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NOTE

Pathology

Bovine abortion and necrotic placentitis by *Aspergillus terreus*

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ABSTRACT. A 31-month-old Japanese Black cow (*Bos taurus*) aborted at 5 months of gestation with no clinical symptoms. Histopathological examination of the placenta and fetus revealed severe necrotic placentitis associated with numerous irregular degenerative fungi and inflammatory cells. Regular filamentous fungi were also detected, without inflammatory response in the fetal digestive and respiratory organs. Both fungi had aleurioconidia and septa in the placenta and fetal organs and immunohistochemically stained with antibodies against *Aspergillus* spp. *Aspergillus terreus* was isolated from the fetal lung and abomasal contents as confirmed using mycological and molecular methods. This is the first immunohistochemical, morphological, and molecular identification of *A. terreus* in bovine placenta and aborted fetuses.

KEY WORDS: abortion, aleurioconidia, *Aspergillus terreus*, cattle, placentitis

Aspergillus infection remains a major cause of morbidity and mortality in humans [2] and in a wide range of animals [16]. In the United States, *A. fumigatus* is the most frequent isolate (62%) in bovine fungal abortion, followed by *A. terreus* (6.7%), *A. nidulans* (3.0%), and *A. flavus* (2.9%) [11].

Species within the genus *Aspergillus* produce asexual conidia that arise from conidiophores (phialidic conidia) [5]. In addition to phialidic conidia, *A. terreus* produces another type of asexual conidia, the accessory conidia –or aleurioconidia–, which arise directly on hyphae [5].

In animals, *A. terreus* infection associated with abortion has been reported in cattle [1, 11] and horses [3, 13]. In 1981, *A. terreus* was a cause of bovine mycotic abortion in the Czech Republic [1]. Unfortunately, those reports were old, and neither molecular analyses nor immunohistochemical studies were performed [1, 11]. Moreover, the histopathological description of bovine *A. terreus* infection was short and insufficient for diagnosis.

In this study, we histopathologically characterized unique fungal lesions in a bovine placenta and aborted fetus, and immunohistochemically and morphologically characterized the suspected fungal species. Furthermore, we genetically analyzed the isolated fungi and compared sequences of part of the β-tubulin gene with those of *Aspergillus* spp. to evaluate their pathogenic potential.

The beef cattle breeding farm was located in the Chiba Prefecture, Japan. A 31-month-old Japanese Black cow aborted 5 months after her second pregnancy in January 2015. The first calving was normal, and the cow exhibited no clinical symptoms, except for abortion. The placenta and aborted fetus were necropsied at the Chiba Prefectural Chuou Livestock Hygiene Service Office.

At necropsy, the allantochorion was diffusely edematous and thickened. Severe necrotic lesions were also observed in the

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numerous placentomes. The fetus presented with cutaneous edema, and the pleural effusion and ascites were bloody and increased (Supplementary Fig. 1). No other gross lesions were observed.

Tissues from the placenta, umbilical cord, and fetal organs (esophagus, rumen, reticulum, omasum, abomasum, duodenum, ileum, cecum, colon, rectum, mesenteric lymph nodes, liver, spleen, kidney, heart, lung, trachea, brain, spinal cord, gall bladder, urinary bladder, thyroid gland, thymus, skin, and muscle) were collected, fixed in 20% phosphate-buffered formalin and embedded in paraffin. Tissue sections (~4-µm thick) were stained with hematoxylin and eosin. To investigate the presence of fungi, periodic acid Schiff (PAS) reaction and Grocott's methenamine silver (GMS) stain were also applied to all tissue sections.

Serial histological sections (placenta, umbilical cord, and fetal organs) were prepared for immunohistochemical staining using a commercial kit (universal immuno-enzyme polymer method using a Histofine simple stain MAX-PO Kit, Nichirei Corp., Tokyo, Japan). The sections were pre-treated with 0.1% actinase E prior to immunostaining, and endogenous peroxidase activity was blocked with methanol and 3% H_2O_2. The primary antibodies used in this study were mouse monoclonal antibodies against *Aspergillus* (Clone Mab-WF-AF-1; Dako, Carpinteria, CA, USA), anti-*Rhizomucor* (Clone WSSA-RA-1; Dako), and rabbit polyclonal antibody against *Candida albicans* (Biogenesis, Dorset, UK). Sections were lightly counterstained with hematoxylin and assessed using light microscopy.

Isolation of the fungi was attempted from the fetal liver, lung, kidney, heart, and abomasal contents, where samples were inoculated onto potato dextrose agar (PDA; Nissui, Tokyo, Japan) containing 50 µg/ml of chloramphenicol and incubated at 36.5°C. Lactophenol wet mounts were used for morphological observation.

Two isolates from the fetal lung and abomasal contents were used for molecular biological identification. Approximately 50 mg of mycelium was mechanically lysed using ceramic beads. DNA was extracted using a commercial kit (QIAamp DNA Mini Kit, Qiagen, Mississauga, Ontario, Canada) and polymerase chain reaction (PCR) was conducted using primers targeting the β-tubulin gene (primer pairs Bt2a and Bt2b) [8]. Direct sequence analysis of the PCR products was performed using a commercial kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Austin, TX, USA) and a sequencer (Applied Biosystems 3130×l Genetic Analyzer, Hitachi, Tokyo, Japan).

Fungal samples for scanning electron microscopy (SEM) were dried, osmium coated (OPC60A; Filgen, Nagoya, Japan), and examined using a SEM (JSM-7600F; JEOL Ltd., Tokyo, Japan).

Histopathologically, severe necrotic placentitis (Fig. 1a) was detected with numerous fungal hyphae in all the placentomes examined. The allantochorion was also congested and edematous with numerous neutrophils, macrophages, and lymphocytes. Fibrinoid necrosis was present in large- to medium-sized arteries. Fungal hyphae and inflammatory cells were also seen in the blood vessels. Numerous fungi with a small number of aleurioconidia were detected in the debris by PAS reaction and GMS staining (Fig. 1b, 1c). The hyphae had various widths (3–12 µm) and were short in length with a septum. Irregular distortions and proliferation were also observed.

Although the hyphae were also detected in the lumen of the fetal digestive tract (esophagus, rumen, reticulum, omasum, abomasum, duodenum, ileum, cecum, colon, and rectum) and trachea, no inflammatory response was observed. The hyphae were of regular diameter (3–4 µm wide), septum, some acute angle branching, and proliferation occurred in parallel arrays as in *Aspergillus* spp. Therefore, fungal hyphae characteristics were compatible with those of *Aspergillus* spp. A small number of aleurioconidia were also detected. Mild purulent pneumonia was observed in the lungs. In the pulmonary lymph nodes, neutrophil infiltration was detected in the lymph sinus. No other lesions were detected in other organs.

Immunohistochemically, the hyphae reacted with a monoclonal antibody against *Aspergillus* in all tissues examined, including the placenta, umbilical cord, and fetal organs. The degrees of reaction were high in the allantochorionic debris (Fig. 1d) and gastrointestinal contents (Fig. 1e), moderate in the tracheal contents, and slight in the bronchia. Neither *Rhizomucor* nor *C. albicans* antigens were detected in any of the tissues examined.

Hyphal growth was observed 2 days later in all inoculated samples (Fig. 1f). All isolates revealed similar colonies, white and cinnamon-colored. The colony reverse was pale yellow. Microscopic morphology was consistent with *Aspergillus* spp., with conidia-producing structures. Conidiophore stipes were smooth-walled and hyaline. The vesicles were subspherical. The phialides were biseriate, and were present in the upper part of the vesicle. The conidial heads were long, fan-shaped, and compact. Conidia were smooth-walled, spherical, and ellipsoidal. Aleurioconidia was also observed in the lateral regions of the hyphae (Fig. 1g). Similarly, SEM micrographs revealed the presence of aleurioconidia, morphologically compatible with *A. terreus*, arising directly on hyphae (Fig. 1h, 1i). The structure was globose to sub-globose with a roughened wall of 4–7 µm in size.

Consequently, the isolate was determined to be *Aspergillus* section *Terrei* by molecular examination. The 467 bp sequences obtained between the two isolates were 100% identical. A homology search for the sequences was conducted using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (Bethesda, MD, USA), and 100% homology was obtained using the β-tubulin gene of a known *A. terreus* strain (Accession No. LC060787). These results confirmed *A. terreus* infection in the aborted fetus and provided an explanation for the occurrence of necrotizing placentitis and subsequent abortion.

Aspergillus spp. hyphae in the infected tissue have been known to exhibit morphological *Mucorales*-like features [14]. In the placenta, the hyphae looked like *Mucorales* with a severe inflammatory response on histopathological examination. However, *Mucorales*-like hyphae were not observed, and typical histopathological features of *Aspergillus* spp. were observed without an inflammatory response in the fetal gastrointestinal contents and respiratory organs. The bovine fetus has a different immune system from adult cows. The major T-cell populations in adult cows are CD4 + T cells and CD8 + T cells [12]. However, the γδ T-cell is the major T-cell population in fetuses and young cows [18, 19]. Therefore, their immune response differs from adult cows [19]. While a *Mucorales*-like change occurred in the placenta, it was not observed in the fetus in the present case. This difference could

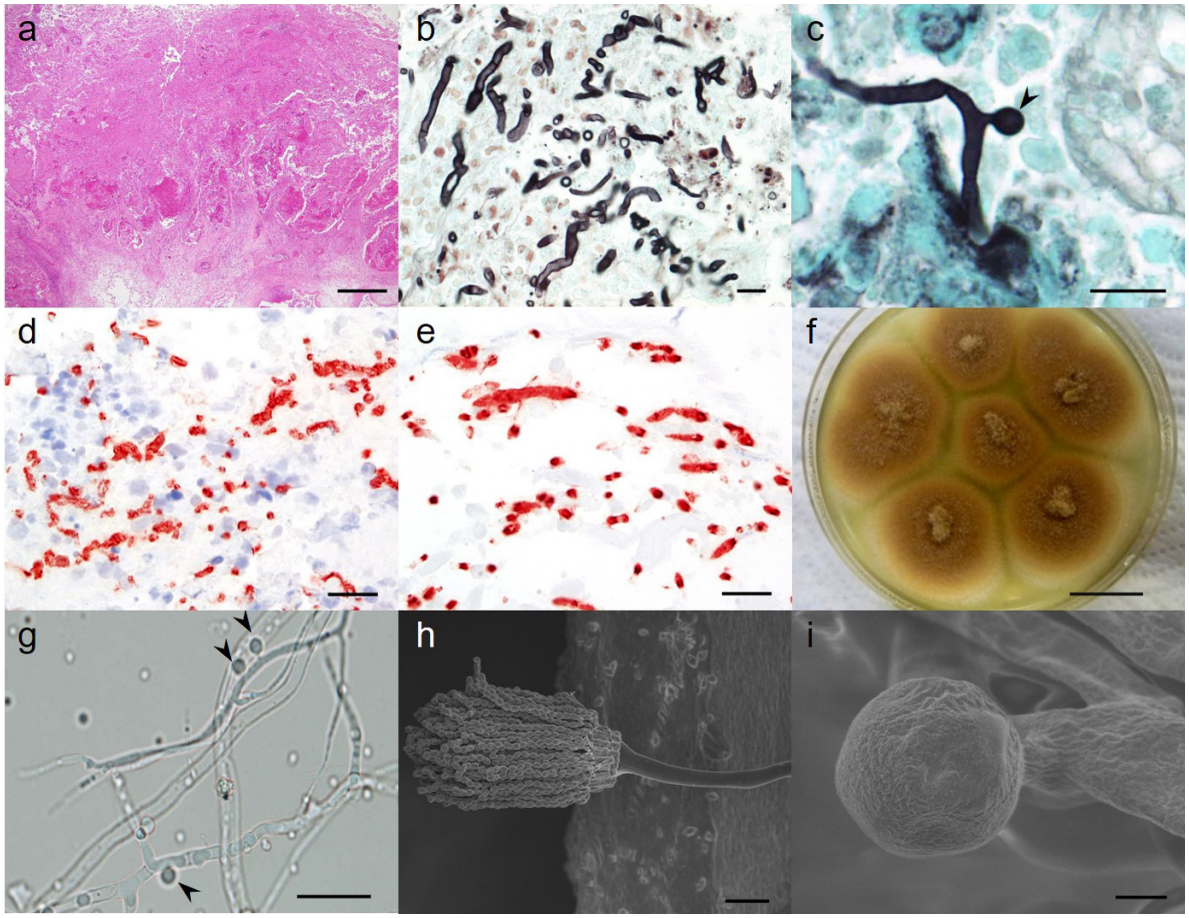

Fig. 1. **a**. Severe necrotizing placentitis. The allantochorion is edematous and swollen and shows severe neutrophil infiltration. Hematoxylin and eosin staining. Bar=500 µm. **b**. Septate fungal hyphae has various widths and are short in length, irregularly distorted, and proliferated in irregular arrays in the placenta. Grocott's methenamine silver (GMS) staining. Bar=10 µm. **c**. Aleurioconidia (arrowhead) and fungal hyphae in the placenta. GMS staining. Bar=10 µm. **d**. The irregular hyphae have reacted with *Aspergillus* monoclonal antibody in the placenta. Immunohistochemistry. Bar=20 µm. **e**. Intact hyphae that reacted with *Aspergillus* monoclonal antibody in the fetus colon contents. Immunohistochemistry. Bar=20 µm. **f**. Isolates revealed white and cinnamon-colored colonies on potato dextrose agar after incubation for 11 days at 36.5°C. Bar=2 cm. **g**. Several aleurioconidia (arrowheads) have formed laterally from isolates' hyphae. Bar=25 µm. **h**. The fungal isolate has compact and densely columnar conidial heads. Scanning electron microscopy (SEM). Bar=10 µm. **i**. Aleurioconidia formed laterally on the isolates' hyphae. SEM. Bar=1 µm.

be due to differences in the immune response of the cow and the fetus.

Because *Mucorales*-like hyphae were observed in *A. terreus* infection, there could be a possibility of misdiagnosis through histopathological examination. For example, an *A. terreus* case might be misdiagnosed as *Mucorales* infection or *Aspergillus* and *Mucorales* mixed infection. In the present study, we performed immunohistochemical examinations, finding that *Aspergillus* spp. was present in both the fetus and the placenta. However, no infection of *Rhizomucor* spp. in these organs was detected. Therefore, immunohistochemical examination can help in the diagnosis of *Aspergillus* spp.

Although an immunohistochemical examination can suggest the genus of the infecting fungi, it is impossible to identify the species by this method. *A. terreus* produces aleurioconidia both *in vitro* and *in vivo* [4, 5]. Thus, confirmation of aleurioconidia in histopathological examination would be important for a definitive diagnosis of *A. terreus* infection. In the present case, aleurioconidia were observed morphologically by SEM, PAS, and GMS staining. Production of aleurioconidia enhances the virulence of *A. terreus* [4, 5], since aleurioconidia can germinate rapidly, have enhanced adherence to microspheres, and are metabolically more active than phialidic conidia [4, 5]. Although no histopathological findings of aleurioconidia were detected in the initial bovine report [1] and previous equine reports [3, 13], they have been described in dogs [6].

Transuterine *A. terreus* transmission and placentitis have been described in dogs [6]. It is very important to confirm the presence of aleurioconidia in aborted fetuses to accurately diagnose mycotic abortion by *A. terreus*, as aleurioconidia has been observed in the uterus and placenta [6]. Although molecular identification of fungi has been the most popular method in recent years [9, 10, 17], in dogs the identification was performed only by morphological observation [6]. In the present case, the identification of *A. terreus* was performed morphologically and molecularly.

In the case of the dog, the most prominent fungal lesion was the necrotic focus in the uterus, which was the most likely source

of dissemination [6]. The cow in the present study did not show any clinical symptoms, and significant fungal growth was observed in the placenta and fetus. There were two possible entry routes to the placenta and fetus. The first possible entry route was hematogenous dissemination from the cow's lung and rumen [7, 15]. Extension from either respiratory or rumen infection is possible [7, 15]. Unfortunately, the cow's lung and rumen could not be examined in the present study. The second possible entry route was vaginal. However, the placental lesions were diffuse, and not localized, in the present case. Although the accurate entry route could not be identified in the present case, the development of severe diffuse lesions in placentomes suggested hematogenous dissemination [7, 15]. Appropriate hygiene management, such as frequent replacement of pregnant cows' bedding, might reduce the frequency of mycotic abortions by *A. terreus*.

We histopathologically characterized the lesions and morphologically characterized the suspected fungal species. This is the first report of genetic, immunohistochemical, and ultrastructural analyses of *A. terreus* associated with bovine abortion. Although *Aspergillus* spp. is known to cause opportunistic infections, there have been few reports of its association with animal abortion. Thus, our study provides important insights into bovine abortion that will benefit both veterinary pathology and livestock veterinarians.

CONFLICTS OF INTEREST. The authors have nothing to disclose.

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