Comparison of Immunohistochemistry Methods for Visualization of Middle Eastern Respiratory Viral Antigen

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Introduction

Middle Eastern respiratory syndrome (MERS), caused by MERS coronavirus (MERS-CoV) **(Figure 1)** first appeared in Saudi Arabia in 2012. **(3)** Although there have been less than 2,400 cases reported, the case fatality rate is 35% **(4)**. Dromedary camels are the known host animal for the virus. However, due to the size, cost, and requirement of specialized facilities for research camels, other potential experimental animals have been sought. In a recent study, alpacas proved to be a suitable experimental animal to further characterize MERS-CoV infection **(1)**. Our work is part of a wider project focused on studying the efficacy of MERS-CoV antigen and RNA labeling in infected alpaca tissues, when these tissues are preserved in novel modular alcohol fixatives compared to 10% neutral buffered formalin (NBF). These new fixatives have superior molecular properties, equivalent histomorpohology **(2)** and like NBF also inactivate the virus. Work with infectious MERS-CoV must be done at biosafety level (BSL) 3. In contrast, preserved samples wherein the virus is inactivated can be worked with in BSL-2. Unfortunately, 10% neutral buffered formalin has poor properties as regards preservation of proteins and nucleic acids **(2)**. In order to examine these new fixatives further, we first validated immunohistochemistry (IHC) for MERS-CoV antigen in formalin-fixed, paraffin-embedded (FFPE) alpaca tissues to serve as an experimental control.

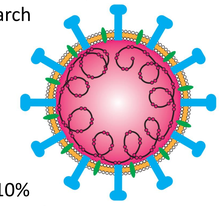

Figure 1

Methods and Materials

Tissues and antibodies:

Formalin-fixed, post-infection day 3 and 6, MERS-CoV infected alpaca trachea and nasal turbinates tissues provided from an alpaca challenge study at Colorado State University in which the animals were inoculated subcutaneously with the Erasmus Medical Center/2012 strain of MERS-CoV were provided by co-author Richard Bowen. These samples were subsampled, processed, embedded in paraffin and unstained sections for IHC according to KS Veterinary Diagnostic Laboratory (KS VDL) standard practices. Formalin-fixed, paraffin-embedded (FFPE) alpaca trachea from the KS VDL served as uninfected control tissue. Although multiple antibodies were tested, here we report our success with rabbit polyclonal anti-MERS-CoV antibody (ab) (40068-RP02, Sino Biological; Wayne, PA).

Immunohistochemistry:

We tried avidin-biotin complex (ABC) **(Figure 2)** and polymer-based horseradish peroxidase (HRP) **(Figure 3)** detection techniques and selected the method with the best initial results for further optimization. The tissue sections were deparaffinized in xylenes and rehydrated through graded (100%-95%-70%) ethanols to distilled water, optionally heat antigen retrieved in pH 6.0 sodium citrate buffer, blocked with 3% H_2O_2 and matched serum. All further steps were performed at room temperature with 1x Tris buffered saline (TBS) and 0.01% tween-20 (TBSt) washes between steps. The primary antibody was initially diluted in 1x TBS to between 1:500-1:2000 (0.5 – 2.0 µg/ml), and detection tried separately with VECTASTIAN® ABC HRP Kit (Peroxidase, Standard) (PK-4000, Vector Labs [VL]; Burlingame, CA) **(Figure 4),** VECTASTAIN® ELITE® ABC HRP Kit (Peroxidase, Rabbit IgG) (PK-6101; VL) **(Figure 5)**, and ImmPRESS® HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit (MP-7401; VL) **(Figure 6)** per manufacturer's instructions. All were visualized with 3,3'- diaminobenzidine (DAB) (PL-4100; VL) or Immpact DAB (SK-4105; VL). The slides were counterstained with Mayer's hematoxylin, dehydrated and mounted with Permount. Later the specificity of the primary antibody was further tested through use of a matched isotype antibody (31235, ThermoFisher Scientific; Rockford, IL) control **(Figure 8)**. Slides were digitally scanned on an Aperio GL (Leica Biosystems Inc.; Buffalo Grove, IL).

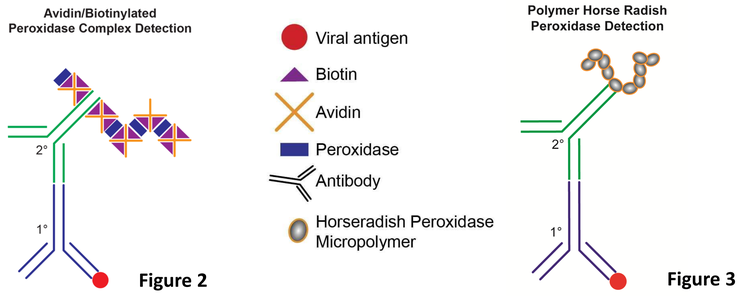

Figure 2 **Figure 3**

Results

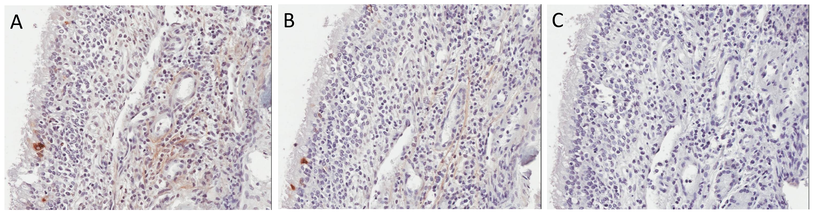

Figure 4. Avidin-Biotin Complex detection of MERS-CoV antigen in infected alpaca nasal turbinates

ABC-HRP detection was tested with different concentrations of anti-MERS-CoV ab. Signal was present with all ab concentrations. There was significant background at 1.0 µg/ml (A) and even more with 2.0 µg/ml (data not shown) compared with 0.5 µg/ml (B). The 0.5 µg/ml signal was weak. The reagent control (C) had no signal or background. The 0.5 µg/ml concentration was chosen for further analysis. Animal #4.

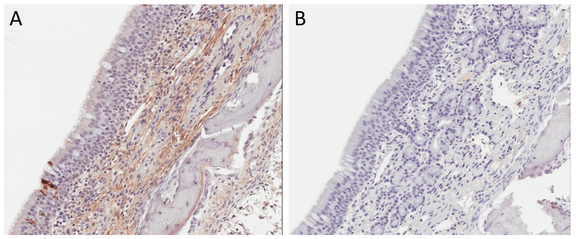

Figure 5. Avidin-Biotin Complex Elite detection of MERS-CoV antigen in infected alpaca nasal turbinates

ABC-HRP Elite detection of 0.5 µg/ml anti-MERS-CoV ab improved the viral antigen signal:noise (A). Minimal background was evident on the reagent control (B). Animal #4.

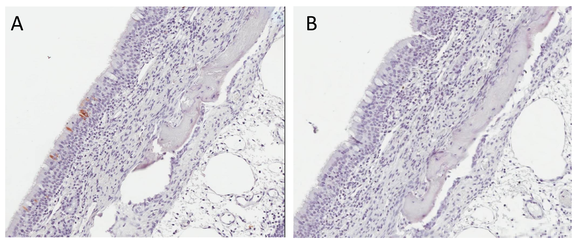

Figure 6. Polymer detection of MERS-CoV antigen in infected alpaca nasal turbinates

Polymer-HRP detection of 0.5 µg/ml anti-MERS-CoV ab provided a reliable, albeit weaker signal, with no background (A). The reagent control (B) had neither non-specific signal or other background. Since the signal was weak, further optimization of this approach included trying stronger DAB and increasing the primary ab concentration. Animal #4.

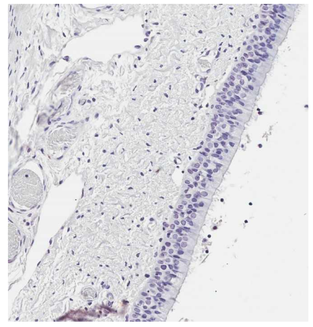

Figure 7. Polymer detection of MERS-CoV antigen in uninfected alpaca trachea

Polymer-HRP detection of anti-MERS-CoV on uninfected control trachea had no specific signal as expected at 1.0 µg/ml anti-MERS-CoV ab.

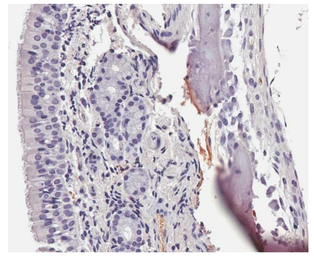

Figure 8. Polymer detection with an isotype control in uninfected alpaca nasal turbinates

Matched isotype control, 1.0 µg/ml rabbit IgG isotype control ab, on MERS-CoV infected nasal turbinate tissue further confirmed the signal specificity. Animal #9.

Discussion

- The best approach was heat antigen retrieval with pH 6 sodium citrate buffer, 20 minute H_2O_2 block, 2.0 µg/ml of anti-MERS CoV antibody, polymer detection and Impact DAB.
- Across all 8 study animals, we detected viral antigen more frequently and abundantly in the nasal turbinate respiratory epithelium compared to the tracheal epithelium. MERS-CoV antigen was present in both ciliated and goblet cell cytoplasm. This signal correlates with expected MERS-CoV antigen distribution in the MERS alpaca model.
- Future directions include:
  - Further analysis of IHC for all the FFPE alpaca tissues (n=8 animals)
  - IHC protocol development fofr matched tissues preserved in the novel alcohol fixatives
  - Correlation of IHC with MERS-CoV *in situ* hybridization, which labels viral RNA instead of antigen

Acknowledgements

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References

1. Adney, D. R., Bielefeldt-Ohmann, H., Hartwig, A. E., & Bowen, R. A. (2016). Infection, Replication, and Transmission of Middle East Respiratory Syndrome Coronavirus in Alpacas. *Emerging Infectious Diseases*, *22*(6), 1031-1037.
2. Chung, J. Y., Song, J. S., Ylaya, K., Sears, J. D., Choi, L., Cho, H., … Hewitt, S. M. (2017). Histomorphological and Molecular Assessments of the Fixation Times Comparing Formalin and Ethanol-Based Fixatives. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, *66*(2), 121–135. doi:10.1369/0022155417741467
3. Vergara-Alert, J., van den Brand, J., Widagdo, W., Muñoz, M., Raj, S., Schipper, D....Segalés, J. (2017). Livestock Susceptibility to Infection with Middle East Respiratory Syndrome Coronavirus. *Emerging Infectious Diseases*, *23*(2), 232-240.
4. https://www.who.int/emergencies/mers-cov/en/ (visited 3/29/2019)