Immunohistochemistry: A Rapid and Specific Diagnostic Tool for Influenza Virus Infection in Pigs

Abstract
During the 2016 spring, a local outbreak of severe dyspnea, seromucous nasal secretion, anorexia and loss of productive performance was identified in an intensive pig farm in southern Chile. Samples of diseased animals were submitted to the Diagnostic Laboratory of the Faculty of Veterinary Medicine, in Chillan, Chile, for pathological and histopathological study. Macroscopically, lungs appeared to be congested, edematous and “non-collapsed”, which could be related to lesions compatible with interstitial pneumonia. Microscopic lesions showed a variable thickening of the septa, epithelial necrosis of the airways, diffuse inflammatory infiltration surrounding bronchial and bronchiolar areas, inflammatory exudate in the alveolar spaces with activate alveolar macrophages and fibrin. All lesions were compatible with Swine Influenza Virus (SIV) infection, and we successfully confirmed through SIV nucleoprotein immunohistochemistry staining of said samples, by utilizing a rapid and specific first-line diagnostic method that allows a rapid decision-making tool to control the spread of the virus. Additionally, the presence of the virus was confirmed by a serological field study.

Keywords: Immunohistochemistry; Diagnostic; Antigen-detection; Swine Influenza Virus; Pigs

Introduction
Influenza A viruses are important in veterinary medicine because they cause influenza in horses, swine and birds, among other species [1]. In humans, horses and swine, influenza is an acute respiratory disease that can become a major epidemic problem [2-5] and cause local problems in production systems [6]. Swine flu is an infectious disease caused by an influenza virus type A, characterized by the sudden onset of cough, dyspnea and fever, and is considered one of the most devastating swine respiratory diseases in intensive production systems [7]. This infection has a significantly negative impact in the productive performance of animals, reducing average daily gains, increasing feed conversion efficiency and causing several other complications related to secondary infections by common agents in the respiratory tract [8]. Additionally, fever in animals could lead to miscarriages and reproductive failure [9]. According to Donovan [10] & Dykhuis et al. [11], the estimated cost of the infection in marked pigs ranges from 3.23 to 10.31 dollars per head. The use of a rapid and specific diagnostic tool, which allows the producer to make the right decision, is essential. The aim of this study was to describe a rapid and specific immunohistochemistry (IHC) method in order to diagnose Swine influenza Virus Infection from field cases in diagnostic laboratories.

Material and Methods
In order to confirm and evaluate the diagnosis of Swine Influenza Virus, the immunohistochemistry technique was performed on lung samples. The specimens, a total of three cuts per affected pig, with a positive control (donated by Dr. Carlos Perfumo, Universidad de La Plata, Argentina), were fixed in 4% buffered formalin solution, embedded in paraffin, and sectioned at 4 μm. The paraffin wax sections were rehydrated and its endogenous peroxidase activity was inhibited by 3% H2O2 in methanol for 30 minutes and washed with phosphate-buffered saline solution (PBS) for 5 minutes. After hydration, sections of tissue were incubated in trepsin solution (0.1%) in presence of calcium chloride dihydrate 3M for 20 minutes at 37°C. Later on, slides were washed in PBS several times, and the samples were incubated overnight at 4°C with primary Anti-Influenza A Antibody, nucleoprotein, clones A1, A3 Blend (Millipore, MAB 8251) diluted 1:500 and the Cell Marque Ancillary Reagents Diamond: antibody diluent (Sigma-Aldrich, 938B). Negative control slides in absence of primary antibody but in presence of mouse IgG were included for each staining. ImmPRESS™ HRP Universal Antibody (Anti-Mouse IgG/Anti-Rabbit IgG, Peroxidase) Polymer Detection Kit (Vector Laboratories, MP-7500) was incubated at room temperature for 30 minutes. Finally, ImmPACT DAB Peroxidase (HRP) Substrate kit (Vector Laboratories, SK-4105) was used to produce the color at the target site antigen to be recognized by the primary antibody. The nuclei were stained blue by the hematoxylin counterstain.

Results
Lung lesions were characterized by severe congestion, edema and interstitial pneumonia. As expected, histopathological analyses showed the same morphological patterns that were observed grossly, characterized by congestive interstitial blood vessels (pulmonary veins) and diffuse inflammatory infiltrate in the interstitial tissue. Additionally, severe bronchial and...
bronchiolar epithelial necrosis with cellular desquamation was observed (Figure 1A). The inflammatory infiltration was a mix of neutrophils and alveolar macrophages, with fibrin in the alveolar spaces forming hyaline membranes between cells (Figure 1B). Immunohistochemical staining of viral nucleoprotein showed intense viral replication in the lungs of pigs, located mainly in the airways, in the bronchial and bronchiolar epithelial cells (Figure 1C). In addition, specific cytoplasmic staining of alveolar macrophages revealed strong uptake of viral particles, either by direct phagocytosis of the virus or by phagocytosis of influenza virus-infected cells (Figure 1D).

Figure 1: Lung histopathology (H&E) of diseased pigs presents different levels of leukocyte inflammatory infiltration consisting predominantly of neutrophils, together with macrophages. In Section A, it is possible to identify severe bronchial epithelial cellular desquamation (→). Section B shows alveolar spaces containing numerous neutrophils and macrophages surrounded by fibrinous exudate (→). The expression and localization of the influenza virus antigen in lung tissue was detected by immunohistochemistry (sections C and D). Section C shows the positive antigen detection in the ciliated epithelia of the bronchus and the macrophages in the lamina propria (→). Section D shows alveolar macrophages with positive staining with the anti-nucleoprotein antibody (→).
11. Dykhuis HC, Painter T, Fangman T, Holtkamp D (2012) Assessing production parameters and economic impact of swine influenza, PRRS and Mycoplasma hyopneumoniae on finishing pigs in a large production system. The American Association of Swine. Vet. Ann. Meeting Denver, Colorado USA, p. 75-76.

12. Casanova T, Van de Paar E, Desmecht D, Garigliany MM (2015) Hyporesponsivity of Alveolar Macrophages and Higher Respiratory Cell Permissivity Characterize DBA/2 Mice Infected by Influenza A Virus. J Interferon Cytokine Res 35(10): 808-820.

13. Qi L, Kach JC, Dugan VG, Jagger BW, Lau YF, et al. (2011) The ability of pandemic influenza virus hemagglutinins to induce lower respiratory pathology is associated with decreased surfactant protein D binding. Virol J 412(2): 426-434.

14. Kim HM, Lee YW, Lee KJ, Kim HS, Cho SW, et al. (2008) Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. J Virol 82(9): 426.

15. Cappuccio J, Dibarbora M, Lozada I, Quirogac A, Oliveraa V, et al. (2017) Two years of surveillance of influenza a virus infection in a swine herd. Results of virological, serological and pathological studies. Comp Immunol Microbiol Infect Dis 50: 110-115.

16. Pantin-Jackwood MJ (2014) Immunohistochemical staining of influenza virus in tissues. Methods Mol Biol 1161: 51-58.