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Fatal *Clostridium novyi* Type B Infection in a Sow

Noriko AKIYAMA[1], Tomoyuki SHIBAHARA[2]*, Kazutada USHIYAMA[1], Haruna SHIMIZU[1], Itsuo KOIZUMI[1] and Makoto OSAKI[3]

[1] Yamanashi Tobu Livestock Hygiene Service Center, Yamanashi Prefecture (Fuefuki, Yamanashi 406-0034, Japan)
[2] Pathology and Pathophysiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, (Tsukuba, Ibaraki 305-0856, Japan)
[3] Bacteriology and Parasitology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, (Tsukuba, Ibaraki 305-0856, Japan)

Abstract

A 33-month-old indoor sow showed a sudden loss of appetite and then died. Necropsy revealed a sponge-like appearance of the liver parenchyma, encephalomalacia, and dark red coloration of the heart. Histologically, extensive necrotic lesions were detected in the liver, brain and heart, and Gram-positive rods were detected in these necrotic lesions. Immunohistochemically, the rods reacted with an antibody against *Clostridium* species. Anaerobic cultures yielded high numbers of *Clostridium novyi* (*C. novyi*) type B. These findings suggested that necrosis and encephalomalacia were associated with *C. novyi* type B. *C. novyi* type B infection was diagnosed as the cause of death, and this was a case of fatal *C. novyi* type B infection with gas gangrene in an indoor sow.

Discipline: Animal health
Additional key words: encephalomalacia, gas gangrene infection, indoor, pig, sponge-like appearance

Introduction

Clostridium novyi (*C. novyi*) are anaerobic, spore-forming Gram-positive rods that vary in size. They can be divided into four types based on toxin production (Sasaki et al. 2002). The α toxin is produced by types A and B, and the β toxin by types B and D (*C. novyi* type D is also called *Clostridium haemolyticum*) (Sasaki et al. 2002). These toxins cause tissue necrosis. Type C is non-toxigenic and therefore avirulent.

Although considered quite rare, swine *C. novyi* infections have been reported in Croatia (Almond & Bilkei 2005), Slovakia (Friendship & Bilkei 2006), Spain (García et al. 2009), Kenya (Friendship & Bilkei 2007) and Japan (Itoh et al. 1987). *C. novyi* type A was isolated from the systemic organs of a 7-month-old sow in Japan (Itoh et al. 1987). The remaining cases were associated with *C. novyi* type B, and occurred in outdoor sows (Friendship & Bilkei 2006, 2007, García et al. 2009). The livers had a honeycomb appearance with gas bubble infiltration (Friendship & Bilkei 2006, 2007) or a sponge-like appearance (García et al. 2009, Itoh et al. 1987). These reports were concerned with the bacteriology or histology of the affected pigs, but these descriptions were short and limited. Histopathological examination was not performed on the brains in previous studies (Friendship & Bilkei 2006, 2007, García et al. 2009). Moreover, there are no available reports on the immunohistochemical and molecular features of fatal systemic gas gangrene associated with *C. novyi* type B in a sow.

This report describes the clinical, microscopic and bacteriological characteristics of an indoor sow with a sponge-like appearance of the liver parenchyma and encephalomalacia due to *C. novyi* in Japan. Our findings are compared with those in swine cases reported in Europe (Almond & Bilkei 2005, Friendship & Bilkei 2007, García et al. 2009) and Africa (Friendship & Bilkei 2006).

Material and methods

The case farm located in Japan's Yamanashi prefecture had 180 female breeding pigs, which were Tokyo X, a new Japanese breed of domestic pig created by combining bloodlines from the Duroc (USA), Berkshire (UK) and Beijing Black (China) breeds. The sows were fed a commercial

*Correspondence author: e-mail tshiba@affrc.go.jp
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feed in an indoor breeding unit, and farrowed twice yearly.

A 33-month-old sow, which had farrowed uneventfully on 14 April 2013, suddenly showed anorexia and depression on 30 April 2013, and died the next morning. The lowest and highest air temperatures on the day of death were recorded as 11.6°C and 23.3°C, respectively. When it was a piglet, the sow was vaccinated against porcine circovirus type 2, enzootic pneumonia, and Glasser's disease.

A necropsy was performed within 10 hours of the sow's death at the Yamanashi Tobu Livestock Hygiene Service Center. Tissue samples of the major organs (liver, spleen, kidney, heart, lung, brain and intestines) were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Tissue sections (approximately 3-µm thick) were stained with hematoxylin and eosin (HE), and then Gram-stained for histological examination.

For immunohistochemistry (IHC) analysis of the tissue sections, a rabbit polyclonal antibody against *Clostridium* species (Virostat Inc., Westbrook, ME, USA) was used. Immunohistochemical detection was performed using a commercial kit (*N*-Histofine Simple Stain MAX PO (R); Nichirei Bioscience Inc., Tokyo, Japan) according to the manufacturer's instructions. Following five minutes of incubation with *N*-Histofine Simple Stain AEC solution, the sections were counterstained for one minute with Mayer's hematoxylin, and then mounted with aqueous mounting medium (Ultramount Aqueous Permanent Mounting Medium; Dako, Glostrup, Denmark). Sections of a piece of liver (into which *Clostridium perfringens* had been injected) were immunolabeled as positive controls.

Bacteriologically, tissue homogenates derived from the liver and brain were inoculated into cooked meat medium containing 1% glucose, and then cultured at 37°C. Cultured supernatant fractions were streaked onto egg-yolk GAM agar medium and incubated at 37°C under anaerobic conditions using the Anearopack Kenki system (Mitsubishi Gas Co. Ltd., Tokyo, Japan) for the isolation of bacteria. Genomic DNA was extracted from the supernatants of cooked meat medium and isolated bacteria by using a DNA extraction kit (InstaGene Matrix; Bio-Rad Laboratories, Hercules, CA, USA), according to the product manual. A partial region of 16S rRNA gene (rDNA) from genomic DNA of the isolated bacteria was amplified by polymerase chain reaction (PCR), and the amplicon was directly sequenced as previously described (Dorsch & Stackenbrandt 1992). The obtained sequences were aligned with sequences in the DDBJ/GenBank/EMBL database.

To discriminate between *Clostridium* species, a PCR amplifying the flagellin gene *fliC*, present on the chromosome, was also performed as previously described (Sasaki et al. 2002). This PCR, using one forward and five reverse primers, has been developed to identify and differentiate between *C. novyi* types A and B, *C. haemolyticum*, *Clostridium chauvoei* and *Clostridium septicum* (Sasaki et al. 2002). In this study, the primer pairs to identify *C. novyi* type B and *C. haemolyticum* were used, respectively.

Results

At necropsy, an excessive amount of bloody ascites were detected. Gross lesions were present in the liver, brain and heart. The surface of the liver was dark red, and coalescing white and pale necrotic areas were also detected. The coalescing white necrotic areas had a honeycomb appearance with gas bubble infiltrations on the cut surface (sponge-like appearance of the liver parenchyma) (Fig. 1). The pale necrotic areas had a light gray-white color with small vacuoles on the cut surface. The liver became extremely friable. Severe encephalomalacia was observed (Fig. 2). Marked bloody pericardial effusion was detected, and the heart was dark red. No gross abnormalities were found in other organs, including the spleen, kidney, lung and intestines.

Histologically, the normal hepatic architecture was disrupted by large vacuoles (intrahepatic spherical non-staining cavities) and necrotic hepatocytes in the hepatic lesion observed macroscopically (Fig. 3). The vacuoles were most prominent in the centrilobular regions. The lesions were characterized by extensive tissue necrosis. Numerous large Gram-positive spore-forming rods, singly or in clusters, were discernible in the extensive severe necrotic lesions. Several rods were also detected in the central vein and interlobular vessels. Most of these rods had

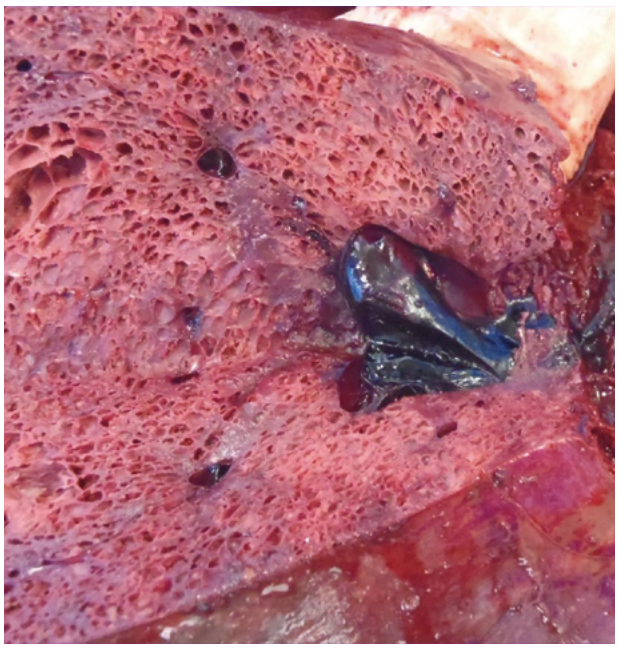

Fig. 1. Sponge-like appearance of the liver parenchyma in a sow.

clear sub-terminal or terminal oval spores that deformed the rods. Similar severe necrotic lesions were detected in the brain and heart.

In other organs such as the spleen, kidney, lung and intestines, similar but mild focal necrotic lesions were observed with the Gram-positive rods. The necrotic lesions were closely related to distribution of the rods. No other significant histological abnormalities were observed in the remaining area. The postmortem artifacts were negligible in the systemic organs, including the intestinal mucosa and renal tubular epithelial cells.

Immunohistochemically, the rods observed in the HE- and Gram-stained sections were strongly positive for *Clostridium* species, as were the rods in the positive control slide containing *C. perfringens* (Fig. 4).

Anaerobic cultures yielded a large number of Gram-positive *Clostridium*-like organisms from the liver and brain. 16S rDNA sequencing of the isolates revealed that the *Clostridium*-like organisms (accession number AB857215) were phylogenetically most closely related to two strains of *Clostridium*; that is, *C. novyi* types B ATCC25758 (accession number AB035087) and *C. haemolyticum* ATCC9650 (accession number NR_024749), whose 16S rDNA sequence was identical. The sequence similarity between the isolates and those two bacterial species was extremely high (both 99.9%, based on a comparison of 1468 base pairs of sequence). In the PCR assay, the species-specific amplicon (approximately 427-bp in size) showed *C. novyi* type B to be present in the liver and brain (Fig. 5).

The high 16S rDNA sequence similarity suggested that the isolates were either *C. novyi* types B or *C. haemolyticum*. The multiplex PCR system based on the *fliC* flagellin subunit gene sequence is able to identify and differentiate between *C. novyi* types B and *C. haemolyticum* (Sasaki et al. 2002). A combination of 16S rDNA gene sequencing and multiplex PCR amplification of the *fliC* gene of bacterial DNA, extracted from the isolates and culture supernatant of liver and brain samples, revealed the unique presence of *C. novyi* type B.

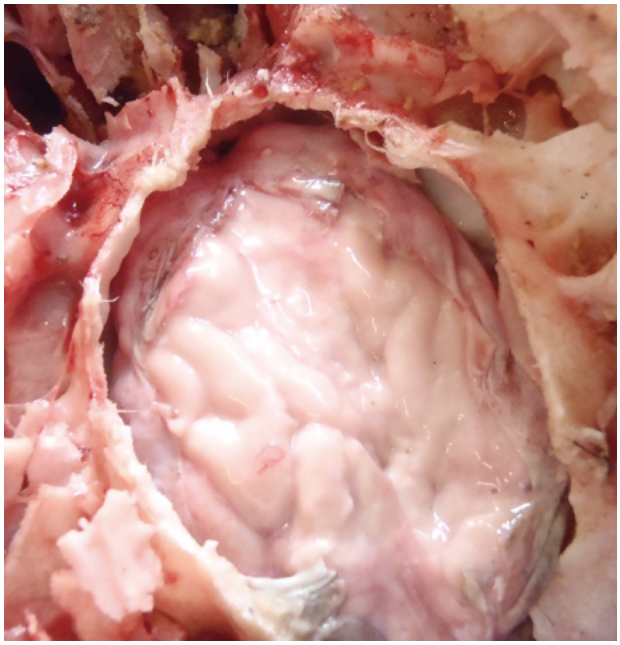

Fig. 2. Severe extensive encephalomalacia in a sow.

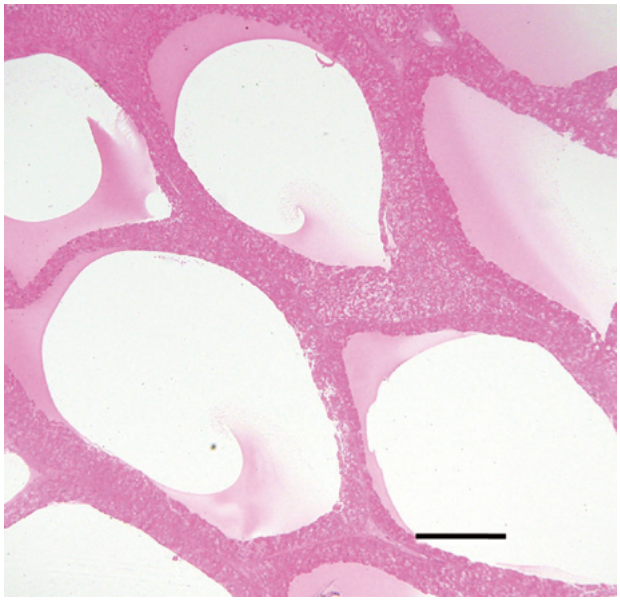

Fig. 3. Intrahepatic spherical non-staining cavities detected in necrotic lesion.
HE. Bar = 200 µm.

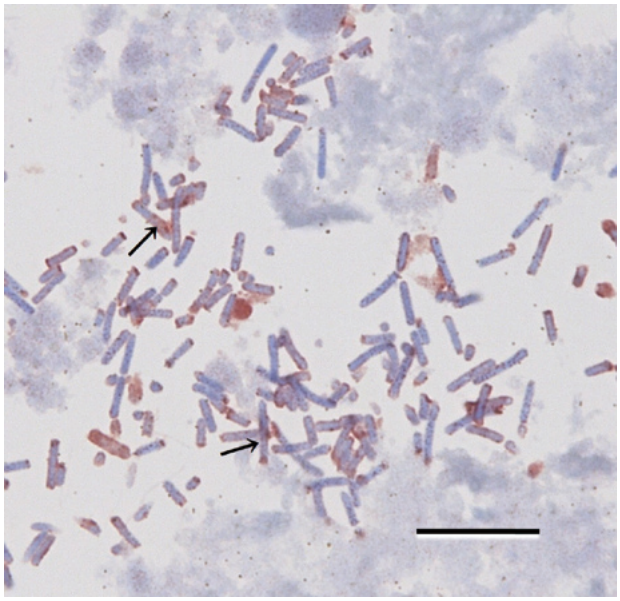

Fig. 4. Rods reacted with antibody against *Clostridium* species (arrows) in the necrotic brain lesion.
IHC, Bar = 10 µm.

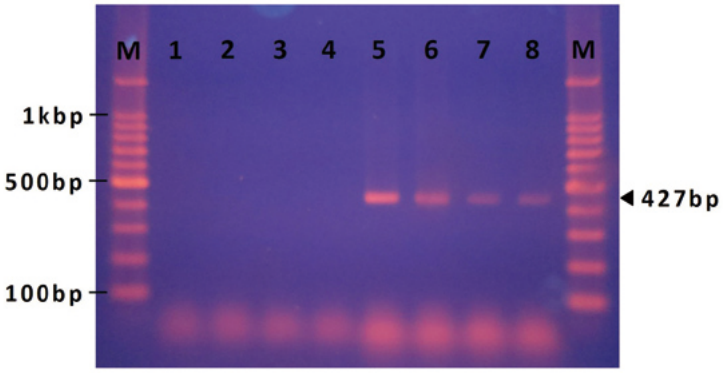

Fig. 5. Gel electrophoresis (2% agarose gel stained with ethidium bromide) showing a species-specific 427-bp band (arrow) identifying *C. novyi* type B in the liver and brain.
Lane M, molecular size marker (100-bp molecular ruler; Bio-Rad Laboratories); lanes 1 and 5, supernatant of culture in cooked meat medium of brain; lanes 2 and 6, supernatant of culture in cooked meat medium of liver; lanes 3 and 7, egg-yolk GAM agar medium of brain; lanes 4 and 8, egg-yolk GAM agar medium of liver; lanes 1-4, primers for *C. haemolyticum* were used; lanes 5-8, primers for *C. novyi* type B were used.

Discussion

These results indicate that the sponge-like appearance of the liver parenchyma and encephalomalacia was caused by *C. novyi* type B in the sow. Diagnosis of *C. novyi* infection in swine is difficult, because suspect cases are usually found dead (Friendship & Bilkei 2007). To achieve a correct diagnosis of *C. novyi* infection in pigs, the carcasses must be examined within 12 hours of death (García et al. 2009). In the present study, an appropriate postmortem examination and sample collection within 10 hours of death, along with microbial isolation and the use of PCR, were conducted and achieved a correct diagnosis of swine *C. novyi* infection. The present sow died on a spring morning, and the air temperature was between 11.6°C and 23.3°C in the interval between death and necropsy. All the tissues examined showed severe (liver, brain and heart) to mild (spleen, kidney, lung and intestine) necrosis with bacteria. The postmortem changes and artifacts were negligible, even in the intestinal mucosa and renal tubular epithelial cells. Although this bacterium has been implicated in the sudden death of sows, the present case is the first to demonstrate *C. novyi* type B as a cause of systemic gas gangrene accompanied by encephalomalacia in Asian pigs reared under indoor conditions.

The presence of extensive brain necrosis was striking in our case. In a sow infected with *C. novyi* type A, Gram-positive rods were detected in blood vessels within the meninges and brain, but necrotic histopathological findings did not detect (Itoh et al. 1987). In previous cases of sows infected with *C. novyi* type B, histopathological examinations only targeted abdominal organs and not the central nervous system (Friendship & Bilkei 2006, 2007, García et al. 2009). Moreover, IHC identification of *Clostridium* antigen was not performed in the previous studies (Friendship & Bilkei 2006, 2007, García et al. 2009, Itoh et al. 1987). Our immunohistochemical and bacteriological findings indicated that the necrotic brain lesions were closely associated with *C. novyi* type B. The most interesting finding of this case was the presence of unique hepatic necrosis, encephalomalacia, and myocardial necrosis associated with *C. novyi* type B, as such combination had not been previously reported. Based on these findings, we speculate that the necrotic lesions were formed when the causative rods were transferred hematogenously from the liver, which may have been the original lesion, to other organs including the brain and heart.

Sows reared outdoors have higher mortality compared with those reared indoors (Akos & Bilkei 2004, Almond & Bilkei 2005, Friendship & Bilkei 2006, 2007, García et al. 2009). The present case and the incidents in Europe and Africa could serve as an early warning for Asian countries using both indoor and outdoor pig production systems.

Much of the sow mortality associated with *C. novyi* appears to occur at or near the critical farrowing period (García et al. 2009). General functional changes in the sow's immune system during gestation may have played an important role in the occurrence of the present *C. novyi* infection, as previously described (García et al. 2009). Chemotherapy for *C. novyi* infection is apparently difficult

due to the involvement of many organs and acute deterioration. Further studies of vaccines and chemotherapy are thus considered essential.

In humans, a similar sponge-like appearance of the liver parenchyma was detected in a 23-year-old woman who died in Berlin in 1953 (Widulin et al. 2013). It was caused by fatal intrauterine gas gangrene following mechanical abortion (Widulin et al. 2013). Histologically, numerous empty cystic spaces, lined with abundant Gram-positive, rod-shaped bacteria corresponding to clostridiae, were the predominant findings (Widulin et al. 2013). Unfortunately, no histological finding was described in other organs. Bacterial isolation was not performed in the human case (Widulin et al. 2013), but *Clostridium* species including *C. novyi* would be suspected as the likely cause of death.

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