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Systemic mucormycosis caused by *Lichtheimia ramosa* in a pregnant cow

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Abstract: Mucormycosis is a life-threatening fungal disease caused by opportunistic pathogens present in the environment. This report presents a case of a *Lichtheimia ramosa* infection that manifested as characteristic lesions in various visceral organs of a pregnant cow in Korea. The post-mortem examination revealed the cause of death to be mucormycosis. The fungi isolated from the liver were found to be *Lichtheimia* spp. Analysis of the fungi using Internal Transcribed Spacer gene sequencing facilitated the identification of *Lichtheimia ramosa.* To the best of our knowledge, this is the first clinical case of angioinvasive mucormycosis in cattle.

Keywords: cattle; fungal disease; angiovasive; Internal Transcribed Spacer

Mucormycosis is a severe fungal infection that mostly affects immunosuppressed individuals. Infections can be characterised by extensive angioinvasion and necrosis. *Mucor* includes zoonotic pathogens present in soil, decaying matter, and cattle feed, such as hay (Ali and Khan 2006; Woo et al. 2008). In humans, fungi from the order Mucorales cause zygomycosis more frequently than do those from the order Entomophthorales. Zygomycosis is subdivided into various infection types according to the distribution of the lesions (Jensen et al. 1994; Garcia-Hermoso et al. 2009). Regardless of the specific type of fungal infection, zygomycosis is a fatal disease that requires an early, accurate diagnosis and treatment (Jensen et al. 1994; Garcia-Hermoso et al. 2009). In humans and cattle, most cases of zygomycosis are caused by mucormycosis. Bovine zygomycosis usually produces focal lesions (mostly lymphadenitis or gastric zygomycosis) and is often incidentally detected during an autopsy or an examination at a slaughterhouse. Mortal-zygomycosis is rarely reported in cattle.

Cases of zygomycosis caused by angioinvasive transmission from other organs along with one aborted case are among the limited number of studies that discuss fatal fungal infections. With the exception of the latter case, these incidences of disseminated bovine mucormycosis were similar to the fatal human mucormycosis.

In this study, a fatal case of systemic mucormycosis caused by *Lichtheimia ramosa* that spread through the alimentary system is discussed.

Case report

A 31-month-old pregnant cow suddenly demonstrating nasal discharge and anorexia was admitted to the veterinary clinic with abnormal clinical signs, including depression.

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She was treated for a suspected intestinal disorder; however, her condition did not improve, and she died. An autopsy was conducted.

The following necropsy findings were observed: the lungs were dark and swollen, and the spleen was three times larger than the normal size. Several irregular yellowish, haemorrhagic foci (0.5–2.0 cm in diameter) with dark red borders were observed in the liver (Figure 1A). Similar lesions were also noted in the rumen, reticulum, and omasum. A small number of lesions were also observed in the abomasum. Tissue samples from the lungs, spleen, heart, kidneys, rumen, reticulum, abomasum, omasum, intestine and liver were collected. The samples were divided and frozen for DNA extraction or fixed in 10% neutral formalin for 24 h before routine wax embedding and histologic processing. The liver tissue was used for the bacterial and fungal culture.

The histopathologic examination revealed severe lesions in the liver, rumen, reticulum, and omasum. These tissues showed marked necrotic changes and severe inflammatory cell infiltration predominantly consisting of macrophages, lymphocytes, and neutrophils. Multifocal haemorrhagic lesions and thrombi were also noted (Figure 1B and 1C). The hyphae were thin and the centre of the wall was empty. Line structures that stretched irregularly and reacted strongly with Gomori's methenamine

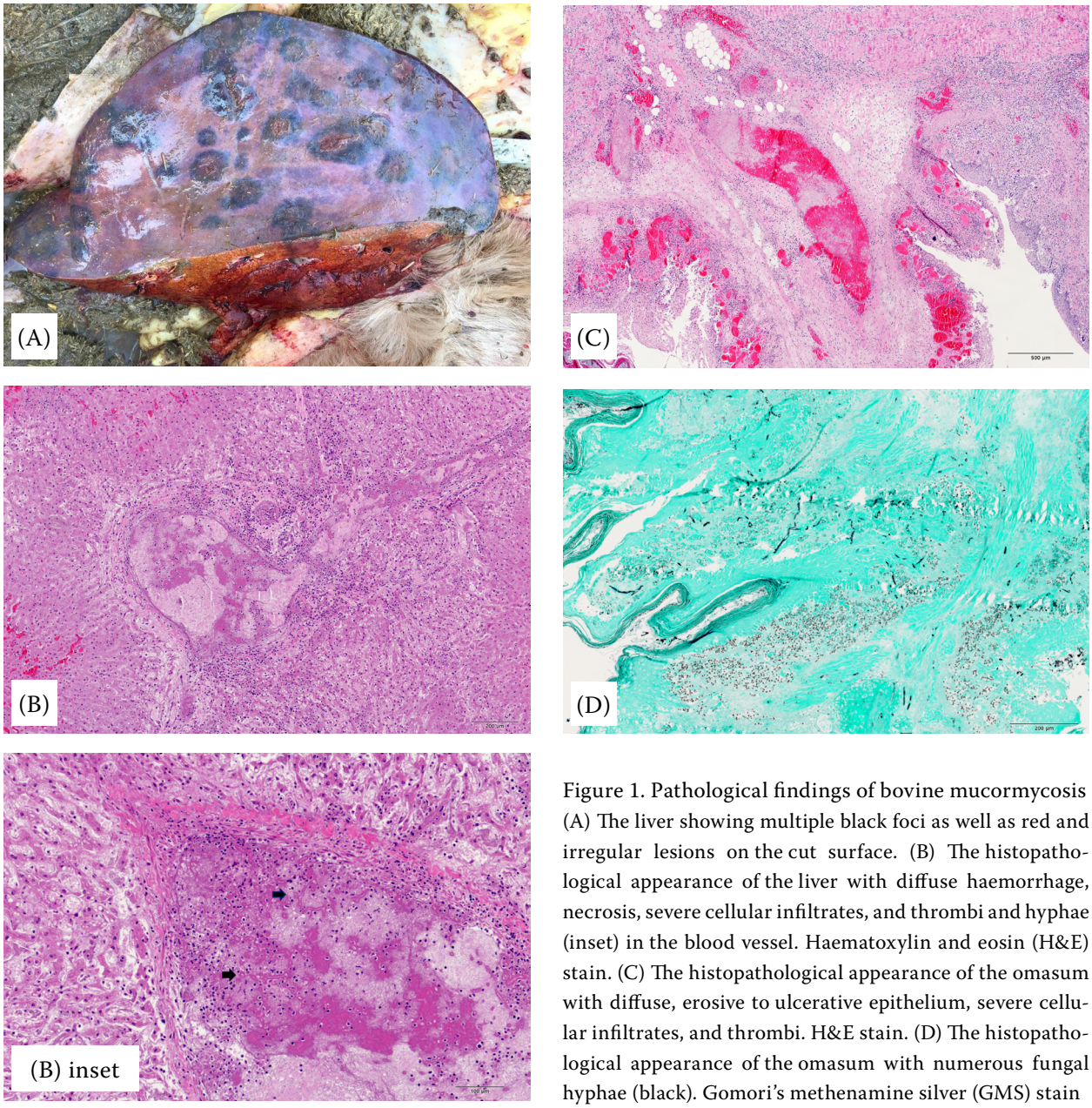

Figure 1. Pathological findings of bovine mucormycosis
(A) The liver showing multiple black foci as well as red and irregular lesions on the cut surface. (B) The histopathological appearance of the liver with diffuse haemorrhage, necrosis, severe cellular infiltrates, and thrombi and hyphae (inset) in the blood vessel. Haematoxylin and eosin (H&E) stain. (C) The histopathological appearance of the omasum with diffuse, erosive to ulcerative epithelium, severe cellular infiltrates, and thrombi. H&E stain. (D) The histopathological appearance of the omasum with numerous fungal hyphae (black). Gomori's methenamine silver (GMS) stain

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silver (GMS) stain were also observed (Figure 1D). The hyphal length ranged from 5 µm to 50 µm. Necrotic foci around the fungal hyphae were found in the liver, rumen, reticulum, and omasum in accordance with the macroscopic findings. Thrombi were found in the affected regions of the liver, rumen, and abomasum, and ulceration with inflammation was observed in the abomasum.

Additionally, thrombi in the blood vessels, thickened alveolar walls, and mild pneumonia were observed in the lungs.

The tissue samples were used for the bacterial culture and viral gene amplification. As a result, no bacteria or viruses were detected. The fungi were consistently cultured on a blood agar. These fungi were subsequently transferred to potato dextrose agar plates and incubated at 25 °C for 4 days. The isolated colonies, named B17Q226, were subjected to a microscopic examination and gene amplification for the mould classification. Microscopically, these fungi had pyriform sporangia with a conical-shaped columella; pronounced apophysis; and smooth, coloured, long elliptical spores measuring 2.18–3.06 µm in diameter. For the molecular analysis, DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) was performed using Internal Transcribed Spacer (ITS) primers (ITS1 and ITS4) (Woo et al. 2008; Woo et al. 2010b), which demonstrated bands at approximately 850 bp. The amplicon was purified using a Quick Purification Kit (QIAGEN, Hilden, Germany), and the purified DNA was used for the direct sequencing. The B17Q226 sequence (633 nt) obtained by Macrogen (Seoul, Republic of Korea) was confirmed as *Lichtheimia ramosa* using the BLAST (basic local alignment search tool) technology from the National Center for Biotechnology Information (NCBI). This nucleotide sequence (GenBank accession number MH675478) included a partial Internal Transcribed Spacer 1 (ITS1), a complete 5.8S ribosomal RNA, and a partial Internal Transcribed Spacer 2 (ITS2), all of which matched the *Lichtheimia* spp. sequences in the NCBI GenBank database. These were identified using the Bioedit software. The analysed nucleotide sequence showed 93% identity with *L. ramosa* (GQ342877). A phylogenetic tree was generated using neighbour-joining algorithms in MEGA v6 (Tamura et al. 2013). The percent frequencies of the groupings were determined after a 1 000 bootstrap evaluation. The phylogenetic tree showed that this isolate (MH675478) clustered with *L. ramosa* strains (GQ342877, MG58398, FJ719386, and FJ719406) (Figure 2). *L. ramosa* belongs to the fam-

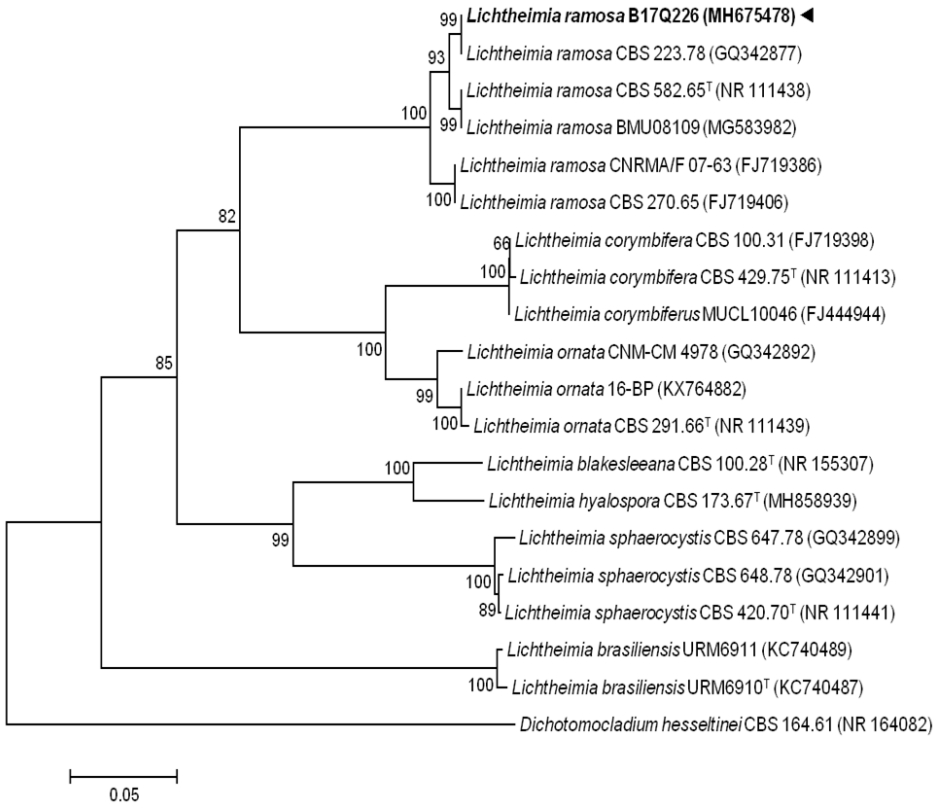

Figure 2. The phylogenetic tree showing the relationship of the *Lichtheimia* species and the *L. ramosa* isolate in the Internal Transcribed Spacer 1, 5.8S ribosomal RNA, and Internal Transcribed Spacer 2 regions. All the reference sequences are indicated by the GenBank accession numbers. *Dichotomocladium hesseltinei* is used as an outgroup. All *Lichtheimia* spp. ex. type strain sequences are marked as ^{T}. Bootstrap values greater than 500 of 1 000 replicates are indicated

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ily *Lichtheimiaceae* in the order Mucorales (Garcia-Hermoso et al. 2009; Tamura et al. 2013). Previous studies have shown that the ITS gene is useful for the identification of the *Lichtheimia* species (Ali and Khan 2006; Woo et al. 2008).

DISCUSSION

After examination of the gross lesions, abdominal foci associated with a fungal infection were found in a pregnant cow. A fungal infection and the corresponding inflammatory foci were also noted in the mucosa of the rumen, abomasum, and liver in the form of gross lesions. It was hypothesised that the fungi entered the liver via the digestive organs, since the hepatic lesions were more severe than those in the abomasum.

Furthermore, many hyphae were also observed in the omasum; thus, it was presumed that the fungal infection in the liver originated from a digestive infection transmitted through the blood vessels from the omasum. In previous studies, three cases of haematogenously transmitted zygomycoses with multiple thrombi, perivascular necrotic parenchyma, and cellular infiltration have been reported (Munday et al. 2006; Davies et al. 2010). These cases, however, involved a cerebral infection, which may have been caused by the inhalation of spores into the respiratory tract (Woo et al. 2010a). A distribution of lesions through the blood vessels was not observed in this study. Further, the transmission of fungi from the lymph nodes and other organs was not observed and no lesions were observed in the lungs. Thus, in this case, the infection was most likely caused by the intake of spores into the digestive system, which subsequently may have spread through the blood vessels.

According to the histopathological analysis, the cow in the current case was diagnosed with heematogenous mucormycosis that developed via the digestive system.

Using the PCR analysis, the fungus which had infected the liver and abomasum was identified as *L. ramosa*, which belongs to the family Lichtheimiaceae in the order Mucorales (Chihaya et al. 1986). As *Lichtheimia* species are often present in feed, such as silage (Ortega et al. 2010), the consumption of asexual spores might cause gastritis and infections, which could progress to haematogenous infections. Additionally, the *Lichtheimia* species are common gastric micro-organisms found in cattle (Chihaya et al. 1986).

Parenteral administration of broad-spectrum antibiotics over three days has been reported to cause gastrointestinal mycosis in cattle (Woo et al. 2010a), and the cow in the present case suffered mycotic abomasitis, hepatitis, and omasitis following the antibiotic treatment for five days.

In humans, mucormycosis usually occurs in patients with diabetes or in patients who are immunocompromised, but it can also arise in apparently healthy individuals (Knudtson et al. 1973; Woo et al. 2010b). In this case, the pregnant cow experienced respiratory problems, did not demonstrate pneumonia and responded to the antimicrobial treatment. There is a possibility that the cow may have been immunosuppressed as a result of the pregnancy; however, no immunological information was available. According to a recent study, mucormycosis often originates from the sinuses and spreads to the ethmoidal and orbital veins to the brain (Knudtson et al. 1973). In the current case, the fungus may have primarily infected the gastric organ without inhalation and subsequently invaded the liver through the blood vessels, rather than through the lymphatic tissue. Previous studies have shown that mucormycosis in humans varies according to the host's immune system. *Lichtheimia* species, as other mucormycetes, invade the vasculature; however, they are not neurotropic infectious agents. Why gastric mucormycosis occurred via a unique invasion route in the present case remains unclear; it may be been related to host factors, such as the cow's immune status, rather than the characteristics of the infectious agent. Among domestic animals, mucormycosis has only been reported in sheep, where it was caused by the *Conidiobolus* species (order: Entomophthorales) (Woo et al. 2010a). Disseminated mucormycosis has been observed in humans and sheep; however, Zygomycetes abortion has only been observed in animals.

Mucormycosis, due to *L. ramosa*, had not been previously reported in adult cattle, but it was reported once in an aborted foetus, which did not demonstrate any pathological abnormalities (Ali and Khan 2006). Similar to reports in other species, the source and route of the infection in this case was unclear (Cheng et al. 2009; Skiada et al. 2009; Woo et al. 2010a). The presence of gastric inflammatory lesions with GMS positive fungi, however, suggests that the oral route was the source

https://doi.org/10.17221/8/2020-VETMED

of the infection. It has been reported that a *Mucor* infection is dependent on the immunity and nutritional status of the host (Woo et al. 2008). It is uncommon in adult cattle; thus, it is thought that the pregnancy may have contributed to the development of the disease in this case.

In conclusion, we suggest that fatal fungal infections should be considered as a differential diagnosis if the cow does not respond to antibiotics and is immunosuppressed, such as in pregnancy.

Conflict of interest

The authors declare no conflict of interest.

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