Immunohistochemistry for detection of infectious agents through antibody-antigen biomarker interaction in formalin-fixed paraffin-embedded tissues

by

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Abstract

Immunohistochemical detection of antigen distribution within infected formalin-fixed paraffin-embedded (FFPE) tissues is an extremely specific and complex procedure that is still relatively new in diagnostic pathology. New and improved techniques that aid to improve the sensitivity and specificity of an immunohistochemical (IHC) test are required for a more accurate etiologic diagnosis. The objective of this research study was to evaluate IHC protocol development techniques and procedures on FFPE biological tissues to improve biomolecular antibody-antigen detection using chromogenic enzymes. This study provides evidence suggesting that multiple factors play a significant role on the outcome of an accurate IHC test; pH and purified antibody concentration being among the most prominent. In addition, diminishing testing development associated costs, while reducing diagnostic turn-around time is attainable through a standardized approach to the protocol development process, resulting in efficient and accurate IHC detection of infectious agents in tissue sections.

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Introduction

The efficacy of IHC as a diagnostic tool is determined by the accurate protocol development according to the antigen characteristics of the infectious agent. There is no uniformity to IHC testing, each test must be specifically designed based on the characteristics of the identifying agent. The protocol process is one of the major pitfalls when utilizing IHC as a tool for diagnosis, it can be time consuming and costly while producing false positive results due to multifaceted steps and insufficient reference material pertaining to the subject.

New and improved IHC development standardization techniques may eliminate superfluous steps in the development process, reducing cost and diagnosis time while increasing overall reliability of IHC as a diagnostic tool. Biomolecular detection is a complex and sensitive technique that requires the knowledge of a trained pathologist to interpret morphology of biological tissues in association with an infectious agent or a disease. IHC interpretation is qualitative by nature and greatly dependent on the subjective analysis of pathological etiology and agent characteristics, resulting in associated vulnerabilities to false interpretation which is exacerbated by inadequate IHC staining quality. Ensuring quality and efficiency of IHC staining as an informative diagnostic tool, comprises solely on the protocol development process. Diminishing background staining due to non-specific binding of primary and secondary antibodies, while improving signal amplification at antibody-antigen binding motifs are crucial for reducing inaccurate interpretation by pathologists. Improved IHC development methods will also aid in dual biomarker immunolabelling, which provides additional information on biomarker co-localization expression patterns within tissues matrices.

Chapter 1 - Literature Review

1.1 IHC staining

Immunohistochemistry (IHC) is a laboratory method that utilizes antibodies to detect and bind to antigens within biologically preserved tissues that have undergone a fixation process to immobilize protein structures. The antibody-antigen interaction is microscopically visualized through a colorimetric reaction of a chromogen substrate and an enzyme-labeled catalyst, producing a color precipitate at the location of a biomolecular target, displaying distribution and/or localization expression patterns of protein antigens in sample tissues (Abcam, 2019).

The two methods of IHC staining are characterized as direct and indirect. A direct IHC method is based on an enzyme-conjugated primary antibody and is generally utilized when biomarker antigen expression is pre-determined as abundant (Abcam, 2019). The indirect IHC method is the most common technique of IHC agent detection as it associates enhanced signal expression with low antigen load, making it indispensable in the detection of unknown or novel biomarkers within biological tissues. Indirect IHC detection is based off an initial un-labelled primary antibody that binds to a target antigen and signal amplification is enhanced with a secondary labelled antibody that must be raised against the host species of the un-labelled primary antibody, focusing on immunoglobulin isotype and if available, subtype (Abcam, 2019).

Pathological diagnosis is established through predetermined knowledge of the location of a target molecule and their immunoreactivity pattern. From this an investigator is able to determine if the microanatomic and cellular distribution of an antigen is a match for a disease (Matos, Trufelli, & Pinhal, 2010).

IHC is used to detect infectious agents as well as cancer specific tumor antigens, through specificity and sensitivity of monoclonal and polyclonal antibody-antigen interactions. This

demonstrates the effectiveness of IHC as a reliable laboratory diagnostic method for determining the etiology of a disease, cytogenesis of a neoplasm or elucidating the pathogenesis of a disease process (Ramos-Vara et al., 2008).

The first IHC stain was developed in 1941. However, the field of IHC is in continuous development due to novel and emerging infectious diseases and/or mutations within the genome of established diseases. In recent years, its application is extended for a number of laboratory immunohistochemical tests designed specifically for the detection of neoplastic and infectious macromolecule proteins (Ramos-Vara et al., 2008).

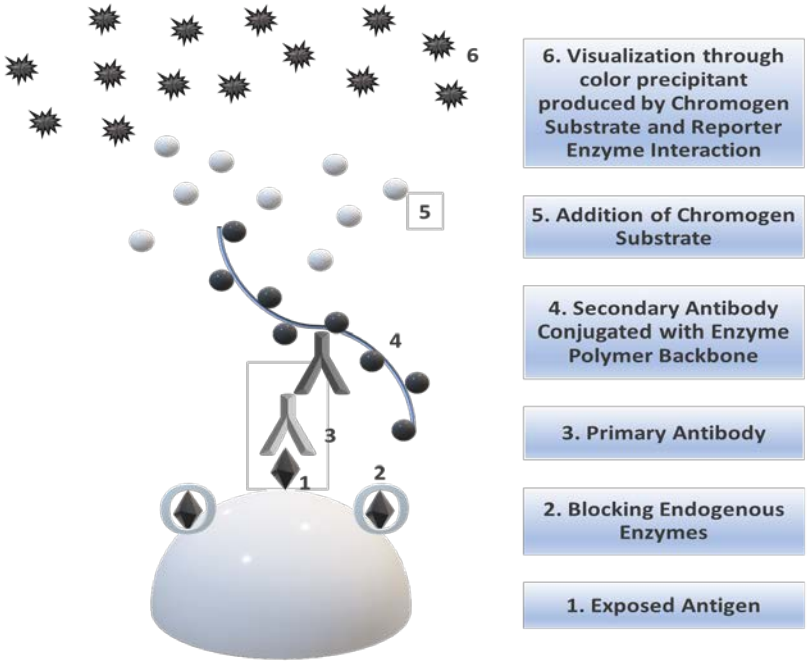

Figure 1 Biomechanical depiction of indirect IHC staining for detection of infectious agents in FFPE tissue sections.

1.2 Process and Challenges of indirect IHC

IHC staining by nature is vulnerable to multiple variables, hence the multifaceted approaches to the protocol development process. The lack of standardization addressing these drawbacks result in delayed or inconsistent experimental test results. Obtaining true-positive test results requires fundamental knowledge of the target proteins, as well as delineating foundational steps to eliminate unnecessary functions in the developmental stages of target design.

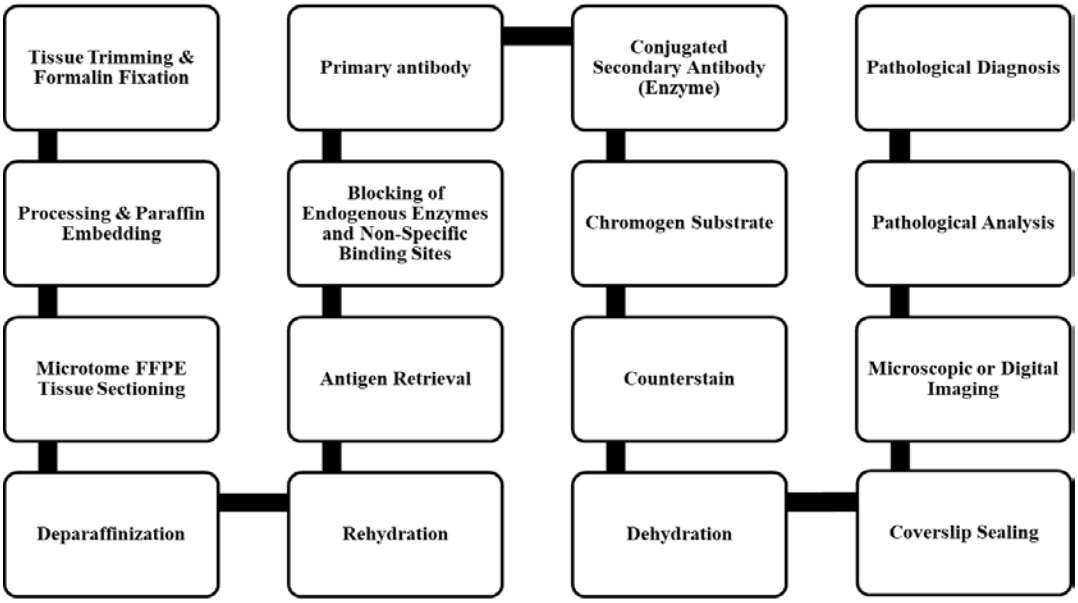

Figure 2 Essential steps in the indirect IHC staining process

Step 1 – Ensure proper fixation of sample tissues, with 24–48-hour fixation time with a sample to fixative ratio of 1:10 respectively (KSVDL, 2021). Improper fixation of tissues will produce non-specific binding from endogenous proteins while presenting possible biosafety concerns. Over-fixed tissues will make antigen retrieval difficult due to extensive protein cross-linking within the tissue matrix which occurs when exposed to a formalin-fixative, resulting in

loss of antigen detection over-time. (Scalia et al., 2017). Complete tissue infiltration of paraffin wax immobilizes the fixed tissues for sectioning on a microtome.

Step 2- Use high quality sections cut at 4 microns and thoroughly dry onto a charged slide. If the section is uneven, displays ribboning, is poorly adhered or retains water, sectioning artifacts will occur, yielding variable staining (Geoffrey, Westra, Steven, Anderson, & James 2019).

Step 3- Ensuring de-paraffinization and preparation of tissue samples is essential, if existing paraffin resides within the tissue matrix, weak to no staining will occur.

Step 4- Antigen Retrieval (AR) is one of the critical steps in ensuring antigen detection and overall IHC success, choosing the appropriate AR method for conserved epitope exposure in tissues that have undergone tissue preservation is imperative due to the hypersensitivity of the biomolecular exposure process. pH, reagent, and reaction conditions all influence the success of antigen retrieval, the most common method being Heat Induced Epitope Retrieval (HIER). The proper AR solution and its pH must be determined with consideration of appropriate temperature and incubation time.

Step 5- Blocking endogenous enzymes such as peroxidase, found primarily in liver, kidney and spleen tissues and Alkaline phosphatase (AP), typically present in kidney, intestine, lymphoid and placental tissues, is key to reducing non-specific binding, leading to a potential false-positive diagnosis (Chen, Cho, & Yang, 2010). The reason behind blocking endogenous AP and peroxidase stems from the detection step; the enzymes used as the catalysts for the color precipitation of the chromogen substrate are horseradish peroxidase (HRP) and AP.

Step 6- Primary antibody detection through direct and indirect methods, is the most crucial step in the IHC staining process, the other steps build upon the quality of the purified

antibody. The decision of monoclonal or polyclonal antibody use is determined by the abundance of infectious agents or the severity of tissue damage and target expression associated with the disease in question, in addition to the availability of commercial or in house reagents. Antibody paratope and antigen epitope binding motifs may require amplification for increased localized visualization by means of a secondary antibody. Enzyme labeled secondary antibodies bind to unlabeled primary, resulting in the amplification of the binding sites and increased detection sensitivity (Frank, 2002; ThermoFisher, 2021).

An enzymatic polymer chain conjugated to the secondary antibody can further amplify immunoreactivity at sites of primary antibody-antigen binding motifs. Due to the specificity of the polymer-based detection system, dual antigen labeling and IHC multiplex staining is achievable, reducing the final staining procedure process (Vector labs, 2021).

Step 7- Signaling binding motif sites are subsequently visualized through a chromogen substrate and enzyme probe reporter colorimetric reaction. After the reporter enzyme is added (AP or HRP) it binds to the secondary polymer, building a backbone of amplification sites that will catalyze the precipitation of chromogen resulting in a colorimetric reaction at the antibody-antigen binding site (ThermoFisher, 2021).

Step 8- Counterstain is utilized after the detection method to obtain contrast of signal and highlight chromogen deposition. An example of a commonly used counterstain is hematoxylin, a nucleic acid stain.

Step 9- Dehydrate and coverslip with mounting media for protection of stain and tissue.

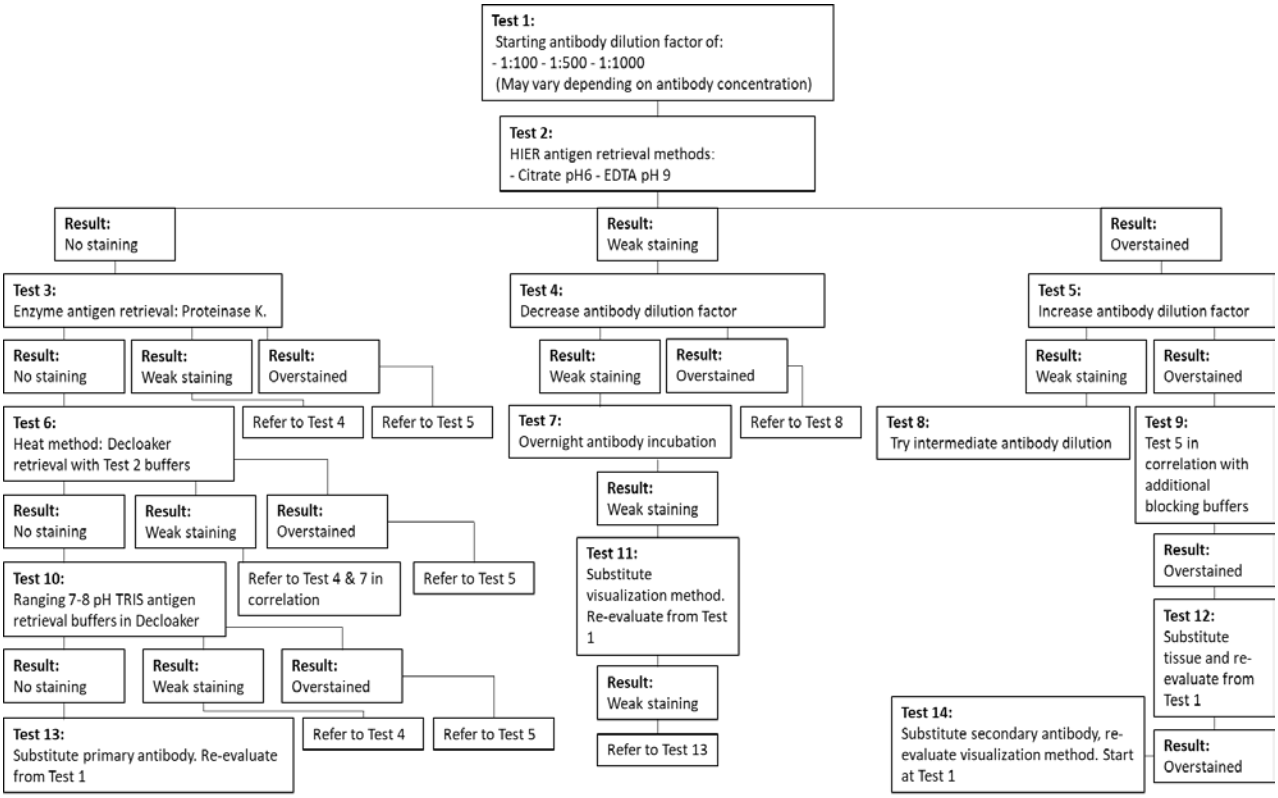

Figure 3 IHC Standardized protocol development process schematic

1.3 Dual staining vs. Single staining of tumor antigens

Immunohistochemical techniques were developed for the detection of a single target antigen, it was not until the last decade that the demand for dual staining appeared due to the correlative informational data it provided. Dual IHC staining enables visualization of co-localized biological markers in a tissue section by binding to two different target antigens, identifying multiple epitope regions. Clinical pathologists rely on accurate and timely IHC laboratory methods to evaluate molecular interaction and reach a diagnosis, hence the increase for improved IHC development techniques and tests (Dixon et al., 2015). Due to the complexity and specificity associated with dual IHC staining, fundamental protocol development and molecular biological immunoreactive knowledge is essential to success. The elimination of antibody cross-reactivity in correlation with specified reporter dyes is accomplished by

determining isotype, clonality and host species for both antibodies utilized in the dual stain (Dixon et al.,2015). The respective antibodies can be stained sequentially or simultaneously, depending on automated stainer capabilities, antibody cross-reactivity and staining pattern intensity requirements (Dixon et al., 2015).

1.4 Viral IHC detection - African Swine Fever Virus

African Swine Fever Virus (ASFV) is a highly contagious arboviral hemorrhagic disease that affects domestic and feral pigs with a high mortality rate for which there is no current vaccine, subsequently spreading throughout Africa, Asia, and Europe. ASF is considered an agriculture transboundary disease threat to the U.S. The viral pathogen is a highly stable DNA enveloped virus, remaining in infected animal by-products and the environment, presenting an additional route of exposure through the spread of fomites (Guinat et al., 2016).

Immunohistochemical detection allows for visualization of antigen distribution through infected tissues, demonstrating pathogenicity and disease characteristics deficient in other laboratory detection methods. ASFV can be detected in the cytoplasm of infected host macrophages and monocytes during late stages of viral replication by IHC (Dixon, Islam, Nash, & Reis, 2019; McCullough, 1990). Lymphoid infected tissues such as: spleen, tonsil, and lymph nodes, should take precedence when considering IHC as the viral detection tool, due to the abundance of peripheral immune cells, especially macrophages.

1.5 Bacterial IHC detection – *Ehrlichia ruminantium*

Heartwater, is a vector-borne disease caused by the obligate intracellular bacterium: Ehrlichia ruminantium. It primarily infects ruminant species throughout Africa and parts of the

Caribbean. *E. ruminantium* is classified under the order of Rickettsiales and family Anaplasmataceae (Allsopp, 2015; Dumler et al., 2001). The current prevention methods for *E. ruminantium* infectious are immunization, antibiotics, vector control and breed resistance; each method presenting separate challenges. The only current available vaccine for *E. ruminantium* is a live attenuated vaccine derived from infected sheep blood which must be cryopreserved and administered intravenously (Allsopp, 2015, Neitz & Alexander, 1945). The reliance on antibiotics as an effective treatment is likely to lead to resistance (Allsopp, 2015). Detection methods utilized for identification of *E. ruminantium* are typically molecular based due to the variable concentrations of bacterial antigens within a respective biological tissue sample, resulting in the relatively elusive identification and detection of *E. ruminantium* through immunohistochemical techniques (Jardine, Vogel, van Kleef, & van der Lugt, 1995; Brown & Skowronek, 1990). Additionally, utilizing IHC as a pathological diagnostic tool provides a testing method to remote areas deprived of laboratory facilities or tools, requiring the preservation of biological tissue samples for future diagnostics (Jardine et al., 1995).

1.6 Prion IHC detection - Chronic Wasting Disease

Transmissible spongiform encephalopathies (TSEs) encompass a spectrum of fatal neurodegenerative diseases that are caused by the hosts cellular prion proteins (PrP) which have undergone a conformational change (Kupfer, Hinrichs, & Groschup, 2009). The newly misfolded prion protein is the proteinaceous causative agent of all prion-related diseases, found in both humans and animals. Human prion disease result in Creutzfeldt–Jakob disease (CJD) and Variant CJD (vCJD) while animals are affected by Bovine Spongiform Encephalopathy (BSE) or “Mad cow disease” in bovine, Scrapie in ovine species and Chronic Wasting disease (CWD) in

Cervidae. (32 Lee, 2013) BSE is classified as a zoonotic disease while scrapie and CWD are a concern for zoonotic potential, categorizing prion diseases as reportable (USDA, APHIS, 2020). Prions are extremely stable infectious particles, allowing them to persist in a host or environment for years while remaining resistant to all forms of disinfectant, with incineration as the only confirmed method of decontamination for all known prion strains (USDA, APHIS, 2020; Saunders, Bartelt-Hunt, & Bartz, 2008). Due to limited understanding of the tertiary misfolding and pathogenesis of infectious prions, detection methods present difficulties, establishing IHC as the gold standard of CWD and Scrapie testing (USDA, APHIS, 2020). Current USDA testing standards for prion diseases require a postmortem IHC of the dorsal nucleus of the Vagus nerve located in the obex of the brainstem in addition to the follicular regions of the medial retropharyngeal lymph nodes (USDA, APHIS, 2020).

Chapter 2 – Methodology

2.1 Introduction - IHC Dual stain detection of CD3/ PAX-5

CD3 or cluster of differentiation 3, is a T cell co-receptor protein complex that aids in the activation of naïve CD8+ cytotoxic T cells and CD4+ helper T cells. (Janeway et al., 2001)

CD3 is expressed at all stages in the T cell development process, therefore establishing its importance as an IHC marker for pathological diagnosis of lymphoproliferative disorders and neoplastic tumors of T cell origin within tissue sections (Salvadori, Gansbacher, Pizzimenti, & Zier, 1994; Vernau & Moore 1999).

PAX5 is a B cell activator protein transcription factor which stimulates activation, proliferation, and differentiation of the B-lineage cell line (Jensen et al., 2007; Palmisano et al., 2003; Schafer, 1998). The PAX5 protein is specifically expressed as a nuclear marker and is detectable during the early stages of the B cell maturation process but remains undetectable in mature plasma cells. (Desouki, Post, Cherry, & Lazarchick, 2010; Adams et al., 1992; Horcher, Souabni, & Busslinger, 2001; Krenacs et al., 1998). For these reasons PAX5 is commonly used as an IHC molecular marker for not only the detection of B cell neoplasms but subclassification identification of lymphomas establishing lineage specific traits (Feldman & Dogan, 2007).

The combination of CD3 and PAX5 as a dual IHC stain for lymphoproliferative disorders has the potential to demonstrate co-localization of specific antigen expression within associated cells. The use of a dual B and T cell biomolecular target method, would provide pathologists with informative disease characteristics and in conjunction, decrease interpretation and diagnosis time, while potentially reducing costs in comparison to single target IHC tests.

2.1.1 Dual staining - Materials and Methods

Soft tissue sections from canine lymph nodes were trimmed and fixed using 10% neutral buffered formalin (NBF) and processed for immunohistochemistry using the Sakura Tissue Tek VIP 6 processor. The paraffin embedded lymphoid tissues were cut on a microtome at 4 microns and placed onto positively charged slides then left to dry overnight at 37℃ to ensure tissue adherence. Slides were stained using a Leica Bond RXm automated research stainer. Slides were deparaffinized at 72℃ for 30 seconds. Antigen retrieval was performed using an EDTA heat induced epitope retrieval (HIER) solution with a pH of 9.0 (Leica Biosystems Epitope Retrieval 2) at 100.0°C for 20 minutes followed by a 3% hydrogen peroxide block for 5 minutes. The slides were then incubated with the first antibody of the dual stain: CD3 mouse monoclonal antibody (Leica Biosystems, clone LN10) for 15 minutes at 15-25℃ followed by a polymerization step with PowerVision Poly- Horseradish Peroxidase (HRP) anti-mouse IgG conjugate (Leica Biosystems Inc., IL), for 15 minutes at room temperature. Color precipitate was developed with Diaminobenzidine (DAB) chromogenic substrate (Leica Biosystems Refine Kit) system for 10 minutes. Omission of counterstain and peroxidase steps prior to addition of subsequent PAX5 antibody. Second antigen retrieval preparation step using a Citrate HIER with a pH of 6.0 (Leica Biosystems Epitope Retrieval 1), incubated for 20 minutes at 100.0°C. PAX5 rabbit monoclonal antibody (ChampDx, clone CHMP0307) diluted 1:300 in Bond Primary Antibody Diluent (Leica Biosystems), was added to the slides and incubated for 15 minutes at room temperature. Polymerized with PowerVision Poly- Alkaline Phosphatase (AP) anti-rabbit IgG conjugate (Leica Biosystems Inc., IL), for 15 minutes at room temperature. Visualization through Fast Red chromogenic substrate (Leica Biosystems Red Refine Kit) for 15 minutes and counterstained with Hematoxylin.

For comparison, the same protocol was carried out, altering the antibody sequence only to determine the effects, if any, that DAB and Fast Red demonstrate when placed over one another. Additional single marker CD3 and PAX5 comparison control stains were run simultaneously for pathological baseline verification of the dual staining. Slides were digitally scanned at 40x magnification using Aperio AT2 digital scanner and uploaded with eSlide Manager (Leica Biosystems Inc., IL).

2.2 Viral - African Swine Fever Virus – Materials and Methods

PCR confirmed ASFV positive lung samples in addition to experimental splenic tissues were fixed in 10% NBF, processed with paraffin, embedded, and cut at 4 microns. The tissue sections were then placed on positively charged slides and incubated at 37°C until completely dry. Staining of the slides were conducted on a Leica Bond RXm automated research stainer. Slides were deparaffinized at 72°C for 30 seconds. Antigen retrieval was performed using an EDTA HIER solution with a pH of 9.0 (Leica Biosystems Epitope Retrieval 1) at 100.0°C for 20 minutes followed by a 3% hydrogen peroxide block for 5 minutes. The slides were then incubated with primary mouse anti-ASFV p30 monoclonal antibody at a 1:100 dilution for 15 minutes at room temperature followed by a polymerization step with PowerVision Poly-AP anti-mouse IgG conjugate (Leica Biosystems Inc., IL), for 15 minutes at room temperature. Color precipitate was developed through Fast Red chromogenic substrate (Leica Red Refine Kit) for 15 minutes and counterstained with Hematoxylin.

The protocol was replicated using a 1:300 dilution to determine background or endogenous antibody binding for pathological interpretation. Additionally, biological and reagent negative controls were conducted simultaneously for viral staining characteristic

verification. Slides were digitally scanned at 40x magnification using Aperio AT2 digital scanner and uploaded with eSlide manager (Leica Biosystems Inc., IL).

2.3 Bacterial - *Ehrlichia ruminantium* – Materials and Methods

Experimental lung tissue samples from sheep were processed according to histological standards for immunohistochemical staining. All staining was conducted on a Leica Bond-Max automated research stainer. Slides were deparaffinized at 72°C for 30 seconds, directly followed by antigen retrieval performed with a Citrate HIER solution with a pH of 6.0 (Leica Biosystems Epitope Retrieval 1) at 100.0°C for 20 minutes followed by a 3% hydrogen peroxide block for 5 minutes. The slides were then incubated with an in-house polyclonal canine sera primary antibody for *E. ruminantium* at a 1:100 dilution for 15 minutes at room temperature followed by an anti-canine secondary-HRP conjugated antibody at a 1:500 dilution for an additional 15 minutes at room temperature. Color precipitate was developed with DAB+ chromogenic substrate (Leica Refine Kit) system for 10 minutes. A biological negative control was run in conjunction with three reagent negative controls consisting of a primary only, secondary only and a complete negative reagent. Due to the nature of in-house antibodies, additional verification was required to corroborate the immunoexpression and pathological associated etiology of the target agent.

2.4 Prion – Chronic Wasting Disease - Materials and Methods

Confirmed CWD positive obex and lymph node cervid tissues were fixed in 10% NBF, processed and paraffin embedded. Sections were taken at 5 microns, placed on positively charged slides and dried at 37°C overnight. Slides were incubated at 80°C for 15 minutes and

exposed to a series of xylene and ethanol baths for deparaffinization, once complete, the slides were placed in 88% formic acid for 5 minutes followed by a pH correcting step of Tris Buffer with a pH of 7.5 over three washes. Antigen retrieval step was conducted in a decloaker (Biocare) for 20 minutes at 120℃ at 21-25 PSI range, with a cool down period of 25 minutes. Slides were placed in bond buffer wash and staining was conducted on a Leica Bond-Max automated stainer. The slides were incubated with F99 Prion antibody (VMRD) at a 1:500 dilution with Leica IHC/ISH Super Blocker 110 reagent for 30 minutes at room temperature followed by a polymerization step with PowerVision Poly-AP anti-mouse IgG conjugate (Leica Biosystems Inc., IL), for 10 minutes at room temperature. Color precipitate was developed through Fast Red chromogenic substrate (Leica Red Refine Kit) for 15 minutes and counterstained with Hematoxylin. Comparative slides were incubated in Tris buffer with a pH of 6.5 following the inactivation step. Slides were run simultaneously to establish the effects of pH on overall staining pattern. Additionally, biological negative controls were required for pathological verification. Slides were digitally scanned at 40x magnification using Aperio AT2 digital scanner and uploaded with eSlide manager (Leica Biosystems Inc., IL).

Results

3.1 CD3-PAX5 Dual staining results

Antibody compatibility selection of the B/T-cell biomarker targets determined prior to the dual IHC development process.

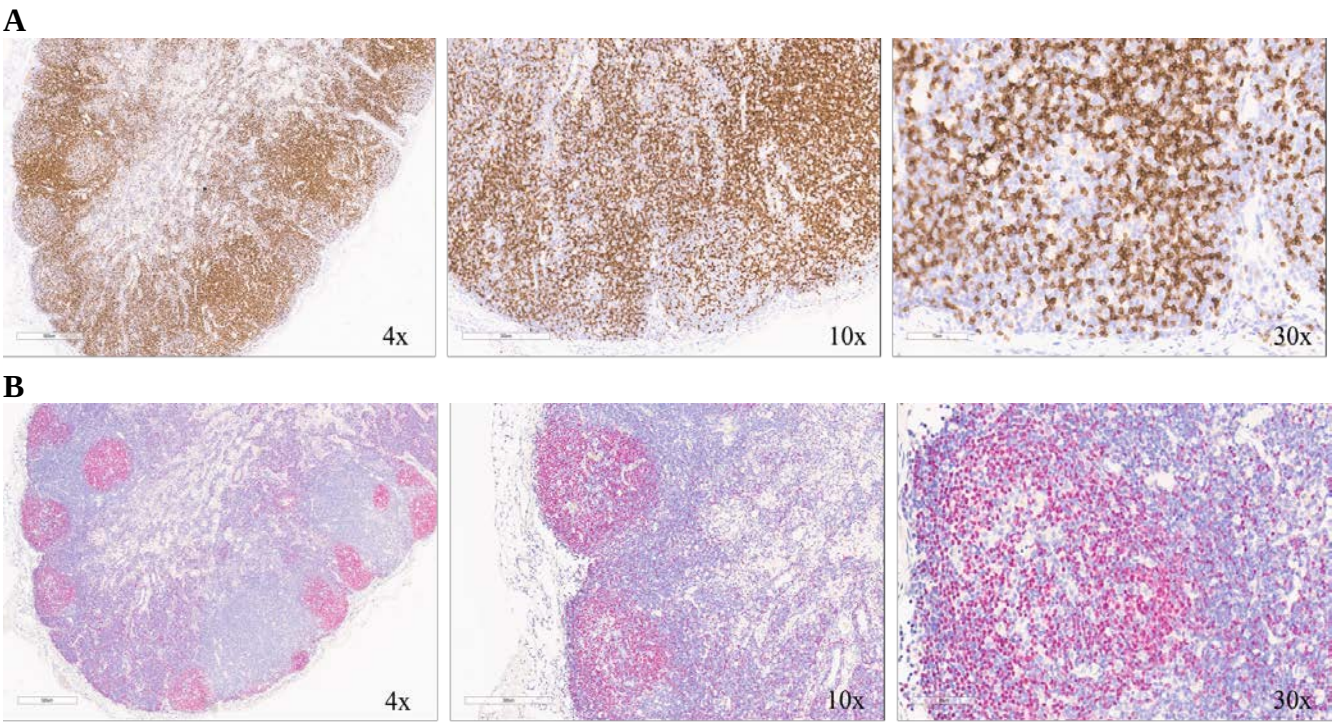

Figure 4 CD3 and PAX5 single staining IHC method on canine lymph node tissue sections.

(A) CD3 staining (T cell marker) with Leica RTU, Refine DAB/HRP, Monoclonal Mouse antibody. CD3 antibody is a RTU reagent and is formulated to be used in direct correlation with the Leica DAB/HRP kit. The CD3 cytoplasmic immunoreactivity appearance was scored as positive and negative. (B) PAX5 staining (B cell marker) on lymph node with Champ DX Monoclonal Rabbit antibody with Refine Red/Alkaline Phosphatase. The PAX5 nuclear immunoreactivity appearance was scored as positive and negative.

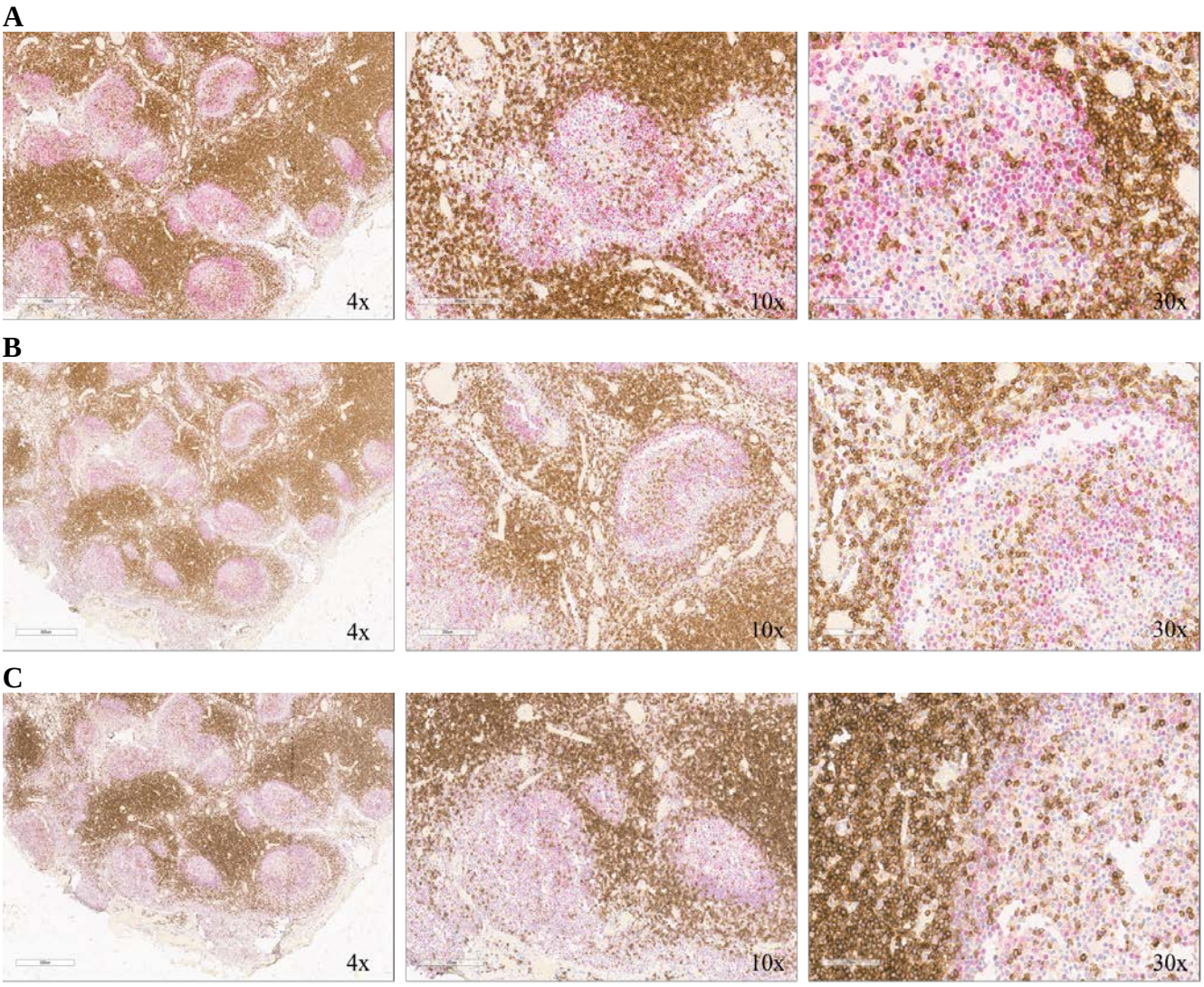

Figure 5 : CD3 PAX5 Dual sequential staining IHC method on canine lymph node tissue sections.

(A) CD3-HRP staining followed by PAX5-AP (20 minutes HIER incubation method set for initial and secondary staining, respectively. (B) CD3-HRP staining followed by PAX5-AP with reduced initial HIER incubation (10 minutes HIER incubation method set for initial CD3 staining with maintained 20 minutes HIER incubation of PAX5. (C) Order of sequential staining altered, PAX5-AP staining followed by CD3-HRP (20 minutes HIER incubation method set for initial and secondary staining, respectively).

3.2 ASFV staining results

Positive ASFV was detected in both lung and splenic tissues, viral antigen characteristics demonstrated peak signal to noise ratio at a primary antibody dilution factor of 1:300 in lung tissue and 1:100 in splenic tissue.

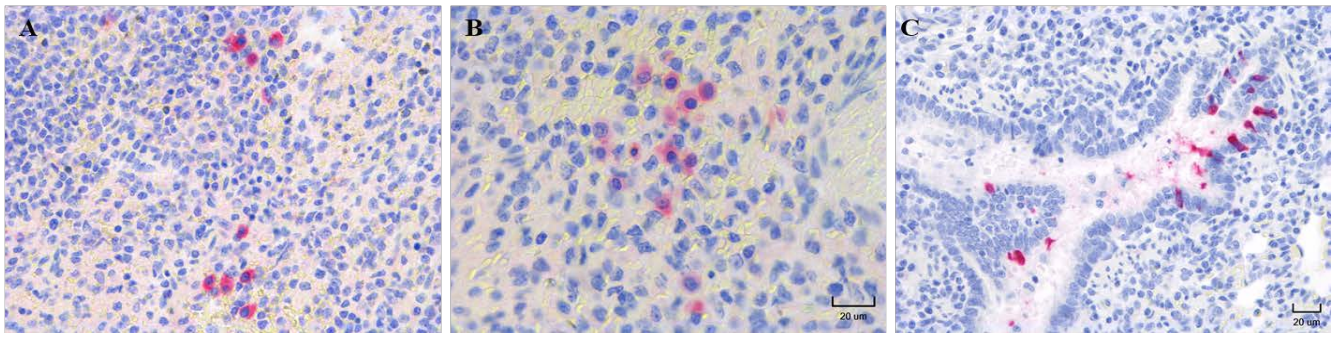

Figure 6 : IHC staining of ASFV in porcine spleen and lung tissues utilizing p30 monoclonal antibody in conjunction with AP/Fast Red for visualization.

(A) Experimental splenic tissue, p30 antibody dilution factor of 1:100 visualized with AP/Fast red (B) Experimental splenic tissue, p30 antibody dilution factor of 1:100 visualized with AP/Fast red. (C) Lung tissue utilized as development control, p30 antibody dilution factor of 1:300 visualized with AP/Fast red.

3.3 *Ehrlichia ruminantium* staining results

Targeted staining detected in lung sections eliminated as *E. ruminantium* based reagent control and bacterial characteristics. Blocking of endogenous peroxidases was pursued to determine cause of staining.

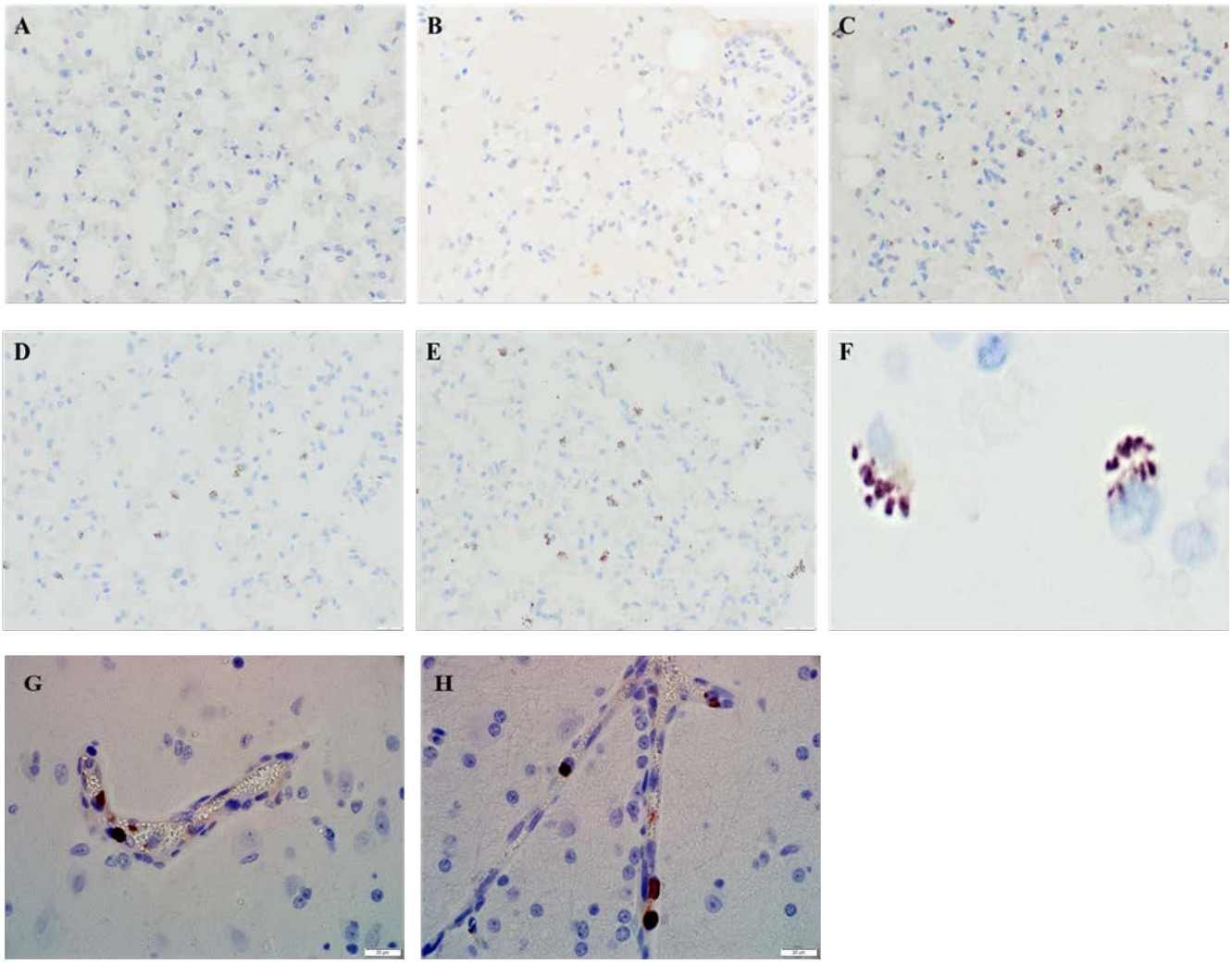

Figure 7 : E. ruminantium IHC staining in sheep lung tissue, visualized with DAB/HRP.

(A) 1:500 dilution, pre-treatment of Proteinase K. (B) 1:500 dilution with HIER pre-treatment with EDTA pH (9). (C) 1:500 dilution with HIER pre-treatment with Citrate pH (6). (D) Reagent negative control with HIER pre-treatment with Citrate pH (6). (E) Peroxidase blocking step prior to addition of chromogen. (F) Non-specific staining (G) & (H) Positive IHC staining of *E.*

ruminantium in sheep brain tissue, (Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria).

3.4 Prion- Chromic Wasting Disease staining results

Positive CWD staining was demonstrated in both the obex and submandibular lymphoid follicles as shown in Figures 8-9. The positive staining intensity decreased when exposed to a lower pH level of Tris buffer following a formic acid inactivation step.

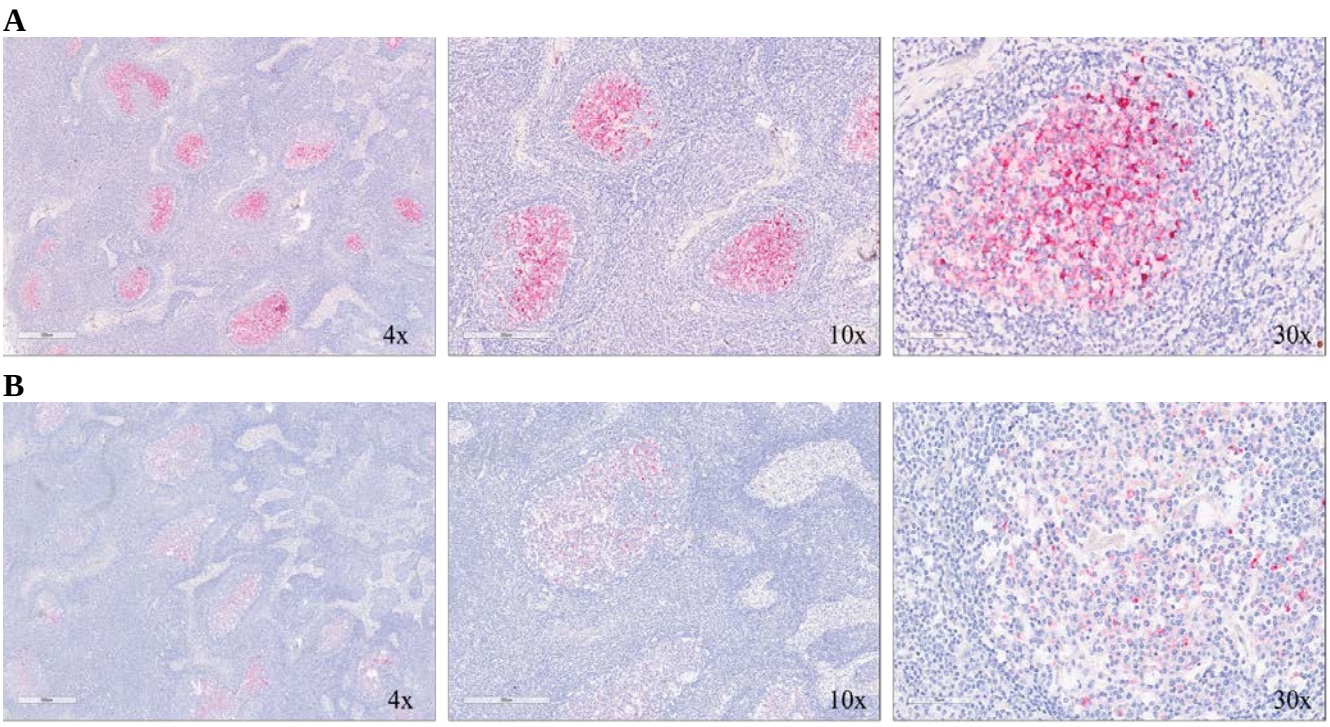

Figure 8 CWD prion IHC staining in cervid submandibular lymphoid tissue with F99 antibody and AP/Fast Red for visualization.

(A) Strong CWD positive staining visualized in lymphoid follicles utilizing Tris Buffer with a pH of 7.5 in staining process. (B) Visibly diminished CWD positive staining in lymphoid follicles utilizing Tris buffer with a pH of 6.5 in staining process.

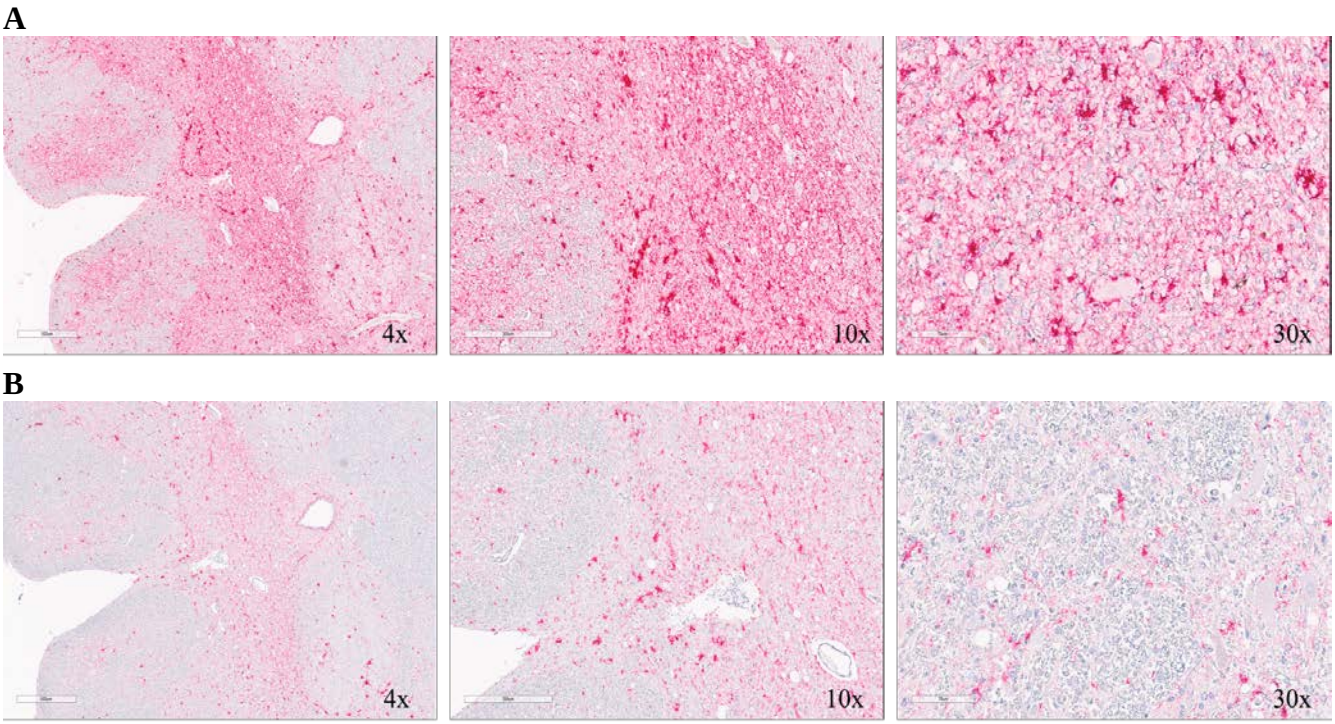

Figure 9 CWD prion IHC staining in cervid brainstem (obex) tissue with F99 antibody and AP/Fast Red for visualization.

(A) Strong CWD positive staining visualized in obex sections utilizing Tris Buffer with a pH of 7.5 in staining process. (B) Visibly diminished CWD positive staining in obex sections utilizing Tris buffer with a pH of 6.5 in staining process.

Discussion

4.1 Dual staining discussion

As shown in Figures 4-5, CD3 and PAX5 demonstrate their effectiveness as comparative T and B cell compatible biomarkers, respectively, through robust and distinctive staining patterns, providing additional pathological information while minimizing interpretive analysis. Chromogenic staining intensity exhibited modifiable expression through antigen retrieval incubation periods, as demonstrated in Figure 5 (B). Due to DAB's superior insolubility, it was chosen as the first antigen biomarker target. Histological comparative analysis demonstrated strong independent CD3 and PAX5 staining in comparison to Fast Red as the initial target identifier in the dual detection process. Dual staining provided co-localized B and T-cell distribution patterns as well as comprehensive cell lineage insight within the tissue matrix, absent in single target use, illustrating multifunctional uses of dual staining. Ensuring antibody compatibility is essential to success of dual staining and perhaps the greatest challenge associated with the development process.

4.2 Viral IHC detection - African Swine Fever Virus discussion

ASFV infected porcine lung and spleen tissue sections exhibited variable viral antigen expression from differing immunological responses from host and tissue type, altering IHC primary antibody dilution. Figure 6 demonstrates true staining of ASFV viral antigens within the cytoplasm of the cell. Implementing a strategic standardized approach to the development of p30 antibody, reduced excess antibody and reagent waste during the protocol development process.

Visualization of p30 and viral antigen biomolecular binding was modified based on tissue target and subsequently the primary antibody dilution factor.

4.3 Bacterial IHC detection - *Ehrlichia ruminantium* discussion

E. ruminantium bacteria replicate within the cytoplasm of the hosts endothelial cells, predominantly in the lungs and myocardium of the infected ruminant species (Jardine, 1995). The pathological staining analysis of the experimental HRP-conjugated antibody performed on lung tissue proved to be false staining. A combination of endogenous protein expression and staining reagents utilized, produced non-specific DAB binding visualization deviating from characteristic *E. ruminantium* bacterial pathogenesis, as demonstrated in Figure 7. The biological and reagent negative controls ran simultaneously, exhibited comparable punctate staining as demonstrated in the experimental lung sections, augmenting the false-positive diagnostic analysis. The *E. ruminantium* experimental antibody development illustrates the importance of collaborating with a diagnostic pathologist in addition to incorporating appropriate developmental controls to ensure the validity of an IHC tests.

4.4 Prion IHC detection - Chronic Wasting Disease discussion

Infectious prion proteins were detected in obex and lymphoid tissue sections of naturally infected cervids as shown in Figures 8-9. IHC testing is sensitive with an exorbitant number of factors influencing staining outcome, directly affecting the qualitative analysis should target staining expression diminish, resulting in potential misdiagnoses. Pre-determined steps critical to the staining process could improve identification of causation for weakened staining; pH of reagents involved in antigen retrieval of sequestered epitopes being among the most prominent in CWD staining. CWD has an extended incubation period and animals in the early stages of the

disease may exhibit low levels of infectious prions. Weakened IHC staining in correlation with low infectious biomolecular expression levels increases the probability of a misdiagnosis by the pathologist. Obtaining a strong antibody-antigen binding signal with clean background staining is a continuous balancing act for all IHC developmental and subsequent tests, particularly regarding prion testing. The conformational protein folding patterns of infectious prions present additional challenges requiring subsequent protocol steps for successful staining. Improved quality of overall staining can be achieved by knowledge of critical sensitive steps and development of strategic approaches to the IHC testing development process.

Conclusion

IHC has established itself as a reliable and efficient diagnostic tool with exemplary specificity in identification of infectious biomolecular expression and cell types in FFPE tissue sections. Relying on biomolecular manipulation, IHC is sensitive to a multitude of factors, presenting a challenge with respect to development of new and improved IHC tests. The implementation of a strategic standardized approach for IHC protocol development is essential to diminishing protocol development associated costs and diagnosis turnaround time while providing reproducible results.

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Appendix A - Supplemental Funding Information

A.1 Project funding supplemental information

A.1.1 Support and materials for CD3-PAX5 dual staining development project was provided through Kansas State Veterinary Diagnostic Laboratory (KSVDL).

A.1.2 Support and materials for ASFV p30 antibody development project was provided by Dr. Giselle Cino through Kansas State University, Department of Diagnostic Medicine/Pathobiology (DMP).

A.1.3 Support and materials for *Ehrlichia ruminantium* antibody development project was provided by Dr. Charan Ganta through Kansas State University, Department of Diagnostic Medicine/Pathobiology (DMP).

A.1.4 Support and materials for CWD staining comparison was provided by Shane Hesting at Kansas Department of Wildlife and Parks (KDWP) and KSVDL.

Glossary of Terms

IHC- Immunohistochemistry

FFPE- Fixed-formalin paraffin-embedded

HIER- Heat Induced Epitope Retrieval

HRP- Horseradish Peroxidase

AP- Alkaline Phosphatase

ASFV- African Swine Fever Virus

TSE's- Transmissible spongiform encephalopathies

CWD- Chronic Wasting Disease

PrP- Prion protein

CJD- Creutzfeldt–Jakob disease

vCJD- Variant CJD

BSE- Bovine Spongiform Encephalopathy

NBF- Neutral buffered formalin

EDTA- Ethylenediaminetetraacetic acid

DAB- Diaminobenzidine