using the following primers: forward, 5′-GCAGATG CATATGTTTTTGTGGG-3′; and reverse, 5′-GATTTC CAGTTGCTTCGAATG -3′).[18] The RT-PCR products were directly sequenced with an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the PCR primers.

Results

Patients

The clinical characteristics of the 20 patients are presented in Table 1. Fourteen patients were male. The median age of the patients was 39.8 years (range, 2–81 years). Thirteen (65%) patients were aged between 15 and 59 years. Thirteen patients (65%) died within 3 days after the onset of influenza symptoms. The mean duration of illness was 5.7 days (range, 1–29 days). The most frequent medical history or underlying disease was cardiovascular disease, which included dilated cardiomyopathy, hypertension, chronic heart failure, atrial fibrillation, and pulmonary hypertension. Five patients were obese with a body mass index exceeding 30 kg/m^2. In terms of clinical symptoms, most patients had fever and cough. Antiviral treatment

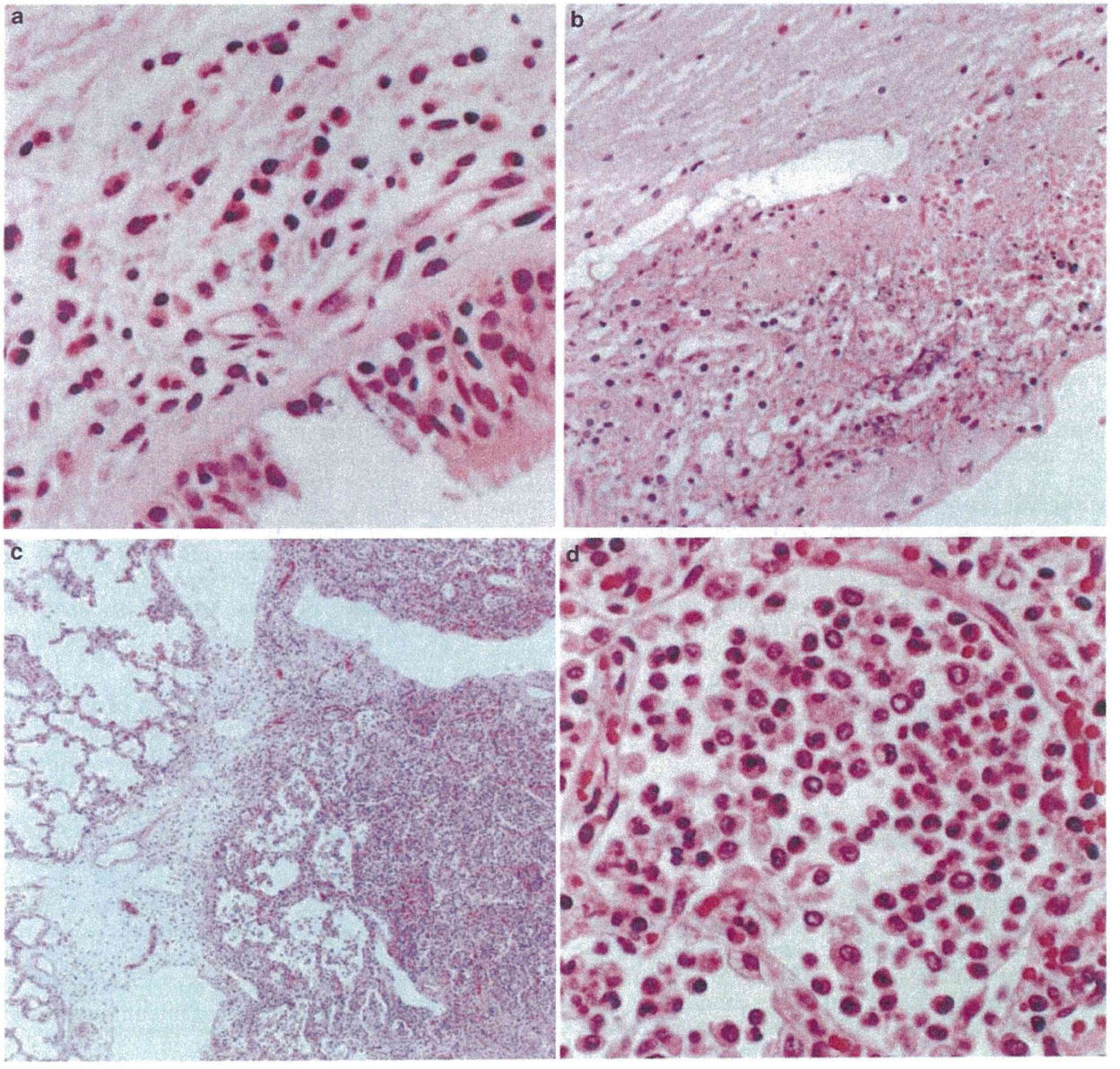
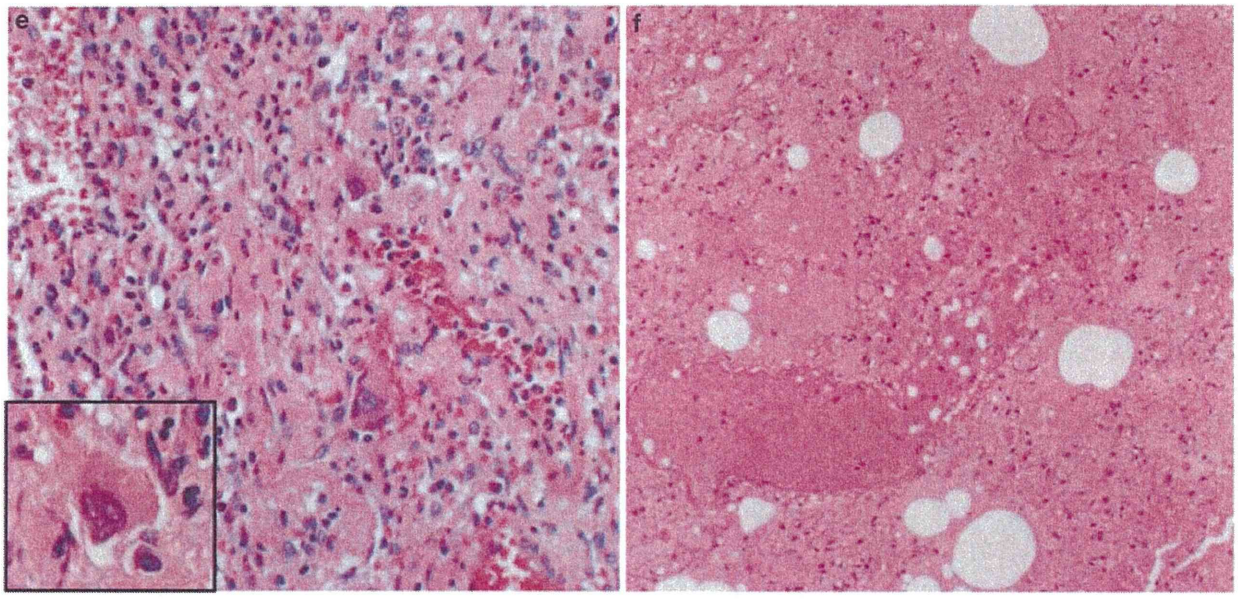


Figure 1 Representative histopathological findings on respiratory tissue sections with hematoxylin–eosin (H&E) staining. (**a**) Mucosal infiltration of eosinophils and (**b**) necrotizing tracheobronchitis (case 15). (**c** and **d**) Acute bacterial bronchopneumonia with massive infiltration of neutrophils in the alveolar spaces (case 13). (**e**) Organized diffuse alveolar damage (DAD) with giant cells (inset) (case 18). (**f**) Acute massive alveolar edema and congestion (case 19). Original magnification, × 400: **a**, **d**, and **e** (inset); × 200: **b** and **e**; × 100: **f**; × 40: **c**.



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was administered in 13 cases (oseltamivir in 11 cases and zanamivir in 2 cases) within 48 h after disease onset. Case 1 received oseltamivir from day 7 after disease onset. Six cases (cases 3–6, 19 and 20) from the medical examiner's office and cases 2 and 9 were found in the state of cardiac pulmonary arrest. Case 8 was dead on arrival without hospitalization. Eleven cases were hospitalized and seven of them were intubated because of hypoxia. Three cases (cases 15–17) were suspected of having fulminant myocarditis associated with influenza virus infection, because they suddenly developed ventricular fibrillation and cardiac arrest 2 or 3 days after disease onset. Bacterial culture was done for 11 cases. In three cases, lung specimens were taken aseptically during autopsy, and *Streptococcus pneumoniae* was cultured in one case and *Pseudomonas aeruginosa* in two cases. In one case, *S. pneumoniae* was cultured from the sputa on admission. The bacterial cultures were negative for the other seven cases.

Histopathological Findings of the Trachea and Bronchi

Histopathological changes in the trachea and bronchi are shown in Table 2. The most frequent histopathological findings were mucosal/submucosal mononuclear cell infiltration, multifocal desquamation of the epithelium, congestion and hemorrhage, mucosal infiltration of eosinophils (Figure 1a), and necrotizing tracheobronchitis (Figure 1b). We also observed inflammation in and around the bronchial glands (Figure 2e and k). The duration of illness in cases 17 and 18 was 29 and 21 days, respectively. The trachea and bronchi in these cases were regenerated and presented no remarkable pathological changes.

Histopathological Findings of the Lung

Histopathological findings of the lung are shown in Table 2. Exudative or organized DAD, hyaline membrane formation, acute intra-alveolar edema, congestion and/or hemorrhage, capillary or small vessel thrombosis, interstitial or intra-alveolar inflammatory cell infiltration, fibrosis, and type II pneumocyte hyperplasia were observed (Figure 2a, c, and g). Cases with massive infiltration of neutrophils in the alveolar spaces and bacterial isolation from a pharyngeal swab or lung specimens were defined as having secondary or coincident bacterial bronchopneumonia (Figure 1c and d). We excluded the pathological findings in case 17 from the pathological assessment of influenza infection, because they were extensively modified by complications caused by the long duration of percutaneous pulmonary support.

According to the most prominent histopathological feature in each case, there were several histopathological patterns. Five cases presented with DAD: acute DAD with a hyaline membrane (cases 1–4; Figure 2a, c, and g) and organized DAD (case 18; Figure 1e). Three cases had acute massive intra-alveolar edema with variable degrees of hemorrhage (cases 5, 6, and 19; Figure 1f) and five cases had secondary or coincident bacterial pneumonia (cases 7, 8, 11, 13, and 20; Figure 1c and d). Four cases had more severe lesions in the upper airway with limited intra-alveolar edema and hemorrhage (cases 9 and 12), or no pathological changes in the lower airway (cases 15 and 16). The main histopathological

findings in cases 10 and 14 included preexisting emphysema with congestion and edema.

Histopathological Findings of Extrapulmonary Organs

The pathological findings of heart tissue specimens in three cases, which were suspected of having myocarditis as described above, did not show inflammatory cell infiltration or myocyte necrosis. Cases 5 and 12 died of acute influenza virus-associated encephalopathy. In brain sections, case 5 showed histopathological changes in the brain, which included leakage of plasma proteins from vessels, activated microglia, and clasmatodendrosis of astrocytes (data not shown), whereas case 12 had

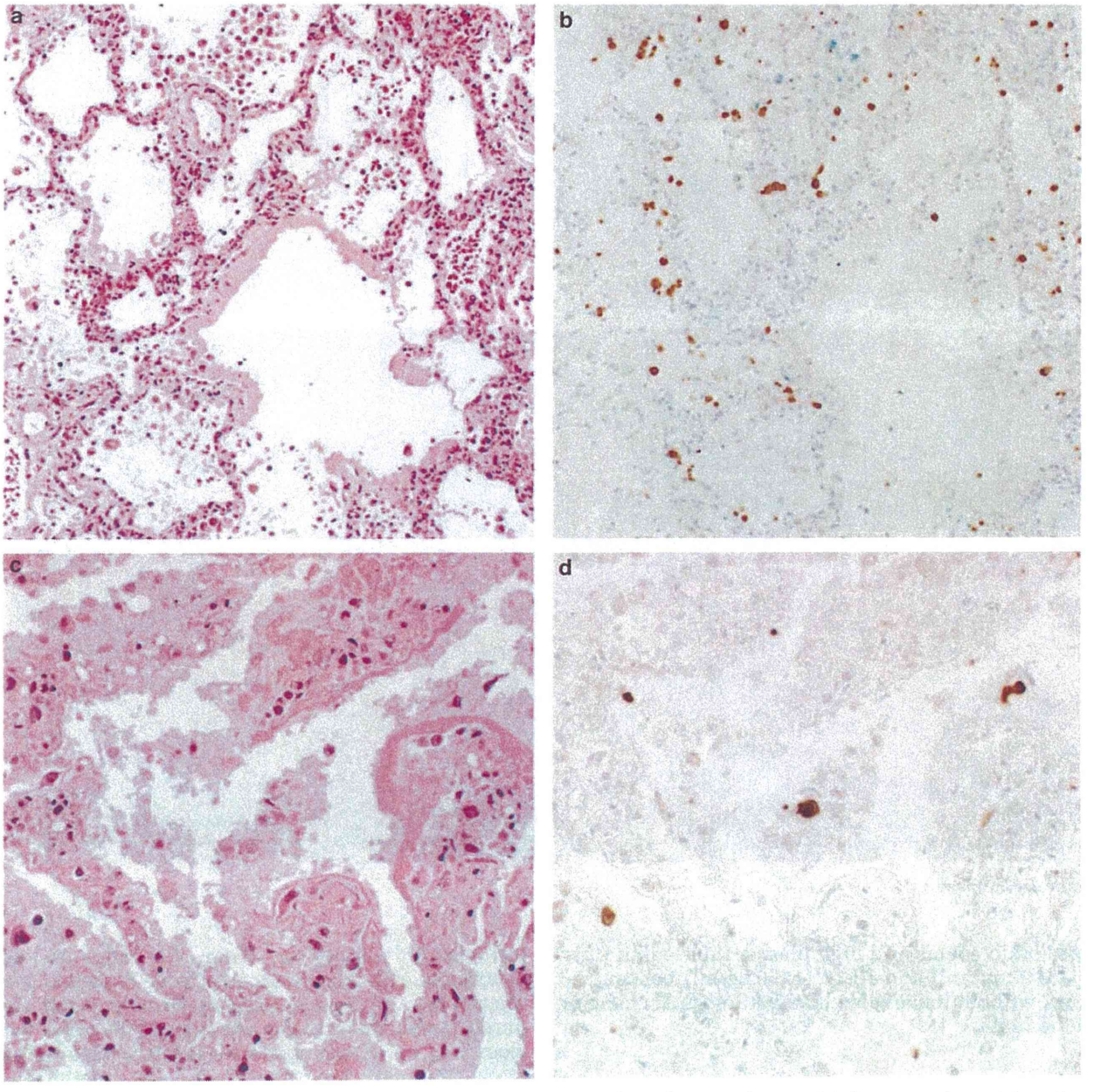

Figure 2 Hematoxylin–eosin (H&E) staining (**a**, **c**, **e**, **g**, **i**, and **k**) and immunohistochemistry for type A influenza nucleoprotein antigen (InfA-NP) (**b**, **d**, **f**, **h**, **j**, and **l**) using consecutive sections. Histopathological findings in the alveoli (**a–d**, **g**, and **h**), in the submucosal glands (**e**, **f**, **k**, and **l**), and in the trachea (**i** and **j**) are shown. InfA-NP antigens were detected in the alveoli presenting with an exudative stage of diffuse alveolar damage (DAD) with hyaline membrane formation and hyperplasia of type II pneumocytes (**a** and **b**, case 1; **c** and **d**, case 3). InfA-NP antigens were detected in both submucosal glands (**e** and **f**), and desquamated alveolar epithelial cells in case 4 (**g** and **h**). In case 7, InfA-NP antigens were detected in the nuclei of epithelial cells of trachea with mucosal mononuclear cell infiltration (**i** and **j**). In case 9, mononuclear cell infiltration around the bronchial submucosal glands (**k**) and viral antigens in submucosal glands (**l**) are shown. Original magnification, ×400: **e**, **f**, **i**, and **j**; ×200: **a–d**, **g**, **h**, **k**, and **l**.

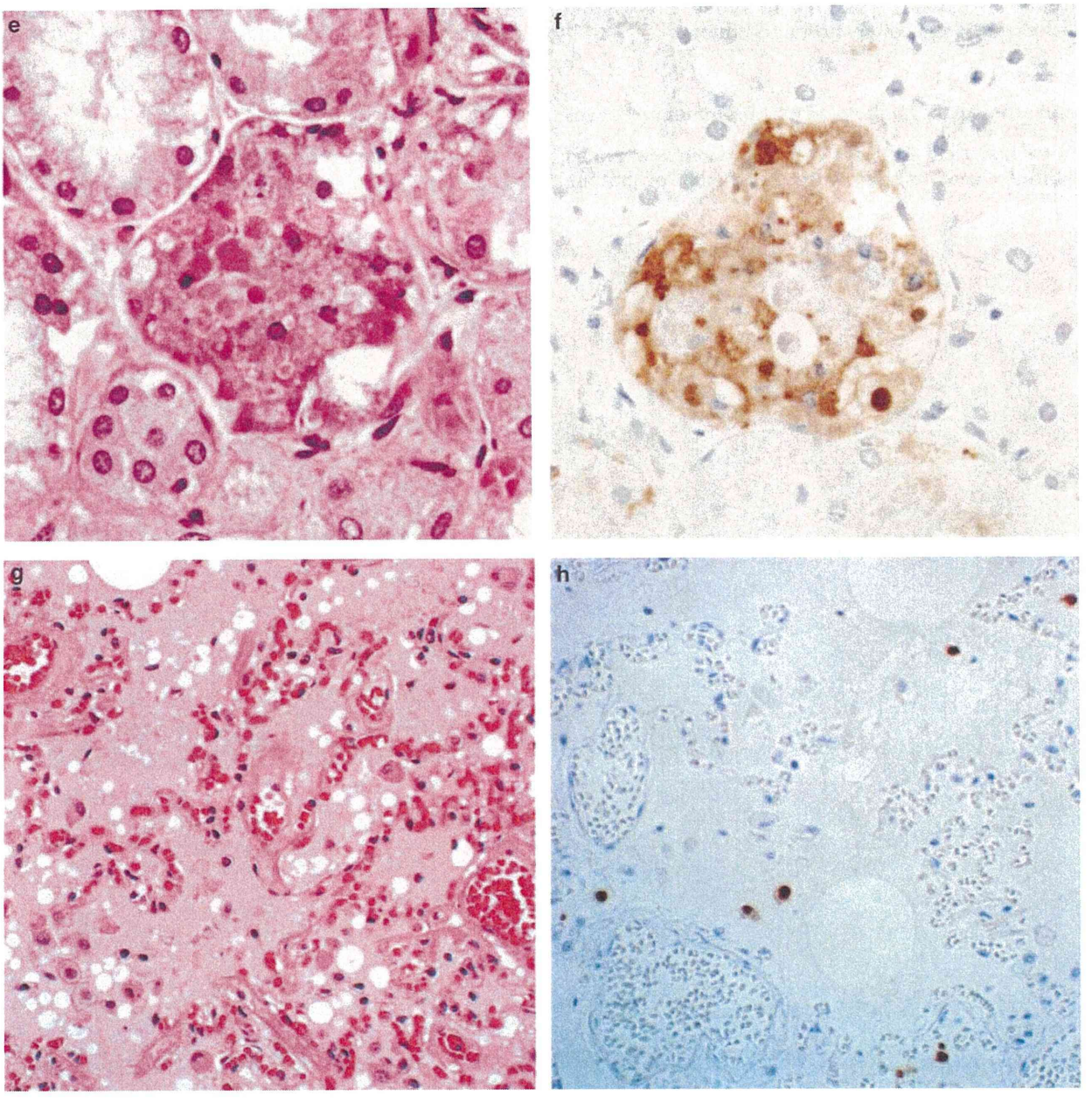

Figure 2 Continued.

gross brain edema and high plasma interleukin (IL)-6 (16197 pg/ml) and IL-10 (1156 pg/ml) concentrations, without remarkable histopathological changes in the brain.

Detection of Viral Antigens and Viral RNA in Formalin-Fixed and Paraffin-Embedded Tissue Sections

The histopathological findings described above are common in cases of seasonal influenza,[24,25] past pandemic influenza,[26,27] and H5N1 avian influenza.[22,28] However, these findings are not specific to influenza virus infection. Therefore, the distribution of virus antigen in formalin-fixed and paraffin-embedded tissue sections of 20 cases was examined by immunohistochemistry, with a monoclonal antibody for InfA-NP, and the relationship between histopathological findings and 2009 H1N1 virus infection was determined. InfA-NP antigen was only detected in the respiratory tract in 10 cases (cases 1–10). In cases 1–3, InfA-NP was detected in type II pneumocytes and in type I pneumocytes in the alveoli (Figure 2a–d). In case 4, it was detected in epithelial cells in the alveoli, bronchiole, glands,

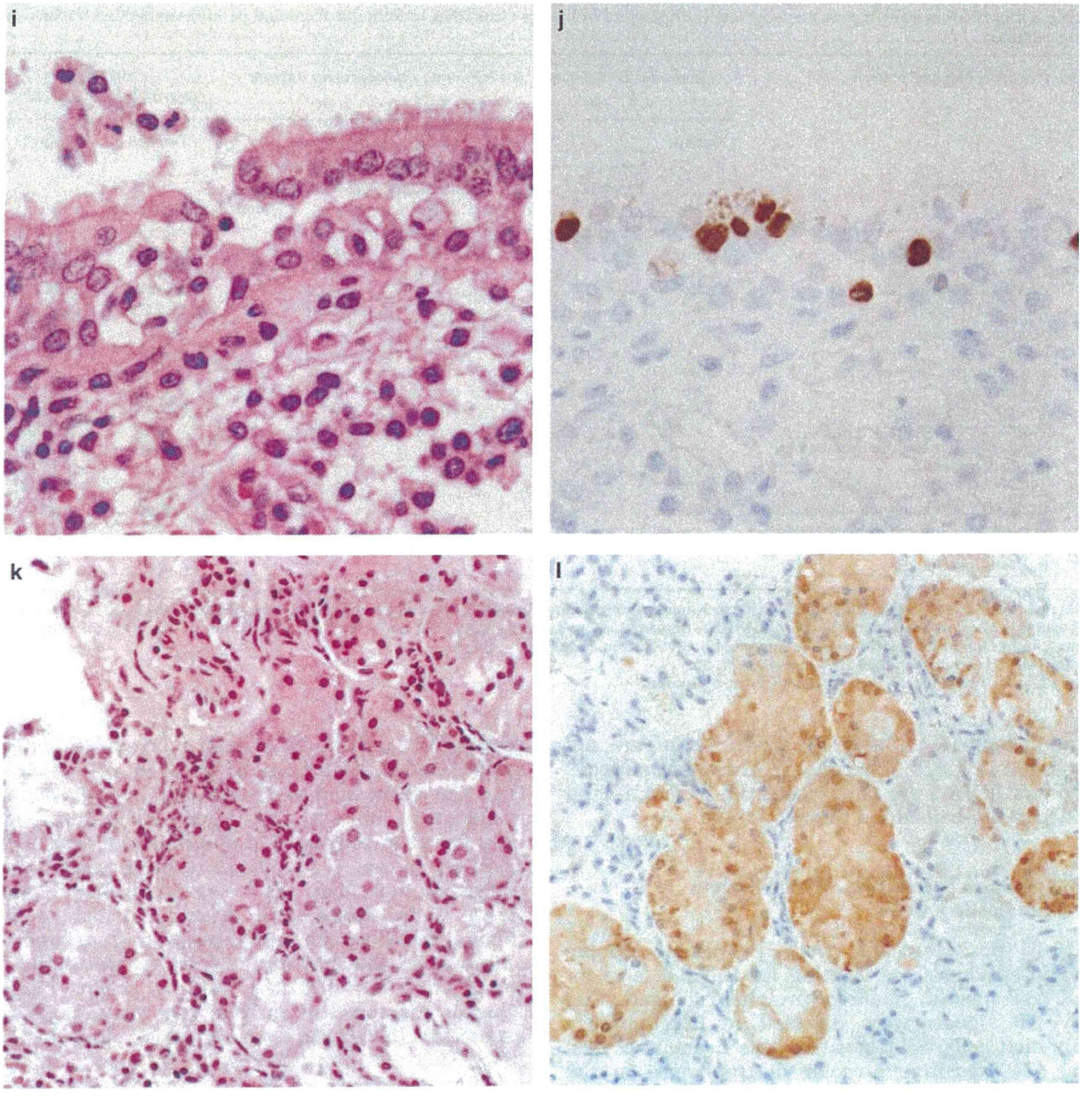

Figure 2 Continued.

bronchi, and tracheae (Figure 2e–h). In cases 5–10, InfA-NP was not detected in epithelial cells in the alveoli, but was detected in the submucosal glands, bronchi, and tracheae (Figure 2i–l). In cases 1, 2, and 8, InfA-NP was also detected in CD-68-positive alveolar macrophages as described below. Acute DAD is not specific for influenza virus infection, as it is caused by many factors; however, the detection of InfA-NP in alveolar epithelial cells appeared to be related to acute DAD with hyaline membrane formation (Table 3).

The 2009 H1N1 viral RNA in formalin-fixed and paraffin-embedded tissue sections was evaluated by rRT-PCR. The relative virus RNA copy number per cell showed the quantitative distribution in each case. The results of rRT-PCR only show that viral RNA is present in the examined section. On the other hand, immunohistochemistry can identify the cells infected by the virus. Immunohistochemistry was more useful than the rRT-PCR method when RNA in the formalin-fixed and paraffin-embedded tissue sections had degenerated and little remained (ULD, Table 3). Viral RNA was detected from either the trachea or lung in 11 cases. RNA in formalin-fixed and paraffin-embedded specimens in cases 3 and 6 was degenerated, and they were not available

Table 3 Distribution of influenza nucleoprotein antigen and influenza virus RNA in formalin-fixed and paraffin-embedded trachea and lung sections

Case no.	*Histopathological pattern*	*Immunohistochemistry for influenza nucleoprotein antigen*						*rRT-PCR (copy/cell)** (a.a. 222)****	
		Trachea	*Lung*					*Trachea*	*Lung**
			Bronchus	*Gland*	*Bronchiolus*	*Alveolus*	*Macrophage*		
1	Acute DAD with HM	−	−	−	+	+	+	0.65 (D)	723 (G)
2	Acute DAD with HM	−	−	−	−	+	+	ULD	12.8 (D)
3	Acute DAD with HM	None	−	−	−	+	−	None	NA
4	Acute DAD with HM	+	+	+	+	+	−	14.8 (D)	143 (D)
5	Massive pulmonary edema, hemorrhage	−	−	+	−	−	−	28.5 (D)	30.9 (D)
6	Massive pulmonary edema, hemorrhage	None	+	+	−	−	−	None	NA
7	Bacterial pneumonia	+	+	+	−	−	−	0.26 (D)	ULD
8	Bacterial pneumonia	+	−	+	−	−	+	ULD	35.5 (D)
9	Hemorrhagic bronchitis, pulmonary edema, congestion	+	+	+	−	−	−	14.5 (D)	22.3 (D)
10	Tracheobronchitis, emphysema	None	−	+	−	−	−	None	37.8 (D)
11	Bacterial pneumonia, plexiform pattern	None	−	−	−	−	−	None	0.04
12	Tracheobronchitis limited pulmonary hemorrhage	−	−	−	−	−	−	0.006	0.1
13	Bacterial pneumonia	−	−	−	−	−	−	0.07	0.65

Abbreviations: Case no.: case number, rRT-PCR: real-time reverse transcription-PCR assay, DAD: diffuse alveolar damage, HM: hyaline membrane, +: positive, −: negative, NA: not available due to RNA degeneration, none: no sample, ULD: under the limit of detection, D: aspartic acid, G: glycine.
Lung*: lung section included bronchus, bronchiole, alveolus, macrophage or glands.
(Copy/cell)**: copy/cell = 2009 H1N1/β-actin × 1500.
(a.a. 222)***: 222nd amino acid of hemagglutinin protein.

for analysis (NA, Table 3). Cases 14–20 are not included in Table 3, because neither viral antigens nor viral RNA were detected in the formalin-fixed and paraffin-embedded tissue sections.

Identification of Influenza Nucleoprotein Antigen-Positive Cells

InfA-NP-positive cells were identified. Double immunofluorescence staining for InfA-NP and for cell-type-specific marker proteins showed that InfA-NP signals were detected in EMA-positive epithelial cells, SP-D-positive type II pneumocytes, and CD68-positive alveolar macrophages. In the respiratory tract, they were detected in AE1/AE3-positive epithelial cells of the trachea, bronchi, bronchioles, and submucosal glands (Figure 3). The InfA-NP signal was not detected in CD34-positive endothelial cells (data not shown).

D222G Substitutions in the Hemagglutinin of 2009 H1N1 Virus

The substitution from aspartic acid (D) to glycine (G) in the hemagglutinin protein enables the virus to bind to SAα2–3Gal, which is abundantly expressed in distal bronchioles and type II pneumocytes.[18–20] The nucleotide sequence of the hemagglutinin gene in the 2009 H1N1 virus was examined by direct sequencing in formalin-fixed and paraffin-embedded trachea, and lung sections to detect amino acid substitutions at position 222 (225 in H3). The amino acid at position 222 in each section is shown in Table 3. The D222G substitution was only found in lung section specimens of case 1. In addition, in case 1, the results of direct sequencing suggested that there were several different 2009 H1N1 virus clones with different nucleic acids (Figure 4). When the sequences were translated to amino acids, the substitution from aspartic acid (D) to glycine (G) (D222G) was a major clone, whereas the substitution from aspartic acid (D) to asparagine (N) (D222N) was a minor clone in the lung. This was consistent with the results of our previous analysis in frozen lung tissues using *de novo* sequencing with a next-generation DNA sequencer,[29] an effective analytical tool, which needs a high number of copies of viral RNA for analysis. In this study, using formalin-fixed and paraffin-embedded specimens, and a direct sequencing method, we also detected different 2009 H1N1 virus clones with different nucleic acids in the tracheal sample from case 1 (Figure 4), where the D222G substitution was a minor clone and 222D was a major clone.

Discussion

Twenty autopsy cases in the present study were confirmed to have 2009 H1N1 virus infection by

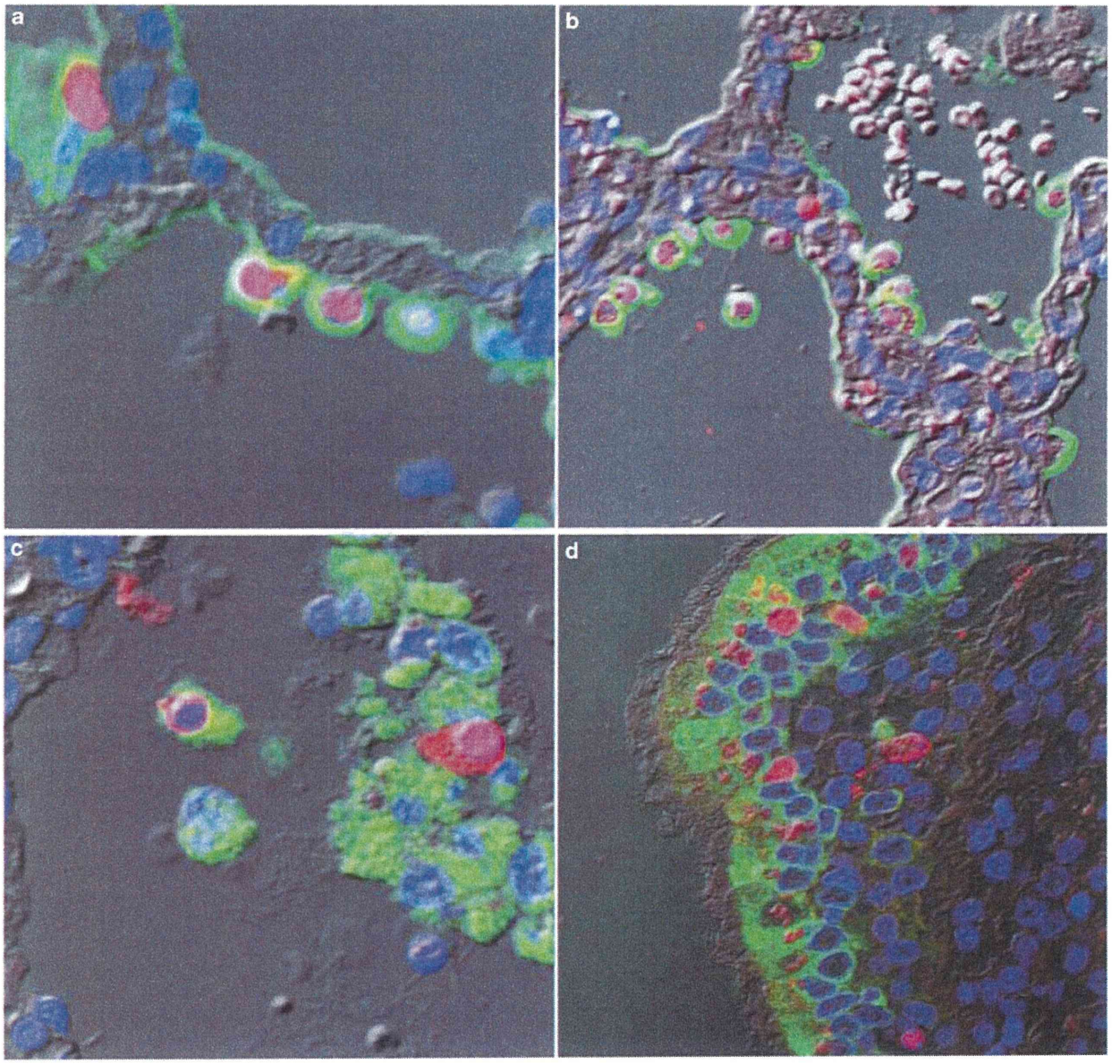

Figure 3 Double immunofluorescence staining. Type A influenza nucleoprotein antigen (InfA-NP; red) and cell-type-specific marker protein (green) are colocalized in the same cells. TO-PRO-3 nucleic acid staining (blue) and differential interference contrast (DIC) images are also shown. (**a**) InfA-NP (red) and epithelial membrane antigen (EMA) are shown in epithelial cells (green). (**b**) InfA-NP (red) and surfactant apoprotein D (SP-D) are shown in type II pneumocytes (green). (**c**) InfA-NP (red) and CD68 (PG-M1) are expressed in alveolar macrophages (green). The Inf-NP (red)-positive cell on the right is a detached type II pneumocyte. (**d**) InfA-NP (red) and cytokeratin AE1/AE3 are shown in epithelial cells in the upper airways (green). Original magnification, × 400.

RT-PCR from a nasopharyngeal swab. The characteristics of the patients were similar to those reported previously,[11–15] except for a higher ratio of males, a lower prevalence of obesity, and a shorter duration of illness.

The 2009 H1N1 virus replicates in epithelial cells in both the respiratory tract in humans and in animal models.[30] Potential infection in alveolar epithelial cells is the main feature that differentiates it from seasonal influenza strains. In the current study, autopsy cases of 2009 H1N1 infection presented with DAD with InfA-NP-positive alveolar epithelial cells, similar to those of avian H5N1 infection in humans.[11–17,28] The first autopsy case in Japan (case 1) also showed that the 2009 H1N1 virus replicated in types I and II alveolar epithelial cells and caused fatal viral pneumonia.[16] However, the present study with pathological analysis of 20 autopsy cases showed that fewer than expected cases showed similarities to case 1. Only five (25%)

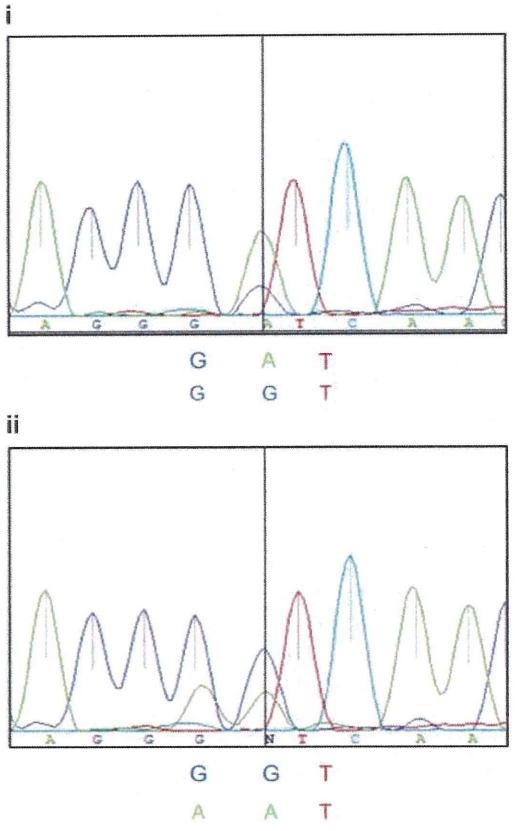

Figure 4 Amino acid residue at position 222 (225 in H3) in hemagglutinin of the 2009 pandemic influenza A (2009 H1N1) virus in formalin-fixed and paraffin-embedded trachea, and lung tissue sections was determined by direct sequencing. The nucleotide sequence around the amino acid residue at position 222 in the hemagglutinin gene of the 2009 H1N1 virus in case 1 is shown. (i) GAT (D, aspartic acid) was the major sequence, and GGT (G, glycine) was the minor sequence in the trachea. (ii) GGT (G, glycine) was the major sequence and AAT (N, asparagine) was the minor sequence in the lung.

cases showed DAD histopathologically, which is much less than that reported in other countries. It was previously reported that 95–100% of cases with fatal 2009 H1N1 influenza showed a spectrum of histopathological changes of DAD.[12–14] In addition, it is noteworthy that four (20%) cases had no particular findings in the lower respiratory tract. The cause of death in at least six cases was not respiratory failure, and instead was encephalopathy (cases 5 and 12), cerebral hemorrhage (cases 9 and 18), and myocardial dysfunction (cases 15 and 16). Encephalopathy[2–4] and myocardial disorders[5,6] have been reported to be associated with 2009 H1N1 virus infection and seasonal influenza virus infection, although the pathogenesis of these diseases and their relationship to influenza virus infection have not yet been elucidated.

There are no histopathological findings specific to 2009 H1N1 influenza infection, such as a specific inclusion body. The detection of InfA-NP in tissue sections is evidence of influenza virus infection and specific findings. In the current study, in 10 cases (50%), InfA-NP was detected in formalin-fixed and paraffin-embedded tissue sections of the respiratory tract (Table 1; cases 1–10). In the other 10 cases, there were several reasons why the antigen was not detected, such as effective antiviral treatment, low virus titer, clearance of viral antigens after a long duration of illness, and the condition of formalin-fixed and paraffin-embedded samples.

In the present study, 10 InfA-NP positive cases were investigated further. In cases 1–4, InfA-NP was detected in alveolar epithelial cells. Common clinical features of these patients included obesity and no antiviral treatment within 48 h after disease onset (Table 1). In addition, they presented with histopathological findings of acute DAD, similar to H5N1 avian influenza virus infection in humans[28] (Table 2). The distribution of InfA-NP in cases 5–10 was similar to that observed in seasonal influenza virus infection.[25] Their histopathological findings and the causes of death, which included secondary bacterial pneumonia, were also similar to those of seasonal influenza (Table 2).

Seasonal influenza virus binds to SAα2-6Gal, which is expressed in epithelial cells in the upper respiratory tract. The avian influenza virus preferentially binds to SAα2-3Gal, which is expressed in distal bronchioles and type II pneumocytes in the lower respiratory tract.[31] It was shown *in vitro* that the 2009 H1N1 virus binds to both SAα2-6Gal and SAα2-3Gal.[32] In this study, cases 1–4 presented with DAD with InfA-NP-positive pneumocytes. We expected to find the D222G substitution in hemagglutinin gene recovered from lung specimens in these cases, but it was only detected in case 1. In case 1, 2009 H1N1 presented with a quasispecies at position 222 in the lung and in the trachea, and the major sequence was different in each location (Figure 4). The D222G clone, which was a minor clone in the trachea, propagated and accumulated in the lung where SAα2-3Gal is abundantly expressed. In the other cases, quasispecies were not found by direct sequencing and the amino acid at position 222 was aspartic acid. The 222D clone rarely binds to SAα2-3Gal, according to the results of an *in vitro* study.[33] This might explain why many more InfA-NP antigens were detected in pneumocytes in case 1 than in cases 2 and 4.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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