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# Immunohistochemistry in Poultry Pathology: Current Practices and Future Prospects

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**Abstract**

IMMUNOHISTOCHEMISTRY (IHC) is a valuable diagnostic and research technique used to identify specific antigens within tissue sections by utilizing the precise binding between antibodies and their corresponding antigens. The principle of IHC involves applying labeled antibodies either directly or indirectly to bind to target antigens, followed by visualization through chromogenic or fluorescent detection systems. Different IHC methods, including direct, indirect, chromogenic, and immunofluorescence techniques, provide flexibility in terms of sensitivity and specificity based on the research or diagnostic objectives. After staining, tissues are examined under a microscope to assess the distribution and intensity of antigen expression. The results are typically interpreted using combined semi-quantitative scoring, which integrates staining intensity and the percentage of positive cells into a single score, enabling objective and standardized evaluation. IHC offers numerous benefits, such as high specificity, the ability to detect proteins within their tissue context, and compatibility with formalin-fixed, paraffin-embedded tissues. In poultry medicine, IHC is essential for detecting and localizing viral and bacterial pathogens, greatly aiding in disease diagnosis, surveillance, and vaccine assessment. It is also extensively used in apoptosis research, including the detection of *Caspase-3*, a key enzyme involved in programmed cell death, providing insights into tissue damage and immune responses. Despite its advantages, IHC has limitations, including the potential for non-specific staining, the need for high-quality antibodies, and technical variability. Future developments in poultry IHC include creating more species-specific reagents, implementing automated digital analysis, and utilizing multiplex staining to improve diagnostic accuracy and research results.

**Keywords:** Immunohistochemistry, poultry, pathology, diagnosis, disease.

## Introduction

Immunohistochemistry (IHC) is a powerful immunological technique employed for the in-situ detection, localization, and visualization of specific antigens (typically proteins) within formalin-fixed, paraffin-embedded (FFPE) or frozen tissue sections [1]. This method leverages the high specificity of antigen-antibody interactions to map the expression of target molecules within preserved tissue architecture, thereby providing both molecular and histological insights [2].

The standard IHC workflow begins with the application of a primary antibody, which selectively binds to the target antigen within the tissue. This primary antibody may be monoclonal or polyclonal, depending on the specificity and application. Subsequently, a secondary antibody, raised against the species of the primary antibody and conjugated to a reporter molecule, most commonly an enzyme (e.g., horseradish peroxidase [HRP] or alkaline phosphatase [AP]) or a fluorophore (for immunofluorescence), is applied [3]. Upon addition of an appropriate chromogenic substrate (e.g., 3,3'-diaminobenzidine [DAB] for HRP), an enzymatic reaction ensues, producing an insoluble, colored precipitate that demarcates the site of antigen expression. Alternatively, in fluorescence-based IHC, signal detection is achieved via fluorescent light emission under a fluorescence microscope, allowing for high-resolution, multi-target visualization [4].

Crucially, IHC allows for the retention of tissue histoarchitecture, enabling researchers to study the spatial and cellular distribution of proteins within their native microenvironment. This contrasts with homogenization-based techniques such as Western blotting or ELISA, which, while quantitative, lack contextual information regarding localization or cell-type specificity [5].

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In both basic and translational research, IHC serves as a cornerstone methodology in fields such as oncology, neurobiology, immunopathology, and infectious disease diagnostics. Its clinical relevance is underscored by its widespread use in human and veterinary histopathology for tumor typing, biomarker validation, and pathogen detection [6].

In the domain of veterinary medicine and poultry sciences, particularly in poultry health and disease research, IHC has proven indispensable [7]. Its applications include:

-Detection and localization of infectious agents, such as avian influenza virus, Newcastle disease virus, Salmonella spp., and Eimeria spp., within specific target tissues.

-Immunophenotyping of inflammatory and immune cells to characterize innate and adaptive immune responses.

-Assessment of apoptotic and stress markers (e.g., cleaved caspase-3, heat shock proteins) in the context of environmental, toxicological, or pharmacological stressors.

-Evaluation of vaccine-induced tissue responses, including antigen expression and immune cell infiltration.

-Pathogenesis studies elucidating host-pathogen interactions, tissue tropism, and lesion development at the cellular level.

Moreover, advances in IHC, such as multiplex immunohistochemistry, automated digital image analysis, and the integration of machine learning algorithms, are enhancing the technique's diagnostic precision and throughput, thereby expanding its utility in large-scale surveillance programs and experimental poultry research [5].

*Core Principles and Technical Approaches in Immunohistochemistry*

Immunohistochemistry (IHC) is fundamentally based on the high-affinity and high-specificity interaction between antibodies and antigens within tissue sections [7]. This molecular interaction enables the visualization of protein expression and localization within the context of preserved tissue architecture, providing crucial spatial and functional insights. The technique bridges the disciplines of immunology, biochemistry, and histopathology and is widely applied in both research and diagnostic settings [8].

*Tissue Preparation and Fixation.*

The IHC workflow begins with the collection and fixation of tissue, most commonly using 10% neutral-buffered formalin**,** which cross-links proteins and stabilizes tissue morphology. The fixed tissues are then processed and embedded in paraffin wax to produce formalin-fixed paraffin-embedded (FFPE) blocks. Sections are cut (typically 4–5 μm thick), mounted onto adhesive microscope slides, and 2eparaffinised using xylene and rehydrated through graded alcohols to water [7].

*Antigen Retrieval*

During fixation, especially with formalin, antigenic epitopes can become masked by cross-linking, reducing antibody accessibility. Therefore, an antigen retrieval step is performed to unmask these epitopes. This is achieved by either:

- Heat-induced epitope retrieval (HIER) using buffers such as citrate or EDTA at high temperature (e.g., microwave, pressure cooker),

-Or enzymatic digestion, using proteolytic enzymes such as proteinase K or trypsin.

The choice of retrieval method is antigen-dependent and requires optimization to balance epitope exposure with tissue integrity [9].

*Blocking of Non-Specific Binding*

To prevent non-specific binding of antibodies to endogenous tissue components (e.g., Fc receptors or charged proteins), a blocking step is carried out. This typically involves incubation with:

-Normal serum or bovine serum albumin (BSA),

-Enzyme inhibitors to block endogenous peroxidase or alkaline phosphatase activity (especially when enzyme-labeled systems are used).

This step minimizes background staining and enhances signal specificity [6].

*Primary Antibody Incubation*

The primary antibody is applied to the tissue section. This antibody is raised specifically against the antigen of interest and may be:

-Monoclonal (high specificity, single epitope recognition),

-Or polyclonal (recognition of multiple epitopes, often more sensitive but less specific).

Incubation times and concentrations must be empirically determined to ensure optimal binding and minimal background [7].

*Secondary Antibody and Detection Systems*

Following primary antibody incubation, a secondary antibody is introduced, which binds to the primary antibody. The secondary antibody is typically species-specific (e.g., anti-mouse, anti-rabbit) and is conjugated to a detection molecule, such as:

*Enzymes*

*Horseradish peroxidase (HRP)* is commonly used with 3,3′-diaminobenzidine (DAB) as a chromogen, producing a brown precipitate.

*Alkaline phosphatase (AP)*—used with chromogens like Fast Red or BCIP/NBT, producing red or blue signals [5].

*Fluorophores*

Used in immunofluorescence (IF) applications, allowing detection via fluorescence microscopy with the possibility of multiplexing [5].

There are two principal types of IHC detection strategies

-Direct IHC: A 3abelled primary antibody directly binds to the antigen (less common due to lower signal amplification).

-Indirect IHC: A labeled secondary antibody binds to an unlabeled primary antibody (more sensitive due to signal amplification).

Additional amplification systems, such as avidin-biotin complex (ABC) or polymer-based systems, are often used to increase signal intensity [7].

*Visualization and Interpretation*

Upon substrate addition, a visible reaction product (in chromogenic IHC) or fluorescent signal (in immunofluorescence) is generated at the site of antigen-antibody interaction [6]. This allows for:

-Qualitative assessment (presence/absence of antigen),

-Semi-quantitative scoring (e.g., intensity and percentage of positive cells),

-And spatial localization within specific cell types, tissue compartments, or organ regions.

Stained slides are evaluated under light or fluorescence microscopy, and may be further 3atalyse using digital imaging and quantitative image analysis software, particularly in research or high-throughput diagnostic applications [10].

*Exploring the Methods of Immunohistochemistry*

Immunohistochemistry (IHC) encompasses a range of methodologies, each tailored to enhance the specificity and sensitivity of antigen detection in tissue sections. The choice of method is influenced by the physicochemical properties of the target antigen, the nature of the antibody-antigen interaction, tissue preservation techniques, and the desired detection system. The principal aim is to achieve precise localization of target proteins within the architectural context of the tissue, with minimal background interference and maximal signal clarity [5].

*Direct vs. Indirect Immunohistochemistry*

*Direct Immunohistochemistry*

In the direct IHC method**,** the primary antibody is conjugated directly to a reporter molecule**,** typically an enzyme (e.g., horseradish peroxidase [HRP] or alkaline phosphatase [AP]) or a fluorophore (e.g., FITC, Alexa Fluor dyes). This antibody binds specifically to the antigen of interest within the tissue section. While this method **is** relatively fast and straightforward, it is limited by lower sensitivity, as it lacks signal amplification—each antigen site is bound by only one 3atalyse antibody molecule. Therefore, direct IHC is generally reserved for the detection of abundantly expressed antigens or for applications requiring rapid screening [7].

*Indirect Immunohistochemistry*

The indirect method is more widely used in both research and diagnostic settings due to its greater sensitivity and flexibility**.** In this approach, an 3atalyse3d primary antibody binds to the target antigen, followed by a 3atalyse secondary antibody that recognizes the species-specific portion of the primary antibody. This method allows for signal amplification**,** as multiple secondary antibodies can bind to a single primary antibody, substantially enhancing signal intensity. Moreover, it enables the use of universal secondary reagents, reducing the need for multiple 3atalyse primary antibodies across experiments [7].

*Detection Modalities*

*Chromogenic Detection*

Chromogenic IHC (C-IHC) employs enzyme-labeled antibodies that 3atalyse colorimetric reactions, resulting in the deposition of an insoluble, light-visible pigment at the site of antigen localization. Common systems include [7]:

*-HRP with DAB (3,3'-diaminobenzidine)**:***

Produces a brown precipitate, widely used due to its stability and compatibility with hematoxylin counterstaining.

*-AP with Fast Red or BCIP/NBT**:*** Yields red or blue reaction products, often selected for dual-staining protocols or when contrast with DAB is needed.

Chromogenic detection is compatible with conventional bright-field microscopy**, allows for** long-term slide storage, and is suitable for routine diagnostic workflows [7].

*Immunofluorescence (IF)*

In immunofluorescence-based IHC, antibodies are conjugated to fluorochromes, and detection is performed using fluorescence or confocal microscopy**.** Fluorophores emit light at specific wavelengths when excited by a light source, permitting high-resolution imaging and multiplex

detection. Multiple antigens can be visualized simultaneously by 4nalyse4g each antibody with a distinct fluorophore, allowing for protein co-localization studies and dynamic spatial analysis within the tissue [7].

However, immunofluorescence is more susceptible to photobleaching and requires specialized imaging systems and appropriate storage conditions to maintain signal integrity.

*Advanced and Enhanced Detection Systems*

*Polymer-Based Detection Systems*

Polymer-based systems utilize dextran or other polymeric backbones to which multiple enzyme molecules and secondary antibodies are attached. This results in significantly increased signal intensity, without the need for biotin, thereby eliminating potential background from endogenous biotin. These systems offer superior sensitivity and specificity, and are particularly effective for detecting low-abundance antigens in complex tissues [11].

*Avidin-Biotin Complex (ABC) System*

The ABC technique is based on the strong non-covalent binding between biotin and avidin (or streptavidin). The secondary antibody is conjugated with biotin, which binds to an avidin-enzyme complex, amplifying the signal. Although highly sensitive, ABC systems may yield a non-specific background in tissues with high endogenous biotin (e.g., liver, kidney, egg yolk), necessitating a biotin-blocking step for accurate interpretation [11].

*Multiplex and Dual Staining Techniques*

To 4nalyse multiple targets within a single tissue section, multiplex IHC or dual staining protocols are employed. These techniques involve:

-Using primary antibodies from different host species,

-Conjugating each antibody to distinct chromogens or fluorophores,

-Or implementing sequential staining with antibody stripping and restaining.

Multiplex IHC enables the study of co-expression, cellular interactions, and spati**al** proximity of different proteins, especially relevant in immunological studies and disease models with complex tissue responses [11].

*Considerations for Method Selection:*

The appropriate IHC method must be selected based on several critical variables [11]:

-Antigen abundance and stability: Rare antigens require higher sensitivity detection systems.

-Tissue type and fixation method: Lipid-rich or necrotic tissues may present higher background or require milder antigen retrieval.

-Antibody characteristics: Host species, monoclonal vs. polyclonal, and antibody affinity affect staining outcomes.

-Analytical goals: Whether qualitative localization, semi-quantitative scoring, or multiplexing is desired.

Meticulous optimization of antigen retrieval, blocking conditions, antibody concentrations, and incubation times is necessary to ensure specific, reproducible, and interpretable results [12].

-*Challenges and Troubleshooting Strategies in Immunohistochemistry*

Immunohistochemistry (IHC) is a complex, multi-step technique in which each stage, ranging from tissue preparation to detection, must be meticulously optimized to ensure accurate, specific, and reproducible staining [13]**.** Despite its widespread utility, IHC is susceptible to a range of technical issues that can compromise data quality, leading to false-positive or false-negative results, increased background, or loss of tissue morphology. These challenges are particularly significant in poultry and veterinary pathology, where species-specific variables such as tissue architecture and antibody compatibility must be accounted for during assay development [14].

*Common Technical Challenges in IHC*

*Weak or Absent Staining*

Potential causes [15]

-Inadequate antigen retrieval (e.g., insufficient heating or incorrect buffer pH).

-Over-fixation leading to excessive cross-linking and epitope masking.

-Low antibody concentration or use of an inappropriate antibody clone.

-Degraded or autolyzed tissue, often due to delayed fixation.

o Solutions:

-Optimize heat-induced epitope retrieval (HIER) or enzymatic digestion.

-Titrate both primary and secondary antibodies.

-Use fresh reagents and ensure proper tissue preservation post-harvest.

*High Background Staining*

o Potential causes

-Inadequate blocking of non-specific sites or endogenous enzyme activity.

-Excessive antibody concentration.

-Prolonged incubation times or incomplete washing steps.

o Solutions:

-Use appropriate blocking agents (e.g., serum, BSA, casein).

-Employ peroxidase or alkaline phosphatase blocking reagents.

-Increase stringency of wash steps with buffered saline solutions containing detergent (e.g., PBS-Tween).

*Non-Specific or Diffuse Staining*

o Potential causes:

-Cross-reactivity of the antibody with off-target proteins.

-Use of non-validated or low-specificity antibodies.

-Inadequate washing or use of suboptimal buffers.

o Solutions:

-Validate antibodies using positive and negative tissue controls.

-Perform adsorption controls if using polyclonal antibodies.

-Utilize species-specific secondary antibodies with minimal cross-reactivity.

*Tissue Damage, Folding, or Loss*

o Potential causes:

-Improper fixation technique or excessive time in fixative.

-Inadequate slide adhesion during antigen retrieval or washing steps.

-Overheating during HIER.

Solutions:

-Optimize fixative concentration and fixation duration (typically 24–48 hours in 10% neutral buffered formalin).

-Use charged or adhesive-coated slides (e.g., poly-L-lysine, silane-coated).

-Control temperature and pH during retrieval to preserve tissue morphology.

*Species-Specific Considerations in Poultry IHC*

Immunohistochemistry in poultry species, particularly poultry, presents unique technical challenges [15]:

-Limited availability of validated poultry-specific antibodies, especially monoclonal reagents.

-Differences in epitope conformation due to poultry-specific protein isoforms.

-Variation in tissue composition (e.g., higher lipid content, yolk proteins) that may affect staining quality.

-Higher endogenous biotin and pigment content, requiring specific blocking strategies.

*Troubleshooting in poultry IHC therefore requires rigorous validation protocols, including*

-Use of species-appropriate controls (e.g., infected vs. uninfected poultry tissues).

-Testing antibody cross-reactivity in poultry tissues, particularly when using antibodies developed against mammalian antigens.

-Careful adjustment of antigen retrieval methods tailored to delicate poultry tissues [15].

*Best Practices for IHC Optimization and Troubleshooting*

-Antibody Validation:

o Employ positive controls (tissues known to express the target protein).

o Include negative controls (isotype controls or omission of primary antibody) [16].

-Standardization

o Use consistent fixation protocols, including time, fixative type, and sample thickness.

o Calibrate and document incubation times, temperatures, and antibody dilutions [17].

-Reagent and Equipment Quality

o Store antibodies and detection reagents under appropriate conditions (e.g., avoid repeated freeze-thaw cycles).

o Regularly calibrate microwave ovens or pressure cookers used for HIER [11].

-Slide Handling

o Prevent slide drying during staining by maintaining humidified chambers.

o Ensure proper mounting and counterstaining to maintain tissue integrity and clarity [11].

*Optimizing Microscopic Examination and Digital Image Presentation in Laboratory Practice*

Microscopy is a fundamental technique in histopathology and IHC that involves the accurate visualization of tissue sections to examine cellular and subcellular structure and the localization of particular antigens [18]. Tissue slides are examined following staining under a light microscope, often with multiple objectives (e.g., 4x, 10x, 40x, 100x oil immersion) to inspect both low-power overview and high-power detail elements [19]. Proper contrast adjustment, focus, and illumination are essential for visualization. Pathologists in IHC analyze staining

pattern, intensity, and distribution to interpret antigen expression compared to tissue morphology and pathological change.

Computer microscopy systems have made it possible to collect high-resolution digital images directly from the microscope through attached cameras. Digital images of precise areas of interest can be stored, quantified, and transmitted without the requirement of physical slides. Image acquisition requires attention to exposure time, white balance, and resolution to accurately represent the stained tissue. Serial images at different magnifications can be taken to provide comprehensive coverage [20].

For visualization, digital images are usually processed via dedicated software programs to adjust brightness and contrast and annotate structures, such as labeling certain cell types, lesions, or regions of antigen positivity. Images are assembled into figures for publication in scientific literature, reports, or educational aids. Integrity of the images should be ensured by avoiding uncontrolled manipulation that can corrupt findings. Also, the image file formats must be selected according to the purpose publication-quality figures through high-resolution TIFF or PNG files, and compressed JPEGs for everyday documentation or on the web [21].

Coupling of digital microscopy with image analysis software also facilitates quantitative measurement of staining, for instance, calculation of percentage positive cells or staining intensity, which lends objectivity and reproducibility to histopathological diagnosis. Overall, microscopy examination coupled with digital imaging enhances diagnostic accuracy, facilitates easier communication of results, and benefits research and education in veterinary pathology and poultry disease investigations [22].

*Combinative semiquantitative scoring*

Combinative semiquantitative scoring is a widespread method in IHC that integrates two critical parameters, the intensity of staining and the percentage of positively stained cells, to obtain a more accurate and standardized assessment of antigen expression in tissue sections **[**23**]**. This approach allows researchers and pathologists to move beyond mere qualitative assessment by assessing both the intensity of staining (e.g., weak, moderate, or strong) and the percentage of stained cells (e.g., <10%, 10–50%, >50%). The two scores are typically summed together, frequently by multiplication, to provide an end result immunoreactive score (IRS) reflecting the general level of target antigen expression [24]. This method enhances the objectivity and reproducibility of IHC analysis and has widespread uses in comparative research, disease diagnosis, and monitoring treatment response. In veterinary and poultry research, combinative semiquantitative scoring is frequently employed to evaluate immune responses, detect pathogens, or assess protein expression patterns across different tissues or experimental groups [25].

*The Diagnostic Value of Immunohistochemistry in Poultry Pathology*

Immunohistochemistry is highly advantageous in poultry disease diagnosis by ensuring specificity, sensitivity, and resolution in space in the identification of disease-related proteins and pathogens within tissue sections [26]. Unlike traditional tests such as culture or PCR, IHC allows for visualization of antigen localization within the context of intact tissue structure, allowing pathologists to relate the presence of an infectious agent or biomarkers to corresponding histopathological alterations [27].

This is especially true in bird diseases where more than one pathogen may cause the same lesion, because IHC can identify viral, bacterial, or parasitic aetiological agents by using particular antibodies. IHC can also identify small quantities of antigen that are below other methods' detection limit, hence increasing the diagnostic sensitivity, particularly in latent or chronic infections. It also facilitates the identification of cellular phenotypes and tumor markers in viral oncogenic diseases and assists in tumor classification and understanding tumor origin [28].

Another advantage is that IHC may be performed on archived formalin-fixed, paraffin-embedded tissue, which allows one to carry out retrospective studies and confirm diagnoses from samples taken previously. Further, IHC allows for the evaluation of host immune responses and disease pathogenesis by identifying the distribution of inflammatory markers and pathogens within infected tissues [29]. Overall, the ability, specificity, and depth of diagnosis provided by IHC make it a very useful tool in poultry pathology, improving disease diagnosis, guiding treatment decisions, and identifying effective control and prevention strategies in poultry farming [30].

*Immunohistochemistry in Detecting Viral Infections in Birds*

Immunohistochemistry is an important diagnostic method in poultry pathology, particularly for the identification and characterization of viral infections in birds [26]. Its ability for the direct localization of viral antigens in tissue sections provides both diagnostic specificity as well as viral pathogenesis information, of particular importance in the discrimination between diseases with equivalent clinical or histopathological presentation [31].

Infectious Bronchitis (IB) caused by Infectious Bronchitis Virus (IBV), IHC can be used to detect viral antigens within the respiratory tract epithelial cells, kidneys, and reproductive organs, which is useful in tissue tropism and infection stage

identification (Fig. 1A) [32]. In Infectious Bursal Disease (IBD) caused by IBDV, IHC is the routine procedure for the detection of viral proteins within the bursa of Fabricius, particularly in lymphoid follicles, where viral replication leads to immunosuppression. This is vital in measuring the efficacy of vaccines and disease severity.

In Newcastle Disease (ND), which is caused by Newcastle Disease Virus (NDV), IHC can identify viral antigens in the brain, lungs, spleen, intestines, and trachea, which is important in establishing systemic infection in velogenic strains (Fig. 1B) [33]. In the case of Avian Influenza (AI), particularly of highly pathogenic forms, IHC can detect viral antigens in many organs such as the lungs, pancreas, heart, brain, and intestines, and is therefore indispensable for differentiating AI from other respiratory or systemic diseases [34]. Infectious Laryngotracheitis (ILT), due to ILT virus, is diagnosed using IHC by showing viral antigens within tracheal epithelial cells, specifically in upper respiratory tract necrotic and inflammatory lesions (Fig. 1C) [35].

In addition to detection, IHC is advantageous for preserving tissue architecture so that researchers and diagnosticians can correlate viral localization with specific pathological changes, an advantage essential for deciphering the disease process. Also, IHC can be used in both acute outbreak investigations and retrospective studies, and hence it is a very useful and essential method in poultry virology and diagnostic histopathology [36].

*Immunohistochemistry in Detecting Oncogenic Viruses in Poultry*

Immunohistochemistry plays a very important role in the diagnosis and research of oncogenic viral poultry diseases, such as Marek's disease virus (MDV), avian leukosis virus (ALV) with its various subgroups, and reticuloendotheliosis virus (REV). These viruses induce tumors, primarily lymphomas, through the transformation of specific populations of immune cells, and their precise identification is essential for disease control and research [37].

Among the most significant advantages of IHC in this case is that it can detect viral antigens and cell tumor markers on formalin-fixed, paraffin-embedded tissue sections with both phenotypic and spatial information about the neoplastic cells. For instance, in Marek's disease (MD), a neoplastic disease induced by an oncogenic herpesvirus, IHC detects viral proteins such as pp38, a phosphorylated early protein linked with viral replication and oncogenesis, and glycoprotein B (gB), in the neoplastic T lymphocytes that infiltrate nerves, spleen, liver, and other tissues [38]. Utilization of tumor markers like CD3, which is a T-cell co-receptor, confirms the T-cell origin of the lymphomas characteristic of MD, ruling out B-cell or other neoplasia (Fig. 2A) **[**36**]**.

In contrast, tumors of mainly B-cell lineage or myeloid nature are induced by avian leukosis virus (ALV) infections, and IHC using the p27 gag protein (viral capsid protein) detects ALV-infected cells within lymphoid organs such as the bursa of Fabricius, spleen, and liver (Fig. 2B) [39]. Subgroup-specific antibodies also distinguish between ALV subgroups (e.g., A, B, J), which aids in epidemiological studies and vaccine strategy [40]. For example, in reticuloendotheliosis virus (REV), a virus that causes both immunosuppression and lymphoproliferative disease, IHC identifies viral antigens in numerous tissues, such as lymphoid organs as bursa and skin, demonstrating its broad tissue tropism and oncogenic potential (Fig. 2C) [41]. Besides viral antigen detection, IHC also utilizes cellular tumor marker proteins expressed by specific cell types to establish tumor lineage and differentiation status. These markers include CD3 for T lymphocytes, Bu-1 for B lymphocytes, and other markers like macrophage markers (e.g., KUL01) or proliferation markers (e.g., Ki-67), which give insight into tumor aggressiveness and growth rates [42].

Coupling viral antigen detection with cellular markers, IHC enables a definitive diagnosis that distinguishes among different viral oncogenic processes and tumor types, knowledge that is necessary to comprehend pathogenesis, guide treatment, and control spread within flocks **[5]**. Furthermore, tumor markers help in the differentiation of primary and metastatic lesions, in addition to assessing the immune microenvironment of tumors, therefore expanding the scope of veterinary oncopathology. Therefore, viral antigen and tumor marker detection by IHC is a powerful approach for unveiling the complex biology of poultry oncogenic viruses, and it aids in improving diagnostic efficacy, disease monitoring, and the development of effective control measures in poultry production [43].

*Tissue-Based Diagnosis of Poultry Bacterial Infections Using Immunohistochemistry*

Immunohistochemistry is an important research and diagnostic tool in the diagnosis and investigation of bacterial disease in poultry, particularly systemic infection or chronic inflammation, since it can detect and localize specific bacterial antigens in tissue lesions **[**44**]**. In contrast to the classical culture techniques that could produce false negatives because of previous antibiotic exposure, contamination, or stringent bacterial growth conditions, IHC gives unequivocal proof of bacterial presence inside infected tissues, thus enhancing the precision of diagnosis.

In *Escherichia coli* (colibacillosis), IHC can identify bacterial antigens in organs such as lungs, liver, airsacs, and pericardium to confirm its role in

fibrinous perihepatitis, pericarditis, and airsacculitis, especially in mixed infections **[**45,46**]**. Salmonellosis caused by Salmonella species like *S. Pullorum, S. Gallinarum, and S. Enteritidis*, IHC illustrates the pathogen's distribution in the liver, spleen, intestines, and cecal tonsils, particularly in macrophages and in necrotic areas, critical for the detection of acute infections and asymptomatic carrier animals [47]. With fowl cholera, caused by *Pasteurella multocida*, IHC is useful for detecting bacterial antigens in the lungs, liver, spleen, heart, and wattles, and is particularly useful in acute septicemic outbreaks when birds rapidly succumb and postmortem materials spoil quickly [48].

For infectious coryza, caused by *Avibacterium paragallinarum*, IHC finds the organism in the infraorbital sinuses and nasal cavities, with mucosal inflammation and serous to purulent exudation. For necrotic enteritis, caused by Clostridium perfringens, IHC enables identification of bacterial cells and toxins in intestinal mucosa, especially in areas of extensive necrosis, differentiating it from other enteric diseases with similar gross lesions [49]. Furthermore, IHC is indispensable in the diagnosis of poultry tuberculosis (T.B), caused by Mycobacterium avium complex. Unlike other bacterial diseases, poultry T.B. is a granulomatous, chronic disease affecting organs like the liver, spleen, intestines, and bone marrow. IHC using mycobacterial antigen-specific antibodies allows the visualization of the bacilli within macrophages and granulomas and can supplement acid-fast staining and provide specificity in formalin-fixed, paraffin-embedded tissues [50]. This is particularly useful for Mycobacterium avium discrimination from other acid-fast diseases or non-diseasing granulomatous disease.

Overall, IHC provides a tissue-contextual, strong, and sensitive diagnostic method for the detection of a wide range of poultry bacterial infections, not only permitting accurate pathogen identification but also providing an improvement in disease pathogenesis, host response, and tissue tropism understanding. Its application is not confined to diagnostics but extends to vaccine testing, outbreak investigation, and retrospective disease surveillance, making it an irreplaceable tool in both research and clinical settings in the management of poultry diseases [51].

*Immunohistochemical Detection of Caspase-3 as a Biomarker of Apoptosis in Poultry: Pathophysiological Insights into Mycotoxins, Drug Toxicity, and Heat Stress*

The immunohistochemical (IHC) detection of caspase-3 antigen in poultry tissues serves as a highly sensitive and specific biomarker for apoptosis a tightly regulated form of programmed cell death essential for maintaining tissue homeostasis. Unlike necrosis, apoptosis proceeds via defined molecular pathways, culminating in characteristic morphological and biochemical changes, including chromatin condensation, DNA fragmentation, and cellular disassembly [52]. Caspase-3, a cysteine-aspartic protease known as an "executioner" caspase, plays a pivotal role in this process by catalyzing the cleavage of numerous intracellular substrates following activation through intrinsic or extrinsic apoptotic pathways [53].

In poultry, exposure to mycotoxins, secondary fungal metabolites predominantly produced by *Aspergillus, Fusarium, and Penicillium spp.*, is a well-documented initiator of apoptotic cell death [54]. These mycotoxins, commonly present in contaminated feed, induce oxidative stress, disrupt mitochondrial membrane potential, and cause direct DNA damage. These events converge on the activation of the intrinsic apoptotic pathway, characterized by cytochrome c release from mitochondria and the subsequent activation of caspase-9, which in turn activates caspase-3 [55]. The immunohistochemical localization of cleaved (active) caspase-3 in various organs, particularly the liver, kidneys, spleen, gastrointestinal tract, and the bursa of Fabricius, correlates with histopathological lesions and provides spatial context to mycotoxin-induced cellular injury (Fig. 4A). Caspase-3 expression mapping thus facilitates the identification of apoptotic foci and the severity of tissue-specific responses to toxin exposure [56].

Similarly, certain therapeutic agents, notably macrolide antibiotics such as tilmicosin and azithromycin, although efficacious against bacterial infections, exhibit dose- and duration-dependent cytotoxic effects. These antibiotics have been implicated in the generation of reactive oxygen species (ROS), mitochondrial dysfunction, and lipid peroxidation in non-target tissues [57]. These deleterious effects may activate the intrinsic apoptotic cascade, culminating in the activation of caspase-3. The presence of caspase-3 in tissues such as myocardium, hepatocytes, and renal tubular epithelium, as demonstrated through IHC, provides mechanistic insight into drug-induced apoptosis, manifesting clinically as cardiotoxicity, hepatocellular necrosis, or nephropathy (Fig. 4B). Thus, caspase-3 serves not only as a marker of apoptosis but also as an indicator of potential adverse drug effects in veterinary pharmacology [58].

Environmental stressors, particularly heat stress, also play a critical role in inducing apoptosis in poultry [59]. High ambient temperatures compromise thermoregulation, leading to cellular injury via protein denaturation, membrane destabilization, and excessive ROS production [60]. These factors activate the mitochondrial pathway of apoptosis, resulting in elevated expression of caspase-3 in thermosensitive tissues, including the central nervous system (e.g., cerebral cortex), skeletal muscle, liver, spleen, thymus and intestinal epithelium (Fig. 4C).

The IHC-based detection of caspase-3 under such conditions allows for the differentiation between apoptosis and other forms of cell death, offering a nuanced understanding of the systemic impact of environmental stress on poultry health and welfare [56].

High-resolution immunohistochemistry permits the cellular localization of apoptotic events within intact tissue architecture, enabling researchers to assess the pattern, distribution, and intensity of caspase-3 expression across affected organs. This is essential not only for evaluating the pathophysiological consequences of various insults but also for assessing the efficacy of intervention strategies aimed at mitigating apoptosis. For instance, supplementation with antioxidants (e.g., vitamin E, selenium), immunomodulators, or thermal acclimation protocols has shown promise in reducing apoptotic indices in stressed or toxified birds [61].

*Limitations and Future Perspectives of Immunohistochemistry (IHC) in Poultry*

Immunohistochemistry has become an indispensable tool in poultry pathology and research, particularly for localizing specific antigens within tissue architecture and elucidating host-pathogen interactions. However, despite its widespread utility, the technique presents several limitations that restrict its broader application and diagnostic precision in the context of poultry health [22].

One of the most critical limitations lies in the availability and specificity of antibodies. For many poultry pathogens, especially emerging, understudied, or non-commercially prioritized species validated and species-specific antibodies are either scarce or completely lacking [14]. This deficit compromises both the sensitivity and specificity of IHC assays in poultry, potentially leading to false-negative or cross-reactive staining. Furthermore, the production of custom poultry-specific antibodies remains costly and time-intensive, limiting accessibility in routine diagnostics and smaller-scale laboratories.

Another major constraint is the technical dependency on optimal tissue processing and fixation protocols. Over-fixation can result in epitope masking due to excessive cross-linking, while under-fixation or autolysis during sample collection can degrade antigenic sites, both of which can yield inaccurate or uninterpretable results. The reproducibility of IHC is also challenged by manual variability in staining procedures and the need for experienced personnel for both staining and interpretation. In settings without advanced imaging systems, IHC may be interpreted in a qualitative or semi-quantitative manner, reducing objectivity and inter-laboratory comparability [62].

Additionally, traditional IHC typically detects only a single or limited number of antigens per tissue section, which may be insufficient in multifactorial diseases or coinfections—common scenarios in poultry production systems. This limitation hinders comprehensive pathogen profiling and restricts the ability to correlate immune or pathological responses to multiple infectious agents simultaneously [22].

Despite these limitations, the future of IHC in poultry diagnostics and research is promising, with several innovations poised to enhance its performance and applicability.

Multiplex Immunohistochemistry (mIHC): The advancement of multiplex IHC technologies, which enable the simultaneous detection of multiple antigens in a single tissue section, represents a significant leap forward. mIHC will allow researchers and diagnosticians to analyze complex host-pathogen interactions, coinfections, and tissue immune responses with greater depth and resolution. This is especially relevant in studies of respiratory, enteric, and systemic diseases where multiple pathogens or immunological markers may be involved [63].

Digital Pathology and Artificial Intelligence (AI): The integration of IHC with digital imaging platforms and AI-driven image analysis is expected to transform the interpretative landscape [64]. Automated quantification of staining intensity, cell counts, and spatial distribution of markers will reduce subjectivity, improve reproducibility, and enable high-throughput analysis. AI models trained on annotated poultry tissue datasets can further aid in pattern recognition and disease classification, enhancing diagnostic accuracy [65].

Development of Recombinant Antibodies and Peptide-Based Probes: Advances in molecular biology now allow for the design and production of recombinant monoclonal antibodies and peptide-based antigen probes, which offer greater batch-to-batch consistency, higher specificity, and adaptability to poultry targets. This will broaden the range of detectable pathogens and cellular markers relevant to poultry, including viral, bacterial, and protozoal agents, as well as immune and apoptotic markers such as cytokines and caspases [14].

Combination with Complementary Molecular Techniques: The combined application of IHC with in situ hybridization (ISH), RNAscope, or mass spectrometry-based proteomics is expected to offer a multidimensional view of disease processes. While IHC localizes proteins, ISH can detect pathogen-specific nucleic acids or host gene transcripts within the same tissue context. These integrative approaches will facilitate better correlation between pathogen presence, gene expression, and host response [66].

Application in Surveillance and Vaccine Evaluation: IHC holds future potential not only in disease diagnosis but also in monitoring vaccine efficacy, surveillance of subclinical infections, and evaluation of therapeutic interventions. Quantitative IHC approaches could be utilized to assess tissue-level immune responses post-vaccination or after treatment with immunomodulators and anti-inflammatory agents [22].

## Conclusion

Immunohistochemistry has emerged as a vital tool in poultry research and diagnostics, providing precise localization and characterization of proteins, pathogens, and cellular processes within tissue sections. By combining morphological evaluation with molecular specificity, IHC enables detailed insights into disease mechanisms, immune responses, and tissue pathology. The use of combinative semiquantitative scoring systems enhances the objectivity and reproducibility of results, making the technique particularly valuable in both experimental and clinical settings. Its applications in detecting viral and bacterial infections, as well as markers such as Caspase-3 in apoptosis studies, underscore its broad utility in veterinary pathology. Despite certain technical challenges such as variability in staining, antibody specificity, and the need for rigorous standardization IHC continues to evolve, with advances in antibody development, digital imaging, and multiplex analysis promising to expand its capabilities. As poultry health and production face increasing demands, immunohistochemistry will remain a critical tool for advancing disease control, vaccine evaluation, and fundamental research in poultry biology.

*Ethics approval*

This review does not involve any human or animal testing.

*Conflict of interest*

The authors declare no competing interests.

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## الكيمياء المناعية النسيجية في أمراض الدواجن: الممارسات الحالية والآفاق المستقبلية

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**الملخص**

الكيمياء المناعية النسيجية، تقنية تشخيصية وبحثية قيّمة تُستخدم لتحديد مستضدات محددة داخل مقاطع الأنسجة، وذلك بالاستفادة من الارتباط الدقيق بين الأجسام المضادة ومستضداتها المقابلة. يعتمد مبدأ الكيمياء المناعية النسيجية على تطبيق أجسام مضادة مُوسومة، إما بشكل مباشر أو غير مباشر، للارتباط بالمستضدات المستهدفة، متبوعًا بتصويرها من خلال أنظمة الكشف الكروموجيني أو الفلوري. توفر طرق الكيمياء المناعية النسيجية المختلفة، بما في ذلك التقنيات المباشرة وغير المباشرة والكروموجينية والمناعية الفلورية، مرونة في الحساسية والنوعية بناءً على أهداف البحث أو التشخيص. بعد التلوين، تُفحص الأنسجة تحت المجهر لتقييم توزيع وكثافة التعبير عن المستضد. تُفسَّر النتائج عادةً باستخدام نظام تسجيل شبه كمي مُركَّب، يجمع بين شدة التلوين ونسبة الخلايا الإيجابية في درجة واحدة، مما يُتيح تقييمًا موضوعيًا وموحدًا. يُقدّم الكيمياء المناعية النسيجيةفوائد عديدة، مثل الخصوصية العالية، والقدرة على اكتشاف البروتينات ضمن سياقها النسيجي، والتوافق مع الأنسجة المُثبَّتة بالفورمالين والمُضمَّنة بالبارافين. في طب الدواجن، يُعدُّ الكيمياء المناعية النسيجيةأساسيًا للكشف عن مُسبّبات الأمراض الفيروسية والبكتيرية وتحديد مواقعها، مما يُساعد بشكل كبير في تشخيص الأمراض ومراقبتها وتقييم اللقاحات. كما يُستخدم على نطاق واسع في أبحاث موت الخلايا المبرمج، بما في ذلك الكشف عن كاسباس-3، وهو إنزيم رئيسي يُشارك في موت الخلايا المُبرمج، مما يُوفّر رؤىً حول تلف الأنسجة والاستجابات المناعية. على الرغم من مزاياه، إلا أن الكيمياء المناعية النسيجية له حدود، بما في ذلك إمكانية التلوين غير المُحدَّد، والحاجة إلى أجسام مُضادة عالية الجودة، والتباين التقني. تشمل التطورات المستقبلية في الكيمياء المناعية النسيجية للدواجن ابتكار المزيد من الكواشف المُخصَّصة للأنواع، وتطبيق التحليل الرقمي الآلي، واستخدام التلوين المُتعدِّد لتحسين دقة التشخيص ونتائج الأبحاث.

**الكلمات الدالة:** الكيمياء المناعية النسيجية، دواجن، علم الأمراض، تشخيص، مرض.