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J. Comp. Path. 2019, Vol. 167, 41–45

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INFECTIOUS DISEASE

An Outbreak of Fatal *Bordetella bronchiseptica* Bronchopneumonia in Puppies

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Summary

Twenty-two newborn puppies that did not receive colostrum exhibited acute respiratory signs and died at a breeding facility. Pathological examinations were performed on four of the puppies. At necropsy examination, the lungs were firm and mottled dark red, consistent with acute bronchopneumonia. Histopathologically, there was marked infiltration of neutrophils and macrophages into the bronchi and alveoli, and gram-negative coccobacilli were attached diffusely to the cilia of bronchial mucosa. Immunohistochemistry for *Bordetella bronchiseptica* antigen revealed positive labelling of the bacterial agents. On electron microscopy, a large number of coccobacilli were observed attaching to the cilia of bronchial epithelial cells. Real-time polymerase chain reaction amplified a *B. bronchiseptica* gene from the affected lung tissue. Based on these findings, the four puppies were diagnosed with fatal *B. bronchiseptica* bronchopneumonia.

Keywords: *Bordetella bronchiseptica*; bronchopneumonia; colostrum; dog

Introduction

Bordetella bronchiseptica infection is associated with respiratory disease in various animal species. In the dog, *B. bronchiseptica* is one of the most common pathogens that cause canine infectious respiratory disease (CIRD) (Ford, 2012). *B. bronchiseptica* may act as the primary cause of CIRD, or infection may be secondary to infection with other pathogens that cause CIRD such as mycoplasmas, canine adenovirus type 2 (CAV-2), canine parainfluenza virus (CPiV), canine herpesvirus (CHV) and canine respiratory coronavirus (CRCoV) (Ford, 2012). The pathogenesis of CIRD is complex, and other factors such as immunological and nutritional status of the dog are also important.

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0021-9975/$ - see front matter
https://doi.org/10.1016/j.jcpa.2018.12.002

B. bronchiseptica is commonly isolated from the upper respiratory tract of healthy dogs and also from dogs with upper respiratory disease (Mochizuki *et al.*, 2008). Typically, *B. bronchiseptica* infection causes tracheobronchitis and dogs clinically manifest nasal discharge and coughing. In addition, *B. bronchiseptica* can be associated with community-acquired infectious pneumonia, especially in puppies (Radhakrishnan *et al.*, 2007). In a retrospective histopathological study of 36 dogs with bronchopneumonia, eight cases were positive for *B. bronchiseptica* by immunohistochemistry (IHC) and/or polymerase chain reaction (PCR) (Taha-Abdelaziz *et al.*, 2016). However, in most cases (6 of 8 cases) bacterial agents had not been noted in the pathology report based on examination of haematoxylin and eosin (HE)-stained sections. Moreover, since *B. bronchiseptica* can be detected from healthy puppies, the key factors in developing fatal pneumonia are unknown.

In the present study, we describe the pathological findings of fatal *B. bronchiseptica* bronchopneumonia in puppies from a breeding facility. Lack of colostrum may have been a factor contributing to the outbreak of this infectious disease at the facility.

Materials and Methods

Cases

A total of 22 newborn puppies exhibited acute respiratory signs and died over a 1-month period at a breeding facility (Table 1). The puppies were from four different litters and did not receive colostrum or milk due because the dams had a Caesarean section or died during natural delivery or because the puppies were smaller than other littermates and could not feed on their own.

Gross and Microscopical Pathology

Necropsy examination was performed on four of the puppies (Table 2) and heart, lung, stomach, intestine, liver, pancreas, spleen, kidney, bladder, thyroid gland, adrenal gland and brain were collected and fixed in 10% neutral buffered formalin. Samples of the lung lesions were snap-frozen for molecular examination. Formalin-fixed tissue samples were processed routinely and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE) and Gram stain.

Immunohistochemistry

Sections of the lungs were subjected to immunohistochemistry (IHC) using rabbit polyclonal antibody specific for *B. bronchiseptica* (CV10, Advanced Technology Development Center, Kyoritsu Seiyaku Corp., Ibaraki, Japan) and mouse monoclonal antibody specific for *Escherichia coli* (clone 2D7/1, Abcam, Cambridge, UK). Antigen retrieval was performed by incubating the sections with 0.1% actinase E at 37°C for 20 min for *B. bronchiseptica* and with citrate buffer at 121°C for 10 min for *E. coli*. Endogenous peroxidase activity was blocked by H_2O_2 3% in methanol. Antibody binding was 'visualized' using peroxidase-labelled secondary antibodies (Histofine simple stain Max-PO Kit; Nichirei Bioscience, Tokyo, Japan) and aminoethyl carbazole substrate solution (Nichirei Bioscience). Sections were counterstained with haematoxylin.

Electron Microscopy

Portions of the formalin fixed lung tissues were washed with 0.1 M phosphate buffer (pH 7.4), post-fixed in1% osmium tetroxide and dehydrated through a graded ethanol series. Tissue samples for scanning electron microscopy (SEM) were then immersed in t-butyl alcohol, freeze-dried (VFD-21S; Vacuum Device, Ibaraki, Japan), coated with Au (E-1030; Hitachi High-Technologies Corporation, Tokyo, Japan) and examined using a scanning electron microscope (S-4800; Hitachi High-Technologies Corporation). Tissue samples for transmission electron microscopy (TEM) were dehydrated and embedded in Luveak-812 resin (Nakaraitesque, Kyoto, Japan). Ultrathin sections were counterstained with uranyl acetate and lead solution. Prepared sections were examined with a transmission

Table 1
Clinical information on affected dams

Dam case number	*Breed*	*Delivery*	*Litter size*	*Number of puppies not fed colostrum*	*Number of puppies died (died/not fed colostrum)*
1	Pug	Caesarean section	9	9	8 (8/9)
2	Mixed	Natural delivery (mother died during delivery)	8	8	5 (5/8)
3	Poodle	Natural delivery	5	2*	2 (2/2)
4	Pug	Caesarean section	8	8	7 (7/8)

*These puppies were fed a milk substitute because they were born smaller than the other littermates and did not take in colostrum on their own.

Table 2
Clinical information on affected puppies

Puppy case number	*Breed (dam case number)*	*Age in days*	*Sex*	*Clinical presentation*
1	Pug (4)	5 days	Female	Debilitation, haemoptysis
2	Pug (4)	19 days	Female	Acute respiratory distress, haemoptysis
3	Mixed-breed (2)	20 days	Male	Acute respiratory distress
4	Mixed-breed (2)	21 days	Male	Acute respiratory distress

electron microscope (JEM-1400Plus; Jeol, Tokyo, Japan).

Real-time Polymerase Chain Reaction

A sample of frozen lung (from case 4) was subjected to real-time polymerase chain reaction (PCR) for detection of 12 CIRD pathogens, including *B. bronchiseptica*, *Mycoplasma cynos*, *Streptococcus equi* subspecies *zooepidemicus*, canine distemper virus, H3N8 canine influenza virus (CIV), H3N2 CIV, H1N1 influenza virus, canine pneumovirus, CAV-2, CPiV-3, CHV and CRCoV. Testing was by the RealPCR CRD panel (Idexx, Tokyo, Japan) as previously described (Schulz *et al*., 2014; Lavan and Knesl, 2015).

Results

At necropsy examination, the lungs were diffusely firm and mottled dark red in all four puppies; consistent with acute bronchopneumonia (Fig. 1A). No other significant gross findings were noted. On histopathological examination of the lungs, severe neutrophil and macrophage infiltration was observed in the bronchi and alveoli of all four puppies examined (Fig. 1B). The lungs were diffusely congested and

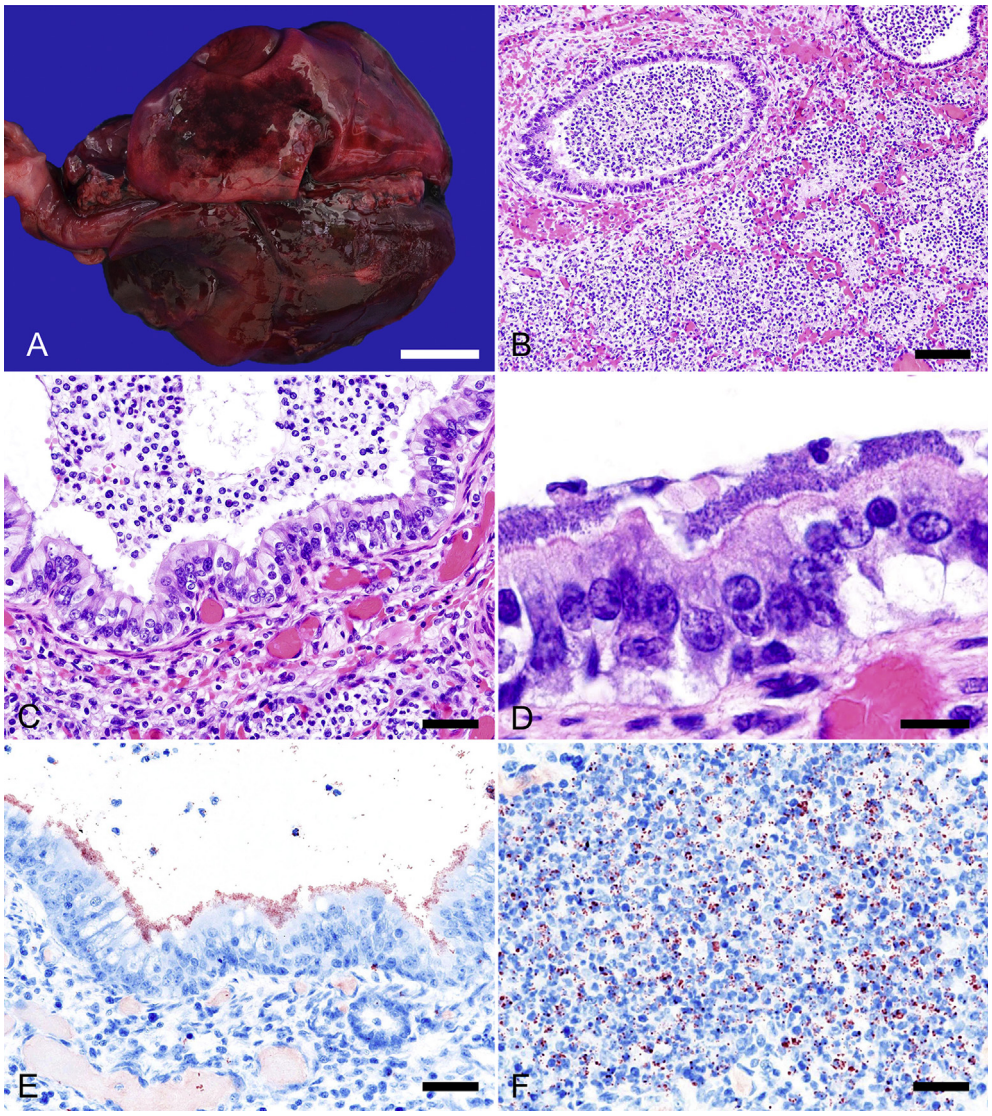

Fig. 1. Pathological findings of *B. bronchiseptica* bronchopneumonia in puppies. (A) The lung is diffusely swollen with dark red areas. Bar, 1 cm. (B) Severe infiltration of neutrophils and macrophages into the bronchi and alveoli. HE. Bar, 200 µm. (C) Basophilic bacteria on the surface of bronchial epithelium. HE. Bar, 50 µm. (D) Large numbers of bacteria are attached to the cilia of bronchial epithelial cells. HE. Bar. 20 µm. (E) IHC for *B. bronchiseptica* antigen. Bacteria on the surface of the bronchial epithelium are positively labelled. Positive signals are also seen in the cytoplasm of inflammatory cells in the bronchus. Bar, 50 µm. (F) IHC for *B. bronchiseptica* antigen. Numerous positive signals in the alveolus with severe inflammation. Bar, 50 µm.

multifocal necrosis and haemorrhage were observed in areas with severe inflammation. Aggregates of basophilic coccobacilli were often observed on the surface of bronchial mucosa (Fig. 1C). At higher magnification, the bacterial agents were seen to be attached to the cilia of bronchial epithelial cells (Fig. 1D). These coccobacilli were negative on Gram staining. No other significant histological lesions were observed in other organs.

IHC for *B. bronchiseptica* antigen revealed positive labelling of the bacteria on the surface of the bronchial mucosa (Fig. 1E). In addition, positive signals were detected in the cytoplasm of neutrophils and macrophages in the bronchi and alveoli (Fig. 1F). Lung tissues were negative for *E. coli* antigen by IHC.

SEM showed numerous coccobacilli on the surface of the bronchial mucosa (Fig. 2A). On TEM, coccobacilli were observed between the cilia of bronchial epithelium (Fig. 2B). The bacteria were 0.5 μm in diameter and 1.0−1.5 μm in length, with pili on the membrane attached to the cilia of the bronchial epithelial cells.

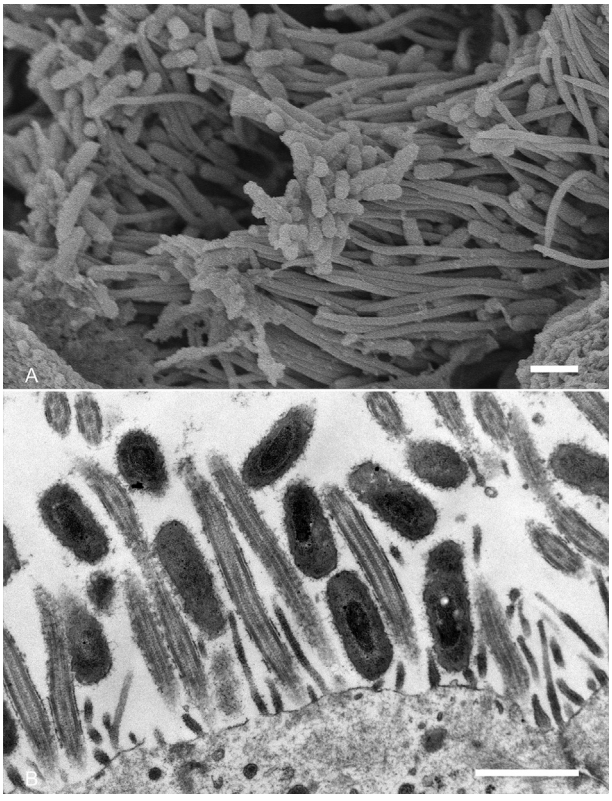

Fig. 2. Electron microscopy of *B. bronchiseptica* bronchopneumonia in puppies. (A) SEM of the bronchial mucosa. Numerous coccobacilli are attached to the cilia of bronchial mucosa. Bar, 10 μm. (B) TEM of the bronchial mucosa. Coccobacilli with membranous pili attached to the cilia of bronchial epithelial cells. Bar, 1 μm.

Real-time PCR of the lung tissue was positive for *B. bronchiseptica*. Other pathogens associated with CIRD were not detected.

Discussion

The four puppies were diagnosed with fatal *B. bronchiseptica* bronchopneumonia. Histopathologically, severe suppurative bronchopneumonia with bacterial ciliary adhesion was observed in the lungs of the puppies. Cilia-adherent bacteria in the respiratory tract are a characteristic histopathological finding of *B. bronchiseptica* infection (Caswell and Williams, 2016). However, this finding may not be evident in cases of fatal bronchopneumonia, and IHC and PCR examination are required in such cases to detect the aetiological agent (Taha-Abdelaziz *et al.*, 2016). In the present study, large numbers of cilia-adherent bacteria were found on HE-stained sections of the lungs, indicating severe infection by *B. bronchiseptica*. Cilia-adhesion, together with production of toxins by *B. bronchiseptica*, induces ciliostasis of respiratory epithelial cells and further causes secondary infections of other pathogens. Additionally, as shown in the present study, *B. bronchiseptica* can enter and survive within inflammatory cells and affect the immune system of the host. In the present study, other pathogens were not detected by histopathology or real-time PCR, indicating that *B. bronchiseptica* infection was the primary cause of the fatal bronchopneumonia. There is genetic diversity in *B. bronchiseptica* strains; however, the relationship between strains and pathogenicity remains uncertain.

Intranasal and oral vaccines against *B. bronchiseptica* are used in veterinary practice for dogs. It is recommended that vaccination is performed on dogs 3 weeks old or older. Maternal antibodies do not interfere with local antibody responses induced by these mucosal vaccines. Maternal antibodies are transferred from dams to puppies via the colostrum, although antibodies are also present in milk, and these may provide some protection of the intestinal mucosa (Decaro *et al.*, 2004). Maternal antibody may persist for up to 14 weeks in dogs (Gooding and Robinson, 1982; Day *et al.*, 2016). In the present study, all of the dogs that died were younger than 3 weeks old. Although we could not determine the serum antibody titre of the puppies, the outbreak implicates lack of maternal antibody as a major factor contributing to the development of the fatal *B. bronchiseptica* bronchopneumonia.

In conclusion, the present study demonstrates the pathological findings of fatal *B. bronchiseptica* bronchopneumonia in dogs and the importance of intake of

colostrum for prevention of this infectious disease in puppies.

Conflict of Interest Statement

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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[*Received, September 5th, 2018*
Accepted, December 10th, 2018]