

Cilia-associated Respiratory (CAR) Bacillus-like Organism (CLO) Identified in a Banded Rock Rattlesnake (*Crotalus lepidus klauberi*)

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ABSTRACT: Cilia-associated respiratory bacillus-like organisms (CLO) are bacteria found intermixed among the cilia of epithelial cells lining the respiratory tract. They are considered moderate pathogens and have been reported in rodents, rabbits, ruminants, cats, and pigs. In this article, we describe respiratory disease associated with a *Mycobacterium* sp. and CLO in a banded rock rattlesnake (*Crotalus lepidus klauberi*). The CLO were confirmed via Warthin-Starry silver stain and ultrastructure examination.

KEY WORDS: cilia-associated respiratory bacillus-like organism, CLO, *Crotalus lepidus klauberi*, granuloma, *Mycobacterium*, rattlesnake.

INTRODUCTION

Cilia-associated respiratory (CAR) bacillus-like organisms (CLO) are filamentous, argyrophilic Gram-negative bacilli with gliding motility that immigrate between cilia of respiratory epithelium and are associated with respiratory disease in laboratory mice, rats, rabbits, cattle, goats, pigs, and cats (Schoeb *et al.*, 1993; Ramos-Vara *et al.*, 2002; Percy and Barthold, 2007). The bacilli have been documented in some wild ruminant species as well: red deer (*Cervus elaphus*), chamois (*Rupicapra rupicapra*), and roe deer (*Capreolus capreolus*) (Bergottini *et al.*, 2005). Both CAR bacilli and CLO are considered moderately pathogenic. Infections in rats have occurred via intranasal inoculation and rarely via contamination of bedding. Clinical signs, characterized by mucopurulent bronchopneumonia, begin within 14 days postinoculation (Percy and Barthold, 2007). In rodents, rabbits, and cats the main histopathologic findings are chronic suppurative bronchitis and bronchiolitis, lymphoplasmacytic peribronchiolar cuffing, and infiltration of the lamina propria and submucosa by moderate to high numbers of lymphocytes and plasma cells with rare to few neutrophils and eosinophils (Ramos-Vara *et al.*, 2002; Percy and Barthold, 2007). There is an association, causal or not, between lymphoid follicles in airway walls and CAR bacilli in calves (Caswell and Williams, 2007). To our knowledge, there are no case reports of a CLO affecting reptiles. In a banded rock rattlesnake (*Crotalus lepidus klauberi*), we describe the respiratory disease associated with multiple bronchial and distal tracheal mycobacterial granulomas and silver-stained filamentous CLO intermixed within the cilia of the respiratory epithelium.

CASE REPORT

A 162-g (0.36 lb), adult male banded rock rattlesnake died of unknown causes at the Louisville Zoological Garden (Louisville, KY) in February 2008. The snake was housed

individually and no obvious clinical signs were noticed. The captive husbandry, including diet, ambient temperature, and humidity, was considered appropriate for this species.

A cursory post-mortem examination was performed prior to collecting representative tissues from each organ system for histological evaluation. The snake was considered to be in good body condition with a normal integument and musculoskeletal structure. A small amount of blood-tinged fluid was present in the coelomic cavity, lightly coating the viscera, and hemorrhage was noted in both kidneys. There were no significant lesions observed in the remaining tissues. The trachea was unopened and the brain was not examined grossly, but both were submitted for histopathology. Fresh tissues were not archived at the time of post-mortem examination.

Representative tissues were fixed in 10% buffered formalin solution, embedded in paraffin, and 4- μ m sections were routinely stained with hematoxylin and eosin. Additional slides of lung were stained using Lillie-Twort, Kinyoun's acid-fast, and Warthin-Starry staining procedures for the presence of infectious organisms.

Polymerase chain reaction (PCR) amplification analysis was performed on formalin-fixed, paraffin-embedded pulmonary and bronchial tissue by the Research Animal Diagnostic Laboratory of the University of Missouri, Columbia, MO. Polymerase chain reaction was performed using two CAR bacillus-specific primer sets; one amplifying mouse and rat isolates and the other amplifying rabbit isolates, as previously described (Cundiff *et al.*, 1995). An additional PCR assay using generic bacterial primers as previously described (Franklin *et al.*, 1999) was also performed. For electron microscopy, formalin-fixed, paraffin-embedded lung had paraffin removed via melting and xylene washes and then was rehydrated from 100% ethanol. Following rehydration, the tissue was osmicated for 1 h and then dehydrated and routinely embedded in epon-araldite. Thin sections were stained with uranyl acetate and lead citrate.

The bronchial and distal tracheal mucosa had multiple granulomas that expanded the lamina propria. These granulomas consisted of a large center of necrosis surrounded by heterophils, epithelioid macrophages, lymphocytes, plasma cells, and rare multinucleated giant cells. One of the granulomas extended into the adventitia, distorting the cartilaginous rings; other granulomas partially blocked the distal tracheal lumen (Fig. 1). In multiple areas of the epithelium, the ciliary border appeared thickened and striated (Fig. 2).

A few acid-fast bacilli were found within macrophages in the granulomas. Due to the low number of acid-fast bacilli and the unlikelihood of obtaining an accurate result, specific *Mycobacterium* PCR was not performed. The Warthin-Starry stained sections had many long, silver-staining bacilli in between and parallel to the cilia along the apical surface of the intrapulmonary bronchus (Fig. 3).



Figure 1. Photomicrograph of the bronchial granuloma composed of epithelioid macrophages protruding into the lumen. Hematoxylin and eosin, bar = 1 mm.

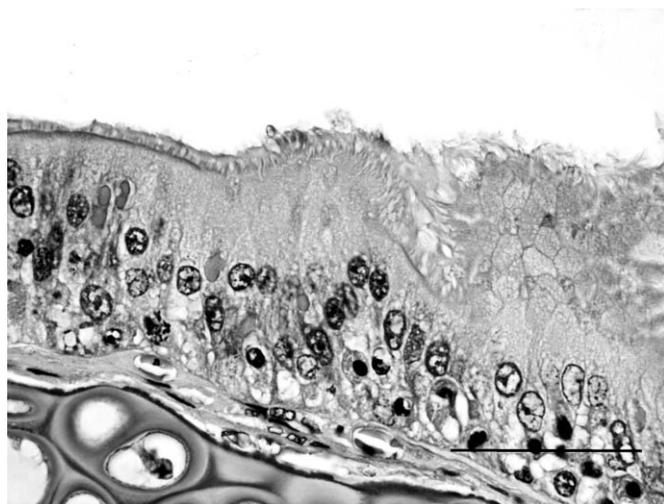


Figure 2. Photomicrograph of the bronchial ciliated epithelium. Note thickened cilia layer lining the respiratory epithelium which has a striated appearance. Hematoxylin and eosin, bar = 50 μ m.

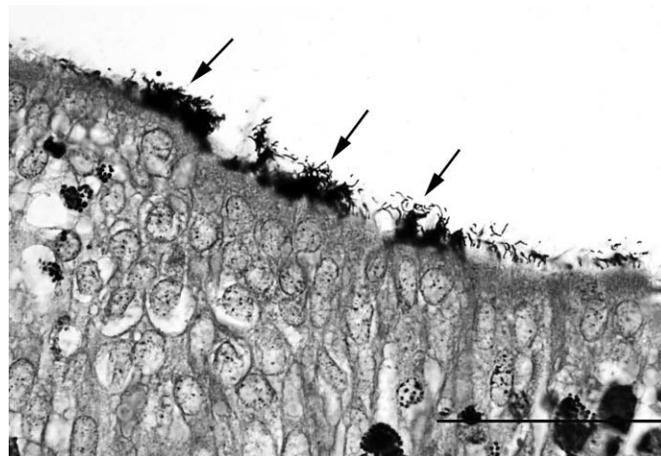


Figure 3. Photomicrograph of cilia lining respiratory tract stained by a Warthin-Starry silver stain. There are numerous silver-staining (black) long bacilli parallel to the cilia (arrows). Warthin-Starry, bar = 50 μ m.

Additional findings included multiple, dilated renal tubules expanded by uric acid and urate crystals that were sharp and radiating and that occasionally formed gout tophi. The liver had atrophied hepatocytes with mild lipodosis. No histopathologic lesions were noted in the testis, thyroid gland, digestive tract, adipose tissue, brain, eye, skin, pancreas, heart, skeletal muscle, or bone.

The PCR analyses were unable to amplify any CAR bacillus DNA fragments or any generic bacterial DNA from the sample. No bacterial cultures or viral fluorescent antibody testing was performed, as no fresh tissues had been obtained. Multiple electron-dense bacteria with a diameter ranging from 250–375 nm and length of 1.9–2.5 μ m intermixed among the cilia were seen by electron microscopy (Fig. 4).

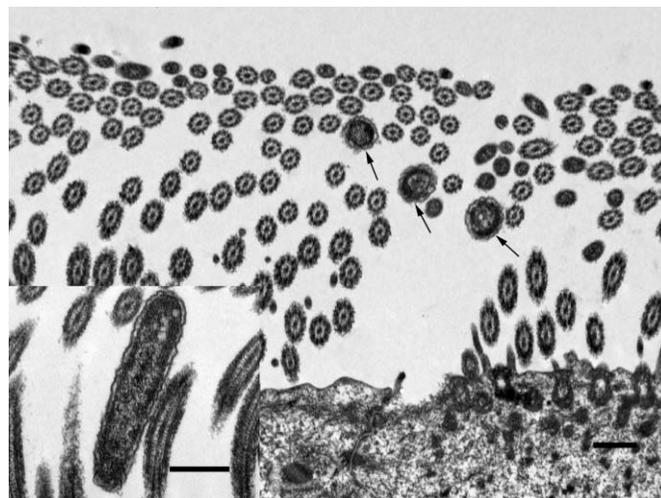


Figure 4. Transmission electron microscopy image of ciliated bronchial epithelial cells and multiple cross-sections of bacteria among the cilia (arrows). Lead citrate–uranyl acetate, bar = 500 nm. Inset: Higher magnification of a bacterium adjacent to cilia in longitudinal section showing the undulating, trilaminar cell membrane. Bar = 500 nm.

These bacilli had a trilaminar, undulating cell membrane and granular cytoplasm and were consistent with CAR bacillus or CLO (Ramos-Vara *et al.*, 2002; Percy and Barthold, 2007).

DISCUSSION

Histologic examination of submitted tissues indicated that the snake had a *Mycobacterium* sp. infection causing multiple bronchial and distal tracheal granulomas and concurrent CLO infection of the ciliary border of the bronchial epithelium. The mycobacterial infection was probably the primary infection, possibly allowing for secondary colonization of the bronchial mucosa by the CLO. Asphyxia due to airway obstruction and decreased oxygen exchange due to the bronchial mycobacterial granulomas is the most likely cause of death in this case. In other species, clinical disease due to CLO is caused by bacterial-associated bronchitis, bronchiolitis, bronchopneumonia, and bronchiectasis (Schoeb *et al.*, 1997; Ramos-Vara *et al.*, 2002). Such features were not present in this case, so it is difficult to ascribe clinical disease and death to the CLO, but pulmonary changes might have developed had the snake lived.

Mycobacterium sp. can be a cause of granulomatous pneumonia in snakes; however, it is usually an extension from a location other than the lung and is considered sporadic (Jacobson, 2007). This case is unusual in that there were tracheal and bronchial luminal granulomas, not lung granulomas, and disseminated mycobacteriosis was not present. The anatomic and physiologic differences in snakes, in conjunction with immunosuppression secondary to possible captive stressors such as inappropriate temperature or humidity, poor nutrition, and poor sanitation, can all predispose to lower airway disease (Schumacher, 2003; Murray, 2006).

Cilia-associated respiratory bacilli-like organisms are generally 0.2 μm wide by 6.0 μm long and are interspersed parallel to respiratory cilia. They were first diagnosed in rats in the early 1980s and, within the past 30 yr, infections have been documented in mice, rabbits, cattle, goats, pigs, cats, and wild deer species (Fallon, 1996; Schoeb *et al.*, 1997; Bergottini *et al.*, 2005). The transmission of CAR bacillus or CLO between rodent species is thought to be via respiratory secretions and close contact. While there is no documentation of cross-species transmission, this may represent a possible mode in cats due to predation on rodents which carry CAR bacillus (Ramos-Vara *et al.*, 2002). Although we could not identify the specific CLO in this case, the potential for cross-species transmission via respiratory secretions from the mice or rats fed to snakes, or via contaminated bedding, needs to be considered.

To the authors' knowledge, silver-staining CLO have not been documented in reptiles. There is also no indication that these organisms are a component of the normal respiratory flora in snakes, as there are no records of observing these organisms in healthy snakes. In addition, a bacteriologic survey performed on boid snakes did not detect the presence of CLO in the upper or lower respiratory tracts of healthy snakes (Hilf *et al.*, 1990).

Other tests to diagnose CLO definitively include PCR assay, immunohistochemistry, electron microscopy, and an antibody-related enzyme-linked immunosorbent assay utilizing the patient's serum (Bergottini *et al.*, 2005). In vitro culture is rarely done due to the expense and difficulty of

growing CAR bacillus in cell culture media (Caniatti *et al.*, 1998). The inability to amplify DNA via PCR in this case may be due to genetic variations between the PCR primers and this CLO in the snake, to prolonged formalin fixation coupled with the use of paraffin embedded tissue (Ramos-Vara *et al.*, 2002), or to both. In general, the Warthin-Starry stain is still the diagnostic tool of choice for both CAR bacillus and CLO.

Cilia-associated respiratory bacillus in rodent species is usually associated with other primary respiratory illnesses; however, respiratory deficiencies due to CLO have been recorded without a concurrent infection. The bacilli have been associated with potentiation of disease caused by *Mycoplasma pulmonis* in rats and may possibly accentuate the effects of this and other infectious agents (Fallon, 1996). A study on rabbit and rat isolates of CAR bacillus using mammalian cell culture and transmission electron microscopy found that the CAR bacillus isolates may be of different strains (Cundiff *et al.*, 1994). In addition, the term CLO encompasses different species, and possibly different genera, that each affect their own distinct species (Bergottini *et al.*, 2005). These interpretations may point to an ability of CLO to infect other hosts, but this remains to be proven.

The less-significant histologic lesions of urate crystals within multiple renal tubules, and the lipid within hepatocytes in the liver, were likely associated with dehydration and anorexia, respectively. This rattlesnake had three separate entities affecting its health including mycobacteriosis, CLO, and dehydration, although the granulomas within the distal trachea, leading to obstruction, were considered the primary cause of death. The most engrossing finding, however, was that of CLO, as this has not been previously documented in reptiles.

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