

# Delayed Clearance of Viral Load and Marked Cytokine Activation in Severe Cases of Pandemic H1N1 2009 Influenza Virus Infection

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**Background.** Infections caused by the pandemic H1N1 2009 influenza virus range from mild upper respiratory tract syndromes to fatal diseases. However, studies comparing virological and immunological profile of different clinical severity are lacking.

**Methods.** We conducted a retrospective cohort study of 74 patients with pandemic H1N1 infection, including 23 patients who either developed acute respiratory distress syndrome (ARDS) or died (ARDS-death group), 14 patients with desaturation requiring oxygen supplementation and who survived without ARDS (survived-without-ARDS group), and 37 patients with mild disease without desaturation (mild-disease group). We compared their pattern of clinical disease, viral load, and immunological profile.

**Results.** Patients with severe disease were older, more likely to be obese or having underlying diseases, and had lower respiratory tract symptoms, especially dyspnea at presentation. The ARDS-death group had a slower decline in nasopharyngeal viral loads, had higher plasma levels of proinflammatory cytokines and chemokines, and were more likely to have bacterial coinfections (30.4%), myocarditis (21.7%), or viremia (13.0%) than patients in the survived-without-ARDS or the mild-disease groups. Reactive hemophagocytosis, thrombotic phenomena, lymphoid atrophy, diffuse alveolar damage, and multiorgan dysfunction similar to fatal avian influenza A H5N1 infection were found at postmortem examinations.

**Conclusions.** The slower control of viral load and immunodysregulation in severe cases mandate the search for more effective antiviral and immunomodulatory regimens to stop the excessive cytokine activation resulting in ARDS and death.

Instead of the anticipated influenza A pandemic with a viral antigenic shift of the surface hemagglutinin subtypes from the seasonal H3 and H1 to either H2 or H5

[1], a swine origin influenza A H1N1 virus is now replacing the seasonal H1N1 as the cause of the present pandemic. Although such antigenic change is expected to be associated with clinical severity due to the general lack of neutralizing antibody, the crude mortality rate in developed countries was comparable to seasonal influenza. Despite that the majority of the deaths were associated with major underlying illnesses, the higher rate of severe disease in the younger age sector merits more investigations [2]. Moreover, this new virus appeared to have recently jumped from swine to human [3] and replicated more efficiently in the lower respiratory tract of laboratory mammals when compared

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with the recent seasonal influenza A H1N1 viruses [4–6]. Although smoking, pregnancy, and morbid obesity were being highlighted as important risk factors for complications in severe cases [7–9], the pathogenesis has not been investigated by serial viral load and immunological studies as in the case of fatal influenza A H5N1 infections, which had persistently high viral load and marked proinflammatory cytokine activation [10, 11].

During the initial containment phase of this pandemic H1N1 2009 influenza epidemic in Hong Kong, all known infected patients were compulsorily isolated in hospitals, which provided the opportunity of taking serial clinical samples [12]. We compared the clinical progression; viral load, cytokine, and chemokine levels; and histopathological findings of laboratory-confirmed cases of different clinical severity. Their implications on the pathogenesis and treatment were discussed.

## PATIENTS AND METHODS

**Patients.** Seventy-four patients, who were admitted to hospitals in Hong Kong between May and September 2009 and tested positive for pandemic H1N1 2009 influenza virus, were included in the study. Twenty-three patients either developed acute respiratory distress syndrome (ARDS) and/or died (ARDS-death group). In this group, 13 patients (56.5%) died and 18 (78.3%) developed ARDS. Another group of 14 patients developed oxygen desaturation to <90% while breathing room air and required respiratory support, but survived and without developing ARDS (survived-without-ARDS group). Thirty-seven patients isolated in the hospital during this period without respiratory decompensation were randomly selected to represent the mild-disease group.

**Methods.** This study was approved by the institutional review board of the Hospital Authority of Hong Kong. Clinical findings including history and physical examination, oximetric measurement, hematological, biochemical, radiological, and microbiological investigation results were entered into a pre-designed database. Their acute physiology and chronic health evaluation II scores were recorded [13]. ARDS and multiorgan dysfunction syndrome were defined with standard criteria [14–15]. We observed all groups until death or discharge from hospital.

All 74 patients had the diagnosis of pandemic H1N1 2009 influenza virus infection confirmed either when the pandemic H1 gene was tested positive by reverse-transcription polymerase chain reaction (RT-PCR) or by viral culture in nasopharyngeal or endotracheal specimens. All laboratory procedures were performed as previously reported [16]. Serial nasopharyngeal, and if intubated, endotracheal viral load, were assessed by quantitative PCR of the influenza A virus M gene [17]. The detection limit of this assay was 900 copies of RNA/mL. Blood, urine, and fecal samples were also tested. Oseltamivir resistance was directly detected in the initial positive nasopharyngeal or en-

dotracheal sample by RT-PCR and sequencing of a 320-bp fragment of the neuraminidase gene spanning the 274 position for a C→T mutation [18]. The test was repeated in respiratory specimens of patients with persistently high viral load despite oseltamivir treatment. All initial respiratory tract samples were cultured in Madin Darby Canine Kidney cell line with trypsin for 7 days.

The respiratory tract samples on admission were also assessed by multiplex PCR (Luminex) with ResPlex II v2.0 assay (Qiagen) for coinfection with respiratory syncytial virus, influenza B virus, parainfluenza viruses 1–4, human metapneumovirus, enteroviruses, rhinovirus, adenovirus, bocavirus, and coronaviruses NL63, HKU1, 229E, and OC43, in accordance with manufacturer's instructions; and by PCR for *Mycoplasma pneumoniae* and *Chlamydomphila pneumoniae* [19, 20]. We investigated blood, sputum or endotracheal aspirates, and urine samples bacteriologically, as clinically indicated. Initial urine samples were tested for pneumococcal and legionella antigen by immunochromatographic enzyme immunoassay (Binax). The host immunological response was monitored by the Luminex enzyme immunoassay (Luminex) for 25 different plasma cytokines and chemokines.

**Statistical analysis.** Clinical, virological, and immunological characteristics were compared. The Fisher exact test and  $\chi^2$  test were used for categorical variables where appropriate, and the Mann-Whitney *U* test was used for continuous variables because the data was not normally distributed. Significant risk factors for severe diseases were not further analyzed by multiple logistic regressions due to the small number of cases. Linear regression was used to assess the trend of mean viral load. The Jonckheere-Terpstra trend test was used to compare cytokine or chemokine level with clinical severity. Pearson correlation was used to assess the relationship between the viral load and the absolute lymphocyte count or plasma cytokine levels. SPSS software, version 17.0 for Windows (SPSS), was used for statistical computation. *P* values <.05 were considered to represent statistically significant differences.

## RESULTS

Patients in ARDS-death group were significantly older and more likely to be obese or to have underlying diseases than mild-disease group (Table 1). Lower respiratory tract symptoms, especially dyspnea, were more common at presentation in ARDS-death group. Comparison between patients in ARDS-death and survived-without-ARDS group did not reveal significant differences in terms of demographic characteristics, presenting symptoms, and vital signs on admission, except that wheezing was more common in survived-without-ARDS group. Chronic pulmonary disease was more common in survived-without-ARDS group than ARDS-death group. Overall, 93.2% of patients received oseltamivir, but nebulized zanamivir was

**Table 1. Demographic Characteristics and Underlying Comorbidities**

Characteristic	ARDS-death group (n = 23)	Survived-without-ARDS group (n = 14)	P <sup>a</sup>	Mild-disease group (n = 37)	P <sup>b</sup>
<b>Demographic characteristic</b>					
Age, median years (range)	49 (23–84)	52 (30–79)	.199	21 (15–53)	<.001
Ratio of male to female patients	13:10	10:4	.365 <sup>c</sup>	14:23	.157 <sup>c</sup>
Obesity (BMI, >27)	4 (17.4)	6 (42.9)	.132	0 (0)	.018
Pregnancy	1 (4.3)	0 (0)	>.99	0 (0)	.383
<b>Underlying disease<sup>d</sup></b>					
No underlying disease	6 (26.1)	2 (14.3)	.683	35 (94.6)	<.001 <sup>c</sup>
Chronic heart disease	3 (13.0)	4 (28.6)	.390	0 (0)	.052
Chronic pulmonary disease	4 (17.4)	9 (64.3)	.006	2 (5.4)	.191
Diabetes mellitus	1 (4.3)	4 (28.6)	.057	0 (0)	.383
<b>Presenting symptoms</b>					
Feverishness	18 (78.3)	8 (57.1)	.268	31 (83.8)	.734
Cough	22 (95.7)	14 (100)	>.99	27 (73.0)	.039
Sputum	13 (56.5)	10 (71.4)	.365 <sup>c</sup>	8 (21.6)	.006 <sup>c</sup>
Sore throat	9 (39.1)	5 (35.7)	.835 <sup>c</sup>	23 (62.2)	.082 <sup>c</sup>
Wheezing	5 (21.7)	8 (57.1)	.039	0 (0)	.006
Myalgia/ arthralgia	5 (21.7)	3 (21.4)	>.99	10 (27.0)	.646 <sup>c</sup>
Dyspnea	21 (91.3)	14 (100)	.517	1 (2.7)	<.001 <sup>c</sup>
Vomiting	4 (17.4)	0 (0)	.276	0 (0)	.018
Diarrhea	2 (8.7)	1 (7.1)	>.99	3 (8.1)	>.99
<b>Exacerbation of underlying disease</b>					
COAD	3 (13.0)	3 (21.4)	.654	0 (0)	.052
Asthma	0 (0)	6 (42.9)	.001	0 (0)	NA
<b>Vital signs on presentation</b>					
Hypotension <sup>e</sup>	9 (39.1)	1 (7.1)	.056	0 (0)	<.001
Pulse, >100 beats/min	15 (65.2)	10 (71.4)	>.99	9 (24.3)	.002 <sup>c</sup>
Respiratory rate >20 breaths/min	14 (60.9)	9 (64.3)	.835 <sup>c</sup>	0 (0)	<.001 <sup>c</sup>
Temperature, >38°C	10 (40.3)	4 (28.6)	.365 <sup>c</sup>	23 (62.2)	.157 <sup>c</sup>
Duration of symptoms before admission, median days (range)	5 (0–12)	3.5 (0–11)	.229	1 (0–4)	<.001
Duration of hospitalization, median days (range)	14 (1–69)	10 (4–27)	.398	7 (2–11)	.025

**NOTE.** Data are no. (%) of patients, unless otherwise indicated. For statistical analysis, Mann Whitney *U*-test was used for continuous variables, whereas the Fisher exact test was used for categorical variables unless otherwise indicated. ARDS, acute respiratory distress syndrome; BMI, body mass index (calculated as weight in kilograms divided by the square of height in meters); NA, not available.

<sup>a</sup> Comparison between ARDS-death and survived-without-ARDS groups.

<sup>b</sup> Comparison between ARDS-death and mild-disease groups.

<sup>c</sup> By  $\chi^2$  test.

<sup>d</sup> For ARDS-death group, other underlying comorbidities included hypertension (in 5 patients), cerebrovascular disease (in 3), malignancy (in 3), chronic liver disease (in 2), epilepsy (in 1), thalassemia trait (in 1), and hypothyroidism (in 1). For the survived-without-ARDS group, other comorbidities include hypertension (in 5 patients), chronic liver disease (in 1), chronic renal disease (in 1), thalassemia trait (in 1), and eczema (in 1).

<sup>e</sup> Systolic blood pressure, <90 mm Hg; or diastolic blood pressure, <60 mm Hg.

only used in patients with more severe diseases. The median time for initiation of oseltamivir treatment was 5 days after symptom onset in the ARDS-death group, which is later than 4 days in the survived-without-ARDS group and 2 days in the mild-disease group. Three patients in the ARDS-death group did not receive oseltamivir because the diagnosis was made after death. Empirical antibiotics were started in all patients in the ARDS-death and survived-without-ARDS group on admission. The regimen was modified according to bacteriological test results or on an individual basis.

As for the initial laboratory parameters at presentation (Table 2), neutrophil count, serum alanine transaminase (ALT), creatine kinase, and lactate dehydrogenase levels were higher, but

the absolute lymphocyte count was lower in the ARDS-death group than the mild-disease group. ARDS-death group also had a significantly lower total white blood cell, neutrophil, and platelet counts and ALT levels than the survived-without-ARDS group. The nasopharyngeal ( $r^2 = 0.0386$ ;  $P = .009$ ) and endotracheal ( $r^2 = 0.1461$ ;  $P < .001$ ) viral loads inversely correlated with the concomitant absolute lymphocyte count.

Initial nasopharyngeal and endotracheal viral loads were similar among patients with different disease severity (Table 3). Peak viral load occurred within 2 days after symptom onset (Figure 1A). Nasopharyngeal viral load decreased after oseltamivir therapy, with the slowest decrease in the ARDS-death group (Figure 1B). Viremia was found by RT-PCR in 3 patients

**Table 2. Investigations, Treatments, Complications, and Clinical Outcomes**

Variable	ARDS-death group (n = 23)	Survived-without-ARDS group (n = 14)	P <sup>a</sup>	Mild-disease group (n = 37)	P <sup>b</sup>
Initial laboratory findings, median (range)					
Total white blood cell count, × 10 <sup>9</sup> cells/L	5.1 (0.8–28.0)	9.0 (4.5–13.4)	.011	5.3 (2.8–8.4)	.994
Neutrophil count, ×10 <sup>9</sup> cells/L	3.9 (0.4–21.7)	7.0 (3.3–13.2)	.017	3.2 (1.1–7.2) <sup>c</sup>	.046
Lymphocyte count, ×10 <sup>9</sup> cells/L	0.6 (0.2–2.8)	0.65 (0.3–1.8)	.758	1.2 (0.5–2.6) <sup>c</sup>	<.001
Platelet count, ×10 <sup>9</sup> platelets/L	178 (68–310)	225 (117–352)	.036	194 (111–329)	.063
Alanine transaminase level, IU/L	45 (13–167)	17 (11–61)	.010	18 (5–45) <sup>c</sup>	<.001
Creatinine level, μmol/L	76 (46–628)	79.5 (49–236)	.851	67 (42–115)	.052
Creatine kinase level, IU/L	221 (20–1561)	127 (55–5077)	.562	73 (42–623) <sup>d</sup>	.017
Lactate dehydrogenase level, IU/L	474 (157–1154)	398 (163–2246)	.346	194 (135–344) <sup>e</sup>	<.001
Clinical outcome					
Mechanical ventilation <sup>f</sup>	22 (95.7)	4 (28.6)	<.001	0 (0)	<.001 <sup>g</sup>
ICU admission	21 (91.3)	7 (50)	.014	0 (0)	<.001 <sup>g</sup>
Median APACHE II score (range) <sup>h</sup>	22 (0–42)	15 (7–34)	.405	NA	NA
Antiviral treatment data					
Oseltamivir treatment	20 (87.0)	14 (100)	.275	35 (94.6)	.362
Duration of symptoms before starting oseltamivir, median days (range)	5 (1–13)	4 (0–11)	.307	2 (0–5)	<.001
Nebulized zanamivir	15 (65.2)	1 (7.1)	.001 <sup>g</sup>	0 (0)	<.001 <sup>g</sup>
Duration of symptoms before starting zanamivir, median days (range)	7 (1–15)	5 (5–5)	.828	NA	NA
Complications					
Progression of infiltrates to all 4 quadrants on chest radiograph	11 (47.8)	4 (28.6)	.247	0 (0)	<.001
Bacterial coinfection on presentation	7 (30.4)	2 (14.3)	.434	0 (0)	.001
Ventilator-associated pneumonia	4 (17.4) <sup>i</sup>	0 (0)	.276	NA	NA
Pulmonary embolism	1 (4.3)	0 (0)	>.99	0 (0)	.383
Acute pulmonary edema	0 (0)	2 (14.3)	.137	0 (0)	NA
Myocarditis	5 (21.7)	0 (0)	.135	0 (0)	.006
Pericardial effusion	4 (17.4)	1 (7.1)	.135	0 (0)	.018
Acute renal failure requiring renal replacement therapy	6 (26.1)	0 (0)	.065	0 (0)	.002
Multiorgan dysfunction syndrome	16 (69.6)	1 (7.1)	<.001 <sup>g</sup>	0 (0)	<.001 <sup>g</sup>
Inotropic support	18 (78.3)	2 (14.3)	<.001 <sup>g</sup>	0 (0)	<.001 <sup>g</sup>

**NOTE.** Data are no. (%) of patients, unless otherwise indicated. For statistical analysis, the Mann Whitney *U* test was used for continuous variables, and the Fisher exact test was used for categorical variables, unless otherwise indicated. APACHE, Acute Physiology and Chronic Health Evaluation; ARDS, acute respiratory distress syndrome; COAD, chronic obstructive airway disease; ICU, intensive care unit; NA, not applicable.

<sup>a</sup> Comparison between ARDS-death and survived-without-ARDS groups.

<sup>b</sup> Comparison between ARDS-death and mild-disease groups.

<sup>c</sup> Data are for 36 patients.

<sup>d</sup> Data are for 18 patients.

<sup>e</sup> Data are for 23 patients.

<sup>f</sup> Patients who did not undergo mechanical ventilation. One patient in the ARDS-death group and 4 patients in the survived-without-ARDS group received bi-level positive airway pressure support only, and 3 patients in the ARDS-death group received extracorporeal membrane oxygenation.

<sup>g</sup> By  $\chi^2$  square test.

<sup>h</sup> Only for patients admitted to the ICU.

<sup>i</sup> Included methicillin-resistant *Staphylococcus aureus*, methicillin-susceptible *S. aureus*, *Klebsiella* species, *Citrobacter* species, *Enterobacter* species, *Pseudomonas aeruginosa*, and *Acinetobacter* species.

in the ARDS-death group and 1 patient in the survived-without-ARDS group. Only 1 stool sample, from a patient in the survived-without-ARDS group, had positive RT-PCR results. All urine samples tested negative by RT-PCR. Oseltamivir resistance with H274Y mutant existing as quasi-species in 3 different specimens were found in 1 patient in the survived-without-ARDS group.

Of the 25 cytokines or chemokines assayed, significantly higher levels of granulocyte colony-stimulating factor (G-CSF), interferon (IFN)- $\alpha$ 2, interleukin (IL)-1 $\alpha$ , IL-6, IL-8, IL-10, IL-15, IFN- $\gamma$ -induced protein 10 (IP-10), monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)- $\alpha$  were found in the initial samples of ARDS-death group than

mild-disease group (Table 3). Initial IL-17 levels were lower in the ARDS-death group than in the mild-disease group, with the difference approaching statistical significance ( $P = .074$ ). Compared with the survived-without-ARDS group, significantly higher levels of G-CSF, IL-1 $\alpha$ , IL-6, IL-10, IL-15, MCP-1, and TNF- $\alpha$  were found in ARDS-death group. When stratified according to days after symptom onset (days 0–3, days 4–6, and days 7–10), only IL-6, IL-10, and IL-15 levels were higher in those with more severe disease throughout all 3 periods, whereas G-CSF, IL-1 $\alpha$ , IL-8, IP-10, and TNF- $\alpha$  levels were higher only during later phase of the disease (Figure 2A–2K).

Coinfections on presentation occurred in ARDS-death and

**Table 3. Initial Plasma Cytokine or Chemokine Levels and Viral Load in Respiratory Specimens**

Variable	ARDS-death group (n = 18)	Survived-without-ARDS group (n = 10)	P <sup>a</sup>	Mild-disease group (n = 29)	P <sup>b</sup>
Cytokine or chemokine, log <sub>10</sub> pg/mL <sup>c,d</sup>					
G-CSF	2.11 (1.33–3.91)	1.13 (0.19–2.03)	<.001	1.31 (0.74–2.43)	<.001
IFN-α2	1.1 (0.71–2.49)	0.79 (0.71–1.79)	.138	0.71 (0.71–2.07)	.024
IL-1α	2.13 (1.38–2.78)	1.66 (1.17–2.19)	.014	1.59 (0.94–2.46)	<.001
IL-6	2.40 (1.32–4.00)	1.19 (0.81–1.97)	<.001	0.08 (0.08–2.14)	<.001
IL-8	2.25 (1.23–4.02)	1.70 (1.18–3.20)	.084	1.21 (0.78–2.66)	<.001
IL-10	1.80 (1.14–2.70)	1.02 (0.19–2.36)	.002	0.48 (0.19–1.60)	<.001
IL-15	0.92 (0.44–1.70)	0.33 (0.20–2.36)	<.001	0.20 (0.20–0.65)	<.001
IL-17	0.20 (0.20–2.94)	0.20 (0.20–0.87)	.298	0.76 (0.20–2.48)	.074
IP-10	3.83 (3.01–4.01)	3.10 (2.69–4.01)	.065	3.00 (1.80–3.36)	<.001
MCP-1	3.11 (2.48–3.74)	2.73 (2.41–3.44)	.017	2.58 (1.94–3.00)	<.001
TNF-α	1.19 (0.62–2.12)	0.90 (0.89–1.15)	.002	0.86 (0.80–1.25)	<.001
Viral load, log <sub>10</sub> copies/mL <sup>e</sup>					
Nasopharyngeal <sup>f</sup>	5.45 (2.65–7.49)	5.68 (2.65–8.65)	.817	5.97 (2.65–10.7)	.484
Endotracheal <sup>g</sup>	4.97 (2.65–7.16)	5.24 (4.83–5.86)	.369	NA	NA

**Note:** Data are presented as median (range). Mann-Whitney *U*-test was used for statistical analysis. ARDS, acute respiratory distress syndrome; G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; NA, not applicable; TNF, tumor necrosis factor.

<sup>a</sup> Comparison between ARDS-death and survived-without-ARDS groups.

<sup>b</sup> Comparison between ARDS-death and mild-disease groups.

<sup>c</sup> The detection limit of the assay was 3.1 pg/mL for G-CSF, 10.16 pg/mL for IFN-α2, 17.31 pg/mL for IL-1α, 2.41 pg/mL for IL-6, 3.19 pg/mL for IL-8, 3.10 pg/mL for IL-10, 3.17 pg/mL for IL-15, 3.15 pg/mL for IL-17, 2.69 pg/mL for IP-10, 3.33 pg/mL for MCP-1, and 3.21 pg/mL for TNF-α.

<sup>d</sup> Other cytokines or chemokines tested were granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-12(p40), IL-12(p70), IL-13, macrophage inflammatory protein 1α, macrophage inflammatory protein 1β, and TNF-β.

<sup>e</sup> The initial nasopharyngeal samples were collected at a median of 4 days after symptom onset for the ARDS-death group (range, 0–13 days), 4 days (range, 0–10 days) for survived-without-ARDS group, and 2 days (range, 0–8 days) for mild cases. The initial endotracheal samples were collected at a median of 6 days (range, 1–12 days) for the ARDS-death group and 7 days (range, 7–12 days) for the survived-without-ARDS group after symptom onset.

<sup>f</sup> Data are for 17 patients in the ARDS-death group, 13 patients in the survived-without-ARDS group, and 37 patients in the mild-disease group.

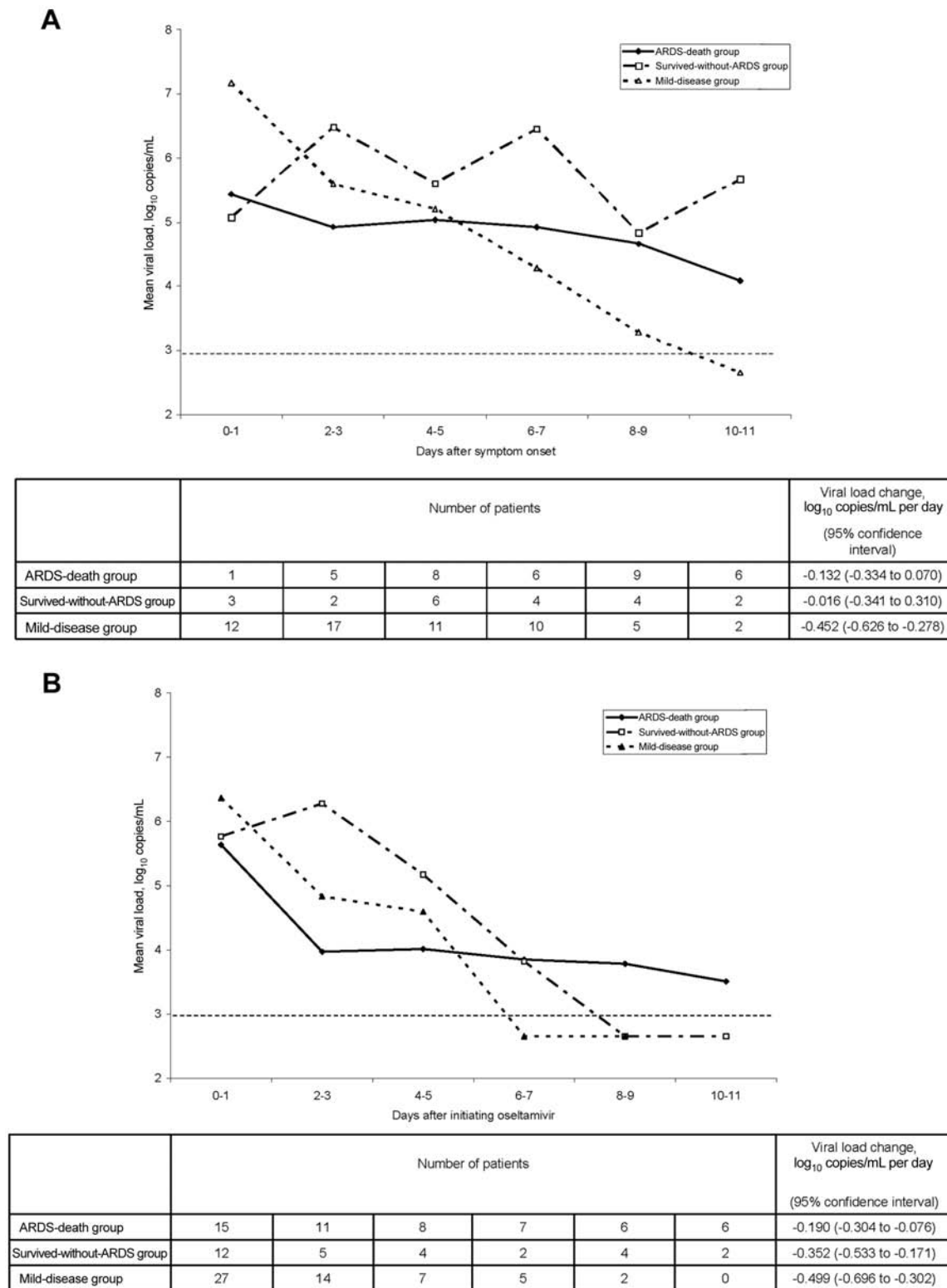
<sup>g</sup> Data are for 16 patients in the ARDS-death group and 3 patients in the survived-without-ARDS group.

survived-without-ARDS groups (Table 2). Among ARDS-death group, community-acquired methicillin-resistant *Staphylococcus aureus* was cultured from blood and pleural fluid samples from 1 patient and from sputum samples from another patient. Methicillin-susceptible *S. aureus* and *Pseudomonas* species were cultured from 2 and 1 patient respectively. One patient had pulmonary tuberculosis, with a sputum smear positive for acid-fast bacilli and PCR positive for *Mycobacterium tuberculosis*. This same patient also had pneumococcal antigenuria. For survived-without-ARDS group, *Haemophilus influenzae* and *Pseudomonas* species were found in 1 patient each. Rhinovirus was detected in 2 patients from ARDS-death group and 1 patient from survived-without-ARDS group. Other respiratory viruses, *C. pneumoniae*, *M. pneumoniae*, and *Legionella* species were not detected.

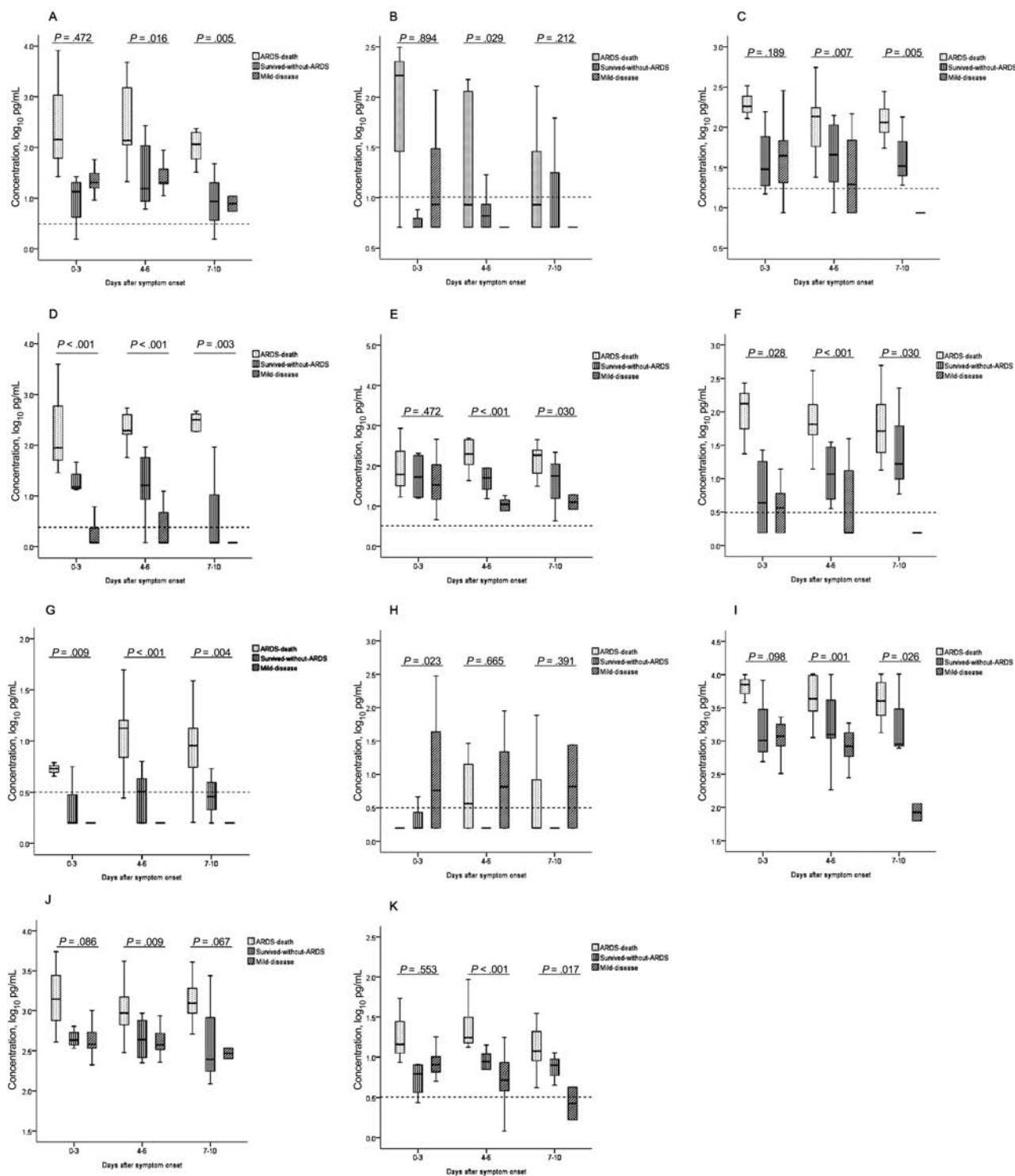
Complications were most common in the ARDS-death group (Table 2). Five patients (21.7%) had clinical, laboratory, and echocardiographic evidence of myocarditis; 3 of these cases were confirmed at postmortem examination (Figure 3E). Multiorgan dysfunction syndrome predominantly occurred in ARDS-death group (69.6%). One patient in the ARDS-death

group developed both ventilator associated pneumonia and *Enterobacter cloacae* bacteremia. No rhabdomyolysis or encephalopathy was found.

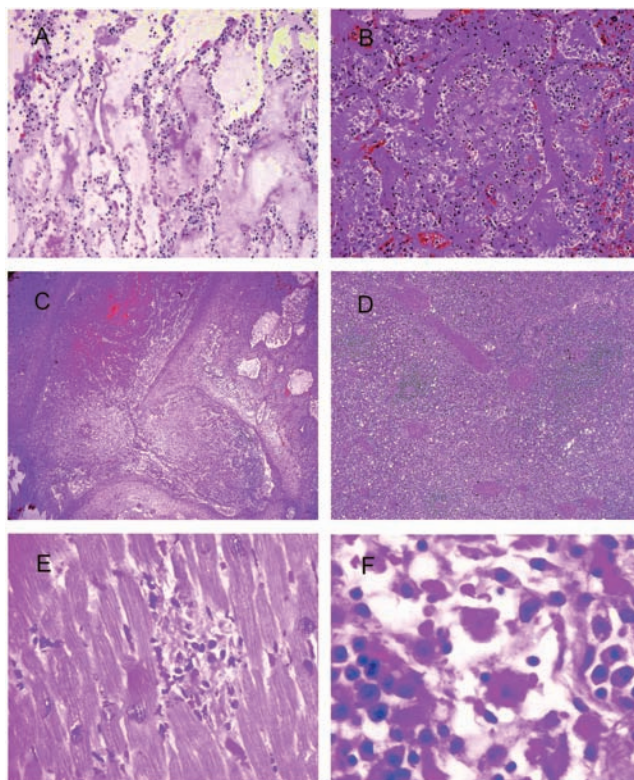
The median time of death was 10 days after symptom onset (range, 3–33 days). The cause of death was respiratory failure in 11 patients (including 5 patients with secondary bacterial pneumonia, 4 patients with myocarditis), heart failure due to myocarditis in 1 patient, and *Escherichia coli* bacteremia in 1 patient. Postmortem examination or paramortem tissue biopsies were performed in 5 patients who died between day 3 and 28 after symptom onset. Examination of 4 patients who died around day 10 after symptom onset showed acute exudative diffuse alveolar damage with proteinaceous exudate in alveolar space and interstitial infiltrate in alveolar septa (Figure 3A). One patient, who died on day 28 after symptom onset, had fibroproliferative changes of diffuse alveolar damage (Figure 3B). Reactive hemophagocytosis was found in the lymph node or bone marrow of 2 patients (Figure 3F). Lymphoid atrophy was also observed (Figure 3D). Three cases had changes of acute lymphocytic myocarditis (Figure 3E). One case had thrombosis in branches of pulmonary artery (Figure 3C) and



**Figure 1.** Nasopharyngeal viral load in patients with different clinical severity, according to number of days after symptom onset (A) and to number of days after initiation of oseltamivir treatment (B). Horizontal dotted line indicates detection limit of reverse-transcription polymerase chain reaction. ARDS, acute respiratory distress syndrome.



**Figure 2.** Plasma cytokine or chemokine level, stratified by days after symptom onset. Median, quartiles, and range are shown. The Jonckheere-Terpstra trend test was used to compare cytokine level with disease severity. *Horizontal dotted line*, detection limit of the assay. *A*, Granulocyte colony-stimulating factor. *B*, Interferon (IFN)-α2. *C*, Interleukin (IL)-1α. *D*, IL-6. *E*, IL-8. *F*, IL-10. *G*, IL-15. *H*, IL-17. *I*, IFN-γ-induced protein 10. *J*, Monocyte chemoattractant protein-1. *K*, Tumor necrosis factor-α.



**Figure 3.** Histopathological changes of pulmonary and extrapulmonary tissue (hematoxylin-eosin stained) of the deceased patients suffering from pandemic influenza A H1N1 2009. *A*, Lung parenchyma showing acute exudative phase of diffuse alveolar damage on day 7 after symptom onset in a 42-year-old previously healthy man with interstitial cellular infiltrate and proteinaceous exudates in alveoli and hyaline membrane formation (original magnification,  $\times 100$ ). *B*, Lung parenchyma showing chronic fibroproliferative phase of diffuse alveolar damage with marked thickening of the alveolar septa and alveolar space being replaced by fibrogranulation tissues on day 28 of symptom onset in a 37-year-old previously healthy woman (original magnification,  $\times 200$ ). *C*, Thrombosis of branches of pulmonary artery in the same patient in panel *B* who died despite extracorporeal membrane oxygenation (original magnification,  $\times 200$ ). *D*, Lymphoid atrophy with no recognizable germinal centre in the spleen of the same patient in panels *B* and *C*. The periarteriolar lymphoid sheath (white pulp), which was markedly reduced in size (original magnification,  $\times 400$ ). *E*, Myocardium showing a microscopic focus of lymphoid aggregate accompanied by myofibril necrosis in a 58-year-old man presenting with elevated creatine kinase isoenzyme CK-MB and echocardiographic evidence of diastolic dysfunction (original magnification,  $\times 200$ ). *F*, Mediastinal lymph node showing reactive hemophagocytosis in the same patient in panel *E* (original magnification,  $\times 400$ ).

splenic artery with wedge-shaped infarct despite heparinization while undergoing extracorporeal membrane oxygenation.

## DISCUSSION

Clinical studies on the severity of pandemic H1N1 2009 influenza virus infection have not utilized patients with mild disease except for pregnant patients [8]. Laboratory study in cell lines showed that this virus was similar to recent seasonal influenza A H1N1 virus in terms of viral replication rate and the ability to induce proinflammatory cytokines but markedly inferior to that of influenza A H5N1 virus [11, 21]. However, unlike seasonal influenza virus, which replicates mainly in the upper respiratory tract, challenge studies in mammals showed that the pandemic virus can replicate to a high level in both the upper and lower respiratory tracts [4–6]. The findings of ARDS with postmortem findings of diffuse alveolar damage, reactive

hemophagocytosis and lymphoid atrophy in our deceased patients were compatible with a cytokine storm and have similarly been reported in H5N1 infection [10]. Therefore, we studied the clinical characteristics, serial viral load in the respiratory specimens, and plasma levels of cytokines and chemokines in the 3 groups of patients with different levels of disease severity.

Irrespective of disease severity, the viral load already peaked within 2 to 3 days of symptom onset. Animal experiments and clinical studies showed that viral load increased steadily within 48 h after inoculation when the host failed to mount any innate immune response [22]. Once innate immune response was mounted, the viral load reached a plateau. Due to the later presentation of the severe cases, data on the viral load in the initial days after symptom onset were scarce. Thus, the nasopharyngeal viral load at presentation appeared to be slightly lower in the more severe groups. The later presentation of the



more severe cases might have allowed more time for the recruitment of cytotoxic T lymphocyte response specific to the highly homologous nucleoprotein antigen present in all influenza A viruses which can suppress the viral load.

A slower decline in viral shedding was observed in all severe patients in the ARDS-death group or the survived-without-ARDS group than in the mild-disease group (Figure 1A and 1B). The finding could be related to a less effective innate and adaptive immune response together with a delay in starting antiviral therapy due to a later presentation. Fatal cases of H5N1 infection had persistently high viral load due to oseltamivir resistance [23], but oseltamivir resistance was not responsible for the slower rate of decline in viral shedding in our series, because only 1 patient was infected with oseltamivir-resistant virus who eventually survived without complication by ARDS. Although uncontrolled viral infection was unlikely to be the only cause of death or ARDS, antiviral therapy that can suppress the viral load more rapidly, such as intravenous zanamivir [24], and neutralizing antibody in convalescent plasma or hyperimmune intravenous immunoglobulin should be considered in randomized, control trials for the treatment of severe cases. Such strategy may accelerate clearance of their viral load, which may also reduce the excessive cytokine release.

As for the role of proinflammatory cytokines or chemokines, which can have both antiviral activity and damage on host tissue, highest levels were seen in the ARDS-death group. Plasma cytokine and chemokine profiles of the severe group were consistent with the challenged macaque model, which showed elevated levels of MCP-1 and IL-6 in their inflamed lungs [6]. When stratified by time after symptom onset, there was a significant correlation between disease severity and the level of IL-6, IL-10, and IL-15 throughout the initial 10 days after symptom onset. Correlation between higher IL-6 levels and severe influenza infection has been well established in both animal and in human [26–27], but this correlation has not been reported for IL-10 and IL-15. As an anti-inflammatory cytokine, IL-10 is essential in dampening inflammatory response to prevent excessive host damage. Of interest, IL-10 deficiency was shown to be protective in influenza infection in mice, and high IL-10 production in elderly was associated with poorer antibody response to influenza vaccine [27, 28]. IL-15 level has been reported to be elevated among many bacterial and viral infections [29]. Despite being regarded as a proinflammatory cytokine, recent animal model suggested that high levels of IL-15 may actually decrease the level of antibody against influenza [30]. In contrast, IL-17 was lower in the more severe groups, consistent with previous studies which showed that IL-17 might help to protect against lethal influenza [31]. The use of immunomodulator in the treatment of influenza is still controversial but appeared effective when an antiviral is given concomitantly [32].

In our series, lymphopenia was more common among patients with severe disease, as seen in SARS coronavirus infection [33]. Furthermore, an inverse correlation between lymphocyte count and viral load in both the nasopharyngeal and endotracheal specimens was observed. Similar findings were found in patients with H5N1 infection [26]. Lymphopenia in infection may be due to lymphocyte apoptosis mediated by TNF- $\alpha$ , which was also elevated. Another possibility was direct infection of T lymphocytes, as shown in histopathological studies of H5N1 infection [34].

Evidence of bacterial coinfection was present in 30.4% of our most severe cases, which resembled the rate in another autopsy study [35]. However, bacterial isolates in our series have been covered adequately during the course of hospitalization. Other nonpulmonary complications, such as myocarditis, might have contributed to the mortality. Chronic pulmonary diseases and exacerbation of underlying pulmonary comorbidities were over-represented in the survived-without-ARDS group, suggesting that although these patients required oxygen supplementation, their prognosis was usually better than those who have respiratory decompensation due to viral pneumonitis. Thrombotic phenomena such as thrombosis in branches of pulmonary artery and splenic artery leading to wedged splenic infarct were found, which resembled those reported during the 1918 pandemic [36]. Although abdominal symptoms were reported to be an important clinical manifestation, and although an *in vitro* study showed that this virus could replicate to a high level in the intestinal cell line Caco-2 [21], gastrointestinal symptoms were not prominent in our cases. As in patients who developed cytokine storm, shock requiring inotropic support and multiorgan dysfunction syndrome were frequent.

Our study had several limitations. The frequent taking of clinical samples was prohibitive to the enrollment of children, and even adult patients occasionally refused serial nasopharyngeal or blood sampling. Viral load can be affected by minor differences in sampling technique, but this problem will affect all groups. The use of nasopharyngeal swabs may result in a lower viral load than nasopharyngeal aspirate, but very few of our samples were obtained by swabs. Because of the changing clinical status of individual patients, treatment regimen could not be unified and may confound the viral load and immunological profiles. In summary, a slower control of viral load and dysregulated cytokine profile are important in the pathogenesis of severe disease by this new pandemic virus. Preexisting immune impairment due to underlying diseases or genetic susceptibility in apparently immunocompetent cases should be investigated. Our findings mandate interventional studies of severe cases by other antiviral and immunomodulatory strategies.

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