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# **Ovine bronchoalveolar lavage cellularity: reproducibility and the effect of multiple repeated lavage**

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Short title: Repeated bronchoalveolar lavage in sheep

## **Summary**

We sought to determine the normal within-sheep variability of bronchoalveolar cellular parameters and to infer the numbers of sheep required to detect a defined significant change within the same. We further sought to examine the effect of repeated bronchoalveolar lavage (BAL) on those parameters. Neither the total cell number nor the absolute numbers of any given cell type changed significantly following repeated BAL. Further, there was no significant change in percentage differential cell populations over time in randomly sampled lobes. However for lobes sampled repeatedly, a significant change in the percentage of lymphocytes was detected. Although the percentages of neutrophils in repeatedly and randomly sampled lobes were significantly correlated, the percentage found in the former tended to be greater and more variable than in the latter. As a consequence, larger group sizes are required to detect given changes in neutrophil percentages in repeatedly sampled lobes over time when compared with detecting equivalent changes in randomly selected lobes.

## **Introduction**

Current British and European legislation surrounding the use of living animals in scientific experimentation embodies the concept of reducing the numbers of animals involved to the minimum necessary to obtain valid results. Practical application of this concept demands prior knowledge of the extent to which the variable under study does indeed vary within and/or between animals.

Sheep are frequently used as models of human lung diseases including asbestosis (Begin et al 1983), ARDS (Wheeler et al 1990), allergic airway disease (Bosse et al 1987, Wegner

et al 1993) and emphysema (Susskind et al 1985). The technique of bronchoalveolar lavage has been extensively used in many of these studies to follow sequential changes in cellular populations within the bronchoalveolar space.

Despite the widespread use of this technique, there is a dearth of information in the literature concerning the normal intrasubject variability of cellular parameters within this species.

Further, it has been reported that repeated bronchoalveolar lavage in sheep causes significant changes in cellular parameters (Woodside et al 1983), effects that may confound the interpretation of sequential changes.

We sought to determine the normal within-sheep variability of bronchoalveolar cellular parameters and further to infer the numbers of sheep required to detect a defined significant change within the same. We further sought to examine the effect of repeated bronchoalveolar lavage on those parameters.

## **Materials and methods**

### ***Animals***

Eight crossbred sheep (3 F: 5 MN)(bodyweight: 39-72 kg) were used in this study. Animals were adjudged to be free from significant pulmonary disease on the basis of clinical examination.

### ***Anaesthesia***

Anaesthesia was induced with intravenous thiopentone sodium (Intraval sodium; Rhône Mérieux Ltd.) and maintained, following intubation, with gaseous halothane (2-2.5%) in air and nitrous oxide.

### ***Bronchoalveolar lavage***

A flexible fibre optic bronchoscope (5.3mm OD)(Model FG-16X; Pentax U.K. Ltd.) was advanced and wedged in selected segmental bronchi. The subtended segments were lavaged by installation and withdrawal of five, 10ml aliquots of normal saline (0.9% NaCl solution). BALF was immediately placed on ice until subsequent analysis.

### ***Sample handling***

Lavage fluid from each lung lobe was separated into supernatant and cells by centrifugation at 400g for 7min and the resultant cell pellets were washed twice in phosphate buffered saline (PBS). Cells were counted using a Neubauer haemocytometer and values expressed per millilitre BALF. Cytocentrifuge slides were prepared and stained using Leishmann's stain for differential counts on 400 cells. Cells were classified as neutrophils, macrophages, eosinophils, lymphocytes or mast cells according to standard morphological criteria. Cells not classified in the aforementioned groups were recorded as 'other' cells.

### ***Study design***

A total of five sequential bronchoscopies at intervals of 4-7 days were performed on individual sheep. During each procedure BALF was obtained from two lung segments. One segment (the posterior right apical) was consistently sampled during each procedure whereas the other segment was chosen at random. The order in which the samples were taken was randomised and no randomly chosen segment was sampled on more than one occasion in an individual sheep.

## Statistics

Changes over time were analysed by repeated measures one-way analysis of variance (ANOVA). Post hoc comparisons on parameters found to be significant by ANOVA were performed by using a Tukey-Kramer honestly-significant-difference (HSD) post hoc mean comparison. The sample sizes necessary for a subsequent study to detect, for given parameters, significant changes in paired data were calculated using the standard deviations of the differences between the repeated measurements (Ward et al 1995). For these calculations, a power of 80% and a significance level of  $p$  equal to 0.05 were specified.

## Results

Total cells did not change significantly over time for either the randomly or the repeatedly sampled lobes. Absolute numbers of any given cell type did not change significantly over time.

There was no significant change in the percentage differential cell populations over time in the randomly sampled lobes. However for the repeatedly sampled lobe the percentage of lymphocytes on day 2 was significantly less than that on days 1 and five ( $p = 0.03$  for both comparisons; paired t-test)(Figure 1).

There was no significant difference in total or differential cell counts from samples taken from repeatedly and randomly sampled lobes on the same day ( $p > 0.05$ ; two-sample t-test). Notwithstanding this fact when all data points from randomly sampled lobes were combined into one data set and summary statistics generated to compare with those from the repeatedly sampled lobes (Table 1), the latter results were characterised by a significant increase in the percentage of neutrophils ( $p = 0.042$ ; two-sample t-tests). In addition, the percentage of neutrophils was more variable in the repeatedly sampled lobe.

The standard deviations of the differences between consecutive total and differential cell counts on repeatedly and randomly sampled lobes were used to generate figures 3 and 4 which depict the estimated sample sizes required to detect a range of specified differences in total and differential cell counts in BALF.

A significant correlation existed between square root transformed neutrophil percentages from randomly and repeatedly sampled lobes (Figure 5)( $p < 0.005$ ;  $r = 0.46$  (Pearson product moment correlation coefficient)).

## **Discussion**

Our interest in examining the effect of multiple repeat small volume (50ml) bronchoalveolar lavage on total and differential cell counts arose from a need to examine time-dependent (4-7 day interval) changes in these parameters following treatments applied at the whole lung or lung segmental level.

Previous studies have failed to demonstrate any significant effect of repeated lavage on total and differential cell counts in sheep. Specifically, in one study (Woodside et al 1983), in which bronchoalveolar lavage was performed and repeated once 4hr - 7d later, there was an early but short-lived influx of neutrophils, accompanied by lymphocytes, and followed 2-4 days later by an influx of alveolar macrophages. By day 7 the numbers of all three cell types had returned to baseline. Further, Chanana et al (1981) reported that, in sheep, repeated BAL at weekly intervals produced no apparent changes in either the number of cells recovered or the differential cell populations.

Respecting that in the first study (Woodside et al 1983) the position of the lavage catheter tip (6cm beyond the carina) and volume infused (520ml) differed from the desired protocol

in the present study and that in the latter study (Chanana et al 1981) it is not clear whether the same, or different, subsegments were lavaged at each time point it was our contention that these results did not provide enough evidence to prove the hypothesis that multiple repeated lavage would affect neither total nor differential cell counts.

Our results demonstrate that, with the exception of the percentage of lymphocytes in repeatedly sampled lobes, multiple bronchoalveolar lavage affects neither total nor the proportion of different cell types within the total cell population. Regarding the changing lymphocyte population, there did not appear to be any underlying trend towards a reduction in the percentage of lymphocytes in BALF over the time course, therefore the significant reduction on day 2 in relation to days 1 and 5 defies explanation and would appear to be an anomalous result. Certainly conclusions based on this observation are justifiably reserved.

If, from preliminary data, it is anticipated that a treatment or experimental intervention will increase bronchoalveolar cellularity by 30% i.e. from  $60 \times 10^3$  to  $80 \times 10^3$  cells/ml, then at least 20 animals would be required to reliably detect this change (Figure 3). With a larger predicted change the numbers required decrease. This type of power calculation highlights the requirement, during experimental planning, of having at least some preliminary knowledge of the variability of the planned measurements in the normal situation.

Neutrophils comprised a significantly greater percentage of cells from the repeatedly sampled lobes and in addition both the proportions of this cell type and also of macrophages from this source were more variable (table 1) than the respective proportions in randomly sampled lobes. This meant that much larger group sizes would be required to detect a given change in the repeatedly sampled lobe when compared to



randomly sampled lobes. The variability in percentage macrophages appeared to be a consequence of the changes in the neutrophil population in that the variability of the absolute numbers of macrophages did not differ between randomly and repeatedly sampled lobes whereas the absolute neutrophil numbers were more variable in the latter (data not shown).

The question obviously arises as to the reasons for such variability in the neutrophil population in the repeatedly sampled (right apical) lobe. It is considered unlikely that the lavage procedure itself makes a significant contribution as there is no significant increase in the neutrophil population in this lobe with time. The question then arises as to whether the changes in the neutrophil population in the right apical lobe are a reflection of changes in the lung in general ie whether they are correlated with percentage neutrophils in the randomly sampled lobes. Examining the correlation between the square root transformed neutrophil percentages from repeatedly and randomly sampled lobes demonstrates that a significant correlation does indeed exist (Figure 5). Thus we make the observation that any tendency towards a neutrophilic response in the lung as measured by sampling random lobes appears to be magnified in the right apical lobe.

The observation may relate to an absolute difference in this particular lobe's capacity to recruit neutrophils in response to an undefined stimulus to the lung in general. In this regard, it is of some interest that there is a predilection towards developing neutrophilic inflammation and lung lesions in the anteroventral lobes of the lung, including the right apical, in sheep naturally or experimentally infected with *Pasteurella* spp. The alternative explanation, that the procedure of bronchoalveolar lavage, whilst in itself insufficient to maintain neutrophilic inflammation over a 4-7 day interval, might increase the potential of a

particular lobe to recruit neutrophils where there is a 'lung-wide' stimulus to do so, is equally plausible. Both hypotheses are amenable to further examination.

In conclusion, our findings should be of value to anyone interested in using bronchoalveolar lavage to monitor changes in ovine lung cell populations over time. They indicate that bronchoalveolar lavage conducted at 4-7 day intervals affects neither total cell numbers nor absolute counts of individual cell phenotypes, whether the BALF is obtained from the same or randomly selected lobes. They highlight the variability of such measurements and as such this information can be used to calculate group sizes required to demonstrate significant change in a particular variable. Lastly, the relative proportion of neutrophils in BALF samples obtained by repeatedly sampling the right apical lobe, was greater and more variable than respective proportions in randomly sampled lobes.

## **Acknowledgements**

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Figure 1.

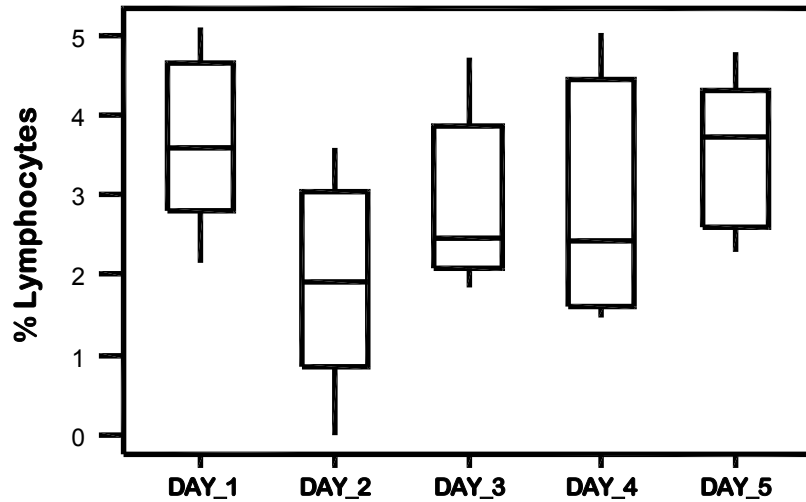


Figure 1: Box-and-whiskers plot of lymphocyte percentages over the time course of the study. The bottom of the box is at the first quartile (Q1), the top is at the third quartile (Q3) and the middle horizontal line depicts the median value, thus the 'box' defines the middle half of the data. The whiskers are the lines that extend from the top and bottom of the box to the lowest and highest observations still inside the region defined by the lower limit  $Q1 - 1.5(Q3 - Q1)$  and the upper limit  $Q3 + 1.5(Q3 - Q1)$ . There were no values outside these limits. The percentage of lymphocytes on day 2 was significantly less than that on days 1 and 5 five ( $p = 0.03$  for both comparisons; paired t-test).

Table 1

	Random					Repeated				
	Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max
Cells/ml of BALF ( $\times 10^3$ )	60.1	35.39	54.3	6.5	133.1	54.7	34.69	44.8	10.3	173.1
% Neutrophils	2.9	3.73	1.4	0	19.0	6.9 <sup>†</sup>	11.56	2.9	0	59.2
% Macrophages	93.4	4.83	95.2	78.7	100	89.5	11.69	93.4	38.5	100
% Lymphocytes	3.1	2.01	2.5	0	10.3	3.0	1.26	2.9	0	5.1
% Mast cells	0.1	0.19	0.1	0	0.8	0.2	0.26	0.1	0	1.5
% Eosinophils	0.4	0.89	0.2	0	5.4	0.3	0.46	0.1	0	1.9
% Other	0	0	0	0	0	0.3	1.00	0	0	5.3

Table 1: Summary statistics relating to total and differential cell counts from repeatedly and randomly sampled lobes. The statistics describe the pooled data (n=40 for repeated and n=40 for random ie BALF samples from both repeatedly and randomly sampled lobes in each of 8 sheep BALF sampled on 5 separate days). † Two-sample t-test results indicate a significantly greater percentage of neutrophils in BALF from repeatedly sampled lobes; p = 0.042).

Figure 2

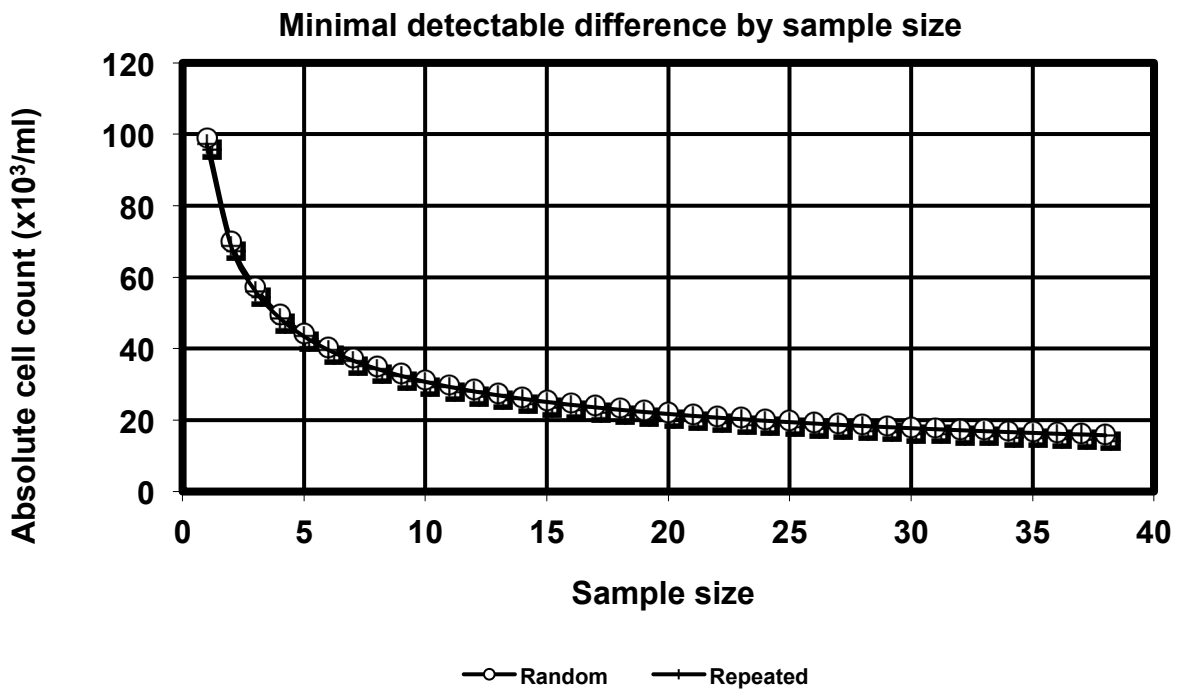


Figure 2. Minimal detectable difference in relation to sample size for total cell counts expressed per ml of BALF from the same lobe sampled repeatedly (-+), and multiple randomly selected lobes (-o-) within the same lung.

Figure 3

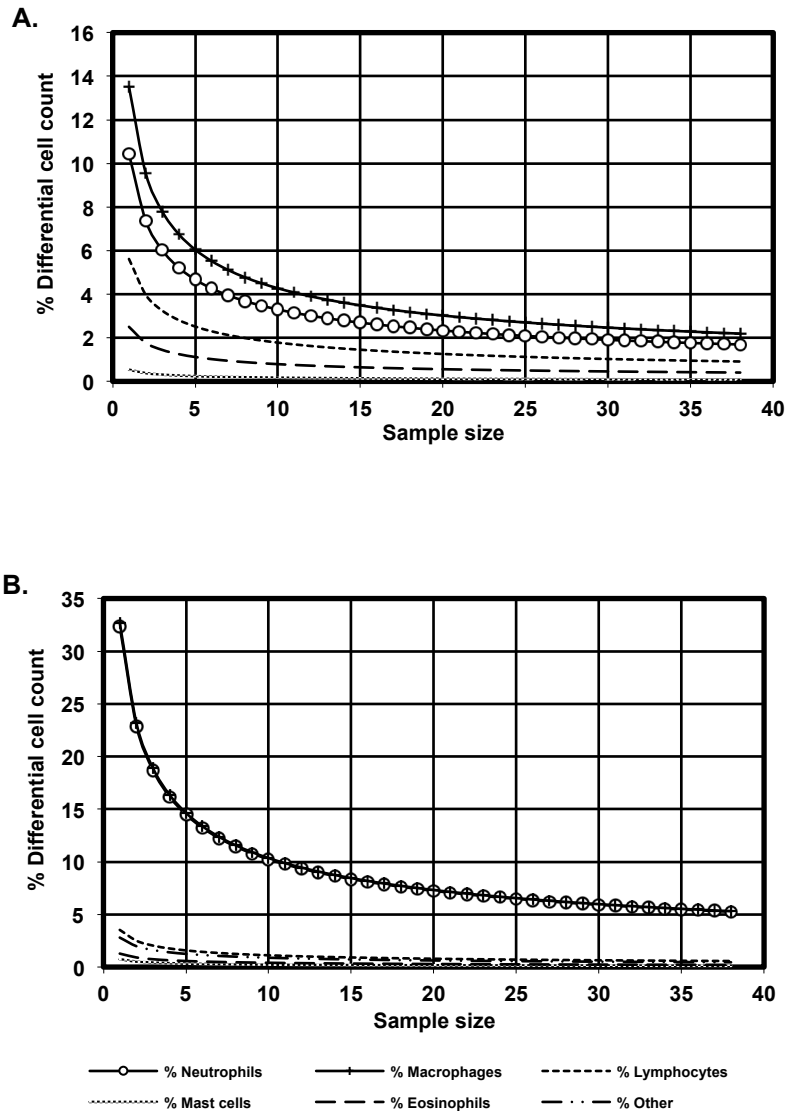


Figure 3. Minimal detectable difference in relation to sample size for percentage differential cell counts in BALF from (A) multiple randomly selected lobes within the same lung, and (B) the same lobe sampled repeatedly.

Figure 4.

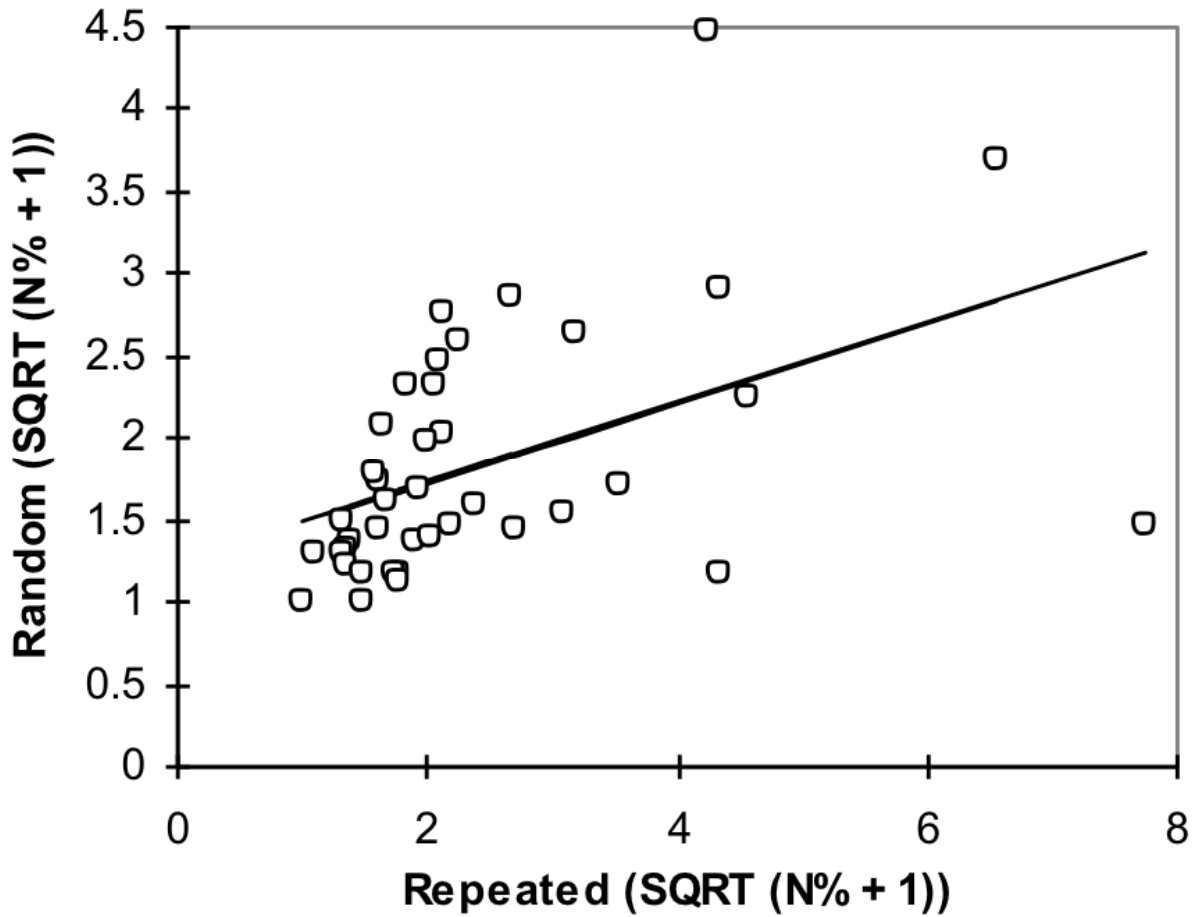


Figure 4. Association between percentage neutrophil counts in repeatedly and randomly sampled lobes. The percentage data (+1) was square root transformed (SQRT). A significant correlation exists between these variables (Figure 5)( $p < 0.005$ ;  $r = 0.46$  (Pearson product moment correlation coefficient)).

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