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Histopathological and Complementary Diagnostic Approaches for Protozoal Infections in Veterinary Medicine: A Narrative Review

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Abstract: Parasitic diseases represent a major challenge in veterinary medicine, significantly affecting animal health, production performance, and overall welfare. In protozoal infections, clinical manifestations are typically nonspecific and variable, depending on the etiological agent and host, thereby necessitating laboratory-based diagnostic confirmation. This review aims to provide a comprehensive evaluation of conventional histopathological methods in conjunction with integrated histopathological, immunohistochemical, and molecular techniques for the detection of protozoa in tissues and the characterization of associated lesions. Emphasis is placed on the diagnostic value, applicability, and limitations of these complementary approaches, highlighting their role in improving diagnostic accuracy and advancing the understanding of protozoal pathogenesis. The literature search strategy was systematically conducted across PubMed and Google Scholar, employing key terms such as "protozoal infections," "histopathology," "immunohistochemistry," and "molecular diagnostics" combined with relevant descriptors pertaining to parasite detection and the characterization of tissue lesions. Beyond histopathological examination, complementary immunohistochemical and molecular techniques have been widely reported to enhance parasite detection, enable precise species identification, and provide deeper insight into host–pathogen interactions, thereby improving diagnostic accuracy and supporting differential diagnosis in parasitic diseases.

Introduction

Histological diagnosis of veterinary protozoal diseases remains essential because it allows the simultaneous assessment of the type and distribution of lesions, as well as the spatial relationship between the etiological agent and the affected tissue. This approach is particularly important in infections caused by tissue-dwelling or intracellular protozoa, such as *Toxoplasma gondii*, *Neospora caninum*, *Leishmania infantum*, and certain *Cryptosporidium* species, where the mere detection of parasitic DNA does not necessarily demonstrate lesion causality.

Given the biological diversity and variable tissue tropism of protozoa of veterinary importance, diagnosis should rely on the correlation of clinical, gross pathological, histological, and laboratory findings. Conventional H&E histology remains the initial method for lesion mapping, allowing evaluation of tissue changes and their distribution. However, its sensitivity may be limited in cases with low parasite burden, focal distribution, or nonspecific parasite morphology, making complementary diagnostic methods necessary in selected cases.

Material and method

The literature search strategy was systematically conducted across PubMed and Google Scholar, employing key terms such as "protozoal infections," "histopathology," "immunohistochemistry," and "molecular diagnostics" combined with relevant descriptors pertaining to parasite detection and the characterization of tissue lesions.

Results and discussions

Hematoxylin and eosin (H&E)

Histology with hematoxylin and eosin (H&E) remains the basic method in the morphological diagnosis of veterinary protozoal diseases, because it allows the evaluation of tissue architecture, lesion patterns, and the relationship between the parasite and the host. It is a rapid, accessible technique that is useful for case screening and for guiding fu

H&E can directly reveal some protozoa or suggest the diagnosis through the lesions they produce, being useful in infections with *Toxoplasma gondii*, *Neospora caninum*, *Eimeria*, *Cryptosporidium*, *Leishmania*, *Babesia*, *Theileria*, and *Sarcocystis*. However, the sensitivity of the method decreases when the parasitic load is low, the distribution is focal, or the organisms are difficult to differentiate from debris and artifacts.

The main limitation of H&E is its reduced specificity for exact species identification. Therefore, the method should be considered mainly a tool for screening and contextualizing lesions, often requiring complementation with IHC, ISH/CISH, PCR/qPCR, or special stains for etiological confirmation.

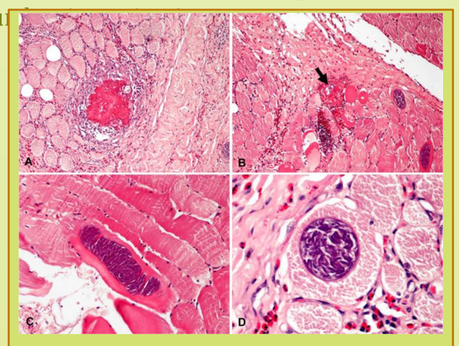

Fig.1: Pectoral muscle of a horse affected by granulomatous eosinophilic myositis caused by *Sarcocystis gigantea*. H&E.

Special stein

In the veterinary literature, most tissue protozoa can be recognized in sections stained with hematoxylin and eosin (H&E). However, special stains become useful in situations where H&E has limited sensitivity: when the parasite is small, superficially located at the epithelial level, masked by inflammation or pigment, or when its morphology may overlap with fungi, processing artifacts, or other protozoa.

Veterinary pathology atlases and studies in fish pathology indicate that the most frequently used and documented special stains for protozoa are Giemsa/May–Grünwald–Giemsa, acid-fast stains such as Kinyoun or modified Ziehl–Neelsen, the Periodic Acid–Schiff (PAS) reaction, and, less commonly, Grocott methenamine silver. In more specialized fields, particularly fish pathology, Feulgen staining and the Alcian blue/PAS combination are also mentioned, while methods such as Warthin–Starry, histological Gram stain, protargol/Klein, methylene blue, toluidine blue, and phloxine tartrazine have more limited or mainly historical use.

From a diagnostic perspective, Kinyoun/modified Ziehl–Neelsen is the most targeted method for cryptosporidiosis, Giemsa is the most versatile for small and intracellular protozoa, PAS is particularly useful for highlighting apicomplexan cysts and their carbohydrate-rich content, while GMS has more of a contrast-staining role than that of a specific protozoological method. The value of these stains is greatest when they are interpreted in correlation with the lesion topography demonstrated by H&E and, when necessary, complemented by etiological confirmation methods such as IHC, ISH, or PCR/qPCR.

Table 1. Comparative table of special stains used in the diagnosis of veterinary protozoal diseases

Stain	Representative FFPE protocol	What it highlights	Documented protozoa/hosts/tissues	Why use it over H&E	Main advantages	Main limitations
Giemsa / MGG	3–4 µm sections; deparaffinization and rehydration; undiluted Giemsa for 15 min; 0.1% acetic acid for 10 sec; water; 2-propanol; xylene; no retrieval/proteolysis.	Small organisms stained dark blue; better nuclear/cytoplasmic contrast; sometimes the kinetoplast or granular structures are easier to visualize.	Leishmania in canine tissues; Theileria annulata in bovine tissues; Cryptosporidium, Hexamita/Spironucleus, Eimeria, Cochlosoma, and Chilomastix in turkey intestine; Cryptosporidium in plastic sections from lambs.	Greatly increases contrast for small parasites, especially intracellular or superficial forms.	Inexpensive, widely available, preserves tissue architecture, and is useful for morphological screening.	Non-specific; lower sensitivity than IHC/ISH/PCR when parasite loads are low; possible confusion with T. cruzi or other small structures; sensitive to ethanol contamination of staining baths.
Kinyoun / modified Ziehl–Neelsen	6 µm sections; deparaffinization; carbol-fuchsin at 56°C for 1 h; 1% acid-alcohol; methylene blue for 2 sec; no retrieval/proteolysis.	Acid-fast oocysts/stages stained red on a blue background.	Cryptosporidium spp. in classical veterinary diagnosis; paraffin-embedded intestinal sections from turkeys, with localization along the epithelium.	Oocysts become readily visible and are better distinguished from yeasts or debris.	Excellent contrast, low cost, and very useful when cryptosporidiosis is suspected.	Not species-specific; sensitivity decreases with over-decolorization; superficial stages may be absent from the section.
PAS	Deparaffinization; 0.5% periodic acid for 5 min; 3 rinses; Schiff reagent for 15 min; tap water for 5 min; Mayer hematoxylin for 1 min; no retrieval/proteolysis.	Polysaccharides, glycogen, mucins; in protozoa, amylopectin granules/bradyzoites and sometimes cyst contents or cyst wall.	Neospora-like protozoal cysts in bovine CNS; Besnoitia besnoiti in bull skin/testicle; Cryptosporidium baileyi-like structures in avian conjunctiva and nasal epithelium.	Useful when H&E suggests an apicomplexan cyst, but additional morphochemical evidence is needed.	Simple, reproducible, very suitable for FFPE samples, and preserves tissue well.	Highly non-specific; positive background in mucins, glycogen, or fungi; variable intensity among bradyzoites, with possible false-negative results.
GMS	4–5 µm sections; oxidation with chromic acid; metabisulfite; heated methenamine silver; gold chloride; hypo; light green; no retrieval, but strong oxidation.	Argyrophilic organisms stained black on a green background.	Cryptosporidium baileyi in the conjunctiva, nose, and bronchi of owls; Besnoitia besnoiti bradyzoites stained only very weakly.	Provides strong contrast when H&E shows only equivocal spherical structures.	Very good optical contrast in selected cases.	Inconsistent for protozoa; silver artifacts; fungi and other structures may interfere; in Besnoitia, staining is very weak.
Feulgen	Deparaffinization; 5 mol/L HCl at 22°C for 50 min; water; Schiff reagent for 60 min; sodium bisulfite; alcohols; xylene.	DNA/nuclei stained red-violet; parasome/kinetoplast in special contexts.	Neoparamoeba and some flagellates in fish pathology.	Highlights nuclear structures that are not visible on H&E.	Very useful for differential diagnosis in specialized aquatic pathology laboratories.	Niche, slow, and highly dependent on exact hydrolysis; of limited usefulness in domestic mammal pathology.
Alcian blue/PAS	Deparaffinization; Alcian blue pH 2.5 for 30 min; 0.5% periodic acid for 5 min; Schiff reagent for 15 min; Mayer hematoxylin for 1–5 min.	Acid mucins blue, neutral mucins/glycogen magenta, mixed mucins violet; may improve recognition of amoebae and the mucous response.	Neoparamoeba pemaquidensis/perurans in fish gills.	Useful when the parasite is embedded in an intense mucous response.	Provides information about both the parasite and the host response.	More lesion-oriented than parasite-specific; very limited usefulness in domestic mammals and birds.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded tissues (FFPE) is, in veterinary protozoology, a lesion-oriented etiological method. It not only detects the parasite antigen but also localizes it in relation to the histological lesion. This localization explains why IHC remains valuable even in the era of PCR/qPCR and ISH: a positive molecular result confirms parasitic DNA, but does not by itself prove that the protozoan is the cause of the lesion.

In contrast, IHC can support this morphological association.In the veterinary literature, the most solid applications of IHC are in ruminant abortions caused by Toxoplasma gondii and Neospora caninum, canine cutaneous leishmaniosis, equine protozoal myeloencephalitis caused by Sarcocystis neurona, and, more selectively, Theileria infections in wildlife and Cryptosporidium infections of the digestive tract.

The practical message for the laboratory is clear: IHC should be used after histological screening of lesions and interpreted together with H&E staining. When the parasitic load is low or distribution is focal, the method should be complemented by PCR/qPCR or ISH, especially in mild Leishmania dermatitis and in unevenly distributed T. gondii/N. caninum lesions.

The major advantage of IHC is the direct localization of the parasite within the lesion, allowing correlation with histological changes. Well-validated antibodies improve specificity and reduce cross-reactions, especially compared with older-generation antibodies. However, IHC is less sensitive than PCR/qPCR in cases with low parasite load or focal distribution, and a negative result does not exclude protozoal disease.

In canine leishmaniosis, Xavier et al. described a simple and cost-effective protocol applied to FFPE skin samples: 4–5 µm sections, deparaffinization and hydration, inhibition of endogenous peroxidase with 4% H_2O_2, blocking with normal goat serum at 1:100, followed by incubation for 18–22 h at 4°C with heterologous immune serum from dogs naturally infected with L. chagasi as the primary antibody. This was followed by a biotinylated secondary antibody and a streptavidin–peroxidase complex, visualization with DAB, and counterstaining with Harris hematoxylin.In this study, IHC significantly increased the detection of amastigotes compared with H&E, with an overall sensitivity of 62.1% versus 44.8% for H&E.

Immunofluorescence (IF)

Immunofluorescence, in contrast, has a more limited role in the veterinary histopathological diagnosis of protozoal infections. In the studies identified, its direct application to tissue sections was less frequent than that of immunohistochemistry (IHC). In the study by Özkaraca et al. on 102 aborted bovine fetuses, IF detected *N. caninum* in only 8 cases, compared with 18 cases by IHC and 26 cases by duplex PCR. The authors noted that IF is technically easy to perform, but more difficult to interpret because of nonspecific staining.In a different context, Erlandsen et al. used immunofluorescence for the detection of Giardia cysts in animal tissues and fecal samples, demonstrating the usefulness of the technique in specimens subjected to freeze–thaw cycles. Finally, in the classical protocol described by Cole et al., IFAT performed on slide spots containing dried tachyzoites was used to validate the specificity of the anti-Neospora antibody before its application to FFPE sections by IHC.

Therefore, in this review, immunofluorescence should be regarded as a valuable but more restricted diagnostic approach for protozoal infections in veterinary pathology. Compared with IHC, it is less standardized for use on FFPE tissue sections and is more commonly applied in serology, cytology, smears, tissue imprints, or antibody validation procedures. Its main advantages are the rapidity of the technique and the potential for specific fluorescent detection when well-validated reagents are available. However, the reviewed veterinary literature indicates that, in some tissue-based applications, IF may have a lower diagnostic yield than IHC and may be more difficult to interpret because of nonspecific fluorescence.

In situ hybridization (ISH)

In situ hybridization (ISH) combines molecular biology with histological examination, allowing the detection of parasitic DNA or RNA directly within tissue sections. Its main variants include chromogenic ISH (CISH), fluorescent ISH (FISH), and newer methods such as RNAscope.

In veterinary protozoal diseases, ISH/CISH has been used to identify agents such as *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis* spp., *Giardia duodenalis*, *Cryptosporidium* spp., *Babesia* spp., *Plasmodium* spp., and *Leishmania* spp. The method is valuable because it shows not only the presence of the parasite, but also its localization and distribution within the lesion.

Its main advantages are high specificity and the ability to correlate the parasite with tissue damage. However, ISH is limited by probe quality, nucleic acid degradation in FFPE samples, higher cost, protocol complexity, and reduced sensitivity in cases with low parasite burden. Therefore, ISH is best used as a targeted complementary method alongside H&E, IHC, and PCR/qPCR.

Polymerase chain reaction (PCR)

Molecular diagnosis by PCR, including qPCR and dPCR, is valuable for detecting protozoa in animals because of its high sensitivity, even in samples with low parasite burden or FFPE tissues. qPCR and dPCR allow parasite DNA quantification, while sequencing enables species-level identification.However, these methods do not show parasite localization within lesions and cannot always distinguish viable parasites from residual DNA. Therefore, PCR/qPCR/dPCR should be used as complementary tools to histology, IHC, and ISH, which confirm the agent directly within affected tissue.

Conclusions

In conclusion, the histological diagnosis of protozoal diseases in veterinary medicine represents a multimodal process. The initial examination of H&E-stained sections remains the starting point, allowing the identification of tissue changes and, in some cases, parasitic forms. For confirmation or quantification of infection, it is complemented by special stains, immunohistochemical and tissue-based molecular methods, such as IHC, IF, and ISH, which increase diagnostic specificity and sensitivity, as well as by genetic techniques, such as PCR/qPCR/dPCR, capable of detecting even low parasite burdens. The integration of these methods into an optimized diagnostic protocol, such as histopathology + IHC/ISH + PCR, provides high diagnostic accuracy, reduces the risk of false-negative or false-positive results, and supports a rigorous diagnosis of protozoal infections in animals.