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Mycoplasma bovis May Travel Along the Eustachian Tube to Cause Meningitis in Japanese Black Cattle

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Summary

Mycoplasma bovis (*M. bovis*) is a common inhabitant of the upper and lower respiratory tracts of cattle and is considered to be the main aetiological agent of otitis media in calves. The eustachian tube appears to be the most common portal for pathogens to enter the middle ear. We investigated the transmission route of *M. bovis* causing otitis media that progressed to meningitis or meningoencephalitis in Japanese Black cattle. *M. bovis* was detected in 10 cases by a loop-mediated isothermal amplification method or by immunohistochemistry. One case of caseonecrotic granulomatous meningoencephalitis, one case of caseonecrotic granulomatous meningitis, one case of suppurative meningoencephalitis, eight cases of eustachitis, nine cases of tonsillitis and six cases of suppurative bronchopneumonia were identified by histopathological examination. *M. bovis* antigen was detected in the eustachian tubes of eight cases. In nine cases, *M. bovis* was also detected in tonsillar epithelial crypts and lumina, in intraluminal inflammatory cells and in the epithelial cells of minor salivary glands located around the eustachian tubes and tonsils. The results suggest that *M. bovis* can infect and colonize the tonsils and enter the eustachian tubes, causing otitis media, which, in cases of chronic infection, can progress to meningitis.

Keywords: eustachitis; granulomatous meningitis; oropharynx; tonsil

Mycoplasma bovis (*M. bovis*) is an important primary pathogen and a common inhabitant of the upper and lower respiratory tracts of cattle (Gagea *et al*, 2006; Radaelli *et al*, 2008; Waites and Atkinson, 2009) following infection by the respiratory or oral route (Maunsell *et al*, 2011, 2012). Bacterial suppurative meningitis and meningoencephalitis in cattle have been associated with several pathogens, including *Escherichia coli*, *Haemophilus somnus*, *Listeria monocytogenes*, *Arcanobacterium pyogenes*, *Manheimia haemolytica*, *Streptococcus* spp and *Pasteurella* spp (Konradt *et al*, 2017). In addition, meningitis or meningoencephalitis caused by *M. bovis* has been reported in progressive cases of otitis media as a result of direct dissemination (Maeda *et al*, 2003; Lamm *et al*, 2004).

Otitis media in calves has been reported as being caused by *H. somnus*, *Pasteurella multocida*, *Streptococcus* spp, *Actinomyces* spp, the ear mite *Raillietia auris* and *M. bovis*. Otitis media causes progressive otitis interna and meningitis characterized by ataxia, recumbency and nystagmus (Walz *et al*, 1997). The main aetiological agent of otitis media in calves is believed to be *M. bovis*, alone or in combination with other bacteria (Maeda *et al*, 2003; Bertone *et al*, 2015). The identified

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routes of transmission are extension of external ear infection, colonization of the oropharynx, extension via the eustachian tube and haematogenous spread (Walz *et al*, 1997; Francoz *et al*, 2004; Lamm *et al*, 2004).

Cattle have five tonsils: the lingual, palatine, pharyngeal and tubal tonsils and the tonsil of the soft palate (Casteleyn *et al*, 2011). The major salivary glands of cattle are the parotid and mandibular glands, which are pure serous glands and mixed seromucous glands, respectively (Mallikarjunappa *et al*, 2019). Minor salivary glands are characterized by mucus-producing acini and are distributed throughout the oral cavity (Hand *et al*, 1999; Mallikarjunappa *et al*, 2019; Porcheri and Mitsiadis, 2019). The purpose of this study was to investigate the distribution of *M. bovis* in the tonsils, eustachian tubes, meninges and brain tissue in natural cases of *M. bovis* infection in Japanese Black cattle in order to better understand the pathogenesis of meningitis and meningoencephalitis associated with otitis media.

Ten Japanese Black cattle in southern Kyushu, aged 2−17 months, which had clinical signs of suspected *M. bovis* infection (Table 1) and had poor prognoses, were necropsied. The tonsils, eustachian tubes, temporal bone (case no. 2), lungs, meninges and brains were collected for *M. bovis* detection and histopathological examination. The Animal Care and Use Committee of the University of Miyazaki approved the experimental procedures (No. 2015−006). All tissue samples were screened for *M. bovis* using a loop-mediated isothermal amplification (LAMP) method specific to *M. bovis* (Higa *et al*, 2016). Other potential bacterial infections of the eustachian tubes were excluded using conventional polymerase chain reaction (PCR) (Supplementary Table 1). Brain and meninges samples or caseous material from the brain and meninges were cultured on *Mycoplasma* NK broth (Kanto Chemical, Tokyo, Japan) and *Mycoplasma* NK agar (Kanto Chemical) for *M. bovis* isolation. Other bacterial isolations were performed on 5% blood agar and colony-forming bacteria were identified using a matrix-assisted laser desorption/ionization biotyper (Bruker Dultovics, Bremen, Germany). For histological examination, the tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE). The formalin-fixed temporal bone (case no. 2) was decalcified using 10% formic acid before processing. Histopathological lesions of inflammation of the eustachian tubes were graded on a scale from I to III, as described by Maunsell *et al* (2012).

Table 1
***Mycoplasma bovis* (*M. bovis*) detection and histopathological changes in tissues of Japanese Black cattle**

Case no.	*Age (Sex)*	*Clinical signs*	M. bovis *detection by LAMP*				*Histopathological changes*					M. bovis *detection by IHC*						
			Tonsil	*Eustachian tube*	*Lung*	*Meninges*	*Tonsillitis*	*Eustachitis*	*Bronchopneumonia*	*Meningitis*[a]*/ meningoencephalitis*[b]	*Tonsil*			*Eustachian tube*		*Lung*	*Meninges*
											A	*B*	*C*	*A*	*B*	*C*		
1	4 m (F)	Neurological signs	ND	ND	ND	P[f]	ND	ND	ND	+++[b]	ND	ND	ND	ND	ND	ND	ND	+++
2	17 m (M)	Ear drooping	P[f]	P[p]	N[f]	P[f]	++	++	+	+++[a]	−	+	+	−	+	−	+	++
3	9 m (M)	Ear drooping	P[f]	P[p]	N[f]	P[f]	++	++	−	+++[b]	+	+	+	−	+	−	+	++
4	2 m (U)	Ataxia	P[f]	P[p]	N[f]	N[f]	+	++	−	−	−	+	−	−	+	−	−	−
5	9 m (F)	Pneumonia	P[f]	P[p]	N[f]	N[f]	++	+	−	−	+	−	++	+	−	−	−	−
6	5 m (F)	Pneumonia	P[f]	P[p]	N[f]	N[f]	+++	+++	+++	−	+	−	++	+	−	−	−	−
7	9 m (M)	Pneumonia	P[f]	ND	P[f]	ND	+++	ND	+++	ND	+	+++	+	ND	ND	+++	+++	ND
8	11 m (F)	Pneumonia	P[f]	P[p]	P[f]	N[f]	+	++	+++	−	++	−	++	−	−	+++	+++	−
9	3 m (F)	Pneumonia, arthritis	P[f]	P[p]	P[f]	N[f]	+++	++	+++	−	++	−	−	+	+	+++	+++	−
10	8 m (M)	Pneumonia	P[f]	P[p]	P[f]	N[f]	+	+	+++	−	+	+++	+	−	−	+++	+++	−

P, positive; N, negative; [f], DNA extracted from fresh sample; [r], DNA extracted from paraffin-embedded sample; [a], meningitis; [b], meningoencephalitis; ND, not done; U, unknown; A, epithelial cells; B, lymphocytes and/or neutrophils; C, minor salivary glands.
Severity of histopathological changes and intensity of immunolabelling graded semiquantitatively as: none, -; minimal, +; moderate, ++; or severe, +++.

Immunohistochemistry (IHC) was performed using a rabbit polyclonal anti-*M. bovis* primary antibody (Kanda *et al*, 2019). Tissue sections from a case of *M. bovis* endocarditis (Kanda *et al*, 2019) was used as a positive control, while normal rabbit serum (Dako Corporation, Carpinteria, California, USA) was used as a negative control. The intensity of IHC labelling was graded semiquantitatively as none (−), minimal (+), moderate (++) or severe (+++).

M. bovis was detected in several organs in all 10 cattle using LAMP detection (Table 1). No other bacteria were detected in any of the eustachian tubes using PCR. In three cases, *M. bovis* colonies were identified in the brain and meninges (cases no. 1–3). Two cases had only *M. bovis* infection (cases no. 1 and 3), while one case had *M. bovis* and *Kocuria rhizophila* infection (case no. 2).

Two cattle developed unilateral or bilateral ear drooping. Caseous material was found at the base of the brain of case no. 3 (Fig. 1a1). This material also filled the parietal meninges and extended to the cerebellum on the other side, covering the brainstem to the medulla oblongata (Fig. 1a2). A nodule (2.0 cm × 1.5 cm) was found on the right side of the medulla oblongata in case no. 2 (Fig. 1b1) and was connected to the facial and vestibulocochlear nerves. The cut surface of the nodule was whitish and irregular with hard mineralized caseous necrosis material in the centre (Fig. 1b2). Partial osteolysis was seen in the right temporal bone of case no. 2 (Fig. 1c1). The cut surface had a whitish, hard texture with caseous necrosis (Fig. 1c2). The right middle ear canal, located in the temporal bone, had an accumulation of yellowish to whitish exudate (Fig. 1c3). There was no exudate in the external ear canal in any of the cases. The anatomical locations of the tonsils, eustachian tubes and brain are shown in Fig. 1d1. The pharyngeal and tubal tonsils cover the opening of the eustachian tubes (Fig. 1d2), which connect to the eustachian canal within the basisphenoid bone (Fig. 1d3). The tonsils had lesions of caseous necrosis, especially the palatine tonsils in case no. 9 (Fig. 1e).

Histological examination revealed two cases of meningoencephalitis and one of meningitis. Caseonecrotic granulomatous meningoencephalitis was seen in case no. 1. Multiple nodules of caseous necrotic material, combined with degenerate neutrophils, neutrophils and macrophages, were surrounded by macrophages and epithelioid cells (Fig. 2a), while mononuclear cell perivascular cuffing was found in the brain. The nodule, seen grossly in case no. 2, comprised caseonecrotic granulomatous inflammation, which was similar to case no. 1 (Fig. 2b), although there were no significant brain changes in this case. Suppurative meningoencephalitis was observed in case no. 3; the meninges were oedematous and had infiltrated neutrophils, degenerate neutrophils and fibrin (Fig. 2c). The temporal bone in case no. 2 also had lesions of caseonecrotic granulomatous inflammation. Necrotic material, neutrophils and macrophages, admixed with bone lysis, were found (Fig. 2d). In addition, eight cases had lesions of eustachitis (Fig. 2e) on at least one side, which were of various degrees of severity (three of grade I, four of grade II, one of grade III) (Table 1). Tonsillitis, characterized by the accumulation of neutrophils and macrophages with cell debris in the crypt or lumen, was also found in nine cases (Table 1 and Fig. 2f). Suppurative bronchopneumonia was present in six cases.

IHC demonstrated *M. bovis* antigen in most neutrophils and in some macrophages, especially at the margins of the meningeal caseonecrotic lesions in three cases (nos. 1–3) (Figs. 3a–c). However, *M. bovis* was not labelled in brain tissue in any case. *M. bovis* was also labelled in neutrophils, macrophages and degenerate cells in the temporal bone of case no. 2 (Fig. 3d). The eustachian tubes contained low numbers of *M. bovis*-labelled inflammatory cells, especially neutrophils, in the lumen and epithelium (Table 1, Figs. 3e and f). The epithelial cells of the minor salivary glands surrounding the eustachian tube were labelled for *M. bovis* in seven cases (Fig. 3f). *M. bovis* was also labelled in the cells in the inflammatory exudate in the tonsil and epithelial cells of the tonsillar crypt (Fig. 3g). However, *M. bovis* antigen was not detected in the major salivary glands in any case. Bronchopneumonia associated with *M. bovis* infection was seen in four cases, but *M. bovis*-labelled cells were identified in the eustachian tubes in only two of these cases.

In this study, two cases of meningoencephalitis and one of meningitis, out of the 10 cases investigated, were observed macroscopically and histopathologically. *M. bovis* antigen was consistently detected using LAMP and IHC, and infection was confirmed by culture of the organism. Interestingly, none of these three cases had pneumonia and *M. bovis* was not detected in lung tissue (case no. 1 was not analysed). Cranial nerves VII and VIII were normal in all cases and no noticeable changes were observed in any other organ. This suggests direct spread of *M. bovis* infection to the meninges by penetration through the adjacent suppurative lesion. Konradt *et al* (2017) hypothesized four pathways of transmission: haematogenous, lymphatic dissemination, direct penetrating lesion and centripetal ascending infection along a peripheral nerve. Our results suggest that direct penetration of *M. bovis* from the infected eustachian tube is a possible transmission

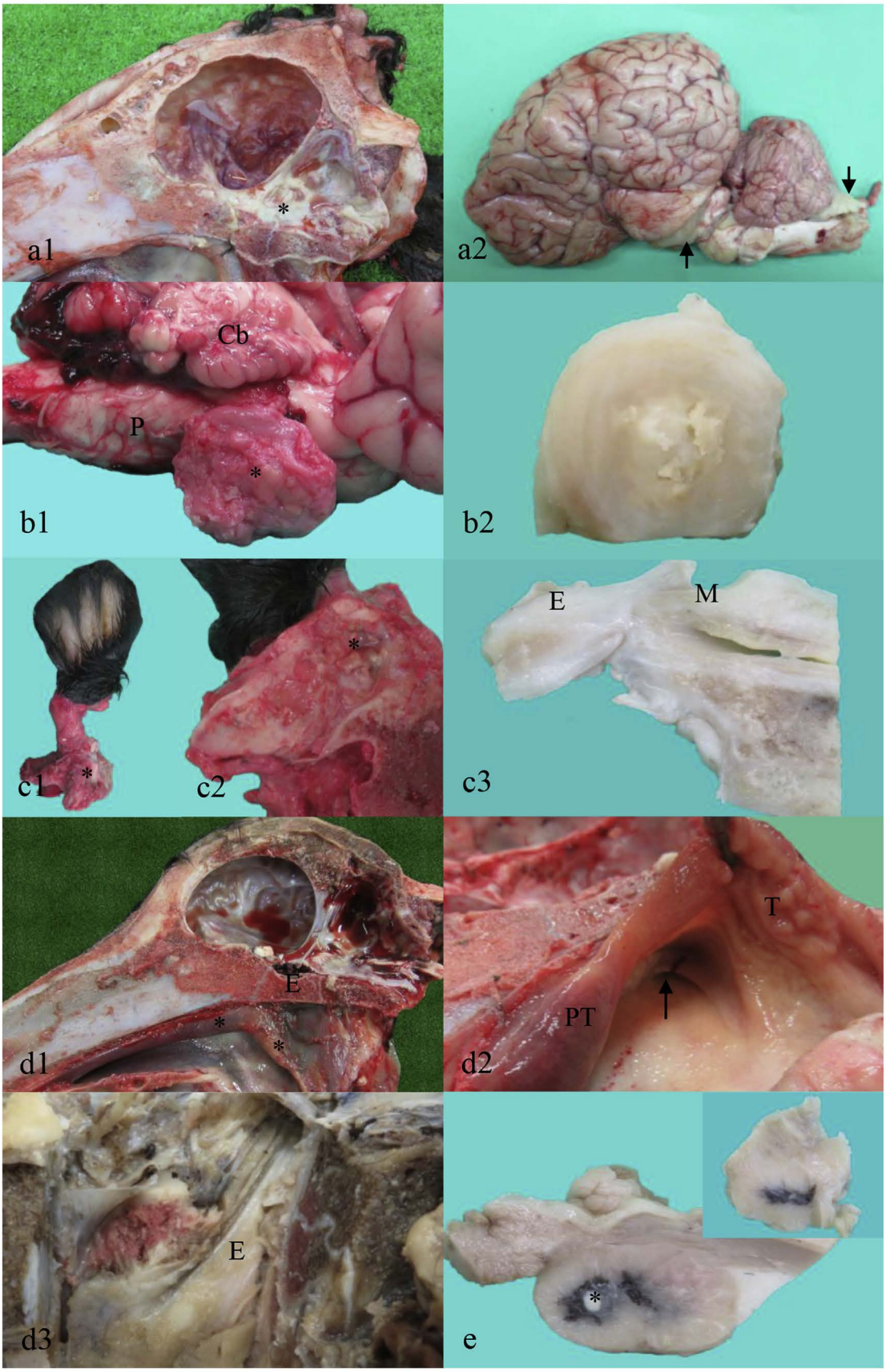

Fig. 1. Meningitis, meningoencephalitis, otitis media, eustachitis and tonsillitis, *M. bovis* infection, calves. (a1) Caseous material in right calvarium at level of cerebellum (*) (case no. 3). (a2) Left hemisphere of brain with yellowish to greenish caseous material in regions

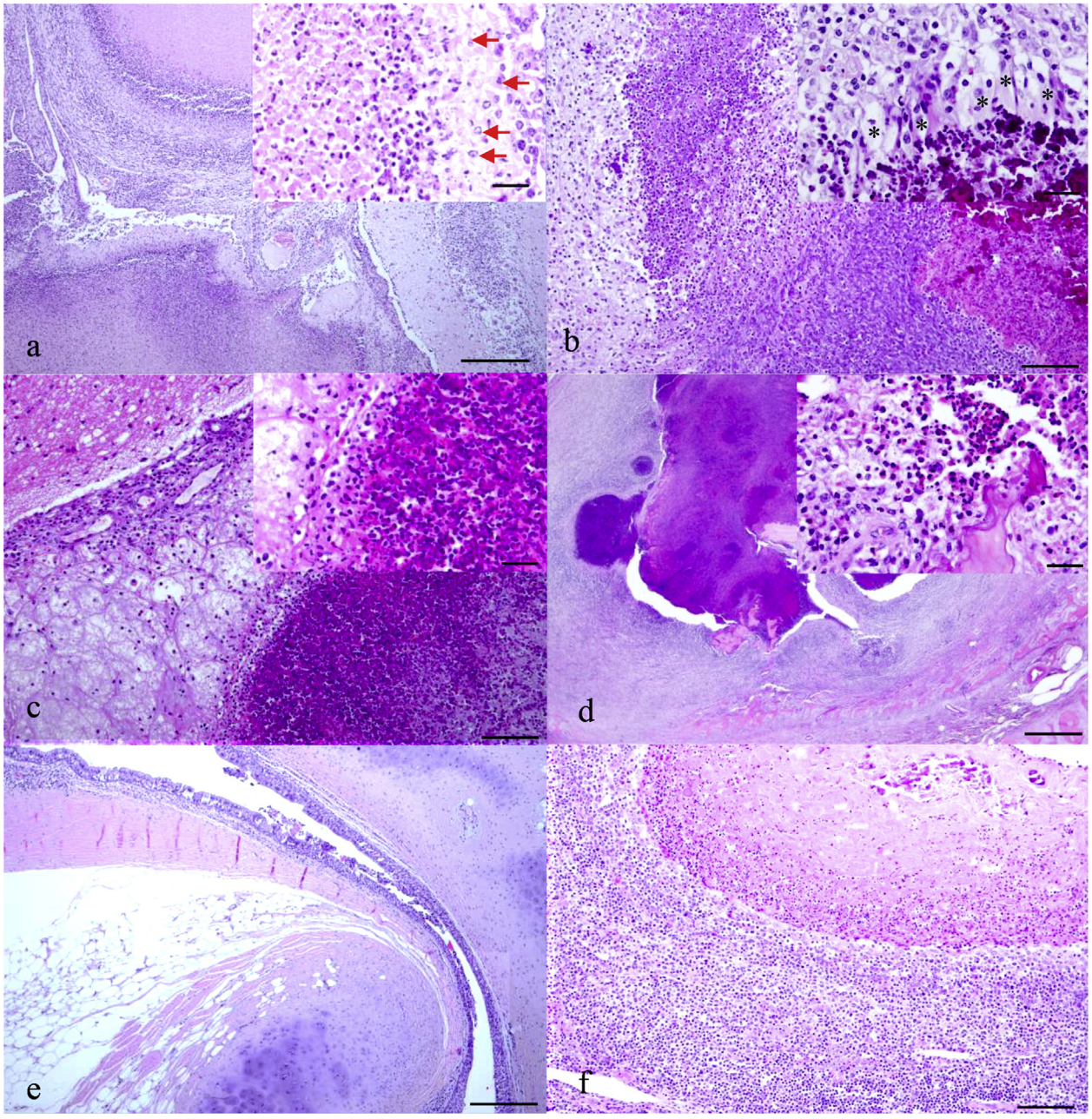

Fig. 2. Meningitis, meningoencephalitis, eustachitis and tonsillitis, *M. bovis* infection, calves. (a) Caseonecrotic granulomatous meningoencephalitis (case no. 1). HE. Bar, 300 µm. Inset: caseonecrotic material surrounded by macrophages (arrows). HE. Bar, 30 µm. (b) Inflammatory cells and mineralization in lesions of caseonecrotic granulomatous meningitis with associated nodule (case no. 2). HE. Bar, 100 µm. Inset: macrophages (*) at periphery of necrotic material. HE. Bar, 30 µm. (c) Suppurative meningoencephalitis with oedema and infiltrated neutrophils, macrophages and degenerate cells in meninges (case no. 3). HE. Bar, 100 µm. Inset: detail of infiltrated inflammatory cells. HE. Bar, 30 µm. (d) Caseonecrotic granulomatous osteomyelitis of right temporal bone (case no. 2). HE. Bar, 1,000 µm. Inset: neutrophils admixed with bone lysis. HE. Bar, 30 µm. (e) Eustachitis grade II with scattered intraluminal neutrophils and cell debris (case no. 8). HE. Bar, 300 µm. (f) Palatine tonsillitis (case no. 9). HE. Bar, 100 µm.

of brainstem and cerebellum (arrows) (case no. 3). (b1) Meningeal nodule (*) at base of cerebellum (Cb) and connected to pons (P) (case no. 2). (b2) Centre of meningeal nodule (case no. 2) heterogeneous in colour. Formalin fixed. (c1) Dissected right external ear connected to temporal bone (*) (case no. 2). (c2) Right temporal bone abscess (*) (case no. 2). (c3) Exudate in right middle ear canal (M) within temporal bone (case no. 2). Right horizontal ear canal (E). Formalin fixation. (d1) Anatomical location of tonsils (*) and eustachian tube (E) in right calvarium (case no. 5). (d2) Anatomical location of pharyngeal tonsil (PT), tubal tonsil (T) and opening of eustachian tube (arrow) (case no. 5). (d3) Location of eustachian tube (E) in eustachian canal after removal of basisphenoid bone (case no. 5). Formalin fixation. (e) Whitish exudate (*) in palatine tonsil (case no. 8). Inset: normal appearance of a palatine tonsil (case no. 10).

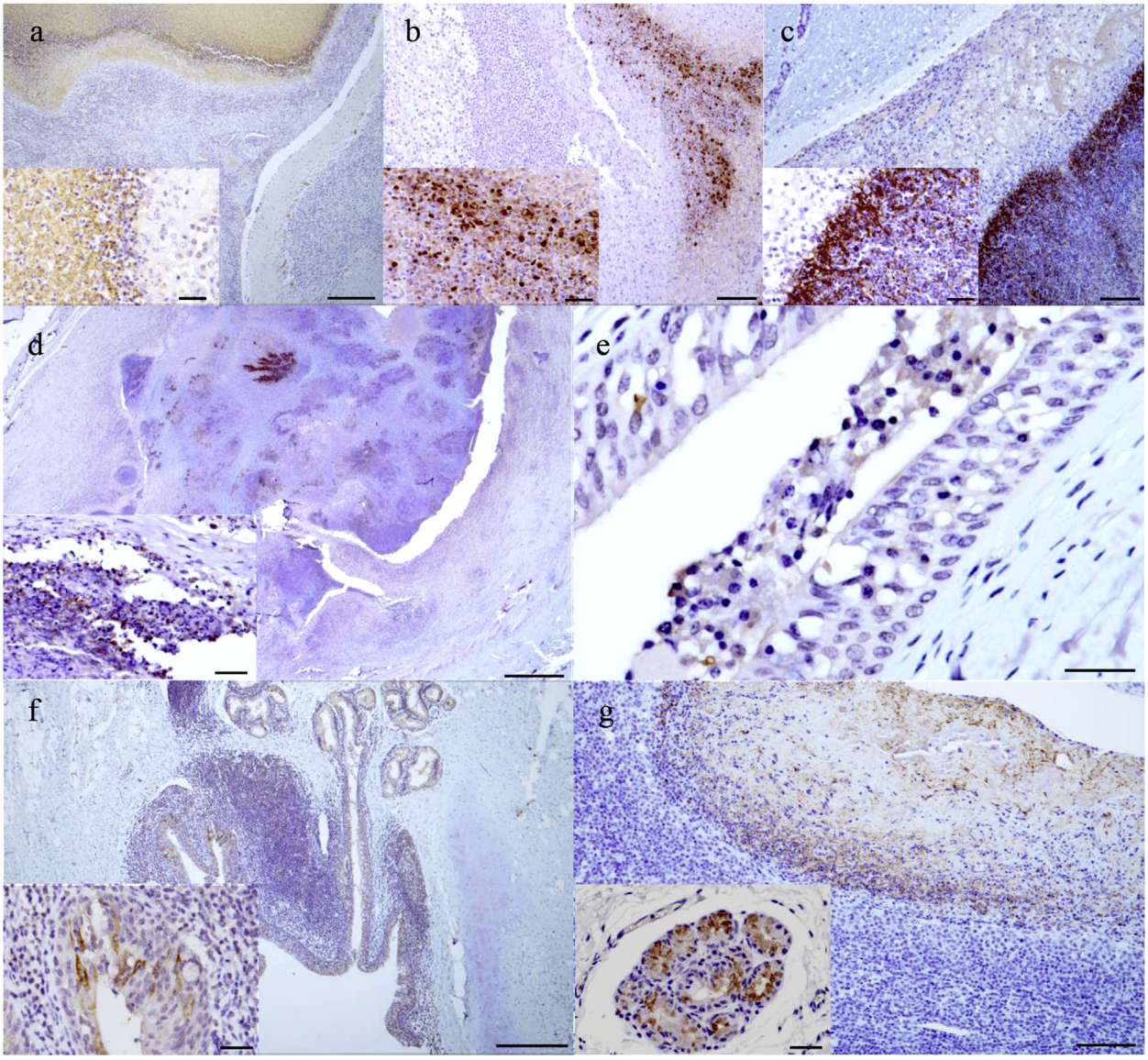

Fig. 3. Meningitis, osteomyelitis, eustachitis and tonsillitis, calf, *M. bovis* infection. Immunolabelling of *M. bovis* antigen. (a) *M. bovis* labelled in lesions of caseonecrotic granulomatous meningoencephalitis (case no. 1). IHC. Bar, 300 µm. Inset: antigen in neutrophils and degenerate cells in meninges. Bar, 30 µm. (b) *M. bovis* labelled in lesions of caseonecrotic granulomatous meningitis (case no. 2). IHC. Bar, 100 µm. Inset: antigens in neutrophils and degenerate cells in associated nodule. Bar, 30 µm. (c) *M. bovis* labelled in lesions of suppurative meningoencephalitis (case no. 3). IHC. Bar, 100 µm. Inset: antigen in neutrophils and degenerate cells in meninges. Bar, 30 µm. (d) *M. bovis* labelled in right temporal bone (case no. 2). IHC. Bar, 1,000 µm. Inset: antigen in neutrophils and degenerate cells. Bar, 30 µm. (e) *M. bovis* labelled in intraluminal inflammatory cells in eustachian tube (case no. 8). IHC. Bar, 30 µm. (f) *M. bovis* labelled in epithelial cells of eustachian tube (case no. 5). IHC. Bar, 100 µm. Inset: *M. bovis* labelled in epithelial cells. Bar, 30 µm. (g) *M. bovis* labelled in neutrophils and degenerate cells in lumen of palatine tonsil (case no. 9). IHC. Bar, 100 µm. Inset: *M. bovis* antigen in cytoplasm of epithelial cells of minor salivary glands (case no. 5). Bar, 30 µm.

route to the brain, followed by the development of meningitis. Otitis media that did not result from the extension of infection from the external ear was found in eight of the 10 cases in this study. *M. bovis* antigen was consistently present at the site. Therefore, it can be concluded that otitis media in the investigated animals was not due to the extension of external ear infection or haematogenous spread. However, these lesions of otitis media might be affected by infection of *M. bovis* in the oropharynx and extension via the eustachian tubes. Our results were consistent with those of others (Walz *et al*, 1997; Maeda *et al*, 2003; Francoz *et al*, 2004; Maunsell *et al*, 2012), who reported otitis media as a result of extension of *M. bovis* infection from the

eustachian tube and which extended to the tympanic bullae, causing suppurative to caseous necrosis and bone lysis.

In this study, *M. bovis* was most frequently detected, either by LAMP or IHC, in the palatine tonsil. This finding suggests that colonization of *M. bovis* in the tonsils at the oropharyngeal region, which are located close to the entrance of the eustachian tubes, is followed by spread of infection to the middle ear and meninges via the eustachian tube. After reaching the meninges and causing meningitis at the level of the cerebellum, cranial nerves VII and VIII might be affected in severe cases and the animal may develop neurological signs. Our results agree with those of Maunsell *et al* (2012), who reported that eustachian tube colonization did not occur without concomitant tonsillar colonization. Moreover, we found otitis media in association with bronchopneumonia in three cases, and in one case of bronchopneumonia with tonsillitis associated with *M. bovis* infection. These findings indicate that the upper respiratory tract and oropharynx were the primary sites of *M. bovis* colonization before infection extended to the lower respiratory system. We speculate that *M. bovis* infection was initiated in the minor salivary glands, followed by colonization of the tonsils with subsequent extension to the eustachian tube, resulting in otitis media. Schibrowski *et al* (2018) reported that shared pen water was the highest risk factor for the transmission of *M. bovis* from infected to susceptible animals. Therefore, farm management practices should prevent fomite contamination with this organism.

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Conflict of Interest Statement

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Supplementary data

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