Background – Histopathology has been essential in advancing our knowledge in veterinary dermatology. However, morphological features and histological patterns cannot always reveal an aetiological diagnosis.

Objectives – Several ancillary techniques can assist in achieving an aetiological diagnosis. Some of these techniques have found their way into routine diagnostic dermatopathology, whereas others are still mainly used in research. This review discusses the utility, strengths, advantages/disadvantages and challenges associated with each technique.

Methods – Digital microscopy, immunohistochemistry, immunofluorescence, salt-split skin, Western blots, electron microscopy, PCR, in situ hybridization, tissue microarrays, next-generation sequencing, DNA microarrays and laser microdissection are discussed.

Conclusions – It is crucial to understand the limitations of each technique and to correlate the results both with pathological findings and the clinical presentation. As such, dermatopathology will remain the important link between benchtop science, available results from ancillary techniques and clinical veterinary dermatology.

Introduction
Histopathology has made significant contributions to vastly advance our knowledge in veterinary dermatology. We routinely use morphological features and histological patterns in association with clinical presentation to achieve a diagnosis. Despite this increased understanding, more frequently than we would like, histopathology alone does not reveal an aetiological diagnosis. As a consequence, the dermatopathologist is confronted with the need to use additional techniques to further characterize a particular lesion in hope of identifying its exact aetiology.

Over the years, many of these techniques have been incorporated in numerous published studies of various skin diseases and the results have been interpreted carefully in tandem with the morphological changes seen in the skin lesions. Although not readily available for each difficult case, these studies have improved our understanding of the aetiology and pathomechanisms of many skin diseases. This acquired knowledge assists the diagnostic dermatopathologist to draw better conclusions from basic histopathology seen on H&E stained sections.

This review will discuss the utility and potential of digital microscopy, immunohistochemistry, immunofluorescence, Western blot, electron microscopy, PCR, in situ hybridization, tissue microarray and DNA microarray, next-generation sequencing (NGS) and laser microdissection. As it is crucial to understand the challenges and limitations of each technique, both advantages and disadvantages are discussed in this review and examples of how our understanding of skin diseases have been expanded by each technique are given.

Digital microscopy
Instead of using the original glass slide with the actual sample, many diagnostic laboratories scan each slide and provide digital scans to the pathologist for evaluation.1

Method
Entire slides are scanned at different magnifications, typically an overview scan (2x) and a scan at higher magnification (40x). These are uploaded on a server and the scanned images accessed over the internet. The customized software allows the scan to be moved around in a similar way to moving a glass slide on the microscope stage. Instead of changing objectives, zooming-in and -out reveals additional details of the lesion.

Advantage
Because digitized cases can be shared easily between pathologists, second opinions can be provided in a very timely manner. Pictures are obtained from the scanned slides and either sent along with the pathology report or used for publication. Teaching from a scanned slide ensures that all students are looking at exactly the same lesion; this may not always be the case with serial sections, which may exhibit differences in morphological features between different slides from the same biopsy sample.2 Moreover, limited numbers of serial sections

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can be produced from small needle or punch biopsy specimens. The computer software also allows quantitative evaluations of various features on images from the tissue sections. For example, immunohistological stains can be evaluated with regards to percentages of positively staining cell populations within a lesion.

Disadvantages and challenges
Each file is on average between 1 and 5 GB, but may go all the way up to 10 GB. Hence, storage of these files requires a large amount of available memory on the receiving computer to evaluate each scanned slide successfully. High quality thin histological sections are crucial to produce high quality full slide scans. Despite meticulous scanning of a sample, certain features such as granules, small inclusion bodies and small pathogens are not necessarily obvious on scanned slides (personal observation). In such instances, the pathologist needs to go back to the actual glass slide or request re-imaging of the glass slide by alternative modalities, such as Z-stack images.

Immunohistochemistry
The use of antibodies in diagnostic pathology has become an indispensable technique that is routinely used by many diagnostic laboratories and pathologists. Pathologists often have to rely on the immunophenotype of a neoplastic cell population to identify its origin. However, antibodies are specific for an antigen in a specific species. Prior to using an antibody in a different species, it has to be evaluated carefully for its potential for correct cross-reactivity in this new species. Cross reactivity is more likely in highly conserved antigens such as CD3.

Anatomic pathologists also use immunohistochemistry (IHC) to highlight certain tissue structures and the location of morphological changes in relation to those particular structures. This approach has become very helpful in differentiating various diseases affecting the basement membrane zone (BMZ). Moreover, many infectious pathogens, in particular viruses, are too small to appreciate in routine histopathology. Immunohistochemistry has proven to be very helpful in identifying such pathogens within affected tissues.

It is important to keep in mind that specimen fixation can affect the potential of an antibody to bind to its epitope (see below). Moreover, it cannot be stressed enough that IHC results have to be viewed together with the actual morphological lesion on the H&E section. It is imperative to know the antigen of interest and its correct location within the cell or tissue in order to evaluate appropriate binding of an antibody, as occasionally irrelevant or spurious staining can occur; this is quite often observed with the commonly used anti-CD79a antibody (personal observation).

Method
Primary antibodies specific for certain epitopes are applied to histology sections or cytology samples. Unbound antibodies are washed off, whereas bound antibodies are identified by a second anti-immunoglobulin antibody. This secondary antibody is specific for the species in which the primary antibody has been produced. For example: if the primary antibody is a mouse–anti-dog leukocyte marker, the secondary antibody will be an antimouse IgG. This secondary antibody is conjugated to an enzyme and detection of antibody binding is based on the enzymatic reaction on a chromogen, resulting in a colour reaction occurring wherever the tagged antibody binds. For example: streptavidin-hydrogen peroxidase (HRP) binds to biotin on secondary antibodies and HRP will initiate the enzymatic reaction on the chromogenic. The costs of IHC lay primarily in the purchase of the antibodies, some of which have a limited lifespan. Hence, most diagnostic laboratories limit their choice of antibodies to the most commonly used ones.

Advantage
Typically IHC is used for immunophenotypic characterization of tumour cells, identification of particular tissue structures or infectious pathogens in both diagnostic dermatopathology and research. Immunohistochemistry can be applied on aspirates, tissue imprints or snap-frozen tissue sections, and some antibodies also recognize antigens in formalin-fixed paraffin-embedded (FFPE) tissue sections. Most diagnostic laboratories limit their IHC to antibodies that can be used on FFPE tissues.

Disadvantages and challenges
Formalin fixation creates crosslinks, which potentially obscure the specific epitopes recognized by a particular antibody. Hence, a limited number of antibodies are currently available for use in formalin-fixed tissues. Typically, an antigen retrieval procedure (enzymatic or heating with citric acid) is required to break the crosslinks in order to make epitopes available for the binding of an antibody. False negative results may occur on inappropriate fixed tissues because subsequent processing of tissue sections through the alcohol series can destroy insufficiently fixed antigens. False positive results may occur as antibodies can bind nonspecifically, in particular to partially damaged tissue. This is readily evident in areas of necrosis. If the procedure is done manually, immunohistochemistry is a rather labour-intensive procedure; automatic immunostainers are available, but costly.

Examples of contributions to dermatology
There are innumerable examples of the use of antibodies to immunophenotype neoplastic processes, including leukocytic tumours (Figure 1), cutaneous glomus tumours and amelanotic melanomas. As intranuclear inclusion bodies are not always evident on H&E, identification of papilloma virus typically is dependent on using the cross-reactive anti-bovine papilloma virus (BPV)-2 antibody. With acute and subacute distemper, canine distemper virus can be identified within keratinocytes of haired skin, footpads and nasal mucosa. Identifying the location of laminin or collagen IV in lesions of subepidermal vesiculation assists the differentiation between bullous pemphigoid (BP), epidermolysis bullosa aquisita (EBA) and linear IgA disease.

Immunofluorescence
Similar to immunohistology, immunofluorescence (IF) is used to identify proteins with the help of specific
antibodies coupled with a fluorophore. Each fluorophore requires a light source of specific wavelength to initiate emission of fluorescence. Examples are fluorescein isothiocyanate (FITC), Texas red (TR) or phycoerythrin (PE). Immunofluorescence is usually used on snap-frozen tissues or on cytology specimens.

Method
Both direct immunofluorescence (DIF) and indirect immunofluorescence (IIF) are used in dermatopathology, in particular in diagnosing autoimmune diseases. Typically, DIF is used to evaluate skin lesions for the presence of intralesional immunoglobulins and/or complement factors using fluorescent-coupled anti-Ig or anti-complement factor antibodies. Alternatively, with IIF sera are applied to a healthy skin specimen (usually lip or tongue) in order to identify tissue-specific circulating autoantibodies. In a second step, a fluorophore-coupled anti-species immunoglobulin heavy chain antibody is applied to identify location of bound primary antibodies. Similar to IHC, costs of IF are mostly associated with the purchase of the antibodies and an immunofluorescence microscope.

Advantage
Immunofluorescence is often used for double labelling of samples for the presence of two different proteins. By exposing the sample to light that excites both fluorophores used, the expression of both proteins can be evaluated simultaneously. For example, FITC labelled proteins will emit a green light, whereas TR labelled proteins will emit a red light, which makes it visually advantageous to distinguish the individual protein expressions. If both proteins are expressed in the exact same location (overlapping), the merge of green and red lights will result in a yellow light emission.

Disadvantages and challenges
Auto-fluorescence is a common challenge with IF on formalin-fixed tissues. Hence, IF is usually performed on frozen sections and is therefore not a common procedure in routine diagnostic dermatopathology. Typically it is performed manually, which is a rather labour-intensive procedure. Also IF stained slides need to be stored in the dark, preferentially at cool temperatures, because fluorophores tend to fade. Hence, IF stained slides cannot be stored for a prolonged period of time. Moreover, certain fluorophores fade very rapidly upon microscopic evaluation. It is therefore very important to document reaction quickly by capturing images.

Examples of contributions to dermatology
Both BP and EBA are characterized by anti-basement membrane antibody that can be identified by both DIF and IIF (Figure 2). With salt-split skin samples, BP is mostly associated with immunoglobulin deposition at the roof of the split (i.e. above the lamina lucida; see Salt-split skin section below). The location of the positive reaction reflects the presence of autoantibodies against collagen XVII in BP, which is a protein located above the lamina lucida. In contrast, with EBA positive IF is mostly at the bottom of the cleft in salt-split skin because circulating autoantibodies are directed against collagen VII, a protein located below the lamina lucida. Dogs with pemphigus foliaceus (PF) have circulating antibodies against desmocollin-1 (DSC1); this has been demonstrated with IIF by applying sera from dogs with PF to canine transfected DSC1 293Tcells.

Salt-split skin (SSS)
The BMZ is a complex structure with many different glycoproteins, several of which are targets for different autoimmune diseases. Antibody binding to any of these BMZ proteins results in dermo-epidermal separation and identifies it microscopically as a sub-epidermal blistering disease. However, light microscopy does not allow identification of the exact location of the separation within the BMZ.
Salt-split skin (SSS) samples consistently reveal a split within the lamina lucida, leaving the hemidesmosomes attached to the epidermis, whereas the lamina densa region of the BMZ will stay attached to the dermis.
Method
Typically, small samples of normal lip or tongue are immersed in 1 M NaCl for 24 h. Subsequently the samples are either embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek; Torrance, CA, USA) and snap frozen for either IHC or IIF. The samples can then be exposed to a patient’s serum to evaluate for presence of circulating autoantibodies. Alternatively, samples are prepared in 1 M NaCl with 5 mM ethylenediamine tetraacetic acid (EDTA), 50 mM phenylmethane sulfonylefluoride (PMSF) and 50 mM N-ethylmaleimide (NEM) at 4% for 96 hours with stirring. Samples are fixed in 2% glutaraldehyde and 1% osmium tetroxide and processed for electron microscopy (EM). Patients’ sera can be kept frozen at −20°C or −80°C for a prolonged period of time.

Advantage
Frozen SSS lip or tongue can be stored at −80°C. Sections from these blocks can be used over a long period of time. It offers a first step to differentiate autoimmune diseases targeting the BMZ.

Disadvantages and challenges
Processing of frozen tissue is still not a routine technique performed in veterinary diagnostic settings. It also requires that clinicians collect patients’ sera before immunosuppressive therapy is initiated.

Examples of contributions to dermatology
SSS has been used to further characterize and differentiate pathomechanisms involved in BP, EBA and mucous membrane pemphigoid (MMP). As mentioned above, IIF using sera from patients with BP and MMP (Figure 2) mostly reveal a positive staining of the top portion of the SSS sample, because circulating antibodies in these diseases target the NC16A ectodomain of collagen XVII (BP180, BPAG2). Alternatively, circulating antibodies in sera of dogs with EBA have antibodies targeting the NC12 domain of collagen VII; consequently, IIF will reveal a positive linear staining along the bottom portion of the SSS.

Electron microscopy
Ultrastructural evaluation of tissues and cell cultures has been used since 1939. However, other techniques such as PCR have often replaced the need for access to EM in daily diagnostic pathology.

Method
Samples need to be fixed in a special fixative such as 2% glutaraldehyde and 1% osmium tetroxide. Previously formalin-fixed tissues can be used also; however, the quality of the samples may be somewhat impaired. With scanning electron microscopy (SEM), a focused beam of electrons interacts with atoms in the sample and is used to identify surface topography and composition of a sample. Resolution >1 nm can be achieved. Specimens can be observed in high vacuum, in low vacuum, in wet conditions (environmental SEM), and at a wide range of cryogenic or elevated temperatures.

With transmission electron microscopy (TEM), a beam of electrons is transmitted through an ultra thin tissue section. As the electrons interact with the specimen, an image is formed, which subsequently is enlarged and visualized on a fluorescent screen or on photographic film. The small samples are put on a fine metallic grid and are evaluated within a vacuum.

Advantages
Electron microscopy allows identification of pathogens (Figure 3) as well as cellular and extracellular matrix changes in the context of the structures of the surrounding tissues. It is a powerful tool to identify new pathogens and their exact location within cellular structures. Conjugating antibodies with gold beads (immuno-gold) have been used to identify a specific protein within extracellular matrix or cellular compartments.

Disadvantages and challenges
The maintenance of an EM facility is costly and requires trained staff to run it. Hence, most diagnostic laboratories do not include an EM service and many anatomical pathology departments at universities no longer have their own EM facility. Therefore, samples will typically need to be sent out to laboratories with EM capabilities for further evaluation.

Examples of contributions to dermatology
Electron microscopy enabled the identification of a reduction of anchoring fibrils in the basement membrane of a young dog with subepidermal blistering disease consistent with dystrophic epidermolysis bullosa (DEB). Although periodicity of collagen fibres was within normal limits, horses with hereditary equine regional dermal asthenia (HERDA) had a higher variability of the cross-sections of the collagen fibrils when compared to healthy horses. Ultrastructurally, fibroblasts of shar-pei dogs with hereditary cutaneous hyaluronosis (mucinosis) differ.
from healthy dogs; cellular protrusions were noted as well as an increase in hyaluronic acid content in several subcellular structures. Innervous viral infections have first been identified with the help of EM. For example, a new poxvirus was identified in skin lesions of two human patients; subsequent DNA analysis revealed a new poxvirus, which could not be classified as either Parapox genus or Molluscivirus genus. Alternatively, EM also assists in ruling out the presence of a viral infection as shown in pododermatitis in flamingos.

**Western blot**

Protein separation by SDS–PAGE and subsequent Western blot is used by many laboratories to investigate and demonstrate the expression of proteins in a lysate. Alternatively, with access to purified proteins, the presence of specific antibodies to certain proteins can be identified in a patient’s serum.

**Method**

Proteins in a lysate are separated by electrophoresis based on their molecular weight (SDS–PAGE); often the proteins are linearized as electrophoresis is performed under reducing conditions. Simultaneously, proteins of known molecular weights are added as molecular weight markers. The proteins subsequently are transferred from the gel to a highly hydrophobic membrane (nitrocellulose or polyvinylidene difluoride (PVDF), charged nylon). The membrane is exposed to specific antibodies. Various techniques are used to detect the presence of bound antibodies directly on the membrane or, alternatively, on radiographic film using enhanced chemiluminescence.

**Advantages**

Western blots allow the identification of molecular weights of proteins and glycoproteins in tissue extracts of the skin. Western blot is a reliable method to evaluate antibodies for specificity and correct cross-reactivity. It is a simple and rather inexpensive method that tends to be easily interpretable.

**Disadvantages and challenges**

This technique requires access to specific antibodies to identify the protein in question, or alternatively, purified proteins to allow detection of circulating antibodies within the patient’s serum. Moreover, it is a rather labour-intensive technique. Hence, Western blots are limited mostly to research settings in veterinary medicine. Several factors may impair the accuracy of Western blots. Occasionally, several bands reflecting proteins of different molecular weights are detected with a particular antibody. This could be the result of allelic differences of a protein, different isoforms or various glycosylation of proteins. Typically the electrophoretic separations of proteins are run under denaturing conditions (SDS–PAGE), which could change the capability of antibodies to identify the appropriate epitope. Moreover, the blotting may not be reproducible for each lysate, because transfer to the membrane may vary between runs. A further challenge is the quantification of the detected protein.

**Examples of contributions to dermatology**

Dogs with BP have circulating antibodies that recognize a 180 kDa hemidesmosome associated glycoprotein, subsequently identified as NC16A of collagen XVII. Circulating antibodies in dogs directed against the major Microsporum proteins have been identified. Western blot analysis identified a long interspersed nucleotide element (LINE-1) insertion in the transglutaminase-1 gene as the cause of decreased levels of TGM-1 in Jack Russell terriers with ichthyosis.

**PCR and quantitative PCR**

PCR is the most commonly used technique for the detection of DNA/RNA in biological samples. It allows amplification of very small amounts of target DNA or RNA. First described in 1983, PCR has become well established in many diagnostic laboratory settings.

**Methods**

PCR is dependent on the quality of the starting DNA/RNA in the sample that is being evaluated. Real-time quantitative PCR (RT-qPCR) has become the gold standard for detection and quantification of nucleic acids from multiple sources. There are different systems of qPCR. One method includes running a dilution series of known independently quantified standard templates parallel to the unknown sample. A DNA-binding dye such as cyber green binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle. Alternatively, qPCR is achieved by the TaqMan technique, which relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-fluorophore labelled probe during hybridization to the complementary target sequence. Quantification relies on a fluorescent signal produced with each cycle of the PCR reaction. It is often used to identify a viral load in a lesion or to identify levels of mRNA for various intralesional growth factors or cytokines.

**Advantages**

It is a very time efficient, inexpensive and sensitive technique that requires very little starting material. DNA or RNA can be extracted from either fresh or frozen material and with variable success from FFPE tissues. Culturing of certain pathogens is labour-intensive and requires special
techniques; PCR may give a much faster result. PCR is also used to further specify cultured pathogens. Another common use for PCR is the identification of mutations, as for example seen with neoplastic cell populations.\textsuperscript{51}

**Disadvantages and challenges**

PCR is a very sensitive technique. On the one hand, false positive results due to cross-contamination are a major challenge. This has been clearly documented by finding papilloma virus in healthy canine skin.\textsuperscript{52} Moreover, it is important to prove causality of a particular pathogen identified by PCR. This often requires application of additional techniques such as immunohistology or in situ hybridization.\textsuperscript{53,54} On the other hand, false negative results may occur due to low quality DNA/RNA in a sample. Moreover, prolonged or inappropriate formalin fixation can interfere with the successful amplification of DNA or RNA, particularly when the amplified segment is long. Furthermore, false negative results can occur in the presence of adverse factors such as contamination, inhibition of the amplification reaction or problems during nucleic acid extraction.\textsuperscript{55}

**Examples of contributions to dermatology**

PCR is used routinely for the detection of pathogens, in particular those infectious agents that are difficult to culture including mycobacteria, fungi, protozoa and viruses.\textsuperscript{56–58} Truncated LINE-1 inserts were identified associated with transmissible venereal tumours of dogs.\textsuperscript{51} The 1378 bp LINE-1 insert can be identified by PCR and, hence, confirm the diagnosis of metastatic neoplastic cells.\textsuperscript{51} Clonality testing or PCR for antigen receptor rearrangement (PARR) and subsequent electrophoresis is used routinely now to differentiate between lymphomas and an inflammatory lymphoid rich process (Figure 4). Clonal rearrangement of T cell receptor gamma (TCR-\(\gamma\)) or immunoglobulin heavy chain (IgH) supports the diagnosis of T cell lymphoma or B cell lymphoma, respectively, whereas polyclonal rearrangements are more consistent with an inflammatory process.\textsuperscript{53–58} It is important to interpret the clonality results in association with morphological and immunophenotypic features and clinical presentation, because occasionally clonal populations can be found in reactive processes. This has been seen in regressing histiocytomas, which may exhibit the presence of a clonal CD8\(^+\) T cell population (personal observation). In addition, if there is a prominent reactive lymphoid infiltrate associated with the neoplastic lymphocytes, the clone may not be evident within the polyclonal background.

**In situ hybridization**

Small complementary molecular probes are used to detect sequences of certain chromosomal regions or genes. This can be applied for identification of host or pathogen DNA/RNA in tissues and cytology specimens.\textsuperscript{65}

**Methods**

Variably labelled small complementary DNA or RNA probes can be applied to fresh snap-frozen tissues, FFPE tissues, smears or microarrays. The complementary molecular probes are either radio-labelled or conjugated to fluorescent compounds (fluorescence in situ hybridization; FISH), horseradish peroxidase or digoxigenin.\textsuperscript{54,65,66} The detection system depends on the label used (digoxigenin: 4-nitroblue tetrazolium chloride (NBT)/15-bromo-4-chloro-3-indolyl-phosphate (BCIP); fluorophore: light of appropriate wavelength; horseradish peroxidase-based signal: using a chromogenic such as 3,3-diaminobenzidine (DAB)). Occasionally, sequence amplification techniques need to be applied to increase the chance of a signal detection from a rare and or small molecule, as for example micro RNA (miRNA).\textsuperscript{65}

**Advantages**

In situ hybridization (ISH) can be applied to cytology specimens (aspirates and samples from cultures), snap-frozen sections and FFPE tissue sections. In tissue sections, the positive results can be visualized in the context of the morphological lesions. Hence, ISH avoids false positive results as can be seen with PCR.\textsuperscript{54} Moreover, ISH and IHC can be applied simultaneously on a single sample.\textsuperscript{67,68} This enables direct visualization of a pathogen or genetic mutation within a particular cell subpopulation within a heterogeneous cell infiltrate. As pathogens can be readily identified within formalin-fixed tissues, it eliminates the need of access to fresh tissue for a variety of culture techniques. Confirmation of direct causation of a particular pathogen in a skin lesion can be confirmed by applying ISH on a transitional zone from lesional to...
nonlesional skin; the former presents a positive result, whereas the latter should be negative.54

Disadvantages and challenges
ISH is a rather expensive technique, and although quite common in human dermatopathology, it is still not well-established in daily veterinary diagnostic dermatopathology. It has to be emphasized that false positive or negative results can be ruled out only by careful interpretation of ISH results in tandem with the morphological features of the sample. For detection of genetic mutations, the probe has to target the specific location of interest to differentiate between affected and nonaffected samples.

Examples of contributions to dermatology
A close relationship of human and equine Molluscum contagiosum virus has been suggested based on the identification of the virus in equine lesions using two human BamHI-restricted fragments of human M. contagiosum type I DNA.69 The pathological effect of equine Herpesvirus-5 on keratinocytes has been documented where the virus in keratinocytes exhibited cytotoxic effects.70 As in many other species, PCR identifies papilloma virus in normal skin and BPV has been claimed to be associated with various non-neoplastic skin lesions in horses.71–73 However, ISH offered proof that the presence of BPV-1 and BPV-2 are very restricted to lesions of equine sarcoid, whereas adjacent keratinocytes and dermal tissues were negative (Figure 5).54 Moreover, ISH demonstrated that equine penile squamous cell carcinomas can be either papilloma virus induced or the result of solar exposure;74 however, none of these cases had evidence of a combination of both.

Tissue microarrays
Tissue microarrays are blocks composed of numerous different small tissue samples, on average 0.6 mm in diameter, which subsequently can be evaluated simultaneously by different techniques, including IHC and ISH.75–77

Methods
Blocks can be prepared manually or obtained from various manufacturers.77 Sections from these blocks are transferred to glass slides and subsequently processed in a similar way to regular paraffin sections (Figure 6).

Advantages
It allows simultaneous standardized screening of a larger number of tissue samples by ISH or IHC.

Disadvantages and challenges
The construction of tissue microarray is labour-intensive. Each actual tissue sample is very small and may not be truly representative of the entire lesion. Hence, careful selection of location for collecting the 0.6 mm sample from a particular lesion is important.

Examples of contributions to dermatology
New tissue reagents can be evaluated easily, using a microarray of normal and diseased tissues. Tissue microarrays are therefore particularly helpful in laboratory quality assurance controls. They are also used in a research setting focused on screening a large number of samples by ISH or IHC.78,79 Alternatively, they can be helpful for training programmes.

Gene microarray or genome-wide association studies (GWAS)
Gene microarrays or GWAS are also referred to as DNA or genome chips. They facilitate screening of the entire genome within a certain population in one single test.80 Systematic analysis of the entire genome includes non-coding areas (introns) as well as coding areas (exons), screening for mutations and polymorphism in biological samples. The technique of microarrays allows detection

![Figure 5. In situ hybridization (ISH) for bovine papilloma virus (BPV) in an atypical equine sarcoid. (a) The proliferating spindle cell population in the superficial and deeper portion of the dermis (black bars) are separated by a band of normal dermal collagen (Haematoxylin and eosin, 2×). (b) Hybridization to BPV is detectable within the nuclei of the proliferating fibroblasts in the superficial and deeper portion of the dermis (black bars), but not within the normal dermal collagen (2×). (c) Presence of BPV within proliferating fibroblasts (10×). ISH probes complementary to regions of the E5, E6 and E7 coding regions for BPV1 (x02346.1) and BPV2 (M20219.1); horseradish peroxidase-based signal amplification system; 3,3′-diaminobenzidine (DAB; Advanced Cell Diagnostics, Newark, CA, USA); haematoxylin counter stain.](image-url)
of inflammatory skin diseases, the mutational analysis of genodermatoses and polymorphism screening.83

Disadvantages and challenges

GWAS requires prior knowledge of a sequence and thus cannot detect novel genes or unknown gene sequences that may be involved. Gene microarrays are still expensive and labour-intensive. Moreover, the genome of many species has still not been completely sequenced and many genes in domestic animals have not been annotated correctly, which makes the interpretation of data achieved by microarrays difficult. DNA microarrays are therefore still limited to a few species and mostly used in a research setting.

Examples of contributions to dermatology

This technique has been introduced to evaluate gene profiles for the development and progression of tumours, in particular melanomas in humans. For example, thrombospondin-2 and desmoglein-2 are enhanced in aggressive melanomas in humans.83 More sophisticated microarrays are generated to evaluate SNPs on a larger scale to evaluate for mutations (insertion and deletions) as the underlying cause for a particular skin disease. Neuroplastic T cells in humans with Száry’s syndrome express PLS3 or plastin-T, an actin-binding protein that is not observed in normal lymphocytes.84 The significance of this expression is not known, but screening for the presence of this protein assists clinical follow-up for residual disease. Although not readily available in a diagnostic setting, cDNA microarrays will shape the diagnostic approach to skin diseases in the future.

Next-generation sequencing (NGS)

Next-generation sequencing allows fast and comprehensive sequencing of the entire genome of an individual. It can be performed on the entire genome, which includes noncoding areas of the DNA (introns), coding areas of the DNA (exons) and mitochondrial DNA, or limited to coding areas of the genome. The former is referred to as whole-genome sequencing (WGS), whereas the latter is referred to as whole-exome sequencing (WES).85,86 The exome involves roughly 1.5% of the human genome, encompassing most exons of about 20,000 human genes; WES is therefore less labour-intensive than WGS.87 WES involves enrichment procedures, such as exon enrichment and, thus, RNA sequencing, also called whole transcriptome analysis or whole transcriptome shotgun sequencing (WTSS), may often be used as an alternative approach to detect variability in protein-coding regions.88 However, it is important to recognize that the majority of genetic diseases in humans are due to mutations and SNPs within the noncoding regions of the genome; identification of these mutations requires WGS.89 Like every other ancillary testing method, it is crucial to demonstrate the causality of such mutations.

Methods

Originally sequencing was performed by labour-intensive selective incorporation of chain-terminating fluorescent labelled dideoxynucleotides by DNA polymerase during

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in vitro DNA replication followed by capillary electrophoresis, referred to as Sanger’s chain termination sequencing.\textsuperscript{90} NGS is a rapid, automated sequencing process performed by either DNA polymerase- or DNA ligase-dependent methods.\textsuperscript{86,91,92}

**Advantages**

Of particular note is that WES and WTSS are relatively fast procedures, which allow identification of genetically based diseases that tend to have a rather phenotypically heterogeneous clinical presentation. In contrast to gene microarrays, these allow the identification of novel genes and absolute gene quantification, as well as the determination of alternatively spliced gene transcripts, which may be important in disease biology.

**Disadvantages and challenges**

NGS is still expensive and requires expertise in handling large data outcome and thus is currently still mostly limited to research settings. However, it has found its way into diagnostics in human medicine.\textsuperscript{85,86} In veterinary medicine, the genomes of many species have still not been sequenced, which limits the use of this method. In a similar manner to the challenge with gene microarrays, interpretation of data can be a challenge due to incorrect annotation of many genes in domestic animals.

**Examples of contributions to human dermatology**

Two cases of X-linked hypohidrotic ectodermal dysplasia were confirmed by WES.\textsuperscript{85} The diagnosis of acrodermatitis enteropathica and localized DEB in siblings was achieved by WES, as clinical presentation of these diseases were not straightforward.\textsuperscript{85}

**Laser capture microdissection**

Laser capture microdissection (LCM) is a very powerful tool with which to isolate cells from a heterogeneous cell population in culture or on histological slides. The cells can be visualized and photographed before collection. The collection process does not disrupt the phenotypic characteristics or molecular state of the cells. Therefore DNA, RNA and protein can be extracted from collected cells (Figure 7).

**Methods**

LCM is performed with direct microscopic visualization. There are two basic methods used to date: infrared (IR) and ultraviolet (UV) light.\textsuperscript{93,94} They both allow direct microscopic selection of tissues/cells in culture and capture into a collection tube. A UV light beam excises the selected cells and a light catapult transports the cells into a collection tube.\textsuperscript{93} Alternatively, an IR laser is used to melt a thermoplastic membrane attached to a cap that overlies the cells of interest.\textsuperscript{94}

**Advantages**

LCM allows selection of cells from cytological specimens, frozen sections and FFPE tissue sections, as well as cell cultures. Tissue sections can be stained with H&E, immunohistochemistry or FISH, before being moved to the dissection chamber, which ensures identification of cell populations of interest. Potentially various subpopulations of cells can be collected from one original specimen and subsequently evaluated separately. The cells left behind remain unaltered in the tissue.

**Disadvantages and challenges**

The method is very expensive and the collection of the samples is a time consuming process. Therefore, LCM is still used mostly in research rather than diagnostic dermatopathology.

**Examples from the literature**

Patients with psoriasiform arthritis have altered miRNA expression in Th17 cells.\textsuperscript{95} LCM was used to identify
altered compartmentalization of miRNAs in epidermal and dermal cells of patients with psoriasis.96 Transition from precancerous to neoplastic cells can be confirmed within a single tissue sample, as has been shown in people with nevi cells transitioning into melanoma.97 LCM also allows evaluation of extracellular matrix proteins; for example, it was reported that decorin, a major protein involved in regulation of collagen fibre diameter, is expressed in reticular dermis, but absent in papillary dermis.98 The difference in decorin mRNA expression may contribute to the age and UV irradiation induced decrease of collagen I and IV by affecting bundle diameter in the superficial dermis. It also has been shown that the immunosurveillance for herpes simplex is due largely to the persistence of CD8+ T cells in the dermo-epidermal junction; these cytotoxic T cells lack expression of chemokines to egress and recirculate.99 Lastly, LCM was used to collect lesional bacterial colonies subsequently characterized as Strepotomycyes species in mycetoma-like lesions in cats.100

Conclusions
The techniques reviewed in this paper have greatly improved our understanding of skin diseases. However, many are labour-intensive and costly, and consequently they are more frequently used in a research setting. Some techniques, including PCR and IHC, are more commonly offered in diagnostic laboratory settings, whereas others such as clonality and ISH are still limited to a few diagnostic laboratories.

The summary of the clinical presentation and morphological features of a lesion guides the clinician and pathologist in their choice of appropriate additional techniques that should be considered to achieve the aetiological diagnosis. It is crucial to know the limitations of each technique to avoid overinterpretation of the results. Moreover, it is imperative that the results of each additional test are not interpreted in a vacuum: each technique contributes a piece to solving the puzzle. Each result has to be viewed in association with the histological characteristics of the lesions as well as clinical presentation of the patient to ensure correct interpretation and ultimately the most appropriate treatment. With that knowledge, dermatopathology will always remain an important link between benchtop science, new techniques and clinical veterinary dermatology.

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Résumé

Contexête – L’histopathologie est essentielle dans les avancées des connaissances en dermatologie vétérinaire. Cependant, les critères morphologiques et les patrons histopathologiques ne peuvent pas toujours révéler de diagnostic étiologique.

Objectifs – Plusieurs techniques ancielles peuvent aider à la détermination d’un diagnostic étiologique. Certaines de ces techniques sont devenues routinières en diagnostic dermatopatologique tandis que d’autres ne sont encore utilisées qu’en recherche. Cette revue discute de l’intérêt, de la force, des avantages et inconvénients et des défi associés à chaque technique.

Méthodes – La microscopie digitale, l’immunohistochimie, l’immuno-fluorescence, le clivage par les sels, les Western blots, la microscopie électronique, la PCR, l’hybridation in situ, les puces tissulaires, le séquençage de dernière génération, les puces à ADN et la microdissection au laser sont discutés.

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Conclusions – It is crucial to comprehend the limits of each technique and to correlate the results to the clinical implications. Since the dermatopathology rests on the principal between the sciences of laboratory, the results available from the ancillary techniques and dermatology veterinary clinic.

Resumen
Introducción – La histopatología ha sido esencial en el avance de conocimientos en dermatología veterinaria. Sin embargo, las características morfológicas y los patrones histológicos no pueden siempre indicar un diagnóstico etiológico.

Objetivos – varias técnicas auxiliares pueden ayudar a obtener un diagnóstico etiológico. Algunas de estas técnicas son utilizadas en la dermatopatología diagnóstica de forma rutinaria, mientras que otras son utilizadas fundamentalmente en investigación. Esta revisión argumenta acerca de la utilidad, valor, ventajas y desventajas y retos asociados con cada técnica.

Métodos – se comentan las técnicas microscopía digital, inmunohistoquímica, inmunofluorescencia, piel separada por sal, western blot, microscopia electrónica, PCR, hibridación in situ, microarrays de tejido, secuenciación de siguiente generación, microarrays de ADN y microdissección con láser.

Conclusiones – es crucial entender las limitaciones de cada técnica y correlacionar los resultados tanto con los hallazgos patológicos como con la presentación clínica. Como tal, la dermatopatología permanecerá como el eslabón de unión entre la ciencia de escritorio, los resultados disponibles de técnicas auxiliares y la dermatología clínica veterinaria.

Zusammenfassung
Hintergrund – Um unser Wissen in der Veterinärdermatologie zu forciert, ist die Histopathologie essentiell gewesen. Nichtsdestotrotz können die morphologischen Merkmale und die histologischen Muster nicht immer die ätiologischen Diagnosen enthüllen.

Ziele – Mehrere ergänzende Techniken können dabei helfen eine etiologische Diagnose zu erlangen. Einige dieser Techniken haben ihren Weg in die Routinediagnostik der Dermatopathologie gefunden, während andere weiterhin hauptsächlich in der Forschung angewendet werden. Diese Review diskutiert die Anwendung, die Stärken, Vor- und Nachteile und Herausforderungen, die mit den einzelnen Techniken verbunden waren.


要約
背景 – 理学組織学は医師皮膚科学において我々の知識を進歩させるために必須である。しかしながら、形態学的な特徴および病理学的なパターンによって病名の診断が明らかにできないことがある。

目的 – 複数の補助的な手法が病因の診断を助けることが可能である。これらの手法のいくつかは病理皮膚学の診断に日常的に取り入れられている一方で、その他の手法はまだも主に研究で使用されているのみである。

この論文はそれらの手法に関与した実用性、長所、利点、欠点および課題について述べる。

方法 – デジタル顕微鏡法、免疫組織化学、免疫蛍光法、食塩剥離皮膚標本、ウエスタンプロット法、電子顕微鏡法、PCR、in situハイブリダイゼーション法、組織マイクロアレイ法、次世代シークエンス法、DNAマイクロアレイ法、ならびにレーザーマイクロダイゼーション法を考察した。

結論 – それぞれの手法の限界を理解すること、および病理学的な所見と臨床症状の両方の結果を関係づけることが非常に重要である。そのようなものとして、皮膚病理学はベンチトップ化学、補助的な手法により得られる結果および臨床既往皮膚科学をつなぐ重要な存在であると考えている。

摘要
背景 – 组织病理学已经成为提高兽医学病理学相关知识的重要组成部分。但是，形态特征和组织学模式并不总是能够确诊病因。

目的 – 一些辅助技术可以帮助确诊病因。其中一些技术已经成为常规的皮肤诊断方法。其他仍然主要用于研究。

方法 – 本篇文章主要讨论数码显微镜、免疫组化、免疫荧光检测法、皮肤病理、蛋白质印迹、电子显微镜、PCR、原位杂交法、组织芯片、新一代技术测序技术、DNA微阵列和激光显微解剖等技术。

总结 – 关键是理解每项技术均有其局限性以及要结合病理学发现和临床表现来进行诊断。正因为如此，皮肤病理学仍然是台式科学的重要环节，可结合辅助技术和兽医临床皮肤病学获得诊断线索。