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Evaluation of a Morphometric Density Approach for Quantitation of Pneumonia Severity using a Murine Influenza Model

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Abstract

Routine 5 µm H&E-stained lung sections were utilized to investigate the capability of histomorphometry for determining pneumonia severity using a murine influenza model. Lung density was measured using the NIH ImageJ software program and the results for binary digital images were compared to corresponding results for semiquantitative pneumonia severity scoring. Lung samples from mice exposed (inoculated controls or IC) or not exposed (non-inoculated controls or NC) to influenza virus were evaluated at 3-, 5-, 7-, 10- and 15-days following exposure. On day 15 the mean density and standard deviation for NC at 25-x were 106 ± 23.6 and for IC 154.9 ± 36.3, and the difference was significant ($p \geq 0.05$). Using 400-x measurements NC values were 68.1 ± 8.5 and IC 96.8 ± 16.1 and significant. Significant differences were present at either magnification in the occurrence of "morphometric pneumonia" (defined as density values above the upper 95% confidence interval of controls). Density measurements @ 25-x were moderately correlation with pneumonia severity scores (correlation coefficient or r=0.55), but measurements @ 400-x were strongly correlation with scores (r=0.75). The approach provides a simple, rapid, and inexpensive method for the quantification of pneumonia using free digital software and routine microscopy. It also provides a quantitative method for validation of semiquantitative severity scoring of pneumonia.

Keywords: Mice • Pneumonia • Lung density • Morphometrics

Introduction

Digital microscopy is often applied for the interpretation and quantification of pathological evaluations using experimental animals [1-9] and humans [10]. However, many studies utilize expensive commercial image analysis systems having proprietary software that preclude routine standard applications or employ labor- and time-intensive procedures. We evaluated the use of a relatively simple inexpensive morphometric method to quantify the histologic lung inflammatory response using a murine pneumonia model. For this purpose, the free, downloadable NIH ImageJ image analysis software program was utilized in conjunction with a standard microscope and attached camera. The results of this study are reported.

Materials and Methods

General methods

The lung sections used in the study were retrieved from our histology archives of previous studies. The samples represented noninfected control (NC) and infected control (IC) populations from a large therapeutic drug trial that utilized a mouse influenza pneumonia model.] A detailed description of the mouse pneumonia model was previously published by other investigators [11]. Briefly, the IC mice were infected intranasally with 5 x 104 H1N1 influenza virus suspended in 50ul media, while the NC mice were given only media. A total of 177 lung samples were included in the study. Groups of mice were sacrificed and evaluated at 3-, 5-, 7-, 10- and 15-days following virus exposure. Samples of lungs were removed and placed in neural buffered formalin for histology slide processing. Our routine histology and morphometric studies utilized standard 5 µm thick H&E-stained sections.

Microscopic evaluations were performed using an Olympus CX31 microscope (Olympus Corporation, Breinigsville, PA). Photomicrographs were taken with an AmScope MU1403 camera (AmScope, Irvine, CA) using the associated software-based program. The microscope lamp setting was 5 and the software was set at medium density (2048 x 1644 pixels).

Morphometrics

The morphometric approach used for quantifying lung inflammation represents a modification of the method we previously reported for quantification of avian bone marrow cellularity using density measurements [9]. The lung sections were first photographed at 25-X (1 image) and 400-X (3 images) magnifications. For each histology section the entire lung area present at 25-X was outlined and measured. Three microscopic fields from the most affected regions were used for the 400-X evaluations, each field measuring 15,510 μm^2 with a total area of 46,530 μm^2.

The colored photographed images were converted to binary format using the downloadable National Institutes of Health (NIH) ImageJ software program. The pixel densities of the converted binary images were then calculated by the image analysis software. The presence of "morphometric pneumonia" (MP) was arbitrarily defined as a density value that was above the upper 95% confidence interval for the NC control values and for a particular magnification. The 95% confidence intervals are given in (Table 1). The percentage of pneumonia area was also determined for some images.

The morphometric results were also compared to those obtained by semiquantitative pneumonia severity scoring. A six -level scoring system for grading pneumonia was employed in which: 0=absent, pneumonia not observed; 1=minimal severity; 2=mild; 3=moderate; 4=marked and 5=severe. When uncertainty existed between scoring groups in assigning a value, half values were given. All evaluations were made in a "blind" manner without reference to experimental treatments.

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Table 1. The results of routine histologic and morphometric evaluations.

25-x RESULTS										
	NC GROUP					IC GROUP				
Day	3	5	7	10	15	3	5	7	10	15
Number	16	6	17	17	18	25	9	26	25	18
Minimum	57	66	43	60	67	73	57	85	100	84
Maximum	140	117	158	161	143	160	140	186	241	209
Mean	110.6	97.8	111.8	115	106.5	118.5	114.4	131.1	157.8	154.9
Std. Dev.	23.7	22.3	27.9	24.1	23.6	24.1	29.4	22.6	38.3	36.3
Lower CI	98	74.3	97.5	102.6	94.8	108.6	91.8	122	142	136.8
Upper CI	123.3	121.2	126.2	127.4	118.3	128.5	137	140.2	173.6	172.9
Mean Density (ANOVA)						>0.99	>0.99	>0.9999	0.007	0.0004
Occurance (Fishers Test)						0.53	0.028	0.13	0.004	0.012
400-x RESULTS										
	NC GROUP					IC GROUP				
Day	3	5	7	10	15	3	5	7	10	15
Number	16	6	17	17	18	25	9	26	25	18
Minimum	58	54	50	42	52	51	73	71	59	61
Maximum	89	80	87	87	86	101	96	119	126	118
Mean	69.9	62.4	70.2	66.7	68.1	79	83.4	92.4	92.6	96.8
Std. Dev.	8	9.8	9.2	11.7	8.5	14.1	6.9	11.9	16.6	16.1
Lower CI	65.6	52.1	65.4	60.6	63.9	73.2	78.1	87.6	85.8	88.8
Upper CI	74.1	72.7	74.9	72.7	72.4	84.8	88.6	97.2	99.5	104.8
Mean Density (ANOVA)						>0.99	0.328	4.00E-04	<0.0001	<0.0001
Occurance (Fishers Test)						0.004	0.002	<0.0001	0.0002	0.0005

CI=95% Confidence Interval

Statistical evaluations

Statistical analysis utilized the GraphPad Prism version 9 software program (GraphPad Software, Inc. San Diego, CA.). Statistical differences between the groups were determined using the Kruskal-Wallis ANOVA test and the Dunn's test for multiple comparisons. Statistical differences in pneumonia occurrence utilized the Fisher's exact test. Correlation coefficients were calculated using the Pearson test.

Results

Microscopic examples of a noninfected normal control lung (NC) and infected pneumonia lungs (Infected control or IC) are shown in (Figure 1A-F). The results for routine histologic and morphometric evaluations are given in (Table 1) and are presented graphically in (Figures 2-4).

Histologically the viral pneumonia was focal to multifocal, but sometimes diffuse (Figure 1). The inflammation was characterized by thickening and hypercellularity of the alveolar walls and flooding of the alveolar lumens with mainly mononuclear inflammatory cells. Cuboidal metaplasia of type II alveolar pneumocytes was sometimes present resulting in an adenomatous appearance (Figures 1C and 1D).

A suggestion of an increase in the mean density or "morphometric pneumonia" (MP) for infected group (IC) compared to the non-infected lungs (NC) group using the 25-X images occurred as early as experimental day 3. A significant difference between means of the IC and NC groups at this magnification was not apparent until day 10 and persisted on day 15 (Table 1 and Figure 2). Significant elevations in mean density in IC compared to NC when using 400-X images occurred from 7 day onward (Figure 3). Note that there was a reduction in density for NC at the 25X compared to 400-X measurements (Table 1).

The strength of correlations between the pneumonia severity scores and mean densities was generally good (Table 1). While the strength of the correlation coefficient using the 25-X density data was moderate (r=0.55), there was a strong correlation using the 400-X evaluation data (r=0.75).

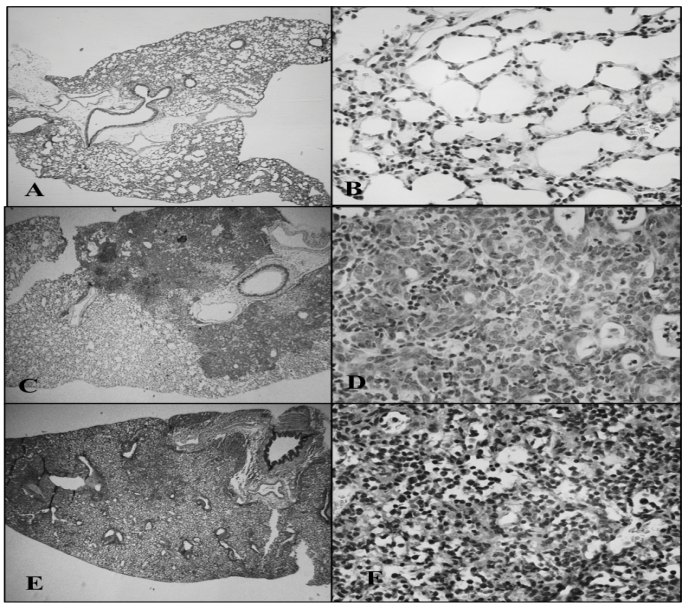

Figure 1A-1F. (A,B) NC lung section 56. Normal lung shown respectively at 25-X and 400-X. The pneumonia severity score is 0, the mean density @ 25-X 60.2 and the density @ 400-X is 42.9. The involved pneumonia lung area was 0%. **(C,D)** Focal pneumonia involved lung IC 76 on day 15 shown respectively at 25-X and 400-X. The pneumonia severity score was 3.5. The mean density @ 25-X measured 87.9 and @ 400-X 103.6. The area involved is 46%. It should be noted that all measurements made at 400-X were from the pneumonia areas. **(E,F)** IC lung section 39. Diffuse pneumonia example 39 on day 7 shown respectively at 25-X and 400-X. Pneumonia score 4.0, mean density @ 25-X 151.1 and @ 400-X 95.6. The involved area is 100%.

Discussion

The general approach of using binary digital image density measurements to quantitate pneumonia severity in mice appears valid. It provides a simple and inexpensive method for quantification of pneumonia parameters using routine

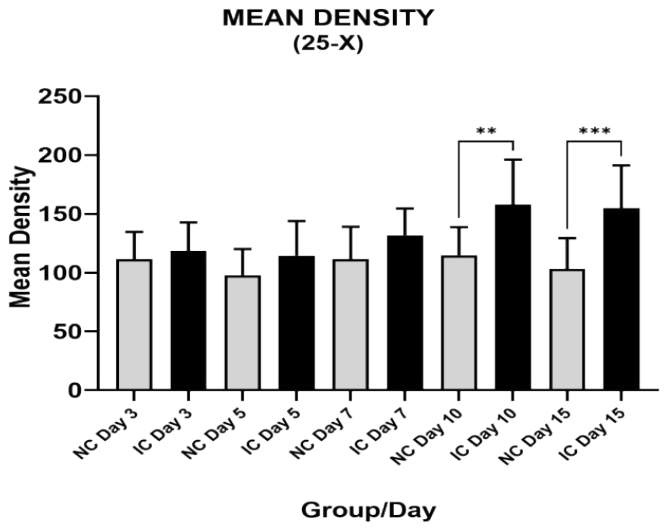

Figure 2. Mean density measurements made from the 25-X binary images. Although there is a suggestion of an elevation in density of the IC relative to the NC group occurring as early as day 5, a significant difference was not seen until day 10. The background density recorded in the NC reflects normal lung density levels.

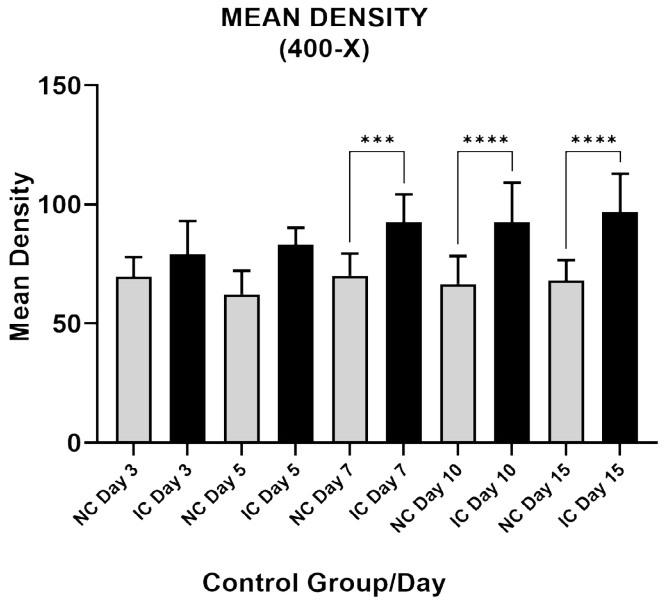

Figure 3. Mean density measurements made from the 400-X binary images. Although there is a suggestion of an elevation in density of the IC relative to the NC group occurring on days 3 and 5, a significant difference was not seen until day 7 and persisted to day 15.

histology sections, standard microscopy and ImageJ, a free downloadable software program. The method is rapid, and results can be obtained in a few seconds. The procedure j provides an approach for evaluating archived histology specimens. The basic morphometric approach reported could be further refined by application of other procedures such as special staining or immunohistochemistry. For example, the % area of pulmonary fibrosis in mice has been measured using special staining (Masson's trichrome) for collagen detection 7. The color-threshold was adjusted for blue to detect collagen staining and the % blue area determined by scanning for blue coloration [7].

Our approach for measuring pneumonia intensity is based on the method we previously developed for quantitation of bone marrow cellularity in chickens [12]. It is also similar to another method reported for quantitation of bone marrow cellularity in chickens with chicken anemia virus (CAV) previously described by others [1]. Those authors used a positive pixel count algorithm for quantification of bone marrow cellularity which measured area and staining intensity. However, that method also requires an image analysis optic system using proprietary software. In contrast, our density approach is performed with

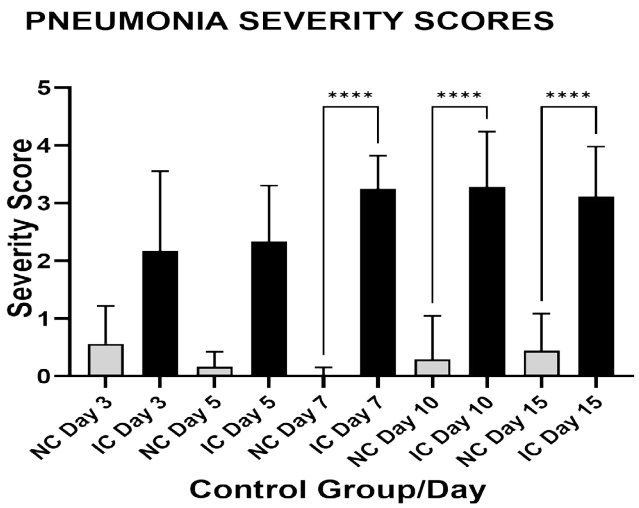

Figure 4. Pneumonia severity scoring results. While there is a suggestion of an elevation in scores of IC relative to the NC group even on days 3 and 5, a significant difference was not seen until day 7 and persisted to day 15.

a standard microscope using a software program provided free for download from NIH.

The density results using 25-X images were generally different from the results with the 400-X images. The difference mainly reflects the incorporation of the entire lung section area for the 25-X density measurements which includes normal lung regions. This allows for estimation of overall pneumonia involvement that reflects both intensity and the lung area involved. In contrast, the 400-X density measurements exclusively reflect the severity or intensity of pneumonia in the involved lung regions. This was also an apparent difference in strength of correlation between density measurements made at different magnifications to severity scores for pneumonia. Thus, measurements at the two magnifications can potentially provide different types of useful quantitative information on pneumonia involvement.

Pneumonia severity scoring in this study was performed by an experienced investigator. However, differences in semiquantitative lesion scoring can vary between observers. Digital density measurements allow for verification or standardization of the severity scoring. The calibration of lesion scoring results with lung density measurements facilitates the establishment of normal ranges and the standardization of severity scoring between different observers.

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Declaration of Conflicting Interests Statement

Dr Wilson has served as a paid consultant to the Center for Natural Products Research at the University of Mississippi who provided samples for this study.

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