

Natural *Mycoplasma bovis* infection associated with epithelioid granulomatous caseonecrotic bronchiolitis at a progressed disease stage in calves

Mathurot Suwanruengsri¹ Ryoko Uemura² Uda Zahli Izzati¹ Takuya Kanda²

Naoyuki Fuke¹ Masahiro Yasuda³ Takuya Hirai¹ Ryoji Yamaguchi^{1*}

Abstract

Mycoplasma bovis (*M. bovis*) is a pathogen that especially effects the respiratory system of cattle. We focused on the histopathological changes in the lungs caused by *M. bovis* because the lesions in the bronchi or bronchioles and alveoli are quite different in natural infections. Thirty-five lung samples from 2-to 12-month-old Japanese Black calves with respiratory symptoms were collected and examined for bacteria presence and pathological changes using immunohistochemistry (IHC). Using the loop-mediated isothermal amplification method, 18 of the 35 samples were found to be positive for *M. bovis*, which was isolated and identified. In 7 of the samples, only *M. bovis* was detected, whereas in the other 11, other bacteria coexisted with *M. bovis*. Anti-*M. bovis*, anti-MAC387, anti-Iba1, anti-CD3, anti-CD20, anti-AE1/AE3 cytokeratin and anti-IL8 were used for antigen detection. Histopathological studies and diagnoses of the lungs were examined in two parts; bronchiolar lesions and alveolar lesions. The bronchiolar findings were divided into three groups: granulomatous, caseonecrotic and suppurative bronchiolitis. The alveolar lesions found were quite mild, except in three cases. IHC staining revealed strong evidence of *M. bovis* in degenerated neutrophils, which was confirmed by electron microscopy. The main inflammatory cell response to *M. bovis* infections was degenerate neutrophils, which caused the destruction of the bronchial epithelium and possibly induced epithelioid granulomatous inflammation, resulting in severe chronic respiratory diseases in cattle.

Keywords: granulomatous bronchiolitis, calves, *Mycoplasma bovis*, neutrophil

¹Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Miyazaki, 1-1 GakuenKibanadai-Nishi, Miyazaki 889-2192, Japan

²Department of Animal Health, Faculty of Agriculture, University of Miyazaki, Miyazaki, 1-1 GakuenKibanadai-Nishi, Miyazaki 889-2192, Japan

³Department of Veterinary Anatomy, Faculty of Agriculture, University of Miyazaki, Miyazaki, 1-1 GakuenKibanadai-Nishi, Miyazaki 889-2192, Japan

*Correspondence: a0d402u@cc.miyazaki-u.ac.jp (R. Yamaguchi)

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Introduction

Bovine respiratory disease complex (BRDC) is the most important respiratory disease in cattle and is caused by multiple factors, such as viral infection, bacterial infection and environmental stress (Szeredi *et al.*, 2010). Some viruses and bacteria can act as triggers or primary causes of respiratory disease in cattle or as coinfection causes. *Mycoplasma bovis* (*M. bovis*) is one of the most important primary pathogens in BRDC (Radaelli *et al.*, 2008).

Only a few recent reports have been made on the pathogenesis of *M. bovis* infections, especially pneumonia in calves. Thus far, many researchers have reported that bronchitis and bronchiolitis are the main pathological changes caused by *M. bovis* and that lymphoid proliferation, which is often characterized as follicles, is the main feature of *M. bovis* infection (Adegboye *et al.*, 1995; Hermeyer *et al.*, 2012; Hermeyer *et al.*, 2011). Several studies have reported that *M. bovis* produces chronic bronchopneumonia characterized by caseonecrotic nodules in the cranioventral lung lobe (Arcangioli *et al.*, 2008; Margineda *et al.*, 2017; Panciera and Confer, 2010). Calf lung lesions associated with *M. bovis* infection are usually called bronchopneumonia by pathologists (Caswell and Archambault, 2008). However, as the degree of inflammation is quite different in severity between the bronchi or bronchioles and alveoli in natural *M. bovis* infection, it is more difficult to diagnose than usual bronchopneumonia, which is commonly an acute or subacute infection that shows mild inflammation in the bronchioles (Caswell and Williams, 2016). Moreover, many studies have reported that *M. bovis* grows mostly in macrophages (Hermeyer *et al.*, 2012; Margineda *et al.*, 2017; Thomas *et al.*, 1986), while others have shown that it proliferates in neutrophils (Gagea *et al.*, 2006; Kleinschmidt *et al.*, 2013; Rodríguez *et al.*, 2015). Thus, there remains controversy about the main inflammatory response to *M. bovis* infections.

A granulomatous inflammation is a distinct type of chronic inflammation that occurs in response to endogenous or extrinsic antigens or idiopathy and it is characterized predominantly by monocytes and macrophages (Ackermann, 2017). Few studies of granulomatous inflammation in the lung associated with *M. bovis* have been reported in cattle (Khodakaran-Tafti and López, 2004) and bison (*Bison bison*) (Janardhan *et al.*, 2010). The lesions were characterized by a central areas of necrosis, which were occasionally mineralized and surrounded by a rim of degenerate neutrophils, plasma cells and macrophages. These characteristics have been described in a few reports (Janardhan *et al.*, 2010). However, to our knowledge, a granulomatous formation in bronchioles in which morphological changes of macrophages resemble bronchiolar epithelial cells associated with *M. bovis* infection and confirmation of the granulomatous formation associated with *M. bovis* using immunohistochemistry (IHC) have not been reported elsewhere.

The purpose of this study was to histopathologically investigate Japanese Black calf lung lesions with antigen distribution in natural *M. bovis* infections to better understand the pathogenesis

of pneumonia associated with *M. bovis* and to clarify the histopathological changes in the bronchiole and alveoli in advanced disease and/or those with poor prognosis. We also observed granulomatous bronchiolitis and inflammatory cell responses to *M. bovis* in lung lesions for deeper insight into their pathology.

Materials and Methods

Sample collection: This study included samples from Japanese Black calves with a history of respiratory symptoms and poor prognosis from different local farms in southern Kyushu subjected to necropsy in the Laboratory of Veterinary Anatomy, University of Miyazaki from 2015 to 2020. Thirty-five Japanese Black calves, aged from 2 to 12 months (19 males and 16 females), were humanely euthanized and routinely autopsied. The cattle's clinical histories showed 24 cases of pneumonia alone, 2 of pneumonia and suspected endocarditis, 2 of pneumonia and emaciation, 3 of pneumonia and arthritis, 2 of pneumonia and otitis, 1 of pneumonia, emaciation and suspected endocarditis and 1 of pneumonia, emaciation and otitis (Supplementary Table 1). Significant gross changes were found only in the lungs but not in other organs, at necropsy. Their lung tissues were subsequently collected for bacterial examination and histopathological studies, while other organs were not examined in the next steps of the examination and analysis. The Animal Care and Use Committee of the University of Miyazaki approved the experimental procedures (No. 2015-006).

Bacterial isolation and detection: For *M. bovis* detection and identification, 35 fresh lung tissue samples were incubated in *Mycoplasma* NK broth (Kanto Chemical, Tokyo, Japan) at 37°C overnight. The DNA was extracted with Lucigen Quick Extract DNA (Lucigen, Wisconsin, USA) and then subjected to loop-mediated isothermal amplification (LAMP) method specific for *M. bovis* (Higa *et al.*, 2016). The cultured broth was incubated and spread on *Mycoplasma* NK agar (Kanto Chemical, Tokyo, Japan). After incubation at 37°C in 5% carbon dioxide (CO₂) for 5-7 days, the typical fried-egg-shaped colonies that grew on the agar were identified by the LAMP method. The lung samples, which were found negative for *M. bovis* by LAMP, were histologically analyzed. The next steps of examination were eliminated due to *M. bovis* being negative.

For general bacteria isolation and identification, the tissue samples were stamped and spread on 5% sheep blood agar (Nissui, Tokyo, Japan) and incubated at 37°C in a 5% CO₂ incubator for 24 to 48 h. Each bacterial colony on 5% sheep blood agar was picked up to be identified by a matrix-assisted laser desorption/ionization (MALDI) Biotyper (Bruker Dultovics, Bremen, Germany). Briefly, each colony exhibiting distinct bacterial morphology and growing on 5% sheep blood agar was smeared like a thin-film layer on a circular spot on the target plate. An alpha-Cyano-4-hydroxycinnamic acid (HCCA) matrix solution was added to each sample, which was then analyzed with MALDI Biotyper system.

Histopathology and electron microscopy: The collected lung tissue samples were fixed in 10% buffered formalin and routinely embedded in paraffin wax. Four μm -thin sections were cut and stained with hematoxylin and eosin (H&E). The Brown-Hopps modification of the Gram stain was used for differentiating between gram positive and gram negative bacteria. The existence of *M. bovis* was ultrastructurally examined using transmission electron microscopy (TEM) (HT7700, Hitachi, Japan), as previously described (Teh *et al.*, 2018), using the paraffin-embedding sample of Case 2.

IHC staining: After deparaffinization and rehydration through grading alcohol, antigen retrieval was performed by microwave at 110°C for 10 mins with a citrate buffer solution pH 6.0, except for the MAC387 antibody, which was retrieved with the proteinase-K enzymatic antigen. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol solution for 15 mins and incubated in Blocking One (Nacalai Tesque, Kyoto, Japan) for 30 mins at room temperature to avoid a non-specific reaction. A 1:3000 dilution rabbit polyclonal *M. bovis* antibody (Kanda *et al.*, 2019) for *M. bovis* antigen detection, 1:400 dilution mouse monoclonal MAC387 (Dako, Glostrup, Denmark) for neutrophils, 1:500 dilution rabbit polyclonal Iba1 (Wako, Osaka, Japan) for macrophage, rabbit polyclonal CD3 (ready to use, Dako, Tokyo, Japan) for T lymphocytes, rabbit polyclonal CD20 (ready to use, Spring Bioscience, California, USA) for B lymphocytes, mouse monoclonal AE1/AE3 cytokeratin (ready to use, Dako, Tokyo, Japan) for epithelial cells and 1:200 dilution rabbit polyclonal IL-8 (Cloud-Clone Corporation, Katy, USA) for interleukin-8 (IL-8) were prepared in Blocking One. These antibodies were used as primary antibodies. The MAC387, an anti-human myeloid/histiocyte antigen, is expressed by neutrophils and monocytes, as well as by a subset of reactive tissue macrophages. This antibody recognizes the intracytoplasmic L1 antigen or calprotectin, which is expressed in neutrophils, monocyte/macrophages and potentially epithelial cells (Fukunaga *et al.*, 2018). Histofine MAX PO (multi) (Nichirei Biosciences, Japan) was used as the secondary antibody. For the chromogenic substance, 3,3' diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, Missouri, USA) was used and hematoxylin was used as the counterstain. Normal rabbit serum (Dako Corporation, Carpinteria, CA, USA) and normal mouse IgG (sc-2025 serum, Santa Cruz Biotechnology, Inc., Oregon, USA) with the same concentrations were used instead of the primary antibody as a negative control. Tissue sections used as positive controls for each antibody were as follows: tissue sections of bovine endocarditis associated with *M. bovis* infection from the previous study (Kanda *et al.*, 2019) for the *M. bovis* antibody, bovine bronchopneumonia without *M. bovis* infection for MAC387 and IL-8, bovine brain for Iba1, bovine lymph node for CD3 and CD20 and bovine intestine for the AE1/AE3 antibody. A positive and negative controls were simultaneously employed each time an antibody was used.

Double IHC staining: Double IHC staining was performed to clarify the co-expression of the *M. bovis* antigen and neutrophils, as well as that of the *M. bovis* antigen and macrophages, in *M. bovis*-infected tissue. The antigens were retrieved with proteinase-K for the dual stain of the *M. bovis* antibody and the MAC387 antibody and the antigen retrieval with a microwave using a citrate buffer pH. 6.0 was performed for dual staining of the *M. bovis* antibody and the Iba1 antibody. The primary antibody-reacted sections were incubated with a secondary Histofine MAX PO (multi) and then a brown substrate DAB for the anti-*M. bovis* antibody and a purple substrate 4-Chloro-1-naphthol (4CN) (Tokyo Chemical Industry, Tokyo, Japan) for the anti-MAC387 antibody. The counterstain with hematoxylin was not performed. The brown color of the DAB for the anti-*M. bovis* antibody and the purple color of the 4CN substrate for the anti-Iba1 antibody were used for visualization. The control was included simultaneously.

Results

Bacterial isolation and identification: *M. bovis* was detected in 18 calf lung tissues out of 35 cases of pneumonia using the LAMP method and the tissue samples from the other 17 of the 35 samples showed no *M. bovis* antigens. Only *M. bovis* was isolated and identified in 7 of the 18 positives in bacterial cultures. In the other 11, other bacteria, such as *Pasteurella multocida* (*P. multocida*), *Manheimia hemolytica* (*M. hemolytica*), *Trueperella pyogenes* (*T. pyogenes*), and/or *Bibersteinia trehalosi* (*B. trehalosi*), were qualitatively isolated together with *M. bovis*, (Supplementary Table 2).

Gross and histopathological findings: Multiple whitish to yellowish nodules were found in the cranioventral regions of the 15 lung samples and 3 cases showed diffuse-like whitish to yellowish lesions (Fig. 1) over the entire lung lobes of *M. bovis*-detected lungs. Yellowish material was slightly oozing out of the nodules (Fig. 2) in the bronchiole and sometimes from the parenchyma of the cut surface, which caused the space-occupying lesion (Fig. 3) and marble-like colors were seen on the cut surfaces of different lung lobes.

Histopathology and diagnoses of the lungs were conducted on two parts of the samples, on the bronchiolar and alveolar lesions, because the major lesions were severe space-occupying bronchiolitis. The severity of the bronchiolar and alveolar lesions was also pathologically quite different. The diagnoses of the bronchiolar lesions were divided into three groups, granulomatous bronchiolitis, caseonecrotic bronchiolitis and suppurative bronchiolitis, depending on the pathological changes in the bronchioles. In 18 cases of *M. bovis*-associated pneumonia, granulomatous bronchiolitis was found in 11 cases, caseonecrotic bronchiolitis in 4 cases and suppurative bronchiolitis in 3 cases. However, mild to moderate suppurative bronchiolitis but no granulomatous bronchiolitis was found in the 17 cases not associated with *M. bovis* (data not shown).

In the 11 cases in which granulomatous bronchiolitis (Figs. 4 and 5) was observed, caseonecrotic material, some neutrophils and degenerative neutrophils were found in the lumen where they occupied space. Many macrophages appeared to have replaced the bronchiolar epithelium near or at the wall of the bronchioles, producing granulomas characterized by epithelioid macrophages, which responded by growing *M. bovis*, degenerative neutrophils and/or destroying bronchiolar epithelial cells (Figs. 4a and 4b). Mineralization was detected within the bronchiolar caseonecrotic lesions in 8 cases and, especially in Case 11, dystrophic mineralization of the bronchiolar lumen was very clearly observed with epithelioid macrophage encapsulation. In some areas with macrophage accumulation, some bronchiolar epithelial cells remained, left by granulomatous changes caused by destroyed and disappearing bronchiolar epithelial cells to react to degenerative neutrophils with *M. bovis* antigens (Figs. 5a and 5b). Lymphoid follicles were found only in two cases. Almost all of the samples in this group showed advanced and developed lesions. Further, the caseonecrotic bronchiolitis found in 4 cases showed that degenerative neutrophils and neutrophils had infiltrated the bronchioles and had destroyed the bronchiolar epithelial cells in some areas. The bronchiolar wall was proliferated by fibrous tissue and infiltrated by lymphocytes. Moreover, mineralization was sometimes shown in the lesions. Notably, the suppurative bronchiolitis observed in 3 cases showed that a significant number of neutrophils and some macrophages had infiltrated the bronchiolar spaces. There were no bronchiolar epithelial destructions or caseonecrotic lesions. Lymphoid follicles were found in Case 17 and mineralization was seen in Case 6.

In the alveoli, there were some neutrophil and macrophage infiltrations in all cases except for one case, as shown in Table 1. Mineralization in the alveolar septa was frequently seen. Bacterial colonies showing Gram-negative coccobacilli were seen in the bronchiolar lumen and alveolar spaces with different severity levels in five other bacteria-coexisting cases (Cases 13 to 16, and 18), while bacterial colonies showing Gram-positive coccobacilli were seen in three cases (Cases 16 to 18) (Supplementary Table 2). The histopathological changes of alveolitis increased from moderate to severe with fibrin and/or necrotic inflammatory cells, including oat cell accumulation in three cases (Cases 14 to 16) with gram-negative and/or gram-positive coccobacilli strongly detected in the lesions.

IHC findings: *M. bovis* antigens were predominantly detected in the bronchiolar lumen in all 18 *M. bovis*-infected cases and, in some cases, they were seen in the alveolar spaces. We initially observed that, in granulomatous bronchiolitis, the *M. bovis* antigen was detected at the periphery of the caseonecrotizing and/or degenerative neutrophilic foci in contact with epithelioid macrophages, as shown in Figs. 6a and 6b, and was also detected in the bronchiolar lumen. MAC387-labeled neutrophils were found in peripheral caseonecrotic material close to the bronchiolar wall, which is probably similar to the *M. bovis*-positive site,

in almost all cases (Figs. 7a and 7b). *M. bovis* was also found in some Iba1-labeled macrophages, as shown in Fig. 8. Iba1-labeled epithelioid macrophages were morphologically similar to dysplastic bronchiolar epithelial cells but were distinguishable by being found in the bronchiolar wall area (Figs. 8a and 8b). The complete disappearance of the bronchiolar epithelium was confirmed by anti-AE1/AE3, as shown in Figs. 9a and 9b. The transitional stage to granulomatous formation was also observed. *M. bovis* antigens (Figs. 10a and 10b) that existed in the caseonecrotic material, neutrophils and/or degenerated neutrophils were detected (Figs. 11a and 11b). The Iba1-labeled macrophages reacted to *M. bovis*-existing degenerative neutrophils and formed epithelioid cells (Figs. 12a and 12b). In comparison, the bronchiolar wall, which was not occupied by *M. bovis*-existed cells, remained, and was confirmed by the anti-AE1/AE3 antibody (Figs. 13a and 13b).

Further observations showed that, in caseonecrotic and suppurative bronchiolitis, the *M. bovis* antigen was detected in the caseonecrotic material and/or MAC387-labeled neutrophils and, to a lesser degree, in some Iba1-labeled macrophages. The macrophages were sometimes seen outside the bronchiolar wall rather than in the bronchiolar lumen. The loss of some bronchiolar epithelium was also confirmed by the anti-AE1/AE3 antibody. *M. bovis* antigen detection and the severity of bronchiolar and alveolar changes are summarized in Table 1. The *M. bovis* antigen was detected at the lesion of the bronchiolar lumen in all 18 cases, consistent with MAC387-labeled neutrophils, while the main Iba1-labeled macrophages were seen at the apical, middle and basal areas of the bronchiolar wall. Moderate to severe CD3-labeled T lymphocytes were found in the middle area of the bronchiolar wall in 11 cases. CD20-labeled B lymphocytes, in contrast, were more recognized than CD3-labeled T cells in only six cases in a fairly similar area. Some IL-8-labeled cells were detected in the bronchiolar lumen in all 18 cases and scattered minimal to moderate IL-8-labeled cells were detected in the luminal surface side of the bronchiolar wall in four cases.

A few *M. bovis* antigens were distributed in the alveolar lesions in 13 of the 18 cases, as shown in Table 1 and Fig. 14. MAC387-labeled neutrophils, Iba1-labeled macrophages, CD3-labeled T lymphocytes, CD20-labeled B lymphocytes and IL8-labeled cells were detected in alveolar spaces. In only a few cases was a large number of positive *M. bovis* antigens observed in many MAC387-labeled neutrophils at the alveoli where severe suppurative alveolitis was found.

Double IHC staining revealed that the *M. bovis* antigen was expressed in the cytoplasm of MAC387-labeled neutrophils, many of which had degenerated, as shown in Fig. 15. The *M. bovis* antigen was also observed in the epithelioid macrophages that form granulomatous bronchiolitis. They sometimes phagocytosed the *M. bovis*-infected neutrophils, as shown in Fig. 16.

TEM examinations revealed *Mycoplasma* spp. located extracellularly within the necrotic lesion. The organisms were also attached to the cell borders of major neutrophils, as well as to the intracytoplasm of phagocytic macrophages in the bronchioles, as shown

in Figs. 17a and 17b. *Mycoplasma* was mostly round to oval shaped and approximately 270–600 nm (mean 360 nm) in diameter, with sparse granules in their

cytoplasm. These findings correspond with those of the *M. bovis*-positive area observed in IHC.

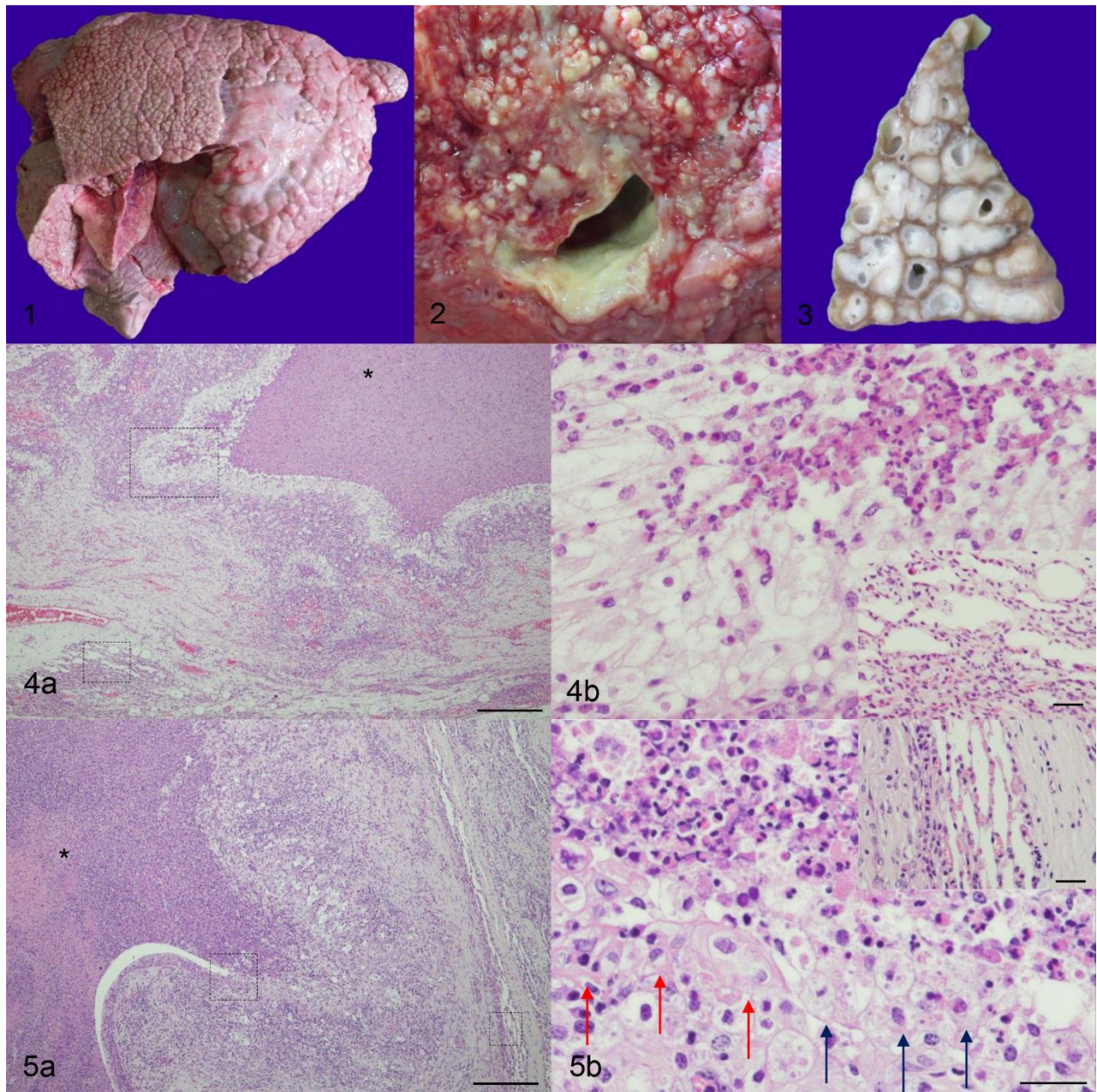


Figure 1 The gross appearance of pneumonia associated with *M. bovis* infections in Japanese Black cattle shows diffuse-like whitish to yellowish lesions in the entire lung lobe in case 2.

Figure 2 The lung cut surface of pneumonia associated with *M. bovis* infections in Japanese Black cattle shows the caseous material exudate oozing from the lung parenchyma in case 9.

Figure 3 The cut surface of the lung shows a nodule-like appearance at the site of the bronchi and/or bronchioles and lung parenchyma in a formalin-fixed sample of case 1.

Figure 4 Granulomatous bronchiolitis and mild alveolitis in case 8, hematoxylin and eosin (H&E) staining.

(4a): The caseonecrotic granuloma at the bronchiolar area; necrotic area (asterisk). The dotted square frames show high magnification of the bronchiolar and alveolar areas in Figure 4b and inset, H&E, bar = 300 μ m.

(4b): The degenerative neutrophils are surrounded by epithelioid macrophages, which are morphologically similar to the bronchiolar epithelial cells at the bronchiolar area, H&E, bar = 30 μ m. Inset: The alveolar area, which is compressed by the bronchioles and show scarce inflammatory cell infiltration, H&E, bar = 30 μ m.

Figure 5 Granulomatous bronchiolitis and mild alveolitis in case 9, hematoxylin and eosin (H&E) staining.

(5a): The caseonecrotic granuloma at the damaged and disappeared areas of the bronchiolar epithelial cells, while some the bronchiolar epithelial cells remain. Cases of necrotic granulomas occur while some bronchiolar epithelial cells remain. The macrophages become epithelioid cells and are activated to transition to the granulomatous formation with the necrotic area (asterisk). The dotted square frames show the high magnification of the bronchiolar and alveolar areas in figure 5b and inset, H&E, bar = 300 μ m.

(5b): The remaining epithelial cells (red arrow) and epithelioid macrophages (blue arrow) are lining the bronchiole, H&E, bar = 30 μ m. Inset: The alveolar area, which shows minimal alveolitis, H&E, bar = 30 μ m.

Table 1 Comparison of histopathological severity and *Mycoplasma bovis* (*M. bovis*) antigenic distribution

Main histopathological changes	Number of detected cases	Number of cases in bronchiolar changed detection								Number of cases in alveolar changed detection							
		Severity of bronchiolitis				<i>M. bovis</i> antigen detection				Severity of alveolitis				<i>M. bovis</i> antigen detection			
		+++	++	+	-	+++	++	+	-	+++	++	+	-	+++	++	+	-
Granulomatous bronchiolitis	11	11	0	0	0	7	4	0	0	1	1	9	0	0	1	7	3
Caseonecrotic bronchiolitis	4	3	0	1	0	2	1	1	0	2	1	0	1	0	1	1	2
Suppurative bronchiolitis	3	2	1	0	0	1	1	1	0	0	2	1	0	0	1	2	0

Remark: The histopathological severity of the pathological lesions in the lung was semi-quantitatively graded as none (-), minimal (+), moderate (++) or severe changes (+++)

Supplementary Table 1 The cattle's clinical signs information

Clinical signs	No. of cases
Pneumonia	24
Pneumonia and suspected endocarditis	2
Pneumonia and emaciation	2
Pneumonia and arthritis	3
Pneumonia and otitis	2
Pneumonia, emaciation, and suspected endocarditis	1
Pneumonia, emaciation, and otitis	1

Supplementary Table 2 *Mycoplasma bovis* (*M. bovis*) associated pathological findings and the antigen distribution of pneumonia and bacterial isolation

No. of cattle	Months of age	Gross appearance	Histopathological changes						Brown-Hopps staining		<i>M. bovis</i> and other bacteria isolation and detection
			Bronchioles			Alveoli			<i>M. bovis</i> lesion area	Other area	
			Diagnosis of bronchiolitis	Destruction of epithelium*	Severity of bronchiolitis	<i>M. bovis</i> antigen detection	Severity of alveolitis	<i>M. bovis</i> antigen detection			
1	5	Cranioventral	Granulomatous	++	+++	++	+	+	-	-	-
2	3	Diffuse-like	Granulomatous ^c	++	+++	+++	+	-	-	-	-
3	6	Cranioventral	Caseonecrotic	-	+	+	-	-	-	-	-
4	6	Cranioventral	Caseonecrotic	+	+++	+++	+++	++	-	-	-
5	3	Cranioventral	Granulomatous ^c	+++	+++	+++	++	++	-	-	-
6	4	Cranioventral	Suppurative	++	+++	+	+ ^c	+	-	-	-
7	5	Diffuse-like	Granulomatous ^c	+++	+++	+++	+	+	-	-	-
8	5	Cranioventral	Granulomatous ^c	+++	+++	+++	+	+	-	-	<i>P. multocida</i>
9	4	Cranioventral	Granulomatous	++	+++	++	+	+	-	-	<i>P. multocida</i>
10	3	Cranioventral	Granulomatous ^c	+++	+++	+++	+	+	-	-	<i>P. multocida</i>
11	9	Cranioventral	Granulomatous ^{c,d}	++	+++	+++	+	-	-	-	<i>P. multocida</i>
12	4	Cranioventral	Granulomatous ^c	+++	+++	+++	+ ^c	-	-	-	<i>P. multocida</i>
13	9	Diffuse-like	Granulomatous ^{c,d}	+++	+++	++	+ ^c	+	+ ^a	+ ^a	<i>P. multocida</i>
14	5	Cranioventral	Granulomatous	+	+++	++	+++ ^{e,f}	+	+++ ^a	+ ^a	<i>P. multocida</i> <i>M. hemolytica</i>
15	12	Cranioventral	Caseonecrotic	+	+++	++	+++ ^f	-	+ ^a	++ ^a	<i>P. multocida</i>
16	11	Cranioventral	Caseonecrotic ^c	++	+++	+++	++ ^f	++	+++ ^a	++ ^b	<i>P. multocida</i> <i>T. pyogenes</i>
17	2	Cranioventral	Suppurative ^d	+	+++	++	++	+	+++ ^b	+ ^b	<i>T. pyogenes</i>
18	8	Cranioventral	Suppurative	+	++	+++	++	++	+ ^a	+ ^b	<i>T. pyogenes</i> <i>B. trehalosi</i>

Remark: The histopathological severity of the pathological lesions in the lung, Brown-Hopps staining, and the IHC was semi-quantitatively graded as none (-), minimal (+), moderate (++) or severe changes (+++); ^a (Gram negative / coccobacilli); ^b (Gram positive / coccobacilli); ^c (with mineralization); ^d (with lymphoid follicle); ^e (with neovascularization); ^f (fibrinonecrotic); * (Confirmed by AE1/AE3 antibody)

Discussion

Eighteen out of 35 pneumonic calves were associated with *M. bovis* infection and were pathologically investigated. Regarding the gross lesions in the 18 *M. bovis*-infected lungs, other organ lesions in these cases might be related to *M. bovis* infections or other factors in BRDC. However, bronchopneumonia associated with *M. bovis* infection was confirmed in all 18 cases with varying degrees of bronchiolitis and alveolitis. Almost all the cases clearly showed cranioventral lesions consisting of varying sizes of multiple white nodules exhibiting pus, except for an entire lobe of diffuse-like pneumonia with pus. There is limited information in the literature regarding the histopathology of natural *M. bovis*-related pneumonia with a growing site, that is, the antigen

distribution in BRDC. Further, a distinction has been highlighted in the degree of inflammation between bronchiolitis and alveolitis, which some researchers call bronchopneumonia (Caswell and Archambault, 2008). Generally, bronchopneumonia has been found to be acute or subacute with less than severe inflammation in the bronchioles. Therefore, we focused on the pathological investigation of lesions of the bronchioles and alveoli associated with *M. bovis* antigens.

In this study, histopathological changes were observed mainly in the bronchiolar region and were extremely mild in the alveolar region, except for three of the 18 cases. Interestingly, 11 cases of granulomatous bronchiolitis, 4 cases of caseonecrotic bronchiolitis, and 3 cases of suppurative bronchiolitis associated with *M. bovis* infections were observed, with mostly mild to

moderate alveolar inflammation. The latter phenomena are similar to previous descriptions elsewhere (Adegboye *et al.*, 1995; Caswell and Archambault, 2008; Gagea *et al.*, 2006). The main histopathological changes associated with *M. bovis* infections were observed in the bronchioles, with varying degrees of change in the alveoli. The expansion of bronchiolitis (bronchiolactasis) was also observed. As the bronchi, including bronchial glands and cartilage connected to the bronchioles, could not be found in the site harboring *M. bovis*, cases in this study might have included bronchitis along with bronchiolitis. This finding is similar to that of a study by Gagea *et al.* (2006), which suggested that *M. bovis* necrotic lesions originated from the bronchioles or small bronchi and that *M. bovis* invaded the bronchioles through the trachea, causing bronchiolitis with necrosis. The lesions sometimes extended into the alveoli, where they displayed antigen expression and inflammation. Existing *M. bovis* in neutrophils were strongly distributed in the bronchioles, resulting in space-occupying necrosis over time and the destruction of the bronchiolar epithelium. *M. bovis* antigens were clearly distributed in the alveolar spaces. Although *M. bovis* infections were commonly diagnosed as bronchopneumonia, bronchiolitis was consistently and completely more severe than alveolitis, leading to a complete obstruction in the bronchiole that prevented air aspiration. Hence, we decided to consider the severity of the lesions in terms of bronchiolitis and alveolitis, rather than in terms of bronchopneumonia, for a better understanding of pathogenesis. Lymphoid follicles were detected in three cases in this study, although it was a common pathological change noted in previous *M. bovis* studies (Adegboye *et al.*, 1995; Hermeyer *et al.*, 2012; Hermeyer *et al.*, 2011).

Granulomatous bronchiolitis was a major histological change in this study. However, to our knowledge, a granulomatous formation in bronchioles in which morphological changes of macrophages resemble bronchiolar epithelial cells associated with *M. bovis* infection and confirmation of the granulomatous formation associated with *M. bovis* using immunohistochemistry (IHC) have not been reported elsewhere. Mainly, *M. bovis*-infected neutrophils accumulated and were later necrotized in the lumen of the bronchioles, causing lesions known as necrotic bronchiolitis. Consequently, the bronchiolar epithelia disappeared, and macrophages accumulated as epithelioid macrophages surrounded the necrotic area, as shown in Figs. 4 and 8. These epithelioid macrophages had large cytoplasm and were morphologically similar to bronchiolar epithelial cells, which are sometimes difficult to differentiate by histology. IHC is valuable and necessary for differentiating these cells. Although these epithelioid cells were morphologically similar to bronchiolar epithelial cells, they were labeled with the anti-Iba1 antibody rather than with the anti-AE1/AE3 antibody, clarifying that they were epithelioid macrophages forming granulomas. However, granulomatous inflammation in the lung associated with *M. bovis* has been reported (Janardhan *et al.*, 2010; Khodakaran-Tafti

and López, 2004). The histological of granulomatous formation did not show an epithelioid morphology. According to the transitional stage of granulomatous formation in this study, epithelioid macrophages with the remaining normal bronchiolar epithelial location were seen, as well as the advanced stage, in which completely epithelioid macrophages instead of bronchiolar epithelium surrounded the caseonecrotic material. This might suggest that epithelioid macrophages are the characteristic of granulomatous inflammation at the bronchioles associated with the advanced stage of *M. bovis* infection.

That *M. bovis* preferentially existed and/or grew in neutrophils and degenerated in these cells in all cases, with fewer occurrences in macrophages, was confirmed by IHC and double IHC staining, as shown in Figs. 6–16. The MAC387 antibody was employed as a useful marker for neutrophils, according to the findings of Grosche *et al.* (2008), who detected calprotectin expression in neutrophils and those of Kadota *et al.* (2017), and Sekiguchi *et al.* (2019), who reported the antibody as a specific marker for bovine neutrophils. Although other antibodies for neutrophil detection, such as the rabbit polyclonal anti-neutrophil elastase antibody (Abcam, Tokyo, Japan) were also tried, only the MAC387 antibody was successful for bovine neutrophil detection. Only a few mononuclear cells of macrophages were labeled with the anti-MAC387 antibody and they were rarely discovered in the Iba1-labeled region. These results revealed that *M. bovis* preferentially exists in neutrophils rather than in intralesional macrophages during infection. These findings are consistent with those of Gondaira *et al.* (2020), who reported that the major cell recruitment of *M. bovis*-associated mastitis is performed by neutrophils. However, our results are inconsistent with those of the previous studies that found *M. bovis* antigen mainly in the cytoplasm of macrophages and at the border of necrotic lesions (Caswell and Archambault, 2008; Hermeyer *et al.*, 2011; Margineda *et al.*, 2017; Rodríguez *et al.*, 1996; Thomas *et al.*, 1986). Moreover, our IHC and double IHC results were consistent with the results of the TEM examination, which revealed that *M. bovis* organisms preferred to locate in cytoplasmic neutrophils more than in macrophages admixing within the necrotic lesion in the bronchiole, as shown in Figs. 15 and 17. Some of the *M. bovis* organisms showed lucent cytoplasm in Fig. 17, reflecting the degenerative process.

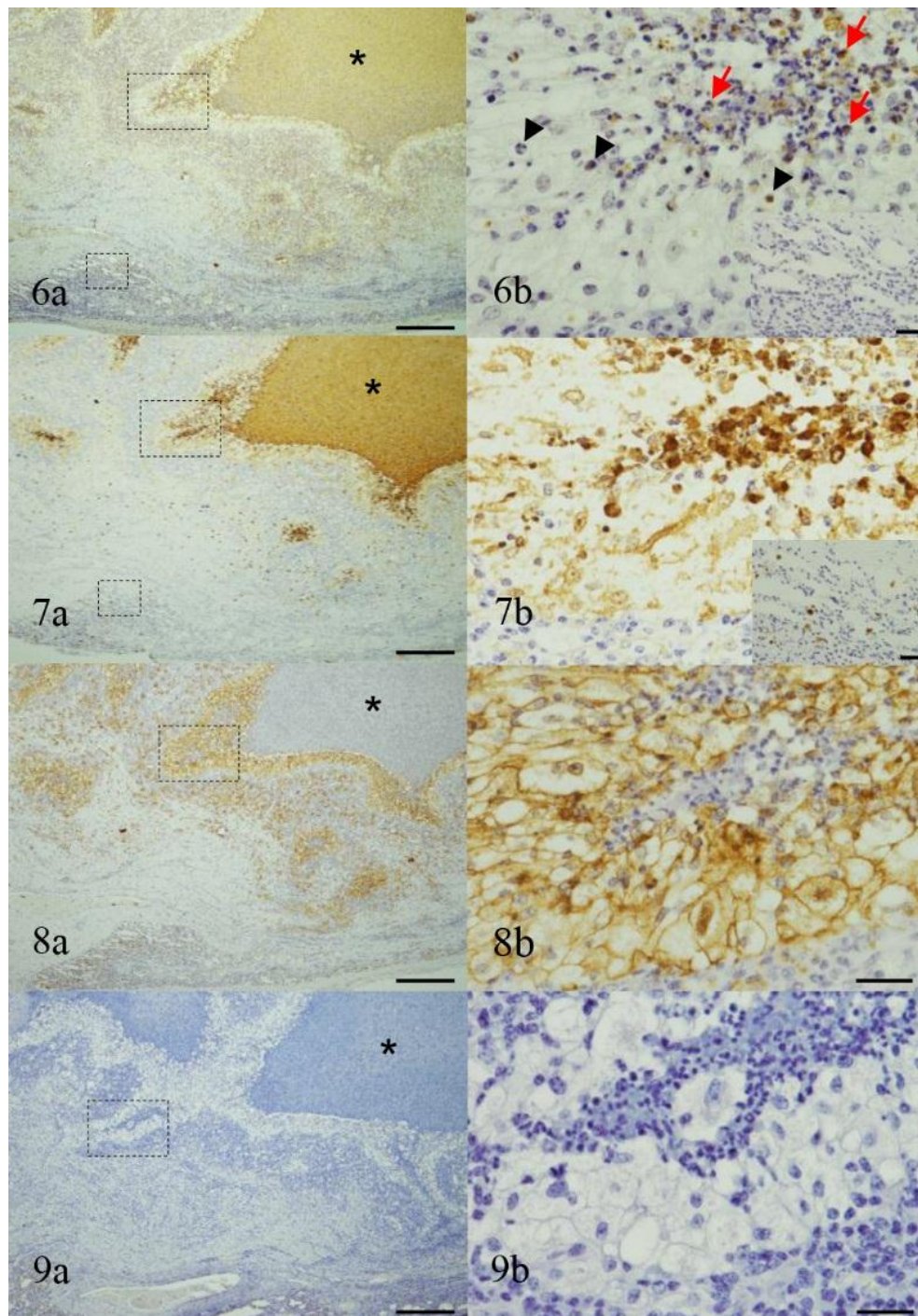


Figure 6-9 Severe granulomatous bronchiolitis and mild alveolitis in case 8, immunohistochemistry (IHC) staining.

(6a): The brown colored *M. bovis* antigens are observed in the lesion of caseonecrosis (asterisk) in the bronchiolar area and in some macrophages, IHC stained anti-*M. bovis*, bar = 300 μ m. The dotted square frames show high magnification of the bronchiolar and alveolar areas in Figure 6b and inset.

(6b): The *M. bovis* antigen is detected at the periphery of the neutrophils (red arrows), degenerative neutrophils and caseonecrotizing foci in contact with epithelioid macrophages (black arrowheads) that contain degenerative neutrophils, IHC stained anti-*M. bovis*, bar = 30 μ m. Inset: *M. bovis* antigen is not observed in the alveolar area, IHC stained anti-*M. bovis*, bar = 30 μ m.

(7a): The MAC387-labeled neutrophils are detected in the same area as *M. bovis* antigenic detection, IHC stained anti-MAC387, bar = 300 μ m; necrotic area (asterisk). The dotted square frames show the high magnification of the bronchiolar and alveolar areas in Figure 7b and inset, IHC stained anti-MAC387.

(7b): High magnification of the bronchiolar area, IHC stained anti-MAC387, bar = 30 μ m. Inset: Some MAC387 positive cells are detected in the alveolar area, IHC stained anti-MAC387, bar = 30 μ m.

(8a): The epithelioid cells of the macrophages are labeled with IHC stained anti-Iba1; necrotic area (asterisk). The dotted square frame shows the bronchiolar's high magnification in Figure 8b, IHC stained anti-Iba1, bar = 300 μ m.

(8b): The Iba1-labeled macrophages show morphology similar to the epithelial cells, IHC stained anti-Iba1, bar = 30 μ m.

(9a): The complete disappearance of the bronchiolar epithelial cells is confirmed by the absence of AE1/AE3 labeling by IHC staining; necrotic area (asterisk). The dotted square frame shows the bronchiolar's high magnification in Figure 9b, IHC stained anti-AE1/AE3, bar = 300 μ m.

(9b): High magnification of the bronchiolar area shows no epithelial lining, IHC stained anti-AE1/AE3, bar = 30 μ m.

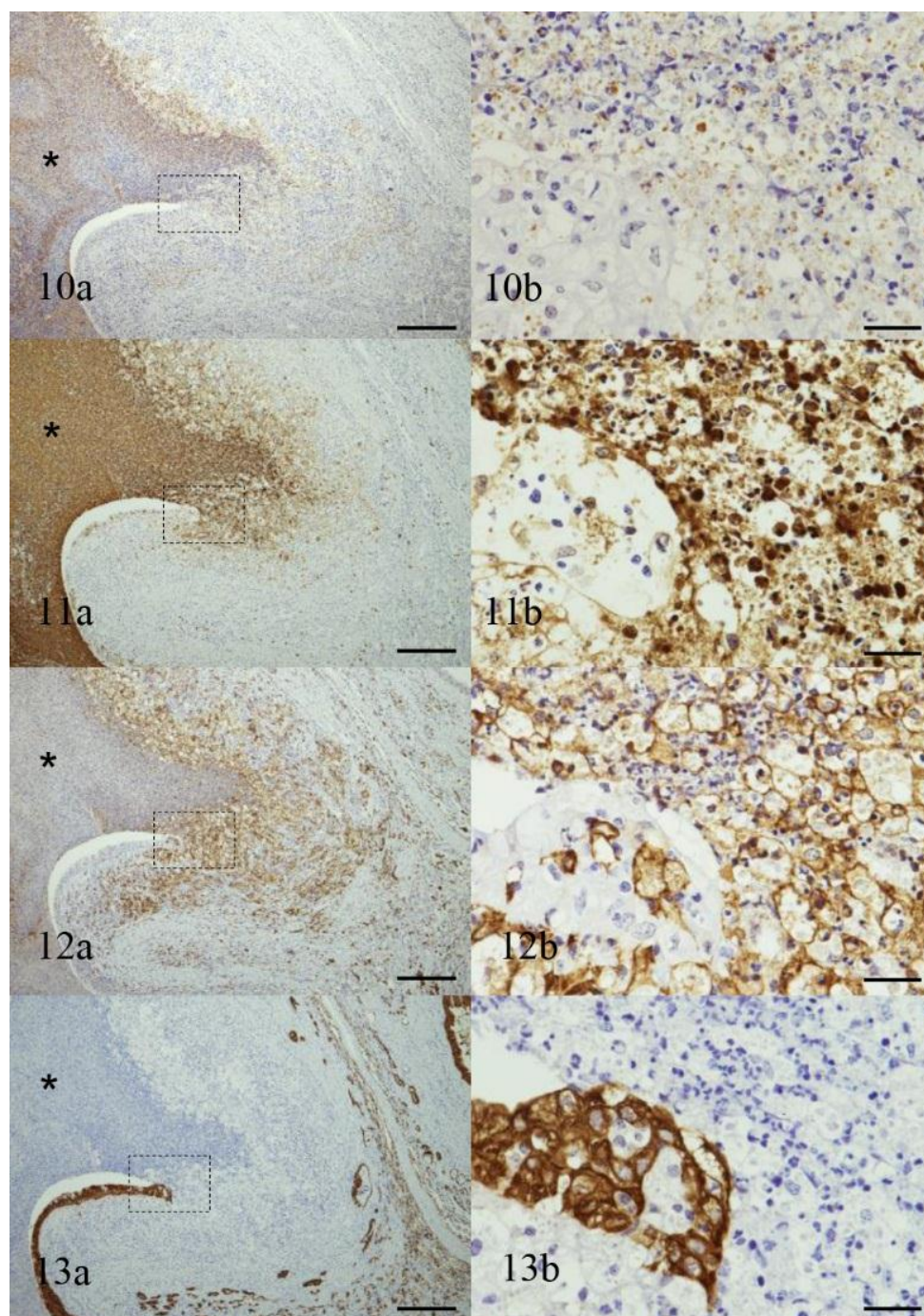


Figure 10-13 Forming of granulomatous bronchiolitis and mild alveolitis in case 9, immunohistochemistry (IHC) staining.

(10a): The brown colored *M. bovis* antigens are observed in the lesions of caseonecrosis in the bronchiolar area; necrotic area (asterisk). Some bronchiolar epithelial cells are destroyed by the *M. bovis*-labeled inflammatory cells. The epithelial cells that are not directly attached to *M. bovis*-labeled inflammatory cells remain. The dotted square frame shows the bronchiolar's high magnification in Figure 10b, IHC stained anti-*M. bovis*, bar = 300 μ m.

(10b): The brown colored *M. bovis* antigens are observed extracellular and/or in the inflammatory cells in bronchioles, IHC stained anti-*M. bovis*, bar = 30 μ m.

(11a): The MAC387-labeled neutrophils are detected in the same area as *M. bovis* antigenic detection, IHC stained anti-MAC387, bar = 300 μ m.; necrotic area (asterisk). The dotted square frame shows the bronchiolar's high magnification in Figure 11b.

(11b): The degenerated neutrophil and neutrophil are positive to the anti-MAC387 antibody, IHC stained anti-MAC387, bar = 30 μ m.

(12a): The epithelioid cells of the macrophages are labeled with anti-Iba1; necrotic area (asterisk). The epithelioid macrophages, which are similar to the bronchiolar epithelial cells, are labeled with anti-Iba1, while some remaining epithelial cells are not labeled with anti-Iba1. The dotted square frames show the bronchiolar's high magnification in Figure 12b, IHC stained anti-Iba1, bar = 300 μ m.

(12b): Iba1-labelled epithelioid macrophages are detected, while the remaining epithelial cells are negative to the anti-Iba1 antibody, IHC stained anti-Iba1, bar = 30 μ m.

(13a): The remaining bronchiolar epithelial cells are labeled with anti-AE1/AE3, IHC, bar = 300 μ m.; necrotic area (asterisk). The dotted square frames show the bronchiolar's high magnification in Figure 13b.

(13b): The AE1/AE3-labeled remaining epithelial cells are detected, IHC stained anti-AE1/AE3, bar = 30 μ m.

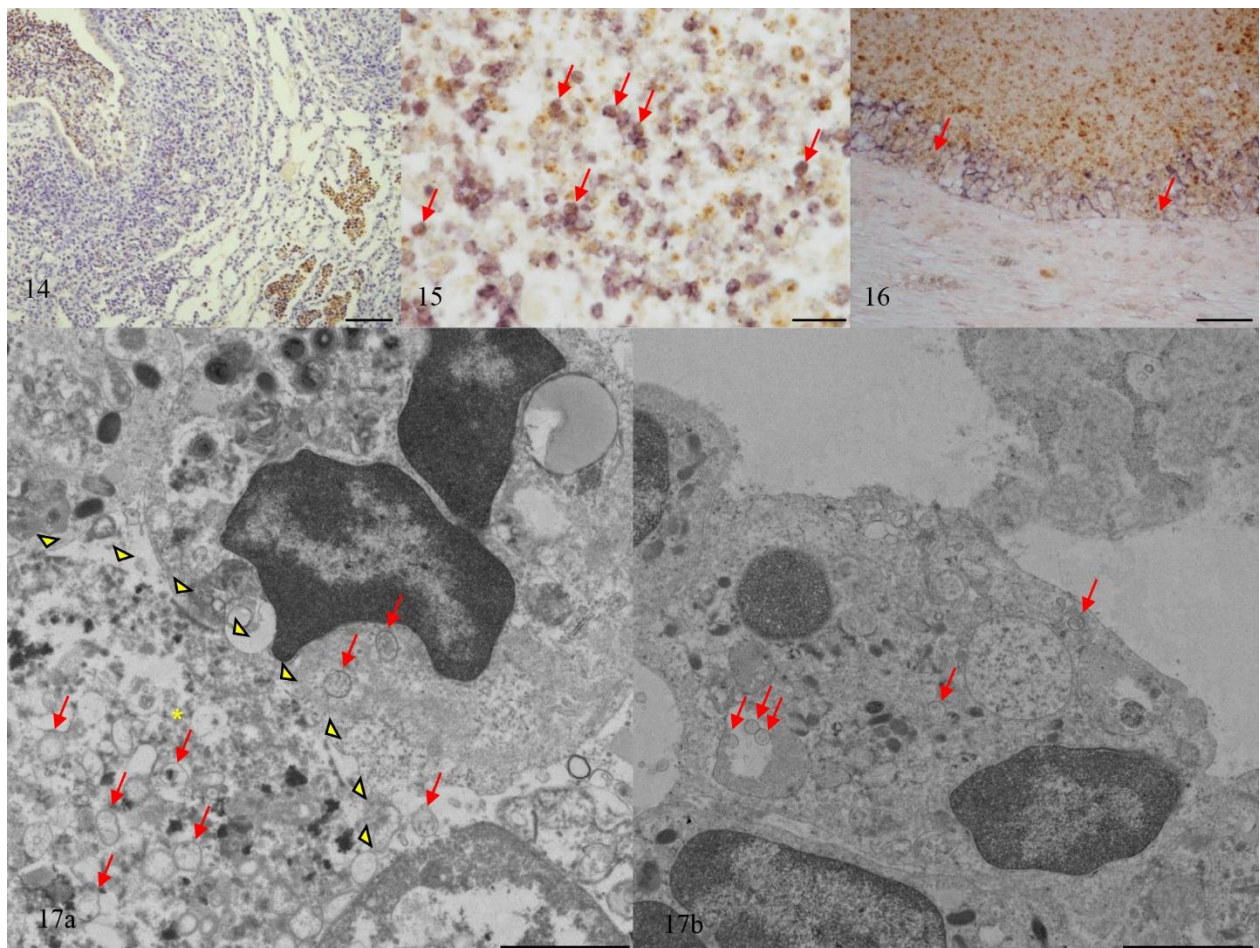


Figure 14 Mild alveolitis associated with *M. bovis* infection in case 1, Immunohistochemistry (IHC) stained anti-*M. bovis*, bar = 100 μ m. The *M. bovis* antigens are observed within the neutrophils in the bronchiolar and alveolar areas. The infiltrated neutrophils did not show strong degeneration in this case, which does not often occur.

Figure 15 Detection of the *M. bovis* antigen in the neutrophils of the bronchiolar caseonecrotic exudate in case 9. Double IHC staining shows a brown colored *M. bovis* antigen and purple colored MAC387-labeled neutrophil. *M. bovis* antigens are detected in the cytoplasm of the neutrophils (red arrow), and some cells have already degenerated so are faintly stained, double IHC, bar = 30 μ m.

Figure 16 Detection of the *M. bovis* antigen and macrophage of the bronchiolar caseonecrotic exudate in case 8. Double IHC staining shows a brown colored *M. bovis* antigen and purple colored Iba1-labeled epithelioid macrophage. *M. bovis* antigens are sometimes seen in the epithelioid macrophages (red arrow), double IHC, bar = 60 μ m.

Figure 17 Detection of the *M. bovis* antigen and macrophage of the bronchiolar caseonecrotic exudate in case 8. Double IHC staining shows a brown colored *M. bovis* antigen and purple colored Iba1-labeled epithelioid macrophage. *M. bovis* antigens are sometimes seen in the epithelioid macrophages (red arrow), double IHC, bar = 60 μ m.

(17a): *M. bovis* organisms (red arrow) are seen in intracytoplasmic neutrophil and degenerative neutrophil (asterisk). The cell membrane of the degenerative neutrophil is located by the yellow arrowhead. Some *M. bovis* organisms show the lucent cytoplasm, which reflects the degenerative process, TEM, bar = 2.0 μ m.

(17b): *M. bovis* organisms are seen inside the cytoplasm of the macrophage. The phagocytic vacuole, which contains possibly degenerated neutrophils containing *M. bovis* organisms, is found in the cytoplasm of the macrophage, TEM, bar = 2.0 μ m.

IL-8 is a cytokine produced by a variety of cells, including macrophages, endothelial cells, fibroblasts, lymphocytes and neutrophils and is primarily a neutrophil-specific chemoattractant (Jiemtaweeboon *et al.*, 2011; Mitchell *et al.*, 2003). The role of IL-8 produced by neutrophils associated with *M. bovis* infections is important because many neutrophils infiltrate the bronchioles and/or alveoli. Furthermore, we found IL-8-positive intracytoplasmic neutrophils containing *M. bovis* antigens in all cases. By contrast, Rodríguez *et al.* (2015), and Valsala *et al.* (2017), reported that the significant expressions of tumor necrosis factor- α , IL-4, IL-10 and interferon- γ activated by macrophages were associated with *M. bovis* pneumonic lesions and that IL-8 expression

contributed less to the lesions. However, Nishi *et al.* (2019), reported that one cytokine expression that significantly increased in cattle arthritis associated with *M. bovis* infections was IL-8. In the present study, the detection of other cytokines was not performed but IL-8 expression was detected in lesions positive for *M. bovis* in all cases. Therefore, IL-8 cytokines might be one of the most important cytokines associated with *M. bovis* infections. Further, a higher degree of T lymphocyte than B lymphocyte infiltration was detected in the middle and basal areas of the bronchiolar wall. This finding remains controversial in previous studies (Dudek *et al.*, 2020; Razin *et al.*, 1998; Vanden Bush and Rosenbusch, 2002), in which both T and B lymphocytes could be activated during *M. bovis*

infections, especially T lymphocytes, whereas suppression of T lymphocytes during *M. bovis* infections has been reported in vivo (Goto et al., 2017; Vanden Bush and Rosenbusch, 2002).

M. bovis was detected with the occurrence of other bacterial pathogens in 11 cases. These other bacteria included *P. multocida*, *M. hemolytica* and *B. trehalosi*, which belong to the family Pasteurellaceae, Gram-negative coccobacilli and *T. pyogenes*, Gram-positive coccobacilli formerly classified as *Actinomyces pyogenes*. The presence of these bacteria in isolation was consistent with the Brown-Hopps stain results, which demonstrated coccobacilli in the lesions. Although IHC staining was not performed to confirm these bacteria in this study, histopathological lesions of fibrinonecrotic alveolitis, which are not characteristic of *M. bovis* infections, were observed in three cases. Although bacterial organisms were found at the site of *M. bovis*-associated lesions in some cases, their degree of severity and main histopathological changes did not differ from single *M. bovis* infection-associated lesions, especially in bronchioles. This finding is inconsistent with those of Ball and Nicholas (2010) and Hemeyer et al. (2012), who reported that severe caseonecrotic lesions mainly occur when other bacteria are present. However, viral coinfection could not be ruled out in this study. Grissett et al. (2015), and Szeredi et al. (2010), reported that virus pathogens in BRDC were commonly isolated within two weeks post-infection, and viral pathogens could already clear up from the lung by the time of examination. Thus, *M. bovis* might be one of the important bacteria that persist and cause lung lesions, a condition related to quite severe bronchiolitis in this study.

Overall, our findings suggest that *M. bovis* associated with severe space-occupying bronchiolitis and mild alveolitis were found in the cranioventral region in almost all cases, and that caseonecrotic granulomatous bronchiolitis occurred in the lungs of 2- to 12-month-old Japanese Black calves with respiratory symptoms. IHC was useful and necessary for determining the stages of the granulomatous reaction and antigen distribution associated with *M. bovis* infection. The severity of bronchiolitis was consistent with the severity of *M. bovis* antigen detection. Notably, neutrophils were the preferred inflammatory cells for the existence and/or growth of *M. bovis* associated with pneumonia, which causes severe chronic respiratory diseases in cattle.

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