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NOTE

Pathology

Bovine peritonitis associated with *Mannheimia haemolytica* serotype 2 in a three-day-old Japanese Black calf

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ABSTRACT. A Japanese Black calf became dehydrated on the first day of life and died on the third day. Gross examination revealed a large amount of yellowish-brown serous fluid in the abdominal cavity and whitish-yellow fibrin in the serosa of the abdominal organs. Patchy red spots were observed throughout the peritoneum, and the outer membrane of the umbilical arteries was dark red. Bacteriologically, *Mannheimia haemolytica* serotype 2 was isolated from the umbilical arteries and vein, liver, and kidney. Histopathology revealed inflammation with *M. haemolytica* serotype 2 in the outer membrane of the umbilical arteries and in the serosa of the bladder and intestinal tract. This is the first case of bovine peritonitis with histopathologic and immunohistochemical identification of *M. haemolytica*.

KEY WORDS: bovine, immunohistochemistry, *Mannheimia haemolytica* serotype 2, peritonitis, umbilical infection

Mannheimia haemolytica is a short Gram-negative bacillus that normally colonizes the nasal cavity and tonsillar crypts in cattle and may transition into a pulmonary pathogen [1, 6, 8]. *M. haemolytica* is the most commonly cultured bacterium associated with bovine respiratory disease complex in feedlot cattle [7]. A variety of stressors, including transport, weaning, and concurrent viral or bacterial infection have been identified as risk factors [5–7].

M. haemolytica is classified into 12 serotypes, i.e., 1, 2, 5, 6, 7, 8, 9, 12, 13, 14, 16, and 17 on the basis of capsular antigenicity [2]. Serotype 1 has been reported to be the serotype most frequently isolated from pneumonic lesions in cattle [2, 5]. The predominant serotype isolated from clinically normal cattle is 2 [8]. In Japan, more than 90% of cases of *M. haemolytica* isolated from cattle with pneumonic pasteurellosis have been classified as serotypes 1, 2, and 6 [10].

Bovine peritonitis is a common condition and is causing an increasing number of deaths in cattle, so has important economic implications [11]. Peritonitis is caused primarily by bacterial infection, which often occurs via the umbilical cord in cattle.

To date, there have been no reports of peritonitis caused by *M. haemolytica*. There has been a report from the US of an isolate of *M. haemolytica* serotype 1 in an umbilical abscess among data collected in Minnesota for 1997–1999 [2]. Unfortunately, no details on clinical condition, findings on gross examination, distribution of *M. haemolytica* lesions, or histopathologic information were described. The present case is the first report of histopathologic and immunohistochemical identification of *M. haemolytica* serotype 2 in bovine peritonitis.

The case was a Japanese Black calf born on February 13, 2017, on a farm in Gunma Prefecture (in the center of Honshu, the main island in Japan) that raises about 800 feedlot cattle and about 310 Japanese Black cattle for breeding purposes. The calf

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showed dehydration from the first day of birth and died on the third day. Gross examination was performed to determine the cause of death on the same day. No clinical abnormalities were detected in the other cattle on the farm.

Diagnostic necropsy was performed at the Gunma Livestock Health Laboratory. Gross examination revealed eyes that were sunken into the orbits and dehydration. There was a large amount of yellowish-brown serous fluid in the abdominal cavity, and white-yellow fibrin was observed in the serosa of the abdominal organs (Fig. 1a). Patchy red spots were observed to be extensively distributed in the peritoneum. The outer membrane of the umbilical arteries was dark red (Fig. 1b). Dark red spots were also found in the costal pleura, and there was a large amount of serous fluid in the thoracic cavity. No gross lesions were found in any of the other organs, including the lung.

For bacterial culture, tissue samples from the umbilical arteries and vein, liver, spleen, kidney, heart, and lung were inoculated into 5% sheep blood agar and deoxycholate-hydrogen sulfide-lactose agar, then incubated at 37°C under aerobic conditions or 5% carbon dioxide gas for 24 hr. Gram-negative rods were isolated from the umbilical arteries and vein and from the liver and kidney.

The isolates were identified biochemically using an ID Test HN-20 Rapid identification kit (Nissui, Tokyo, Japan) and by 16S rRNA sequence analysis [3]. The isolates were identified as *M. haemolytica*.

The isolates were serotyped using the indirect hemagglutination test [4] with *M. haemolytica* serotype-specific antisera [10]. Antiserum against the newly described serotype 17 was not included in the test. All isolates were identified as serotype 2.

Tissue samples from the umbilical arteries, umbilical vein, liver, spleen, kidneys, heart, lungs, rumen, reticulum, omasum, abomasum, intestines (duodenum, jejunum, ileum, cecum, colon, and rectum), bladder, and lymph nodes (hepatic, renal, hilar lungs, mesenteric, and ileocecal) were fixed in 10% neutral phosphate-buffered formalin. The fixed tissues were embedded in paraffin wax, sectioned (at a thickness of approximately 3 μm), and stained using hematoxylin-eosin and Gram's method for histopathologic examination.

Immunohistochemistry was performed to detect the presence of *M. haemolytica*. The formalin-fixed tissues from the umbilical arteries, umbilical vein, liver, spleen, kidneys, heart, lungs, rumen, reticulum, intestines (jejunum, colon, and rectum) and bladder were cut into 3-μm thick sections and treated with 3% hydrogen peroxide in methanol. The dewaxed sections in 0.01 M citrate buffer pH 6 (S2031, Dako REAL Target Retrieval Solution, Dako North America Inc., Carpinteria, CA, U.S.A.) were heated twice in a microwave oven for 5 min for antigen retrieval. The tissues were then incubated with rabbit anti-*M. haemolytica* serotype 2 [10] as the primary antibody for 30 min at room temperature, followed by incubation with a secondary antibody (Dako Envision$^+$ Dual Link System-HRP, Dako North America Inc.). After rinsing in phosphate-buffered saline, the specimens were incubated with aminoethyl carbazole substrate solution (Histofine Simple Stain aminoethyl carbazole solution; Nichirei Bioscience Inc., Tokyo, Japan) at room temperature for 5 min, followed by counterstaining with hematoxylin. Sections of a piece of liver (into which *M. haemolytica* serotype 2 had been injected) were immunolabeled as positive controls.

Histopathologic examination revealed inflammation in the outer membrane of the umbilical arteries with numerous colonies of Gram-negative rods. A band-like lesion was observed around the entire outer membrane of each artery (Fig. 1c). Degenerative neutrophils, debris, and fibrin were observed in the bacterial mass (Fig. 1d) and in the outer membrane surrounding the nutrient blood vessels. These vessels also contained areas of hemorrhage (Fig. 1e). Inflammation was also observed in the umbilical vein, spleen, intestinal tract (duodenum, jejunum, cecum, colon, and rectum) and bladder. Many colonies of Gram-negative rods were observed in the bladder, with some colonies in the umbilical vein, spleen, and intestinal tract. This finding was consistent with the degree of inflammation. Hemorrhage was also observed in the outer membrane of the umbilical vein, the capsule of the spleen, part of the lung pleura, and the serosa of the bladder.

On immunohistochemistry, the Gram-negative rods reacted positively with antibodies against *M. haemolytica* serotype 2 (Fig. 1f, 1g). The strongest positive reactions were in the umbilical arteries and bladder, with lesser amounts of *M. haemolytica* serotype 2 antigen detected in the blood vessels of the kidney, spleen, and lung pleura.

M. haemolytica was not detected in nasal swabs taken for the mother of the infected calf about 9 months after delivery. In 2003 and 2006, respiratory diseases caused by *M. haemolytica* occurred on the same farm. We examined the relationship between the present isolates and those obtained in 2003 and 2006. On serotyping testing, the isolates in 2003 were serotype 2 and those in 2006 were serotype 6. Multilocus sequence typing was conducted for isolates in 2003 and the present isolates, which were of the same serotype. The allele types of the isolates in 2003 were adk/aroE/deoE/gapDH/gnd/mdh/zwf=2/1/1/1/1/1/2, whereas those of the present isolates were adk/aroE/deoE/gapDH/gnd/mdh/zwf=1/2/1/2/1/2/1. This finding indicates that the epidemiologic relevance of these isolates is low, and it is likely that the isolate in the present case entered the farm after 2006.

Our investigations indicated that the peritonitis in this three-day-old Japanese Black calf was associated with *M. haemolytica* serotype 2. Serotype 1 was previously isolated from a bovine umbilical abscess but there was no information on the clinical presentation, results of gross examination, or the distribution of lesions [2]. Although *M. haemolytica* has often been isolated in cases of bovine lung disease [6, 7], there are no reports of severe peritonitis being caused by *M. haemolytica*.

A striking feature in the present case was the presence of inflammation in the form of bands in the outer membrane of the umbilical arteries. *M. haemolytica* serotype 2 was detected on immunohistochemistry in the outer membrane of the arteries and serosa of the bladder. Furthermore, *M. haemolytica* serotype 2 was detected in the serosa of the intestinal tract (jejunum, colon, and rectum) and to a lesser extent in the capsule of the spleen and outer membrane of the umbilical vein. Considering these findings, we speculate that the peritonitis that developed in this calf was caused by a *M. haemolytica* serotype 2 infection of the umbilical artery. In a newborn calf, the bladder lies between the two umbilical arteries and is connected to these vessels by mesenchymal tissue. Anatomically, the bladder is the closest organ connected by a membrane to the umbilical arteries, which may explain why a large number of bacteria were also detected in the serosa of the bladder. This finding also suggests that *M. haemolytica* invaded

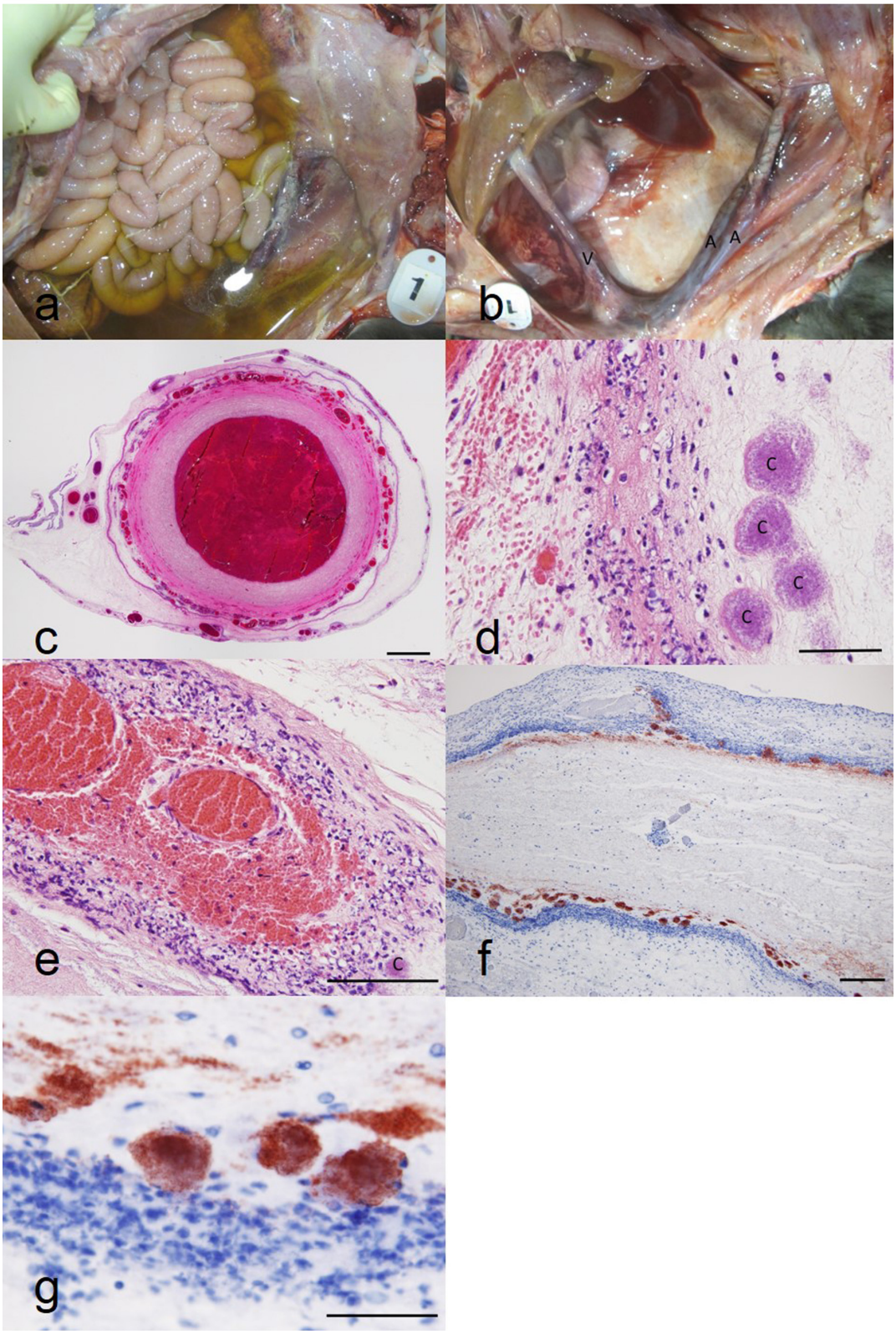

Fig. 1. Post mortem photographs show (a) increased ascitic fluid and whitish-yellow fibrin deposits and (b) the two umbilical arteries (A) and umbilical vein (V). The outer membrane of the umbilical arteries is dark red. (c) A photomicrograph shows a band-like lesion around the entire outer membrane of an umbilical artery. Hematoxylin-eosin staining. Bar, 1 mm. (d) Higher magnification of the same field shown in Fig. 1c shows four colonies of bacteria (C), degenerate neutrophils, and debris on the outer membrane. Hematoxylin-eosin staining. Bar, 50 μm. (e) Another higher magnification of the same field shown in Fig. 1c reveals colonies of bacteria (C), degenerate neutrophils, and debris surrounding the nutrient blood vessels. Hemorrhage is present in the nutrient vessels. Hematoxylin-eosin staining. Bar, 100 μm. (f) Anti-*M. haemolytica*-targeted immunohistochemistry revealed numerous positive reactions. Bar, 200 μm. (g) Higher magnification of the same field shown in Fig. 1f revealed numerous positive reactions consistent with the bacterial mass. Bar, 50 μm.

via the umbilical arteries. Although inflammation was not detected in the liver, kidney, heart, lungs, rumen, reticulum, omasum, or abomasum in the present case, small colonies of *M. haemolytica* were detected in the blood vessels of the kidney, spleen, and lung pleura. Therefore, the possibility of sepsis was considered in this calf.

M. haemolytica lipopolysaccharide has typical endotoxic and proinflammatory properties and causes vasculitis in lung tissue [1, 6, 12]. Leukotoxin can induce lysis of leukocytes and release of proinflammatory cytokines in ruminants [1, 6, 9, 12]. The net result of these virulence factors is alveolar and vascular damage with fibrinous pleuropneumonia [6]. Hemorrhage was observed in the outer membrane of umbilical arteries, umbilical vein, bladder, capsule of the spleen, and pleura of the lung in the present case. We speculated that the hemorrhage in these organs was a result of the actions of lipopolysaccharide and leukotoxin.

Peritonitis associated with *M. haemolytica* in cattle is a rare occurrence and has not been previously reported. Further evaluations of the pathogenicity of *M. haemolytica* are necessary.

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