A Standardized Bronchoalveolar Lavage (BAL) Technique Using Large Volume of Infused Fluid in Young Foals under Intravenous General Anesthesia

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ABSTRACT

The purpose of this paper is to describe a standardized bronchoalveolar lavage (BAL) technique used in 11 clinically healthy foals from one week of age to2 months of age. All foals successfully underwent this procedure five times at different ages. This technique was performed under intravenous general anesthesia with no apparent complications. In our experience, performing transendoscopic BAL in foals under general anesthesia yields a consistent fluid sample with regard to quantity and quality, and allows a thorough observation of the upper and lower airways. This BAL technique can be used for collecting samples for clinical research, and potentially for diagnosing pulmonary diseases in young foals.

INTRODUCTION

The two most common diagnostic aids used to investigate a pathologic process in the lower respiratory tract in horses are tracheal wash and bronchoalveolar lavage. The fluid collected by tracheal wash is a mixture of cells and secretions pooled in the

distal trachea, and therefore, is the preferred sample for bacterial/microbiologic culture.[1-5] Besides bacterial pneumonias, there are non-infectious causes of lung disease that sometimes warrant ancillary diagnostic testing. Bronchoalveolar lavage (BAL) is an excellent method for detecting pathologic processes in the terminal airways and alveoli.[1,5] Compared to tracheal wash, it has the advantage of sampling a more distal area of the lung for detection of distal bronchial and alveolar pathogens and collection of immune cells and surfactant fluid for analysis.[6]

BAL is not a common clinical technique used in young and neonatal foals, although is oftentimes performed for research purposes. Some indications for performing BAL in neonatal and young foals include diagnosis of non-infectious inflammatory lung diseases.[7] evaluation of pulmonary disposition of antibacterial drugs (eg, azithromycin, clarithromycin),[8] immunophenotypic analysis of BAL cells,[9] analysis of surfactant protein composition,[10-12] and occasionally for the diagnosis of pneumonia in conjunction with tracheal wash fluid analysis. [7]

Although there is extensive published literature discussing indications and BAL techniques in adult horses,[1-5, 13-16] there is scant information about this technique in neonatal and young foals. The technique described in this paper was successfully performed in a clinical research setting at our University, and it can also be potentially used for diagnosing pulmonary diseases in neonatal and young foals.

The purposes of this paper are:

1. To describe a standardized BAL technique performed under intravenous general anesthesia, using large volume of infused fluid

2. To report BAL fluid differential cell counts in addition to subjective assessment of the number of erythrocytes, ruptured cells and cell density upon cytocentrifugation slide examination.

MATERIALS AND METHODS

Foals

The BAL technique described in this paper was used in a research study investigating the characterization of the foal's pulmonary immune system conducted at the WCVM, University of Saskatchewan, during 2007. As part of the research study BAL fluid cytological examination was performed. All procedures were approved by the Animal Research Ethics Board, University Committee on Animal Care and Supply (UCACS), University of Saskatchewan, and followed the guidelines of the Canadian Council on Animal Care (CCAC).

The study population comprised a total of 11 healthy draft-type neonatal foals. All dams and foals were kept together in one large pen, with free choice access to grass hay and water. As the foals were born, they all were monitored by distant examination twice daily including respiratory rate and effort, attitude, suckling behavior, and presence of cough. All foals were determined to be healthy at the times the BALs were performed based on distant and physical examination findings, including assessment of rectal temperature, respiratory rate and pattern, thoracic and abdominal auscultation, assessment of mucous membranes, and external umbilical stump. Bronchoalveolar lavage samples were obtained in all foals at 1-2 week of age and every 2 weeks thereafter for 2 months (5 time-points total, ie, 1-2, 3-4, 5-6, 7-8, and 9-10 weeks of age).

1. Anesthetic protocol Premedication

Xylazine hydrochloride (Anased; Novopharm Limited, Toronto, ON, Canada) was administered at 0.2 - 1.0 mg/kg by intravenous (IV) injection for sedation. Xylazine at 0.2 mg/kg was used in foals at 1 week of age, as they were easy to handle. As they became older, the dose of xylazine was increased up to 1.0 mg/kg. For foals that were difficult to handle, xylazine was administered intramuscularly and topped-up intravenously if needed.

Induction

Ketamine hydrochloride (Ketaset; Wyeth Animal Health, St. Laurent, QC, Canada) at 2 mg/kg in combination with diazepam (Di-

azepam; Sandoz, Boucherville, QC, Canada) at 0.1 mg/kg by IV administration was used for induction. An 18 gauge 1.88 inch catheter (BD Angiocath; Becton Dickinson. Infusion Therapy Systems Inc., Sandy, UT, USA) was placed in a jugular vein once the foal was induced, for ease of further drug administration. A dose of 0.02 – 0.05 mg/kg butorphanol tartrate (Torbugesic; Wyeth Animal Health, St. Laurent, QC, Canada) was given IV through the catheter to decrease coughing and to help increase the duration of the anesthesia. One top-up dose of ketamine and diazepam (1/4 to 1/2 of the induction dose) was required in some foals. All foals received between 2-5 L/minute of supplemental oxygen via a nasal oxygen line. A pulse oximeter was placed on the tongue to monitor oxygen saturation and the goal was to maintain saturation values above 95%. Heart rate, respiratory rate, pulse quality, mucous membrane color, capillary refill time, and depth of anesthesia were also monitored by physical examination.

Performing the BAL

A 1 meter or a 2.1 meter, 0.5 inch diameter fiber optic video-endoscope (Olympus; Olympus, Center Valley, PA, USA) was used depending on the age and size of the foal. The end of the endoscope was lubricated with sterilized lubricating jelly (Triad; H&P Industries, Inc, Triad Disposables, Inc, Mukwonago, WI, USA) and guided along the ventro-medial nasal meatus and passed through the nasal passage. Once the pharynx was seen, the head of the foal was slightly extended, and the endoscope was inserted into the trachea and then advanced until the tracheal bifurcation was reached. At that point the endoscope was advanced either into the right or left lung. The endoscope was further advanced until it was wedged into the distal airways.

Five hundred ml of warm saline solution (sodium chloride 0.9%, sodium bicarbonate 0.06%, pH 6.5) was infused through the biopsy channel of the endoscope using a pressure bag (Infu-Surg; Ethox Corp, Buffalo, NY, USA). This solution was obtained by adding 7 ml of 8.4% sodium bicarbonate to a 500 ml 0.9% sodium chloride bag (Abbott Laboratories, Saint-Laurent, QC, Canada). The BAL fluid sample was then retrieved through the biopsy channel using a suction pump (Schuco-Vac: Model S 132; Schuco Inc. Allied Healthcare Products Inc, St Louis, MO, USA). In order to prevent airway collapse and damage, the suction pressure never exceeded 40 cm Hg or 15 inches Hg of vacuum. After aspiration, the procedure was repeated to sample the contralateral lung. The BAL fluid was collected into an Erlenmeyer flask.

Once the BAL was completed, the foals were left to recover on their own on a soft mat. They were assisted once they were attempting to stand or if they appeared to be in danger of injuring themselves. Once able to stand and walk, they were returned to their dams.

Processing the BAL Fluid Sample

Due to the need to perform multiple assays and specific tests for other concomitant research studies run with the same group of animals and BAL samples , ie, cytokine profile, lipid rafts analysis, R. equi PCR, and bacterial culture, the BAL samples were processed in a distinctive manner as follows. The BAL fluid was kept on ice in 1L glass flasks until submitted to the laboratory within 2 hours of collection. Once in the laboratory, it was mixed by swirling before removing a 1 mL aliquot for microbiological plating. The fluid was poured into 225 mL conical centrifuge tubes, the total volume was recorded, and then it was centrifuged at 400 Xg for 10 minutes at 10°C. The supernatant was stored at -20°C. The pellet was suspended in 5 mL PBS and transferred to a 50 mL centrifuge tube. The pellet was washed once with total volume 45 mL PBS (same centrifuge speed as above). The pellet was suspended in RPMI-10%-complete (RPMI; Gibco/Invitrogen Corporation, Carlsbad, CA, USA) containing 10% horse serum, L-glutamine, HEPES, gentamicin, and 2-mercaptoethanol. An aliquot of the cell suspension was used for cell count using

trypan blue dye. Cells were diluted to 4 x 106 cells/mL for cytospin and functional assays.

Twenty-five µL of the cell suspension plus 75 µL media were centrifuged onto a microscope slide using a Thermo Shandon Cytospin 4 (Thermo Fisher Scientific Inc.; Waltham, MA, USA) (ie, ~105 cells), at 500 rpm for 4 minutes at room temperature. The slides were air-dried and stained with Wright Giemsa stain using an Ames Hema-Tek slide stainer (Ames; Elkhart, IN, USA).

Cytological examination

Fifty five cytocentrifugation slide preparations of BAL fluid samples obtained from 11 clinically healthy foals were evaluated. Differential cell counts including percentages of macrophages, neutrophils, lymphocytes, and eosinophils with subjective assessment of the number of erythrocytes, ruptured cells, and cell density were performed (Table 1). Cell density estimates were assigned using the following criteria: a "high cell density" was considered when the cells were contacting a neighboring cell with overlap of cells noted in every field. A "moderate-high cell density" referred to a slightly lower cell population in the cytospin preparation, where cell overlap occurred in 50% of the high power fields (hpf or x50 oil immersion field), thus in the remaining 50% of the

Table 1: *Cytological analysis and differential cell count results of BAL fluid (BALF) cytospin preparations from 11 clinically healthy neonatal foals. The BALF samples were obtained using the standardized BAL technique described in the text. BALs # 1-5 were performed in all foals at one to two week of age and every two weeks thereafter (BAL #1: 1-2 weeks; BAL #2: 3-4 weeks; BAL #3: 5-6 weeks; BAL #4: 7-8 weeks; BAL #5: 9=10 weeks).*
Percentages of macrophages, neutrophils, lymphocytes, and eosinophils varied between foals and between sampling time-points. Overall the proportion of RBC was low indicating minimal iatrogenic bleeding, and the amount of ruptured cells was overall small to moderate. Note that the majority of the samples had a very good cell density.

Foal ID	BAL#	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	RBCs	Ruptured Cells	Cell Density
1	1	94	4	2	0	scant	small	moderate
	2	73	16	11	0	scant	moderate	high
	3	88	7	5	0	scant	moderate	high
	4	77	11	12	0	scant	small	high
	5	85	5	10	0	scant	small	mod-high
2	1	86	11	3	0	scant	small	high
	2	98	2	0	0	none	moderate	low
	3	87	11	1	1	scant	small	high
	4	84	14	2	0	scant	moderate	high
	5	91	7	2	0	scant	small	mod-high
3	1	81	18	1	0	scant	small	moderate
	2	98	2	0	0	none	moderate	low
	3	79	19	2	0	scant	small	moderate
	4	82	12	3	3	scant	moderate	high
	5	92	3	3	2	scant	small	moderate
4	1	70	24	6	0	mod-erate	small	high
	2	91	5	4	0	scant	small	moderate
	3	72	21	4	3	scant	small	moderate
	4	62	32	5	1	scant	small	low-mod

	5	91	9	1	0	none	small	high
5	1	82	16	2	0	scant	small	high
	2	78	17	5	0	scant	small	moderate
	3	89	7	4	0	scant	small	mod-high
	4	92	7	1	0	none	small	low
	5	67	4	29	0	scant	small	mod-high
6	1	89	8	3	0	scant	small	high
	2	92	3	5	0	scant	small	moderate
	3	72	14	3	11	scant	small	moderate
	4	81	14	3	2	scant	moderate	high
	5	82	9	7	2	scant	small	mod-high
7	1	91	8	1	0	none	large	high
	2	98	2	0	0	scant	small	moderate
	3	90	8	2	0	scant	small	low-mod
	4	94	5	1	0	scant	small	mod-high
	5	94	5	1	0	scant	small	mod-high
8	1	73	22	5	0	moderate	moderate	high
	2	91	5	4	0	scant	small	moderate
	3	84	12	4	0	scant	small	moderate
	4	89	5	6	0	scant	moderate	high
	5	78	9	13	0	scant	small	high
9	1	51	37	12	0	scant	small	mod-high
	2	88	10	2	0	scant	small	moderate
	3	64	20	7	9	scant	small	high
	4	76	13	2	9	scant	moderate	high
	5	82	17	1	0	none	small	low
10	1	88	4	8	0	scant	small	high
	2	93	6	1	0	scant	small	moderate
	3	87	9	3	1	scant	small	high
	4	82	11	6	1	scant	small	mod-high
	5	68	7	25	0	scant	small	mod-high
11	1	72	24	4	0	scant	moderate	high
	2	87	6	7	0	scant	small	moderate
	3	89	8	3	0	none	small	low
	4	78	8	0	14	scant	small	high
	5	84	8	3	5	scant	small	high

fields there were free spaces between the cells and the cells did not necessarily contact neighboring cells. The term "moderate cell density" referred to the finding that cells in the majority of fields were not in contact with neighboring cells although contact was observed occasionally. The term "low cell density" was considered when the majority of cells had abundant space between them though, inevitably, cells in contact with one another could be found somewhere on the slide.

Assessment of ruptured cells was based on the presence of nuclear and cytoplas-

mic remnants in the cytospin preparation. The term "large amount" was used when ruptured debris was found in every high power field (hpf), the term "moderate amount" indicated that ruptured debris was present in every 1 to 4 hpf, and the term "small amount" referred to finding ruptured debris in such small quantities that it would require searching greater than 4 hpf's to find it. The number of red blood cells was categorized as "scant" when there were less than 10 erythrocytes per hpf, "moderate" if there were between 10 and 30 erythrocytes per hpf , and "marked" when more than 30 erythrocytes per hpf were found.

RESULTS

Bronchoalveolar lavage in neonatal foals under general anesthesia was used for studying the pulmonary immune system of the foal including cytological examination of the BAL fluid. A total of 11foals successfully underwent this procedure five times at different ages (ie, 1-2, 3-4, 5-6, 7-8, and 9-10 weeks of age). Duration of recumbency using the aforementioned anesthetic protocol was between 15 and 30 minutes depending on the administered dose of induction and anesthetic drugs. In general the recovery was smooth and once the foals were standing they appeared normal and responsive, showing no adverse clinical signs.

Complications included occasional mild nasal bleeding when passing the endoscope through the nasal passage. Oxygen saturation, as assessed upon pulse oximetry on the tongue, often decreased to 88 -95 % as saline solution was infused through the endoscope and typically returned to normal as soon as the fluid was removed.

Using this technique, between 200 and 400 ml of bronchoalveolar fluid was recovered for each 500 ml instilled fluid with a total cell count approximately ranging from 1.5 x 106 to 2.26 x 108 cells, and between 5.0 x 103 to 6.22 x 105 cells/ml. Serial BAL fluid cytological analysis results from 11 healthy neonatal foals from 1 week of age to 2 months of age are shown in Table 1. The cytocentrifugation preparations varied in cell density and were subjectively categorized from low-density to high-density. Most of the samples had a very good cell density (23/55; 41.8% had high density, and 10/55; 18.1% had moderate–high density), and only a few varied in cell density (2/55; 3.6% had low-moderate density and 5/55; 9% had low density) (Figure 1).

Percentages of macrophages, neutrophils, lymphocytes, and eosinophils varied between foals and between sampling time-points. Overall the proportion of RBC was low (7/55; 12.7% did not contain any red cells, 46/55; 83.6% had scant amount of RBC, and 2/55; and 3.6% had only moderate amount of RBCs), indicating minimal iatrogenic bleeding (Figure 2), and the amount of ruptured cells was overall small to moderate (43/55; 78.1% small amount, and 11/55; 20% moderate amount). Only one sample (1/55; 1.8%) yielded a large amount of cellular debris. Ruptured cells with associated cellular debris were present in many of the specimens though, overall, the proportion of rup-

Figure 1: *Cell density on cytocentrifugation slide preparations of BAL fluid samples. Data is presented as a percentage of the total number of samples analyzed. 23/55; 41.8% had high density; 10/55; 18.1% had moderate–high density; 2/55; 3.6% had low-moderate density, and 5/55; 9% had low density.*

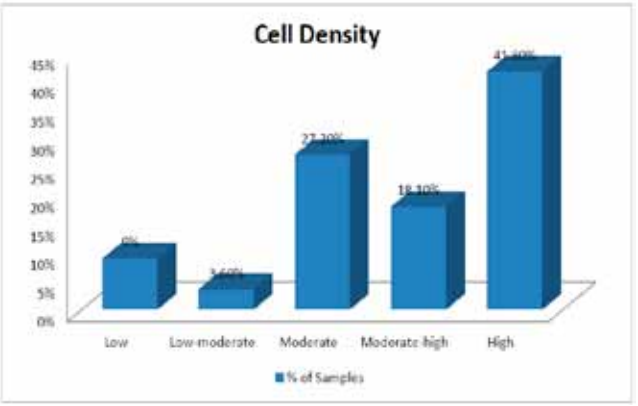


Figure 2: *Overall proportion of Red Blood Cells on cytocentrifugation slide preparation of BAL fluid samples. Data is presented as a percentage of the total number of samples analyzed. 7/55; 12.7% did not contain any red cells, 46/55; 83.6% had scant amount of RBC, and 2/55; 3.6% had only moderate amount of RBCs.*

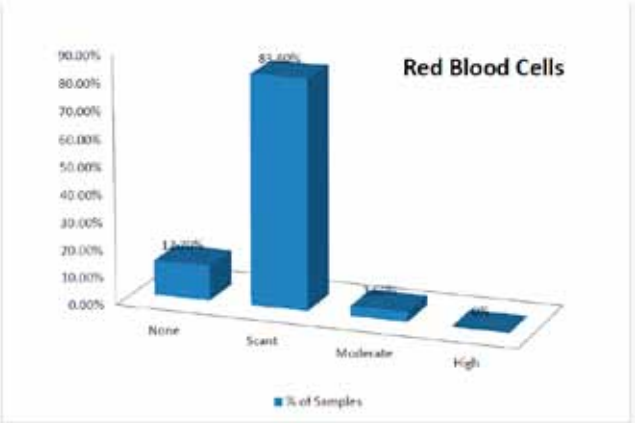
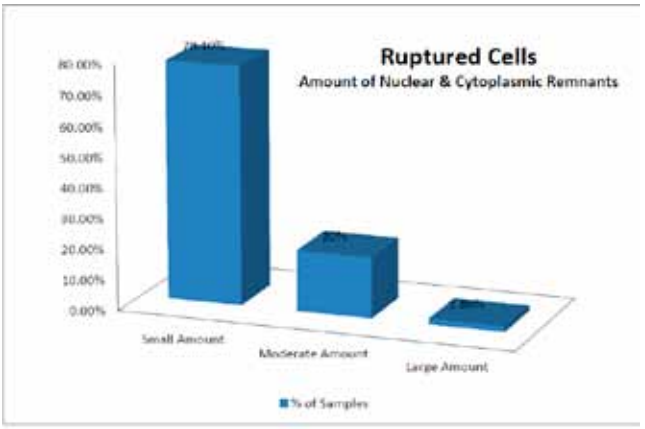


Figure 3: *Ruptured cells with associated cellular debris on cytocentrifugation slide preparation of BAL fluid samples. Data is presented as a percentage of the total number of samples analyzed. 43/55; 78.1% of samples had small amount, 11/55; 20% had moderate amount, and only one sample (1/55; 1.8%) yielded a large amount of cellular debris.*



tured cells was low (78.1% of samples), and this feature was not deemed to significantly interfere with cytological evaluation or the cell differential (Figure 3). Likewise, several samples contained minimal contamination from keratin debris, scant plant fibers and occasional environmental pollen, though this did not impede cytological evaluation. When present, respiratory epithelial cells were satisfactorily preserved.

DISCUSSION

BAL is considered a safe procedure and sensitive method to diagnose diffused pulmonary inflammatory processes at the cellular level close to the peripheral airways and alveoli.[1,18] Furthermore, BAL fluid analysis allows investigation of a wide variety of cellular and functional characteristics of the distal bronchi and alveolar pulmonary tissue. Fluid analysis may include gross examination, total cell count and differential count, microbiological studies (semi quantitative and quantitative bacterial cultures, fungal cultures, PCR, etc), and cell function testing. Using BAL in neonatal foals also has potential for characterizing the normal pulmonary immune system in addition to studying and understanding a variety of pulmonary diseases in the foal. It can be used as a diagnostic tool and as a prognostic indicator of response to treatment in a clinical setting.[20]

Developing a standardized BAL technique among veterinary centers worldwide is needed to allow an accurate comparative evaluation of BAL samples in neonatal foals, because using different techniques with different lavage fluid volumes, and/or sampling different lung sites, may influence the total BAL cell number and the cellular composition of the BAL fluid sample. A previous study performed in healthy adult horses showed an increased percentage of neutrophils and mast cells in the BAL fluid when using a small-volume (50 ml) lavage, com-

pared to a large-volume (350 ml) lavage. The same study revealed a higher number of mast cells in fluid retrieved from the left lungs compared to the right lungs.[21]

For our BAL protocol we used a high volume lavage (500 ml) as an attempt to sample a bigger area of the lung, and therefore, to obtain a more representative sample, and also to compensate for any potential difference in cellular sample composition due to a small-volume lavage. Using this technique between 40% and 80% of the infused volume is retrieved yielding consistent sample cellularity (48/55; 87.2% moderate to high cell density), in addition to minimal bleeding, low red blood cells (RBCs) (46/55; 83.6% had scant amount of RBC, and 2/55; 3.6% had only moderate amount of RBCs) and ruptured cells counts (43/55; 78.1% small amount, and 11/55; 20% moderate amount) (Table 1).

Although a decrease in oxygen saturation was detected at the time the fluid was instilled in the lungs, this effect was transient, lasting only less than a minute until the fluid was retrieved, and no respiratory distress or other apparent complications were seen. The chance of developing complications during and after the procedure, such as decrease in baseline PaO2, bronchospasm, and decrease in lung function, is higher in patients with pre-existent severe lung disease, and therefore BAL should be avoided in these patients.[18]

A proper sedation and restraint method is important to perform the BAL technique in a dependable manner, and to allow retrieving a consistent BAL fluid sample quantity and quality. [18,19] In our experience, performing transendoscopic BAL in foals under general anesthesia consistently yields a very good fluid sample quantity and quality, allows a thorough observation of the airways, and thus allows the endoscope to be directed easily towards a desired lung area for direct inspection and sampling. Furthermore, general anesthesia decreases the chances of foals' injuries and distress, and is safer for both the foal and the personnel performing the BAL. Using our protocol, the recovery from anesthesia was relatively fast, the procedure was very well tolerated, and we did not observe obvious complications. The foals did not develop respiratory distress or other complications detected by distant exam and by physical examination. Foals showed a normal bond with their dams once they were standing after recovery.

An additional 12 neonatal foals used for a Rhodococcus equi vaccine trial in 2005,17 and 15 neonatal foals in 2008 in a similar study successfully underwent three BAL procedures over time (1-2, 3-4, and 4-5 weeks of age) (Lohmann et. al.; manuscript in preparation). These foals were vaccinated once or twice, and then challenged intrapulmonary with R. equi at 3 or 4 weeks of age. Although the foals did not display overt clinical signs of pneumonia during the study, necropsy examination at day 50 revealed varying degrees of macroscopic pulmonary lesions consistent with R. equi pneumonia in most of the foals. Interestingly all 27 foals tolerated the BAL procedure well, with no clinical complications detected by physical examination.

The quantity and quality of the BAL samples were the same as the ones obtained from the healthy foals used for the study described in this paper. In conclusion, and based on our results, this BAL technique can be effectively and safely used in healthy neonatal foals, yielding a consistent BAL fluid sample with regard to quantity and quality, allowing accurate comparative evaluation of samples. Although an additional [27] foals challenged with R. equi tolerated the BAL procedure well and with no apparent complications, further investigation is needed to establish the safe use of this technique in foals with clinically important respiratory disease. This BAL technique can be used for collecting samples for clinical research and, potentially, for diagnosing pulmonary diseases in neonatal and young foals.

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