Veterinary Medical Journal-Giza (ISSN 1110-1423)
Faculty of Veterinary Medicine,Cairo University
Accredited from national authority for Quality Assurance and Accreditation
Giza, 12211, Egypt

Cytological, Biochemical and Bacteriological Analysis of Bronchoalveolar Lavage Fluids in Healthy Cosmopolitan Dogs

A.A. Kubesy, M.R. Khattab*, S.E. Salem**, N.Y. Salem* and M.E. Ali**

* Dept. of Med. and Infect. Dis., Facult. of Vet. Med., Cairo University
** Dept. of Clinical Pathol., Facult. of Vet. Med., Cairo University

Abstract

Epithelial lining fluids (ELF) from alveoli and airways can be diagnostic for lower respiratory diseases. However few studies are concerning in establishing reference range for cytological and non-cytological constituents of bronchoalveolar lavage (BAL) especially after urea adjustment to overcome the lack of uniformity of ELF fraction in the retrieved fluid. This study was conducted to analyze different BAL constituents with urea adjustment. For this purpose 8 dogs were used in this and BALF collected was examined cytologically, biochemically and bacteriologically. The result showed predominance of alveolar macrophage (78% ± 1.85) followed by lymphocytes (9% ± 2.07) in the cytological axis and the range of different biochemical constituents including ALP (197 U/L), LDH (113 U/L), Ca (15 mg/dl), and phosphorous (4 mg/dl), as well as MMP-2, MMP-9 and total gelatinases determination using gelatin zymography.

Bacteriological investigations revealed isolation of *Aceintobacter spp.* and non-mannitol fermenter *Staphylococcous* from 3 healthy dogs.

key words: Cytological, Biochemical , Bacteriological, Analysis of Bronchoalveolar Lavage, dogs.

Introduction

Collection of bronchoalveolar lavage fluids (BALF) is an easy technique for sampling of the canine lower respiratory system. Analysis of BALF provides valuable information in the diagnosis of lower respiratory tract disease **(Woods, 2013)**. BALF provides a minimally invasive method of sampling the fluids lining alveolar epithelium which can be used for cytological, biochemical, immunological and microbial analyses **(Hawkins *et al.*, 2006)**. Defining reference intervals for BAL fluid retrieved from dogs remains challenging **(Hawkins *et al.*, 1990)**. BALF enzymes can be considered as sensitive markers of cellular damage which induced by pathological conditions **(Cobben, 1999)**. Regarding MMPs, gelatinases A (MMP-2) and B (MMP-9) take a principal part in lung tissue remodeling and repair **(Cataldo *et al.*, 2003)**. Bacterial isolates can be retrieved form BALF of normal dogs as the canine tracheobronchial tree is not sterile site **(McKiernan *et al.*, 1984)**. Inconstant concentrations of ELF within the retrieved BAL fluid limit the utility of BALF in evaluation of biomarkers, cytological or biochemical conistetuents of pulmonary disease **(Mercier *et al.*, 2011)**. So determination of ELF fraction in BAL fluid using constant lavage technique and urea adjustment calculation allows a more accurate interpretation of cellular and non-cellular components of ELF **(Mills and**

establish results for cytological, biochemical and microbiological examination of ELF with adjustment of results using urea.

Materials and methods

a- Animals and experimental design:

Eight cosmopolitan (mixed breed) dogs of different ages (3 month to 2 year) and both sexes (4 male and 4 female) were used in this study with great consideration to animal welfare. The dogs were kept in teaching hospital kennel-faculty of Veterinary Medicine, Cairo University. Each animal was exposed to complete clinical examination including; general inspection, physical examination for respiration, pulse, temperature, mucous membrane, superficial lymph nodes and fecal examination (**Houston 2000**).

b- BAL fluid collection:

The animals were generally anaesthetized using ketamine/xylazine combination according to **Humphrey (1971)**. BAL collection was performed according to **Hawkins (2004)**. With animals in sternal recumbancy, routine bronchoscopic examination of the airways was performed first. The bronchoscope is passed into successively smaller airways until a snug fit is achieved between the scope and the airway. Sterile, non-bacteriostatic, 0.9% sodium chloride (saline) solution was instilled through the biopsy channel of the bronchoscope into the airway by preloaded syringe. Immediately on completion of instillation, suction is applied to the same syringe to recover BAL fluid. After retrieval of as much fluid as possible from the first bolus of saline, the process is repeated for one more additional bolus. We routinely used two boluses of 25 ml (50 ml total).

c- Cytological analysis:

Total nucleated cell count, differential nucleated cell count and parasitological examination were examined according to **Hawkins (2004)**. Total nucleated cell counts are performed on undiluted BAL fluid using a hemocytometer. Volume of 200 ul BALF per slide was used to prepare cytological slide. Quick Romanowsky stain (Diff-3 stain® SCICO Diagnostics) was used for staining of cytological preparation. Cytological characterization includes the performance of differential cell counts. A minimum of 200 cells were counted, and qualitative changes were noted. The entire slide was carefully examined for the presence of infectious agents.

d- Biochemical analysis:

BALF and serum Urea were determined according to **Tietz (1990)** using **Urea/BUN – LS** (Modified Urease-Berthlot Method) manufactured by spectrum diagnostics (Egyptian Company for Biotechnology (S.A.E)). for calculation of ELF fraction in lavage fluid.

$$\textbf{Volume}_{ELF} = \textbf{Volume}_{BAL} * \textbf{Urea}_{BAL} / \textbf{Urea}_{serum}$$

BALF alkaline phosphatase (ALP) were determined according to **Tietz (1986)** using **Alkaline Phosphatase Liqui-Color®** Test (P-nitrophenyl-phosphate Methodology) manufactured by Stanbio Laboratory, USA., BALF lactate dehydrogenase (LDH) was determined according to **Kachmar and Moss (1976)** using **LDH Liqui-UV®** Test (Modified Wacker Methodology) manufactured by Stanbio Laboratory, USA., BALF Calcium

(Ca) and phosphorous were determined according to **Bauer (1981)** and **Dryer and Routh (1963)** respectively using **Inorganic Phosphorus Visible** (Fiske - Subbarow method) manufactured by Química Clínica Aplicada S.A. (QCA) spain and **Calcium (CPC) LiquiColor®** Test (Cresolphthalein Complexone Methodology) manufactured by Stanbio Laboratory, USA.

e- Matrix metalloproteinase:

Matrix metalloproteinase – 9 (MMP-9) and Matrix metallo-proteinase – 2 (MMP-2) activities of BALF were determined by gelatin zymography according to the method described by **Hawkes *et al.*, (2001).** Zymography involves the electrophoretic separation of proteins under denaturing (SDS), non reducing conditions through a polyacrylamide gel containing gelatin. The resolved proteins were renatured by exchange of the SDS with a nonionic detergent, such as Triton X-100 and the gel was incubated in an appropriate buffer for the particular proteinases under study. The gel was stained with Coomassie Blue and the proteolytic activities were detected as clear bands against a blue background of un-degraded gelatin.

f- Bacteriological examination:

BAL fluid was cultured for bacterial isolation according to **Johnson *et al.*, (2013).** Standard biochemical methods were used to identify cultured bacteria according to **Jang *et al.*, (2000).** The RapID NF Plus and RapID onE system were used for identification of gram negative bacteria beside traditional identification methods according to **Kitch *et al.*, (1992)** and **Kitch *et al.*, (1994).**

g- Statistical analysis:

The obtained values were presented as means ± SE of the mean. Comparisons between different groups were carried out by independent-samples T test. The level of significance was set at $P < 0.05$ using SPSS software (version 16.0).

Results and discussion

- Clinical examination:

A total number of 8 dogs of different ages (3 month to 2 year) and both sexes (4 male and 4 female) were exposed to comprehensive clinical examination. Body temperature was in normal range, oral, ocular and nasal mucous membranes were slightly pale rosy red without lesions or abnormal discharges, while tonsils, pre-scapular and popliteal lymph nodes were normal.

- Cytological analysis:

The cytological analysis was shown in table (1) and Figure (2). Our results showed that TNCC was 290 cell/ul while by urea adjustment became 1862 cell/ul and consequently the absolute number of differential nucleated cell count will completely changed. On the other hand our differential nucleated cell count showed predominance of alveolar macrophages 78% followed by lymphocytes 9%, neutrophils and eosinophils by 5% each, reactive macrophages 2% then epithelial cell 1%. These result coincided with **Vail *et al.*, (1995)** and **Rajamäki *et al.*, (2001)** who stated that reference intervals used for canine BAL fluid were 300–500 cells/ul composed of 65–85% macrophages along with the absence of intracellular bacteria and less than 5–8% eosinophils, neutrophils, or lymphocytes. **(Mills and**

Lister 2006) Cell counts in lavage fluid are not reflective of cell counts in ELF and may lead to incorrect assumptions of health or disease as range of TNCC of lavage fluid from normal cat 80-580 while TNCC of ELF of these cats ranged from 1660 to 48,100.However disagreed with **Mayer *et al.*, (1990)** who mentioned that cytological findings for 18 healthy dogs infused with 30ml saline/dog and the return percentage was 58.7-71% with percent of 50% macrophages, 30% lymphocytes, 1.5% neutrophils and 15 % epithelial cells. However, all references agreed with predominance of alveolar macrophage in normal BALF specimens.

- Biochemical analysis:

Table (2) showed values of non-adjusted alkaline phosphatase, lactate dehydrogenase, calcium and phosphorous (71 U/L, 34 U/L, 3 mg/dl, 1mg/dl) respectively, while after urea adjustment were (197 U/L, 113 U/L, 15 mg/dl, 4 mg/dl) respectively. As **Maden *et al.*, 2001** measured non adjusted biochemical parameters as LDH 14.15 U/L, ALP 25.8 U/L, Ca 0.3 mg/dl and phosphorous 0.05 mg/dl, **Mills and Lister 2006** reported that LDH of BAL was 1.9 U/L while with urea adjustment become 114.6 U/L. Measuring ALP and LDH reflect cellular injury of respiratory tract while Ca and phosphorous reflect disturbance of mineral metabolism in BAL fluid.

It has been suggested that an influx of Neutrophils and increased enzyme activity in BAL fluid reflect pathologic changes in the lungs and that these are the most sensitive indicators of inflammatory response (**Capelli *et al.*, 1997a, b** and **Cobben *et al.*, 1999**).

- Matrix metalloproteinase:

The matrix metalloproteinase results were shown in Table (2) and Figure (1). Establishing normal value of MMP is very important because when acute inflammation occurs, polymorphonuclear cells are recruited to the inflammation site and help tissue repair by modulating MMPs secretion. (**Corbel *et al.*, 2000**). Excessive expression of MMPs contributes to the pathogenesis of different pulmonary and nonpulmonary diseases. They are believed to play a role in the pathogenesis through degradation of extracellular matrix (ECM) and basement membrane (BM). BM protein degradation might cause influx of inflammatory cells and perturbation of the epithelial/endothelial structure, whereas degradation of elastin and collagen could predispose to airspace enlargement (**Shapiro and Senior 1999**).

- Bacteriological examination:

The resulted isolates were: Non mannitol-fermenter *Staphylococcus spp.* (2/8, 25%) and *Acientobacter spp.* (1/8, 12.5%) all isolates were single isolate per dog and this result come in accordance with previous reports as the healthy tracheobronchial tree and lung are not continuously sterile. Airways down to the first bronchial division are contaminated with low numbers of organisms in clinically healthy animals. Bacterial isolates from tracheal swabs and lungs of clinically healthy dogs were *Staphylococcus* (coagulase positive and negative), *Streptococcus* (α - and nonhemolytic), *Pasteurella multocida*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Acinetobacter spp.*, *Moraxella spp.* and *Corynebacterium spp.* were in accordance with **Lindsey and Pierce**

(1978), McKiernan *et al.,* (1984) and Lee-Fowler and Reinero (2012).

In conclusion, urea adjustment of cytological and biochemical parameters eliminate error of interpretation arises from inconstant ELF fraction within BAL fluid, this provides a useful basis for other studies concerning canine lower respiratory diseases.

Table (1): Cytological constituents of BALF of healthy Cosmopolitan dogs.

Parameter	Non urea adjusted	Urea Adjusted
Total Nucleated Cell Count (TNCC) (cell/ul)	290 (± 68.48)	1062 (± 379.50)
Reactive Macrophage %	2 (± 1.11)	
Alveolar Macrophage %	78 (± 1.85)	
Neutrophil %	5 (± 1.03)	
Eosinophil %	5 (± 3.06)	
Lymphocyte %	9 (± 2.07)	
Epithelial cells %	1 (± 0.39)	

Table (2): Biochemical Constituent of bronchoalveolar lavage of healthy Cosmopolitan dogs

Constituent	Non urea adjusted	Urea Adjusted
ELF dilution factor	0.21 (±0.01)	
ALP (U/L)	7 (±2.68)	187 (±57.86)
LDH (U/L)	34 (±4.57)	125 (±25.01)
Ca (mg/dl)	3 (±0.64)	15 (±2.98)
Phosphorus (mg/dl)	1 (±0.22)	4 (±0.72)
MMP2 gelatinases A (AU)	26.46 (±1.24)	223.56 (±44.83)
MMP9 gelatinases B (AU)	25.24 (±10.22)	215.69 (±47.80)
Complex form (AU)	0 (±0)	0 (±0)
Total gelatinases (AU)	61.64 (±14.51)	526.95 (±150.27)

AU= arbitrary unit

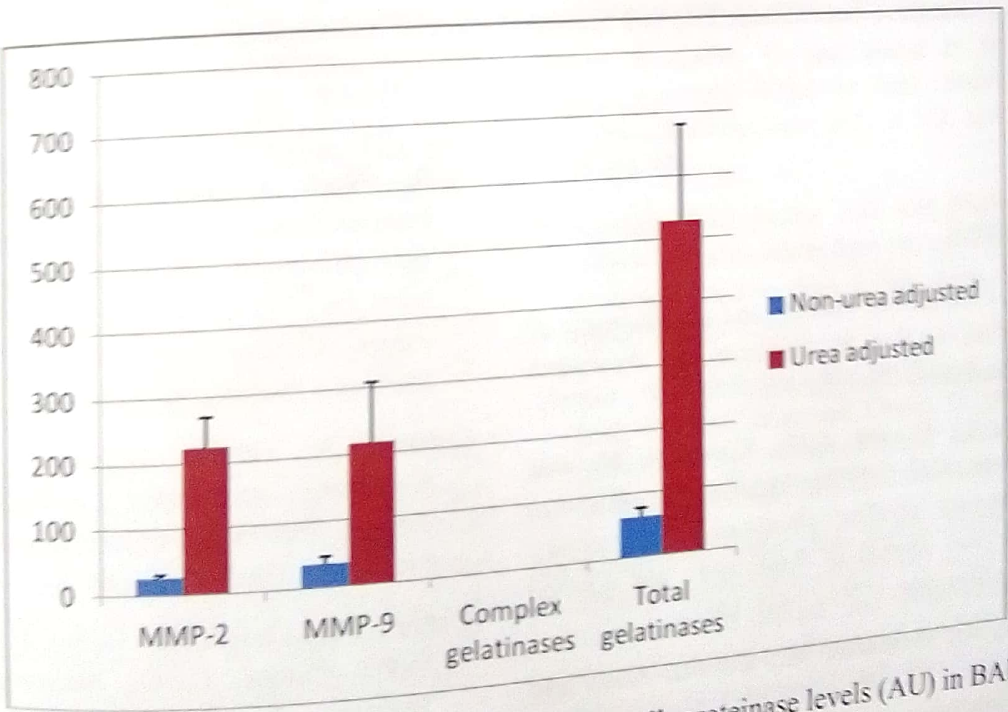

Fig (1): Non-urea adjusted and urea adjusted matrix metalloproteinase levels (AU) in BALF of healthy cosmopolitan dogs

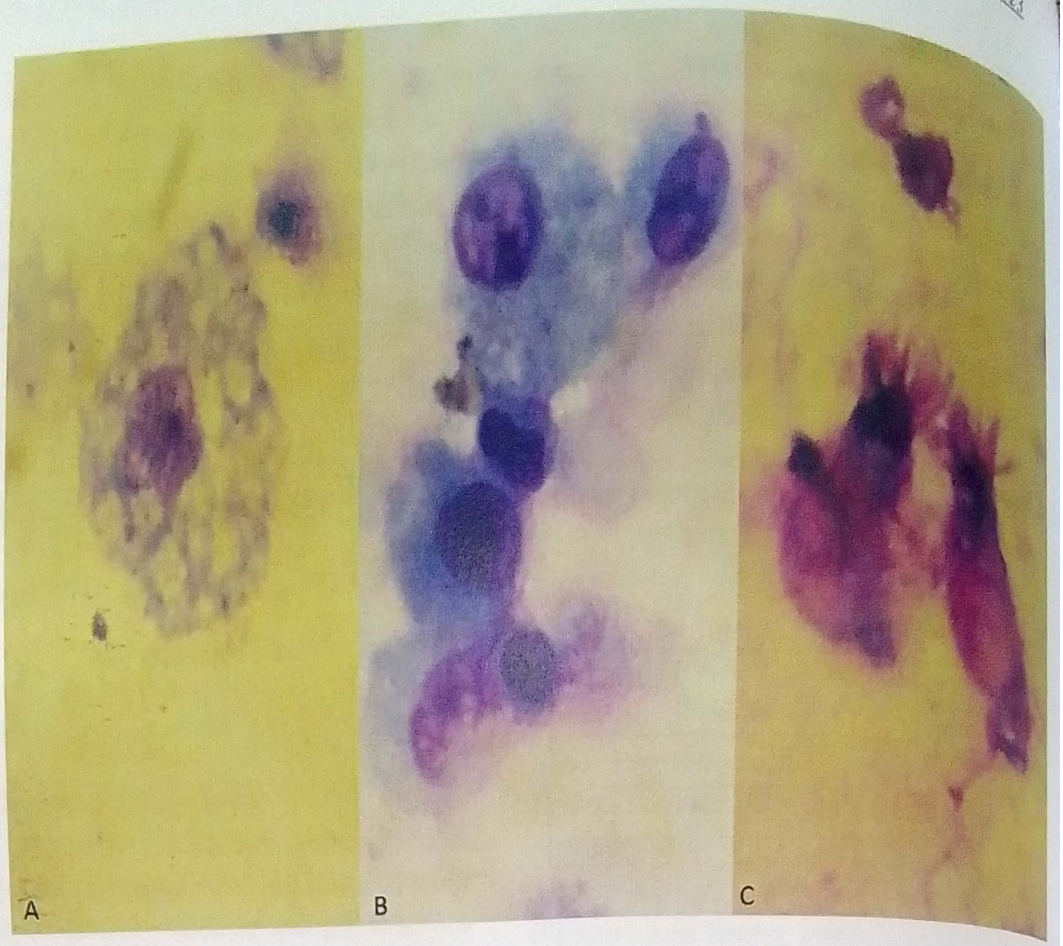

Fig (2): Cytological preparation stained with Diff-3 showing cytological finding of BAL (A) Reactive macrophage, (B) non-activated alveolar macrophage and (C) Epithelial cells.

References

Bauer, P. J. (1981): Affinity and stoichiometry of calcium binding by arsenazo III. Analytical biochemistry, 110(1):61-72.

Capelli, A.; Cerutti, C.G.; Lusuardi, M. and Donner, C.F. (1997b): Identification of human pulmonary alkaline phosphatase isoenzymes. American Journal of Respiratory and Critical Care Medicine, 155(4): 1448-1452.

Capelli, A.; Lusuardi, M.; Cerutti, C.G. and Donner, C. F. (1997a): Lung alkaline phosphatase as a marker of fibrosis in chronic interstitial disorders. American Journal of Respiratory and Critical Care Medicine, 155(1):249-253.

Cataldo, D.D.; Gueders, M.M.; Rocks, N.; Sounni, N.E.; Evrard, B.; Bartsch, P.; Louis, R.; Noel, A. and Foidart, J.M. (2003): Pathogenic role of matrix metalloproteases and their inhibitors in asthma and chronic obstructive pulmonary disease and therapeutics relevance of matrix metalloproteases inhibitors. Cellular and Molecular Biology, 49: 875– 884.

Cobben, N. (1999): Enzymatic markers in monitoring pulmonary inflammation and damage. Phd. thesis, Eijsden ,Universitaire Pers Maastricht, The Netherlands.

Cobben, N. A.; Drent, M.; Jacobs, J. A.; Schmitz, M.P.; Mulder, P. G.; Henderson, R. F.; Wouters, E. F. and Vandieijen-Visser, M.P.(1999): Relationship between enzymatic markers of pulmonary cell damage and cellular profile: a study in bronchoalveolar lavage fluid. Experimental Lung Research, 25(2):99-111.

Corbel, M.; Boichot, E. and Lagente, V. (2000): Role of gelatinases MMP-2 and MMP-9 in tissue remodelling following acute lung injury.

Brazilian Journal of Medical and Biological Research, 33: 749–754.

Dryer, R.L., Routh, J.I., (1963): Determination of Serum Inorganic Phosphorus. Clin. Chem., 4:191

Hawkes, S.P.; Li, H. and Taniguchi, G. T. (2001): Zymography and Reverse Zymography for Detecting MMPs, and TIMPs. In "Matrix metalloproteinase protocols"(Ed., Clark I.M.), Humana Press, 399-410.

Hawkins, E.C. (2004): Bronchoalveolar lavage. In: "Textbook of Respiratory Disease in Dogs and Cats" (Ed., King L.G.), Saunders, St. Louis, Missouri, 118–128.

Hawkins, E.C.; DeNicola, D.B. and Kuehn, N.F. (1990): Bronchoalveolar lavage in the evaluation of pulmonary disease in the dog and cat. J. Vet. Intern. Med., 4:267-274.

Hawkins, E.C.; Rogala, A.R.; Large, E.E.; Bradley, J.M. and Grindem, C.B. (2006): Cellular composition of bronchial brushings obtained from healthy dogs and dogs with chronic cough and cytologic composition of bronchoalveolar lavage fluid obtained from dogs with chronic cough. Am. J. Vet. Res., 67(1): 160-167.

Houston, D.M. (2000): Clinical examination of dogs and cats. Veterinary clinical examination and diagnosis. Harcourt, London, 125-138.

Humphrey, W.J. (1971): Ketamine HCl as a general anesthetic in dogs. Modern Veterinary Practice, 52(6):38-39.

Jang, S.S.; Biberstein, E. and Hirsh, D.C. (2000): A Diagnostic Manual of Veterinary Clinical Bacteriology and Mycology. Davis, CA: University of California Press.

Johnson, L.R.; Queen, E. V.; Vernau, W.; Sykes, J. E. and Byrne, B.A. (2013): Microbiologic and cytologic assessment of bronchoalveolar lavage fluid from dogs with lower respiratory tract infection: 105 cases (2001-2011). Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine, 27(2): 259–67.

Kachmar, J.F. and Moss, D.W. (1976): Enzymes. In:"Fundamentals of Clinical Chemistry." (Ed. Tietz N.W.) Philadelphia: WB Saunders Company, pp. 684-689.

Kitch, T.T.; Jacobs, M.R. and Appelbaum, P.C. (1992): Evaluation of the 4-Hour RapID NF Plus Method for Identification of 345 Gram-Negative Nonfermentative Rods. Journal of Clinical Microbiology, 30(5): 1267-1270.

Kitch, T.T.; Jacobs, M.R. and Appelbaum, P.C. (1994): Evaluation of RapID onE System for Identification of 379 Strains in the Family Enterobacteriaceae and Oxidase-Negative, Gram-Negative Nonfermenters. Journal of Clinical Microbiology, 32(4): 931-934.

Lee-Fowler, T. and Reinero, C. (2012): Bacterial respiratory infections. In: "Infectious Diseases of the Dog and Cat, 4th ed." (Ed. Greene, C.E.), Elsevier, St. Louis, M.I.,pp. 936-950.

Lindsey, J.O. and Pierce, A.K. (1978): An examination of the microbiologic flora of normal lung of the dog. Am. Rev. Respir. Dis., 117:501-505.

Maden, M.; Altunok, V.; Birdane, F. M.; Aslan, V. and Nizamlioğlu, M. (2001): Specific enzyme activities in bronchoalveolar lavage fluid as an aid to diagnosis of tracheobronchitis and bronchopneumonia in dogs. Research in Veterinary Science, 71(2): 141–145.

Mayer, P.; Laber, G. and Walzl, H. (1990): Bronchoalveolar lavage in dogs: analysis of proteins and respiratory cells. J. Vet. Med. A., 37:392-399.

McKiernan, B.C.; Smith, A.R. and Kissil, M. (1984): Bacterial isolates from the lower trachea of clinically healthy dogs. J. Am. Anim. Hosp. Assoc., 20:139–142.

Mercier, E.; Bolognin, M.; Hoffmann, A.C.; Tual, C.; Day, M.J. and Clercx, C. (2011): Influence of age on bronchoscopic findings in healthy Beagle dogs. Vet .J., 187:225–228.

Mills, P.C. and Litster, A.L. (2006): Using urea dilution to standardise cellular and non-cellular components of pleural and bronchoalveolar lavage (BAL) fluids in the cat. Journal of Feline Medicine and Surgery, 8(2):105–10.

Rajamäki, M.M.; Järvinen, A.K.; Saari, S. A. M. and Maisi, P. S. (2001): Effect of repetitive bronchoalveolar lavage on cytologic findings in healthy dogs. American Journal of Veterinary Research, 62(1): 13–16.

Shapiro, S.D. and Senior, R.M. (1999): Matrix metalloproteinases. Matrix degradation and more. Am. J. Respir. Cell Mol. Biol., 20:1100-1102.

Tietz, N.W. (1986): Fundamentals of clinical chemistery. 2nd ed., W.B., Saunders, Philadelphia, PA, p.p.622

Tietz, N.W. (1990): Clinical guide to laboratory tests. 2nd ed., W.B., Saunders, p.p.566.

Vail, D.M.; Mahler, P.A. and Soergel, S.A. (1995): Differential cell analysis and phenotypic subtyping of lymphocytes in bronchoalveolar lavage fluid from clinically normal dogs. Am. J. Vet. Res., 56:282-285.

Woods, K.S. (2013): Standardization of Bronchoalveolar Lavage Aspiration Techniques to Optimize Diagnostic Yield of Canine Lower Respiratory Tract Samples. PhD. Thesis, The University of Guelph, Ontario, Canada.

الملخص العربى

السائل المبطن للخلايا الظهارية المجمع بواسطة غسل القصيبات والحويصلات الهوائية من الممكن أن يستخدم في التشخيص لأمراض الجهاز التنفسي السفلي .لكن دراسات قليلة أسست معلومات مرجعية للمكونات الخلوية وغير الخلوية لسوائل غسل القصيبات والحويصلات الهوائية خاصة بعد تعديل اليوريا للتغلب على عدم الاتساق في جزء السائل المبطن للخلايا الظهارية في السائل المسترجع .وقد أجريت هذه الدراسة لتحليل المكونات المختلفة لسوائل غسل القصيبات والحويصلات الهوائية مع استخدام اليوريا لتعديل هذه المكونات في الكلاب السليمة .لهذا الغرض تم استخدام 8 كلاب في هذه الدراسة. سوائل غسل القصيبات والحويصلات الهوائية التي تم جمعها فحصت خلوياو كيميائيا وبكتريولوجيا .أظهرت نتيجة غلبة بلاعم الحويصلات الهوائية (78٪ ± 1.85)، يليه الخلايا الليمفاوية (9٪ ±2.07) في المحور الخلوي ومجموعة من مختلف المكونات الكيميائية الحيوية بما في ذلك ALP (197 U / L)، LDH (113 U / L)، كالسيوم 15)ملغ / دل)، والفوسفور (4 ملغ / دل)، وكذلكMMP-2 ، MMP-9 ومجموع الانزيمات المحللة للجيلاتين باستخدام . gelatin zymography وكشفت التحقيقات البكتريولوجية عن عزل Aceintobacter.و البكتريا المكورات العقدية غير مخمرة المانيتول من 3 كلاب سليمة.