabolite of methylene blue, and the second is Eosin Y (Tetrabromofluorescein), an acid dye. With this type of stain, it is necessary for slides to be air-dried before being dipped

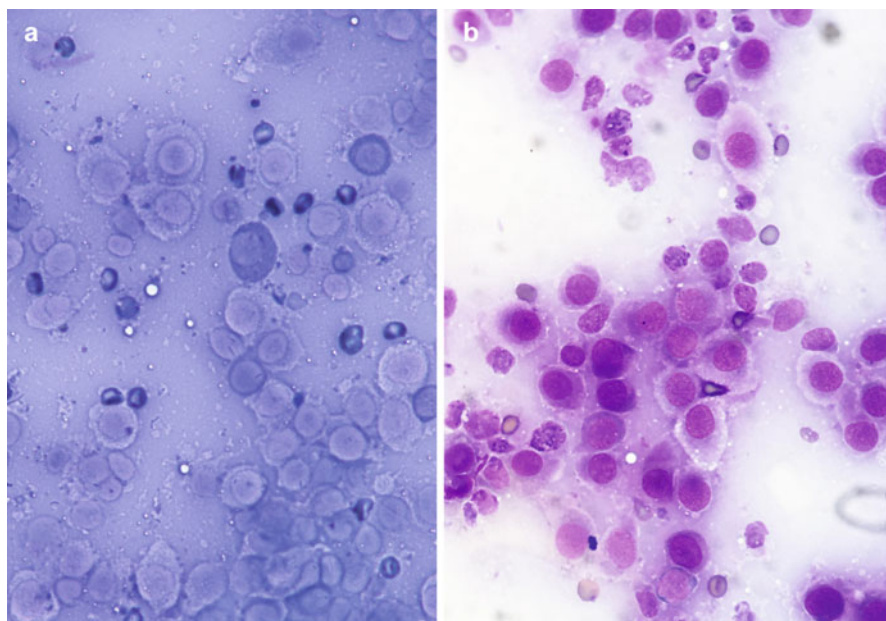


Fig. 2.35 Romanowsky dye: (a) unstained slides because of contact with the formalin vapours; (b) the same sample not exposed to formalin fumes

in the alcoholic fixative. Romanowsky-type staining was conceived for blood and bone marrow smears, but over time and with the expansion of cytopathology, it has found widespread use for staining any type of cytological sample.

Romanowsky is a panchromatic stain that allows the adequate display of nuclear characteristics and is excellent at highlighting cytoplasmic details, such as granules and most microorganisms. The nuclei take on a *purple* colour, whereas cytoplasm stain *azure blue*.

Beginners should be aware that when using this stain, cells must be distributed in a thin, single layer, as specimens that are too thick do not permit the dyes to properly penetrate into the cells.

The technique is very simple and rapid: the slide must be air-dried and immersed into the three aforementioned solutions. There is no predetermined number of dips or seconds to be met for each pass, as much depends on how the dyes are used and how often they are filtered or renewed. In general, the number of dips for each colour depends on how long it takes both the fixative and the dyes to permeate the cells. Three to four dips of 1–2 s each may be sufficient for good staining. In time, the operator acquires the expertise to perform efficient staining.

The Romanowsky method is also useful when transparent acetate tape must be stained. As some tapes are of poor quality, they may curl or become opaque once immersed in the fixative; it is possible to obtain good coloration using only the third dye (blue) or other stains such as blue lactophenol or crystal violet. It is sufficient to deposit only a few drops of the dye on a slide to which the lower surface of the

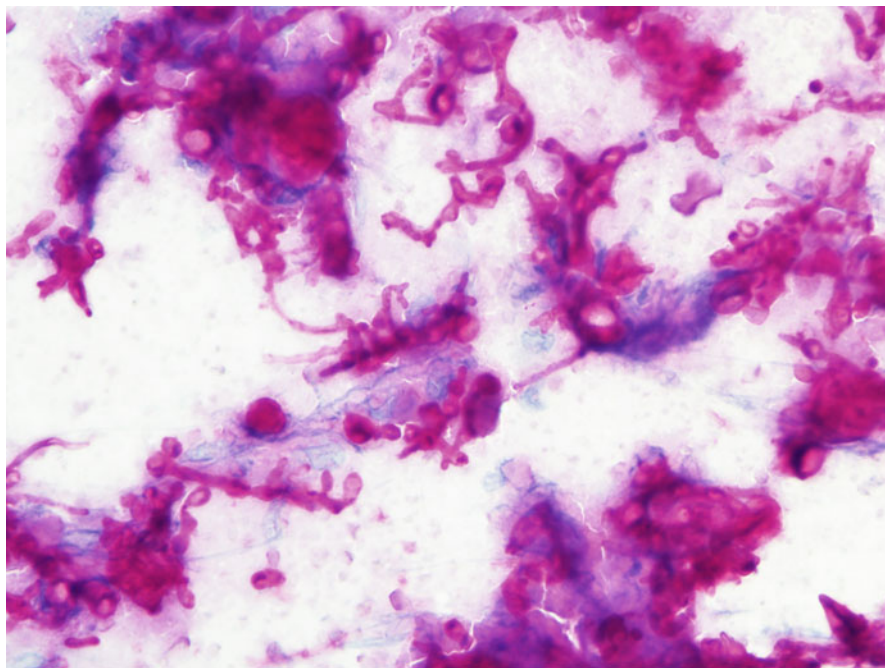


Fig. 2.36 Periodic acid–Schiff staining: fungal bodies stained magenta

adhesive tape where the cells are attached is placed. Before looking at the slides under the microscope, the excess dye, which would make it difficult to view the cellular details, is removed with a piece of absorbent paper.

2.6.2 Periodic Acid–Schiff

Periodic acid–Schiff (PAS) is a dye commonly used in histology to indicate molecules that contain a high percentage of carbohydrate, such as, glycogen, mucins and fungi. In skin cytology, it is most commonly used to highlight fungi that take on a characteristic *magenta* colour (Fig. 2.36). As it does stain different substances, slides can have a PAS-positive background and for this reason, in specimens composed of many inflammatory cells and only a few fungi, the latter are not always easily recognisable (Fig. 2.37).

2.6.3 Grocott's Methenamine Silver

Like PAS, *Grocott's* is also a dye that is used to better visualise fungal organisms. In most fungi, the cell wall is composed of chitin, a polymer of N-acetylglucosamine, which can be linked to polymers of D-glucose, D-mannose, proteins and lipids.

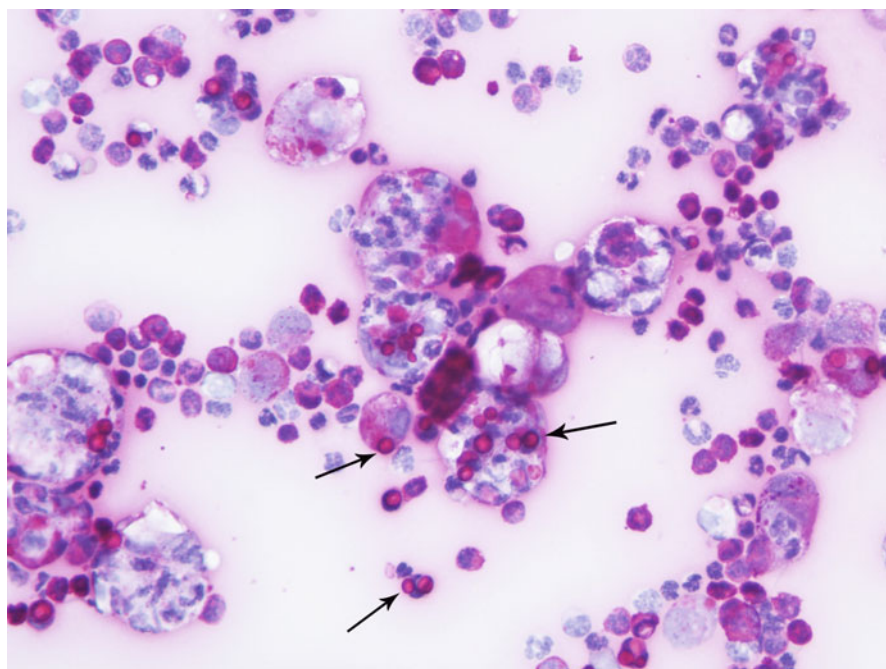


Fig. 2.37 Periodic acid–Schiff staining: in highly cellular specimens, fungi can be hardly detected. The fungal bodies are evident as round, deeply magenta-stained cells (*arrows*)

With use of Grocott's dye, the aldehydic groups that are produced by the oxidation of mucopolysaccharides present in the fungal wall reduce the silver chloride to metallic silver, permitting their visualisation. Fungi are coloured *black* and the rest of the cells and the background *green* (Fig. 2.38). In the author's experience, Grocott's dye is preferable to PAS, as the fungi are more easily detectable and it is particularly useful in cases of dermatophyte kerion, in which only a few arthroconidia are usually present (Fig. 2.39).

2.6.4 Ziehl–Neelsen

Ziehl–Neelsen (Z–N) staining is used to colour *mycobacteria*.

Acid resistance is a particular characteristic of the mycobacteria consisting of the ability of these micro-organisms, once stained with carbol fuchsin, to maintain their red colour, even when subjected to energetic bleaching treatment. The acid resistance is due to the characteristic composition of the cell wall of mycobacteria, which is very rich in lipids. With Z–N staining, mycobacteria are coloured *bright red*, whereas the nuclei take on a *blue* colour (Fig. 2.40).

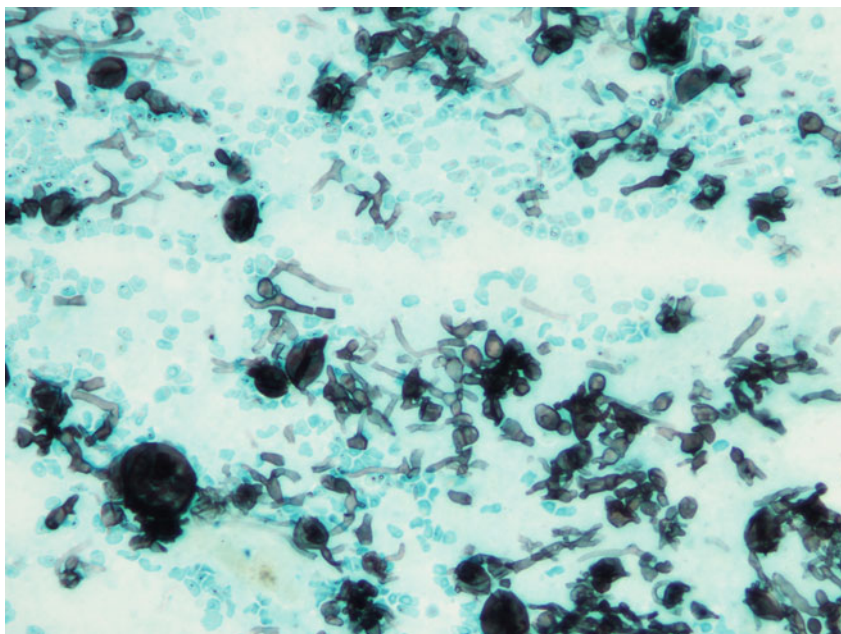


Fig. 2.38 Grocott's staining: many fungal bodies are easily detected because of their black colour against a green background

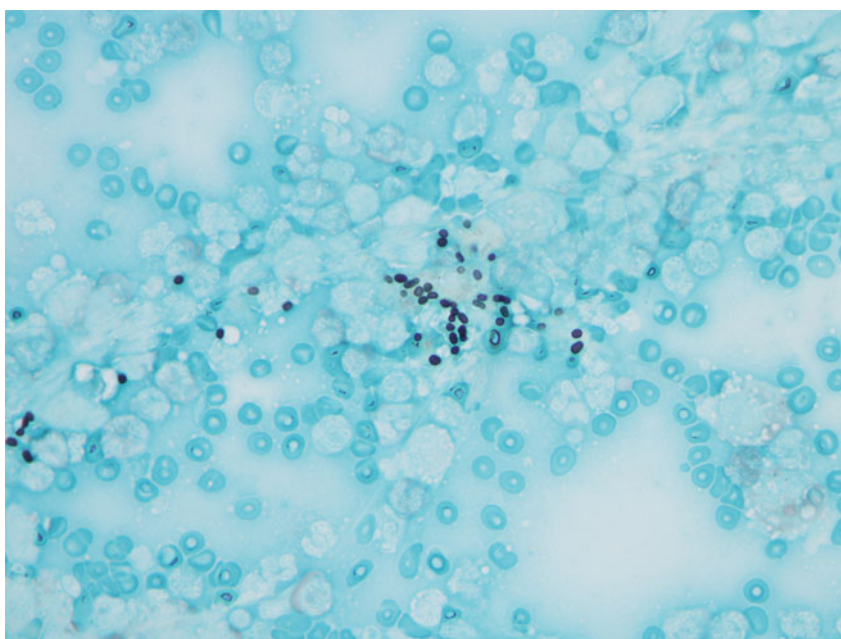


Fig. 2.39 Grocott's staining: small, round, black-stained arthroconidia are easily detectable against a green background

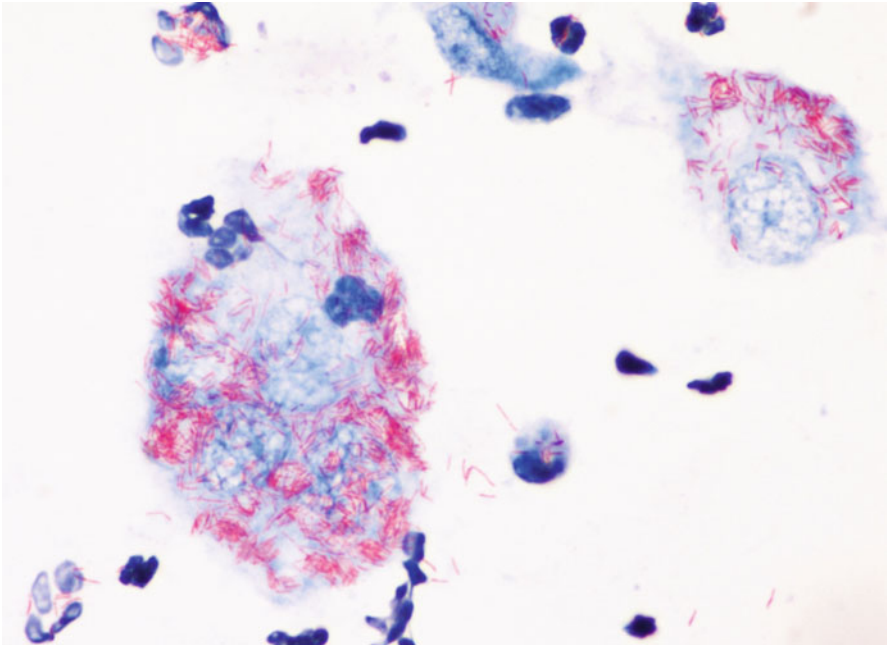


Fig. 2.40 Ziehl-Neelsen staining: mycobacteria are detected as bright red bacilli in the cytoplasm of macrophages

2.6.5 *Oil- Red-O*

The dye *Oil-red-O* is fat-soluble and therefore selectively binds to the lipid structures of cells. The staining can only be performed on fresh samples, as alcohol fixation removes most of the lipids; for this reason, slides must not be dipped in the alcoholic solution. *Oil-red-O* is used for staining fatty substances and in practice it is very useful to confirm the presence of fat in the vacuoles of macrophages and giant cells, as happens in cases of panniculitis and xanthomatosis, or when, in poorly differentiated liposarcomas, we want to detect intracellular lipid material. The lipids assume a *red-orange* colour (Fig. 2.41).

2.6.6 *Von Kossa*

Usually, *Von Kossa* staining is used to highlight *calcium* ions in histological sections. A substitution reaction is the basis of this stain. Cells are treated with a solution of silver nitrate that replaces the calcium salt. In specimens, the areas of *calcium* are coloured in *black*, while the nuclei stain *red* (Fig. 2.42). Such coloration may be useful for confirming the presence of *calcium* salts in specimens from dogs with

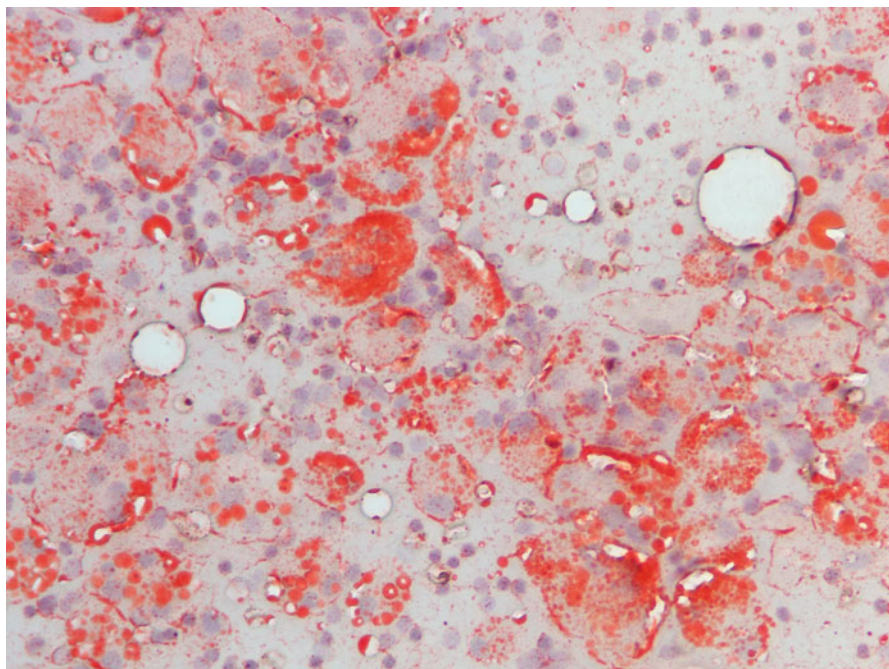


Fig. 2.41 Oil-red-O staining: bright red-stained lipids are evident in the cytoplasm of macrophages

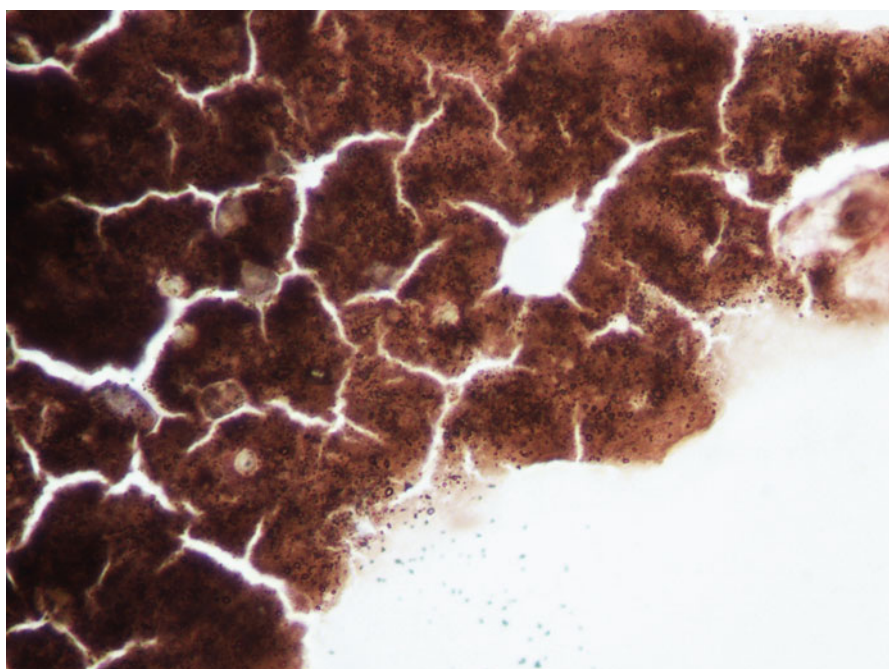


Fig. 2.42 Von Kossa dye: the area where *calcium* salts were present stains black

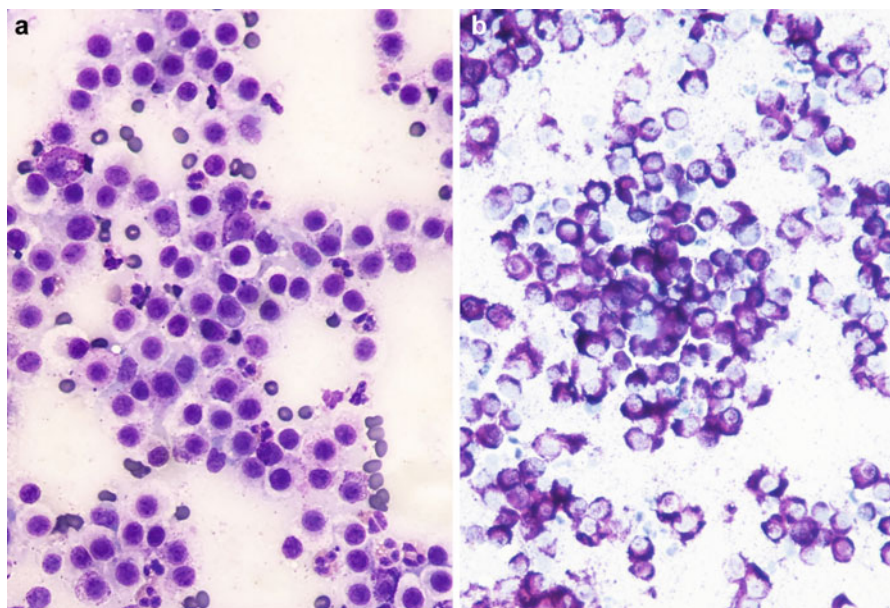


Fig. 2.43 (a) Romanowsky dye: mast cell tumour in which few purple granules are evident in the cytoplasm of neoplastic cells; (b) toluidine blue dye highlights many red-stained granules in the same specimen

cutaneous mineralisation, as in cases of *calcinosis cutis* or *calcinosis circumscripta*.

2.6.7 Toluidine Blue

Toluidine blue dye is used for the metachromatic staining of acidic substances. In practice, it is utilised in poor granular mast cell tumours, where the low number of granules make identification of the mast cells less obvious. The granules of the mast cells stain *red* (Fig. 2.43).

2.6.8 Prussian Blue, or Perls' Reaction

Prussian blue is a dye commonly used in histopathology to detect ferric iron and ferritin in the tissue.

It is a histochemical reaction rather than a true staining technique. Hydrochloric acid splits the protein, allowing the potassium ferrocyanide to combine with the ferric iron, forming ferric ferrocyanide or *Prussian blue*. In skin cytology, it can be

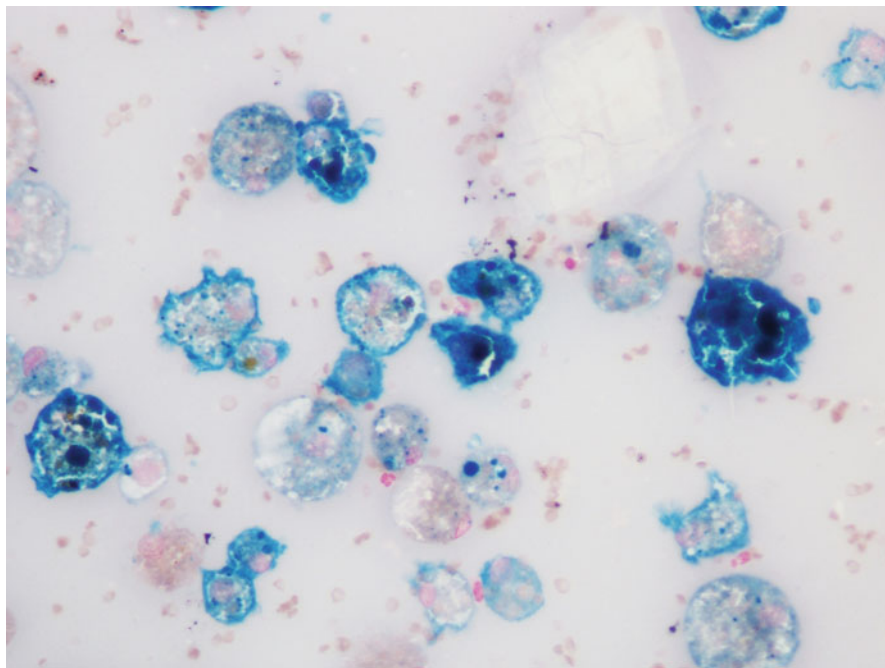


Fig. 2.44 Perl's reaction: with Prussian blue dye, the iron component of apocrine gland secretion is evident in the macrophages sampled from an apocrine cyst

used to confirm the presence of iron in the granular material observed in the cytoplasm of apocrine cells or when only macrophages filled with coarse dark blue material are collected from a cystic lesion suspected of being of apocrine origin. With Prussian blue staining, the ferric iron takes on an *azure blue* colour, whereas nuclei stain *red* (Fig. 2.44).

2.6.9 Congo Red

Amyloid is an acidophil and hyaline (glassy) substance stained by acid dyes. Amyloid is not definitively identified by routine haematoxylin and eosin staining and therefore, special stains are required. Among these, *Congo red* dye is most frequently used. It is an anionic dye that is capable of depositing in amyloid fibrils. With Romanowsky staining, the amyloid cytologically appears as a fibrillar to globular, bright pink extracellular matrix that cannot be differentiated from other substances. With Congo red staining, the amyloid takes on an *orange-red* colour (Fig. 2.45).

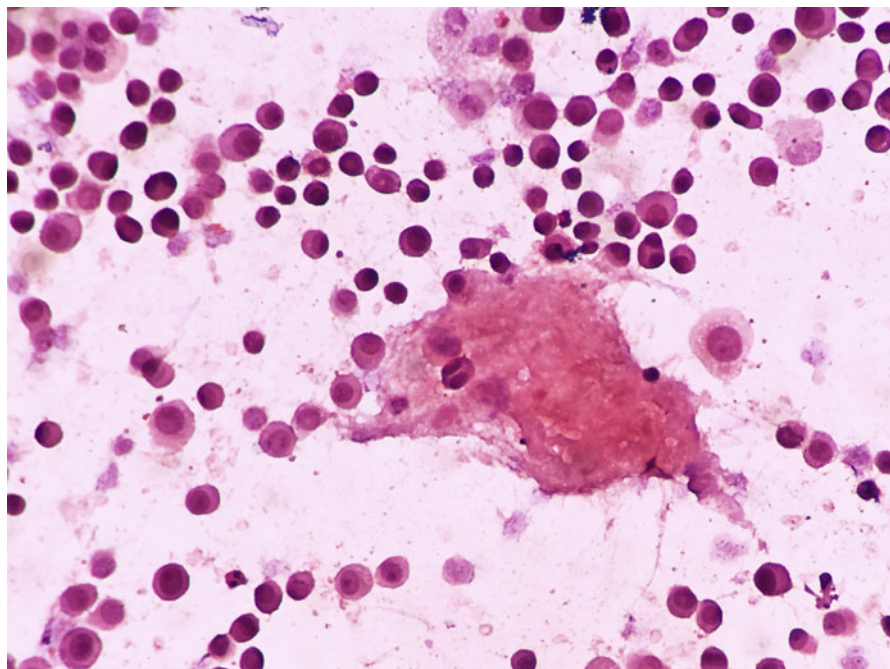


Fig. 2.45 Congo red staining: amyloids can be detected using Congo red. In the pictures, the amyloid is stained orange among many neoplastic plasma cells

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