The Journal of Veterinary Medical Science

NOTE
Pathology

Bovine esophageal and glossal ulceration associated with *Pseudomonas aeruginosa* and *Fusobacterium* spp. in a 10-month-old Holstein heifer

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J. Vet. Med. Sci.
80(7): 1174–1178, 2018
doi: 10.1292/jvms.17-0616

Received: 16 November 2017
Accepted: 15 May 2018
Published online in J-STAGE: 25 May 2018

ABSTRACT. An underweight 10-month-old Holstein heifer presented with anorexia and ananastasia and was euthanized. Postmortem examination revealed extensive ulceration in the esophagus, tongue, and omasum. Histopathological examination revealed severe necrotic esophagitis, glossitis, and omasitis. Many Gram-negative bacilli were detected throughout the necrotic area in the digestive tract; these were identified as *Pseudomonas aeruginosa* on the basis of isolation tests, molecular examinations, and immunohistochemistry. Gram-negative long filamentous organisms in the superficial layers of the necrotic lesions reacted positively with antibodies against *Fusobacterium necrophorum* subsp. *necrophorum*. Thus, the necrotic lesions were confirmed to be associated with *P. aeruginosa* and *Fusobacterium* spp. This is the first detection of *P. aeruginosa* in bovine esophageal and glossal ulcers associated with *Fusobacterium* spp.

KEY WORDS: bovine, esophageal and glossal ulceration, *Fusobacterium* spp., immunohistochemistry, *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic Gram-negative bacillus and an opportunistic pathogen that is abundant in various habitats. It has been implicated in different opportunistic infections and nosocomial outbreaks in human hospitals [21]. A large number of hospital outbreaks have been linked to environmental sources, particularly water systems [3, 27]. The species can evidently rapidly acquire resistance to various antimicrobial agents, and is capable of forming a biofilm [18, 21], which acts as a direct barrier to phagocytic cells and offers innate resistance to antibiotics and disinfectants [10]. Most *P. aeruginosa* infections are difficult to treat because of the pathogen's ability to resist many structurally unrelated antibiotics via both intrinsic and acquired antibiotic resistance mechanisms [13]. Among these mechanisms, a notable one involves the production of various classes of beta-lactamases that mediate the bacterial resistance to beta-lactam drugs. In recent decades, numerous enzymes belonging to this category have been detected in *P. aeruginosa*, including extended-spectrum beta-lactamases such as OXA, VEB, PER, SHV and TEM [14, 16]. Although *P. aeruginosa* is one of the commonly isolated bacteria infecting the skin and respiratory tract in humans [21, 29], few reports have described the histopathological features of infectious lesions caused by this species.

P. aeruginosa is not only a recognized human pathogen but also a verified animal pathogen. It is one of the major causes of diseases such as otitis [12], mastitis [2, 17, 18, 22, 31], endometritis [1, 9], and hemorrhagic pneumonia [5, 20] in both livestock and domestic pets. Recent reports associated with *P. aeruginosa* infection in cattle are restricted to those describing mastitis without histopathological analysis [2, 17, 18].

Fusobacterium spp. are constituents of the normal flora of the oropharynx and the gastrointestinal tract in cattle. *Fusobacterium* spp. have been associated with hepatic abscesses and necrotic laryngitis in feedlot cattle, and foot rot and lameness in dairy and beef cattle [15, 24]. This is the first report on the molecular and immunohistochemical identification of *P. aeruginosa* in bovine

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esophageal and glossal ulcers associated with *Fusobacterium* spp.

An 8-month-old Holstein heifer was moved from a farm to a public ranch in Tochigi Prefecture, in the center of Honshu Island (the main island), Japan in March 2017. The heifer appeared slightly thin when it arrived at the public ranch. At the age of 10 months, on May 11, 2017, it was moved to another public ranch. Ten days later, the heifer exhibited fever, anorexia, and depression, and its condition worsened despite antimicrobial therapy with benzylpenicillin procaine 10,000 U/kg and dihydrostreptomycin sulfate 12.5 mg/kg. On June 5, 2017, it was euthanized because of ananastasia and a poor prognosis. The other cattle on the ranch showed no clinical abnormalities.

Diagnostic necropsy was performed at the Tochigi Prefectural Kenou Livestock Hygiene Service Center. Gross necropsy examination showed multifocal ulceration of the esophagus, tongue, and omasum. Extensive, multifocal lesions of varying sizes (maximum diameter, approximately 4 cm) were observed on the mucosal surface of the esophagus; these were well defined and consisted of ulcerative necrotic tissue that was pale yellow to tan in color (Fig. 1a). Similar multifocal lesions were also found on the mucosal surface of the tongue. A necrotic and ulcerative lesion was also detected on the mucosal surface of the omasum. The mucosa from the palate to the pharynx exhibited petechial hemorrhage, and the mucosal surface from the duodenum to the rectum exhibited hyperemia. Pulmonary, superficial cervical, mandibular, and parotid lymph node enlargement was observed. The mesenteric and colic lymph nodes also showed moderate enlargement. Gelatinous infiltration was found in the renal pelvic region, and the lungs showed hyperemia in part of the accessory lobe and the entire right caudal lobe. Subcutaneous edema in the chest and abdomen and retained ascitic and pericardial fluid were also noted. No gross lesions were found in the other organs.

Tissue samples of the liver, spleen, kidneys, heart, lungs, brain, rumen, reticulum, omasum, abomasum, intestines, esophagus, tongue, and lymph nodes (superficial cervical, mandibular, parotid, subiliac, superficial inguinal, mesenteric, colic, ileocecal, and pulmonary) were fixed in 10% neutral phosphate buffered formalin. The fixed tissues were embedded in paraffin wax, sectioned (approximately 3-μm thick), and stained with hematoxylin and eosin (HE) and Gram's method for histopathological examination.

Immunohistochemistry was performed for the detection of *P. aeruginosa* and *Fusobacterium necrophorum* antigens. All formalin-fixed tissues were cut into 3-μm thick sections, treated with 3% hydrogen peroxide in methanol followed by 0.1% actinase E solution, and incubated at 37°C for 5 min for antigen retrieval. The tissues were then incubated with rabbit anti-*P. aeruginosa* O-antigen serotype group G serum (Cat. No. 213648, Denka Seiken Co., Ltd., Tokyo, Japan) and rabbit anti-*F. necrophorum* subsp. *necrophorum* strain ATCC25286 [23] as primary antibodies for 30 min at room temperature, followed by incubation with a secondary antibody (Dako Envision$^+$ Dual Link System-HRP, Dako North America Inc., Carpinteria, CA, U.S.A.). After rinsing with PBS, 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.

Histopathological examination revealed severe esophageal and glossal ulceration, with focal ulceration in the omasum. Severe multifocal necrotizing esophagitis (Fig. 1b) was observed, with lesions detected from the mucosal epithelium to the lamina propria. The degenerative and necrotic cells were demarcated by numerous neutrophils, a few lymphocytes, and fibrous tissue (Fig. 1c). In the lamia propria and submucosa, necrotic neutrophils and fibrin deposition were detected (Fig. 1d). Angiitis was also observed. The tongue and omasum ulcers were similar to the esophageal ulcers. Lymphocytes were decreased in the lymphoid follicles of the mandibular, superficial inguinal, mesenteric, colic, ileocecal, and pulmonary lymph nodes. Lymphocyte depletion was also observed in Peyer's patch in the ileum, the ileocecal region, the cecum, and the lower jejunum. Coccidial infestation was observed in the ileum and cecum.

Numerous Gram-negative bacilli were found throughout the necrotic regions in the esophagus, tongue, and omasum. The necrotic lesions were closely associated with the distribution of the Gram-negative bacilli. In addition, several Gram-negative long filamentous organisms (Supplementary Figs. 1 and 2) and Gram-positive cocci were seen in the superficial layers of the necrotic lesions. Several Gram-negative bacilli were also detected in the debris and cytoplasm of macrophages in the lumen of the rumen, reticulum, jejunum, and cecum. Moreover they were found in the cytoplasm of macrophages in the lymphatic sinus and follicles in the superficial cervical lymph node, and in the cytoplasm of macrophages in the blood vessels in the rumen and kidney. Immunohistochemically, the Gram-negative bacilli in the necrotic lesions and the superficial cervical lymph node, rumen, reticulum, jejunum, cecum, and kidney reacted positively with antibodies against *P. aeruginosa* O-antigen serotype group G (Fig. 1e and 1f), while the Gram-negative long filamentous organisms in the superficial layers of the necrotic lesions reacted positively with antibodies against *F. necrophorum* subsp. *necrophorum* (Supplementary Fig. 3).

For bacterial culture, tissue samples of the liver, spleen, kidney, heart, lung, brain, and esophageal mucosa were inoculated into normal blood agar and deoxycholate-hydrogen sulfide-lactose agar, then incubated at 37°C under aerobic or anaerobic conditions for 24 hr. Gram-negative rod shaped bacteria were isolated from the esophageal mucosa. A Simple Identification Kit (API 20 NE, bioMérieux, Tokyo, Japan) was used to identify the isolates. The isolates were identified as *P. aeruginosa* by API 20 NE (profile number 1054575, 99% identify).

For *P. aeruginosa* O-antigen serotyping, we utilized Quick Slide Agglutination and test tube precipitation methods using *P. aeruginosa* antisera kits (Denka Seiken Co., Ltd.) in accordance with the manufacturer's instructions. The isolated *P. aeruginosa* was identified as O-antigen group G. The isolate was identified as *P. aeruginosa* biochemically and by 16S rRNA sequencing analysis [19].

The agar dilution method was used as recommended by the Clinical and Laboratory Standards Institute (CLSI) subcommittee on Veterinary Antimicrobial Susceptibility Testing for determination of the susceptibility of the *P. aeruginosa* isolate to 7 different antimicrobials (CLSI 2010). The antimicrobial agents used were ampicillin, cefazolin, streptomycin, gentamicin, enrofloxacin,

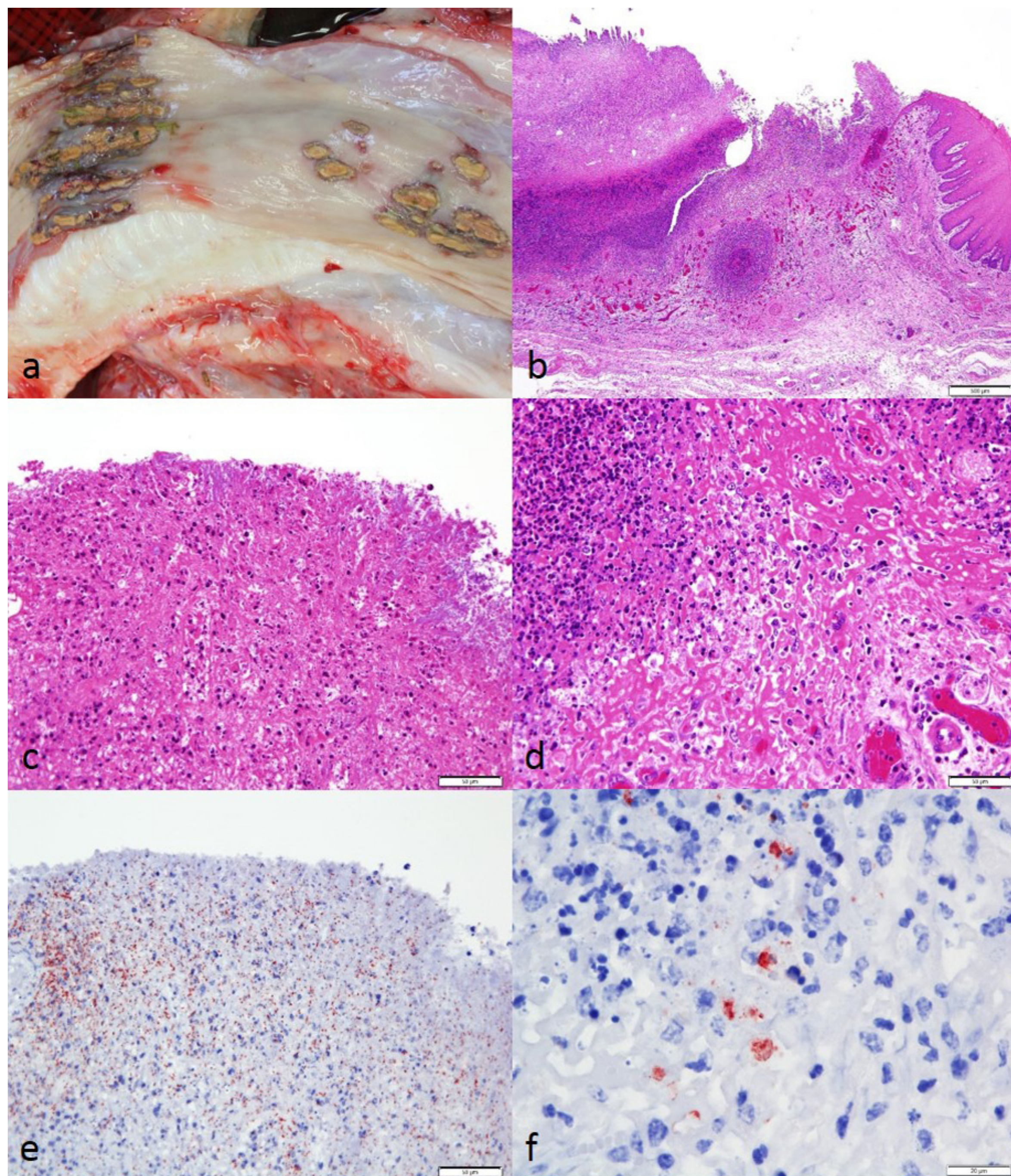

Fig. 1. a. Multifocal ulcers involving the esophageal mucosa and pseudomembranes are evident. b. The esophagus shows severe necrotic ulceration. Hematoxylin and eosin staining. Bar=500 μm. c. Severe necrosis is detected in the esophageal ulcer, and many long filamentous organisms are located in the superficial layers of the necrotic lesions. Hematoxylin and eosin staining. Bar=50 μm. d. The image shows necrotic neutrophils and fibrin deposition in the lamina propria and submucosa in the esophagus. Hematoxylin and eosin staining. Bar=50 μm. e. The same field shown in Fig. 1c, but subjected to anti-*P. aeruginosa* O-antigen serotype group G-targeted immunohistochemistry. The necrotizing lesions in the esophagus show numerous positive results. Bar=50 μm. f. Higher magnification of the same field shown in Fig. 1d, but subjected to anti-*P. aeruginosa* O-antigen serotype group G-targeted immunohistochemistry. Positive reactions are detected in the necrotizing lesions involving the esophageal submucosal tissue. Bar=20 μm.

meropenem, and colistin. The dilution range of all antimicrobial agents used for minimum inhibitory concentration (MIC) testing was 0.063 μg/m*l* to 512.0 μg/m*l*, in two-fold serial dilutions. The following quality control strains were also tested: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25923, and *P. aeruginosa* ATCC 27853. The strain showed sensitivity to streptomycin (MIC 32 μg/m*l*), gentamicin (2.0 μg/m*l*), enrofloxacin (1.0 μg/m*l*), meropenem (0.5 μg/m*l*), and colistin (1.0 μg/m*l*), and resistance to ampicillin ($\geq$512 μg/m*l*) and cefazolin ($\geq$512 μg/m*l*).

For virologic testing, PCRs were performed for malignant catarrhal fever virus, bovine papular stomatitis virus, pseudocowpox virus, orf virus, and bovine viral diarrhea virus. None of the PCRs yielded positive results.

The present results indicated that the observed ulcers involving the esophagus and tongue of the 10-month-old Holstein heifer were associated with *P. aeruginosa*, *Fusobacterium* spp., and Gram-positive cocci. Previously, *F. necrophorum* subsp. *necrophorum* was identified in severe focal necrotizing glossal lesions in a 37-day-old male Japanese black calf [23]. The necrotic lesion in the present case is similar that of previous cases affected with *Fusobacterium* [23]. In the present case, *Fusobacterium* spp. and Gram-positive cocci were found in limited portions of the lesions. Moreover, *Fusobacterium* spp. are obligate anaerobic organisms. Furthermore, there is a possibility that *Fusobacterium* spp. could not be cultured from the present lesions.

Previous studies on bovine *P. aeruginosa* infection, only conducted epidemiological and bacteriological examinations for mastitis, without histopathological analyzes. Histopathological findings have been reported for horses and minks [9, 20]. Moreover, immunohistochemical identification of *P. aeruginosa* antigens has only been reported in one horse [9], with no reports in cattle.

P. aeruginosa has been reported to cause endometritis [1, 9] and keratitis [26] in horses, external otitis in dogs [12], hemorrhagic pneumonia in minks [5, 20], and mastitis in cows [2, 17, 18] and goats [22, 31]. Although the organism has been infrequently isolated in cases of bovine and goat mastitis [2, 17, 18, 22, 31], there are no reports of severe esophageal and glossal ulceration in agricultural livestock, including cattle.

P. aeruginosa is capable of surviving in a broad range of natural environments. It causes bovine mastitis and is acquired via washing with contaminated water or the insertion of contaminated intermammary antibiotic tubes [8]. Therefore, the source of *P. aeruginosa* infection is difficult to identify. An environmental source, possibly water, may have been the source of *P. aeruginosa* infection in the present case.

The presence of severe esophageal and glossal ulceration was a striking feature of the present case. *P. aeruginosa* was immunohistochemically detected in the ulcerous lesions and the superficial cervical lymph node, rumen, reticulum, jejunum, cecum, and kidney. On the basis of these findings, we speculate that *P. aeruginosa*, *Fusobacterium* spp., and Gram-positive cocci proliferated in the oral cavity and esophagus, following which the ulcers developed. Subsequently, *P. aeruginosa* was carried from the ulcerous lesions to other organs via the bloodstream and the digestive tract as previously described [7]. Although necrotic lesions were not detected in the rumen, reticulum, jejunum, or cecum in the present case, *P. aeruginosa* was detected in the debris and cytoplasm of macrophages in the lumen. The findings indicate two possibilities: (1) *P. aeruginosa* was phagocytosed by macrophages in necrotic lesions in the upper digestive tract, including the esophagus, tongue, and omasum, and was transported to the lower digestive tract; or (2) *P. aeruginosa* proliferated in necrotic lesions in the upper digestive tract and was transported to the lower digestive tract where it was phagocytosed by macrophages. It was not possible to delineate which of the two was the case in this study, although the former seems more plausible. In humans, the presence of *P. aeruginosa* in stool culture is usually considered to be of no clinical significance [6]. However, severe gastrointestinal disease such as necrotizing enterocolitis is reportedly associated with *P. aeruginosa* infection in humans [6, 7]. Further studies are needed to fully understand the localization and pathogenicity of *P. aeruginosa* in cattle.

P. aeruginosa is an opportunistic pathogen and a common cause of nosocomial infections in immunocompromised human patients [4, 29]. Fever, diarrhea, pneumonia, skin lesions, and shock can occur in immunocompromised children infected with *P. aeruginosa* [4, 25]. Furthermore, *Pseudomonas* organisms may trigger a transient neutropenic state via the production of toxins. These toxins may inhibit granulocyte migration and cause bone marrow suppression in healthy children [28, 30]. The heifer was thin, and lymphopenia was observed in several lymph nodes. This indicated that the immune system was compromised. Considering that bacterial species other than *P. aeruginosa*, including *Fusobacterium* spp. and Gram-positive cocci, were also detected, we surmised that the severity of the symptoms was a result of mixed infection.

Antimicrobial therapy for bovine mastitis caused by *P. aeruginosa* infection has been reported, and susceptibility was reduced at the biofilm status on bovine mastitis [18]. In the antimicrobial susceptibility tests performed in the current study, the *P. aeruginosa* isolates showed sensitivity to generally efficacious drugs (aminoglycoside, fluoroquinolone, and carbapenem). Therefore, early treatment with these antimicrobial drugs would have been effective. Because the possibility of disease exacerbation by microbial substitution also exists, it is desirable to test the antimicrobial sensitivity of *P. aeruginosa* before establishing a treatment plan. Moreover, measures to reduce *P. aeruginosa* in the environment and elimination by autoimmunity are also considered effective, as observed in humans. It was recently reported that slightly acid electrolyzed water effectively reduced the incidence of mastitis in a cattle herd contaminated with *Pseudomonas* species [11].

Esophageal ulceration associated with *P. aeruginosa*, *Fusobacterium* spp., and Gram-positive cocci in cattle is a rare occurrence, and has not been previously reported. Further evaluations of the pathogenicity of *P. aeruginosa* are necessary.

ACKNOWLEDGMENTS. The authors thank Mr. M. Kobayashi and Ms. M. Shimada for their assistance with the histopathological procedures.

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