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An overview of the detection of bovine respiratory disease complex pathogens using immunohistochemistry: emerging trends and opportunities

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Abstract. The bovine respiratory disease complex (BRDC) is caused by a variety of pathogens, as well as contributing environmental and host-related risk factors. BRDC is the costliest disease for feedlot cattle globally. Immunohistochemistry (IHC) is a valuable tool for enhancing our understanding of BRDC given its specificity, sensitivity, cost-effectiveness, and capacity to provide information on antigen localization and immune response. Emerging trends in IHC include the use of multiplex IHC for the detection of coinfections, the use of digital imaging and automation, improved detection systems using enhanced fluorescent dyes, and the integration of IHC with spatial transcriptomics. Overall, identifying biomarkers for early detection, utilizing high-throughput IHC for large-scale studies, developing standardized protocols and reagents, and integrating IHC with other technologies are some of the opportunities to enhance the accuracy and applicability of IHC. We summarize here the various techniques and protocols used in IHC and highlight their current and potential role in BRDC research.

Keywords: antigens; cattle; diagnosis; immunohistochemistry; lung.

Bovine respiratory disease complex (BRDC) is a multifactorial and multi-etiologic syndrome that causes significant economic losses in beef and dairy cattle operations. The syndrome is caused by bacterial and viral pathogens, and is facilitated by environmental and host factors (Fig. 1). BRDC can cause decreased weight gain, productivity, and reproductive efficiency, alongside increases in treatment-related costs and changes in feed conversion ratio.[88,97] BRDC can be caused by a range of viral pathogens such as bovine adenovirus (BAV-1, -3, -7, -10), bovine alphaherpesvirus 1 (BoHV-1; *Varicellovirus bovinealpha1*; syn. infectious bovine rhinotracheitis virus,), bovine coronavirus (BoCoV; *Betacoronavirus 1*), bovine parainfluenza virus 3 (BPIV-3; *Respirovirus bovis*), bovine respiratory syncytial virus (BRSV; *Orthopneumovirus bovis*), bovine viral diarrhea virus 1 (BVDV-1; *Pestivirus bovis*), and influenza D virus (IDV; *Deltainfluenzavirus influenzae*), as well as bacterial pathogens, including *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida*.[39,44,113]

Clinical signs and outcomes of BRDC infection depend on the type of risk factor involved, and require multiple assay-based detection methods to identify the causative agent.[116] Although nucleic acid–based detection and antigen ELISAs are affordable and scalable with fast throughput, they do not provide antigen localization or indicate disease severity. A comparison of the different BRDC etiology detection methods was published in 2012.[38]

Immunohistochemistry (IHC) serves an important role as a complementary detection method, facilitating the discernment of etiologic antigens as opposed to incidental ones. Furthermore, IHC permits quantification and spatial localization of the antigen within lesions. These elements are vital to population-scale epidemiologic surveillance and formulation of preventive strategies.[4,116] The distribution and localization of the infectious agent in tissues can be observed by combining IHC with other tests, which adds new information about the scope and severity of BRDC. Hence, we summarize here the various techniques and protocols used in IHC and highlight their current and potential future roles in diagnosing BRDC.

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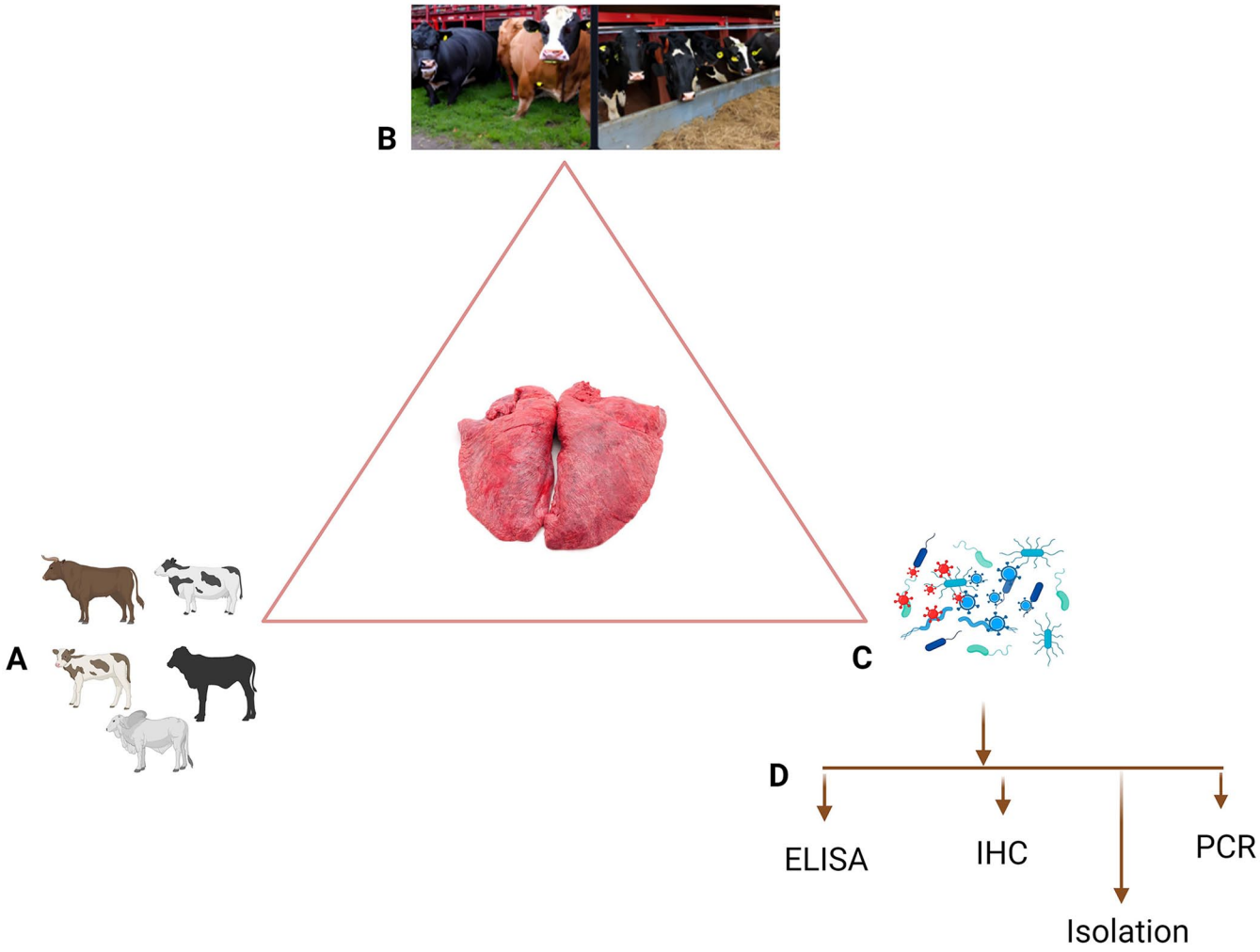

Figure 1. Bovine respiratory disease complex (BRDC) results from an imbalance among host factors, pathogen exposure, and environmental factors. **A.** Host factors, such as age, genetics and/or epigenetics, and immune status of cattle, influence susceptibility to infection. **B.** Environmental factors, such as stress caused by transportation, poor housing conditions, overcrowding, and rapid change in nutrition, compromise the immune system and predispose cattle to BRDC pathogens. **C.** Pathogens, including bacteria and viruses, infect the respiratory tract and induce inflammation. **D.** Detection of BRDC pathogens typically involves antigen-detection techniques, such as antigen ELISA and immunohistochemistry (IHC), nucleic acid–detection methods, such as PCR assay, and/or pathogen isolation.

Principles and procedures of immunohistochemistry

IHC is a complex multiparameter technique that combines histology, immunology, and chemistry to identify and localize specific antigens within tissue.[73] Typically, the process of IHC involves tissue fixation, antigen retrieval, the addition of antigen-specific antibodies, visualization via microscopy, and interpretation. Briefly, tissue samples are fixed, sectioned, and placed on slides, then treated to unmask the antigen. Primary antibodies that bind to the specific antigen are applied, followed by secondary antibodies with chromogenic or fluorescent tags. Tissue sections are examined under a microscope to visualize the signal.[76,106,109,117,122] To ensure assay sensitivity and specificity, IHC tests must be validated in the subject species.[35,41] IHC has evolved from a basic one-step procedure into more sensitive multistep methods such as the avidin-biotin complex (ABC), peroxidase-antiperoxidase (PAP), biotin-streptavidin (BSA) methods,[76] and tyramide signal amplification (TSA) resulting in significant signal amplification.[6] As well, the introduction of robotic automation and advanced detection chemistry has significantly improved the analysis of large sample sets, enhancing throughput, reproducibility, and analytical efficiency.

Tissue preparation

Tissue preparation is one of the most important steps for successful IHC assay development given that the integrity and antigenicity of the target protein must be retained.

Tissue preparation steps include fixation, dehydration, embedding, sectioning, and mounting.[14] The 2 main methodologies involve either formalin-fixed, paraffin-embedded (FFPE) or fresh-frozen tissue. The use of FFPE tissue is the most practical given its relative simplicity and ability to maintain the ultrastructure of the tissue, while also enabling long-term archival storage. However, prolonged contact with heat can readily modify the proteins within the tissue, resulting in alteration of the conformational epitopes of interest and therefore masking antigens and hindering their detection with antibodies.[45,76] In contrast, fresh-frozen tissue, as a result of ice crystal formation during freezing, can be challenging to section and mount onto slides, plus the ultrastructure of tissue may be altered significantly.[4] However, because this method avoids heat treatment or exposure to formalin, the native conformation of the proteins is preserved, enabling antibody detection without subsequent antigen retrieval.

Dehydration, paraffin-embedding, elevated temperature, and fixation onto microscopic slides can all lead to the loss of antigen reactivity.[13,46] Therefore, it is crucial to optimize protocols for fixing each tissue type and antigen of interest, taking into account factors such as histopathology requirements, antigen stability, and storage needs.

Antigen retrieval

The choice of antigen retrieval, or epitope retrieval (ER), depends on the target antigen, tissue type, and staining technique. Heat-induced ER (HIER)[121] and enzyme-induced ER (EIER)[54] are the two most common ER strategies used in IHC. HIER utilizes high temperatures; EIER uses proteolytic enzymes, such as trypsin, to denature and expose antigens. EIER requires tissue-specific optimization processes to ensure minimal structural damage to the tissue or epitope.[20] HIER is the most widely utilized ER method, given that it is simple, effective, and maintains tissue ultrastructure; however, it is not suitable for antigens that contain labile epitopes that could be destroyed during heating.[94,121] HIER depends on the pH and ionic strength of the solutions used[32,92]; most antigens can be retrieved within a pH range of 8–9. However, certain nuclear antigens have enhanced staining in lower pH conditions,[92] highlighting the importance of tailoring ER based on the properties of the antigen of interest to prevent tissue degradation or false-negative results.

The optimal ER strategy depends on the type of antigen and tissue, and the test-battery approach is often used to determine the most suitable method.[93] To identify the optimal method for a specific antigen within a particular tissue, the test-battery approach involves applying multiple ER techniques in sequence to the same tissue section and examining them under a microscope. This approach can improve the sensitivity and specificity of the assay and is used when the antigen of interest is difficult to detect using a single ER technique.[29,93]

Antigen detection using specific primary antibodies

When using IHC to detect antigens, cross-reactivity with other proteins must be avoided. It is therefore crucial to select primary antibodies with high specificity and affinity for the antigen of interest. The antibody must also be of high purity with minimal cross-reactivity while maintaining host species compatibility.[81,117] In a multifactorial disease such as BRDC, there may be genetically similar pathogens as well as commensal organisms within a tissue sample. Thus, well-characterized and validated antibodies that are optimal for the intended application must be used, along with appropriate controls.

Signal amplification and visualization

The specificity and sensitivity of an assay can be enhanced by amplifying the signal generated during antibody–antigen binding with enzymes or other molecules that catalyze a reaction to produce a measurable signal. Signal amplification techniques, such as PAP,[100] ABC,[47] BSA,[37] TSA,[6] fluorescent IHC (FIHC),[62,106] and gold or silver nanoparticles,[25] are commonly utilized in IHC.

The PAP method is based on the use of a peroxidase enzyme to catalyze the formation of a colored product from a substrate (Fig. 2A).[100] For increased sensitivity and specificity, attempts have been made to enhance the PAP technique by incorporating alkaline phosphatase, resulting in a double-labeling approach using both alkaline phosphatase and peroxidase.[69]

In the ABC method, ABC molecules bind to both the biotinylated secondary antibody and each other through the biotinylated enzyme, such as horseradish peroxidase, which improves antigen detection (Fig. 2B).[47,53] This amplification technique has proven to be more sensitive and specific than the PAP method.[30,47,53] Alternatively, streptavidin can be used for amplification instead of avidin.[37]

FIHC uses fluorescently labeled antibodies to detect antigens with higher sensitivity and greater signal amplification compared to traditional chromogenic IHC (Fig. 2C).[62,106] This method is based on the principle of fluorescence, which produces a bright signal where the antigen is present and enables multiplexing.

TSA uses a tyramide molecule labeled with a reporter molecule to bind to proteins at the site of HRP and produce a highly visible signal (Fig. 2D, 2E).[6] Compared to ABC, TSA enables sensitive detection of antigens and multiplexing.[99]

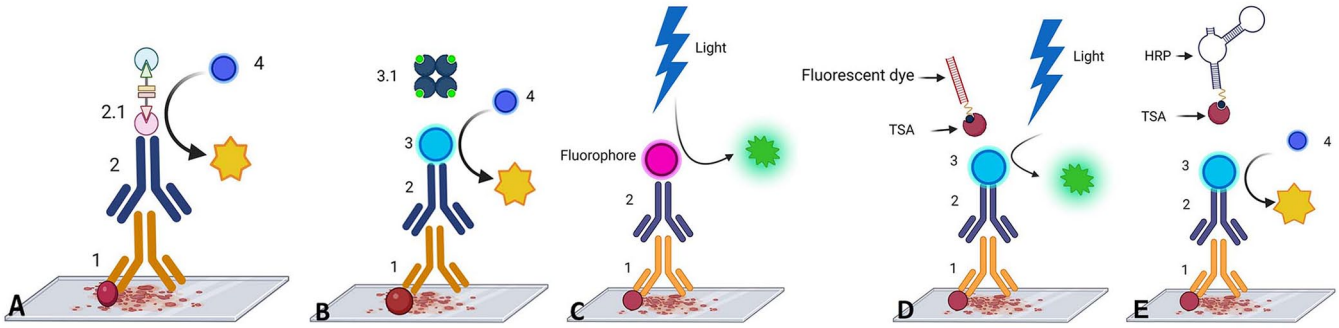

Figure 2. Signal amplification and visualization methods used in immunohistochemistry (IHC). **A.** Peroxidase–antiperoxidase (PAP). **B.** Avidin–biotin complex (ABC) or biotin–streptavidin (BSA) methods. **C.** Fluorescent IHC (FIHC). **D.** Tyramide signal amplification (TSA) method using fluorescent dye. **E.** TSA method using horseradish peroxidase (HRP). 1 = primary antibody; 2 = secondary antibody; 2.1 = secondary antibody conjugated to PAP complex; 3 = biotin, 3.1 = avidin(streptavidin)–biotin conjugated with an enzyme such as peroxidase; 4 = substrates such as diaminobenzidine (DAB) used to visualize antigens in tissue samples after signal amplification.

Gold or silver nanoparticle–labeled secondary antibodies are utilized mostly for scanning electron microscopy IHC.[105]

Immunohistochemistry of BRDC pathogens

In the presence of pathogens, several complex and usually hard-to-control environmental and host factors predispose cattle to the development of BRDC (Fig. 1; Table 1).[96]

IHC has the potential to play a vital role in detecting the most important agents responsible for subclinical BRDC, particularly in identifying the presence and distribution of various pathogens within the lung tissues of affected cattle.[17,95,115] However, the application of IHC for BRDC is not without obstacles. For example, BRDC pathogens can cause localized foci within the lung, meaning the animal must be euthanized to obtain the sample. Similarly, samples obtained by biopsy may only contain healthy tissue resulting in false-negative IHC staining. Cross-reactivity between antigens, particularly when distinguishing closely related bacterial etiologies of BRD, such as those within the *Pasteurellaceae* family (Table 1), can lead to false-positive results. Despite these inherent complexities, IHC remains an important tool in BRDC studies given its ability to detect and visualize the distribution of pathogens within tissues, including the localization of inflammatory cells and other markers.[17,56,95,115]

Viruses and members of the *Mycoplasmataceae* family are often considered primary causes of BRDC, with other bacterial species typically acting as secondary invaders. Most of these bacteria are gram-negative commensal organisms that infiltrate the respiratory system via inhalation or breaches in the mucosal barrier.[79] Once established in the bovine upper respiratory tract, these bacteria proliferate, instigate tissue inflammation, and ultimately lead to pneumonia. This progression can manifest as a spectrum of clinical signs, ranging from subclinical to severe. The interaction dynamics among pathogens impact the severity of BRDC cases.[19,23] Coinfections may have either synergistic or antagonistic effects, depending on the involved pathogens and the host's immune response.[23,122] For example, in vitro studies have demonstrated that the replication of BoHV-1 is adversely affected by *M. haemolytica*.[19] Furthermore, *Bibersteinia trehalosi* (formerly *Pasteurella trehalose*) was found to inhibit the growth of *M. haemolytica*,[23] underlining the intricate nature of BRDC pathogenesis. The roles of most of the bacterial agents in BRDC infections have been discussed in detail elsewhere.[7,18,79] IHC has been used to identify and localize the pathogens of BRDC in tissue samples and are therefore described here.

Immunohistochemical investigation of bacterial causes of BRDC

Histophilus somni

IHC has shown that the level of *H. somni* colonization in lung tissue specimens from cattle is related to the severity of BRDC, even in mixed infections.[77] *H. somni* antigens have been detected in the lungs of calves[40] and in myocardial capillaries, venules, and small veins of the heart in cattle.[68] Unlike other BRDC bacteria, *H. somni* does not seem to have developed resistance to commonly used antimicrobials,[2,42] at least in Australia, making it difficult to culture the organism from cattle that had been treated with antimicrobials before sample collection. In such cases, IHC is a preferred tool for the detection of *H. somni* in tissues from treated cattle, given that it allows the detection of residual antigens within tissue samples,[11,107] even if the bacteria are dead.

H. somni was detected in association with suppurative and chronic suppurative bronchopneumonia.[7] Similarly, in *H. somni*–infected lung tissues, IHC was utilized to visualize and locate bacteria in endothelial cells,[24] neutrophils, and macrophages.[5] The use of IHC to determine the presence of

Table 1. Infectious etiologies and their role in the bovine respiratory disease complex (BRDC).

Pathogen	Family	Description	Reference
Bibersteinia trehalose *Histophilus somni* *Mannheimia haemolytica* *Pasteurella multocida*	*Pasteurellaceae*	Commensals of the upper respiratory tract and opportunistic pathogens in immunocompromised cattle—secondary cause of BRDC.	[39]
Mycoplasma sp.	*Mycoplasmataceae*	Suspected commensal of the upper respiratory tract and opportunistic pathogen—primary cause of BRDC.	[44, 80]
Bovine adenovirus	*Adenoviridae*	Primary cause of BRDC.	[39, 113]
Bovine alphaherpesvirus 1	*Orthoherpesviridae*		
Bovine coronavirus	*Coronaviridae*		
Bovine parainfluenza virus 3	*Pneumoviridae*		
Bovine respiratory syncytial virus	*Pneumoviridae*		
Bovine viral diarrhea virus	*Flaviviridae*		
Influenza D virus	*Orthomyxoviridae*		

H. somni in the respiratory tract of cattle with BRDC has been crucial for assessing the interaction between *H. somni* and host cells and for understanding the pathogenesis of BRDC.

Mycoplasma bovis

IHC has been used to detect *M. bovis* in tissue samples from cattle with BRDC; correlation between the severity of the disease and the level of bacterial colonization in the respiratory tract has been demonstrated. Furthermore, *M. bovis* antigens have been detected in the epithelial cells of bronchi and bronchioles, alveolar macrophages, and syncytial cells,[28,71] the alveoli of the lung,[51,83] heart tissues,[28,71] kidney and nerve tissues,[65] and hepatocytes and axons in facial nerves.[65,71] Although most often associated with pulmonary necrosis and fibrosis,[90] *M. bovis* was also found within abscessed tissues and in other areas of the lung.[1] Interestingly, abscess formation is a strain-related phenomenon,[1] and abscessed airways that have lost their epithelium become encapsulated and then develop coagulative necrotic foci that contain greater concentrations of *M. bovis* antigens.[1]

M. bovis–induced pulmonary lesions can be classified as acute, subacute, or chronic based on histopathologic characteristics, inflammatory response type, and chronicity markers such as fibrosis.[90] Acute lesions are characterized by intact bronchiolar epithelium and hypereosinophilic exudate. Subacute lesions have focal-to-multifocal loss of bronchiolar epithelium. Chronic lesions have coagulative or liquefactive necrosis with eosinophilic debris.[90] *M. bovis* detection was associated with suppurative bronchopneumonia, fibrinonecrotizing pneumonia, fibrinous pleuritis, chronic suppurative pneumonia, and proliferative interstitial pneumonia, depending on lung tissue sampling location.[7] In infections involving pulmonary abscessation, *M. bovis* antigens have been found in association with coagulative necrosis and surrounding necrotic exudates at the periphery of the lumen in the lesions, as well as in close proximity to infiltrating macrophages and neutrophils.[83,90] In addition, caseonecrotic granulomatous inflammation, meningitis-meningoencephalitis, endocarditis, and bronchopneumonia have been linked to *M. bovis* antigens.[103] These findings suggest that histopathologic characteristics and IHC could be used for determining the severity of infection and detection of *M. bovis*, and can provide important diagnostic information for identifying the presence and progression of the infection.

Mannheimia haemolytica

In cattle, *M. haemolytica* antigens have been found in coagulative necrotic foci, lymphatic vessels, alveoli, bronchial epithelium, and bronchioles.[7,49,74,120] In BRDC cases, *M. haemolytica* is often found mixed with other bacteria or viruses in bovine lung tissues. Together, *M. haemolytica* and *P. multocida* antigens have been detected in various locations within lung tissue, including the cytoplasm of neutrophils and macrophages in alveolar and bronchial lumens,[48] the cytoplasm of cells in bronchial epithelium and peribronchiolar areas,[78] and the wall of necrotic alveoli, fibrin, serous exudate, and degenerate leukocytes.[49] The presence of fibrinonecrotizing pneumonia and fibrinous pleuritis was positively correlated with the detection of *M. haemolytica* in bovine lung tissues. On the other hand, there was a negative correlation between the presence of *M. haemolytica* and conditions such as chronic suppurative pneumonia, bronchiolitis obliterans, bronchiectasis, and acute interstitial pneumonia.[7] These results suggest an association between the type of lesion and the presence of M. *haemolytica* in bovine lung tissues. In goats, *M. haemolytica* antigens have been found within epithelial cells of small bronchi and bronchioles, alveoli, and pneumocytes; the pathogenesis and disease course changed in the presence of BPIV-3.[122]

Pasteurella multocida

Enhanced detection of *P. multocida* in birds[102] and experimentally infected calves[21] has been reported through the use of IHC techniques. From cattle with BRDC, a relationship between the degree of bacterial colonization in the respiratory tracts and the severity of the disease has been observed. In one study, the presence of *P. multocida* antigens in experimentally infected calves was observed both within alveolar cells and in the air spaces of the lungs. *P. multocida* antigens were detected at day 1 after infection and became more noticeable in the periphery of lung abscesses by day 10,[21] indicating a time-based increment in bacterial colonization. Similarly, in naturally infected cattle, antigens of *Pasteurella* spp. were detected within the epithelial cells of bronchi, bronchioles, and alveoli.[120] Moreover, an antiserum made from chickens was able to detect *P. multocida* antigens in rabbits,[111] indicating host diversity.

Experimental coinfection of cattle with *P. multocida* and *M. haemol*ytica or with BRSV showed different outcomes.[120] Coinfection of *Pasteurella* spp. with BRSV led to exacerbated pneumonia, exhibiting pathologic features, such as a combination of fibrinous pleuropneumonia from *Pasteurella* infection and interstitial pneumonia from BRSV infection, potentially accompanied by necrosis and hemorrhage.[120] In coinfections involving *Pasteurella* spp. and *M. haemolytica*, type II epithelial cell proliferation occurred within the alveolar walls.[120] Such coinfection-based differences in lesions may indicate the presence of interaction among the pathogens that could potentially change the course of infection and disease pathways.

Immunohistochemical detection of viral causes of BRDC

Bovine adenovirus

BAdV was identified in the lungs of cattle with respiratory disease using IHC. In naturally infected cattle, BAdV antigens have been detected in bronchiolar and alveolar epithelial cells and peribronchiolar mononuclear cells.[15] In a subsequent study of experimentally infected calves, BAdV antigens were detected in the epithelial cells of bronchioles, alveoli, and alveolar macrophages.[55,75] Additionally, BAdV antigens were observed in intranuclear inclusion bodies (IBs), alveolar epithelial cells, and the intracytoplasmic area of the bronchiolar epithelium,[15] indicating viral cellular specificity and localization during BRDC.

Bovine alphaherpesvirus 1

In cattle, in contrast to immunofluorescent and virus isolation techniques, IHC demonstrated a higher rate of BoHV-1 antigen detection.[72] BoHV-1 antigens were detected in various parts of the airways, including nasal concha and trachea, bronchial epithelium, bronchial-associated lymphoid tissue, bronchial epithelium, bronchioles, peribronchiolar inflammatory cells, bronchiolar lumen, macrophages, and interstitial regions.[17,22] In neonatal calves, foci of coagulative necrosis, of various sizes and randomly located in various organs, have been found to contain BoHV-1 antigens.[10] Similarly, experimentally infected calves had lesions such as necrotizing bronchitis, bronchiolitis, and intranuclear IBs. The degree and distribution of BoHV-1–induced pneumonic lesions become much more prominent in the presence of *M. haemolytica* coinfection,[74] suggesting that the virus is a primary causative agent of BRDC. Moreover, BoHV-1 antigen has also been detected in extrapulmonary infections.[66]

Bovine coronavirus

BoCoV antigens were detected in the bronchial epithelium of naturally infected calves.[57] In another study of experimentally infected calves, BoCoV antigens were only found in epithelial cells of the trachea but not in the lungs.[31] The difference in tissue localization of BoCoV during natural and experimental infection could be attributed to factors such as viral strain, method of infection, and other external factors, such as coinfection, which can impact distribution. In another experimental study, a difference was observed in BoCoV antigen localization during a single infection and/or coinfection with BVDV in bovine lungs. In single infection, BoCoV antigens were observed in macrophages and, to a lesser extent, in bronchial epithelium. When coinfected with BVDV, BoCoV antigens were observed in tracheal glands, interstitial cells, and macrophages,[82] showing synergy between BVDV and BoCoV infections.

Bovine parainfluenza virus 3

BPIV-3 antigens have been detected by IHC in the respiratory tissues of cattle suffering from BRDC.[8,71] Coinfection of BPIV-3 with BRSV and *M. bovis* can lead to antigen-specific localization in lung tissues.[71] BPIV-3 antigen can be typically found at the apex of ciliated epithelial cells; BRSV antigen is predominantly present in alveolar epithelium, with some in the bronchial and bronchiolar epithelium. *M. bovis* antigens can be found in bronchial and bronchiolar epithelial cells, alveolar macrophages, and syncytial cells.[71] In experimental infection, time-based BPIV-3 infection distribution has been observed.[8] At 3 d post-inoculation, BPIV-3 antigens were observed in numerous respiratory epithelial cells. However, at 5 d post-inoculation, as viral titers decreased, the antigens spread extensively throughout the airway and alveolar epithelia, correlating with bronchiolitis and multifocal interstitial pneumonia.[8]

In another study, BPIV-3–infected calves had bronchointerstitial pneumonia, including necrotizing bronchiolitis, alveolitis, and intracytoplasmic IBs.[31] Furthermore, in one study, ~50% of the BPIV-3 IHC–positive lung samples were

coinfected with *Mycoplasma* spp., providing further evidence for a high prevalence of *Mycoplasma* spp. within severe and, more importantly, chronic cases of BRDC.[71]

Bovine respiratory syncytial virus

BRSV and BPIV-3 are closely related viruses with similar viral morphology and replication strategy; hence, BPIV-3 and BRSV coinfect the same cell types and share pathobiologic and epidemiologic features.[31] BRSV has been detected in both FFPE[36] and frozen[115] bovine lung tissue sections using IHC, but given the labile nature of BRSV,[58] frozen tissue samples are preferred over FFPE samples.[16] Although BRSV antigens have been observed localized predominantly in bronchioles, bronchi, and alveoli of the lungs of naturally infected cattle,[36,71,120] BRSV antigens have also been detected in alveolar macrophages, epithelial cells, interalveolar septa, and bronchiolar and alveolar lumens.[36,120] Differences in BRSV localization might be attributed to factors such as infection stage, coinfection type, and host immune response.

Bovine viral diarrhea virus

IHC has been used on ear-notch tissue samples from live animals or lung tissue samples from dead cattle.[4] However, in cattle recently infected with BVDV or recently vaccinated with a modified-live BVDV vaccine, detecting viral antigens by IHC may be challenging because the virus is often only present in the skin for a short time.[9] The strength and duration of the immunomodulatory effects of BVDV on cattle after an acute infection remains unclear. However, the ability to identify BVDV by IHC, even momentarily, in lung tissue during a BRDC episode may reveal new pathways in the development of BRDC. BVDV can cause persistent infections in which calves infected in utero shed the virus continually (persistently infected, PI),[12,33] complicating detection via traditional serologic methods. In such conditions, IHC serves as a vital tool by directly visualizing the virus in tissues and bypassing immune response–based limitations, which are observed in serology. For PI cattle, given the convenience of sample collection, cost-effectiveness, and reliability, IHC is one of the preferred methods for detecting BVDV.[52,63]

Influenza D virus

IHC was used to observe replication of IDV in the ciliated cells of the epithelium of the nose and trachea and, to a lesser extent, in the alveolar macrophages of the lung,[50,70] indicating the preference of the virus for the upper respiratory tract. IDV also infects bronchiolar and alveolar epithelial cells and mononuclear cells of the lungs.[86] In a guinea pig model, IDV antigens were detected in alveolar septa, bronchi, and bronchiolar epithelium, and gross histologic lesions were found to be related to the antigen intensity of detection,[98] indicating the role of IDV in BRDC pathogenesis.

Advances in immunohistochemistry

Multiplexing

Multiplex IHC (MIHC) employs multiple fluorophores with distinct spectral properties[60,91] or spectral unmixing to avoid spectral overlap,[85] which occurs when the emission spectra of ≥2 fluorochromes significantly overlap, making it challenging to distinguish different signals.[3,91,99] Spectral unmixing is a process used in fluorescence microscopy to separate and quantify the individual components of mixed or overlapping spectral signals. MIHC allows for the differentiation and localization of multiple targets simultaneously.

Although the current threshold for detection is 8 pathogens,[43] fluorescent MIHC has its own limitations. The limitations of MIHC are antibody specificity, background noise, chromogen interference, cross-reactivity, signal overlap—a situation in which signals from closely located antigens overlap spatially—spectral overlap, technical complexity, and the need for high-resolution imaging.[3,67,104]

Spatial transcriptomics

Spatial transcriptomics is a technique that allows the simultaneous analysis of localization and expression levels of thousands of genes in a tissue sample. Spatial transcriptomics has been used in humans to study tissue architecture, molecular alterations, disease-specific pathways, and biomarker identification.[108,112] Similarly, in BRDC-affected cattle lungs, spatial transcriptomics has the potential to identify the type, degree, and location of damaged cells, as well as host immune response, molecular markers, and pathways. The combination of spatial transcriptomics and IHC would allow the identification of specific cell types and the measurement of gene activity in those cells, revealing cellular and molecular changes in infected tissues.[26,87] Spatial transcriptomics can be combined with MIHC to study BRDC in lung tissue by identifying cell populations, mapping them to gene expression changes, and exploring the spatial distribution of multiple proteins during coinfections, which current detection methods lack.

Enhanced imaging

Tissue clearing enables entire tissues to become optically transparent, allowing for 3-dimensional analysis and combining IHC with other imaging modalities, such as in situ hybridization. Certain hydrophilic tissue-clearing techniques, such as delipidation, allow large molecules, including antibodies, to enter tissues more quickly and deeply, permitting 3-dimensional IHC in samples.[110] Moreover, super-resolution microscopy techniques, such as stimulated emission depletion or single-molecule localization microscopy, offer higher spatial resolution for detecting antigens and protein interaction than does traditional IHC. Methodologies such as these enable identification and subcellular antigen location.[64,89] To quantitatively measure intensity,[84,119]

as well as target protein quantification and distribution,[59] high-resolution images of IHC-stained tissue sections can be captured using digital imaging technology and analyzed with computational algorithms. Furthermore, computer-assisted detection systems using machine-learning algorithms are being developed to improve IHC detection accuracy and efficiency by identifying patterns and features that may be overlooked by the human eye.[34]

Novel detection methods

Another enhancement to the sensitivity of IHC methodologies can be achieved by using quantum dots and/or nanoparticles, as they can produce bright and stable signals, even at low-antigen levels. Quantum dots are small inorganic molecules that emit fluorescence based on size and composition, allowing for the simultaneous detection of multiple antigens.[61] Quantum dots have unique optical properties that allow them to emit fluorescence at different wavelengths, making them a good alternative to traditional fluorescent dyes in IHC. The dots offer high sensitivity and specificity and can be tuned to detect multiple antigens simultaneously.[114,118]

Similarly, nanoparticles are small 1–100-nm molecules that are engineered to bind to specific biologic targets, enhancing the detection and visualization of specific proteins or antigens in tissue samples.[101] Their small size enables them to penetrate tissue sections more quickly, resulting in stronger signals in locations that larger molecules cannot reach.[27,114] Nanoparticle-based probes that bind to specific targets have been developed and can be multiplexed with other markers.[27] These techniques could improve the accuracy and reliability of IHC and are continuously being refined and developed to expand capabilities.

Conclusion and future perspectives

IHC provides a valuable and cost-effective detection approach for identifying BRDC pathogens in fresh, frozen, or FFPE tissue samples. Given its specificity, direct visualization, tissue preservation, and high sensitivity, IHC could play a crucial role in underpinning the control and prevention of BRDC within cattle herds. Moreover, IHC can also be utilized for studying antigen localization and immune response, providing insight into the distribution and interaction of antigens and the immune system.

The potential of using IHC for routine detection of BRDC etiologies holds great promise for the future, with ample room for continued research and advancements. Improving the sensitivity and specificity of the method, availing standardized IHC protocols and reagents, and identifying new biomarkers for BRDC may improve the accuracy of IHC-based detection and increase understanding of the underlying mechanisms of BRDC. Setting up standard protocols and methods may increase the reproducibility of results and reduce variability in methodology. In addition, developing universally accepted standards and integrating such standards with other technologies, such as spatial transcriptomics, artificial intelligence, and big-data analytics, may lead to more efficient and effective BRDC diagnosis and management that could lead to a deeper understanding of the disease and its progression.

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