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Immunohistochemical and Molecular Detection of pH1N1 in Necropsied Pulmonary Tissues of Fatal Cases with Indeterminate Conventional Testing

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Abstract

The rapid emergence of a novel influenza A/H1N1 virus designated pH1N1 2009 caused one of the fastest pandemics of the twentieth century. The rapid development of an accurate detection test for this pandemic virus using reverse transcription-real time polymerase chain reaction (rRT-PCR) helped in timely diagnosis. In India this pandemic peaked between August to October 2009. The r-RT PCR for pH1N1 2009 was the main diagnostic test used on throat/nasopharyngeal swabs in all cases. While in majority of the cases this test provided reliable confirmation of the virus, it gave negative or indeterminate results in a subset of cases meeting the standard case definition for the pandemic infection and negative for seasonal flu. In the present study we examined 4 such fatal cases where microscopic pathology of the lung was consistent with viral bronchopneumonia for the presence of pH1N1 2009 using r-RT PCR on nucleic acid extracted from the paraffin sections that showed presence of viral antigens by immunohistochemistry. In all 4 cases pH1N1 sequences could be identified. These findings therefore emphasize the important role of microscopic pathology techniques in conjunction with molecular tools in the diagnostic confirmation of novel agents during a public health emergency.

Keywords Influenza; Immunohistochemical; Molecular detection; Pulmonary tissues

Short Communication

The investigations into the identification of an etiologic agent during an outbreak or pandemic have multiple components that includes conventional detailed clinicopathologic characterization of the disease; seroepidemiology, laboratory testing of representative clinical specimens using various conventional and modern techniques [1]. However, in many situations, especially in pandemics where the public health machinery is hard pressed for a rapid response, symptom based clinical case descriptions can overlap significantly with other ongoing illnesses having different etiologies leading to indeterminate laboratory findings. This becomes more challenging during a pandemic of acute respiratory illness. Classical pathology studies like autopsy and microscopic histology of the affected organs have always played a vital role in the possible cause of disease based on the nature and pattern of microscopic tissue lesions. The 2009 flu pandemic caused by a novel influenza. A virus of swine origin designated as pH1N1 2009 showed a very rapid global spread with morbidity and mortality pattern that showed fatal disease was mostly in the younger age group with pre-existing clinical conditions like obesity, chronic obstructive pulmonary and cardiovascular disease. The rapid and timely implementation of reliable laboratory diagnosis using a reverse transcriptase real-time polymerase chain reaction (rRT-PCR), international networking and implementation of recommended public health response strategies resulted in fast containment of the pandemic spread. In India, the 2009 pandemic pH1N1 affected a large population and showed significant morbidity and mortality values [2]. During this period, four clinically suspected fatal cases of pH1N1 from Pune city,

India, were found to be negative by the standard r-RT PCR but histopathology of necropsied lung tissue was suggestive of viral bronchopneumonia and tested positive for pH1N1 antigens by immunohistochemistry. In the present study we examined whether the viral RNA sequences could be detected from paraffin embedded sections using r-RT PCR.

Case 1

A 65-year-old female had a history of fever, chills, cough and dyspnea for eight days. Her past medical history was not significant and had no contact with pH1N1 positive cases. On examination she was febrile (39.1°C), heart rate was 98/min, blood pressure 100/60 and a respiratory rate of 28 breaths per minute. Chest auscultation revealed bilateral crackles. The patient was treated empirically with intravenous fluids, antibiotics, bronchodilators and oxygen using non-invasive mechanical ventilation therapy. She tested negative for pH1N1. The patient succumbed on the second day of admission.

Case 2

A three and half months old male child was admitted with complaints of fever, runny nose, and dyspnea. On presentation the child was febrile (39.5°C), tachycardia, and a respiratory rate of 46 breath/minute. Nasopharyngeal swabs tested negative for pH1N1. The patient was treated with intravenous fluids, antibiotics and oxygen but died of progressive hypoxemia and respiratory failure on the second day of admission.

Case 3

A 27-year-old seven-month pregnant female was admitted with a 4-day history of fever, vomiting, myalgia and dyspnea. Chest auscultation showed bilateral bronchial breath sounds. She received symptomatic treatment and was put on mechanical ventilator support from the 10th day of admission. Influenza A (H1N1) viral infection was suspected in this patient, but laboratory test for pH1N1 was negative. Her condition deteriorated and she died on 18th day of admission.

Case 4

A 21 years old male was admitted with a history of fever and dyspnea. Physical examination presented with a temperature of 39.3°C; tachycardia, respiratory rate 22 breaths / minute and blood pressure, 106/72 mm Hg. Chest auscultation showed decreased breath sounds and chest radiograph revealed bilateral lower lobar consolidation. His nasopharyngeal swab sample tested negative for pH1N1. He was treated with intravenous fluid, antibiotics and oseltamivir and died of progressive respiratory failure.

Autopsy was performed in three cases (Cases 1, 2 and 4) where tissue pieces of cerebrum (Case 4), lung (Cases 1, 2 and 4), liver (Cases 1, 2 and 4), trachea (Cases 2 and 4), and muscle (Cases 1 and 2) were fixed in 10% formalin and referred to NIV. Necropsy samples from lung and liver were obtained from case 3. Formalin fixed tissues were processed in a routine way as described elsewhere [3]. Five micron thick sections were stained with haematoxylin and eosin, Gram's, Ziehl-Neelsen and Gomori's methamine silver stains and microscopic changes examined by light microscopy. Previously described procedure was followed for immunohistochemical (IHC) labelling of pH1N1 antigens [3]; briefly, an anti pH1N1 2009 antisera raised in-house was used along with a commercially available kit (Novolink, Leica Microsystems, USA) as per the manufacturer's instructions. Lung sections from other non pH1N1 cases showing diffuse alveolar damage (including patients with seasonal influenza virus infections) were used as controls (Figures 1-3).

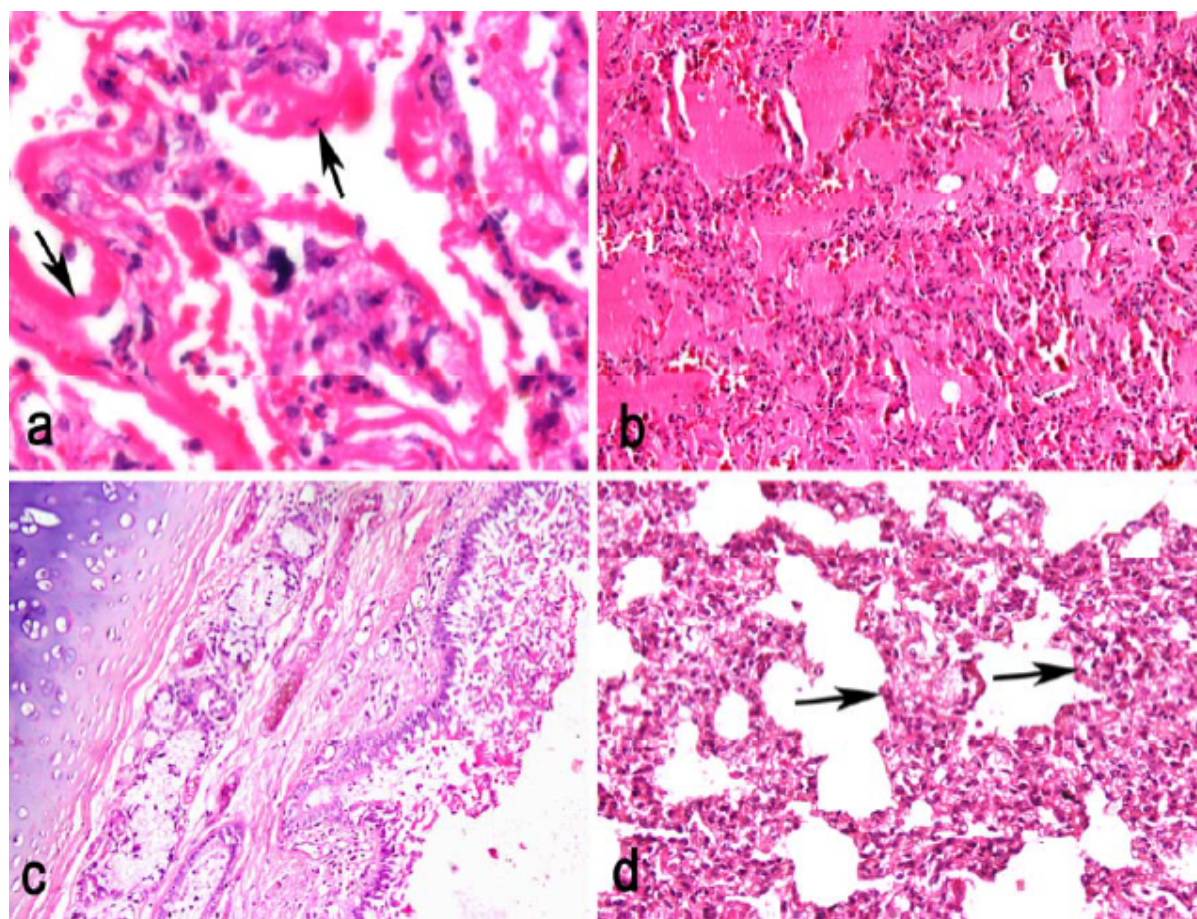


Figure 1: Representative photomicrograph of histopathological features in pulmonary necropsies. (a) Diffuse alveolar damage in lung tissue section with hyaline membrane formation (arrows). (b) Intra-alveolar edema and haemorrhage, (c) Section from tracheal wall showing desquamated epithelial cells and mononuclear cells infiltration in the submucosa. (d) Organizing DAD characterized by intraseptal fibrosis. All sections are H-E stained. Original magnification 100X, (b, c) 200X, (d) 400X (a).

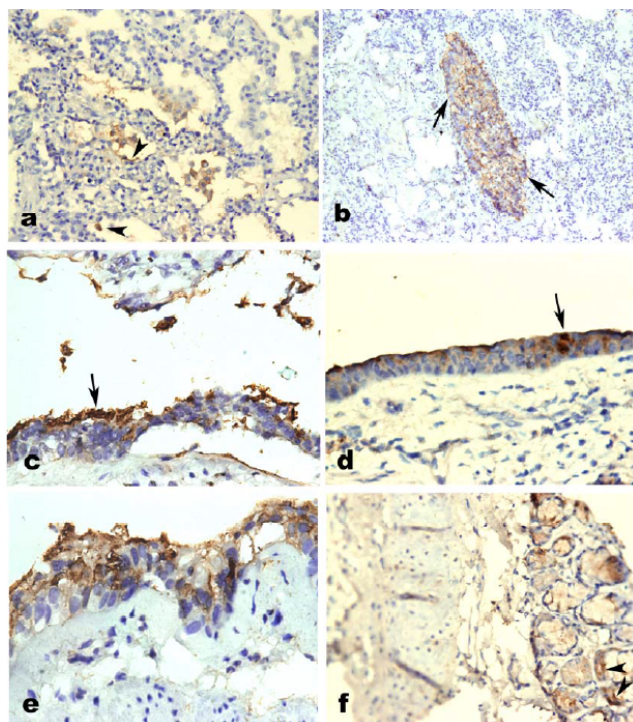


Figure 2: Immunohistochemistry for detection of pandemic influenza A p(H1N1) 2009 viral antigen: Viral antigen is stained red-brown on a haematoxylin background. (a) Positive immunohistochemical stain is present in intraalveolar cells and pneumocytes (arrow heads), (b and c) Bronchiolar epithelial cells showing strong IHC positivity for viral antigen. (d and e) The strong signals are found in tracheo-bronchial epithelial cells. (f) Virus antigens are also detected in subepithelial mucus glands of large airways. Original magnification 200X, (a, b and f) 400X, (c and d) 600X (e).

RNA was extracted from dewaxed sections using the Purelink™ FFPE total RNA isolation kit (Invitrogen USA) as per the manufacturer's instructions and subsequent rRT-PCR for pH1N1 2009 done as per the CDC protocol [4]. The main histopathological findings in all cases was diffuse alveolar damage (DAD) that included alveolar edema, haemorrhage, hyaline membrane formation, interstitial and septal edema with various degrees of mononuclear cell infiltration. There was minimal and focal chronic inflammation in tracheal and bronchial sub mucosa. Cytopathic effect in cells presented in the form of irregularly enlarged hyper chromatic nuclei. Case 3 also showed type 2 pneumocyte hyperplasia and alveolar septal fibroblast proliferation suggesting areas of transition towards organizing DAD (Figures 1a-1d). No microscopic features of vasculitis, thrombosis, hemophagocytosis or microorganisms were detected. Viral antigen was detected predominantly in the bronchial and bronchiolar epithelial cells, intra-alveolar macrophages, pneumocytes (Figures 2a-2f) and sub mucosal mucus-secreting glands of bronchus and trachea. Both nuclear as well as cytoplasmic positivity were noted. There was no staining in the control sections. The rRT-PCR detected pH1N1 viral RNA in all 4 cases (Figures 3a-3b).

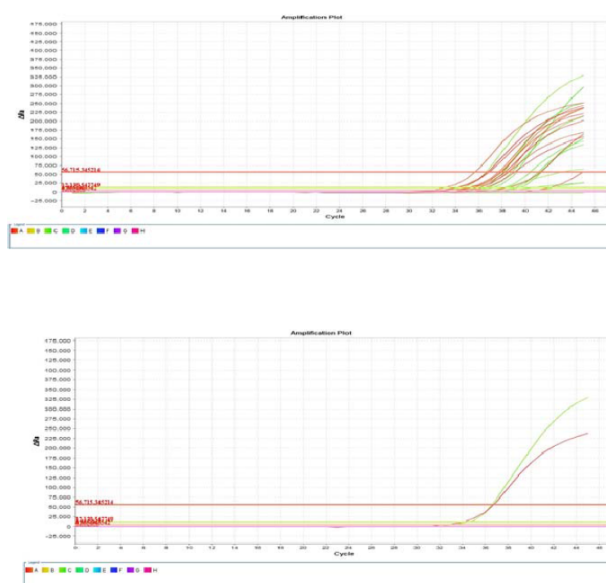


Figure 3: Amplification curve for reverse-transcriptase real time PCR curves for pH1N1 2009 virus in extracted RNA from sections (a) Amplification profile of all samples (b) single specimen from one representative case.

The pathologic anatomy of influenza associated bronchopneumonia was studied as early as in 1919 by Le Count and several major subsequent studies gave extremely well defined profiles on the nature of gross and microscopic pathology associated with pandemic and seasonal influenza deaths [5-7] including the recent 2009 influenza pandemic [3,7-12]. Interestingly, a comparative analysis of the nature of pathologic lesions seen in fatal cases from the past influenza epidemics show differences in presentation. Both the 1919 H1N1 and 2009 H1N1 pandemic presented with epithelial necrosis of large airways and diffuse alveolar damage. In contrast, the histologic findings in fatal seasonal H3N2 and H1N1 showed upper respiratory involvement with lesser extent of damage to alveoli. Immunohistochemical labeling for viral antigens were seen predominately in mucosal epithelial cells. In cases of the H1N1 of 1919 and pH1N1 2009 virus was detected in alveolar cells, predominantly pneumocytes and macrophages [7] a finding similar to those seen in pathology of human infections with H5N1 virus [13].

Interestingly, the pulmonary microscopic pathology in all the 4 cases having negative laboratory diagnosis for pH1N1 showed very consistent features of the H1N1 pathology that prompted us to do the r-RT PCR on the sections in these cases. Earlier studies on archived formalin fixed paraffin embedded tissue sections had isolated human DNA and carried out sequence analysis on it for manifold purposes ranging from ancient DNA studies on Egyptian mummies to more modern forensic medicine applications [14]. Later studies had used direct RNA extraction procedures from formalin-fixed and paraffin embedded tissue sections and showed the presence of viral sequences, especially where conventional testing methods are not available [15]. A case in point being the detection of Borne disease virus in brain sections [16]. The results from our present studies are highly consistent with a recent study where 222 post-mortem tissues were examined using a similar technique for influenza virus sequences [17].

The novelty of our study is that we could demonstrate the same in cases where the conventional laboratory testing on nasopharyngeal aspirate was negative. Interestingly, previous studies from our laboratory had demonstrated the presence of replicating pH1N1 2009 virus in Type II alveolar pneumocytes in pulmonary needle biopsy specimens by electron microscopy [18]. This study brings out an important point that even in the absence of a conclusive conventional diagnostic procedure for a viral agent like influenza, microscopic pathology and IHC with direct PCR on nucleic acids extracted from the sections have the potential to confirm identity of the infecting virus in suspected fatal cases. Further, this also strengthens the ground for using such molecular analysis from tissue sections directly for detecting other agents that may be cofactors and not routinely detected in the first-line diagnostic panel.

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