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Detection of *Mycoplasmopsis* (*Mycoplasma*) *bovis* in formalin-fixed, paraffin-embedded bovine lung sections by immunohistochemistry and in situ hybridization

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Abstract. The bovine respiratory disease complex (BRDC) is a multifactorial disease of economic importance in cattle involving viral and bacterial agents and several environmental and host-associated predisposing factors. *Mycoplasmopsis* (*Mycoplasma*) *bovis* is frequently detected in BRDC cases, but the role of this bacterium in the pathogenesis of BRDC is not completely understood. We explored the utility of routine histopathology and compared immunohistochemistry (IHC) and in situ hybridization (ISH) in pneumonic bovine lung tissue samples for the detection of *M. bovis* infection. Samples were analyzed for *M. bovis* using mycoplasma bacterial culture (screening test), H&E staining, IHC, and ISH. We found that "compatible histologic lesions" are not entirely predictive of the presence of *M. bovis* on culture, IHC, or ISH, and also that there was no statistical difference between IHC and ISH for detecting *M. bovis*. We conclude that IHC and ISH can be used interchangeably to detect *M. bovis* infections in pneumonic bovine lung.

Keywords: bronchopneumonia; bovine respiratory disease complex; *Mycoplasmopsis* (*Mycoplasma*) *bovis*; pneumonia.

The bovine respiratory disease complex (BRDC) is a disease of great economic importance that involves environmental and nutritional factors, a weakened host immune system, and the participation of several viral and bacterial pathogens.[7] The bacterial pathogens most commonly associated with BRDC are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasmopsis* (*Mycoplasma*) *bovis*.[5,7,12] Common viruses involved in BRDC include bovine alphaherpesvirus 1, bovine respiratory syncytial virus, and bovine parainfluenza-3 virus. While the role of *M. bovis* in BRDC remains poorly understood, this bacterium is frequently detected in cases of pneumonia in juvenile cattle in southwest Virginia, prompting the need for a more thorough investigation of its involvement in this complex.[3]

M. bovis and other mycoplasmas typically inhabit mucosal surfaces, such as those of the respiratory, urogenital, and gastrointestinal tracts, as well as the conjunctiva and mammary gland.[6,13,14] *M. bovis* colonizes the upper respiratory tract of calves early in life and is typically a secondary opportunistic pathogen, often contributing to polymicrobial infections in the lung.[4,10,12,18] *M. bovis* is often found in large numbers in the lungs of cattle with BRDC, most likely due to impairment of the mucociliary apparatus.[22] Gross lesions in the lung caused by *M. bovis* are those of a caseonecrotic bronchopneumonia. Histologically, the affected lung has conspicuous round-to-irregular areas of caseous necrosis within airways surrounded by well-delineated areas of granulation tissue.[11,12]

M. bovis can be detected with several techniques. Microbial culture can be used to grow *Mycoplasma* spp. with their characteristic fried-egg appearance, but speciation within the genus requires additional testing.[8,17] PCR can be used to detect the pathogen through amplifying bacterial DNA from samples such as lung, nasal swabs, and bronchoalveolar lavage.[16] Histopathology, immunohistochemistry (IHC), and in situ hybridization (ISH) can also be used to detect *M. bovis*.[1,9,16] Histopathology detects the lesions produced by *M. bovis*, providing a presumptive diagnosis but does not confirm the infection. IHC detects and localizes *M. bovis* proteins within tissue; ISH detects and localizes *M. bovis* DNA

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Table 1. Aerobic bacteria isolated from the lung of each case of bronchopneumonia included in our study.

Case	Isolate 1	Isolate 2
1	*Mannheimia haemolytica*	*Trueperella pyogenes*
2	*Mannheimia haemolytica*	
3	*Pasteurella multocida*	
4	*Pasteurella multocida*	
5	*Pasteurella multocida*	
6	*Pasteurella multocida*	*Trueperella pyogenes*
7	*Trueperella pyogenes*	
8	No growth	
9	No growth	
10	*Pasteurella multocida*	
11	*Pasteurella multocida*	*Trueperella pyogenes*
12	*Mannheimia haemolytica*	
13	*Moraxella osloensis*	
14	No growth	
15	*Pasteurella multocida*	

in tissue.[1,9] We explored the utility of histopathology for the detection of lesions compatible with *M. bovis* infection and compared IHC and ISH for detection of *M. bovis* in lung tissue samples collected from bovine bronchopneumonia cases.

Materials and methods

Case selection

In our retrospective study, we used tissue samples collected from animals submitted to the Wytheville Regional Animal Health Laboratory, Virginia Department of Agriculture and Consumer Services (Wytheville, VA, USA) in 2020. Fifteen cases were selected from 1–12-mo-old animals with bronchopneumonia from a previous pilot study. By a *Mycoplasma* spp. culture (screening test), 10 of 15 cases were positive and 5 were negative. Briefly, fresh lung specimens collected from the right cranial lobe were used for routine aerobic culture (Table 1) and also inoculated onto mycoplasma agar with cefoperazone (Hardy) and incubated in a humidity chamber at 37°C in a 5% CO_2 incubator, for up to 7 d. *Mycoplasma* spp. were identified by colony morphology and Diene stain. In addition, sections from the right cranial lung lobe were collected in formalin and processed routinely for histopathology. One lung block per animal was analyzed. Deep recuts of each lung block were obtained and evaluated side-by-side with the 3 detection methods (routine H&E, IHC, and ISH).

Histopathology

We evaluated the diagnostic value of histopathology using H&E-stained slides by carefully examining each slide for the presence or absence of lesions compatible with *M. bovis* infections (M. Brown, Z. Laney, L. Tabatabai, F. Carvallo). We defined "compatible lesions" as lung sections with one or more rounded areas of caseous necrosis within the airways, surrounded by a mixed inflammatory infiltrate and fibrosis (Fig. 1A). A slide was categorized as positive when one or more compatible lesions were present, or as negative, when no compatible lesions were found.

Immunohistochemistry

Following automated deparaffinization of formalin-fixed, paraffin-embedded (FFPE) tissue sections (EZ Prep 1X; Roche), antigen retrieval was accomplished with an application of Cell Conditioning 1 (CC1; Roche) solution for 16 min at 100°C. Endogenous peroxidases were blocked with an application of Inhibitor CM (Roche) for 8 min. The *M. bovis* primary antibody (1:800, pool MYB57, MYB87, MYB163; obtained from Dr. R. Rosenbusch, Iowa State Diagnostic Laboratory, 2005)[1] was then applied for 28 min at 37°C and detected with a conjugated multimer (OmniMap anti-rabbit HRP multimer, Roche; 16 min). A standard application of each of Discovery ChromoMap DAB (Roche), hematoxylin (8 min), and bluing reagent (4 min) completed the staining process. Slides were removed from the automated IHC stainer (Discovery Ultra; Roche) and automatically dehydrated and coverslipped (Tissue-Tek Prisma Plus stainer and film coverslipper; Sakura). A positive IHC result was identified as brown granular staining within the tissue sections (Fig. 1B). Positive controls were obtained from the California Animal Health and Food Safety Laboratory (San Bernardino, CA, USA), from a calf with *M. bovis* infection confirmed by positive PCR and culture.

In situ hybridization

For cellular localization of *M. bovis* nucleic acid in FFPE tissue sections, a validated ISH method (RNAscope; Advanced Cell Diagnostics) was performed using a standard protocol and an automated ISH research platform (Discovery Ultra; Roche). After deparaffinization and target retrieval (cat. 760-248, mRNA sample prep kit; Roche), 4-µm tissue sections mounted on charged slides were hybridized with 200 µL of a 14ZZ ISH probe targeting a 1,660-nucleotide region of the *M. bovis UVRC* gene encoding a deoxyribodipyrimidine photolyase (GenBank AF003959.1; 508289, B-M. bovis-*UVRC*, Advanced Cell Diagnostics). This was followed by signal amplification (cat. 760-236, mRNA RED probe amplification kit; Roche), detection (cat. 760-234, mRNA RED detection kit; Roche), and counterstaining with hematoxylin. Positive signal was identified as red staining within infected cells (Fig. 1C).

Grading of intensity of IHC and ISH staining

We evaluated the intensity of IHC and ISH staining using nonparametric and subjective scales of 0–3 (Fig. 2; M.

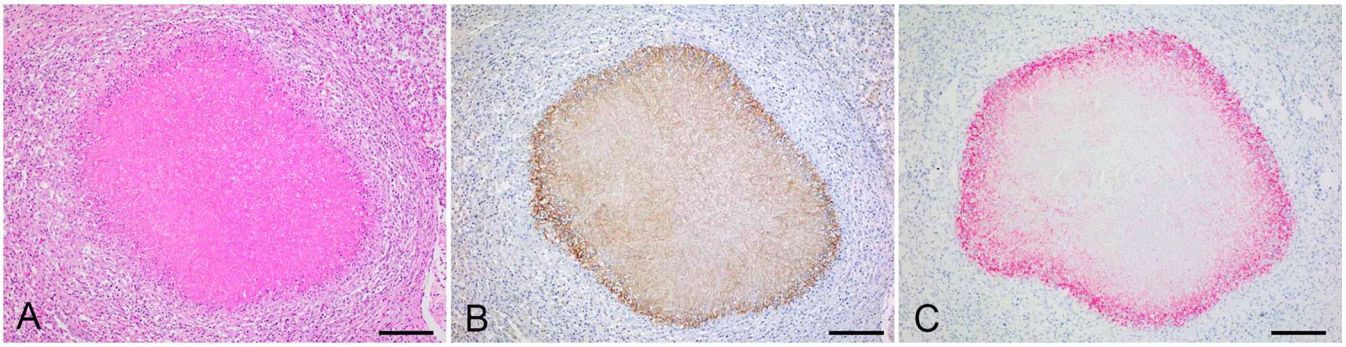

Figure 1. "Compatible lesions" of *Mycoplasmopsis* (*Mycoplasma*) *bovis* infection in calves with pneumonia. **A.** A round structure (presumptive bronchiole) with abundant eosinophilic necrotic cellular debris in the center, surrounded by fibrous connective tissue containing neutrophils and macrophages. H&E. **B.** Deeper section of the same bronchiole, with immunopositivity along the margins of the lesion, between the necrotic cellular debris and the cellular component. *M. bovis* immunohistochemistry, diaminobenzidine chromogen, hematoxylin counterstain. **C.** A strong signal along the margins of the necrotic cellular debris and the cellular component. *M. bovis* in situ hybridization, mRNA RED probe amplification kit, hematoxylin counterstain. Bars = 100 µm.

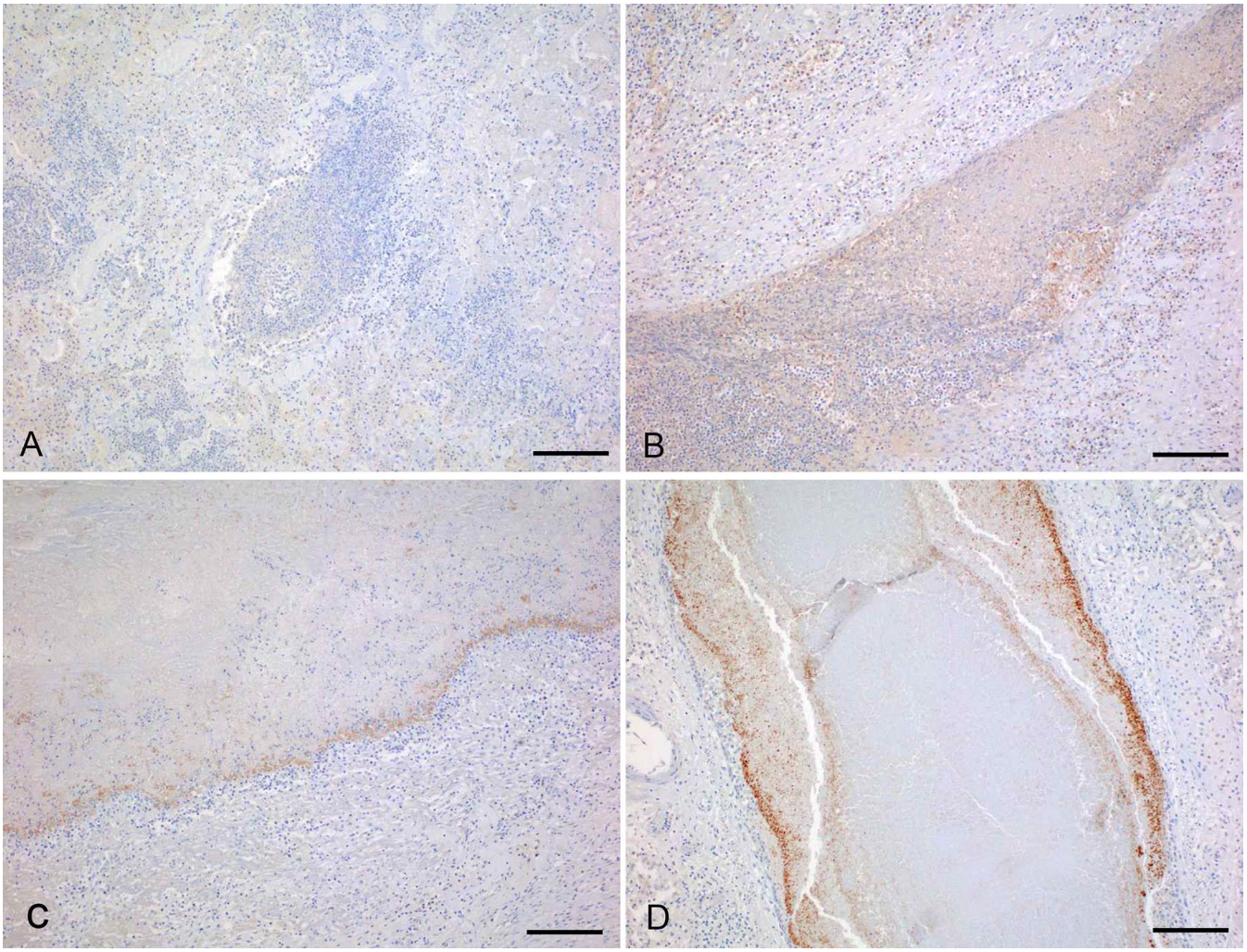

Figure 2. Grading of immunohistochemistry for *Mycoplasmopsis* (*Mycoplasma*) *bovis*. **A.** Grade 0 = no staining could be found. **B.** Mild intensity or grade 1 = staining, but in clusters and faintly visible using 10× magnification. **C.** Moderate intensity or grade 2 = staining could be seen using 4× magnification, but the intensity was variable, and identification of staining required more extensive searching across the slide. **D.** Strong intensity or grade 3 = intensity was strong across most of the visible lesions on the slide, and the stain could very easily be visualized using 4× magnification. Hematoxylin counterstain in all slides. Bars = 100 µm.

Table 2. Scoring of immunohistochemistry (IHC) and in situ hybridization (ISH) results for *Mycoplasmopsis* (*Mycoplasma*) *bovis* bovine bronchopneumonia cases positive by mycoplasma culture (screening test).

			IHC grade		ISH grade	
Case	*Mycoplasma* spp. cultured	H&E compatible lesions	Airways	Alveoli	Airways	Alveoli
1	Yes	Yes	3	3	1	3
2	Yes	Yes	3	3	3	3
3	Yes	Yes	2	3	3	3
4	Yes	Yes	2	3	2	3
5	Yes	Yes	3	3	3	3
6	Yes	Yes	1	0	1	0
7	Yes	Yes	2	0	1	0
8	Yes	No	2	3	3	3
9	Yes	No	2	1	3	2
10	Yes	No	0	3	0	3
11	No	Yes	0	0	0	0
12	No	No	0	0	0	0
13	No	No	0	0	0	0
14	No	No	0	0	0	0
15	No	No	0	0	0	0

M. bovis detection intensity grading system for both IHC and ISH: 0 = absent; 1 = mild; 2 = moderate; 3 = strong.

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Statistical analysis

A weighted kappa statistical method was used to test for agreement between the 2 grading scales used to evaluate the intensity of IHC and ISH staining. A value of 0 = agreement equivalent to chance, <0.2 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = good agreement, 0.81–1.00 = very good agreement, and 1 = perfect agreement between the 2 scales.[20]

Results

Of the 5 of 15 animals that were negative on mycoplasma culture, 4 did not have H&E-compatible lesions and 1 animal did have H&E-compatible lesions (Table 2). Of the 5 samples with no mycoplasma-positive culture, *M. bovis* was not detected through IHC or ISH. In case 11, abundant acid hematin was present in IHC and ISH samples. Of the 10 samples with positive mycoplasma culture, 7 had *M. bovis*–compatible lesions with H&E, and 3 did not have compatible lesions. In all 10 samples with positive mycoplasma culture, *M. bovis* was detected with IHC and ISH in either airway and/or alveolar areas of the lung (Fig. 3). Histologic findings in alveolar areas included neutrophils and macrophages, necrotic cellular debris, and small amounts of fibrin. The alveolar interstitium was variably expanded with similar inflammatory infiltrates and occasional thrombi. *M. bovis* was detected in 7 samples in both the airway and alveolar areas of the lung, in 2 samples only in the airways, and in 1 sample only in alveoli. In total, using IHC and ISH, *M. bovis* was detected in airways in 9 samples and in alveoli in 8 samples.

The weighted kappa for agreement of IHC and ISH in airways is 0.71 (95% CI: 0.49–0.93%; Table 3), which indicates good agreement. *M. bovis* was not detected by ISH in any of the 6 cases with no immunoreactivity for *M. bovis* on IHC. Among subjects with immunoreactivity for *M. bovis* by IHC, there was variable and partial agreement in grading of *M. bovis* by ISH.

The weighted kappa for the agreement of IHC and ISH alveoli is 0.95 (95% CI: 0.87–1.00%; Table 4), which indicates very good agreement. All 7 patients with strong immunoreactivity for *M. bovis* on IHC also had strong detection of *M. bovis* by ISH. *M. bovis* was not detected by ISH in any of the 7 cases with no immunoreactivity for *M. bovis* on IHC.

Discussion

We found suboptimal sensitivity in identifying calves with *Mycoplasma* spp. in the lung when relying on H&E staining alone; we detected lesions in only 7 of 10 culture-positive cases. Studies focusing on histologic lesions associated with

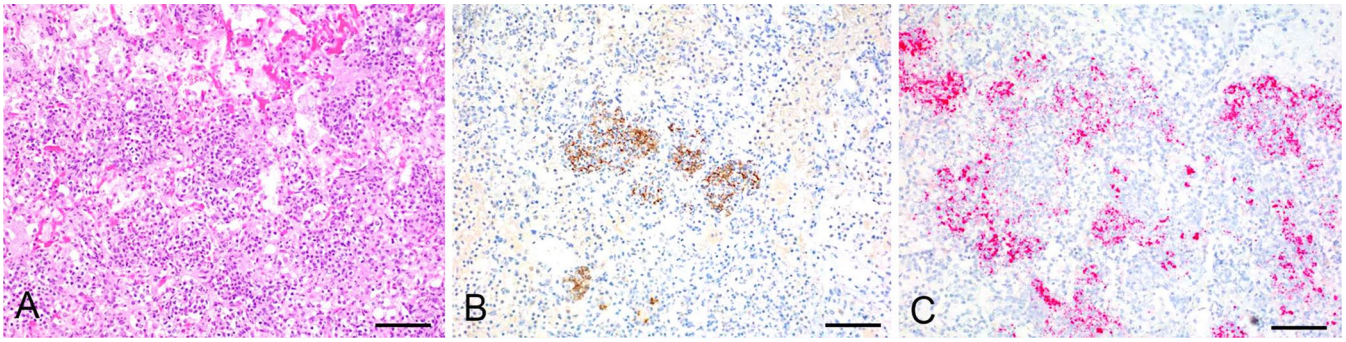

Figure 3. Alveolar detection of *Mycoplasmopsis* (*Mycoplasma*) *bovis*. **A.** In this area, a dense inflammatory infiltrate in alveoli is composed of neutrophils and macrophages, together with necrotic cellular debris. H&E. **B.** A deeper section of the same topographic area, with the presence of *M. bovis* immunopositivity in alveolar inflammatory cells and free in the alveolar lumens. *M. bovis* immunohistochemistry, diaminobenzidine chromogen, hematoxylin counterstain. **C.** Deeper section of the same topographic area, with a strong signal in inflammatory cells and free in the alveolar lumens. *M. bovis* in situ hybridization, mRNA RED probe amplification kit, hematoxylin counterstain. Bars = 50 µm.

Table 3. Comparison of immunohistochemistry (IHC) grade to in situ hybridization (ISH) grade for *Mycoplasmopsis* (*Mycoplasma*) *bovis* in the airways of lungs from calves with bronchopneumonia.

	ISH			
IHC	Not present	Mild	Moderate	Strong
Not present	6	0	0	0
Mild	0	1	0	0
Moderate	0	1	1	3
Strong	0	1	0	2

Table 4. Comparison of immunohistochemistry (IHC) grade to in situ hybridization (ISH) grade for *Mycoplasmopsis* (*Mycoplasma*) *bovis* in the alveoli of lungs from calves with bronchopneumonia.

	ISH			
IHC	Not present	Mild	Moderate	Strong
Not present	7	0	0	0
Mild	0	0	1	0
Moderate	0	0	0	0
Strong	0	0	0	7

M. bovis pneumonia have found variable reliability associated with compatible lesion presentation. In one study, 18 of 18 naturally infected cattle had compatible histologic lesions[11]; an earlier study only demonstrated such lesions in 12 of 25 cases confirmed positive for *M. bovis* via culture.[2] Therefore, it is not a guarantee that H&E-compatible lesions are present in all cases from which *Mycoplasma* spp. are cultured. On the other hand, we found compatible lesions in one case with negative *Mycoplasma* spp. culture, which indicates either that culture is a less sensitive method or that compatible lesions could also be identified in cases caused by agents other than *M. bovis*, such as *M. haemolytica* and *H. somni*.[12]

The use of IHC for the detection of *M. bovis* in FFPE bovine lung tissue samples has been reported in several studies,[2,11,21] which demonstrated that IHC had value in identifying these infections. On the other hand, the use of ISH for the detection of *M. bovis* has only been reported once in an aborted bovine fetus and a neonatal calf, where it was shown to be an effective detection technique.[9]

We found good agreement between IHC and ISH for the detection of *M. bovis* in airways and very good agreement for detection in alveoli. Therefore, IHC and ISH could be used interchangeably for reliable detection of *M. bovis* in bovine lung tissue. However, we only had a few cases in the mild-to-moderate categories; IHC and ISH may be equivalent at detecting strongly positive or negative cases but may not perform as well in detecting less distinct lesions or lower bacterial loads. Larger validation studies are needed to confirm these findings and rule out these concerns.

We detected *M. bovis* in the airways by IHC and ISH in 9 of 10 cases in which *Mycoplasma* spp. were isolated. *M. bovis* antigen and DNA were particularly abundant within the bronchioles. This finding agrees with the expected localization of *M. bovis* within the lungs, as the earliest lesions are believed to begin in small airways, especially bronchioles.[5] Compatible lesions identified on H&E staining were detected by IHC and ISH as well. Only case 10 had a positive mycoplasma culture but absence of any lesions within the airways, as well as absence of any compatible lesions detected on H&E staining. We hypothesize that the lesion was missed in the analyzed section, and that this may be a case of more acute *M. bovis* infection or of coinfection with another bacterium.

Regarding the detection of *M. bovis* in the rest of the pulmonary parenchyma, 8 of 10 samples in which *Mycoplasma* spp. were cultured had IHC and ISH positivity within alveolar spaces. Case 10 had *M. bovis* detected within alveoli only. In cases 2, 8, and 10, IHC and ISH positivity was within foci of coagulative necrosis, and alveolar architecture was still discernable within the lesion. Previous studies have demon-

strated *M. bovis* both on the surface of alveolar epithelium and within alveolar macrophages and neutrophils.[8,21] It has been postulated that areas with coagulative necrosis can also be found with *M. bovis* infection, but it is not clear if this lesion is a transitional form to caseonecrotic "compatible" lesions or if coagulative necrosis and caseonecrotic foci are 2 separate lesions.[2,11] It has also been theorized that the lesions are distinct and may be due to different strains of *M. bovis* or due to coinfection with other pathogens.[11,21] We corroborate that there are 2 sets of lesions caused by *M. bovis* infection: 1) the compatible lesion, which is likely due to bronchiolar necrosis, chronic inflammation, and granulation tissue, and 2) irregular areas of acute necrosis, which first manifest as small foci of inflammation in alveoli that may progress into tortuous areas of coagulative necrosis in the pulmonary parenchyma. Areas of coagulative necrosis do resemble lesions caused by other pathogens associated with BRDC, thus the interpretation of coagulative necrosis should be made with caution.[12] We also conclude that routine H&E stain may serve as an initial screening for the detection of *M. bovis*, but the final detection must be performed with an additional confirmatory test, such as culture, PCR, IHC, or ISH. Also, the absence of compatible lesions is not absolute evidence of negative *M. bovis* status.

We found some challenges and limitations during our study. The interpretation of the IHC sample from case 11 was complicated by the presence of acid hematin. Acid hematin is a brown-to-black amorphous pigment that is produced by an acid acting on hemoglobin in the sample when the pH of the formalin that is used to fix the sample is low.[19] The color and shape of this pigmented material closely resemble positive signaling of *M. bovis* in IHC revealed with diaminobenzidine, making interpretation of the samples more difficult and increasing chances for an incorrect clinical diagnosis. In addition, thrombi within blood vessels were present in a few samples (cases 1, 3, and 11). Thrombosis may resemble the rounded areas of caseous necrosis caused by *M. bovis*, which can be confused as a compatible lesion by an inexperienced eye. Another limitation is the fact that our mycoplasma cultures were not followed up by speciation within the genus, so it is impossible to know if all of the animals with positive mycoplasma cultures were in fact *M. bovis*. There are 13 other *Mycoplasma* spp., such as *M. bovirhinis*, that could also be detected with *Mycoplasma* culture.[15] However, our IHC and ISH procedures were specific for *M. bovis*. It is also possible that areas of coagulative necrosis due to early infection with *M. bovis* were missed in some sections that included only compatible or more chronic lesions. We suggest that the analysis of *M. bovis*–associated lesions should always use a confirmatory test, and we recognize that acute lesions can be confused with other bacterial infections. Another limitation of our study is the unknown status of viral infectious causes of pneumonia in the analyzed specimens.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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