Standardization of Bronchoalveolar Lavage Aspiration Techniques to Optimize Diagnostic Yield of Canine Lower Respiratory Tract Samples

by

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ABSTRACT

STANDARDIZATION OF BRONCHOALVEOLAR LAVAGE ASPIRATION TECHNIQUES TO OPTIMIZE DIAGNOSTIC YIELD OF CANINE LOWER RESPIRATORY TRACT SAMPLES

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University of Guelph, 2013

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Dr. Alice Defarges

Bronchoalveolar lavage (BAL) is a minimally invasive technique utilized in human and veterinary medicine to sample the lower generation bronchi and alveolar spaces. The basic technique for BAL involves infusion of sterile saline into the lower airways and re-aspiration of the fluid (bronchoalveolar lavage fluid; BALF). Certain aspects of BAL technique and BALF processing affect sample quality, and sample quality is important to ensure meaningful cytology. Aspiration techniques for retrieval of BALF have not been critically evaluated in companion animal medicine. This research project compared three aspiration techniques for retrieval of BALF in dogs [manual aspiration without tubing (MA), manual aspiration through polyethylene tubing (MAPT) and suction pump aspiration (SPA)] and their effect on sample quality in healthy dogs and dogs with respiratory tract disease. SPA consistently retrieved a higher proportion of BALF than MA and MAPT. In addition, SPA yielded improved sample quality compared to MA and MAPT. The improved BALF retrieval and cellularity scores in SPA samples did not significantly increase the diagnostic rate achieved from BALF cytology in dogs with pulmonary disease. The results indicated that both MA and SPA are suitable for BAL in dogs with respiratory tract disease. Yet, for the purpose of creating a standardized BAL technique in dogs, SPA is recommended for BALF retrieval due to the improved sample quality parameters.
ACKNOWLEDGEMENTS

Thank you to the Ontario Veterinary College Pet Trust for providing funding for this project.

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To my co-advisor, Dr. Anthony Abrams-Ogg, and the members of my advisory committee, Drs. Dorothee Bienzle, Laurent Viel and Brigitte Brisson, I thank you for your patience, invaluable input and suggestions.

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To my co-resident, Allison: I am so glad that OVC enticed you to return for a residency. I cannot imagine what these past three years would have been like without you and our many meals! I am already excited about visiting you in Montreal.

To the other graduate students (and friends) that I have had the pleasure of working with – Adam, Maggie, Jon, Kim, Shannon, Vincent, Shawn, Michelle, and the “Path crew” – thank you for making my work days so enjoyable.

And lastly, but certainly not least, I want to thank my Mom for supporting me and for supporting my crazy notion to leave general practice and go back to school. I am here today because of your unconditional love and guidance.
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ABBREVIATIONS

BAL – Bronchoalveolar lavage
BALF – Bronchoalveolar lavage fluid
CBC – Complete blood count
CRI – Constant rate infusion
CT – Computed tomography
ECG – Electrocardiogram
EIPH – Exercise induced pulmonary hemorrhage
ELISA – Enzyme-linked immunoassay
ELF – Epithelial lining fluid
IM – Intramuscular
IQR – Interquartile range
IV – Intravenous
MA – Manual aspiration
MAPT – Manual aspiration via polyethylene tubing
MRI – Magnetic resonance imaging
RBC – Red blood cell
SD – Standard deviation
SPA – Suction pump aspiration
SQ – Subcutaneous
TNCC – Total nucleated cell count
TTW – Trans-tracheal wash
CHAPTER 1:
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction
Determining the underlying cause of lower respiratory tract disease may be a challenge for veterinarians. While history and physical examination findings provide valuable information (Miller, 2007), they rarely provide an etiologic diagnosis in patients with respiratory disease and ancillary testing is almost always warranted (Silverstein, 2010). Ancillary testing for patients with lower respiratory tract disease involves a combination of imaging and sampling modalities.

Available imaging techniques for the respiratory tract include survey thoracic radiographs, ultrasonography, computed tomography (CT scan), and magnetic resonance imaging (MRI). Thoracic radiographs have limited sensitivity, with a lower limit of detection of 3 to 5 mm for solitary pulmonary nodules (Mai, 2008; Silverstein, 2010). In addition, visible changes on thoracic radiographs are non-specific and non-sensitive (Mai, 2008; Miller, 2007; Silverstein, 2010) and may represent inactive, clinically insignificant lesions. Thoracic ultrasonography may better differentiate thoracic soft tissue or fluid-filled masses than survey radiographs; however, the sound wave is attenuated by air within the lungs and this significantly limits the utility of thoracic ultrasound (Mai, 2008). Thoracic CT scan is more sensitive than thoracic radiography due to higher contrast resolution, which provides resolution of 1 mm for solitary, metastatic pulmonary nodules (Johnson, 2007; Mai, 2008; Miller, 2007). Using computer-aid detection algorithms, sensitivities of 73 to 96% are reported for the detection of human pulmonary nodules ≥3mm (Fraioli, 2007; Rubin, 2005). As CT scans provide three-dimensional images, which are free from superimposition of thoracic structures, they are considered the gold standard for pulmonary imaging in human medicine (Mai, 2008). MRI also provides three-dimensional images; however, thoracic MRI is rarely performed to assess pulmonary parenchyma as respiratory movement induces imaging artifact and the lungs provide low imaging signal (McConnell, 2008). Limitations of thoracic CT scans in veterinary medicine include increased cost, the need for general anesthesia, and that they are less widely available than survey radiographs. In addition, even when clinical findings are assessed in conjunction with imaging, lesions often require sampling for etiologic diagnosis.

1.2 Modalities for Sampling the Lower Respiratory Tract:
Modalities for sampling the respiratory tract (Table 1.1) include trans-tracheal wash (TTW), trans-thoracic aspiration, lung biopsies, and bronchoscopy with bronchoalveolar lavage (BAL) and/or bronchial brushings. TTW is a minimally invasive technique whereby a sterile catheter is inserted into the trachea via a skin incision distal to the larynx (Creevy, 2009; Silverstein, 2010). Retrieved TTW fluid is hypocellular compared to bronchoalveolar lavage fluid (BALF) since it only provides information about the trachea and major bronchi (Creevy, 2009; Hawkins, 1995; Hawkins, 2004). Thus, cytologic interpretation of TTW and BALF samples differ in 68% of patients (Hawkins, 1995). Trans-thoracic lung aspiration has poor sensitivity for diffuse disease (Hawkins, 2004), but is helpful in the diagnosis of focal pulmonary parenchymal lesions. Sensitivities of trans-thoracic needle aspiration for carcinoma and blastomycosis are 88% and 83%, respectively, with 100% specificity for both diseases (Wood, 1998). It is reported in a canine and feline review that unguided pulmonary fine needle aspiration is associated with a 31% incidence of pneumothorax (6.2% requiring intervention) and
a 12% mortality rate (Teske, 1991). However, ultrasound guided trans-thoracic needle aspiration appears to be safer, with one study reporting no clinically recognized adverse effects (Wood, 1998). Trans-thoracic, trans-bronchial and surgical lung biopsies provide good diagnostic information, but involve higher patient risk (Jara-Palomares, 2009; Silverstein, 2010; Pusterla, 2006). Complications of trans-thoracic lung aspirates and biopsies include pneumothorax, hemorrhage and seeding of neoplastic cells along the biopsy tract (Mai, 2008). In people, trans-bronchial lung biopsy has a complication rate of 7-10% with a 13% complication rate for open lung biopsy (Klech, 1990; Suda, 2008). In horses, complications from trans-thoracic lung biopsy include epistaxis, hemorrhage, respiratory distress, pneumothorax and death (Pusterla, 2006). In addition to the risks of pneumothorax and hemorrhage, surgical lung biopsy in dogs and cats requires post-operative hospitalization and analgesia. Bronchoscopy allows visualization of the lower airways, bronchial brushings provide information about the large airways, and BAL provides a non-invasive method of sampling the alveolar spaces for cytologic, biochemical, immunological, and microbial analysis (Hawkins, 2006).

Table 1.1 – Comparison of pulmonary sampling techniques utilized in small animal veterinary medicine (Cole, 2004; Creevy, 2009; Hawkins, 1990b; Hawkins, 2004; Hawkins, 2006; Johnson, 2007; Klech, 1989; Miller, 2007; Norris, 2001; Silverstein, 2010; Syring, 2004).

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<th>Benefits</th>
<th>Limitations</th>
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<tr>
<td>TTW</td>
<td>General anesthesia not required</td>
<td>Hypocellular sample (compared to BALF)</td>
</tr>
<tr>
<td></td>
<td>Minimally invasive</td>
<td>Samples trachea and upper bronchi only</td>
</tr>
<tr>
<td></td>
<td>Provides samples for cytology and culture</td>
<td>Less sensitive than BAL for pulmonary disease</td>
</tr>
<tr>
<td></td>
<td>Low rate of complications</td>
<td>Contraindicated in fractious patients or those with tracheal collapse, dermatitis on ventral neck or a coagulopathy</td>
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<tr>
<td>BAL</td>
<td>Minimally invasive</td>
<td>Requires general anesthesia</td>
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<td></td>
<td>Provides samples for cytology and culture</td>
<td>Relative contraindication for patients with pre-existing dyspnea</td>
</tr>
<tr>
<td></td>
<td>Relatively safe</td>
<td>Low sensitivity for interstitial pulmonary disease and non-exfoliating neoplasia</td>
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<tr>
<td></td>
<td>Mortality/euthanasia rate of 2% (dogs)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mortality/euthanasia rate of 6% (cats)</td>
<td></td>
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<tr>
<td>Trans-thoracic Needle Aspiration</td>
<td>Light sedation</td>
<td>Hypocellular samples and higher risk for blood contamination (compared to BALF)</td>
</tr>
<tr>
<td></td>
<td>Minimally invasive</td>
<td>Less sensitive than BAL for diffuse disease</td>
</tr>
<tr>
<td></td>
<td>Can be performed under ultrasound guidance with minimal complications</td>
<td>Higher rate of complications than BAL and TTW</td>
</tr>
<tr>
<td></td>
<td>Sensitivity of 83-88% for focal pulmonary diseases</td>
<td>Mortality rate of 12% (unguided; dogs) and 17% (for fluoroscopic-guided)</td>
</tr>
<tr>
<td>Lung Biopsy</td>
<td>Gold standard for diagnosis of pulmonary parenchymal disease</td>
<td>Aspiration through normal lung may induce pneumothorax</td>
</tr>
<tr>
<td></td>
<td>Provides samples for histology and culture</td>
<td>Requires general anesthesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Invasive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Higher morbidity and mortality rate than BAL</td>
</tr>
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<td></td>
<td></td>
<td>Complication rate of 13% (humans)</td>
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The basic technique for BAL involves infusion of sterile isotonic saline into the alveoli with re-aspiration of the fluid (Hawkins, 2004; Silverstein, 2010). Bronchoscopy and BAL are considered relatively safe: in humans, they are outpatient procedures with the majority of side-effects being minor and transient in nature (American Thoracic Society, 2012; Yoneda, 2008) (Table 1.2). Coughing, transient decrease in oxygen saturation, and fever are not considered complications of human BAL since they do not require intervention (Yoneda, 2008). Minor complications may include hemorrhage, persistent hypoxemia, bronchospasm and vasovagal
syncope (Yoneda, 2008). Major complications include pneumonia, arrhythmias, pneumothorax, pneumomediastinum, respiratory failure, and cardiac arrest (Yoneda, 2008). In human medicine, major complication rates of 0-5% have been reported (American Thoracic Society, 2012; Dreisin, 1978; Sampsonas, 2011; Ward, 2001; Yoneda, 2008) with a mortality rate of 0.001-0.1% (Pedreira, 2007). In people, a higher rate of complications is associated with age (young or geriatric patients) and patients under heavy sedation (de Blic, 2002; Morrow, 2001). A similar safety profile has been reported in canine medicine for bronchoscopy and BAL (Hawkins, 1995). Studies involving massive lung lavage (using 6 L of sterile saline) in healthy, purpose-bred dogs have identified only transient changes in cardiopulmonary function with occasional hyperthermia and leukocytosis (Dubin, 1971; Muggenburg, 1972; Muggenburg, 1980). In addition, these studies report that no dogs required intervention during or following the procedure (Dubin, 1971; Muggenburg, 1972, 1980). A mortality/euthanasia rate of 2% (2/101) is reported for BAL in clinical canine cases at a veterinary referral center (Hawkins, 1995). In this study, mortality rate appears to be associated with pre-existing respiratory distress prior to the BAL procedure (Hawkins, 1995). This finding leads to the recommendation that pre-existing dyspnea be a relative contraindication for BAL (Hawkins, 2004). Severe bronchoconstriction has also been reported following BAL in a dog with eosinophilic airway disease, but appears to respond to treatment with bronchodilator drugs (aminophylline 3 mg/kg, IV, q12h; albuterol 180 µg, inhaler, q8h; magnesium sulfate 30 mg/kg, IV, q8h), and oxygen supplementation (Cooper, 2005). In a retrospective review of flexible bronchoscopy and BAL in cats at a veterinary referral center, it is reported that 6% of feline patients required overnight hospitalization and supplemental oxygen therapy, 3% developed pneumothorax, and a 6% mortality/euthanasia rate was associated with inability to restore ventilation following the procedure (Johnson, 2007). No preoperative conditions are associated with mortality following feline BAL in this study; however, pre-anesthetic pulse oximetry and blood gas analysis were not available for all cats (Johnson, 2007). A significantly lower rate of complications is reported in cats that are pre-treated with terbutaline sulfate (0.01 mg/kg SQ q8-12h) for 12 to 24 hours prior to bronchoscopy and BAL (8%), when compared with cats that are not pre-treated (40%) (Johnson, 2011). Pre-treatment with inhaled bronchodilators (salbutamol and ipratropium bromide) prior to BAL prevents bronchoconstriction in allergen-sensitized cats (Kirschvink, 2005). For these reasons, pre-treatment with bronchodilators is currently recommended in cats prior to bronchoscopy (Hawkins, 2004; Padrid, 2011). Brush samples for cytology are another minimally invasive technique that may be performed under bronchoscopic guidance; however, they sample only the bronchi and not the alveolar spaces. In summary, ease of technique, minimal invasiveness, low patient risk, and good diagnostic yield justify the use of BAL as a primary tool for sampling the canine and feline lower airways.


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<th>Side Effect</th>
<th>Rate</th>
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| Bronchospasm | Human: 0.6% to 1%  
Canine: rare  
Feline: increased PenH (associated with bronchoconstriction) in 100% of healthy cats following BAL |
| Pyrexia | Human: 2.5-30% |
Canine: 0% to 17%
Feline: NR

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<th>Condition</th>
<th>Human</th>
<th>Canine</th>
<th>Feline</th>
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<td>Transient Decrease in Pulmonary Function*</td>
<td>2% to 22%; 62.9% of pediatric patients</td>
<td>rare</td>
<td>&lt;24%</td>
</tr>
<tr>
<td>Acute Hemorrhage</td>
<td>0.3% to 3%</td>
<td>rare</td>
<td>rare</td>
</tr>
<tr>
<td>Pneumothorax</td>
<td>0.07% to 0.9%; 100% of 4 pediatric patients (non-bronchoscopic BAL)</td>
<td>NR</td>
<td>3%</td>
</tr>
<tr>
<td>Respiratory Failure+</td>
<td>0.3% to 11%</td>
<td>&lt;2%</td>
<td>&lt;6%</td>
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<tr>
<td>Mortality and/or Euthanasia Rate Following BAL</td>
<td>0.001 to 5%</td>
<td>0-2%</td>
<td>0-6%</td>
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*Increased respiratory effort and/or requirement for supplemental oxygen therapy following the BAL procedure.
+Requiring mechanical ventilation following the BAL procedure. NR = not reported.

1.3 Indications for and Clinical Utility of Bronchoalveolar Lavage:

Indications for bronchoscopy and BAL (Table 1.3) include chronic cough, unexplained or lack of changes on thoracic radiographs, and lung masses. BAL has been shown to have a higher diagnostic yield than TTW for diseases involving the small airways (Hawkins EC, 1990a; Hawkins, 1993; Hawkins, 1995; Miller, 2007). Recovered cells in BALF accurately reflect histologic changes in experimentally induced acute lung injury (Weiland, 1989), therefore the evaluation of alveolar epithelial lining fluid (ELF) can be diagnostic for pulmonary disease (Mayer, 1990; Mills, 2005; Mills, 2006). A fair correlation between BALF and histologic evaluation for inflammatory disease is identified in a retrospective analysis of 16 canine patients with lower respiratory tract disease (Norris, 2001). In this study, 8 BALF samples out of 11 dogs with histologically confirmed inflammatory disease also demonstrate inflammatory BALF cytology (Norris, 2001).

Table 1.3 – Indications and contraindications for BAL in dogs and cats (Hawkins, 1990b; Hawkins, 2004; Padrif, 2011, Silverstein, 2010).

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<thead>
<tr>
<th>Indications for Bronchoscopy and BAL</th>
<th>Contraindications for BAL</th>
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<td>• Acute or chronic cough</td>
<td>• Pre-existing dyspnea (relative contraindication)</td>
</tr>
<tr>
<td>• Unexplained changes on thoracic radiographs</td>
<td>• Coagulopathy</td>
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<tr>
<td>o Alveolar or bronchial pattern</td>
<td></td>
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<tr>
<td>• Normal thoracic radiographs despite clinical signs consistent with respiratory disease</td>
<td></td>
</tr>
<tr>
<td>• Thoracic mass</td>
<td></td>
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<tr>
<td>• Suspicion of pneumonia</td>
<td></td>
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<td>o Samples for microbial culture</td>
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<td>• Stridor</td>
<td></td>
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<tr>
<td>o When laryngeal examination is unremarkable</td>
<td></td>
</tr>
<tr>
<td>• Unexplained abnormal respiratory pattern</td>
<td></td>
</tr>
<tr>
<td>• Removal of bronchial mucoid obstruction</td>
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</tbody>
</table>
Bronchoscopy and BAL can provide definitive diagnoses in cases of inflammatory airway disease, bronchiectasis, eosinophilic pneumonia, pulmonary parasites, bacterial pneumonia, mycotic pneumonia and neoplasia (Barcante, 2008; El-Bayoumi, 2008; Hawkins, 1995; Hawkins, 1990a; Hawkins, 1993; Hawkins, 2006; Norris, 2001). For example, BAL has a diagnostic yield of 95% for *Pneumocystis carinii* pneumonia in people (Strausz, 1998). In dogs with multicentric lymphoma, BALF is more sensitive than thoracic radiographs for the detection of pulmonary involvement (Hawkins, 1993). It is reported in a study of 47 dogs with multicentric lymphoma that while only 34% of thoracic radiographs exhibit evidence for pulmonary involvement, BALF provides cytologic evidence for pulmonary involvement in 66% of cases (Hawkins, 1993). Analysis of BALF also diagnoses fungal pneumonia in (6/9) 67% of canine cases (Hawkins, 1990a). In particular, *Blastomyces dermatitidis* and *Histoplasma capsulatum* organisms, but not *Coccidioidomyces* spp., are identified in BALF in one study (Hawkins, 1990a). A retrospective review of 68 cases of canine and feline BAL reports that BALF provides a definitive diagnosis in 25% of cases (Hawkins, 1995). When BALF cytology is assessed in conjunction with other clinical data (history, thoracic radiographs, bacterial cultures, and/or histology of lung biopsy), it is supportive of the diagnosis in an additional 50% of cases (Hawkins, 1995). It is also reported that BALF cytology is more likely to provide useful information when an alveolar, bronchial pattern, or mass lesion is identified on thoracic radiographs (Hawkins, 1995). In people and dogs, BALF has a lower diagnostic yield for interstitial fibrosis disorders and non-exfoliative neoplasias (e.g. sarcomas) (Hawkins, 1995; Wells, 2010). Recent work in humans, cats and dogs suggests that immunologic analysis of BALF may allow further differentiation of certain conditions (Jara-Palomares, 2009; Norris, 2003; Rafft, 2011). Research teams are currently investigating whether biomarkers, such as immunoglobulins and endothelin-1, can be measured in BALF to support diagnoses of feline asthma and idiopathic pulmonary fibrosis, respectively (Norris, 2003; Rafft, 2011). Serial BAL can also be used to monitor response to treatment (Ettensohn, 1988; Hawkins, 2004). When performing serial BAL, the procedures must be performed greater than 48 hours apart as infusion of saline into the alveoli induces a transient influx of neutrophils (Damiano, 1980; Hoffman, 2008). However, most veterinary patients do not undergo repeat BAL if they are responsive to treatment due to costs and risk of general anesthesia associated with the procedure. In summary, analysis BALF provides valuable information in the diagnosis and management of lower respiratory tract disease.

### 1.4 Bronchoalveolar Lavage Technique:

The basic technique for BAL consists of infusion of isotonic sterile saline into the bronchi and alveoli with re-aspiration. However, many variations on the BAL procedure have been reported. The technique can be performed “blindly” (blind BAL; unguided BAL) by passing a tube into the lungs via the endotracheal tube or with bronchoscopic guidance (Hawkins, 1999; Hawkins, 2004; McCauley M, 1998). Bronchoscopic guided BAL allows visualization of the lower airways and direction of BAL sampling sites to areas with gross lesions. Human prospective studies have shown that bronchoscopic BAL provides samples of higher diagnostic quality and reliability (Perkins, 2006; Strausz, 1998) than the unguided technique. Unfortunately, bronchoscopic guided BAL may not always be possible in smaller veterinary patients. Evidence exists in human and equine medicine that other variations in BAL technique and sample processing also alter the cytologic and biochemical results of BALF (Pickles, 2002a; Pickles, 2002b). Therefore, attempts have been made in human and equine
medicine to standardize the BAL protocol to allow for comparison between clinicians and institutions (Klech, 1989; Baughman, 2007; Hewson, 2002). Such a uniform approach is lacking in small animal medicine. This is demonstrated by a recent survey of board certified veterinary small animal internists that identified that the type of endoscope used, wash fluid volume, and aspiration technique vary greatly for canine BAL (Appendix 1).

1.5 Recommendations for Diagnostic Bronchoalveolar Lavage Fluid Samples:

BALF contains cells and acellular solutes from the bronchoalveolar epithelial environment. In order for BAL to provide meaningful results and allow for objective interpretation, it is imperative that the retrieved infusate samples the bronchoalveolar ELF, provides cells in adequate number and preservation for assessment, and is free of contamination from other anatomic areas. Specific criteria have been suggested in the human and veterinary medical literature, and these will be discussed below.

Three criteria ensure that the infusate samples the bronchoalveoli: percentage of fluid retrieved, presence of surfactant, and measurement of ELF (Lam, 1985; Rennard, 1986; Rennard, 1990; Hawkins, 2004). A higher percentage of retrieved infusate supports sampling from the lower airways, as two prospective studies in human medicine identify that small volume aliquots (<20 mL) and small amounts of retrieved fluid only represent the large airways (Lam, 1985; Rennard, 1990). These small volume samples are considered “bronchial washings”, not BALF (Rennard, 1990), and contain a higher percentage of neutrophils and columnar epithelial cells with fewer alveolar macrophages (Lam, 1985; Rennard, 1990; Cherniak, 1990). Recovery of 46% to 69% (human), 40% to 90% (canine) and 51% to 73% (feline), and 20% to 60% (equine) of BALF has been previously reported (Doucet, 2002a; Doucet, 2002b; Hawkins, 1990b; Hawkins, 2004; Hewson, 2002; Ward, 2001; Wilcox, 1998). In humans, no change in fluid recovery is expected as patients age; however, less BALF retrieval is expected from subjects with obstructive airways diseases (e.g. asthma in people, chronic obstructive pulmonary disease in horses) (Hewson, 2002; Ward, 2001). Using appropriate aliquot volumes and ensuring adequate BAL infusate retrieval (≥40%) support sampling from the alveoli.

Surfactant is a phospholipid, protein, and ion mixture secreted by type II pneumocytes into the epithelial alveolar surface to reduce alveolar surface tension (Boothe, 2004; Zuo, 2008). As pulmonary surfactant within the respiratory tract is only present in the alveolar ELF, the presence of surfactant in BALF confirms that the alveoli have been sampled during the lung lavage. Surfactant is considered to appear as the presence of foam in BALF (Figure 1.1) (Creevy, 2009; Hawkins, 2004; Hewson, 2002; Hoffman, 2008). Enzyme-linked immunoassays (ELISA) for various mammalian species (mice, rats, dogs, and humans) and lipid extraction techniques (Bligh, 1959) exist for measurement of pulmonary surfactant, but they are not routinely used outside of research settings.
Figure 1.1 – The presence of foam (black arrow) within the retrieved BALF supports successful sampling of the alveoli.

Quantification of ELF in BALF has been suggested as a method for confirming that the alveoli have been sampled (Melamies, 2011). Quantifying the amount of ELF in BALF may also aid in the assessment of acellular solutes in BALF. The degree of ELF dilution in BALF varies based on the original volume of the infusate used for BAL and the amount of infusate retrieved, and cannot be linearly predicted by total nucleated cell counts (TNCC) within BALF (Mills, 2005; Mills, 2006). Exogenous substances, such as methylene blue added to BAL infusate or intravenously administered radioisotopes, have been proposed as markers of dilution. A technique using intravenous technetium-99m diethyleneetriaminepenta-acetic acid (99mTc-DTPA) has been validated for the measurement of ELF in the dog (Bayat, 2004); however, this technique requires a novel saturation BAL technique. Administration of a radioisotope also involves increased radiation exposure (for the patient and staff), risk of hypersensitivity reaction, and special housing – all of which limits its use to a research setting. Methylene blue as an exogenous additive to BAL infusate leads to overestimation of ELF due to preferential binding to respiratory cells (Marcy, 1987). In the clinical setting, efforts have been made to quantify ELF in BALF by measuring endogenous substances present in ELF. However, many endogenous substances, such as electrolytes and albumin, have proven to be invalid markers of dilution (Marcy, 1987; Rennard, 1986; Ward, 1997). For example, the concentration of potassium is too easily altered in BALF by cell rupture (Marcy, 1987; Rennard, 1986). It is reported in a study of healthy and diseased infants that total protein, albumin, and sphingomyelin are not valid dilution markers since the ELF concentration of these substances varies between diseased and normal lungs (Dargaville, 1999). In addition, the inability to precisely quantify the concentration of albumin in ELF precludes its use (Marcy, 1987; Ward, 1997). Urea is the most commonly reported endogenous marker of ELF dilution (Bayat, 1998; Melamies, 2011; Ward, 1999; Ward, 2000). Since urea is a freely diffusible molecule, the concentration of urea in ELF should be equal to that of plasma (Rennard, 1986). This equilibrium allows for calculation of volume of ELF when the amounts of urea are known in both BALF and plasma (Figure 2) (Hawkins, 2004). However, the use of urea as a marker of dilution remains controversial, as large influxes of water and urea occur from plasma into the alveolar space during BAL (Duddridge, 1989; Kelly, 1989; Marcy, 1987; Rennard, 1987). Labeling studies in rats have shown that the
majority of urea in BALF originates from plasma (Feng, 1992; Walters, 1989). This influx of urea and water from the circulation leads to overestimation of ELF, especially with progressive aliquots and longer dwell times during BAL (Duddridge, 1989; Kelly, 1989; Marcy, 1987; Rennard, 1986). Other experimental studies suggest that the overestimation of ELF is clinically insignificant with procedure times of less than two minutes (Mills, 2005; Mills, 2006; Ward, 1999); however, in two of these studies, the BAL procedure is performed post mortem (Mills, 2005; Mills, 2006). Since urea passes quickly into newly instilled BAL lavage fluid from the circulation, this effect is not likely to be appropriately assessed in deceased animals. In addition, while a recent study suggested that using urea to estimate ELF volume is more accurate in diseased lungs, when compared with healthy lungs (Bayat, 2004), the rate via which urea diffuses is not likely to be constant between various disease states (Duddridge, 1989; Marcy, 1987; Ward, 2000; Ward, 2001). Therefore, the European Respiratory Society considers urea an unsuitable dilution marker (Haslam, 1999), and therefore recent human and equine consensus panels recommend quantifying the amount of ELF dilution by reporting results by mL of BALF retrieved (Baughman, 2007; Cherniak, 1990; Goldstein, 1990; Hewson, 2002).

\[
\text{Volume}_{\text{ELF}} = \text{Volume}_{\text{BAL}} \times \left( \frac{\text{urea}_{\text{BALF}}}{\text{urea}_{\text{plasma}}} \right)
\]

**Figure 1.2 – Equation for calculating the volume of ELF using urea as a marker of dilution**

It has been shown that sample handling by the clinician influences BALF results. A selective loss of phagocytes can occur with the use of non-siliconized glass collection tubes (Hawkins, 2004). Cilia are lost from canine columnar epithelial cells within 30 min of suspension in saline (Rebar, 1980). Significant decreases in mast cell counts in equine BALF have been reported as early as 8 hours, despite refrigeration (Pickles, 2002b). Decreased cell viability in human BALF has been identified after 12 hours of refrigerated storage (Klech, 1989). Significant alterations in cell counts are identified in feline BALF after 48 hours, regardless of storage temperature (DeClue, 2007). Therefore timely sample processing (within 1 hour) is recommended to minimize cellular degeneration (Wilcox, 1988). If this is not possible, the BALF sample should be refrigerated and processed within 4 hours of collection. Controversy also exists regarding pooling of sequential BALF aliquots. Human BALF from the initial 20 mL aliquot contains a lower TNCC and higher proportion of neutrophils and epithelial cells than BALF of further aliquots (Lam, 1985). However, pooling the initial BALF aliquot with subsequent aliquots from the same lavage location does not significantly change the overall cytology results (Lam, 1985). A higher proportion of mast cells is identified in the initial 20 mL of recovered equine BALF compared to subsequent aliquots; however, the clinical relevance of this difference remains uncertain (Pickles, 2002c). It is reported in a study of feline BAL cytology that there is no significant difference in results of a pooled sample versus three individual aliquots (Hawkins, 1994). As separate analysis of sequential BAL aliquots from the same location is unlikely to be clinically relevant and, as pooling of samples may limit the error associated with any single aliquot (Hoffman, 2008), the majority of consensus statements in human and veterinary medicine recommend pooling BALF samples from the same location.

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Laboratory processing prior to analysis significantly affects BALF results. For example, filtering of BALF through sterile surgical gauze to remove mucus also removes an unpredictable amount of cells (Kelly, 1989; Klech, 1989; Sutinen, 1995). In humans, BALF TNCC is most accurate when determined on unprocessed, uncentrifuged fluid (Wilcox, 1988). Multiple studies have reported that cell viability decreases after centrifugation and cell washing (Mordelet-Dambrine, 1984; Rajamaki, 2001; Wilcox, 1988). Loss of up to 88% of cells has been reported following two centrifugations and one washing (Rajamaki, 2001). Therefore, filtering and cell washing are not recommended for processing of human BALF samples. While cytocentrifugation may result in lower lymphocyte counts, it is the recommended method for equine BALF cytology (Pickles, 2002a; Hewson, 2002). Fixation and staining protocols can also affect differential cell counts (Moumouni, 1994). It is reported in one study that all staining methods result in a preferential loss of lymphocytes when compared to unprocessed BALF, with higher losses in aqueous based stains versus alcohol based stains (Moumouni, 1994). No significant difference exists between cytocentrifuge or air dried preparations, and it is suggested that spray-fixed preparations stained by the Papanicolaou method is the most accurate way to assess lymphocytes in BALF (Moumouni, 1994). One study recommends Wright-Giemsa stain for better delineation of mast cells (Rebar, 1980). Given the documented effects of handling and processing of BALF on results, standard BALF processing is important for repeatable and comparable results between studies and institutions (Table 1.4).

**Table 1.4 – Recommendations for handling and processing BALF samples for cytologic analysis (Andreasen, 2003; English, 2008; Hoffman, 2008; Kelly, 1989; Mordelet-Dambrine, 1984; Pickles, 2002b; Rajamaki, 2001; Rebar, 1980; Sutinen, 1995; Wilcox, 1988).**

<table>
<thead>
<tr>
<th>Processing Variable</th>
<th>Current Recommendations</th>
</tr>
</thead>
</table>
| Pooling of BALF Samples | 1) Cytology: assess each BAL site separately, but pool sequential lavages from the same site.  
2) Microbial culture: pool samples from all sites lavaged.  |
| Time for Processing     | Ideally, process for analysis within 0.5-1 hour. If this is not possible, place on ice (or store at 4°C) and process within 4 hours. |
| Filtering of BALF TNCC  | Do not filter  
Hemocytometer on uncentrifuged BALF |
| Differential Cell Count | 1) Cytocentrifuge at 23 g for 5 min  
2) Stain with Wright-Giemsa  
3) State whether or not epithelial cells are included in the differential count |
| BALF Additives          | Fixatives and/or preservatives are not recommended  
No studies have assessed the effects of adding serum to BALF in veterinary medicine |
Cytologic analysis remains the mainstay of BALF evaluation. Alveolar macrophages are
the predominant cells from BALF in normal animals (Figure 1.3) (English, 2008; Hawkins,
2004). Other cell types commonly identified in normal BALF include lymphocytes, neutrophils,
eosinophils, and mast cells (English, 2008) (Table 1.5). Defined limits for cellularity and cell
preservation are extremely important to allow for accurate diagnosis from BALF. A consensus
statement from the European Society of Pneumonology recommends counting 300 to 500 cells
for the differential cell count in human BALF (Klech, 1989). A minimum 200 cell differential
count is required for high reproducibility of the proportion of neutrophils, alveolar macrophages
and eosinophils in cytocentrifuged canine BALF samples (De Lorenzi, 2009). A 500 cell
differential count is required for adequate reproducibility of canine lymphocytes (De Lorenzi,
2009). There is conflicting information regarding the effect of age on normal BALF of healthy
dogs. One study reported that there is no difference by age (Mayer, 1990), whereas another
study of healthy beagles found that there is a significantly higher neutrophil and lymphocyte
count in young dogs (<4.5 years old) (Mercier, 2010). Some parameters also have wide standard
deviations for “normal” values, which may be partially related to different BALF processing
protocols used in the various studies. For example, McCauley’s protocol (1998) discards the
first of three BALF aliquots from cytologic analysis. Vail’s protocol (1995) filters all BALF
samples through gauze prior to analysis, which, as previously discussed, has been shown to
remove an unpredictable number of cells. No evidence-based recommendations could be found
in the veterinary literature regarding limits for acceptable cell preservation.

Figure 1.3 – Cytology of canine BALF with alveolar macrophages (arrowhead) and an
eosinophil (arrow).
Table 1.5 – Reference ranges for total and differential cell counts in veterinary species (Brown, 1983; English, 2008; Hawkins, 2004; Hewson, 2002; Hoffman, 1997; Hoffman, 2008; Johnson, 2013; Rebar, 1980).

<table>
<thead>
<tr>
<th>Species</th>
<th>TNCC</th>
<th>AM</th>
<th>Neut</th>
<th>Lymph</th>
<th>Eos</th>
<th>Mast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine (unguided)</td>
<td>&lt;400 cells/µL</td>
<td>40-70%</td>
<td>&lt;5%</td>
<td>30-60%</td>
<td>&lt;0.1%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Canine (unguided)</td>
<td>352 ± 115 cells/µL</td>
<td>81 ± 11</td>
<td>15 ± 12%</td>
<td>2 ± 5%</td>
<td>2 ± 3%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Canine (bronchoscopic)</td>
<td>200-500 cells/µL</td>
<td>70 ± 11%</td>
<td>5 ± 5%</td>
<td>7 ± 5%</td>
<td>6 ± 5%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Feline (unguided)</td>
<td>337 ± 194 cells/µL</td>
<td>78 ± 15%</td>
<td>5 ± 5%</td>
<td>0 ± 1%</td>
<td>16 ± 14%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Feline (bronchoscopic)</td>
<td>241 ± 101 cells/µL</td>
<td>71 ± 10%</td>
<td>7 ± 4%</td>
<td>5 ± 3%</td>
<td>16 ± 7%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Legend: TNCC = total nucleated cell count; AM = alveolar macrophages; Neut = neutrophils; Lymph = lymphocytes; Eos = eosinophils; Mast = mast cells.

BALF specimens should be considered unacceptable if they have been contaminated from other areas of the respiratory tract and do not represent the bronchoalveolar milieu. It is reported in a multicenter prospective study of BAL in people that up to 30.4% of BALF samples are unsuitable for analysis (Chamberlain, 1987). The proportion of unsuitable specimens is correlated with the underlying disease, with the highest number of unsatisfactory BALF samples acquired from patients with collagen vascular disease (e.g. idiopathic pulmonary fibrosis) (Chamberlain, 1987). Sample contamination can be assessed via the following criteria: low TNCC, presence of epithelial cells (Figure 1.4), paucity of alveolar macrophages, Simonsiella bacteria, and hemorrhage (Chamberlain, 1987; English, 2008; Hawkins, 2004). Low overall cellularity may indicate a large airway wash (Chamberlain, 1987; Hawkins, 2004). Large numbers of epithelial cells (>5% of nucleated cells) in BALF may be present due to excessive suction, abnormal exfoliation, trauma or viral infections (English, 2008; Silverstein, 2010). Predominance of cuboidal and columnar epithelial cells indicates sampling from the upper airways (trachea and main stem bronchi), as opposed to the lower bronchi and alveoli (English, 2008; Rebar, 1980). Decreased numbers of alveolar macrophages (<10 alveolar macrophages/hpf) has also been suggested as an indication of bronchial sampling in people (Chamberlain, 1987). Squamous epithelium cells (>1% of differential cell count) and large bacteria (Simonsiella spp.) indicate oropharyngeal contamination (English, 2008; Peeters, 2000). The presence of RBCs, without evidence of erythropagocytosis, can also indicate an unsatisfactory BALF sample (Chamberlain, 1987). Acute hemorrhage is rarely found in BAL unless iatrogenic trauma from the BAL procedure results in hemorrhage (Chamberlain, 1987; English, 2008). The presence of erythropagocytosis and/or hemosiderin on cytology can be used to differentiate in vivo pulmonary bleeding from iatrogenic trauma (English, 2008).
Good diagnostic yield has been documented when BAL is used to investigate infectious pneumonia (Goldstein, 1990; Martin, 1987; Peeters, 2000). However, concern exists that nasal or oropharyngeal bacterial contamination could occur during insertion of the bronchoscope, which may result in a high rate of false positive bacterial retrieval. Nasopharyngeal contamination of the endoscope via collection of respiratory secretions within the biopsy channel of the bronchoscope is reported in foals secondary to an unguarded bronchoscopic technique (Hoffman, 1991). Guarded endoscopy, where the endoscope tip is protected by a sterile cover during passage through the oro- or nasopharynx, is now recommended for microbial assessment foals with pneumonia (Hoffmann, 1993). In contrast, Hirt et al (2010) report that the risk of contamination of BALF by canine oropharyngeal microflora is negligible with careful placement of the endoscope into the trachea. Current recommendations for the diagnosis of bacterial pneumonia involve assessing cytology for suppurative inflammation and intracellular bacteria, in combination with semi-quantitative or quantitative bacterial cultures (Peeters, 2000). In human medicine, the suggested cut-off of $10^4$ cfu/mL for pathogenic bacterial growth corresponds to a specificity of 100% for bacterial pneumonia (Rasmussen, 2001; Zedtwitz-Liebenstein, 2005). Quantitative BAL cultures of $>10^5$ cfu/mL are considered true infections in horses (Pusterla, 2006). A retrospective analysis of canine BALF cultures reports sensitivity of 86% and specificity of 100% for bacterial pneumonia with bacterial growth of greater than or equal to 1.7x$10^3$ cfu/mL (Peeters, 2000). When the presence of intracellular bacteria on cytology is included with quantitative cultures, the sensitivity increases to 87% with a specificity of 97% (Peeters, 2000). The use of quantitative bacterial cultures is hampered by the variable dilutional effect by the BAL procedure itself. Using urea to further define the dilution content and express bacterial growth as cfu/mL of ELF has been described in human medicine to increase sensitivity of BALF cultures (Zedtwitz-Liebenstein, 2005); however, as previously discussed, the use of urea as a marker of dilution for BALF is not reliable.
1.6 Review of Human Bronchoalveolar Lavage:

Soon after the development of the flexible bronchoscope by Ikeda in 1971, BAL was described in the human research setting by Reynolds and Newball (Baughman, 2007). Since then, BAL and analysis of BALF have been applied to various human clinical respiratory conditions. General indications for BAL in humans include non-resolving pneumonia, ventilator associated pneumonia, pulmonary hemorrhage, pulmonary infiltrates on thoracic radiographs, and for treatment of protein alveolitis (Goldstein, 1990; Reynolds, 2011; Wells, 2010). BAL is especially helpful in diagnosis of pneumonia in immunocompromised patients (Martin, 1987). Although differential cell counts in BALF can overlap with various pulmonary conditions, newer immunologic studies are helping to differentiate between diseases with similar clinical and radiographic findings, such as idiopathic pulmonary fibrosis and sarcoidosis (Jara-Palomares, 2009).

The difficulties comparing BALF between different patients and institutions has long been acknowledged in human medicine (Baughman, 2007; Ettensohn, 1988; Ward, 2001). Demographic factors have a significant effect on results of human BALF (Cherniak, 1990). It is reported in a multicenter prospective study of healthy human volunteers that BALF retrieval changes with the age, race, and smoking habits of human patients (Cherniak, 1990). A recent review of the human BAL literature (Baughman, 2007) reports that at least nine factors affect the results of BALF: underlying disease process, aspiration suction pressure, aliquot number and volume, proportion of BALF retrieved, processing of BALF, pooling of samples from the same lavage site, lung lobe lavaged, patient position during BAL, and storage of BALF samples prior to analysis. These findings have resulted in the publication of multiple expert consensus statements, most notably the guidelines from the European Society of Pulmonology (Klech, 1989) and the American Thoracic Society (2012). Both groups recommend pre-treatment of patients with atropine and sedation (diazepam and/or an opioid) to minimize respiratory secretions and coughing, respectively. Coughing during BAL can result in bronchial trauma, which may alter BALF composition, and decreased fluid retrieval (Klech, 1989; Reynolds, 2011). The use of bronchodilators in patients with hyperactive airways is also recommended by the American Thoracic Society (2012). The nose and larynx should be topically anesthetized with a sodium channel blocker, such as 1% to 2% lidocaine solution or spray (American Thoracic Society, 2012; Ward, 2001). A maximum dose of 4 to 5 mg/kg of lidocaine is recommended due to the potential bacteriostatic and adverse cell viability effects of lidocaine (Klech, 1989). Patients should be monitored by electrocardiogram and pulse oximetry, and they should receive supplemental oxygen during the procedure (Klech, 1989; Ward, 2001).

Bronchoscopes used to evaluate adult human patients generally have an outer diameter of 5.2 mm and a working length of 55 to 60 cm (Goldstein, 1990; Pedreira, 2006; Robinson, 1988). A bronchoscope with outer diameter of 5.2 mm allows for wedging in the 3rd or 4th generation bronchus in most adult humans (Goldstein, 1990). The bronchoscope is inserted nasally into the trachea and a routine bronchoscopic examination is performed. If trans-nasal passage of the bronchoscope is not possible, or if the patient is under general anesthesia, the bronchoscope may be passed orally (or via an endotracheal tube) into the trachea (Rennard, 1990). The site for BAL is directed by bronchoscopic findings and radiographic changes; however, the right middle and lingular lung lobes are standard BAL sites in diffuse disease (Goldstein, 1990; Klech, 1989) since greater BALF recovery has been documented from these sites when compared to lower lung lobes (Pingleton, 1983). The bronchoscope is then gently wedged into a subsegmental
bronchus. Fluid for BAL should be warmed to body temperature (37.0°C) to reduce the risk of bronchospasm (Klech, 1989). In addition, the fluid should be sterile and isotonic; 0.9% saline is the most commonly reported fluid used for human BAL. The American Thoracic Society (2012) recommends using a total of 100 to 120 mL of infusate, in 20 mL aliquots. The European group makes wider recommendations for fluid volume, with a range of 100 to 300 mL per lavage site (Klech, 1989). It is reported in two studies that low infusate volumes (<20 mL) do not reach the alveoli in people (Lam, 1985; Rennard, 1990). In addition, higher cell viability is identified in with BAL infusate volumes ≥20 mL (Lam, 1985). Other studies have shown that volumes of BAL infusate >50 to 60 mL are required to sample the alveoli (Kelly, 1987; Robinson, 1988); hence, the current recommendations to use at least 100 mL in total. However, the infusate volume data is based on relatively low sample numbers (Kelly, 1987; Lam, 1985; Robinson, 1988) and the results may have limited applicability to a wider general population. In regards to the European recommendations to lavage with up to 300 mL (Klech, 1989), it has been suggested that higher total infusate volumes (200 to 300 mL) may be associated with increased side effects (Costabel, 1991). No prospective studies have evaluated this hypothesis in human medicine. Once the saline has been infused via the bronchoscope’s biopsy channel, either by gravity or syringe, BALF can be retrieved with suction pump aspiration, gravity drainage or manually with a syringe. The American Thoracic Society (2012) suggests negative pressures of 50 to 80 mmHg for suction pump retrieval of BALF compared to negative pressures of 25 to 100 mmHg by the European group (Klech, 1989). Both panels advise that airway collapse should be avoided and, if airway collapse is identified, negative pressure should be reduced. Re-aspiration is continued until no further fluid is retrieved, with a goal of retrieving 40 to 70% of the total instillate. A lower percentage of BALF recovery is expected in patients with asthma, even if they have been pre-treated with bronchodilators, and in patients older than 50 years (Lam, 1985; Cherniak, 1990). If BALF return is poor and more than 100 mL of the original infusate remains in the lungs, it is recommended to abort the BAL procedure (Baughman, 2007). Stable patients should be observed for a minimum of 1 hour following BAL (American Thoracic Society, 2012); however, it is typically an outpatient procedure (Ward, 2001).

Diversity among BAL techniques persists in human medicine despite the existence of the above guidelines. As the guidelines are based on expert opinion, it is possible that different techniques may provide improved diagnostic yield from BALF. For example, higher BALF retrieval and rate of diagnosis, and a lower complication rate are reported in a multicenter prospective clinical trial comparing the use of tubing for manual aspiration to a handheld syringe attached directly to the bronchoscope’s biopsy channel (Rosell, 2006). A prospective study has assessed the diagnostic yield of BAL for pneumonia in 101 immunosuppressed human patients, using a standard BAL protocol based on American Thoracic Society guidelines, and a diagnostic yield of 51.6% is reported (Velez, 2007). Another recent study in 284 human cancer patients has prospectively evaluated the American Thoracic Society BAL protocol and its effect on rate of diagnostis (diagnostic yield) and safety (Sampsonas, 2011). A 90% compliance with the BAL protocol is reported in this study, with the most common alteration in protocol being instillation of a lower fluid volume due to decreased oxygen saturation (Sampsonas, 2011). Diagnostic yield for infectious pneumonia and/or pulmonary malignancy in this study is 33.8% (Sampsonas, 2011). Interestingly, the diagnostic yields from these studies (Sampsonas, 2011; Velez, 2007) are similar to previously reported BAL diagnostic yield in human patients, where a variety of BAL techniques were assessed (El-Bayoumi, 2008; Goldstein, 1990; Martin, 1987). However,
the investigators (Sampsonas, 2011; Velez, 2007) only consider the identification of infectious pneumonia and/or pulmonary malignancy as definitive diagnoses and it is possible that the diagnostic yield could have been higher if other disease processes were included in the list of possible diagnoses. In addition, 87% of patients with hematologic neoplasia in Sampsonas’ study (2011) were receiving antibiotics at the time of BAL, which may have resulted in false negative results for bacterial pneumonia. Further prospective studies are required in human medicine to evaluate the effect of BAL technique on BALF samples and their diagnostic utility.

**1.7 Review of Equine Bronchoalveolar Lavage:**

Equine BAL was first described by Dr. Laurent Viel in 1980 and has since been validated for detection of airway inflammatory disorders (Couetil, 2007; Hoffman, 1997; Couetil 2007; Hoffman, 2002; Hoffman, 2008). Indications for bronchoscopy and BAL in horses include recurrent fever, unexplained changes on thoracic radiographs, chronic obstructive pulmonary disease, exercise induced pulmonary hemorrhage (EIPH), and poor performance. Cytology of BALF can help differentiate inflammatory airway disease from recurrent airway obstruction (Couetil, 2007) and is a sensitive method for identifying occult EIPH (Hoffman, 2002). In addition, hemosiderin scores have been developed to increase specificity of BALF cytology for diagnosis of EIPH (Doucet, 2002a; Doucet, 2002b). When performed in stable patients, equine BAL is very safe (Hoffman, 2008). Transient decreases oxygen saturation, which occur frequently in other species, have not been reported in horses (Hoffman, 1997). In fact, BAL has been shown to transiently improve airway function in horses with recurrent airway obstruction, likely secondary to mechanical clearance of obstructive mucoid respiratory secretions (Leguillette, 2006). Contraindications for BAL in horses include marked tachypnea or dyspnea, cardiovascular abnormalities and hypoxemia (Hewson, 2002). As BAL can be performed on the farm with owner assistance, as well as in the hospital, it is frequently utilized in equine patients.

Equine clinicians have had the most success in implementing a standardized protocol for BAL in their field. Prospective studies have evaluated the effect of aliquot volume, pooling of sequential aliquots, site of lavage and sampling processing on equine BALF (Pickles, 2002a; Pickles, 2002b; Pickles, 2002c). For example, smaller lavage volumes of 50 mL have a higher percentage of neutrophils than larger lavage volumes of 300 mL in healthy horses (Sweeney, 1992). As bronchial washings have a higher percentage of neutrophils (Lam, 1985), this suggests that more than 50 mL is required for BAL infusate to sample the equine alveoli. In addition, a higher proportion of mast cells is identified in BAL from the left lung of healthy horses, when compared to the right lung (Sweeney, 1994). A standard equine BAL technique was agreed upon during an international workshop (Robinson, 2000) in order to acquire consistent cytologic differential cell counts. This protocol has been published (Hewson, 2002; Hoffman, 2008) and is used in multiple prospective studies (Doucet, 2002a; Doucet, 2002b; Perkins, 2008). The standardized equine BAL technique (Hewson, 2002) is as follows:

The horse is chemically and physically restrained for the procedure. Sedation protocols involve alpha-2 antagonists (xylazine, detomidine, romifidine) with or without butorphanol. Pre-treatment with inhaled bronchodilators (albuterol or ipratropium bromide) can reduce coughing during the procedure (Hoffman, 2008). The horse is then physically restrained using a nose twitch and standing stocks (if available/required). BAL is performed with endoscopic guidance using a bronchoscope with minimum working length of 160 cm and outer diameter of 10 to 13
mm (Hewson, 2002). This allows wedging of the bronchoscope in the 4th to 6th generation bronchi (Hewson, 2002). The bronchoscope is passed nasally through the nasopharynx into the trachea. Once the trachea is entered, the horse’s head can be extended to facilitate manipulation of the bronchoscope during bronchoscopy (Hoffman, 2008). The carina is numbed via instillation of 60 to 120 mL of lidocaine solution (0.2% to 0.5%) through the biopsy channel. After routine bronchoscopic examination, the endoscope is gently wedged into the area of interest. Two consecutive aliquots of 250 mL warmed (37.8°C) sterile isotonic saline are infused per site through the endoscope’s biopsy channel. Pre-warmed saline to 37.8°C has been shown to reduce bronchospasm (Hewson, 2002). Infusion of saline can be achieved using preloaded syringes directly attached to the biopsy channel or by using a pressure bulb on a saline bottle, attached to an intravenous solution administration line inserted through the biopsy channel (Hoffman, 2002; Hoffman, 2008). The infusate is immediately retrieved after each bolus into a siliconized glass flask. The most commonly reported method for re-aspiration of BALF is with a medical suction pump set at negative pressures of 30 to 100 mmHg (Hewson, 2002). If a suction pump is not available, BALF retrieval can be achieved manually using 60 or 120 mL syringes (Hoffman, 2002). Excessive negative pressure should be avoided as it may result in airway collapse, decreased fluid retrieval, and trauma to the bronchial mucosa (Hewson, 2002; Klech, 1989). To ensure a representative BALF sample, 40 to 60% infusate retrieval is recommended (Hewson, 2002). Horses should be rested for a minimum of 24 hours following BAL due to subclinical regional loss of alveolar gas exchange (Hoffman, 2008).

1.8 Review of Feline Bronchoalveolar Lavage:
The smaller size of the feline respiratory tract makes bronchscopy difficult (Johnson, 2007; Johnson, 2011). This, coupled with a higher reported rate of complications (Johnson, 2007; Johnson, 2011), has resulted in less available information regarding feline bronchscopy and BAL in the veterinary literature. Yet, bronchoscopy and BAL are useful in the evaluation of feline lower respiratory tract disease (Johnson, 2011). Bronchoscopic abnormalities which have been reported in spontaneous feline lower respiratory tract disease include exudate, hyperemia and epithelial irregularities of the bronchial mucosa, airway collapse and airway stenosis (Johnson, 2011). Differential cell counts from BALF cytology can be used to classify airway inflammation in feline inflammatory airway disease (Hawkins, 1990b; Johnson, 2007) and BALF cultures can definitively diagnose pneumonia (Hawkins, 2004). Analysis of BALF has also been used to diagnose Toxoplasma gondii infection (Hawkins, 1997) and to characterize pulmonary changes in cats experimentally infected with FIV (Hawkins, 1996). While biomarkers in BALF have not yet been able to differentiate chronic bronchitis from asthma in cats with spontaneous respiratory disease (Nafe, 2010), increased immunoglobulin G and A activity has been shown in BALF of cats with experimental induction of sensitization to Bermuda grass allergens (Norris, 2003). Johnson (2011) reports a 91.7% rate of diagnosis when BALF cytology and culture results are assessed in conjunction with physical examination, bronchoscopic findings, and response to treatment.

Historically, a higher rate of complications has been reported in cats following bronchoscopy and BAL than in other species. For example, a retrospective review of flexible bronchoscopy and BAL in cats at a veterinary referral center identifies an overall complication rate of 38% (Johnson, 2007), compared with complication rates of <5% in people (American Thoracic Society, 2012). The majority (24%) of the complications in this review are considered
mild in nature (e.g. including hemoglobin desaturation). However, 6% of cats required overnight hospitalization and supplemental oxygen therapy, 3% experienced pneumothorax, and a 6% mortality/euthanasia rate was associated with inability to restore ventilation following the procedure (Johnson, 2007). Bronchoscopy and BAL induced airflow limitations, characterized by enhanced PenH and bronchoconstriction, have been reported in healthy and experimentally allergen-sensitized cats (Kirschvink, 2005). Pre-treatment with inhaled bronchodilators (salbutamol and ipratropium bromide) prior to BAL prevents airflow limitations in allergen-sensitized cats (Kirschvink, 2005). The benefits of pre-treatment with bronchodilators prior to BAL have also been suggested in cats with lower respiratory disease. A significantly lower rate of complications has been recorded retrospectively in cats that are pre-treated with terbutaline (0.01 mg/kg SQ q8-12h) for 12 to 24 hours prior to bronchoscopy and BAL (8%), when compared with cats that are not pre-treated (40%) (Johnson, 2011). While causation cannot be proven through a retrospective analysis, a standard protocol performed by a single clinician is used for bronchoscopy and BAL in this study (Johnson, 2011).

The protocol for feline BAL is not well established. Both bronchoscopic and non-bronchoscopic (unguided; blind) BAL techniques have been described in the literature (Hawkins, 1994; Johnson, 2011). Unlike in humans and horses, feline BAL requires general anesthesia due to patient anxiety and risk of laryngospasm (Padrid, 2011; Silverstein, 2010). In bronchoscopic BAL, the bronchoscope is inserted orally into the trachea. After systematic bronchoscopic examination of the lower airways, the bronchoscope is wedged gently in a subsegmental bronchus and aliquots of sterile 0.9% saline are infused through the bronchoscope’s biopsy channel (Johnson, 2007). In unguided techniques, the patient is placed in lateral recumbency with the dependent lung lobe the one to be lavaged (Mills, 2006). A sterile propylene canine urinary catheter (6 to 8 F) is passed through the endotracheal tube until resistance is felt (Mills, 2006; Foster, 2004). Sterile 0.9% saline is infused through the urinary catheter. Infusion of the BAL infusate directly down a sterile endotracheal tube has also been described (Hawkins, 1990b; Hawkins, 1994). Following infusion of the BAL infusate, fluid is retrieved using manual aspiration with a hand-held syringe (Hawkins, 1994). Occasionally, raising the cat’s hind-end may increase infusate retrieval (Hawkins, 2004). Mean BALF retrieval ranges from 51 to 73% (Johnson, 2007; Hawkins, 1990). Suction pump aspiration (SPA) to retrieve BALF has not been described in the feline literature. The use of warmed sterile saline (37.0°C) is recommended to reduce the risk of bronchospasm, based on information in human and equine medicine (Hewson, 2002; Klech, 1989). Infusate volume and number of boluses have not been standardized in feline medicine. Infusate volumes can be fixed (3 to 20 mL) (Nafe, 2010; Norris, 2003; Johnson, 2007) or weight adjusted (ranging from 3 to 5 ml/kg) (Hawkins, 2004). Single boluses up to 5 boluses per site have been described (Hawkins, 1996; Hawkins, 2004; Johnson, 2007; Johnson, 2011), although 2 to 3 boluses per site appears to be the most commonly performed technique. There is currently no information critically assessing infusate volume or number of boluses for BAL in cats. Given the available information in other species, it follows that a standard protocol for feline BAL would reduce variation and allow for better comparisons between different institutions.

1.9 Review of Canine Bronchoalveolar Lavage:
Lower respiratory tract disease in dogs results in structural changes in the bronchi (e.g. thickened irregular mucosa, increased exudate) and alterations in the normal ELF cell population
Similar to feline BAL, canine BAL is performed under general anesthesia. As some degree of respiratory compromise is expected in patients with lower respiratory tract disease (Hawkins, 1990b), supplemental oxygen therapy is recommended during BAL and following the procedure until the patient can maintain normal oxygen saturation on room air (Hawkins, 2004). Both bronchoscopic and non-bronchoscopic BAL techniques have been described in the canine literature (Hawkins, 1990b; McCauley, 1998). During non-bronchoscopic BAL, a catheter (sterile urinary catheter, double lumen catheter or modified stomach tube) is inserted orally into the trachea through a sterile endotracheal tube until it is gently wedged into a distal bronchus (Hawkins, 1990b; Hawkins, 1999; McCauley, 1998). Three to 5 x 25 mL aliquots or 5 mL/kg of isotonic sterile saline are then infused and gently re-aspirated with a hand-held syringe (Barcante, 2008; Hawkins, 1999; McCauley, 1998). Unguided BAL techniques often lavage the caudal lung lobes (Hawkins, 2004). During bronchoscopic BAL, the bronchoscope is inserted orally into the trachea. The size of endoscope used depends on the size of the patient. Flexible pediatric bronchoscopes with an outer diameter of 3.5 to 3.7 mm and working length of 40 to 60 cm are recommended, whereas a flexible bronchoscope with an outer diameter of 4.8 to 6 mm and a working length of 60 cm is adequate to reach the 3rd generation bronchi in small to medium sized dogs (Padrid, 2011). In medium to large dogs, a gastroscope with a working length of 100 to 150 cm is often required to fully evaluate the respiratory tract (Padrid, 2011). If patient size allows, the bronchoscope can be passed through a sterile endotracheal tube via a T-adapter port (Hawkins, 1990b; Padrid, 2011). In small patients, supplemental oxygen can be provided with a sterile canine urinary catheter inserted orally into the trachea (Woods, 2013). A complete bronchoscopic examination is performed prior to BAL. Once a lavage site has been identified, the bronchoscope is gently wedged into a subsegmental bronchus. When a tight fit within the bronchus is achieved, warmed (37.0°C) isotonic saline (Hawkins, 2004; Melamies, 2011) is infused through the bronchoscope’s biopsy channel. The number of aliquots used in canine BAL ranges from one to four (Creevy, 2009; Hawkins, 2006; Rafft, 2011). Fixed aliquot volumes of 5 to 50 mL to weight adjusted aliquot volumes (1 to 2 mL/kg) have all been reported in the literature (Andreasen, 2003; Creevy, 2009; Melamies, 2011; Padrid, 2011). The concern regarding low volume infusate BAL is that it might not be sufficient to reach the alveoli. A prospective study of 18 purpose-bred beagles evaluating BALF acquired using 3 x 10 mL boluses of fluid found a high proportion (mean 15%) of epithelial cells in their samples (English, 2008; Mayer, 1990). As ≤5% epithelial cells is currently considered acceptable in BALF (Hawkins, 2004; Rebar, 1980), this brings into question whether a lower volume BAL (30 mL total compared to a typical 50 mL total volume) samples the alveoli milieu. A prospective study in healthy dogs reports a lower number of alveolar macrophages when several large (5 x 100 mL) or multiple small aliquots (10 x 50 mL) are compared with a single 50 mL bolus (Pinsker, 1980). This study also reports a high proportion of neutrophils (15%) in the BALF from a single 50 mL BAL volume (Pinsker, 1980), which is considerably higher than what is considered normal for healthy dogs (Rebar, 1980). Another prospective study in purpose-bred dogs reports
that a weight-adjusted aliquot volume (2 x 1 mL/kg) provides more accurate BALF samples than a fixed aliquot volume (2 x 25 mL) as based on ELF dilution estimated by urea (Melamies, 2011). Previous research has shown that there exists a significant variation of urea influx from the circulation into the alveolar space during BAL (Kelly, 1988). The authors attempt to minimize this effect by using a dwell time of less than 30 seconds (Melamies, 2011), and therefore it appears that using a weight-adjusted aliquot volume will provide good standardization in canine BAL. However, further studies are required to evaluate use of a weight-adjusted aliquot volume in dogs of various weights and in patients with lower respiratory disease. Once saline has been infused into the airways, BALF retrieval is instituted immediately using a handheld syringe (manual aspiration; MA) or with a suction pump connected in series with the bronchoscope aspiration valve via a sterile sample collection trap (Creevy, 2009; Hawkins, 2004; Hirt, 2010; Padrid, 2011; Silverstein, 2010). Negative pressures of 37.5 to 100 mmHg have been reported for canine suction pump aspiration (Mayer, 1990; Woods, 2013). The manual aspiration technique may be modified by attachment of the handheld syringe to sterile polyethylene tubing. The tubing is inserted through the bronchoscope’s biopsy channel (Silverstein, 2010) and wedged in a bronchus for BAL. Fluid retrieval of 40 to 90% of the total aliquot volume is expected (Miller, 2007). Poor BALF retrieval can occur if aspiration results in airway collapse or if the bronchoscope is improperly wedged (Creevy, 2009; Hawkins, 2004). A 35 mL syringe is most commonly reported syringe size used for manual aspiration (Hawkins, 1990; Hawkins, 2004; Norris, 2003). No studies have critically assessed the effect of aspiration technique on canine BALF. Lavage of multiple lung lobes is recommended, even with diffuse disease (Hawkins, 1993; Hawkins, 1995).

1.10 Potential Effects of Aspiration Technique on Canine Bronchoalveolar Lavage Fluid:

One technical aspect of BAL, which has not been previously evaluated in the veterinary literature, is the method of retrieving BALF. In human medicine, aspiration is usually performed with a suction pump with negative pressures of 25 to 100 mmHg. In equine medicine, suction pump aspiration is also commonly reported, with negative pressures of 30 to 100 mmHg. In canine and feline medicine, manual aspiration (MA) with a handheld syringe attached directly to the bronchoscope is the most commonly used technique; however, MA with a handheld syringe attached to sterile tubing and suction pump aspiration (SPA) have also been described. MA cannot be standardized as each clinician may aspirate at different pressure levels. SPA via a disposable aspiration tube has not been standardized in small animal medicine, either. If excessive negative pressure is applied, fluid retrieval may be limited due to dynamic airway collapse and cell preservation may be poor. In addition, bronchial collapse and high negative pressure may result in mucosal trauma and increased proportions of epithelial cells and RBCs in the retrieved sample (Ward, 2001). We hypothesize that the method of retrieving BALF will affect cytology and quality of the sample. In particular, we hypothesize that SPA will provide BALF with improved sample quality, when compared with forms of MA. Therefore, the purpose of this research project is to compare three previously reported techniques for BALF retrieval [MA, manual aspiration via polyethylene tubing (MAPT), and SPA] in healthy dogs and dogs with lower respiratory tract disease.
2.1 Introduction:

Determining the cause of pulmonary disease can be a challenge for veterinarians. Clinicians are limited in that evaluation of thoracic radiographs does not always reveal lesions, diffuse radiographic changes are nonspecific, and radiographic lesions may represent old, clinically inactive lesions (Mai, 2008; Silverstein, 2010). Ancillary diagnostic tests are almost always warranted in patients with respiratory tract disease (Silverstein, 2010). Bronchoscopy allows visual examination of the bronchi, and BAL can be used to collect samples from the alveolar for cytologic, biochemical, and microbiological evaluation. These techniques are relatively safe, and they have been commonly used for evaluation of the bronchi and alveoli of dogs for >20 years (Brown, 1983; Creevy, 2009; Hawkins, 1990b; Hawkins, 2004; Silverstein, 2010).

Indications for bronchoscopy and BAL include chronic cough, unexplained changes evident on thoracic radiographs, and pulmonary masses (Hawkins, 1990b; Hawkins, 2004; Silverstein, 2010). The basic technique for BAL consists of infusing sterile saline (0.9% NaCl) solution into the bronchi and alveoli, which is then followed by aspiration of the infusate (Hawkins, 2004; Silverstein, 2010); however, the procedure varies (Creevy, 2009; Hawkins, 1990b; Rajamaki, 2001) and there are no standardized protocols for small animals. Responses of 28 Diplomates of the American College of Veterinary Internal Medicine to an email survey conducted in September 2011 revealed that anecdotally there was great variation for the type of endoscope used, fluid volume for BAL, and aspiration technique (Appendix 1). Analysis of the available evidence has indicated that technical aspects of BAL, such as the interval from collection until sample processing and analysis, affect the quality of the sample (Mordelet-Dambrine, 1984; Rajamaki, 2001). Sample quality is important to ensure meaningful cytologic analysis and diagnosis. One aspect of BAL, which has not been previously evaluated in dogs, is aspiration techniques for retrieval of BALF: MA and SPA (Hawkins, 1990b; Silverstein, 2010). In human medicine, SPA with negative pressure of 25 to 100 mmHg to collect BALF in a fluid trap is the most commonly used method (American Thoracic Society; Klech, 1983; Ward, 2001). In contrast, MA with a syringe is the most commonly reported technique in veterinary medicine. There are variations in MA with regard to the size of syringe and use of sterile polyethylene tubing passed through the biopsy channel of a bronchoscope (Hawkins, 1990b; Hawkins, 2004; Silverstein, 2010).

The purpose of the study reported here was to compare MAPT and SPA via suction trap connection for collection of BALF in healthy dogs and the effect of these techniques on BALF sample quality. We hypothesized that the method of BALF aspiration would influence sample quality and therefore potentially influence the diagnostic utility of examination of BALF in dogs.

2.2 Materials and Methods:

**Animals** – Twelve healthy adult Beagles were used in the study. To be included in the study, dogs were required to be free of pulmonary disease. The health status of each dog was assessed via the medical history and results of physical examination, a CBC, serum biochemical analysis, and thoracic radiographs (lateral and ventrodorsal views). A board-certified veterinary radiologist (HD) evaluated the thoracic radiographs. Dogs did not receive any medications for at least 30 days before the study, but regularly received prophylactic anthelminthic treatment. Dogs were cared for in accordance with the Canadian Council on Animal Care guidelines at a Canadian Council on Animal Care-accredited facility. The study protocol was approved by a university institutional animal care and use committee.

**Anesthesia** – Dogs were sedated with an IM injection of butorphanol tartrate (0.2 mg/kg) combined with acepromazine maleate (0.02 mg/kg). Anesthesia was induced by IV administration of propofol (4 mg/kg) and maintained with a constant-rate infusion (CRI) of propofol (400 µg/kg/min, IV) and boluses of propofol (2 mg/kg, IV) as required to provide an adequate plane of anesthesia. All dogs received fluids IV, and supplemental oxygen was provided via a sterile soft urinary catheter placed orally into the trachea. Physical examination, a continuous ECG, and pulse oximetry were used to monitor dogs during anesthesia. Normal oxygen saturation was defined as ≥95%. Experimental procedures were discontinued and dogs were allowed to recover from anesthesia if oxygen saturation decreased to <90% for 10 minutes, oxygen saturation decreased to <85% for 5 minutes, or a dog developed bradycardia (<60 beats/min) that was unresponsive to standard interventions.

**Bronchoscopy and BAL** – Dogs were positioned in sternal recumbency for bronchoscopy. A routine bronchoscopic examination was performed and recorded for each dog. The trachea and all lung lobes, up to the second- and third-generation bronchi, were visually examined.

Two BAL techniques were performed in each dog. The caudal lung lobes were lavaged with a weight-based volume (2 mL/kg, divided into 2 aliquots) (Melamie, 2011), with the second aliquot infused immediately after aspiration of the first aliquot. To perform SPA, the tip of the bronchoscope was wedged into a second-generation bronchus. Sterile saline solution was infused through the biopsy channel, which was then followed by infusion of 4 mL of air to empty the channel. Pulsatile aspiration with a maximum negative pressure of 5 kPa (37.5 mmHg) was applied immediately after infusion; aspiration was applied with a suction pump connected directly to the suction valve of the bronchoscope via a disposable aspiration trap (Figure 2.1). The MAPT technique was performed on the opposite caudal lung lobe by wedging the

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2 Arnolds dog catheter with female Luer mount, 8F X 50 cm, Smith Medical International, Hythe, Kent, England.
3 Olympus BG type 1T160 video bronchoscope, outer diameter 6.0 mm, working length 60 cm, biopsy channel inner diameter 2.8 mm, Olympus Canada Inc., Richmond Hill, ON, Canada.
4 Olympus SSU-2 endoscopic aspiration pump, Olympus Canada Inc., Richmond Hill, ON, Canada.
5 Kendall Luki 20-mL (6.25-inch) disposable aspirating tube, Tyco Healthcare Group, Mansfield, MA.
bronchoscope into a second-generation bronchus, passing a sterile polyethylene tube\(^6\) through the biopsy channel of the bronchoscope, and advancing the tube until palpable resistance was met. Sterile saline solution was infused through the polyethylene tube, which was then followed by infusion of 2 mL of air to empty the tube. A 35-mL syringe was attached to the polyethylene tube via a 20-gauge, 1-inch hypodermic needle (Figure 2.2). Gentle pulsatile MA with a 35-mL syringe was performed immediately after infusion (Silverstein, 2010).

![Figure 2.1 - Photograph of the equipment used for BAL with the SPA technique. The disposable aspiration tube is connected directly to the suction valve of a bronchoscope (black arrowhead). Standard polyvinyl chloride tubing (black arrow) is used to connect the disposable aspiration tube to the stem valve of the suction pump (not shown).](image)

\(^6\) Intramedic Clay Adams polyethylene tubing (inner diameter, 0.034 inches; outer diameter, 0.060 inches; wall thickness, 0.013 inches), Becton Dickinson, Franklin Lakes, NJ.
Aspiration via each technique was continued until fluid could no longer be recovered. For MAPT, the syringe was emptied as necessary and then used to continue aspiration. The order of the aspiration techniques and the lung lobe lavaged with each technique were determined with a random number table. The bronchoscope was cleaned and sterilized in accordance with a standard cold sterilization method\textsuperscript{7,8,9} between dogs.

The amount of fluid retrieved, duration of BAL (time from infusion of saline solution until the end of aspiration for the second aliquot) and lowest oxygen saturation were recorded for each technique. Dwell time (time from infusion of saline solution to the first attempted aspiration) was <20 seconds for each collection.

**Examination of BALF** – Macroscopic surfactant was indirectly assessed by measuring the volume of bubbles in each sample immediately after sample collection. A surfactant score of 0 was assigned if no bubbles were present, a score of 1 was assigned for 0.10 to 0.70 mL of bubbles, a score of 2 was assigned for 0.71 to 1.50 mL of bubbles, and a score of 3 was assigned for >1.51 mL of bubbles. Each BALF sample was identified by a unique code of 3 letters, immediately placed on ice after collection, and processed for analysis within 120 minutes after collection. Samples were not filtered during processing. TNCC were determined with electrical

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\textsuperscript{7} Video bronchoscope cleaning and cold sterilization protocol, Olympus Canada Inc., Richmond Hill, ON, Canada.

\textsuperscript{8} Endozime, dual enzymatic cleaning, Ruhof Corp., Mineola, NY.

\textsuperscript{9} Glutacide, Pharmax Limited, Etobicoke, ON, Canada.
impedance. An aliquot (200 µL) of each sample was cytocentrifuged for 6 minutes at 180 X g, and additional slides were prepared from fluid centrifuged for 5 minutes at 500 X g. Slides were stained with Wright stain, and differential cell counts of 400 leukocytes were performed at 400X magnification on cytocentrifuge preparations by a board-certified veterinary clinical pathologist (DB) who was not aware of the technique used to obtain each sample.

**Microscopic assessment** – In addition to performing the differential cell counts, the board-certified veterinary clinical pathologist (DB) also semi-quantitatively assessed 5 variables reflective of sample and slide quality (Appendix 2). Cellularity and number of clusters and sheets of epithelial cells were assessed at 100X magnification; cell preservation, number of bacteria, and differential counts were determined at 400X magnification. Scores of ≥2 for cellularity and cellular preservation were required for a BALF sample to be considered of diagnostic quality (English, 2008). Cells were evaluated for erythrophagocytosis and presence of intracellular pigment during differential counting. Scores >2 for epithelial cells or RBCs were considered to indicate excessive suction or trauma and thus a BALF sample of lesser quality (English K, 2008).

**Statistical analysis** – Statistical analyses were performed with a statistical software program. Nonparametric analyses were performed because none of the BALF data had normal distributions. Comparisons of the percentage of fluid retrieval, total and differential cell counts, lowest oxygen saturation, duration of BAL, and sample quality were analyzed with Wilcoxon matched-pairs signed rank tests. For all analyses, values of $P \leq 0.05$ were considered significant.

2.3 **Results:**

**Dogs** – The study group consisted of 12 healthy Beagles (5 neutered males and 7 spayed females) that ranged from 5 to 8 years of age (mean ± SD, 7.2 ± 1.0 years). Body weight of the dogs ranged from 9.2 to 13.5 kg (mean, 11.2 ± 1.5 kg). Vital parameters of all dogs were within anticipated limits. No respiratory abnormalities were detected during thoracic auscultation. Results of biochemical analyses and CBCs were unremarkable. One of the dogs had a grade 2/5 left-sided systolic heart murmur and evidence of mild left atrial enlargement during examination of thoracic radiographs. Considering that pulmonary vessels and pulmonary tissues appeared normal and the dog did not have abnormal clinical signs, compensated left heart disease was suspected. No dogs were excluded from the study on the basis of the results of physical examination, hematologic analysis, or thoracic radiography. One dog developed a decrease in oxygen saturation to 72% for 5 minutes immediately after induction of anesthesia. In accordance with the study protocol, the procedure was discontinued and that dog was excluded from the study.

**Bronchoscopy and BAL** – Bronchoscopic examination revealed no abnormalities in 9 of the 11 remaining dogs. An oral mass deep in the oropharynx and a generalized, nodular appearance of the bronchial mucosa was identified in 1 dog. In another dog, the bronchial

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10 Z2 Coulter counter, Beckman Coulter, Mississauga, ON, Canada.
11 Shandon Cytospin 4, Thermo Fisher Scientific Inc., Waltham, MA.
12 Olympus BX53 system microscope, Olympus Canada Inc., Richmond Hill, ON, Canada.
mucosa of the right middle and right caudal lung lobes was thickened and irregular. The right middle and right caudal lung lobes in that dog were also sensitive, and bronchoscopy induced considerable coughing during the BAL procedure.

The duration of BAL by use of MAPT ranged from 106 to 372 seconds, and the duration of BAL by use of SPA ranged from 140 to 317 seconds; these values did not differ significantly (Table 2.1). There was sudden loss of negative pressure during aspiration in 2 of the 11 dogs during MAPT.

**Adverse effects of BAL** – Transient (<1-minute) decreases in oxygen saturation were detected in some dogs during both the MAPT and SPA techniques. These decreases in oxygen saturation were self-limiting and resolved without intervention. The lowest oxygen saturation during MAPT was 87% and the lowest oxygen saturation during SPA was 88%; these values did not differ significantly. Iatrogenic linear abrasions were identified along the bronchial walls in 2 dogs after wedging of the polyethylene tubing during MAPT.

| Table 2.1 – Comparison of results for BALF samples obtained via SPA and MAPT from 11 healthy Beagles. |
|-------------------------------------------------|-------------------------------------------------|------------------------------------------|----------------|------------|
| Variable                                        | Median MAPT | Median SPA | Median Difference* | IQR | P value † |
| **BAL procedure parameters**                    |             |            |                  |     |           |
| Duration of BAL (seconds)                       | 208         | 184        | -39              | 98  | 0.87      |
| Lowest oxygen saturation (%)                    | 90          | 94         | 0                | 4   | 0.37      |
| Surfactant score                                | 1           | 2          | 1                | 1   | 0.004     |
| Percentage of retrieved infusate                | 40.4        | 63.1       | 16.2             | 8.8 | 0.001     |
| **Cytology Quality Score‡**                     |             |            |                  |     |           |
| Cellularity score                               | 4           | 4          | 0                | 0   | 1         |
| Cellularity preservation score                  | 4           | 4          | 0                | 0   | 0         |
| Red blood cell score                            | 0           | 0          | 0                | 0   | 1         |
| Epithelial cell score                           | 1           | 0          | 1                | 1   | 0.05      |
| Bacteria score                                  | 1           | 1          | 1                | 1   | 0.59      |
| **BALF parameter**                              |             |            |                  |     |           |
| Total nucleated cell count (No. cells/µL)       | 600         | 900        | 300              | 670 | 0.15      |
| **Differential cell count (proportion)**        |             |            |                  |     |           |
| Macrophages (%)                                 | 65          | 51         | -11              | 13  | 0.007     |
| Neutrophils (%)                                 | 7           | 19         | 7                | 14  | 0.008     |
| Round eosinophils (%)                           | 3           | 2          | -1               | 3   | 0.91      |
| Segmented eosinophils (%)                       | 2           | 1          | -1               | 1   | 0.23      |
| Mast cells (%)                                  | 1           | 0          | 0                | 13  | 1         |
| Lymphocytes (%)                                 | 19          | 20         | -1               | 17  | 1         |
| **Differential cell count (absolute)**          |             |            |                  |     |           |
| Macrophages (No. cells/µL)                      | 365         | 459        | -106             | 260 | 0.46      |
| Neutrophils (No. cells/µL)                      | 42          | 133        | 74               | 217 | 0.014     |
| Round eosinophils (No. cells/µL)                | 28          | 18         | 0                | 0.1 | 0.9       |
| Segmented eosinophils (No. cells/µL)            | 11          | 10         | 2                | 1   | 0.76      |
| Mast cells (No. cells/µL)                       | 4           | 0          | 0                | 13  | 0.81      |
| Lymphocytes (No. cells/µL)                      | 154         | 189        | 18               | 94  | 0.32      |

* A positive value for median difference indicates that the value was higher in samples obtained via SPA than in samples obtained via MAPT, and a negative value for median difference indicates that the value was lower in samples obtained via SPA than in samples obtained via MAPT. † Values were considered significantly different at P≤0.05. ‡ Within this category, each variable was scored on a scale of 0 to 4, except for RBCs, which were scored on a scale of 0 to 3.
**BALF analysis** – Surfactant was macroscopically present in all samples obtained with SPA but was absent in 2 samples obtained with MAPT. Surfactant scores for samples obtained via SPA were significantly ($P=0.004$) higher than scores for samples obtained via MAPT (median difference, 1.0; IQR, 1.0). Percentage of retrieved infusate ranged from 18.5% to 67.7% for MAPT and from 38.0% to 76.3% for SPA. The percentage of BALF retrieved was significantly ($P=0.001$) higher for the SPA technique, compared with the percentage of BALF retrieved for the MAPT technique (median difference, 16.2%; IQR, 8.8%). Median TNCC for samples obtained via MAPT was 600 cells/µL (range, 400 to 1,400 cells/µL), and median TNCC for samples obtained via SPA was 900 cells/µL (range, 400 to 2,000 cells/µL); these values did not differ significantly.

**Microscopic assessment of BALF** – All microscopic scores for cellular preservation and cellularity were 3 or 4. Analysis of sample-quality variables revealed identical values for cellular preservation scores for both MAPT and SPA in individual dogs. Cellularity and RBC scores were not significantly different between MAPT and SPA. Erythrophagocytosis and intracellular pigment in macrophages were not detected. A significantly ($P=0.050$) higher epithelial cell score was identified in BALF obtained via MAPT (median difference, 1.0; IQR, 1.0). Evidence of oropharyngeal contamination (squamous epithelial cells) was identified in 3 of 22 BALF samples.

Bacteria observed on cytologic examination were extracellular and single or in small colonies. There was no significant difference in bacterial scores between BALF obtained with the MAPT and SPA techniques.

No significant differences were identified in percentages or absolute differential cell counts of eosinophils with round nuclei (round eosinophils), eosinophils with segmented nuclei (segmented eosinophils), mast cells, or lymphocytes. Samples obtained via SPA had a significantly higher percentage (median difference, 7%; IQR, 14%; $P=0.008$) and absolute number (median difference, 74 cells/µL; IQR, 217 cells/µL; $P=0.014$) of neutrophils, compared with values for samples obtained via MAPT. Samples obtained via SPA had a significantly lower percentage (median difference, 11%, IQR, 13%; $P=0.007$), but not the absolute number (median difference, 106 cells/µL; IQR, 260 cells/µL; $P=0.0460$), of macrophages, compared with values for samples obtained via MAPT. When data were excluded from the analysis for 1 dog because unilateral localized pulmonary disease was identified during bronchoscopy, differential cell counts still differed significantly.

2.4 **Discussion:**

Studies (Baughman, 2007; Melamies, 2011; Mordelet-Dambrine, 1984) in human and veterinary medicine have indicated the manner in which BAL techniques may influence sample quality and interpretation. For example, filtering and centrifugation of human BALF can decrease sample cellularity by up to 34% and may alter the differential cell count (Mordelet-Dambrine, 1984). Analysis of results of a recent clinical trial performed in healthy Beagles suggested that use of weight-based aliquot volumes provided more consistent BALF samples than did use of fixed volume boluses (Mordelet-Dambrine, 1984). Protocols for BAL in horses were standardized a few years ago with widespread implementation (Hoffman, 2008), which
allows for better comparisons among studies. Such a unified approach has been lacking in small animal medicine; hence, we performed the present study to critically assess 2 currently used methods for fluid retrieval.

We chose to assess the percentage of fluid retrieved, presence of surfactant, sample cellularity, cellular preservation, and presence of RBCs and epithelial cells as our main indicators of BALF quality. These criteria were selected on the basis of currently available evidence in the human and veterinary literature (Baughman, 2007; English, 2008; Hawkins, 2004; Hawkins, 1995).

A higher percentage of retrieved infusate supports the contention that the sample represents fluid from the alveoli (Lam, 1985). Small amounts of retrieved fluid in humans may only represent a sample from the large airways and therefore be considered bronchial wash and not BALF (Klech, 1989; Lam, 1985). A minimum of 33% fluid retrieved has been suggested as an adequate BAL procedure in human medicine (Ward, 2001). Recovery of 40% to 75% of the BAL infusate has been reported in small animal medicine (Creevy, 2009; Hawkins, 2004).

Three of 11 samples obtained via MAPT in the present study comprised <33% of the original lavage volume; therefore, these samples would have been considered non-diagnostic. In contrast, all samples obtained via SPA comprised >33% of the infusate; these values differed significantly.

A sudden loss of negative pressure was reported with the MAPT for 1 dog, and the BALF sample for that attempted collection was less than the 33% retrieval criterion. In a clinical patient, a third BAL aliquot would have been used at this site, and it is possible that a third aliquot would have resulted in a diagnostic sample for MAPT for this dog. Decreased fluid retrieval for the MAPT technique may have been related to punctures in the polyethylene tubing made by the hypodermic needle, which would result in sudden loss of negative pressure. The polyethylene tubes of the 2 dogs in which MAPT was associated with sudden loss of negative pressure were visually inspected after the procedure, and no punctures were detected.

Decreased fluid retrieval could also result from the inability to wedge the polyethylene tubing in a bronchus. For example, if the diameter of the polyethylene tubing was too narrow to occlude a bronchus, the ability to achieve negative pressure during aspiration would rely on wedging of the bronchoscope in the bronchus. It is possible that the sudden loss of negative pressure when aspirating during MAPT was secondary to the bronchoscope being withdrawn from the bronchus with loss of occlusion. In addition, if the polyethylene tubing was not occluding a bronchus, some of the BAL aliquot could move proximal to the tube opening instead of distal into the alveoli. This would decrease infusate retrieval. Occlusion of the tube against the bronchus wall would also greatly hamper fluid retrieval. A final possibility for decreased infusate retrieval is kinking of the tubing, but we were careful to ensure that there was no kinking of the tubing proximal to the bronchoscope biopsy channel.

Retrieval of fluid with the SPA technique may not be dependent on occlusion created by the bronchus around the tip of the bronchoscope (Creevy, 2009). If the bronchoscope tip is not wedged perfectly in a bronchus, the SPA technique may result in increased fluid retrieval as a result of increased suction, compared with the lower achievable negative pressure of the MAPT.
The increased suction associated with SPA may result in collapse of the airways proximal to the bronchoscope tip, thus creating an occlusion at the area of airway collapse. Therefore, SPA has the potential to increase the diagnostic yield of BAL in larger canine patients where the bronchoscope tip cannot be completely wedged in a bronchus.

Surfactant is secreted by type II pneumocytes into the alveoli and reduces alveolar surface tension (Boothe, 2004). The presence of surfactant, as assessed via the presence of foam, in BALF confirms that a sample has been obtained from the alveoli (Creevy, 2009; Hawkins, 2004). In the present study, the presence of surfactant was assessed indirectly by measuring the volume of bubbles present in BALF samples immediately after collection. It is currently unknown whether the method of fluid retrieval influences the amount or characteristics of foam and therefore the volume of bubbles in BALF. However, the presence of foam rising to the top of BALF has been reported as a consistent finding for both manual and SPA techniques (Creevy, 2009; Hawkins, 2004). On the basis of this assessment, all samples obtained via SPA had positive results for the presence of surfactant; however, 2 of 11 samples obtained via MAPT had negative results for the presence of surfactant and would have been considered non-diagnostic. It is possible that BALF obtained via MAPT contained surfactant but that it was not grossly visible as bubbles. Direct measurement of surfactant in BALF samples would have strengthened the analysis for the present study; however, after allocating BALF for other analyses, inadequate volumes of BALF were available to measure phospholipid via the Bligh and Dyer method (Bligh, 1959).

In the present study, limits for cellularity and the cellular preservation scores were extrapolated from values reported in the veterinary clinical pathology literature (De Lorenzi, 2009; English, 2008; Hawkins, 2004). A differential count on a minimum of 200 cells is required for high reproducibility of the proportion of neutrophils, alveolar macrophages, and eosinophils in cytocentrifuged canine BALF samples (De Lorenzi, 2009). A differential count on 500 cells is required for high reproducibility of the proportion of lymphocytes (De Lorenzi, 2009). However, there is no information available in the veterinary literature on what constitutes a minimally acceptable quality of preparation. A score of 2 for cellularity and cell preservation would be marginal. However, given that most cells in such a preparation can still be classified despite less-than-perfect cell preservation, we considered such preparations adequate. The MAPT and SPA techniques yielded BALF samples of adequate cellularity and cellular preservation, and there was no significant difference in scores between the techniques. It has been hypothesized that SPA may result in increased cellular damage, compared with cellular damage for samples obtained via MA and MAPT, because of higher and more variable negative pressures (Hawkins, 2004). It is interesting that individual cellular preservation scores for samples obtained via MAPT and SPA were identical for this group of healthy Beagles. Analysis of these results suggested that SPA was an adequate method for recovery of BALF for cytologic analysis.

Epithelial cells and RBCs were considered markers of BALF samples with poor quality (English, 2008). Large numbers of epithelial cells (>5% of nucleated cells) (Klech, 1989) may be present in BALF because of excessive suction, abnormal exfoliation, or trauma. Predominance of cuboidal and columnar epithelial cells indicates samples collected from the trachea and bronchi, whereas superficial squamous epithelium cells and large bacteria
(Simonsiella spp.) indicate oropharyngeal contamination (English, 2008). Evidence of hemorrhage is rarely found in BALF unless iatrogenic trauma from the BAL procedure results in bleeding. Detection of erythrophagocytosis or hemosiderin during cytologic evaluation can be used to differentiate in vivo pulmonary bleeding from iatrogenic trauma (English, 2008). There was no difference between the 2 aspiration techniques with regard to the proportion of RBCs in the BALF. A higher proportion of epithelial cells was present in samples obtained via MAPT. This could have resulted from trauma secondary to the polyethylene tubing, considering that iatrogenic linear abrasions were identified along the bronchial walls in 2 dogs following wedging of the polyethylene tubing. It could also have been related to the lower amount of infusate retrieved via MAPT, considering that bronchial washings and low-volume BAL aliquots have a higher proportion of epithelial cells and neutrophils (English, 2008; Lam, 1985).

The percentage of neutrophils and alveolar macrophages was significantly different in the BALF obtained via MAPT and SPA. A higher proportion of neutrophils and a lower proportion of macrophages were identified in the BALF obtained via SPA. The absolute neutrophil count was also significantly increased in BALF obtained via SPA. Increased numbers of neutrophils may result from underlying inflammatory pulmonary disease (English, 2008); however, if any dogs in the present study had pulmonary inflammation, we would have expected an increased number of neutrophils in BALF obtained via both MAPT and SPA because other studies (English, 2008; Vail, 1995) have revealed similar differential cell counts among different lung lobes. Therefore, we suspect that the difference in differential cell counts was related to aspiration technique. Polymorphonuclear cells may adhere to tubing, thus decreasing the number of neutrophils in samples obtained via MAPT. Alternatively, evidence in humans indicates that bronchial washings contain a higher proportion of neutrophils and epithelial cells than does fluid from the alveoli (Baughman, 2007; Klech, 1989; Lam, 1985). It is possible that the higher proportion of neutrophils in samples obtained via SPA reflected samples predominantly from the bronchi; however, we believe that this was unlikely, given that a concurrent increase in epithelial cells was not identified in BALF obtained via SPA.

Strengths of the present study included randomization of sample sites and order of sample collection for the aspiration techniques. The same team of investigators (KSW and AMND) performed the BAL procedures to reduce error and enhance precision. Although the investigators were aware of the order of the BAL techniques and location from which the samples were obtained, the veterinary clinical pathologist (DB) was not aware of the origin of the samples when performing the analysis.

The 2 BAL aliquots for each aspiration technique were pooled for analysis in the present study. There is controversy in the human and veterinary medicine regarding pooling of sequential BALF aliquots. In humans, the initial BALF aliquot is considered to represent the bronchial airways more than the alveoli; however, pooling the initial BALF aliquot with subsequent aliquots does not significantly change the overall results (Baughman, 2007). In an equine study, investigators identified a higher proportion of mast cells in the initial 100-mL BALF aliquot; however, the clinical relevance of this difference is unknown, and the equine standardization group continues to recommend pooling of samples for analysis (Hoffman, 2008). In a cytologic study (Hawkins, 1994) of feline BAL samples, investigators found no difference in results of a pooled sample versus results for 3 aliquots. Furthermore, pooling of samples was
recommended in a recent review on airway evaluation in small animals (Creevy, 2009); hence, we decided to pool sequential BAL aliquots in the present study.

Other limitations of the present study included the relatively small sample size and that the dogs were healthy and of similar age, body weight, and breed. We chose middle-aged dogs for the study because age affects composition of BALF (Mercier, 2010). This might limit applicability of the results to dogs with substantial differences in signalments and dogs with pulmonary parenchymal disease. For example, bronchoscopy and BAL in larger dogs may require the use of a longer gastrointestinal scope instead of a bronchoscope. In addition, an increased propensity for airway collapse is observed with many diseases (such as bronchomalacia and chronic bronchitis) of the lower portion of the respiratory tract (Hawkins, 2004; Silverstein, 2010). Infusate retrieval may be hampered if excessive negative pressure associated with aspiration results in airway collapse. Indeed, decreased fluid retrieval has been reported in humans with obstructive lower respiratory tract disease (Ward, 2001). Also, the maximum negative pressure was empirically set at 5 kPa (37.5 mmHg) on the basis of the technique used in human patients (Baughman, 2007), but it is possible that negative pressures of 10 to 25 mmHg could yield better samples because of a decrease in incidence of airway collapse.

Another potential limitation of the present study was the use of sterile polyethylene tubing as part of the MAPT technique. Most reports of clinical trials that involve BAL in small animals define MA as a syringe attached directly to a bronchoscope biopsy channel. However, both techniques for MA are described in a veterinary internal medicine textbook (Silverstein, 2010), and investigators for a prospective clinical trial in humans reported that MAPT resulted in a greater amount of BALF retrieved and a higher diagnostic yield than did MA (Rosell, 2006). Further studies are warranted to compare the use of MA without sterile polyethylene tubing in healthy dogs and to evaluate SPA and MAPT in larger dogs and in dogs with respiratory tract disease.

2.5 Conclusions:
Analysis of results for the present study indicated that in healthy dogs, SPA provided better retrieval of BALF and BALF of higher quality than did MAPT. The SPA technique may improve the rate of diagnostic success for BAL in dogs, compared with the rate of diagnostic success for samples obtained via MAPT; however, further evaluation of these aspiration techniques is required in dogs with respiratory tract disease. In addition, analysis of the results suggested that expected differential cell counts between samples obtained with the MAPT and SPA techniques differ, with a lower percentage of neutrophils for samples obtained with MAPT. These findings further support that BAL protocols should be standardized to obtain results comparable among studies.
CHAPTER 3:
COMPARISON OF BRONCHOALVEOLAR LAVAGE YIELD AND CYTOLOGY OBTAINED FROM MANUAL ASPIRATION WITH A HAND-HELD SYRINGE AND AUTOMATED SUCTION PUMP ASPIRATION IN HEALTHY DOGS

3.1 Introduction:
Bronchoscopy and BAL are minimally invasive techniques used in human and veterinary medicine to visualize the trachea and bronchi, and to sample the bronchoalveolar spaces (Hawkins, 2004; Klech, 1989; Silverstein, 2010). Common indications for BAL include chronic cough, unexplained changes on thoracic radiographs, and pulmonary neoplasia (Hawkins, 1990b; Hawkins, 2004; Silverstein, 2010). After wedging the bronchoscope in a bronchus, BAL is performed by infusing sterile isotonic saline into the small bronchi and alveoli, and then retrieving the infusate (BALF) for analysis (Hawkins, 1990b; Hawkins, 2004; Silverstein, 2010). However, certain technical aspects of the BAL procedure in dogs vary in the current published literature and there is no standardized canine protocol (Hawkins, 1990b; Hawkins, 2004; Silverstein, 2010). These technical aspects, such as infusate volume and sample processing, affect the quality of BALF from healthy dogs (Melamies, 2011; Mordelet-Dambrine, 1984). The methods for infusate retrieval have not been completely critically evaluated in small animal medicine; however, it has been hypothesized that SPA may result in decreased cellular viability due to excessive negative pressure leading to secondary cell lysis (Hawkins, 2004). It is reported in a previous study that automated SPA via suction trap connection provided BALF of higher quality in healthy dogs than did manual aspiration via polyethylene tubing (Woods, 2013). Therefore, the purpose of the present study was to compare 1) MA without polyethylene tubing and automated SPA with suction trap connection for collection of BALF in healthy dogs; and 2) the effect of these techniques on BALF sample quality and volume. We hypothesized that the method of BALF aspiration would influence sample quality and potentially influence the diagnostic utility of examination of BALF in dogs.

3.2 Materials and Methods:

Animals – Thirteen clinically healthy adult Beagles were evaluated for inclusion in the study and were required to be free of pulmonary disease. The health status of each dog was determined by assessment of medical history, physical examination, CBC, biochemical profile, and orthogonal thoracic radiographs (lateral and ventrodorsal views). A board-certified veterinary radiologist (HD) evaluated the thoracic radiographs. Dogs had not received any medications for at least thirty days prior to the study, but regularly received prophylactic anthelmintic treatment. Dogs were cared for in accordance with the Canadian Council on Animal Care guidelines at a Canadian Council on Animal Care-accredited facility. The University of Guelph’s Animal Care Committee approved the study protocol.

Anesthesia – Dogs were sedated, induced and maintained under anesthesia using a previously described protocol (Woods, 2013). All dogs received intravenous fluids, and supplemental oxygen was provided via a sterile semi-rigid catheter placed orally into the

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1 Chapter submitted to the American Journal of Veterinary Research (April 2013).
2 Arnold’s dog catheter with female Luer mount, 8F x 50 cm, Smith Medical International, Hythe, Kent, England.
trachea. The dogs were monitored by physical examination, continuous ECG, and pulse oximetry during anesthesia. Normal oxygen saturation was defined as ≥95%. The experimental investigations were discontinued, and dogs recovered from anesthesia if: oxygen saturation decreased to <90% for 10 minutes, oxygen saturation decreased to <85% for 5 minutes, and/or bradycardia (<60 beats per minute) developed with lack of response to standard interventions.

**BAL** – Dogs were positioned in sternal recumbency for the experimental procedures. For each dog a routine bronchoscopic examination (Padrid, 2011) was performed and recorded with a video bronchoscope\(^3\). The trachea and all lung lobes, up to the second- and third-generation bronchi, were visually examined. Two mL of 0.2% sterile lidocaine solution were infused at the carina to abate coughing during bronchoscopy.

Two BAL aspiration techniques were performed in each dog. The caudal lung lobes were lavaged with a weight-based volume (2 mL/kg, divided into two aliquots), with the second aliquot infused immediately after retrieval of the first aliquot (Melamies, 2011). To perform SPA, the tip of the bronchoscope was wedged into a second-generation bronchus. Sterile isotonic saline solution, warmed to 37.0 degrees Celsius, was infused through the biopsy channel, which was then followed by injection of 4 mL of air to empty the channel. Pulsatile aspiration with a maximum negative pressure of 5 kPa (37.5 mmHg) was performed immediately after infusion using a portable automated surgical suction pump\(^4\) connected directly to the suction valve of the bronchoscope via a disposable suction trap\(^5\). The MA technique was performed on the contralateral caudal lung lobe by wedging the tip of the bronchoscope into a second-generation bronchus and lavage performed as above. A 35-mL syringe was inserted into the bronchoscope’s biopsy channel (Figure 3.1) and gentle pulsatile MA with the 35-mL syringe was applied. Aspiration with each technique was continued until fluid could no longer be recovered. For MA, the same syringe was emptied of air as necessary to continue aspiration. The order of lung lobes lavaged with each aspiration technique was determined with a random number table and blocking. The bronchoscope was cleaned and sterilized in between dogs in accordance with a standard cold sterilization method\(^6,7,8\) (Appendix 3).

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3 Olympus BF type 1T160 video bronchoscope, outer diameter 6.0 mm, working length 60 cm, biopsy channel inner diameter 2.8 mm, Olympus Canada Inc., Richmond Hill, ON.
4 Olympus SSU-2 endoscopic aspiration pump, Olympus Canada Inc., Richmond Hill, ON.
5 Kendall Luki 20-mL (6.25-inch) disposable aspirating tube, Tyco Healthcare Group, Mansfield, MA.
6 Video bronchoscope cleaning and cold sterilization protocol, Olympus Canada Inc., Richmond Hill, ON.
7 Endozime, dual enzymatic cleaner, Ruhof Corp., Mineola, NY.
8 Glutacide, Pharmax Limited, Etobicoke, ON.
The duration of BAL (time from infusion of saline until the end of aspiration for the second aliquot), lowest oxygen saturation, and amount of fluid retrieved were recorded for each technique. A minimum retrieval of 33% of the BAL infusate volume was defined as a diagnostic BALF sample (Ward, 2001). Dwell time (time from infusion of saline to the first attempted aspiration) was <20 seconds for each collection.

**Examination of BALF** – Macroscopic surfactant was indirectly assessed by the presence or absence of foam in each sample immediately after collection. A surfactant score of 0 was assigned if no visible bubbles were present and a surfactant score of 1 was assigned if bubbles were present. Each BALF sample was identified by a unique code of 3 letters, immediately placed on ice after collection, and processed for analysis within 120 minutes. Samples were not filtered during processing. TNCCs were determined by Coulter counter using electrical impedance. An aliquot (200 µL) of each sample was cytocentrifuged (180 X g for 6 minutes), and additional direct smears were prepared from fluid centrifuged for 5 minutes at 500 X g. Slides were stained with Wright stain, and differential cell counts of 400 leukocytes were performed at 400X magnification on cytocentrifuge preparations by a board-certified veterinary clinical pathologist (DB) who was blinded to the technique used to acquire each sample. After performing other analyses, insufficient amounts of BALF were available to measure phospholipid by the Bligh and Dyer method (Bligh, 1959).

**Microscopic assessment** – The pathologist (DB) also assessed five semi-quantitative variables reflective of sample and slide quality using previously reported criteria (Woods, 2013; Appendix 2). Scores of ≥2 for cellularity and cell preservation were required for a BALF sample to be considered of diagnostic quality. Cells were evaluated for erythrophagocytosis during differential counting. Scores for epithelial cells or RBCs (without erythrophagocytosis)

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9 Z2 Coulter counter, Beckman Coulter, Mississauga, ON.
10 Shandon Cytospin 4, Thermo Fisher Scientific Inc., Waltham, MA.
11 Olympus BX53 system microscope, Olympus Canada Inc., Richmond Hill, ON.
>2 were considered to indicate excessive suction or trauma and thus a BALF sample of lesser quality.

**Statistical analysis** – Statistical analyses were performed with a statistical software program[^12]. Data was assessed for normality by Shapiro-Wilk test, with values of \( P > 0.05 \) considered normal distribution. Normally distributed data (duration of BAL, percentage of fluid retrieval, oxygen saturation, and percentage of lymphocytes and macrophages) were compared using paired Student’s \( t \)-test. Non-parametric data (TNCC, percentage of neutrophils and eosinophils, and scores for epithelial cells, cellularity and cell preservation) were compared using Wilcoxon’s Signed Rank test for matched pairs. McNemar’s test was performed to assess binary data (percentage of mast cells, and scores for surfactant, RBCs and bacteria). For all analyses, values of \( P < 0.05 \) were considered significant.

### 3.3 Results:

**Dogs** – Thirteen dogs were evaluated for inclusion in the study. One dog was excluded due to bilateral purulent nasal discharge identified during physical examination. The final study group consisted of 12 healthy adult Beagles (3 intact males, 5 neutered males, and 4 spayed females) that ranged from 8.1 to 10.8 years of age (mean ± SD was 9.7 ± 0.8 years). Body weights of the dogs ranged from 8.1 to 15.5 kg (10.8 ± 2.1 kg). Vital parameters of all dogs were within anticipated limits. No pulmonary abnormalities were identified during thoracic auscultation. Results of the CBCs and biochemical profiles were unremarkable. Three dogs had left-sided systolic heart murmurs (one grade II/VI, one grade III/VI and one grade IV/VI) on physical examination with evidence of mild-to-moderate left atrial enlargement on thoracic radiographs. As none of these dogs had abnormal clinical signs and there was no evidence of pulmonary edema on thoracic radiographs, compensated left mitral valve disease was suspected. No dogs were excluded based on the results of thoracic radiographs.

**Bronchoscopy and BAL** – Bronchoscopic examination was normal in 8 of 12 dogs. In dogs 3 and 5, a mild nodular appearance to the bronchial mucosa was identified in the left lung lobes, but not the right lung lobes. In dog 10, a mild diffuse increase in bronchial mucus was noted. In dog 9, airway collapse was visualized during BAL with both aspiration techniques.

The duration of BAL using MA ranged from 46 to 107 seconds, while SPA ranged from 43 to 137 seconds; these time variations did not differ significantly (Table 3.1).

<table>
<thead>
<tr>
<th>Category</th>
<th>Variable</th>
<th>Mean/median for MA samples</th>
<th>Mean/median for SPA samples</th>
<th>Mean/median distribution of differences between MA and SPA*</th>
<th>SD/IQR for distribution of differences</th>
<th>( P ) Value† for distribution of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL procedure</td>
<td>Duration of BAL (s)</td>
<td>78.4</td>
<td>65.9</td>
<td>-12.5</td>
<td>21.5</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>Lowest oxygen saturation (%)</td>
<td>93</td>
<td>92</td>
<td>-1.0</td>
<td>0.04</td>
<td>0.52</td>
</tr>
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</table>

**Surfactant score**

<table>
<thead>
<tr>
<th>Percentage of retrieved infusate (%)</th>
<th>1</th>
<th>1</th>
<th>n/a</th>
<th>n/a</th>
<th>1.0</th>
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<tbody>
<tr>
<td>46.5</td>
<td>69.5</td>
<td>23.0</td>
<td>8.8</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

**Quality score for cytologic evaluation**

<table>
<thead>
<tr>
<th>Cellularity‡</th>
<th>3.5</th>
<th>4</th>
<th>0</th>
<th>1.0</th>
<th>0.12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell preservation‡</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0.5</td>
<td>0.62</td>
</tr>
<tr>
<td>RBCs§</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
<td>n/a</td>
<td>1.0</td>
</tr>
<tr>
<td>Epithelial cells‡</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.5</td>
<td>0.91</td>
</tr>
<tr>
<td>Bacteria§</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
<td>n/a</td>
<td>0.48</td>
</tr>
</tbody>
</table>

**BALF parameter**

| TNCC (No. of cells/µL)‡ | 200 | 200 | 100 | 50 | 0.02 |

**Differential cell count**

<table>
<thead>
<tr>
<th>Macrophage (%)</th>
<th>72</th>
<th>75</th>
<th>3</th>
<th>8</th>
<th>0.24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil (%)‡</td>
<td>2</td>
<td>2</td>
<td>-1</td>
<td>2</td>
<td>0.37</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>24</td>
<td>19</td>
<td>-5</td>
<td>9</td>
<td>0.12</td>
</tr>
<tr>
<td>Eosinophil (%)§</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>0.56</td>
</tr>
<tr>
<td>Mast cell (%)§</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
<td>n/a</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*A positive value for mean/median distribution of differences indicates that the value was higher in samples obtained via SPA than in samples obtained via MA, and a negative value indicates that the value was lower in samples obtained via SPA than in samples obtained via MA. †Distribution of differences were considered significantly different at P<0.05. Data were distributed in a normal, non-parametric (‡), or binary (§) fashion. Data from three dogs were excluded from analyses for TNCC and differential cell count. Legend: SD = standard deviation, IQR = interquartile range, n/a = not applicable.

**Adverse effects of BAL** – Mild bruising of the bronchial mucosa was identified bilaterally following BAL in dog 9. Transient (<2-minute) decreases in oxygen saturation were observed in eight dogs (66.7%) and six dogs (50%) during MA and SPA, respectively. These decreases were self-limiting, as none of the dogs required intervention. The mean lowest oxygen saturation achieved during MA was 93%, compared with 92% during SPA; these values did not differ significantly.

**BALF analysis** – Surfactant was macroscopically present in all samples obtained with SPA, but was absent in one sample obtained with MA. There was no statistically significant difference between surfactant scores of BALF obtained with SPA or MA (P=1.0). Mean percentage of retrieved infusate ± SD was 46.5% ± 10.2% for MA and 69.5% ± 6.5% for SPA. The percentage of BALF retrieved via the SPA technique was significantly higher (P<0.0001) than for the MA technique (mean distribution of differences ± SD, 23.0% ± 12.6%). Three BALF samples (2 acquired by MA and 1 acquired by SPA) were acellular (TNCC = 0 cells/µL), as per electrical impedance detection of cells. Adequate cellularity on cytocentrifuge preparations allowed for estimation of TNCC of these three BALF samples, and these results were included for analysis. Three dogs were excluded from TNCC and differential cell count analyses. Dogs 3 and 5 were excluded from TNCC analysis due to asymmetrical bronchoscopic abnormalities, and dog 12 was excluded as marked elevation in TNCC with septic suppurative inflammation was identified in one BALF sample. Median TNCC for samples obtained from the remaining nine dogs by MA was 200 cells/µL (range 100 to 300 cells/µL; IQR, 100 cells/µL) and for samples obtained by SPA was 200 cells/µL (range 200 to 400 cells/µL; IQR, 100 cells/µL). The distribution of differences for TNCC in SPA BALF was significantly higher (median distribution of differences, 100 cells/µL; IQR, 50 cells/µL; P=0.02) than for TNCC in MA BALF.
Microscopic assessment of BALF – Despite three BALF samples reported as acellular based on TNCC, adequate numbers of cells were identified on all cytocentrifuge preparations for microscopic analysis. Median cellularity score for samples acquired by MA was 3.5 (range 2 to 4) and for samples acquired by SPA was 4 (range 3-4); these values did not differ significantly. All scores for cell preservation were 3 or 4; these values were not significantly different between the two aspiration techniques. Scores for RBCs, bacteria or epithelial cells were not significantly different between MA and SPA techniques.

Data from dogs 3, 5, and 12 were excluded from differential cell count analysis, due to asymmetrical bronchial mucosa lesions in two dogs, and unilateral septic suppurative inflammation in one dog. There were no significant differences identified in differential cell counts of macrophages, neutrophils, lymphocytes, eosinophils or mast cells.

3.4 Discussion:
In this study, we compared two methods for collecting BAL: MA and SPA. We compared these two aspiration techniques as MA was the most commonly reported method of retrieving BALF in veterinary medicine, and because a previous study identified that SPA provided BALF of higher quality than manual aspiration via polyethylene tubing (Woods, 2013). We assessed sample quality using previously published criteria (Woods, 2013), which included percentage of fluid retrieved, presence of surfactant, cellularity, cell preservation, presence of RBCs (without erythrophagocytosis), presence of bacteria, and the presence of epithelial cells (De Lorenzi, 2009; English, 2008; Hawkins, 1990b; Lam, 1985; Silverstein, 2010). The surfactant score in the present study was refined from the previous study (Woods, 2013) to indicate only the presence or absence of surfactant in BALF samples, since it is currently unknown whether the method of fluid retrieval influences the amount or characteristics of foam, and therefore the volume of bubbles in BALF. It should also be emphasized that no evidence-based recommendations currently exist regarding minimally acceptable limits for cell preservation or RBCs, and these scores were created based on expert opinion.

We found that SPA yielded significantly higher fluid retrieval than MA. A higher percentage of retrieved fluid increases the likelihood that the BAL procedure successfully sampled the alveoli, in addition to the bronchi (Hawkins, 1990b; Lam, 1985). In human medicine, the use of small volume aliquots, with associated small volume aliquot recovery, is considered a bronchial, versus alveolar, wash (Baughman, 2007; Lam, 1985). Recovery of a minimum of 33% to 40% of the original BAL infusate volume has been reported in human and canine medicine (Hawkins, 2004; Klech, 1989; Silverstein, 2010; Ward, 2001). Therefore, we considered BALF retrieval of <33% to be non-diagnostic. The MA technique retrieved <33% of the infusate in one of 12 samples. In contrast, all of SPA BALF samples retrieved >59% of the original infusate volume, and the proportion of fluid retrieval was significantly higher for the SPA technique. In healthy lungs, decreased fluid retrieval could occur secondary to improper placement of the bronchoscope, such as abuttal of the bronchoscope against the airway wall or improper wedging of the bronchoscope tip (Creevy, 2009; Hawkins, 1990b; Hawkins, 2004). Care was taken to visually ensure that the bronchoscope was not occluded against the bronchial wall for both aspiration techniques, which makes this an unlikely cause of decreased fluid retrieval with MA. If a seal is not achieved between the bronchoscope’s tip and the bronchus, negative pressure will retrieve air preferentially over infusate (Creevy, 2009). The seal may be
lost with movement of the bronchoscope during aspiration. Since MA was performed by an assistant, whereas SPA was performed by the primary clinician, movement during BALF retrieval may have been more likely during the MA technique. It is also possible that when compared with MA, SPA allowed for creation of a better seal between the wedged bronchoscope and the bronchus due to creation of increased negative pressure. It has been reported that occasionally no BALF fluid is retrieved despite adequate wedging of the bronchoscope (Creevy, 2009; Hawkins, 2004). In these cases, it is recommended that the bronchoscope be retracted a few mm (Creevy, 2009; Hawkins, 2004). This was attempted prior to discontinuation of BAL for both aspiration techniques and, therefore, is also considered an unlikely cause of one MA BALF sample retrieving a non-diagnostic amount of infusate. It is also unlikely that insufficient negative pressure could be generated through the bronchoscope’s biopsy channel with a handheld 35-mL syringe for fluid retrieval as this technique has been successfully described previously (Hawkins, 1990b). However, if the increased fluid retrieval with SPA is independent of wedging the bronchoscope, it could occur because the SPA technique creates more consistent negative pressure, without airway collapse, than can MA.

Three BALF samples had TNCC of 0. However, despite apparent “acellular” BALF samples based on TNCC, all cytocentrifuge preparations had a sufficient number of cells for analysis. This suggested that laboratory error might have resulted in lack of cell counting. Mucus within BALF can falsely decrease cell counts if it results in cell clumping and occlusion of the Coulter counter aperture. One of the apparently acellular BALF samples, acquired by MA, had a corresponding low fluid retrieval (25%) and low cellularity score (2) on cytocentrifuge preparation, and would have been considered non-diagnostic based on the level of infusate retrieval. The SPA technique provided BALF with significantly higher TNCC counts than the MA technique; however, overall cellularity scores were not significantly different between MA and SPA. Despite previously reported concerns that increased negative pressure achieved with SPA would result in increased cellular damage (Hawkins, 2004), cell preservation scores were not significantly different between MA and SPA. In addition, no significant differences were found between the differential cell counts, which indicated that one type of leukocyte was not preferentially lost during aspiration with either MA or SPA, despite the fact that the disposable suction trap and handheld syringe were made of different materials (polystyrene and polypropylene, respectively). There were no significant differences between RBC, epithelial cell or bacterial scores between MA and SPA. These results suggested that one technique did not result in more trauma to the bronchial mucosa that the other. In summary, MA and SPA were equivalent in regards to quality and preservation of cells in BALF samples from healthy dogs, and their cytology results were comparable.

The question remains as to whether increased BALF retrieval and/or a small increase in TNCC will be clinically relevant in dogs with respiratory disease. An increase in TNCC will not likely result in a corresponding increase in likelihood of identifying neoplastic cells or infectious organisms in BALF, as these changes are considered either present or absent on cytologic analysis (English, 2008). In regards to the association between diagnostic yield and BALF retrieval, there exist conflicting reports in humans (Rosell, 2006; Sampsonas, 2011). One study comparing manual aspiration via tubing to MA reports a higher diagnostic yield with the manual aspiration with tubing technique, which also correlates with increased BALF retrieval (Rosell, 2006). Conversely, a prospective clinical trial of human cancer patients undergoing
bronchoscopy and BAL for new thoracic radiographic infiltrates reports that increased fluid retrieval does not improve diagnostic yield (Sampsonas, 2011). However, this study compares diagnostic yield and BALF retrieval between two disease groups (non-hematologic malignancy group and hematologic malignancy group), and only assesses BALF for evidence of either infectious pneumonia or malignancy (Sampsonas, 2011). These criteria may have biased the results (Sampsonas, 2011). Further evaluation of BAL aspiration techniques is required in dogs with respiratory tract disease to assess whether aspiration technique affects the diagnostic yield of BAL.

Strengths of the present study were randomization of sample sites and order of sample aspiration techniques, having the same team of operators (KSW and AMND) perform all procedures, and blinding of the pathologist to aspiration technique during analysis of BALF samples for cytology scores. Another strength of this study was the comparison of a handheld syringe attached directly to a bronchoscope biopsy channel (MA) to the SPA technique, as MA is the aspiration technique most often reported for canine BAL (Creevy, 2009; Hawkins, 1990b; Hawkins, 2004; Melamies, 2011).

Limitations of the present study included the relatively small sample size and use of healthy dogs of similar age, body weight and breed. This might limit applicability of results to dogs with respiratory disease or different signalments, as wedging the bronchoscope is more difficult in larger dogs (Hawkins, 2004; Creevy, 2009). As such, some clinicians will use a larger diameter endoscope for BAL in larger dogs, and different working channel diameters and lengths (e.g. with longer endoscopes) may alter overall negative aspiration pressures achieved during BAL. Another limitation was that surfactant was not directly measured in BALF samples.

3.5 Conclusions:
The results of this study indicated that in healthy dogs, SPA provided higher percent retrieval of BALF and TNCC than did MA. Sample quality was equivalent between the SPA and MA techniques. The SPA technique may improve the diagnostic yield of BAL in dogs; however, further evaluation of these aspiration techniques is required in dogs with respiratory tract disease.
CHAPTER 4:
COMPARISON OF MANUAL AND SUCTION PUMP ASPIRATION TECHNIQUES FOR PERFORMING BRONCHOALVEOLAR LAVAGE IN 23 DOGS WITH RESPIRATORY TRACT DISEASE

4.1 Introduction:
BAL is a minimally invasive technique that is widely used in veterinary medicine to investigate pulmonary disease (Hawkins, 1990b; Silverstein, 2010). By retrieving infused saline from the airways, a sample representative of the lower generation bronchi, bronchioles and alveolar spaces is collected for analysis (Hawkins, 1990b). However, studies in both human and veterinary medicine have shown that BALF sample quality, and therefore the clinical utility, are affected by certain technical aspects of the BAL procedure (Baughman, 2007; Klech, 1989; Melamies, 2011; Mordelet-Dambrine, 1984; Woods, 2013). As such, standardized BAL protocols have been implemented in human and equine medicine (Baughman, 2007; Hewson, 2002); however, such an approach is not generally used in small animal medicine. We have reported that, in healthy dogs, SPA yielded BALF samples of higher quality than manual aspiration via polyethylene tubing (Woods, 2013) and that SPA retrieved a higher amount of BALF than did manual aspiration without polyethylene tubing (MA)\(^1\). As diseased lungs are more prone to collapse than healthy lungs, the purpose of the study reported here was to compare MA and SPA via suction trap connection for collection of BALF in dogs with respiratory tract disease and the effect of aspiration technique on BALF sample quality. We hypothesized that SPA would yield BALF of better sample quality than MA, and that samples collected with SPA would therefore be of greater diagnostic value.

4.2 Materials and Methods:

Study Population – Client-owned dogs that presented to the Ontario Veterinary College Health Sciences Centre, and for which bronchoscopy and BAL were recommended as part of their diagnostic work-up were eligible for enrolment in this randomized, prospective, single-blinded clinical trial. Written client consent was required for enrolment. Dogs were excluded if bronchoscopic-guided BAL could not be performed. The study protocol was approved by the University of Guelph Animal Care Committee.

Information was collected from the medical record of each dog, including signalment, presenting complaint, duration of clinical signs, previous and current treatments, and interpretation of orthogonal thoracic radiographs.

Anesthesia – Dogs were anesthetized with protocols tailored individually for each patient by the hospital’s Anesthesia Service. Dogs were monitored by physical examination, blood pressure measurement (Doppler method), continuous ECG, and pulse oximetry. Dogs of sufficient size to be intubated with a size 10 (or larger) cuffed endotracheal tube were also monitored with capnography and received supplemental oxygen via the anesthetic circuit. Smaller dogs received supplemental oxygen therapy during bronchoscopy and BAL via a sterile, 

semi-rigid urinary catheter\(^2\) placed transglottally into the trachea. The procedure was discontinued for appropriate treatment if the patient’s clinical condition became unstable during general anesthesia or if unforeseen complications arose.

**Additional Procedures** – Whether the dog underwent other procedures under the same general anesthesia was recorded.

**Bronchoscopy and BAL** – Dogs were positioned in sternal recumbency. In intubated dogs, the procedures were performed through the endotracheal tube via a T-port connection\(^3\). In smaller dogs, the endoscope was passed transglottally into the trachea. A routine bronchoscopic examination was performed in each dog, whereby the trachea, main stem bronchi, and second and third-generation bronchi of all lung lobes were visually examined. Four different video endoscopes\(^4,5,6,7\) were used in this study, and were chosen by the supervising clinician based on patient size. One to 4 mL (based on patient size) of sterile 0.2% lidocaine solution was infused at the carina to reduce bronchospasm.

Two BAL aspiration techniques were performed in contralateral lung lobes of each dog. The order of and the side lavaged with each aspiration technique were randomized using a random number table. The sites for BAL were directed by pulmonary abnormalities visualized on thoracic radiography and/or bronchoscopy. A weight adjusted BAL volume (2 mL/kg, divided into two aliquots) was used at each site (Melamies, 2011). The second aliquot was infused immediately after retrieval of the first aliquot. To perform SPA, the tip of the bronchoscope was gently wedged in a distal bronchus, based on feeling resistance to advancement as well as visualization into a distal bronchus. Sterile 0.9% saline solution, warmed to 37°C, was rapidly infused through the biopsy channel of the endoscope, followed by 4 mL of air to clear the channel. Pulsatile aspiration with a maximum negative pressure of 50 mmHg was applied immediately after infusion using a wall mounted suction unit with pressure regulator\(^8\) connected directly to the suction valve of the bronchoscope via a disposable suction trap\(^9\). Disposable suction traps were replaced as needed if BALF recovery exceeded their maximum capacity (20-mL). To perform MA, the tip of the bronchoscope was similarly wedged into a distal bronchus of the contralateral lung lobe. Sterile 0.9% saline solution, warmed to

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\(^2\) Arnolds dog catheter with female Luer mount, 8F X 50 cm, Smith Medical International, Kent, England.

\(^3\) Right angled swivel connector, Vygon Corp., Lansdale, PA.

\(^4\) Olympus BF-P40 video bronchoscope, outer diameter 5.0 mm, working length 55 cm, biopsy channel inner diameter 2.0 mm, Olympus Canada Inc., Richmond Hill, ON.

\(^5\) Olympus GIF-130 video endoscope, outer diameter 9.5 mm, working length 103 cm, biopsy channel inner diameter 2.8 mm, Olympus Canada Inc., Richmond Hill, ON.

\(^6\) Olympus GIF-140 video endoscope, outer diameter 8.9 mm, working length 140 cm, biopsy channel inner diameter 2.8 mm, Olympus Canada Inc., Richmond Hill, ON.

\(^7\) Olympus GIF-XP160 video endoscope, outer diameter 5.9 mm, working length 103 cm, biopsy channel inner diameter 2.0 mm, Olympus Canada Inc., Richmond Hill, ON.

\(^8\) Vacuum tracheal suction regulator with Ohmeda adapter, Western Medica, Westlake, OH.

\(^9\) Kendall Luki 20-mL (6.25-inch) disposable aspirating tube, Tyco Healthcare Group, Mansfield, MA.
37°C, was rapidly infused through the biopsy channel, followed by 4 mL of air to clear the channel. A 35-mL syringe was inserted through the biopsy valve of the bronchoscope’s biopsy channel and gentle pulsatile aspiration was performed with the hand-held syringe. For MA, the same syringe was emptied of air as necessary to continue aspiration. Aspiration with each technique was continued until fluid was no longer retrieved. The bronchoscope was cleaned and sterilized between each patient with a standard cold sterilization method\textsuperscript{10,11,12}. The time from infusion of saline to the first attempted aspiration was <20 seconds for each BAL aliquot.

**Adverse Effects of Bronchoscopy and BAL** – The lowest oxygen saturation during BAL was recorded for each aspiration technique. Patients that did not regain normal oxygen saturation (≥95%) on room air after recovery from general anesthesia were transferred to the Intensive Care Unit for supplemental oxygen therapy. Whether or not supplemental oxygen therapy was required following recovery was recorded. Patient outcome, defined as survival to discharge from hospital, was also recorded.

**Evaluation of BALF** – The amount of fluid retrieved was recorded for each aspiration technique. For the purpose of this study, low retrieval of BALF was defined as recovery of <40% of the original aliquot volume (Melamies, 2011). Each BALF sample was identified by the patient’s hospital number, and processed for analysis within 40 minutes of collection. Samples were not filtered during processing. TNCCs were determined with electrical impedance\textsuperscript{13}. A 200 µL aliquot of each BALF sample was cytocentrifuged (180 X g for 6 minutes)\textsuperscript{14}, and direct smear slides were prepared from fluid concentrated by centrifugation for 5 minutes at 500 X g and decanting of supernatant. Slides were stained with Wright stain\textsuperscript{15}. The cytologic preparations were assessed by the board-certified veterinary clinical pathologist on duty and results were reported to supervising clinicians. Slide preparations were stored for up to 12 months until analysis by a board certified veterinary clinical pathologist (DB) who was blinded to the patient history, original interpretations and aspiration technique used to collect each BALF sample. Differential cell counts of a minimum of 400 leukocytes were performed at 400X magnification\textsuperscript{16} on cytocentrifuge preparations. Cells were evaluated for erythrophagocytosis, and presence of intracellular bacteria and pigment during differential counting. The presence of erythrophagocytosis or hemosiderin was considered to be consistent with chronic and clinically relevant intrapulmonary hemorrhage. Samples were also assessed microscopically for five variables reflective of sample quality (Woods, 2013; \textbf{Appendix 2}). Semi-quantitative scores were applied for cellularity, cell preservation, and the presence of RBCs (without erythrophagocytosis), extracellular bacteria and epithelial cells. Scores ≥2 for cellularity and cell preservation were considered adequate for diagnostic utility (Woods KS, 2013). Pre-determined cut-offs for RBC and epithelial cell scores were not defined, as increased

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\textsuperscript{10} Video bronchoscope cleaning and cold sterilization protocol, Olympus Canada Inc., Richmond Hill, ON.
\textsuperscript{11} Endozime, dual enzymatic cleaner, Ruhof Corp., Mineola, NY.
\textsuperscript{12} Glutacide, Pharmax Limited, Etobicoke, ON.
\textsuperscript{13} Z2 Coulter counter, Beckman Coulter, Mississauga, ON.
\textsuperscript{14} Shandon Cytospin 4, Thermo-Fisher Scientific Inc., Waltham, MA.
\textsuperscript{15} Hematek Slide Stainer 4488C, Siemens Healthcare Diagnostics Inc., Tarrytown, NY.
\textsuperscript{16} Olympus BX53 system microscope, Olympus Canada Inc., Richmond Hill, ON.
bronchial epithelial cell sloughing and intrapulmonary hemorrhage can occur in dogs with pulmonary disease (Silverstein, 2010). However, an overall trend for increased epithelial cell and/or RBC scores (without erythrophagocytosis or hemosiderin) was considered to indicate increased trauma to the bronchial mucosa during BAL.

**Diagnosis** – The final diagnosis for each patient was based on a combination of history, and findings from physical examination, thoracic radiographs, bronchoscopic examination, BALF cytology and BALF microbial cultures. Inflammation identified in BALF was defined as TNCC >500 cells/µL. Inflammatory BALF was further classified as suppurative (>12% neutrophils), eosinophilic (>14% eosinophils), lymphocytic (>16% lymphocytes) or mixed (elevated proportions of ≥2 types of leukocyte) (Hawkins, 1995; Johnson, 2013). The information obtained from cytologic analysis of BALF was compared with the final patient diagnosis and was coded into three categories, based on a previous retrospective assessment of diagnostic yield of BAL in dogs (cytologic diagnosis score) (Hawkins, 1995). A cytologic diagnosis score of 0 indicated a non-helpful sample (Hawkins, 1995). A score of 1 indicated that the cytologic analysis was supportive of the overall diagnosis (Hawkins, 1995). A score of 2 indicated that a definitive diagnosis was achieved from cytology alone (Hawkins, 1995). For example, a score of 2 was allocated for the presence of intracellular bacteria, neoplastic cells or fungal organisms.

**Statistical Analysis** – Descriptive statistics for patient and BAL parameters were determined with spreadsheet software. Remaining statistical analyses were performed with a statistical software program. All data were assessed for normality by Shapiro-Wilk test, with P>0.05 considered to have a normal distribution. Normally distributed data (percent of retrieved BALF) was assessed using paired Student’s t-test. Non-parametric data (oxygen saturation, TNCC, proportional differential cell counts, semi-quantitative cytology scale, and cytologic diagnosis score) were compared using Wilcoxon Signed Rank test for matched pairs. ANOVA was used to assess influence of endoscope on BALF retrieval. Chi-square testing was used to assess the effect of thoracic radiographs on cytologic diagnosis score. Agreement between classification of BALF for MA and SPA samples was assessed using Cohen’s unweighted kappa coefficient. Agreement between cytologic diagnosis scores for MA and SPA were assessed using Cohen’s weighted kappa coefficient. For all analyses, values of P<0.05 were considered significant.

4.3 **Results:**

**Study Population** – The study group consisted of 23 dogs, with mean age of 6.75 ± SD of 4.39 years and mean weight of 23.4 ± SD of 17.8 kg. Fourteen dogs were male (10 castrated, 4 intact) and 9 were female (8 spayed, 1 intact). Breeds represented in the study population included mixed breed canine (6), Labrador Retriever (3), German Shepherd Dog (2), and one of each of the following: Beagle, Boston Terrier, Cane Corso, Coonhound, Dachshund, Havanese, Portuguese Water Dog, Rhodesian Ridgeback, Shih Tzu, Toy Poodle, Whippet, and Yorkshire Terrier. The most common, not mutually exclusive, presenting complaints in the present study were chronic cough (13), lethargy (6) and tachypnea (5). Gagging (3), acute productive cough

17 Microsoft Excel for Mac 2011, version 14.3.1.
(3), chronic nasal discharge (2), stertor (2), hemoptysis (1), reverse sneezing (1), and exercise intolerance (1) were also reported. Duration of clinical signs prior to presentation ranged from 1 day to 2 years (median 3 months, IQR of 6.25 months). Fifteen dogs (65.2%) were receiving medications at the time of BAL, and 9 of these dogs were receiving ≥2 medications. Current medications included antibiotics [enrofloxacin (3); doxycycline (3); marbofloxacin (1); cefazolin (1); cephalixin (1)], corticosteroids [fluticasone (3); prednisone (3); budesonide (1)], bronchodilators [salbutamol (3); theophylline (1)], analgesics [gabapentin (1); mavacoxib (1)], tracheal collapse elixir\textsuperscript{19} (2), fenbendazole (2), phenobarbital (1), famotidine (1), omeprazole (1), furosemide (1), benazepril (1), and levothyroxine (1). Nine dogs (39.1%) had a history of previous medical therapy for their respiratory signs. Historical treatments, administered within 90 days prior to presentation, included antibiotics [amoxicillin or amoxicillin-clavulanate (4), doxycycline (2), enrofloxacin (1), marbofloxacin (1)], prednisone (4), and fenbendazole (1).

Thoracic radiographs acquired prior to bronchoscopy were assessed by the board-certified veterinary radiologist on duty, and identified diffuse bronchial pulmonary pattern in 4 dogs, interstitial pulmonary pattern in 5 dogs (1 focal, 4 diffuse), diffuse mixed broncho-interstitial pattern in 2 dogs and alveolar pulmonary pattern in 4 dogs (3 focal, 1 diffuse). The remaining 8 dogs had thoracic radiographs without radiographic changes.

**Additional Procedures** – Nine (39.1%) of the study dogs had additional procedures performed during the anesthesia for bronchoscopy and BAL. Three dogs underwent rhinoscopy with nasal biopsies following BAL, and one of these dogs also underwent magnetic resonance imaging of the head. Histologic examination identified severe diffuse lymphocytic-plasmacytic rhinitis, a benign nasopharyngeal polyp, and nasal lymphoma, respectively. Two dogs underwent upper gastrointestinal endoscopy, which identified a severe esophageal stricture in one dog. In this dog, BAL cytology and thoracic radiographs were normal; therefore the owner’s presenting complaint of coughing was judged to be confusion with gagging. Upper gastrointestinal endoscopy and biopsies were unremarkable in the other dog. One dog had an esophageal foreign body retrieved. One dog had a laryngeal examination performed prior to bronchoscopy, which was consistent with laryngeal paralysis. One dog had ovariohysterectomy performed. One dog had a punch biopsy of a digital mass performed, and histologic examination identified perivascular lymphocytic-plasmacytic dermatitis.

**Bronchoscopy and BAL** – A human pediatric gastroscope\textsuperscript{7} (12 dogs) and video bronchoscope\textsuperscript{4} (8 dogs) were used for the majority of the procedures (20/23, 87.0%). In general, the video bronchoscope was used in patients weighing <30 kg and the pediatric gastroscope was used in patients weighing ≥30 kg due to increased working length. However, the pediatric gastroscope was used in 3 dogs weighing <10 kg as further endoscopic evaluation (nasopharyngeal retroflexion or upper gastrointestinal endoscopy) was performed following BAL, and the pediatric gastroscope provided an improved image. Bronchoscopic abnormalities were grossly identified in 15 of the 23 dogs, with tracheal and/or bronchial collapse and increased airway secretions being the most common changes, each identified in 8 dogs. Other abnormalities included erythema of the bronchial mucosa (6), hemorrhage (2), and nodular

\begin{footnotesize}
\textsuperscript{19} Tracheal elixir (per mL): phenobarbital 0.4 mg, ephedrine 0.83 mg, isoproterenol 0.004 mg, theophylline 3 mg, and potassium iodide 10 mg.
\end{footnotesize}
bronchial mucosa (2). Multiple suction trap connections were required for SPA in 7 dogs (>24 kg) when BALF exceeded 20 mL.

**Adverse Effects of Bronchoscopy and BAL** – Seventeen dogs (74.0%) maintained normal oxygen saturation during the procedure. Three dogs (13.0%) had transient decreases in oxygen saturation (<2 minutes), which resolved without intervention. There was no significant difference in the lowest oxygen saturation between techniques ($P=0.82$) (**Table 4.1**). The remaining 3 dogs (13.0%) required oxygen supplementation following recovery from anesthesia. One dog did not survive to discharge from hospital, resulting in a mortality/euthanasia rate of 4.3% (1/23). This dog had presented for a chronic esophageal foreign body and secondary aspiration pneumonia, and experienced an acute decline in respiratory function 4 hours after recovery from anesthesia. This decompensation was consistent with acute respiratory distress syndrome, based on arterial blood gas analysis and clinical condition. Mechanical ventilation was declined and the dog was euthanized. Post mortem examination was declined.

**Table 4.1 – Comparison of results for BALF samples obtained via MA and SPA from 23 client-owned dogs with naturally occurring respiratory tract disease.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Variable</th>
<th>Mean/Median for MA samples</th>
<th>Mean/Median for SPA samples</th>
<th>$P$ value* for distribution of differences between MA and SPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL Procedure</td>
<td>Lowest oxygen saturation (%)</td>
<td>99</td>
<td>99</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Percentage of retrieved infusate (%)†</td>
<td>29.2</td>
<td>50.0</td>
<td>0.0002</td>
</tr>
<tr>
<td>Quality score for cytologic evaluation‡</td>
<td>Cellularity</td>
<td>4</td>
<td>4</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Cell preservation</td>
<td>4</td>
<td>4</td>
<td>0.705</td>
</tr>
<tr>
<td></td>
<td>RBC</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Epithelial cells</td>
<td>0</td>
<td>0</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Extra-cellular bacteria</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>BALF parameter</td>
<td>TNCC (No. of cells/µL)</td>
<td>475</td>
<td>615</td>
<td>0.322</td>
</tr>
<tr>
<td>Differential cell count (proportion)</td>
<td>Macrophage (%)</td>
<td>54</td>
<td>70</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Neutrophil (%)</td>
<td>4</td>
<td>6</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte (%)</td>
<td>8</td>
<td>11</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Eosinophil (%)</td>
<td>2</td>
<td>3</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>Mast cell (%)</td>
<td>0</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Basophil (%)</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytologic diagnosis</td>
<td>Cytologic diagnosis score</td>
<td>0.5</td>
<td>1</td>
<td>0.096</td>
</tr>
</tbody>
</table>

*Values <0.05 indicate that results were significantly different between the MA and SPA techniques; † Normally distributed data; ‡ Within this category, each variable was scored on a scale of 0 to 4, except for RBC, which was scored on a scale of 0 to 3. One dog was excluded from BALF analysis due laboratory error in slide preparation. Three dogs were excluded from differential cell count analysis.

**Examination of BALF** – The percentage of infusate retrieved with MA ranged from 6.6-61.4%. The percentage of infusate retrieved with SPA ranged from 13.6-73.3%. A significantly higher percentage of BALF was retrieved using the SPA technique, when compared with MA (mean distribution of differences 15.7 ± SD 17.1%, $P=0.0002$). The type of endoscope used for the procedure had no significant effect on the amount of BALF retrieved (MA, $P=0.89$; SPA,
Slides from dog 15 were excluded from further analysis, as a laboratory error resulted in acellular slides from the MA sample.

Neither TNCC (P=0.23), nor differential cell counts (neutrophil, P=0.65; macrophage, P=0.81; lymphocyte, P=0.53; eosinophil, P=0.68; mast cell, P=0.12) were significantly different between aspiration techniques. Five of 22 (22.7%) cytocentrifuge preparations from BALF acquired by MA were acellular. These five BALF samples had low TNCC and/or lack of bronchoalveolar cells on the direct smear, and were considered to be non-diagnostic. In contrast, all 22 BALF samples acquired by SPA had adequate cell numbers for analysis. The distribution of differences for cellularity scores was significantly higher for SPA, when compared with MA (P=0.018). There were no significant differences between cell preservation (P=0.70), epithelial cell score (P=0.75), extracellular bacteria score (P=1.0) or RBC score (P=0.5) between MA and SPA.

Diagnosis – Four dogs had final diagnoses unrelated to pulmonary disease (nasal lymphoma, laryngeal paralysis, nasopharyngeal polyp, and esophageal stricture) and were excluded from the final diagnostic yield calculations. When history, physical examination findings, bronchoscopic findings, and microbial analysis of BALF were assessed in conjunction with BALF cytology, a diagnosis was achieved in 17/19 (89.5%) dogs with pulmonary disease. In dogs with pulmonary disease, definitive diagnoses were achieved from cytologic analysis of 3/19 (15.8%) and 4/19 (21.1%) of the BALF samples acquired by MA and SPA techniques, respectively. Definitive diagnoses included bacterial pneumonia (4) and blastomycosis (1). Cytology was considered supportive of the diagnosis in 8/19 (42.1%) and 11/19 (57.9%) of samples acquired by MA and SPA, respectively; these diagnoses included chronic bronchitis (6), bacterial pneumonia (5), and eosinophilic bronchopneumopathy (3). There was no significant difference between cytologic diagnosis score achieved with either technique. Excluding the five dogs with non-diagnostic BALF samples, moderate agreement was found between the classification of inflammatory BALF between MA and SPA (unweighted kappa = 0.541, P<0.001). Substantial agreement was found between the cytologic diagnostic score achieved with SPA and MA (weighted kappa = 0.653, P<0.0001).

No significant associations were found between the different patterns identified on thoracic radiographs and acquiring a definitive diagnosis from BALF cytology.

4.4 Discussion:
While SPA had consistently resulted in increased BALF retrieval from healthy dogs in previous studies when compared with MA, this had not yet been confirmed in dogs with respiratory tract disease (Woods, 2013). In this group of dogs with pulmonary disease, an increased frequency of airway collapse and an overall decreased BALF retrieval was observed with both aspiration techniques, when compared to previous groups of healthy dogs. However, SPA retrieved a significantly higher amount of BALF than MA. An explanation for this finding could be that SPA creates a better wedge between the bronchoscope and bronchial mucosa by collapsing the bronchi proximal to the bronchoscope’s tip.

Recovery of 40-90% of BAL infusate has been reported in canine medicine using various BAL techniques, and currently a minimum 40% BALF retrieval is recommended in dogs to
maximize chances of a diagnostic sample (Hawkins, 2004; Melamies, 2011). In this study, 56.5% (26/46) of BALF samples (17 MA and 9 SPA) comprised <40% of the original aliquot volume. Of the 11 BALF samples with cellularity scores of <4, all but one of them was associated with <40% infusate retrieval. As the majority of cells in BALF originate from the alveoli, this finding agreed with previous reports in human and veterinary medicine that decreased fluid retrieval and low TNCC in retrieved infusate were consistent with bronchial, and not bronchoalveolar, washes (Chamberlain, 1987; Lam, 1985). However, in the present study of dogs with pulmonary disease, only six (6/26, 23.1%) of the low-retrieval BALF samples had inadequate cell numbers for analysis and the majority (15/26, 57.7%) of the low-retrieval BALF samples still yielded a definitive or supportive cytologic diagnosis. In general, maximal infusate retrieval should be attempted for any BAL to maximize the likelihood of retrieving alveolar cells. If patient condition allows, further lavages may be performed as higher fluid retrieval has been reported with subsequent lavages (Hawkins, 1994). However, in cases where low BALF retrieval persists, the samples should be assessed as they may still contain adequate cell numbers for analysis.

The other variables of the semi-quantitative quality score did not significantly differ between techniques. Similar cell preservation, RBC and epithelial cell scores indicated that SPA at maximum negative pressure of 50 mmHg did not result in increased cell lysis or bronchial mucosa trauma, compared with MA. While only moderate agreement was found between the cytologic classification of BALF from the aspiration techniques, a higher level of agreement between BALF cytology between different lung segments was not necessarily expected since a study reported that BALF cytologic assessment from cats with lower respiratory disease often differed between different pulmonary segments, even with diffuse disease (Ybarra, 2012). Similarly, a retrospective review of canine BALF cytology identified that 37% of samples had different types of inflammation among different lung lobes in the same dog (Hawkins, 1995). These findings indicated that cytology results obtained by both MA and SPA were comparable.

A relationship between increased BALF retrieval and improved rate of diagnosis from cytologic assessment has been inconsistently identified in human medicine (Rosell, 2006; Sampsonas, 2011). While SPA yielded higher BALF retrieval, there was no significant difference between the cytologic diagnosis score for the two techniques and thus this study failed to demonstrate a relationship between increased BALF and improved diagnostic yield in dogs.

The adverse effects identified in the current study of canine bronchoscopy and BAL were similar to those in previous reports, in that the techniques were generally safe; however, some dogs required supplemental oxygen therapy following the procedure (Hawkins, 1990b; Hawkins 1995). A retrospective review of canine BAL identified a mortality/euthanasia rate of 2% within five hours of the procedure, with each patient overtly dyspneic prior to BAL (Hawkins, 1995). This finding led to the recommendation that respiratory distress be considered a relative contraindication for BAL. One of 23 dogs was euthanized in this study, within a similar timeframe as the retrospective study. This dog was tachypneic, but not dyspneic, prior to anesthesia. While clinical decompensation of this patient may have been secondary to natural progression of disease or to general anesthesia, the risk of adverse effects should be discussed with all owners prior to performing BAL.
In this study, a weight-adjusted aliquot volume was used based on a report that they provided more uniform epithelial lining fluid recovery in healthy, purpose-bred Beagles (Melamies, 2011). Compared with previous reports, we did not experience an increased mortality rate associated with the weight-adjusted aliquot volume, compared with previous reports (Hawkins, 1995); however, we did experience technical challenges associated with 1 mL/kg aliquots in larger dogs. Multiple suction traps were used during the BAL procedure with SPA, which required equipment changes during the procedure. Suction trap connections with larger capacities (40-mL and 80-mL) exist and their use should be considered for BAL with SPA. In addition, the use of weight-adjusted BAL aliquots in large dogs (>20 kg) (Melamies, 2011) or in dogs with respiratory tract disease has not been critically evaluated. This warrants further investigation, as 1 mL/kg BAL aliquots may be unnecessary.

A limitation of this study was that the majority of dogs received prior or concurrent medications at the time of BAL, which may have affected the nature of BALF. In addition, none of our diagnoses were confirmed by pulmonary histopathology. Other limitations included the lack of long-term patient follow-up and absence of cases with pulmonary neoplasia. A high diagnostic yield had been previously reported from BALF cytologic assessment for dogs with lymphoma and carcinoma (Hawkins, 1993; Hawkins, 1995). Inclusion of patients with pulmonary neoplasia may have altered the rate of definitive diagnoses achieved from BALF cytology.

4.5 Conclusions:

The results identified that SPA with a maximum negative pressure of 50 mmHg resulted in higher infusate retrieval and improved cellularity scores for BALF in dogs with respiratory tract disease when compared to MA. These findings, however, did not correspond to a higher diagnostic yield from BALF cytology. There was moderate agreement between cytology of BALF acquired by both techniques and substantial agreement between the cytologic diagnosis scores. Both the MA and SPA techniques were suitable for BAL in dogs with respiratory tract disease. Further evaluation of weight-adjusted and fixed volume BAL aliquot volumes may be required in dogs with respiratory tract disease.

20 40-cc and 80-cc sterile mucus specimen traps, Stevens Company Limited, Brampton, ON.
CHAPTER 5:
MICROBIAL CULTURE RESULTS FROM CANINE BRONCHOALVEOLAR LAVAGE FLUID

5.1 Introduction:
The canine tracheobronchial tree is not continuously sterile, as the act of inhalation and aspiration of external or normal oropharyngeal microflora expose the lower respiratory tract to pathogens. Up to 50% of tracheal swabs from clinically healthy dogs are positive for bacterial growth (Lindsey, 1978; McKiernan, 1984). However, the lower respiratory tract has physical defense mechanisms to prevent colonization of pathogens in the lungs (Cohn, 2007) and, subsequently, bacterial pneumonia is uncommon in healthy animals. When normal defense mechanisms fail, or when animals are immunosuppressed, this pathogen exposure can lead to infectious pneumonia (Lee-Fowler, 2012).

Clinical signs of infectious pneumonia in dogs include cough, exercise intolerance, mucopurulent nasal discharge, increased respiration rate or effort, changes in pulmonary auscultation, variable fever, inappetence, depression, weight loss, dehydration, and/or hemoptysis (Lee-Fowler, 2012; Kogan, 2008; Tart, 2010). Canine bacterial pneumonia can be single or mixed pathogen infections, with the majority of infections caused by resident microflora (Lindsey, 1978). Anaerobic bacterial pneumonia is often, but not always, associated with a pulmonary foreign body and can occur in up to 41% of canine bacterial pneumonia cases (Lee-Fowler, 2012).

Mycoplasmas are the smallest free-living organisms, which lack cell walls and depend on the environment for nourishment (Greene, 2012). Pathogenic mycoplasmas have tropisms for different tissues, such as erythrocytes, and the respiratory and genital tracts (Greene, 2012). *Mycoplasma* spp. are pulmonary pathogens in cats and humans (Greene, 2012); however, a similar pathogenic role for *Mycoplasma* spp. has not been consistently reported in dogs. While certain species of *Mycoplasma* have failed to result in experimental infection in dogs, some *Mycoplasma* spp. have been identified as the sole pathogen in canine lower respiratory disease (Chandler, 2002). *M. cynos* is associated with increased severity of canine infectious respiratory disease (Chalker, 2004) and mycoplasmal growth has been isolated in up to 78% of young dogs with *Bordetella bronchiseptica* infection (Randolph, 1993). In addition, *M. cynos* has been cultured as the primary pathogen in canine pneumonia from shelter dogs and a litter of puppies (Chalker, 2004; Zeugswetter, 2007).

The purpose of this study was to describe aerobic bacterial and mycoplasmal growth in BALF from clinical healthy dogs and dogs with respiratory tract disease. We hypothesized that aerobic bacterial growth and mycoplasmal growth would be associated with clinical respiratory tract disease in dogs.

5.2 Materials and Methods:
Aliquots of surplus canine BALF, collected via bronchoscopic guidance during three studies reported in chapters 2, 3, and 4 were submitted for aerobic bacterial and mycoplasmal cultures at the time of sample collection. The original study protocols were approved by the University of Guelph’s Animal Care Committee. Purpose-bred Beagles were cared for in
accordance with Canadian Council of Animal Care guidelines and housed in a Canadian Council of Animal Care-accredited facility. Purpose-bred Beagles were determined to be free from respiratory tract disease based on the results of history, physical examination, CBC, biochemical profile, and orthogonal thoracic radiographs. In the groups of healthy purpose-bred Beagles, BALF samples from two lung lobes were placed immediately on ice, processed for microbial cultures within 120 minutes of collection, and cultured separately. Client-owned dogs that presented to the Ontario Veterinary College Health Sciences Centre and for which bronchoscopy and BAL were recommended as part of their diagnostic work-up were eligible for enrolment in the original prospective study. Written client consent was also required for enrolment. Dogs were excluded if bronchoscopic guided BAL could not be performed. In the group of client-owned dogs with respiratory tract disease, BALF samples from two lung lobes were pooled, and processed for microbial cultures within 40 minutes of collection. Microbial cultures were performed at a veterinary reference laboratory\textsuperscript{1}. Aerobic bacterial cultures and susceptibility testing was performed according to standards established by the Clinical Laboratory Standards Institute\textsuperscript{2} using disk dilution technique. Mycoplasmal cultures were performed on Hayflick’s medium as per standard protocols\textsuperscript{3}. Susceptibility testing was not performed on mycoplasmal species.

Semi-quantitative culture results were reported as follows: no growth, R+ for bacteria isolated on replating, 1+ for occasional organisms isolated, 2+ for few organisms isolated, 3+ for moderate amount of organisms isolated, and 4+ for large number of organisms isolated. For aerobic bacterial cultures, the bacteriologist considered 3+ to 4+ growth clinically relevant, extrapolated from previously reported data for specificity of quantitative canine BAL bacterial cultures for bacterial pneumonia (Peeters, 2000).

Aliquots of BALF were routinely processed and assessed cytologically by a board-certified clinical pathologist as part of the previously reported studies (Woods, 2013; chapters 3 and 4). Inflammation identified in BALF (TNCC>500 cells/µL) was classified as suppurative (>12% neutrophils), eosinophilic (>14% eosinophils), lymphocytic (>16% lymphocytes) or mixed (elevated proportions of ≥2 types of leukocyte) (Hawkins, 1995; Johnson, 2013). As the canine oropharynx is not sterile, culture results from samples with cytologic evidence of oropharyngeal contamination (squamous epithelial cells and/or Simonsiella sp. bacteria) were excluded from analysis (English, 2008). For dogs with available culture results from two BALF samples (acquired during the same procedure), results were reported together on a “per dog” basis.

\textsuperscript{1} Animal Health Laboratory, University of Guelph Laboratory Services, Guelph, ON.
Statistical Analysis – Statistical analyses were performed with statistical software programs\textsuperscript{4,5}. Associations between groups were assessed using logistic regression. For all analyses, $P<0.05$ was considered significant.

5.3 Results:

The study populations included 23 healthy purpose-bred Beagles and 23 client-owned dogs with respiratory tract disease. None of the healthy Beagles were receiving or had received antimicrobial drugs within 30 days of BALF collection. The group of healthy Beagles consisted of 12 male dogs (9 castrated males, 3 intact males) and 11 female dogs (11 spayed females, no intact females). Mean age and weight of the healthy Beagles was $8.6 \pm SD 1.4$ years and $11 \pm SD 1.8$ kg, respectively. Seven ($7/23 = 30.4\%$) of the client-owned dogs were receiving antimicrobial drugs (doxycycline, enrofloxacin, or cephalexin) at the time of BALF collection. Six of these dogs were receiving antibiotics with known anti-mycoplasmal activity. The group of client-owned dogs consisted of 14 male dogs (10 castrated males, 4 intact males) and 9 females (8 spayed females, 1 intact female). Mean age and weight of the client-owned dogs was $6.75 \pm SD 4.39$ years and $23.4 \pm SD 17.8$ kg, respectively. Breeds represented in the group of client-owned dogs included mixed breed canine (6), Labrador Retriever (3), German Shepherd Dog (2), and one of each of the following: Rhodesian Ridgeback, Portuguese Water Dog, Toy Poodle, Shih Tzu, Havanese, Whippet, Boston Terrier, Coonhound, Cane Corso, Beagle, Dachshund, and Yorkshire Terrier. The most common, not mutually exclusive, presenting complaints for the group of client-owned dogs were chronic cough (13), lethargy (6) and tachypnea (5). Gagging (3), acute productive cough (3), chronic nasal discharge (2), stertor (2), hemoptysis (1), reverse sneezing (1), and exercise intolerance (1) were also reported as presenting complaints. Duration of clinical signs prior to presentation ranged from 1 day to 2 years (median 3 months, IQR 6.25 months).

Aerobic bacterial cultures were performed on all BALF samples. Mycoplasmal cultures were not performed on BALF samples from two client-owned dogs, at the discretion of the attending clinician. Seven out of 46 BALF samples from the 23 healthy Beagles had cytologic evidence of oropharyngeal contamination, and their culture results were excluded from analysis. All but one healthy Beagle had culture results from at least one BALF sample for analysis. One pooled BALF culture from the group of client-owned dogs was excluded based on evidence of oropharyngeal contamination.

Fourteen of 22 (63.6\%) healthy Beagles were positive for *Mycoplasma* spp., all with 1+ level of growth, and five (5/14, 35.7\%) of these dogs were also positive for aerobic bacterial growth. All Beagles with aerobic bacterial growth (5/22 = 22.7\%) were also positive for mycoplasmal growth. All five Beagles with aerobic bacterial growth showed low levels of growth (3 dogs with 1+ growth, 2 dogs with 2+ growth). Organisms isolated from healthy purpose-bred Beagles are listed in Table 5.1. The remaining 8 healthy Beagle dogs (36.4\%) were negative for both bacterial and mycoplasmal growth.

\textsuperscript{5} IBM® SPSS® Statistics, IBM Corporation, Markham, ON.
Table 5.1 – Bacterial and mycoplasmal recovery from 22 healthy purpose-bred Beagles.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma spp.</td>
<td>M. edwardii</td>
<td>7 (31.8%)</td>
</tr>
<tr>
<td></td>
<td>M. spumans</td>
<td>6 (27.3%)</td>
</tr>
<tr>
<td></td>
<td>M. canis</td>
<td>4 (18.2%)</td>
</tr>
<tr>
<td></td>
<td>M. maculosum</td>
<td>3 (13.6%)</td>
</tr>
<tr>
<td></td>
<td>M. molare</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>Untyped</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td>Pasteurella spp.</td>
<td>P. multocida</td>
<td>3 (13.6%)</td>
</tr>
<tr>
<td></td>
<td>P. canis</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td>Neisseria spp.</td>
<td>NR</td>
<td>2 (9.1%)</td>
</tr>
</tbody>
</table>

Legend: NR = not reported.

Seven of 22 (31.8%) of client-owned dogs with respiratory tract disease were positive for aerobic bacterial growth. Three dogs had 3+ level of growth, three dogs had 2+ level of growth and one dog had 1+ level of growth. Two of the three client owned dogs with 2+ aerobic bacterial growth were receiving concurrent antibiotic therapy. Four dogs (4/20 = 20%) were positive for Mycoplasma spp., three of which had 1+ level of growth and one had 4+ level of growth. All of the dogs with positive mycoplasmal cultures also had positive aerobic bacterial growth. One of the dogs with positive aerobic bacterial culture did not have mycoplasmal culture performed. Organisms isolated from dogs with respiratory tract disease are listed in Table 5.2. No dogs with respiratory tract disease were positive for only Mycoplasma spp. The remaining sixteen dogs (72.7%) client-owned dogs were negative for both bacterial and mycoplasmal growth.

Table 5.2 – Bacterial and mycoplasmal recovery from 22 client-owned dogs with respiratory tract disease.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma spp*.</td>
<td>M. maculosum</td>
<td>2 (9.1%)</td>
</tr>
<tr>
<td></td>
<td>M. spumans</td>
<td>2 (9.1%)</td>
</tr>
<tr>
<td></td>
<td>M. edwardii</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>M. canis</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>M. molare</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td>Pasteurella spp.</td>
<td>P. canis</td>
<td>2 (9.1%)</td>
</tr>
<tr>
<td>Bordetella spp.</td>
<td>B. bronchiseptica</td>
<td>2 (9.1%)</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>E. faecalis</td>
<td>1 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>E. avium</td>
<td>1 (4.5%)</td>
</tr>
</tbody>
</table>

Legend: NR = not reported; * Mycoplasmal culture results were based on samples from 20 dogs.

There was no statistically significant association between age or sex and bacterial growth (P=0.42 and P=0.45, respectively) or mycoplasmal growth (P=0.53 and P=0.39, respectively) on BALF culture. There was no significant difference in the likelihood of aerobic bacterial growth in dogs with respiratory tract disease, when compared with healthy dogs (P=0.74). Dogs with respiratory tract disease had 0.151 lower likelihood of having positive mycoplasmal growth from BALF fluid, when compared with healthy dogs (P=0.01).
Cytology of BALF from healthy Beagles identified mixed inflammation (6), lymphocytic inflammation (4), and suppurative inflammation (3). Cytology of BALF from dogs with respiratory tract disease identified suppurative inflammation (5), mixed inflammation (5), eosinophilic inflammation (3), and lymphocytic inflammation (2). Dogs with mixed inflammation on BALF were 6.7 times more likely to have positive aerobic bacterial growth than dogs with normal BALF cytology ($P=0.039$). There were no significant associations between BALF cytology and mycoplasmal growth.

5.4 Discussion:
In the current study, we assessed aerobic bacterial and mycoplasmal growth from BALF samples of clinically healthy dogs and dogs with lower respiratory tract disease in an effort to determine the role of these pathogens in health and disease.

The tracheobronchial tree is not sterile in healthy dogs. As such, assessment of BALF cytology for the presence of intracellular bacteria, in combination with semi-quantitative or quantitative bacterial cultures is recommended to distinguish true infection from airway colonization. Unfortunately, *Mycoplasma* spp. may not be identified by cytology due to their lack of staining characteristics. A threshold of $1.7 \times 10^3$ colony forming units per mL has been reported to have 86% sensitivity and 100% specificity for bacterial pneumonia in canine BALF samples (Peeters, 2000). Quantitative bacterial cultures were not performed on BALF at our institution; however, a semi-quantitative assessment of growth was reported by the bacteriologist. All healthy dogs in our study with positive bacterial cultures exhibited a low level of growth (1+ or 2+), consistent with temporary airway colonization. Intracellular bacteria were not identified cytologically in BALF from healthy dogs with positive aerobic bacterial growth. One client owned dog exhibited 1+ aerobic bacterial growth and was also considered to have temporary airway colonization. The majority of client-owned dogs with positive bacterial cultures exhibited higher levels of aerobic bacterial growth than the healthy dogs, and four of these six dogs (66.7%) also had intracellular bacteria identified on cytology. Bacteria isolated in our study were consistent with isolates previously reported in the veterinary literature (Lee-Fowler, 2012).

*Mycoplasma* spp. are the smallest free-living organisms, which rely on host secretions for nourishment. A variety of infections in various hosts have been attributed to mycoplasmal infection (Greene, 2012); however, the role of mycoplasmal infections in canine lower respiratory disease remains uncertain. *Mycoplasma* spp. are normal inhabitants of the oropharynx (Randolph, 1993), and a variety of *Mycoplasma* spp. have been isolated from the lower respiratory tract of up to 34% of healthy dogs (Greene, 2012; Randolph, 1993). Conflicting reports exist as to whether a higher prevalence of mycoplasmal growth from the lower respiratory tract is associated with canine lower respiratory tract disease. In one study, an increased prevalence of *M. canis* is reported in dogs with respiratory disease (30%, versus 21% in healthy dogs) (Chalker, 2004). Other studies have reported similar prevalence of mycoplasmal and ureaplasmal growth from pulmonary samples between groups of healthy and diseased dogs (Randolph, 1993). In the current study, we identified a lower risk of mycoplasmal growth in dogs with respiratory tract disease, when compared with a group of healthy purpose-bred Beagles. *M. cynos*, which is the only mycoplasmal strain consistently reported as a primary pathogen in canine lower respiratory tract disease (Chalker, 2004; Hong, 2012; Zeugswetter,
was not isolated from dogs with lower respiratory tract disease in the present study. In addition, mycoplasmal isolates were not identified as the sole microorganism in any of our client-owned dogs with respiratory tract disease. These results suggested that either the *Mycoplasma* spp. isolated in this study were not associated with respiratory tract disease in dogs or that they were secondary pathogens. However, the inability to find a significant difference in mycoplasmal growth could be due to the fact that the group of purpose-bred Beagles was not an appropriate control group for client-owned dogs with respiratory tract disease. The purpose-bred Beagles were not age-matched the group of client-owned dogs, with the Beagles being older, since microbial analysis of BALF from the Beagles was supplemental information from the initial studies in chapters 2 and 3 (Woods, 2013). Both higher and lower mycoplasmal prevalence were reported in older dogs in previous studies evaluating pulmonary mycoplasmal colonization (Randolph, 1993; Jameson, 1995). It was also suggested that group housing of dogs allowed for maintenance of mycoplasmal strains within kennel situations (Mannering, 2009). Since the Beagles in this study were group housed, it is possible that they had a higher prevalence of mycoplasmal colonization than dogs living in a private household (like the client owned patients included in this study). Finally, many of the client-owned dogs were receiving antibiotic therapy with known anti-mycoplasmal activity at the time of BALF collection. This likely interfered with both aerobic bacterial and mycoplasmal culture, resulting in false negative results.

Bacterial and mycoplasmal pneumonia had been previously associated with suppurative and mixed inflammation on BALF cytology (English, 2008; Greene, 2012; Hawkins, 1995). Normal BALF cytology had also been reported in dogs from which *Mycoplasma* spp. were isolated (Jameson, 1995). Results from the current study were able to substantiate an association between mixed inflammation on BALF cytology and aerobic bacterial growth. No other significant associations were identified between other types of inflammatory BALF or with mycoplasmal growth.

Limitations of this study included small sample size, which limited our ability to detect significant associations, and that anaerobic bacterial cultures were not performed in any dogs. This may have resulted in false negative bacterial culture results. In addition, the group of healthy purpose-bred Beagles may not represent an adequate control group for the group of client owned dogs with respiratory tract disease, due to dissimilarities in housing and age, and antibiotic treatment on client owned dogs

5.5 Conclusions:

Oral microflora, including bacterial and *Mycoplasma* spp. were commonly isolated from BALF of clinically healthy dogs. The use of semi-quantitative or quantitative bacterial cultures, in addition to the presence of intracellular bacteria on cytology, was useful for distinguishing bacterial pneumonia from colonization. Dogs with mixed inflammation on BALF cytology were more likely to have positive aerobic bacterial culture results. A role for *Mycoplasma* spp. as a primary respiratory pathogen was not identified in this study. Further studies are required to define the role of *Mycoplasma* spp. in canine respiratory tract disease.
BAL is a minimally invasive technique for sampling the lower respiratory tract; however, the analysis of BALF is difficult to standardize due to the differences in BAL protocols and BALF sample handling. These differences can affect sample quality, TNCC, and differential leukocyte counts within BALF (Melamies, 2011; Mordelet-Dambrine, 1984). Variable concentrations of ELF within the retrieved BALF combined with a lack of reliable markers of dilution further limit the utility BAL in the search for biomarkers of pulmonary disease. In addition, the composition of BALF in dogs has been shown to change with age and with environment (Mercier, 2010; Collie, 1997; Baldwin, 1993). For example, younger dogs (<4.5 years) have a higher proportion of neutrophils in BALF and older dogs (>11 years old) have a higher proportion of lymphocytes in BALF when compared with middle-aged dogs (6-7.8 years) (Mercier, 2010). In another study, the proportion of eosinophils in BALF is reported to be variable and unrelated to airway reactivity in healthy group-housed purpose-bred Beagles (Collie, 1997). In some of these Beagles, the proportion of eosinophils in BALF is higher than the reference interval for healthy dogs (Collie, 1997; English, 2008). Therefore, defining reference intervals for canine BALF parameters remains challenging, despite the fact that BAL has been performed in veterinary medicine for over 20 years (Hawkins, 1990b). While a standardized procedure for canine BAL and BALF processing would not reduce patient-origin variability, it might aid in the development of reference intervals transferable between clinicians and institutions.

The main objective of this dissertation was to assess the effect of different methods for retrieving BAL infusate on sample quality in order to determine an optimal procedure. To that end, our main findings were that SPA consistently yielded more BALF than MA and MAPT. In addition, SPA improved sample quality as indicated by higher surfactant and lower epithelial cell scores when compared to MAPT. A comparison of MAPT and SPA in dogs with respiratory tract disease was initiated, but aborted after three samples were collected because MAPT appeared to induce moderately severe hemorrhage in one sample, and resulted in bronchial mucosal abrasions in another patient (Appendix 4). The SPA technique when compared to MA resulted in significantly higher BALF cellularity scores in dogs with respiratory tract disease and moderate agreement between the types of inflammation. There was no significant difference in the rate or nature of cytologic diagnosis.

These findings confirmed the hypothesis that aspiration technique significantly affects BALF samples. The results indicated that the MAPT technique cannot be recommended for BAL in dogs, due to the increased rate of iatrogenic trauma to the bronchial mucosa in healthy dogs. A limitation of SPA compared to MA was that the former requires specialized equipment (disposable suction trap and suction pump and/or suction pressure regulator). Yet, the cost associated with this equipment was minimal and, therefore, the SPA technique should be feasible for application in general practice. Despite lack of significant difference in the rate of cytologic diagnosis between BALF acquired by MA and SPA techniques, SPA consistently provided BALF samples with higher infusate retrieval and better sample quality. Therefore, it is recommended that for a standardized canine BAL protocol, the SPA technique should be applied.
In this research, other aspects of the BAL protocol and sample handling were standardized, in addition to aspiration techniques. For example, a standard weight-adjusted infusate volume was used. In addition, the site of lavage in healthy dogs was standardized as this may affect BALF retrieval. In people, greater BALF recovery was reported from the right middle and lingular lung lobes (Klech, 1989; Goldstein, 1990; Pingleton, 1983). It is unknown why these lobes would yield higher infusate retrieval in humans. Non-bronchoscopic BAL in dogs resulted in more frequent sampling from the dependent caudal lung lobe (Hawkins, 1999), likely secondary to the effects of gravity. In a study evaluating the effect of BAL site in healthy dogs, an increased cell recovery from the left caudal lung lobe compared to the right middle lung lobe was reported (Carré, 1985). Based on this information, the bilateral caudal lung lobes were selected as sites for BAL in the healthy purpose-bred dogs in order to minimize variability of infusate retrieval due to anatomic and positional differences. In the group of dogs with respiratory tract disease, sites of BAL were chosen based on abnormalities visualized on thoracic radiography and bronchoscopy, in order to retrieve BALF samples that were relevant to the patient. This precluded having a standardized site for BAL in these patients; however, a contralateral lung lobe was sampled in each case.

Once submitted to the veterinary reference laboratory, all BALF samples were processed in an identical fashion, as it was reported that time until processing and different protocols for cytocentrifugation altered differential cell counts in BALF (Dehard, 2008; Mordelet-Dambrine, 1984; Rebar, 1980). Despite the use of a standard BAL protocol, some of our BALF samples from healthy dogs had adequate TNCC via electrical impedance, but acellular cytocentrifuge slides. In most of these BALF samples, low numbers of alveolar macrophages and/or the presence of epithelial cells were consistent with an inadequate BAL procedure, and oropharyngeal contamination or bronchial, rather than alveolar, washes. However, the acellular slide in one dog was the result of laboratory error, likely associated with failure of the cytocentrifuge to prepare a properly concentrated sedimented slide. Since the laboratory did not prepare additional cytocentrifuge slides, and the slides from this dog were excluded from analysis.

Despite the use of a standardized protocol for BAL in the healthy dogs, creation of a reference interval from these data is not recommended because the composition of BALF from group-housed purpose-bred dogs may differ significantly from that of client-owned dogs (Collie, 1997). For example, increased dog-to-dog contact secondary to group housing can facilitate transmission of respiratory pathogens (e.g. *Mycoplasma* spp.) (Chalker, 2004; Mannering, 2009), and mycoplasmal pulmonary infection has been correlated to suppurative and mixed inflammation in canine BALF (English, 2008; Rosendal, 1977). In addition, group-housed dogs may be exposed to increased environment allergens within kennels, which can result in eosinophilic inflammation in BALF secondary to reactive airway disease (Baldwin, 1993). These hypotheses were supported by the findings that BALF from some of the healthy purpose-bred dogs had elevated TNCCs, elevated proportions of lymphocytes compared to historical canine BALF differential cell count reference intervals, and/or increased *Mycoplasma* spp. isolation when compared with the dogs with respiratory tract disease. In addition, some of the healthy dogs also had pulmonary osteomas, mild bronchial and/or interstitial changes on

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pulmonary radiographs (consistent with aging changes), and/or compensated left heart disease (Lamb, 2002). Since pulmonary histology and pulmonary function testing were not performed in these dogs, it was not possible to confirm that these dogs were truly normal and thus it was not possible to rule out an effect from these changes on the composition of BALF.

Isolation of *Mycoplasma* spp. was significantly higher in our group of healthy purpose-bred dogs when compared to dogs with respiratory tract disease. This was not surprising, since higher prevalence of mycoplasmal growth had been reported from the lungs of group-housed dogs (Chalker, 2004) and because many of the dogs with respiratory tract disease were receiving antimicrobial drugs with anti-mycoplasmal activity at the time of BAL. However, since *Mycoplasma* spp. are normal flora of the oropharynx, it is possible that our results were influenced by oropharyngeal contamination of the bronchoscope during trans-glottal passage of the bronchoscope into the trachea. Guarding the bronchoscope tip with a sterile cover during intranasal passage was recommended for collection of BALF samples for bacterial culture in foals (Hoffman, 1991). However, in a recent canine study it was found that careful trans-glottal passage of the bronchoscope resulted in negligible contamination of BALF by oropharyngeal flora (Hirt, 2010). Guarding the bronchoscope tip during trans-glottal passage and the effect on microbial culture results has not been evaluated in dogs. In addition, despite the use of cold sterilization protocols of bronchoscopes between patients, there was anecdotal concern about microbial contamination when BALF for culture is acquired directly through the bronchoscope’s biopsy channel (e.g. via SPA or MA). In data acquired in chapter 1, no significant difference between positive aerobic culture results acquired by MAPT or SPA was found ($P=0.083$). This indicated that there was no significant increase in bacterial growth from BALF samples collected directly through the bronchoscope’s biopsy channel when compared to BALF samples collected through sterile tubing.

A limitation of the studies reported here was relatively small sample size. This limitation might be most relevant to the study in chapter 4 where the small number of dogs hampered our ability to detect associations between thoracic radiographic changes and diagnostic utility of BALF cytology. In particular, it was not possible to assess for differences associated with focal versus diffuse radiographic lesions. The studies reported here were also limited by the fact that acceptable definitions for cell preservation and RBC scores within cytology quality score were based on expert opinion, rather than evidence-based recommendations. In addition, cell preservation in these studies was determined from microscopic features of cellular degeneration (e.g. cell membrane rupture); however, reproducibility of the cell preservation score could have been strengthened by use of a dye viability test to quantify the proportion of viable cells in the BALF samples. A final limitation was that multiple clinicians performed the BAL procedures in the group of dogs with respiratory tract disease. Some of the clinicians were residents in training, and having variably experienced clinicians perform bronchoscopy and BAL may have influenced BALF retrieval and sample quality in samples acquired by both aspiration techniques in chapter 4.

Aspects of the canine BAL technique that warrant further investigation include the evaluation of SPA at varying negative pressures on BALF sample quality, the modification of
MA so that a Luer-lock syringe$^2$ is attached directly to the ridge of the bronchoscope biopsy channel, weight adjusted versus fixed volume BAL infusate in dogs >20 kg, and how guarding the bronchoscope during oropharyngeal passage affects mycoplasmal recovery. In addition, there are no studies assessing the effects of infusate volume or aspiration technique in cats. As BAL in feline patients is associated with higher rates of adverse effects and bronchoconstriction, it is possible that MA would be the preferred aspiration technique in this species or that SPA at lower negative pressures may be required.

Conclusions:
In healthy dogs and in dogs with respiratory tract disease, our results indicated that SPA resulted in retrieval of a significantly higher volume of BALF than did MA and MAPT. The SPA technique also yielded improved sample quality and cellularity scores, without negatively affecting cell preservation when compared to MA and MAPT. However, these results did not correspond to a higher rate of diagnosis in dogs with pulmonary disease. These results indicated that both MA and SPA can be used when performing BAL in dogs with respiratory tract disease. Yet, for the purpose of creating a standardized BAL technique in dogs, SPA is recommended for BALF retrieval due to improved sample quality.

$^2$ Kendall Monoject\textsuperscript{TM} Luer lock tip 35 mL syringe, Tyco Healthcare Group, Mansfield, MA.
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APPENDIX 1

Appendix 1. Responses from a survey of veterinary internal medicine specialists via the American College of Veterinary Internal Medicine e-mail listserv regarding techniques used for canine bronchoalveolar lavage. Three multiple-choice questions were e-mailed to the American College of Veterinary Internal Medicine e-mail listserv in September 2011. There were 28 respondents, out of 1328 members, within four weeks (12 internists from academia and 16 from private referral practice).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Selection</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoscopy</td>
<td>Bronchoscope Only</td>
<td>14/28 = 50.0%</td>
</tr>
<tr>
<td></td>
<td>Gastroscope Only</td>
<td>1/28 = 3.6%</td>
</tr>
<tr>
<td></td>
<td>Endoscope Based on Patient Weight</td>
<td>13/28 = 46.4%</td>
</tr>
<tr>
<td>BAL</td>
<td>Estimated Volume</td>
<td>14/28 = 50.0%</td>
</tr>
<tr>
<td>Infusate Volume</td>
<td>Weight-Adjusted Volume</td>
<td>11/28 = 39.3%</td>
</tr>
<tr>
<td></td>
<td>Fixed Volume Independent of Weight</td>
<td>3/28 = 10.7%</td>
</tr>
<tr>
<td>Aspiration Technique</td>
<td>Manual Aspiration Directly to Endoscope</td>
<td>13/28 = 46.4%</td>
</tr>
<tr>
<td></td>
<td>Manual Aspiration Via Polyethylene Tubing</td>
<td>7/28 = 25.0%</td>
</tr>
<tr>
<td></td>
<td>Suction Pump Aspiration Via Suction Trap</td>
<td>6/28 = 21.4%</td>
</tr>
<tr>
<td></td>
<td>Suction Pump Aspiration Directly to Endoscope</td>
<td>2/28 = 7.2%</td>
</tr>
</tbody>
</table>
**APPENDIX 2**

**Appendix 2.** Criteria used for microscopic assessment of quality of cytocentrifuge preparations of BALF collected from healthy Beagles.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Scale</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellularity (No. of cells/slide)</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10 to 100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100 to 200</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>200 to 500</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

| Cell preservation              | 0     | <10            |
| (% of well-preserved cells/slide)| 1     | 10 to 25      |
|                                 | 2     | 25 to 50       |
|                                 | 3     | 50 to 80       |
|                                 | 4     | >80            |

| Epithelial cells (No. of cells/slide) | 0     | Absent         |
|                                       | 1     | <5             |
|                                       | 2     | 51 to 100      |
|                                       | 3     | 101 to 200     |
|                                       | 4     | >201           |

| RBCs (% of cells/slide)           | 0     | ≤1             |
|                                   | 1     | 2 to 3         |
|                                   | 2     | 4 to 5         |
|                                   | 3     | ≥6             |

| Bacteria (No. of cells/slide)     | 0     | Absent         |
|                                   | 1     | <5             |
|                                   | 2     | 6 to 10        |
|                                   | 3     | 11 to 20       |
|                                   | 4     | >21            |
APPENDIX 3

Appendix 3. Video bronchoscope cleaning and cold sterilization protocol.

1. Pre-cleaning
   • Following the patient procedure, flush sterile water through the bronchoscope biopsy channel and wipe the outer working length with moistened gauze until there is no visible debris.

2. Leakage testing
   • Place water resistant cap onto the videoscope cable connector.
   • Attach leak tester to the water resistant cap.
   • Turn on the leak tester and confirm expansion of the angulation rubber (at bronchoscope tip).
   • Submerge the bronchoscope in clean water and monitor for bubbles.
     • Bubbles indicate damage to the bronchoscope and cleaning should be aborted if damage is identified.

3. Manual cleaning
   • Add 45-mL of enzymatic detergent to a container of clean water.
   • Submerge the bronchoscope in enzymatic detergent solution and attach cleaning lines to the aspiration valve and biopsy channel.
   • Flush 180-mL of enzymatic detergent solution through the cleaning lines.
   • Wipe the outer working length of the bronchoscope.
   • Remove cleaning lines and pass the cleaning brush through the biopsy channel until no visible debris is identified (minimum of three passes).
   • Re-attach cleaning lines and flush 180-mL of enzymatic detergent solution through the lines
   • Remove the bronchoscope from enzymatic detergent solution and flush 180-mL of air through the cleaning lines.
   • Submerge the bronchoscope in a container of clean water.
   • Flush 180-mL clean water through cleaning lines.
   • Flush 180-mL air through cleaning lines.

4. High level disinfection
   • Using personal protection equipment, submerge the bronchoscope into glutaraldehyde solution.
   • Flush 180-mL of glutaraldehyde solution through the cleaning lines.
   • Let stand for 10 minutes.
   • Flush 180-mL of air through the cleaning lines.
   • Transfer the bronchoscope to a container of sterile deionized water.
   • Flush 180-mL of deionized water through the cleaning lines.
   • Flush 180-mL of air through cleaning lines.
   • Remove cleaning lines and hang bronchoscope to dry.

5. Alcohol flush (end-of-day cleaning)
   • Flush 60-mL of 70% isopropyl alcohol through the cleaning lines.
   • Flush 180-mL of air through the cleaning lines.
   • Remove cleaning lines and hang bronchoscope to dry.
Appendix 4. Comparison of results for BALF samples obtained by MAPT and SPA from three client owned dogs with naturally occurring respiratory tract disease.

<table>
<thead>
<tr>
<th>Category</th>
<th>Variable</th>
<th>Median for MAPT samples</th>
<th>Median for SPA samples</th>
<th>P value* for distribution of differences between MA and SPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF Procedure</td>
<td>Lowest oxygen saturation (%)</td>
<td>90</td>
<td>94</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Percentage of retrieved infusate (%)†</td>
<td>19.2</td>
<td>30.0</td>
<td>0.60</td>
</tr>
<tr>
<td>Quality score for cytologic evaluation‡</td>
<td>Cellularity</td>
<td>4</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Cell preservation</td>
<td>4</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>RBC</td>
<td>3</td>
<td>3</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Epithelial cells</td>
<td>0</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Extra-cellular bacteria</td>
<td>0</td>
<td>1</td>
<td>0.18</td>
</tr>
<tr>
<td>BALF parameter</td>
<td>TNCC (No. of cells/µL)</td>
<td>3500</td>
<td>3500</td>
<td>0.18</td>
</tr>
<tr>
<td>Differential cell count (proportion)</td>
<td>Macrophage (%)</td>
<td>16</td>
<td>37</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Neutrophil (%)</td>
<td>72</td>
<td>61</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte (%)</td>
<td>3</td>
<td>2</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Eosinophil (%)</td>
<td>1</td>
<td>2</td>
<td>0.18</td>
</tr>
<tr>
<td>Cytologic diagnosis</td>
<td>Cytologic diagnosis score</td>
<td>0</td>
<td>1</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Values <0.05 indicate that results were significantly different between the MA and SPA techniques; ‡ Within this category, each variable was scored on a scale of 0 to 4, except for RBC, which was scored on a scale of 0 to 3.