



Histopathologic Evaluation and Scoring of Viral Lung Infection

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Abstract

Emergent coronaviruses such as MERS-CoV and SARS-CoV can cause significant morbidity and mortality in infected individuals. Lung infection is a common clinical feature and contributes to disease severity as well as viral transmission. Animal models are often required to study viral infections and therapies, especially during an initial outbreak. Histopathology studies allow for identification of lesions and affected cell types to better understand viral pathogenesis and clarify effective therapies. Use of immunostaining allows detection of presumed viral receptors and viral tropism for cells can be evaluated to correlate with lesions. In the lung, lesions and immunostaining can be qualitatively described to define the cell types, micro-anatomic location, and type of changes seen. These features are important and necessary, but this approach can have limitations when comparing treatment groups. Semiquantitative and quantitative tissue scores are more rigorous as these provide the ability to statistically compare groups and increase the reproducibility and rigor of the study. This review describes principles, approaches, and resources that can be useful to evaluate coronavirus lung infection, focusing on MER-CoV infection as the principal example.

Key words MERS-CoV infection, Lung, Scoring, Pathology, Immunostaining

1 Introduction

Emergent coronaviruses such as severe acute respiratory syndrome (SARS-CoV) and Middle East respiratory syndrome (MERS-CoV) have caused significant impacts on human health, especially during their initial outbreaks [1, 2]. People infected with these coronaviruses often have significant lung disease that contributes to clinical morbidity and mortality [3–5]. Histopathologic examination and immunostaining (e.g., immunohistochemistry) of lung tissues are essential to better understand disease pathogenesis and evaluate novel treatments of these current (and future) virus outbreaks [6–10]. Here, we will focus on MERS-CoV infection to present important principles for valid qualitative and quantitative evaluation of infected lung tissues.

1.1 Factors that Influence Evaluation

Preparation of quality lung tissue samples is important for histopathologic examination to optimize preservation of fine pulmonary architecture and, in the case of immunostaining, antigenicity of target epitopes [11–13]. A study by Engel and Moore identified more than 60 variables in this time frame, beginning with proper sample collection and handling and including multiple aspects of tissue collection, fixation, processing, embedding, slide drying, and storage [14]. Thus, attention to details and quality early will greatly aid the subsequent evaluation, interpretation, and impact of tissue examination.

To collect lungs for histology, samples should be harvested as soon as possible following death to minimize autolysis [11]. Autolysis (“self-digestion”) is a postmortem change characterized by degradation of cellular constituents (DNA, RNA, protein) and dissolution of the tissue [15]. Not only can this cause degradation of epitopes and increased nonspecific staining with immunohistochemistry, autolytic regions can be morphologically confused with foci of necrosis and edema [15–17]. If animals will be euthanized, it is preferable to select a method that does not target the lungs such as an intravenous agent. Even use of inhalational overdose of carbon dioxide, as is commonly used in rodents, can potentially cause minor edema/hemorrhage [11, 18, 19]. Evaluation of controls should be standard to evaluate for antemortem or euthanasia-related variables affecting lung evaluation. When examining rodents versus lungs from larger animals or humans, sampling becomes a relevant variable. For instance, mice have small lungs that can be sectioned onto one glass slide for widespread evaluation. Larger sized lungs cannot be sampled adequately using only one slide without introducing sampling bias. Therefore, several samples will need to be collected in larger lungs. The collection method will need to be defined in the methods of publications and should include collection site (standardized vs. lesions sites) and total number, the latter of which depends on the size of the lungs, distribution of lung lesions, and overarching goals of the study.

Proper and adequate fixation of the tissues is essential to retain optimal tissue morphology and cellular antigenicity for immunostaining techniques [11, 20]. However, it is important to remember that if lungs are to be assessed or scored for macroscopic (gross) indicators of disease (such as color, surface texture, and consistency), this must be done prior to fixation, which will affect all of these parameters. Macroscopic evaluation and scoring can be a nice tool to complement histopathology lesions [21, 22]. For tissues that will be paraffin embedded, sections are typically fixed in 10% neutral buffered formalin or 4% paraformaldehyde, though other fixatives may be employed based on the desired analysis endpoints. Collected lung samples can be placed in a minimum of 20:1 volume of fixative:tissue with a maximal thickness of tissue of no more than ~5 mm in at least one dimension to be consistently fixed [20]. For

rodents, inflation of the lungs via intratracheal instillation of fixative is recommended to best preserve lung morphology and reduce artifactual atelectasis [15]. However, this approach is contraindicated for lung infection as this can alter the anatomic location of inflammation and cellular debris [22]. The lungs and heart of rodents can be removed en bloc for fixation. Freezing of tissue may be an alternative approach to preserve specific antigens, but this process typically results in suboptimal retention of cellular and tissue architectural detail [11].

After processing to dehydrate the fixed lungs, samples must be embedded and sectioned in a consistent manner. Due to the relatively small size of mice, all lung lobes can be embedded en bloc with the ventral lobar surfaces oriented down in the cassette, which results in sections showing longitudinal views of major conducting airways. An alternative approach for mice, or standard approach for larger species, lung lobes can be collected as multiple sections that fit into a cassette, with each sample embedded separately [11]. Slides are typically stained with hematoxylin and eosin (HE) for routine histologic evaluation. If immunostaining is desired, it is essential to optimize and validate each new antibody utilizing appropriate positive and negative controls to ensure accurate staining results [20, 23]. Similarly, if special histochemical stains will be employed, appropriate control slides and tissues should also be utilized for each batch.

Awareness of normal anatomy and morphology is necessary to recognize any type of change and when utilizing animal models of human disease, this includes knowing differences between the species [24, 25]. For example, there are a number of morphologic differences between the respiratory tract structures of mice and humans. Lobation is distinct, in that mice have four right lung lobes (cranial, middle, caudal, and accessory) and only one left lobe, while humans have three right lung lobes (upper, middle, and lower) and two left lobes (upper and lower) [15, 22]. Rats and mice lack intralobular septa, intrapulmonary bronchi, intrapulmonary submucosal glands, and respiratory bronchioles. Mice also have more club cells extending to the trachea, a thinner blood-gas barrier, and a smaller alveolar diameter than humans [11, 26]. These anatomic variations do not mean that rodents cannot be very valuable models of lung disease; rather they are highlighted here as an example of the type of knowledge necessary for correct interpretation of experimental models.

Inclusion of experienced board-certified pathologists, who are specially trained to examine and interpret tissues changes, as part of the multidisciplinary team can greatly enhance the quality of tissue evaluation [22, 27]. By histopathology, a skilled eye (ideally a pathologist familiar with the model) can not only define the types of inflammatory processes, but also corroborate these findings to clinical signs and/or data from other analyses [22, 27–30]. In addition, pathologists have knowledge of correct lesion

nomenclature, as well as potential effects of such variables as strain-related background lesions, husbandry, the microbiome, and diet on the interpretation of results [25]. If pathologists are not involved in designing translational experiments and interpreting lesions in animal models, bias may be introduced and the accuracy of the data and conclusions may be questionable. This approach, which lacks the expertise of a pathologist trained in tissue interpretation, has been labeled as “do-it-yourself pathology” and is linked to multiple publications containing erroneous interpretations [22, 25, 31, 32]. While observations made by biomedical personnel may be biologically accurate in some cases, it is important to note that tissue examination by non-pathologists (even those who are “scientific experts” for a particular disease) is prone to false-positive and false-negative errors and not recommended [33]. Ideally, tissues should be examined by a pathologist familiar with histopathology of the model (*see Note 1*). It is recognized that not all labs have access to pathologists for this role and in many situations a member of the investigational team is assigned to the role. In these situations, if possible, it helps to have a pathologist review the study findings prior to publication or have the examiner meet with a pathologist to screen the slides and data for accuracy.

Lungs have unique features compared to other organs that are important for consideration in designing experiments or when making interpretations. For study of infectious diseases, distribution and histologic appearance of lung lesions depends on a variety of factors including the viral inoculate concentration, route of exposure, regional deposition, cellular uptake, chronicity, and host immune response. For instance, inbred mouse strains can have variably sized airways that may affect viral droplet delivery or clinical disease manifestations such as airway obstruction [34]. Inbred mouse strains can also exhibit biased (e.g., Th1 vs. Th2 immune responses) or deficient immune signaling pathways that might influence infection susceptibility or severity [35, 36]. Sex can also be an influencing factor for infection and needs to be considered in the experimental design [37]. Even actions as simple as laying an animal in lateral recumbency to recover from anesthesia following viral inoculation may lead to more prominent lesions in certain lobe(s) [22]. For many of these features, inclusion of appropriate control animals (i.e., strain-, age-, and sex-matched, housed under identical husbandry conditions and free from confounding pathogens) is necessary and important to tease out any lesions unrelated to the treatments. Unlike the other organs in which the size is relatively static, the lung has dynamic size changes during normal respiration. Handing of the postmortem lung in a standardized manner is useful to prevent postmortem atelectasis or variable inter-animal insufflation. Right ventricular perfusion of fixative into the lungs prior to extraction can help with fixation as well as insufflate the airspaces without dislodging inflammation or mucocellular debris [22].

1.2 Histopathology

Histopathology is the microscopic examination of tissues for morphologic or structural changes that differ from normal and these changes are called lesions. Histopathology of coronavirus-infected lung in humans and animal models can be a useful tool to help define affected cells, illuminate the structural cause(s) of clinical signs, and clarify potential therapies. During disease outbreaks, clinical data including autopsy cases can be studied in parallel with animal model investigations to better define lung disease pathogenesis and therapies. For instance, in 2012 the novel human coronavirus known as MERS-CoV was first isolated from a patient dying in Saudi Arabia [2, 38]. In the region of the outbreak, local burial rituals along with the requirement for high biosecurity constrained autopsy studies from being performed until the first report in early 2016 [4]. Within a few years of the first reported MERS-CoV case in humans in 2012, several animal models were being studied and these models provided much of the initial critically important lung pathology data [39–44].

Histopathologic examination of viral lung infection requires awareness of any anticipated lesions from clinical or published data, as it is available. Examples of MERS-CoV lesions are listed in Table 1. For instance, acute diffuse alveolar damage (DAD) is a common feature of MERS-CoV lung lesions and it is composed of lesions such as edema, inflammation, and alveolar septal injury [4, 48–50]. While awareness of reported lesions can help guide the pathologist in examination, it is also useful to have a consistent method for examination of experimental tissues to avoid unintentional bias that might cause a failure to detection of unexpected lesions [51]. Consistent examination of all tissues from control and treatment groups can reduce the chances of mistakenly diagnosing nonspecific model background phenotype as a MERS-CoV-specific

Table 1
Examples of lesions seen in MERS-CoV lung infections

Lesions	Necrosis/cell death [45]
	Edema [8, 21, 45]
	Hyaline membranes/fibrin [21]
	Inflammation [8]
	Thrombi [8, 46]
	Congestion [8]
	Hemorrhage [45, 46]
	Pneumonia [46, 47]
	Type II hyperplasia [47]
	Syncytia [47]

Table 2
Methods of masking to prevent observer bias [22, 52–54]

Method	Approach	Usage
Comprehensive	Samples are labeled without group identification (1, 2, 3, 4 . . .), minimal background information provided	Allows for experienced observers to score well-defined models, otherwise susceptible to errors
Grouped	Samples are labeled according to de-identified groups (A1, A2, A3, B1, B2, B3 . . .)	Allows for masked evaluation of groups while observer is informed about experimental context
Post-examination	Samples are examined in a transparent manner to determine the type and scope of tissue changes, samples are then masked for scoring	Allows for full examination and disclosure of experimental context; groups with small N may let observer recall sample group assignment

lesion [22, 25, 29]. For instance, a lesion that is present in the controls and treatment groups can be defined as a background model/technique phenotype and should not be reported as a MERS-CoV specific lesion. Masking of the pathologist to the group assignments is useful to avoid observer bias and each type of masking method has certain advantages and limitations (Table 2, *see Note 2*) [22]. A common approach for histopathologic examination is to start at low magnification to screen for any obvious lesions and assess quality of the tissue section (*see Note 3*). This allows examination of microscopic structures such as airways, alveoli, alveolar septa, air spaces, vessels, and pleura. Examination at high magnification allows for screening of cellular and interstitial components of each structure for lesions (e.g., injury, inflammation, necrosis). Most slides will be examined using HE, but additional stains can be used on serial sections to further define any changes. For instance, mucus in goblet cells or secreted into air spaces can be highlighted by special stains like Periodic acid Schiff in glycogen-depleted tissues or Alcian blue [55, 56].

After the slides and stains have been examined for all groups, the results will need to be prepared for publication. Qualitative characterization of the findings is very important to understand features of the disease including cellular tropism, anatomic predisposition, and nature of lesions leading to clinical signs (*see Note 4*) [10, 21]. Qualitative descriptions of lesions include type (e.g., epithelial sloughing/necrosis), location (e.g., alveoli), distribution (e.g., locally extensive), inflammation (e.g., neutrophilic), and cell types involved (e.g., type I pneumocytes). Qualitative features can be sufficiently described in the text and exemplified in representative figures. Use of arrows and other forms of annotation are valuable in figures to clarify and guide readers through the images. High-quality descriptions will help the reader (including reviewers) better understand what was seen and allow for others to reproduce the study.

1.3 Immunostaining

Immunostaining (immunohistochemistry) is a valuable tool in viral lung disease investigations as it can be used to study cellular localization of receptors and viral targets. For instance, detection of the MERS-CoV receptor dipeptidyl peptidase 4 (DPP4) virus receptor can give insights to cell tropism to help explain disease pathogenesis [4, 6, 21, 42, 57–59].

There are several tissue handling (preanalytical) factors that can significantly affect the quality and specificity of immunostaining and its analysis. These have been discussed earlier sections of the paper and in several reviews [20, 52, 60–63]. Similarly, there are many factors during the staining procedure that itself can also influence the results. Deparaffinization, lack of control tissues, optimization/validation techniques, species, batch effects, and chromogens can all influence the final quality and assessment of immunostaining methods. Standard operating procedures for each of the technical steps, if used by all biomedical staff, can significantly mitigate many of these issues. Use of positive and negative control tissues for each batch of immunostained tissues can help in validating appropriate staining and also making clear any potential nonspecific immunostaining. After the stained slides have been examined for all groups, qualitative statements about the immunostaining can be made and prepared for publication text and images. Descriptive text of immunostaining (receptor or virus) could include cell types (e.g., type I pneumocytes), cell integrity (necrotic vs. intact cells), and subcellular location (e.g., diffuse cytoplasmic). Demonstration of immunostaining using annotated images can strengthen the qualitative data.

1.4 Scoring

As shown above, qualitative descriptions of tissue changes are useful and necessary, but they are less applicable in terms of group comparisons. More robust and reproducible methods are desirable and these criteria can be sought in tissue scoring systems (semiquantitative and quantitative) that produce data that allow for statistical analyses for evaluation of group differences (*see Note 5*) [52, 53]. Importantly, these scoring principles can be applied to tissue lesions (gross and/or histopathologic) as well as immunostained sections.

1.4.1 Nominal Approaches

Nominal approaches do not score or make quantitative measurements on tissue samples, but rather each sample is assigned to well-defined categories [52, 54]. The numbers of samples assigned to each category are recorded and evaluated with appropriate statistical tests. As a simple mock example, consider examining the lungs of wild-type (WT) or mutated mice for the presence or absence of edema, a common feature of DAD. Each mouse would be assigned to either “no edema” (Fig. 1a) or “edema” (Fig. 1b, c) categories. If 10 mice per group were evaluated, the WT group might have nine with edema and one without, while the mutated group has

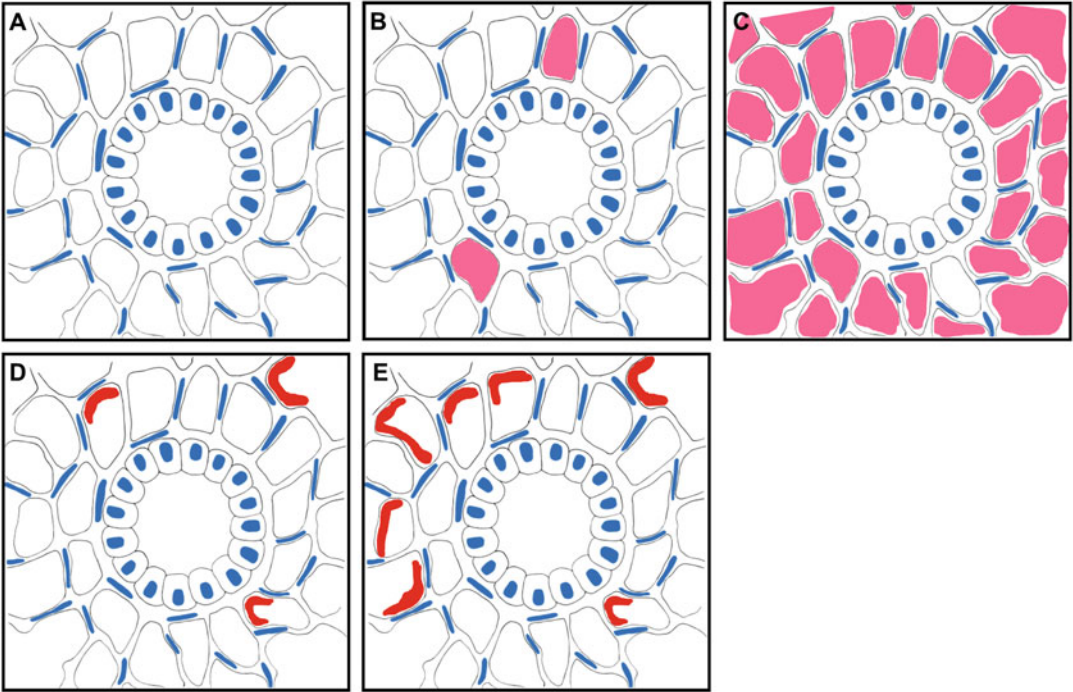


Fig. 1 Mock example of mouse lung lesions during MERS-CoV infection. (a) Normal bronchiole and alveolar structures. (b, c) Pulmonary edema (pink color filling alveoli). (d, e) Hyaline membranes (red crescents lining alveolar walls)

three with edema and seven without. Evaluating these data using a Fisher exact test results in a significant difference ($P = 0.02$) between WT and mutated mice. The presence of any lesion or immunostaining can be similarly assessed in this manner, but it is important to have clear guidelines or thresholds to distinguish the categories.

1.4.2 Semiquantitative Approaches

Semiquantitative approaches are used to transform qualitative tissue changes into numerical scores using specific morphologic criteria [52, 53]. Semiquantitative methods have several advantages in that they can be done with minimal technical resources, quickly at the microscope for small to medium studies, provide guidance for future quantitative studies, and provide complementary data for publication [52–54]. The most commonly used semiquantitative methods produce ordinal scores. Ordinal implies there is an order or progression of severity in the assigned grades that define each score, with typically four to five grades being optimal (e.g., 0, 1, 2, 3, 4). Each grade should be well defined so there is minimal ambiguity in assigning samples. Use of simple descriptive modifiers such as normal, rare, mild, moderate, and severe is discouraged as these have different meanings for each observer and thus limit reproducibility of the scoring. As a mock example of ordinal

scoring, WT and mutated mice might be evaluated for the extent of hyaline membranes lining alveolar walls. The scoring grades might look like: “0”—none, “1”—<25% (*see* Fig. 1d), “2”—26–50% (*see* Fig. 1e), “3”—51–75%, and “4”—>75% of alveolar walls in the lung section. If the ordinal scoring for seven mice per group produced the following results for WT (3, 3, 2, 3, 4, 3, 4) and mutated mice (1, 2, 1, 1, 1, 1, 2), then the data can be statistically analyzed. Importantly, ordinal scores do not meet the assumptions required for parametric tests; thus nonparametric tests should be used [33]. For the mock example, the difference between groups using a Mann-Whitney U-test was significant ($P = 0.002$).

1.4.3 Quantitative Approaches

Quantitative methods are tissue techniques that measure specific tissue components (length, area, volume, number, percentage, etc.) [52]. Quantitative methods tend to have greater precision and sensitivity than semiquantitative methods. These methods often require high-quality images and specialized software to properly analyze the tissues, which can make the methods costlier for some labs than semiquantitative techniques. The growing interest in automation and artificial intelligence may increase future efficiency and cost-effectiveness of quantification of tissue parameters, especially for large projects [64–67].

Quantification of viral lesions and immunostaining in tissues is an option; however, quantification is not commonly performed in tissue sections due to potential confounding factors such as random distribution of viral inoculum and difficulty in objectively quantifying lesions. If choosing to perform quantitative scoring, evaluation of clinically relevant anatomic compartments (airways or alveoli) can help standardize the assessment. As a mock example, viral immunostaining could be evaluated as a percent of cell number in mouse bronchioles (Fig. 2a–c; 0%, 12.5%, and 43.8%, respectively) or as an alternative one could also assess the area of immunostaining as a percent of the bronchiolar epithelium area. In contrast, the alveolar compartment can be more difficult to assess than airways because of their thin walls, which makes evidence of necrosis/sloughing or immunostaining a challenge. To normalize analysis, one could assess the percent of alveoli with immunostaining (Fig. 2d–e). However, this would likely require extensive time/labor or specialized software. If quantitation is not feasible but is an important variable, one could revert to semiquantitative scoring to assess immunostaining as a percentage of affected alveolar walls. Using the distribution scoring system defined for Fig. 1, one could score the samples in Fig. 2d–e, as ordinal scores of 1 and 4, respectively. While the mock example is simple, reality often paints a more complex portrait of lesion or immunostaining distribution (Fig. 2f).

When it comes to tissue scoring, each project is unique. Investigators will have to evaluate the lung samples to determine the best scoring approaches in relation to the breadth of lesions and goals of

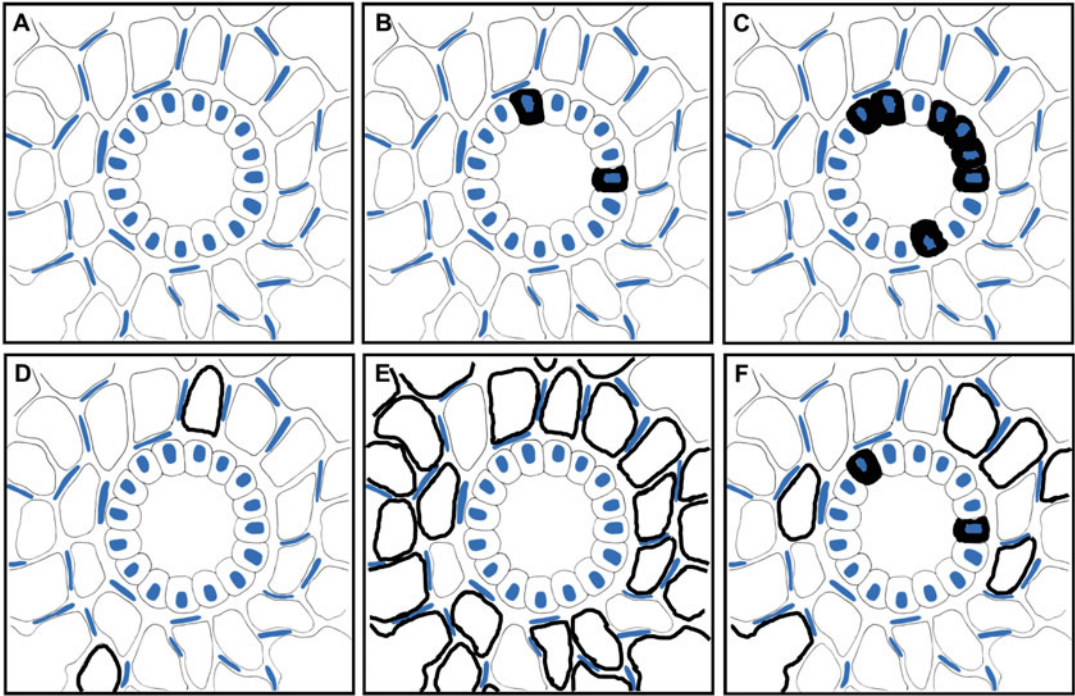


Fig. 2 Mock example of viral immunostaining during MERS-CoV infection. (a) No immunostaining in control lung. (b, c) Immunostaining (black color) in airways. (d, e) Immunostaining in alveoli. (f) Immunostaining in airway and alveoli

the project. Most importantly, any scoring that is performed should be corroborated, when possible, with other data to validate the findings [22, 52, 53]. For instance, if group A has more immunostaining than group B, this could be validated by ELISA or Western blots of whole lung homogenates. Alternatively, lesion severity could be corroborated to measurements of clinical data (*see Note 6*). Validation can help give more confidence in the data rigor and reproducibility.

1.4.4 Statistical Analyses

Inappropriate use of paired t-tests and shopping for significance are two issues that have slipped into the published literature and potentially compromise the interpretation and reproducibility of studies [33]. For the various scoring methods, statistical analyses of the data should involve the collaborative expertise of a statistician to be able to identify the most relevant tests to confidently evaluate for group differences [22, 33, 52, 53].

2 Summary

Examination of infected lung tissues for histopathology and immunostaining are common and needed approaches to study viral lung infection, especially in emergent coronaviruses like MERS-CoV.

Following the principles and concepts above will help guide and lead studies to more valid and reproducible data.

3 Notes

1. Ideally a pathologist familiar with the model is available for the lab to evaluate experimental tissues. If not, then a pathologist collaborator should be sought to perform or review of the results of examination prior to submission for publication. This prevents publication of data that is flawed or needs subsequent retraction.
2. Masking is important to prevent potential bias by the observer pathologist (Table 2). For new projects, the post-examination is preferred as this helps the pathologist understand the goals/experimental design of the project as well as see quality and scope of lesions/stains. For most other research projects where the pathologist is familiar with the model, these can be masked in grouped fashion to maximize the interpretative power of the pathologist to screen for biologically relevant changes in a group-specific manner. Comprehensive masking is often discouraged as it effectively constrains the ability of the pathologist in defining relevant versus unconnected data and therefore limits the sensitivity and specificity of the pathology data.
3. Evaluation of slides from all treatment and control groups prior to detailed examination is useful to give the pathologist an overview and primer of the type, scope and severity of lesions/stains.
4. Detailed examination of the tissues allows for extrapolation of qualitative descriptive data. If there are questions regarding the cells/tissues that can be addressed by specific stains—these could be done at this time to corroborate/clarify descriptive findings.
5. When biologically relevant lesions are defined in the project, group-specific changes may be evaluated for by semiquantitative or quantitative scores. Semiquantitative approaches are often done initially and the results can be used as screening tools to set up primary scoring approaches or be used as primary/supplemental data for reporting group differences in lesions or stains. Quantitative approaches may be performed by at the microscope (e.g., cell counts) or automated on digital images by specialized software.

Regardless of the masking method (*see Note 2*), it is often useful to score the slides in a random masked fashion and in one sitting to prevent diagnostic drift. After scoring, it is sometimes beneficial to take scoring data to see if these same differences

are morphologically detectable in the respective groups. If the pathologist can see these differences, it gives further confidence to the scoring approach and final interpretations. If not, it can raise questions as to the scoring methods.

6. Effective reporting of pathology data requires transparency of methods, numbers of animals, statistical analyses, etc. Producing graphs of scoring data with matching images that are annotated can be very powerful tools in conveying the results to readers.

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