NOTE  Pathology

Immunohistochemical and Ultrastructural Identification of Fusobacterium necrophorum subsp. necrophorum in Bovine Fatal Necrotizing Glossitis

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ABSTRACT. A 37-day-old male Japanese black calf showing marked salivation and leucocytosis died and was examined the tissues histologically. Histological lesions were characterized by severe focal necrotic glossitis on the ventral side of the root of the tongue. Immunohistochemically, Fusobacterium necrophorum subsp. necrophorum antigen was detected in the necrotic tissues and its distribution corresponded to that of the gram-negative, nonsporeforming, long filamentous organisms. Ultrastructural similarities between the organism and F. necrophorum subsp. necrophorum, but not subsp. funduliforme were observed. These findings clearly demonstrated that the fatal necrotic glossitis was caused by F. necrophorum subsp. necrophorum. This is the first report of bovine fatal necrotizing glossitis with leucocytosis caused by F. necrophorum subsp. necrophorum infection, and this organism may be an important fatal pathogen in calves with glossal lesions.

KEY WORDS: calf, fatal necrotizing glossitis, Fusobacterium necrophorum subsp. necrophorum.

Fusobacterium necrophorum, a gram-negative, non-poreforming anaerobe, is a normal inhabitant of the alimentary tract of animals and humans [5, 6, 15, 16]. Two types of F. necrophorum, subsp. necrophorum (biotype A) and funduliforme (biotype B), have been recognized. They differ morphologically, biochemically, and biologically [1, 4, 12–14]. Studies have indicated that the strains of F. necrophorum subsp. necrophorum are more pathogenic, hemolytic and enzymatically active than those of subsp. funduliforme [1, 4, 13, 14]. The pathogenic role of F. necrophorum is complex and not well defined. Several toxins, such as leukotoxin, endotoxin, hemolysin, hemagglutinin and adhesion, have been implicated as virulence factors [16].

Fusobacterium necrophorum infection is known to cause sporadic diseases in ruminants. In cattle, it is most commonly associated with interdigital necrobacillosis, hepatic abscesses and rumenitis, calf diphtheria (stomatitis, laryngitis and pharyngitis) and anterior vena cava thrombosis [2, 3, 5, 7, 10–12, 17]. These conditions are often diagnosed from the clinical manifestation and the typical macroscopic appearance of lesions [16]. Bacteriology often is inconclusive despite the use of anaerobic techniques, and overgrowth by contaminating bacteria is common [16, 18]. It is therefore probable that the true incidence of disease caused by F. necrophorum is unknown [18].

This communication describes fatal necrotizing glossitis in a calf with leucocytosis resulting from F. necrophorum subsp. necrophorum infection, a condition that, to the authors’ knowledge, has not previously been reported in cattle.

The subject, a 28-day-old male Japanese black calf, and 34 other cattle were kept in an enclosed stall that had access to an outdoor, unpaved yard. The animal showed anorexia, salivation, dehydration and very fetid breath. Detailed examinations of the oral cavity revealed focal ulcerative lesions on the ventral side of the root of the tongue. At this stage, hematological examinations revealed marked leucocytosis and neutrophilia (26,500 leukocytes, 19,610 neutrophils, 6,890 lymphocytes and no eosinophils and basophils per µL). The animal was treated with kanamycin, dihydrostreptomycin and lactated Ringer’s solution, but the condition did not improve. After eight days, the calf was debilitated, dull, anorexic and reluctant to stand. There had been some loss of weight. After a period of 10 days, the animal died and was subjected to necropsy. No clinical abnormalities were found in any of the other cattle.

Several tissue samples taken from the tongue and other organs were collected, and fixed by immersion in 10% phosphate buffered formalin and embedded in paraffin. The tissue sections (approximately 3 µm thick) were stained with hematoxylin and eosin (HE) and Gram’s method for the histological examinations. The specimens were also stained by the Warthin-Starry technique. Serial histological sections from the tongue tissues were prepared for the streptavidin-biotin immunoperoxidase method with Histofine SAB regents (Nichirei Corp., Tokyo, Japan). After deparaffinization of the tongue section, endogenous peroxidase activity was blocked by methanol and 3% H2O2 (Sigma Chemical Company, St. Louis, MO, U.S.A.). The primary antibody was a rabbit polyclonal antibody against F. necrophorum subsp. necrophorum strain ATCC25286 (kindly provided by Dr. Y. Nakjima, National Institute of Animal Health, Ibaraki, Japan) at 1:5,000-fold dilution. The sections were lightly counterstained with Mayer’s hematoxylin and assessed under a light microscopy. The specificity of the

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antisera was ascertained by using sections of the liver from cattle experimentally infected with *F. necrophorum* subsp. *necrophorum*. To ascertain the specificity of the staining, anti-*Actinobacillus lignieresii* rabbit serum (kindly provided by Dr. M. Nakazawa, National Institute of Animal Health, Ibaraki, Japan) and normal rabbit serum were used for staining of control sections.

Small blocks taken from the 10% formalin-fixed tongue were post-fixed in 1% osmium tetroxide, embedded in epoxy resin, sectioned (approximately 60 nm thick) and stained with uranyl acetate and lead citrate. The sections were then examined with a JEOL JEM-1010 transmission electron microscope (TEM).

At necropsy, the gross lesions were confined to the ventral side of the root of the tongue, and were 5.5 × 4.5 × 3.0 cm, focal well-defined, ulcerative elevated plaques of yellow-gray necrotic tissue (Fig. 1). No lesions were observed in other parts of the oral cavity, larynx, pharynx, esophagus and the remainder of the respiratory and digestive tracts.

Histologically, the glossal lesions were characterized by severe focal necrotizing glossitis (Fig. 2). They showed degeneration and coagulative necrosis of skeletal muscle and degenerative cells surrounded by inflammatory cells, including neutrophils and macrophages (Fig. 3). Numerous gram-negative, long filamentous argyrophilic nonspore-forming organisms were found mainly at the boundary between necrotic and viable tissue (Fig. 4). Clubs were not formed around the microbial clumps in these lesions.

Immunohistochemically, *F. necrophorum* subsp. *necrophorum* antigen was demonstrated corresponding to the gram-negative bacilli in the necrotic lesions (Fig. 5). There was no evidence of *A. lignieresii* antigen in the tissue exam-
Electron microscopy showed that the organisms possessed a cell wall structure typical of gram-negative bacteria. The organisms were 0.65 µm in diameter (Fig. 6). The cytoplasm appeared electrondense due to abundant distribution of ribosomes, while nucleus were less electrondense. Unlike *F. necrophorum* subsp. *funduliforme*, extracellular vesicles and particles of diverse shapes and sizes were not observed despite of careful examinations of ultrathin sections.

The disease reported here was closely related to a *Fusobacterium* infection. The organisms seen in the necrotizing glossitis, while not cultured and positively identified, resembled to *F. necrophorum* in terms of their histological morphology and immunohistochemical reactivity [16]. Detailed ultrastructural features of two types of *F. necrophorum* have been reported: *F. necrophorum* subsp. *necrophorum* is distinguished from *F. necrophorum* subsp. *funduliforme* on the basis of the presence or absence of extensively disseminated extracellular vesicles [4]. Such
vesicles were absent in our case, and the considerations favored the inclusion of the microorganisms to be *F. necrophorum* subsp. *necrophorum* rather than subsp. *funduliforme*.

Necrobacillosis is a sweeping term applied to a number of lesions or disorders associated with *F. necrophorum*. Although there is no doubt that *F. necrophorum* can be recovered from various lesions in animals, there is a question as to whether it is a primary pathogen. It is often impossible to make an accurate diagnosis of *F. necrophorum* infection because of difficulties in anaerobic cultural procedures [9, 16, 18]. Our case was clearly distinguished from macroscopically similar diseases such as actinobacillosis (woody tongue), actinomycosis, nocardiosis and staphylococcal infection (botryomycosis) on the bases of location, size, histopathology and etiology [6, 16]. Profuse growths of *F. necrophorum* subsp. *necrophorum* in the glossal lesions strongly suggested that the fatal necrotic glossitis was the result of fusobacterial infection.

In cattle, *F. necrophorum* is the principal etiologic agent of bovine liver abscesses [6, 8, 12, 16]. The organism is also implicated in calf diphtheria and interdigital necrobacillosis (foot rot), and is frequently isolated from the cases of mastitis and metritis [2, 3, 7, 17]. In calves, a depressed immune system together with granulocytopenia is considered to allow *F. necrophorum* infection to become generalized [9], but granulocytopenia was not observed in our case. We therefore agree with one speculation that unknown factors, perhaps viral, caused granulocytopenia [9].

Recently, a case of unusual necrotic glossitis and sinusitis with *F. necrophorum*-like organisms was reported in a 7-year old cow, and its clinical and histological findings were similar to those of our case [18]. However, attempts to isolate the organisms failed and immunohistochemical and ultrastructural examinations were not performed. Moreover, there was no explanation for the development of the lingual lesions. *F. necrophorum* is a normal inhabitant of the gastrointestinal tract in mammals [5, 6, 15]. In our case, the organisms may have invaded the glossal tissue from a traumatic injury formed during eating and rumination. This is the first report of bovine fatal necrotic glossitis with leukocytosis caused by *F. necrophorum* subsp. *necrophorum*, and it is suggested that this organism should be included in the list of possible pathogens of glossitis in calves.

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