



NOTE

Pathology

Systemic mycobacteriosis caused by '*Mycobacterium avium* subspecies *hominissuis*' in a 14-month-old Japanese black beef steer

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ABSTRACT. A 14-month-old Japanese black beef steer presented with severe chronic diarrhea and emaciation and was euthanized. Postmortem examination showed thickened and corrugated intestinal mucosa and enlarged granulomatous mesenteric lymph nodes with caseating necrosis. Numerous epithelioid cells and multinucleated giant cells infiltrated in the lamina propria and the submucosal tissue of the intestines. These cells were also observed in the systemic organs. Many acid-fast bacilli were detected in the cytoplasm of these cells and were identified as '*Mycobacterium avium* subsp. *hominissuis*' (*Mah*) on the basis of the results of molecular examinations and immunohistochemistry. These findings indicate that *Mah* can cause systemic mycobacteriosis, and this unique infection needs to be distinguished from Johne's disease and tuberculosis in cattle.

KEY WORDS: caseating granuloma, cattle, chronic diarrhea, *Mycobacterium avium* subsp. *hominissuis*

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Mycobacterium avium subsp. *hominissuis* (*Mah*), subordinated the *Mycobacterium avium* complex (MAC), is frequently isolated from patients with lung disease [17] and from tissue samples of slaughtered pigs [15]. *Mah* causes serious systemic infection in humans infected with human immunodeficiency virus (HIV) [10] and immunocompromised children [7], and it plays an important role as a zoonotic pathogen in MAC infection in humans and pigs [1]. In pigs, the pathogen has been found in the granulomatous lesion in the mesenteric lymph nodes, liver, spleen and jejunum [15, 30]. Most *Mah*-infected pigs usually have no clinical signs, and lesions are often detected at meat inspection [6].

In cattle, MAC has been mostly isolated at slaughterhouses in Europe [3], Africa [24] and South America [11], serving as an early warning for Asian countries. Previous reports were concerned with the epidemiology and bacteriology of the affected cattle, but descriptions were short and limited. Furthermore, histopathological examinations were not performed in the previous studies [3, 11, 24]. Therefore, there is limited information on systemic *Mah* infection in cattle and the expression of clinical symptoms. Moreover, there are no available reports on the immunohistochemical or molecular features of systemic mycobacteriosis associated with *Mah* in cattle within the global literature.

This report describes the clinical, microscopic and bacteriological characteristics of a beef calf with systemic granuloma due to *Mah* in Japan. We compare our findings with other cases of mycobacterial infection including Johne's disease and tuberculosis in cattle.

A 9-month-old Japanese black beef steer was moved from one farm to another farm with 44 Japanese black beef cattle in Aichi Prefecture, located on the Pacific coast on central Honshu island (main island), Japan in March 2016. The steer was housed in a free-stall barn with sawdust. The steer showed watery diarrhea immediately after the introduction. The condition was slightly improved by antibiotics, an antiprotozoal drug, an antidiarrheal drug, probiotics and steroids. The steer showed recurrent bouts of

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diarrhea for 5 months. In August 2016, at 14 months of age, the steer showed anorexia, severe diarrhea and wasting. Due to poor prognosis and difficulty in standing, the calf was euthanized for postmortem examination in Aichi prefectural Chuo Livestock Hygiene Service Center. No clinical abnormalities, such as diarrhea, were detected in other cattle on the farm.

The mucosa from the jejunum to the colon was grossly thickened and showed extensive hemorrhage (Fig. 1a). The mesenteric lymph nodes were remarkably swollen and contained large caseous necrotic foci. The hepatic and pulmonary lymph nodes were also enlarged. Mottled discoloration of the liver, enlarged spleen, edema of the lung and atelectasis were noted. No visible lesions were found in the other organs.

At necropsy, tissue samples of the liver, spleen, kidney, heart, lung, stomachs, intestines, brain and lymph nodes (mesenteric, hepatic and pulmonary) were fixed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin wax, sectioned (approximately 3- μ m thick) and stained for histological examination with hematoxylin and eosin and Ziehl-Neelsen (ZN) staining.

Immunohistochemistry was performed to detect the *Mycobacterium* antigen. All formalin-fixed tissues were cut in 3- μ m thick sections, treated with 3% hydrogen peroxide in methanol, followed by 0.1% actinase E solution, and then, incubated at 37°C for 20 min for antigen retrieval. The tissues were then incubated with rabbit anti-*Mycobacterium bovis* serum (Dako, Carpinteria, CA, U.S.A.) as a primary antibody for 30 min at room temperature and then reacted with a secondary antibody (Histofine Simple Stain MAX-PO (R); Nichirei Bioscience Inc., Tokyo, Japan). After rinsing with phosphate buffered saline, the specimens were incubated with aminoethyl carbazole substrate solution (Histofine Simple Stain aminoethyl carbazole solution; Nichirei Bioscience Inc.) at room temperature for 5 min and then counterstained with hematoxylin. In order to examine the construction of acid-fast bacteria, formalin-fixed jejunum tissue was examined by transmission electron microscopy as described previously [22].

Histologically, granuloma formation was severe in the jejunum, ileum, cecum, liver, spleen, and the mesenteric, hepatic and pulmonary lymph nodes, and mild in the lung and abomasum. Numerous epithelioid cells and multinucleated giant cells and a few lymphocytes infiltrated in the lamina propria, muscularis mucosae and especially, the submucosa beneath the thickened intestinal mucosa, which was composed of multifocal granulomas (Fig. 1b). The degenerative mucosal membrane was thin and partially ruptured at the site of the granuloma; macrophages and lymphocytes were observed. Villous atrophy was also observed in the small intestine. Numerous epithelioid cells and multinucleated giant cells infiltrated in the germinal center and the sheathed artery in the spleen, hepatic sinusoids, Glisson's sheath in the liver, peribronchial lymphoid follicles, and hepatic and pulmonary lymph nodes. Necrotic tissues were surrounded by epithelioid cells, lymphocytes, neutrophils and fibrous tissue in the mesenteric lymph nodes.

Numerous acid-fast bacteria were detected in the epithelioid cells infiltrating the tissues with granulomas in the systemic organs, including jejunum, ileum (Fig. 1c), cecum, liver, spleen, lymph nodes, lung and abomasum. Acid-fast bacteria were also detected in the cytoplasm of monocytes and were present freely in the plasma in the blood vessels within the lung, liver (Fig. 1d) and hepatic lymph nodes. Immunohistochemically, acid-fast bacteria positively reacted with the antibody against *M. bovis* (Fig. 1e). Ultrastructurally, numerous bacteria were detected in the cytoplasm of the epithelioid cells and macrophages in the jejunum. An electron-transparent layer, comprising an arabinogalactan-mycolic acid complex, was observed in its cell wall (Fig. 1f), which corresponded to previously described *Mycobacterium* features [29].

Tissue stamp smears prepared from ileal mucosa and mesenteric lymph nodes were examined by direct ZN staining, and many acid-fast bacilli were detected.

For direct molecular analysis, genomic DNA was extracted from liver, lung, spleen, ileum and mesenteric lymph nodes using a DNA extraction kit (JohneSpin; Fasmac Co., Atsugi, Japan). All DNA samples from tissues were negative in a specific polymerase chain reaction (PCR) targeting the *Mycobacterium tuberculosis* complex (MTC) IS6110 [4] and in a real-time quantitative PCR assay to detect *Mycobacterium avium* subsp. *paratuberculosis* IS900 [16] (Table 1). A multiplex real-time PCR assay for rapid identification of mycobacteria including MTC and MAC [25] suggested involvement of MAC in the tissues examined (Table 1).

For bacterial culture, tissue homogenates prepared from liver, spleen, lung, mesenteric lymph nodes and intestinal mucosa were inoculated into normal blood agar and deoxycholate-hydrogen sulfide-lactose agar, and then incubated at 37°C under 5% CO₂ in aerobic or anaerobic condition. Ogawa's medium (Nissui Pharmaceutical, Tokyo, Japan) were also used for isolation of mycobacteria. Acid-fast bacilli was isolated from all tissue samples examined, and no other bacteria were detected. DNA samples were prepared using Instagene Matrix (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's instructions. The following molecular examinations were performed for identification of the isolates: sequencing based on *Mycobacterium* sp. 16S rRNA [8], *Mycobacterium avium* subspecies-specific PCRs [18, 23], PCR of IS1245 [20], PCR and sequencing of *hsp65* [28] and MTC-MAC multiplex real-time PCR [25] (Table 1). All isolates were identified as *Mah* (Table 1) (Accession Number: LC224326).

These results indicate that the systemic granulomatous lesion in the steer was caused by *Mah*. In previous studies on bovine *Mah* infection, only epidemiological and bacteriological examinations were conducted, and provided limited histopathological information [3, 11, 24]. Moreover, immunohistochemical identification of the *Mycobacterium* antigen was not performed [3, 11, 24]. Our immunohistochemical and bacteriological findings indicated that the systemic granulomatous lesions were closely associated with *Mah*. Of note was the presence of the unique systemic granuloma associated with *Mah*, and to the best of our knowledge, this combination has not been previously reported.

Systemic *Mah* infection is often detected in slaughtered pigs [15, 30] and immunocompromised patients [7, 10], whereas it has been reported in only two horses [18] and a kiang (*Equus kiang*) [2]. The occurrence of *Mah* granulomas in the systemic organs is rare in cattle, but not reported in sheep [27] and goat [26].

Granulomas were detected in the digestive tracts and several abdominal organs in this steer, and the distribution of the present case is similar to that of the kiang [2]. In pigs infected with *Mah*, the lesions were detected in the liver, spleen and small intestines

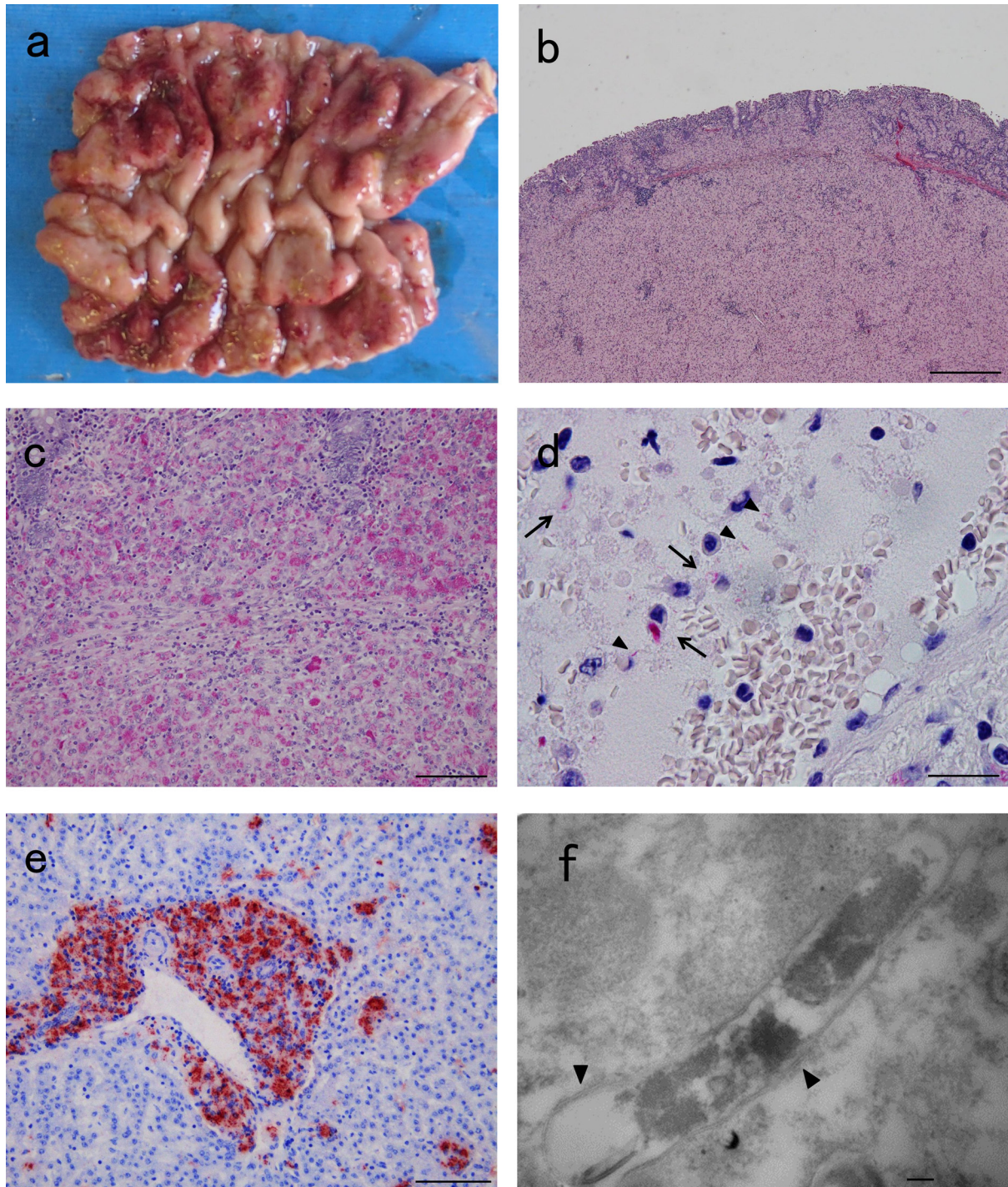


Fig. 1. a: Marked thickening of the ileum. Extensive hemorrhage is observed in the intestinal mucosa. b: Numerous epithelioid cells are present in the lamina propria, muscularis mucosae and submucosa in the ileum. Villous atrophy is also observed. Hematoxylin and eosin staining. Bar=500 μ m. c: Numerous acid-fast bacteria are found in the cytoplasm of epithelioid cells and multinucleated giant cells in the ileum. Ziehl-Neelsen staining. Bar=100 μ m. d: Numerous acid-fast bacteria are observed in the cytoplasm of monocytes (arrows) and are present freely (arrowheads) in the lobular hepatic vein in the liver. Ziehl-Neelsen staining. Bar=20 μ m. e: Immunohistochemistry showing that bacilli in the epithelioid cells and macrophages react with an antibody against *M. bovis* in the sinusoids and Glisson's sheath in liver. Bar=100 μ m. f: Transmission electron microscopy examination of *Mah* in the cytoplasm of epithelioid cells in the jejunum. The thick electron-transparent layer (arrowheads) is visible in the bacterial cell wall. Bar=100 nm.

Table 1. Results of molecular examinations

PCR	Target	Results		References
		Tissue	Colony	
1. <i>Mycobacterium</i> sp.	16S rRNA	N/A	<i>M. avium</i>	[8]
2. MTC	IS6110	-	N/A	[4]
3. MAC	IS900 (<i>M. paratuberculosis</i>)	-	N/A	[16]
	IS901 (<i>M. avium</i>)	N/A	-	[19, 23]
	IS1245	N/A	+	[20]
	<i>hsp65-645C/T</i>	N/A	C	[28]
4. MTC-MAC multiplex PCR		MAC	MAC	[25]

MAC; *Mycobacterium avium* complex, MTC; *Mycobacterium tuberculosis* complex, N/A; not applicable, PCR; polymerase chain reaction, C; cytosine (Results of sequence analysis of 645th nucleotide in *hsp65* region).

[30]. Several granulomas were only located in the digestive tract and mesenteric lymph nodes in horses [18]. In contrast, principle granulomas were mostly detected in the lung and cervical lymph nodes in human *Mah* infection [13, 14]. This difference in anatomic distribution correlates with the likely routes of infection, which include direct airborne transmission in humans and indirect ingestion of contaminated milk and food in animals including cattle, kiang, pig and horses.

The source of MAC infection is sawdust [21] and peat [12] in pigs, water [5] and the environment [9] in humans. In the present steer, sawdust was used for bedding in the free-stall barn. Therefore, environmental sources including sawdust and/or water appear to be the source of *Mah* in the present case.

The presence of extensive granuloma was striking in our case. Acid-fast bacteria were detected in the granulomatous lesion and within blood vessels in many organs. On the basis of these findings, we speculate that the granulomatous lesions were formed when the causative pathogen was transferred by blood circulation via the portal vein from the intestines, which may have been the original lesion location, to other organs including the liver and spleen. Therefore, cattle seem to be at potential risk of systemic *Mah* infection.

Most MAC are associated with subclinical granulomas in the lymph nodes of pigs [6] and cattle [3] detected in slaughterhouses. Diarrhea can occur in immunocompromised children [7] and HIV infected patients [13]. In the kiang, social stress, as indicated by the incidences of intra-species aggression, multiple skin wounds and a healing rib fracture, was likely a major direct or indirect immunocompromising factor, leading to the establishment of systemic *Mah* infection [2]. In view of the previous reports regarding *Mah* infection, granulomas of the digestive tract appear to be the primary cause of the symptoms in the present case, and the stress of transportation is a likely predisposing factor for the infection.

Therapy for bovine *Mah* infection is apparently difficult due to the involvement of many organs and chronic deterioration. Therefore, to prevent the spread of *Mah* in farms, further studies on vaccines and therapy should be considered essential.

In the present case, Johne's disease, tuberculosis, nocardiosis and actinomycosis were also suspected as a differential diagnosis; however, immunohistochemical analysis and isolation with identification and genetic sequencing of isolates excluded these diseases and confirmed the diagnosis of *Mah* infection. The present findings, including caseating necrosis and partially ruptured muscularis mucosae in the intestines, were unique, and they are not detected in Johne's disease. Distinguishing MTC from MAC is important for the immediate prevention of an epidemic; however, it can take a long time to diagnose MTC or MAC due to their long incubation period. We conducted MTC-MAC quantitative PCR using organs in cattle instead of bacterial colonies for the first time. All results of studies using organs correspond to those using colonies. These results suggest that MTC-MAC quantitative PCR is useful and can determine MTC or MAC more quickly than the sequence analysis of colonies.

The occurrence of *Mah* granulomas in the systemic organs of cattle is rare and has not been previously reported globally. Therefore, additional investigations will be necessary to confirm the difference in pathogenicity among the conventional strains of *Mah* and the isolate in this study.

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