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Ovine Fetal Necrobacillosis

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Summary

The association of *Fusobacterium necrophorum* with late term abortion in sheep is reported. The bacterium was not culturable, but was identified in five cases by fluorescence in-situ hybridization (FISH) with an oligonucleotide probe specifically targeting 16S rRNA in *F. necrophorum*. Gross lesions were found in several tissues. Histologically, placental lesions were characterized by locally diffuse infiltration of neutrophils, closely associated with abundant small Gram-negative and FISH-positive rods, thrombosis and necrosis. Lesions in the fetal–maternal interface were multifocal and consisted of villous necrosis and suppurative inflammation. Spread to the fetus from the placenta appeared to occur in two ways. Some fetuses had multifocal necrotizing hepatitis consistent with haematogenous spread through the umbilical vein; further dissemination to other organs occurred. Transplacental spread and infection of the fetus through the amniotic fluid was characterized by development of multifocal suppurative dermatitis and suppurative bronchopneumonia. Localization of FISH-positive bacteria in necrotic lesions was restricted to the periphery. *F. necrophorum* would seem to have been unrecognized previously as a cause of abortion. The value of culture-independent diagnostic methods is emphasized.

Keywords: Fusobacterium necrophorum; abortion; necrobacillosis; sheep

Introduction

Fusobacterium necrophorum, a Gram-negative, strictly anaerobic pleomorphic bacterium, is an opportunistic pathogen in several animal species (Langworth, 1977). In ruminants, the bacterium may act as a principal pathogen or as an important constituent in mixed bacterial infections. The most significant diseases associated with *F. necrophorum* in ruminants are the "rumenitis-liver abscess complex" (Scanlan and Edwards, 1990; Nagaraja and Chengappa, 1998), necrobacillosis of the foot (Parsonson *et al.*, 1967; Yager and Scott, 1985), and necrotizing stomatitis (Barker and Van Dreumel, 1985; McCourtie *et al.*, 1990). In neonatal ruminants, necrotizing hepatitis may be the result of ascending infection from omphalophlebitis (Kelly, 1985).

Abortion due to *F. necrophorum* was reported in a bovine fetus showing widespread serosal inflammation (Otter, 1996); other reported cases (Pattnaik *et al.*, 1994), have lacked histopathological confirmation. Isolation of the bacterium from aborted fetuses is not sufficient to establish a diagnosis, as *F. necrophorum* is a common invader of the bovine uterus *post partum* (Bekana *et al.*, 1994, 1997). Opening of the cervical canal before abortion may lead to colonization of the fetus and its membranes, or these structures may become contaminated during passage through the caudal parts of the genital tract. Consequently, diagnosis must be based on histopathological recognition of inflammation in association with *F. necrophorum*.

Kirkbride *et al.* (1989) reported ovine abortion due to *Fusobacterium nucleatum*, but fetal infection with *F. necrophorum* in sheep has not been reported. The present report describes ovine fetuses spontaneously infected with *F. necrophorum*.

Materials and Methods

Fetuses Infected with F. necrophorum

The animals were obtained from a larger study on the causes of prenatal mortality in Danish sheep (Agerholm

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et al., 2006). Initially, six fetuses consisting of three pairs of twins (cases 1A,B – 3A,B) with an unidentified bacterial infection were selected, forming a group of cases with similar inflammatory lesions closely associated with microcolonies of Gram-negative bacteria. These bacteria were identified as *F. necrophorum* by laser capture microdissection of bacterial colonies from formalin-fixed tissue, followed by polymerase chain reaction (PCR) amplification of bacterial 16S rDNA and sequencing. An rRNA-targeting oligonucleotide probe specific for *F. necrophorum* was designed and used in a fluorescent in-situ hybridization (FISH) assay (see below). This examination identified a further pair of *F. necrophorum*-infected twin fetuses (cases 4A,B) and an infected placental specimen from a sheep (case 5) that aborted in late gestation. Placental material was not available from cases 2A, 3A and 4A.

Pathology

The fetal age was calculated from breeding data and compared with the stage of development. The fetus and corresponding fetal membranes were examined *post mortem* and tissues including placenta, lung, myocardium, liver, kidney, skeletal muscle, brain and cervical spinal cord were sampled for histopathology. Multiple areas were sampled from placenta, lung, liver, and brain and other tissues were taken if showing gross lesions. The specimens were fixed in 10% neutral buffered formalin, dehydrated through graded alcohols, embedded in paraffin wax and sectioned at 7 μm (brain) or 3 μm (other tissues). Initial histological examination was performed on haematoxylin and eosin (HE)-stained sections. Additional staining methods were applied when considered appropriate. These included Mallory's phosphotungstic acid haematoxylin method (PTAH), Masson's trichrome method, the periodic acid-Schiff (PAS) technique, and the Brown-Hopps method for Gram-positive and Gram-negative bacteria.

Immunohistochemistry

Immunohistochemical examination for *Listeria monocytogenes* antigen was performed on all tissues of any pair of twins from which this organism was cultured. Selected sections from the other cases were also examined. A commercially available polyclonal antibody against *L. monocytogenes* types 1 and 4 (Difco Laboratories, Detroit, MI, USA) was used in a streptavidin-biotin immunoperoxidase assay.

Bacteriology

This was performed on samples of lung, liver, fetal placenta and abomasal contents, as previously described (Agerholm *et al.*, 2006). Briefly, each sample was spread on two blood agar plates (Blood Agar Base CM0055; Oxoid, Basingstoke, UK, containing sterile bovine blood 5%) of which one was incubated aerobically and the other anaerobically for 48 h at 37 °C. Colonies were subcultured and identified by standard methods. Selected isolates were characterized by the API-20-E kit (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's recommendations. Examination for *Campylobacter* spp. was performed as previously described (Agerholm *et al.*, 2006).

In-situ Hybridization

This was performed with the general bacterial probe eub338: S-D-Bact-0338-a-A-18 5′-GCTGCCTCCC-GTAGGAGT-3′ (Amann *et al.*, 1990) and a probe specific for *F. necrophorum* (F.necrophorum183: S-S-F.necrophorum-0183-a-A-18, 5′-GATTCCTCCATGC-GAAAA-3′ [Boye *et al.*, 2006]).

Epifluorescence Microscopy and Scanning

An Axio Imager M1 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence with a 100-W HBO lamp and filter set XF53, (Omega Optical, Brattleboro, VT, USA) was applied for simultaneous detection of red and green fluorescence. Filter sets 43 HE and 38 HE (Carl Zeiss) were used to "visualize" CY3 and fluorescein, respectively. Images were obtained with an AxioCam MRm version 3 FireWire monochrome camera (Roper Scientific, Ottobrunn, Germany) and the software AxioVision version 4.

Entire tissue sections were examined by scanning fluorescence in-situ hybridization using an ArrayWoRx^{e} scanner (pixel resolution of 3.25 μm) (Applied Precision, Issaquah, WA, USA).

PCR and Sequencing

DNA was extracted from dermal lesions of fetus 1A kept at −20 °C until used. Subcutaneous tissue was removed and material scraped with a scalpel from the dermis was suspended in 1 ml of 0.9% NaCl. DNA was extracted with the Easy-DNA kit (InVitrogen, Paisley, UK) and "protocol #3," according to the manufacturer's instructions. Primers (MWG-BIOTECH AG, Ebersberg, Germany) used for 16S rRNA gene amplification consisted of S-D-Bact-0008-a-S-20 (5′-AGAGTTT-GATCMTGGCTCAG-3′) and S-Univ-*-1492-a-A-21 (5′-GTTACCTTGTTACGACTTCAG-3′), modified from Weisburg *et al.* (1991). Oligonucleotide probes were designated according to Alm *et al.* (1996). Reaction conditions were as follows: 1 × PCR Buffer II (Roche Applied Science, Indianapolis, IN, USA), 3 mM $MgCl_2$,