The Veterinary Journal 232 (2018) 52–56

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Original Article

Detection of specific bacterial agents by quantitative PCR assays in the bronchoalveolar lavage fluid of dogs with eosinophilic bronchopneumopathy vs. dogs with chronic bronchitis and healthy dogs

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ARTICLE INFO

Article history:
Accepted 16 December 2017

Keywords:
Bordetella bronchiseptica
Bronchoalveolar lavage
Dogs
Eosinophilic bronchopneumopathy
Mycoplasma spp.

ABSTRACT

In humans, *Mycoplasma pneumoniae* and *Bordetella pertussis* infections are suggested to trigger or exacerbate asthma. Whether *Mycoplasma* or *Bordetella* are associated with chronic inflammatory bronchial diseases in dogs has not been investigated. The aim of this study was to assess detection rates of *Mycoplasma canis* (*M. canis*), *M. cynos* and *Bordetella bronchiseptica* (*Bb*), in dogs with eosinophilic bronchopneumopathy (EBP) and chronic bronchitis (CB), compared with healthy dogs. Specific quantitative PCR (qPCR) analysis for *M. canis*, *M. cynos* and *Bb* were retrospectively performed on bronchoalveolar lavage fluid (BALF) collected from 24 dogs with EBP, 21 dogs with CB and 15 healthy dogs. Possible associations between qPCR results and age, BALF cytology or clinical severity scores (CSS) in dogs with EBP were investigated.

There was no difference in *M. canis*, *M. cynos* and *Bb* detection rates in dogs with EBP ($n = 6$, $n = 2$ and $n = 6$, respectively) and dogs with CB ($n = 2$, $n = 2$ and $n = 2$, respectively) compared with control dogs ($n = 4$, $n = 2$ and $n = 2$, respectively). In dogs with EBP, the proportion that were qPCR-positive for *Bb* was higher in dogs with higher CSS ($P = 0.014$) and BALF from *Bb*-positive dogs had higher percentage of neutrophils ($P < 0.001$). Among dogs that were qPCR-positive for *Bb*, moderate to high loads were only detected in dogs with EBP. *M. canis* and *M. cynos* detection was not associated with EBP or CB; higher *Bb* loads were only present in dogs with EBP and high CSS. A possible cause and effect relationship between *Bb* infection or load and EBP remains unclear and requires further investigation.

Introduction

Idiopathic eosinophilic bronchopneumopathy (EBP) is a chronic disease characterized by eosinophilic infiltration of the lung and bronchial mucosa in young adult dogs (Corcoran et al., 1991; Clercx et al., 2000; Rajamäki et al., 2002). Dogs with non-specific chronic bronchitis (CB) also present with chronic cough, but are usually older. The etiology of both inflammatory conditions remains unclear. In EBP, an underlying immunologic hypersensitivity is suspected, but the inciting antigens remain mostly unidentified (Clercx et al., 2002; Peeters et al., 2005; Clercx and Peeters, 2007). For both of these bronchial diseases, current treatments consist of long-term oral and/or inhaled glucocorticoids (Clercx et al., 2000; Bexfiels et al., 2006; Rozanski, 2014; Canonne et al., 2016b).

In humans, infections with *Mycoplasma pneumoniae* (*M. pneumoniae*) have been associated with asthma for decades (Hansbro et al., 2004; Atkinson, 2013; Ye et al., 2014). *M. pneumoniae* infection is associated with acute exacerbation of adult asthmatics and future development of asthma in children (Hansbro et al., 2004) and specific treatment improves pulmonary function in asthmatics (Kraft et al., 2002). However, *Bordetella pertussis* (*B. pertussis*) has been also discussed as potential trigger in human inflammatory bronchial disease and asthma (Harju et al., 2006; Wakashin et al., 2008; Nicolai et al., 2013; Yin et al., 2017).

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https://doi.org/10.1016/j.tvjl.2017.12.014
1090-0233/

Furthermore, in a mouse model of allergic asthma, prior *B. pertussis* infection was documented to exacerbate airway signs (Ennis et al., 2004; Kavanagh et al., 2010).

Although *Mycoplasma cynos* (*M. cynos*) has been identified as an emerging, possibly contagious, and lethal pathogen in dogs with canine infectious respiratory disease (CIRD, kennel cough or canine infectious tracheobronchitis; Rycroft et al., 2007; Zeugswetter et al., 2007; Mannering et al., 2009; Priestnall et al., 2014), but the exact role of *M. canis* and *M. cynos* as primary respiratory pathogens still remains unclear (Chandler and Lappin, 2002; Chalker et al., 2004; Chan et al., 2013). Moreover, a recent study demonstrated the impact of oral bacterial contamination on *Mycoplasma*-specific PCR results of bronchoalveolar lavage fluid (BALF; Chan et al., 2013), making the investigation of a potential primary role of *Mycoplasma* spp. in canine lower respiratory conditions even more complex.

Bordetella bronchiseptica (*Bb*) is recognized as one of the primary causative pathogen agents of CIRD. *Bb* can exist in respiratory tract of dogs as either commensal or pathogen (Bemis et al., 1977; Bemis, 1992; Schulz et al., 2014). Currently, quantitative PCR (qPCR) assays provide highly sensitive and specific detection of of *Bb*, *M. canis* and *M. cynos* (Chan et al., 2013; Schulz et al., 2014; Lavan and Knesl, 2015; Canonne et al., 2016a). However, the potential role of these infectious agents in chronic inflammatory bronchial diseases in dogs have not yet been investigated and whether these bacterial agents could act as triggers or exacerbating agents in dogs with EBP is unknown.

The aim of the present study was to evaluate the presence and bacterial load of *M. canis*, *M. cynos* and *Bb* in steroid-naïve dogs newly-diagnosed with EBP and compare the results to those for dogs with non-specific CB and healthy dogs, using specific qPCR analysis performed on BALF samples. Additionally, potential associations between positive qPCR results for any of these bacterial species and the clinical severity of EBP were assessed.

Materials and methods

Dogs

Client-owned dogs presented to the National Veterinary School of Alfort Companion Animal Hospital between March 2009 and February 2016 and diagnosed with EBP or CB were prospectively recruited.

Definitive diagnosis of idiopathic EBP and CB was made in accordance with criteria previously described (Clercx et al., 2000; Rozanski, 2014). Briefly, this was based on compatible respiratory clinical complaints, radiological changes, bronchoscopic findings, analysis of the BALF including culture and standard cytological examination and, in some cases, histopathological examination of endoscopic bronchial mucosal biopsies. In dogs with CB, bacterial involvement in neutrophilic airway inflammation was excluded based on cytology (lack of degenerative neutrophils, absence of intra-cellular bacteria), culture and positive response to steroids. In dogs with EBP, exclusion of other causes of eosinophilic airway inflammation, including cardiopulmonary parasites, was required to definitively diagnose idiopathic EBP by using fecal and BALF analysis (Baermann method, cytology and qPCR on BALF). All dogs were newly-diagnosed and steroid-naïve.

In dogs with a final diagnosis of idiopathic EBP, a clinical severity score (CSS; 1-5/5) was assessed at the time of diagnosis based on severity and frequency of cough, evidence of exercise intolerance or lethargy, as previously defined (Canonne et al., 2016b). Cough was scored from 1-3/3 depending on frequency and severity; presence of retching or exercise intolerance was recorded with 1 additional point. Points obtained from cough scoring, retching and exercise intolerance were added to a maximum total of 5 points.

Bronchoscopy, BALF procedure and processing were performed as described earlier (Clercx et al., 2000). Briefly, dogs were anesthetized using various anesthetic protocols after a 5 min pre-oxygenation period. Five to 20 mL aliquots (depending on bodyweight) of sterile saline (NaCl 0.9%) were instilled twice into a same bronchus and a third time into a different lung lobe via a flexible pediatric endoscope (Fujinon, Pediatric video-bronchoscope EB-530S), followed by immediate aspiration by gentle automated suction. The recovered BALF was immediately processed. Aliquots of naïve BALF were used for semi-quantitative bacterial culture (Collard Laboratories, according to methods previously described; Peeters et al., 2000), total cell count determination using a hemocytometer, and cytospin preparation (centrifugation at 1400 rpm, 197 *g*, for 4 min at 20 °C, Thermo Shandon Cytospin 4). Differential cell count calculations were established by counting a total of 300 cells viewed on high power fields on the cytospin preparation; 400–600/μL was considered a normal cell count. BALF cytology was considered normal if there were <12% neutrophils and <7% eosinophils. The remaining recovered BALF was centrifuged at 1300 *g* for 15 min at 4 °C. The resulting supernatant and cell pellet were stored separately at −80 °C. Additionally, angiostrongylosis was excluded in all dogs with EBP based on a negative quantitative PCR (qPCR) test results in BALF (Canonne et al., 2016b). Additionally, all dogs were treated at the time of diagnosis with anthelmintics (fenbendazole 50 mg/kg q24 h for 5 days, Panacur Intervet International via MSD Animal Health, or milbemycin oxime 1 mg/kg once, Milbemax Novartis), while results were pending.

Control group

Stored BALF samples collected from 15 healthy dogs were available from previous studies for which ethical approval had been previously obtained from the University Local Ethical Committee (University Local Ethical Committee Approval number 1435; Approval date 30th April 2013). The stored samples were collected from healthy dogs belonging to veterinary staff or students ($n = 13$) or from shelters ($n = 2$), and none had either history or clinical signs of respiratory disease. Bronchoscopy, BALF and laboratory processing and storage had been performed as for dogs with EBP and CB and both total cell counts and cytological examination of cytocentrifuged preparations of BALF were within normal limits in each dog.

Quantitative PCR analysis

Quantitative PCR analysis was performed on stored BALF for *M. canis*, *M. cynos* and *Bb* in dogs with EBP, CB and healthy dogs. The frozen pelleted cells were thawed and, after re-suspension in a small volume of sterile saline (0.9% NaCl), samples were sent to the laboratory for qPCR analysis. The qPCR results were expressed as Ct values. Ct values were arbitrarily further categorized into five groups: very high load (Ct < 20), high load (20.1–24), moderate load (24.1–28), low load (Ct 28.1–32) and very low load (>32.1).

Statistical analysis

Statistical analyses were performed with a commercially available software (XLstat software). Data were expressed for continuous and categorical variables as median with range or as proportions, respectively.

Chi-square or Fisher's exact (when necessary) tests were used to compare the proportions of qPCR detection of *M. canis*, *M. cynos* and *Bb* between dogs with EBP and healthy dogs and between dogs with EBP and dogs with CB. Dogs with EBP were grouped according to CSS into two groups: dogs with CSS ≤ 3 (mild clinical severity) and dogs with CSS > 3 (moderate-to-severe clinical signs). The proportion of dogs with EBP and positive qPCR results for *M. canis*, *M. cynos* and *Bb* were compared between dogs with CSS ≤ 3 and dogs with CSS > 3 by using exact Fisher's exact test (for $n \leq 5$). For each bacterial agent, the median age of dogs with EBP with positive qPCR was compared to the median age of dogs with EBP with negative qPCR using Wilcoxon–Mann Whitney tests. For each bacterial agent, median total cell count and percentage of neutrophils in the BALF of dogs with EBP that were qPCR-positive were compared to BALF parameters from dogs with EBP that were qPCR-negative by using Wilcoxon–Mann Whitney's test.

Values of $P \leq 0.05$ were considered statistically significant.

Results

A convenience sample of 24 dogs with EBP (median age, 4.2y [0.9–13.2]) and 21 dogs with non-specific CB (median age, 8.0 [0.9–14.0]) were selected.

Table 1 reports the proportions of dogs with positive qPCR result for *M. canis*, *M. cynos* and *Bb* in each of the three groups. For each bacterial agent, no significant difference in qPCR detection rates were found between dogs diagnosed with EBP and healthy dogs and between dogs with EBP and dogs with CB (Table 1). Among dogs with positive qPCR for *M. cynos* on BALF, Ct values corresponding to very high load were only found in dogs with inflammatory bronchial disease. Among dogs with positive qPCR for *Bb* on BALF, moderate or high load were only found in dogs with EBP.

In dogs with EBP, CSS varied between two and five at the time of diagnosis; total BALF cell counts were 700–6000/mL; % eosinophils was 20–85; and % neutrophils was 1–30 (Table 2). The proportion of dogs with *Bb* in the BALF was significantly higher in dogs with CSS > 3 (5/9, 56%) than in dogs with CSS ≤ 3 (1/15, 7%; $P = 0.015$).