Prolonged viral shedding in pandemic influenza A(H1N1): clinical significance and viral load analysis in hospitalized patients

M. Giannella[1], M. Alonso[1,2], D. Garcia de Viedma[1,2], P. Lopez Roa[1], P. Catalán[1,2], B. Padilla[1], P. Muñoz[1,2] and E. Bouza[1,2]

1) Department of Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Madrid and 2) CIBER de Enfermedades Respiratorias (CIBERES), Spain

Abstract

The clinical significance of prolonged viral shedding (PVS) and viral load (VL) dynamics has not been sufficiently assessed in hospitalized patients with pandemic 2009 influenza A(H1N1). We performed a prospective study of adults with confirmed influenza A(H1N1) virus infection admitted to our hospital from 20 September 2009 to 31 December 2009. Consecutive nasopharyngeal swabs were collected every 2 days during the first week after diagnosis, and then every week or until viral detection was negative. Relative VL was measured on the basis of haemagglutinin and RNaseP gene analysis. PVS was defined as positive detection of influenza A(H1N1) virus by real-time RT-PCR at day 7 after diagnosis. We studied 64 patients: 16 (25%) presented PVS. The factors associated with PVS were admission to the intensive-care unit (69% vs. 33%, p 0.02), purulent expectoration (75% vs. 44%, p 0.04), higher dosage of oseltamivir (62.5% vs. 27%, p 0.016), corticosteroid treatment (50% vs. 21%, p 0.05), mechanical ventilation (MV) (50% vs. 12.5%, p 0.004), and longer stay (34 vs. 7 median days, p 0.003). Multivariate analysis revealed the factors independently associated with PVS to be immunosuppression (OR 5.15; 95% CI 1.2–22.2; p 0.03) and the need for MV (OR 11.7; 95% CI 2.5–54.4; p 0.002). VL at diagnosis correlated negatively with age and septic shock. VL dynamics of patients with acute respiratory distress syndrome and/or mortality were very different from those of other patients. PVS was detected in 25% of hospitalized patients with pandemic 2009 influenza A(H1N1) and was strongly associated with immunosuppression and the need for MV. Diagnostic VL and viral clearance varied with the clinical course.

Corresponding author: M. Giannella, Department of Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Doctor Esquerdo 46, 28007 Madrid, Spain
E-mail: maddalena.giannella@libero.it

Introduction

Influenza is a well-known cause of acute respiratory disease. It is generally a self-limiting infection with systemic and respiratory symptoms, and usually resolves after 3–6 days in most patients. Viral clearance in the respiratory tract occurs after 3–5 days [1]. However, a complicated course consisting of severe respiratory illness, exacerbation of underlying diseases and the need for intensive care has been reported for some patients [2,3]. The recently described pandemic influenza A(H1N1) virus had a more aggressive course in specific populations, such as young patients and pregnant women [4,5].

Persistence of viral shedding and the dynamics of viral load (VL) have received little attention in hospitalized patients with seasonal or pandemic influenza. Most reports assess the impact of antiviral therapy [6–8], and data regarding the significance of prolonged viral shedding (PVS) and VL as markers of poor outcome are anecdotal [6,9].

We conducted a prospective study of the frequency of PVS and the dynamics of VL in order to assess epidemiological and prognostic data among hospitalized patients with pandemic influenza A(H1N1).

Materials and Methods

Study design and setting

We performed a prospective study of adult patients (>16 years) with laboratory-confirmed influenza A(H1N1) virus infection who were admitted consecutively to our hospital from 20 September to 31 December 2009.

Ours is a large, general, tertiary teaching hospital currently serving a population of approximately 715 000 inhabitants in Madrid, Spain. The hospital has 1550 beds and includes all medical and surgical specialties, including solid organ (heart, liver, and kidney) and bone marrow transplant programmes.

Study patients had laboratory-confirmed influenza A(H1N1) virus infection and had been hospitalized for at least 48 h. Written informed consent was obtained, and consecutive nasopharyngeal swabs were collected every 2 days during the first week after diagnosis, and then every week or until viral detection was negative. Clinical data were recorded with the use of a pre-established protocol that included the following: demographic characteristics; underlying diseases; influenza vaccination status; clinical, laboratory and radiographic findings at presentation; data on treatment; influenza-related complications; and outcome. The hospital ethics committee approved the study.

During the study period, patients with influenza-like illness were diagnosed and treated according to a standard protocol. Nasopharyngeal swabs were taken in all suspected cases, and patients were admitted if they developed serious medical conditions or presented severe underlying diseases. Droplet precautions were implemented, and oseltamivir was started until the results of viral detection were available. Patients were then treated and discharged according to usual clinical practice.

Definitions

We defined PVS as the detection of influenza A(H1N1) virus by real-time RT-PCR on day 7 after diagnosis.

On the basis of the underlying diseases and the severity of illness at presentation, we divided our patients into four risk groups: (i) patients presenting acute respiratory failure or septic shock that required admission to the intensive-care unit; (ii) immunocompromised patients (i.e. patients with haematological malignancy (with or without bone marrow transplantation), human immunodeficiency virus infection, inflammatory diseases under biological or immunosuppressant treatment, and with solid organ transplant); (iii) patients with chronic comorbid conditions, such as older age (>65 years), a body mass index ≥30, chronic obstructive pulmonary disease, including asthma, cardiovascular disease excluding isolated hypertension, active cancer, chronic renal failure, chronic liver disease, diabetes mellitus, haemoglobin disease, and altered mobilization of respiratory secretions; and (iv) pregnant women.

Acute respiratory distress syndrome (ARDS) and septic shock were defined with the use of standard criteria [10,11].

Sample processing and influenza A(H1N1) virus detection

The nasopharyngeal swabs were preserved in 1 mL of viral transport medium and stored at 4°C for no more than 48 h until processing. Viral RNA was extracted from 200 μL of the sample in a Nuclisens EasyMag device (bioMérieux, Boxtel, The Netherlands), following the manufacturer's instructions, eluted in 60 μL of elution buffer, and maintained at 4°C if it was analysed immediately or at −70°C until assay. Influenza A(H1N1) virus was detected by real time RT-PCR, following the WHO/CDC protocol, in a Stratagene MX3000 thermocycler (Stratagene, La Jolla, CA, USA).

Viral quantification

VL was determined retrospectively in diagnostic specimens and in most of the consecutive specimens.

Quantification of influenza A(H1N1) virus was performed by real-time RT-PCR in a LightCycler 2.0 device, with the RealTime ready Influenza A/H1N1 Detection Set and Real-Time ready RNA virus Master (Roche Diagnostics, Mannheim, Germany). Two independent reactions were performed to quantify the viral haemagglutinin and human RNaseP genes by means of specific standard curves included in each of the assays. Relative quantification of influenza A(H1N1) virus was achieved by normalizing the number of haemagglutinin targets to the number of RNaseP targets in the specimen. VL was expressed as relative quantification units.

To evaluate the reproducibility of the assay, three aliquots of a selection of specimens were analysed on three consecutive days, leading to a standard deviation of 0.018 ± 0.018. The analytical sensitivity of the assay was 2.83 $\log_{10}$ copies/mL.

To analyse VL dynamics over time, we divided patients into six groups (0, 2–4, 5–7, 8–10, 11–13, and 14–16) according to the number of days after diagnosis. The available values for each specific time-point were averaged.

Statistical analysis

In the univariate analysis, categorical variables were compared by use of the chi-square test. The non-normally distributed continuous variables were compared by use of the Mann–Whitney U-test, and expressed as the median and interquartile range (IQR). The normally distributed continuous variables were compared by use of the t-test, and expressed as the mean and standard deviation (SD). The Spearman rank correlation coefficient was used to assess the correlation

between initial viral concentration and age, duration of symptoms, time of oseltamivir initiation after illness onset, days of viral shedding, and length of hospital stay. Stepwise logistic regression models were used in the multivariate analysis to analyse risk factors for PVS. Variables with a p-value <0.1 in the univariate analysis were included in the multivariate models. Differences were considered to be significant for p-values <0.05. The analysis was carried out with SPSS 15.0 (SPSS, Chicago, IL, USA).

Results

During the study period, 91 patients with laboratory-confirmed influenza A(H1N1) virus infection were hospitalized and followed up until discharge. Of these, 64 patients agreed to participate in the study.

We detected PVS at day 7 in 16 patients (25%), of whom six (9.3%) continued to present PVS on day 14 after the initial positive sample.

Patients with PVS were compared with those who did not present PVS (Table 1). The univariate analysis showed the factors associated with PVS to be admission to the intensive-care unit (69% vs. 33%, p 0.02), purulent expectoration (75% vs. 44%, p 0.04), higher dosage of oseltamivir (150 mg/12 h) (62.5% vs. 27%, p 0.016), corticosteroid treatment (50% vs. 21%, p 0.05), mechanical ventilation (MV) (50% vs. 12.5%, p 0.004), and a longer hospital stay (34 vs. 7 median days, p 0.003). We were unable to demonstrate a significant correlation between PVS and mortality (12.5% vs. 8.3%, p 0.63).

The multivariate analysis showed the factors that were independently associated with PVS to be immunosuppression (OR 5.15; 95% CI 1.2–22.2; p 0.03) and the need for MV (OR 11.7; 95% CI 2.5–54.4; p 0.002).

TABLE 1. Comparison of hospitalized patients with pandemic influenza A(H1N1) who presented prolonged viral shedding (PVS) and those who did not

	Patients with PVS, N = 16 (25%)	Patients without PVS, N = 48 (75%)	p	OR (95% CI)	p
Demographic data					
Sex					
Male	8 (50)	19 (39.6)	0.56		
Female	8 (50)	29 (60.4)			
Age (mean ± SD)	47.6 ± 17.5	43.9 ± 13	0.37		
Risk groups					
ICU	**11 (68.8)**	**16 (33.3)**	**0.02**		
Immunocompromised	8 (50)	13 (27.1)	0.12	5.15 (1.2-22.2)	**0.03**
Chronic comorbidity	9 (56.2)	35 (72.9)	0.23		
Pregnant women	2 (12.5)	4 (8.3)	0.63		
Charlson comorbidity score (median, IQR)	6, 3–7.75	2, 1–6	0.06		
Presenting symptoms					
Fever	15 (93.8)	45 (93.8)	1		
Cough	14 (87.5)	40 (83.3)	1		
Sputum production	**12 (75)**	**21 (43.8)**	**0.04**		
Headache	0	8 (16.7)	0.18		
Myalgia	6 (37.5)	21 (43.8)	0.77		
Diarrhoea	4 (25)	8 (16.7)	0.47		
Days of symptoms before diagnosis (median, IQR)	2, 1–4.7	2, 0–4	0.59		
Initial radiographic findings					
Any infiltrate	5 (31.2)	14 (29.8)	1		
One-segmented infiltrate	1 (6.2)	7 (14.9)	0.66		
Bilateral infiltrates	6 (37.5)	13 (27.7)	0.53		
Interstitial infiltrates	2 (12.5)	1 (2.1)	0.15		
Treatment					
Days of illness at initiation of treatment (median, IQR)	2.5, 0–5	2, 0–4	0.48		
Oseltamivir 75 mg/12 h	7 (43.8)	35 (72.9)	0.06		
Oseltamivir 150 mg/12 h	**10 (62.5)**	**13 (27.1)**	**0.016**		
Antibiotic treatment	16 (100)	40 (83.3)	0.18		
Corticosteroids	**8 (50)**	**10 (20.8)**	**0.05**		
Inotropic support	3 (18.8)	2 (4.2)	0.09		
Oxygen supplementation	8 (50)	14 (29.2)	0.14		
Mechanical ventilation	**8 (50)**	**6 (12.5)**	**0.004**	11.7 (2.5-54.4)	**0.002**
Complications					
COPD exacerbation	4 (25)	3 (6.2)	0.06		
Asthma exacerbation	1 (6.2)	7 (14.6)	0.66		
Bacterial co-infection	6 (37.5)	16 (33.3)	0.77		
ARDS	6 (37.5)	6 (12.5)	0.06		
Septic shock	3 (18.8)	2 (4.2)	0.09		
Outcome					
Death	2 (12.5)	4 (8.3)	0.63		
Days of hospital stay (median, IQR)	**34, 9–50**	**7, 4.7–20**	**0.003**		
Days of ICU stay (median, IQR)	21, 2–49	4.5, 2–14.5	0.20		

ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; ICU, intensive-care unit; IQR, interquartile range; SD, standard deviation.
Bold values indicate factors with p < 0.05.

TABLE 2. Relationship between diagnostic relative viral load and clinical characteristics of hospitalized patients with pandemic influenza A(H1N1)

	Viral load with this feature (median, IQR)	Viral load without this feature (median, IQR)	p
Risk groups			
ICU admission	0.15 (0.02–4.1)	0.10 (0.007–1.7)	0.55
Immunocompromised	0.15 (0.02–1.8)	0.10 (0.016–2.5)	0.73
Chronic comorbidity	0.14 (0.02–2.4)	0.10 (0.007–1.64)	0.96
Pregnant women	4.1 (0.08–58.1)	0.1 (0.02–1.08)	0.07
Vaccination status			
Seasonal influenza vaccination	0.2 (0.02–1.6)	0.1 (0.01–2.7)	0.99
Pandemic influenza vaccination	12.1 (4.3–19.8)	0.1 (0.02–1.5)	0.06
Prolonged viral shedding	0.56 (0.03–5.9)	0.08 (0.007–0.85)	0.08
Complications			
COPD exacerbation	0.45 (0.16–0.45)	0.10 (0.01–2.3)	0.21
Bacterial co-infection	0.05 (0.005–1.44)	0.14 (0.02–2.7)	0.38
ARDS	0.07 (0.01–1.98)	0.12 (0.02–2.3)	0.71
Septic shock	**0.006 (0.0005–0.07)**	**0.14 (0.02–2.7)**	**0.03**
Outcome			
Death	0.02 (0.0008–0.17)	0.12 (0.02–2.9)	0.06

ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; ICU, intensive-care unit; IQR, interquartile range.
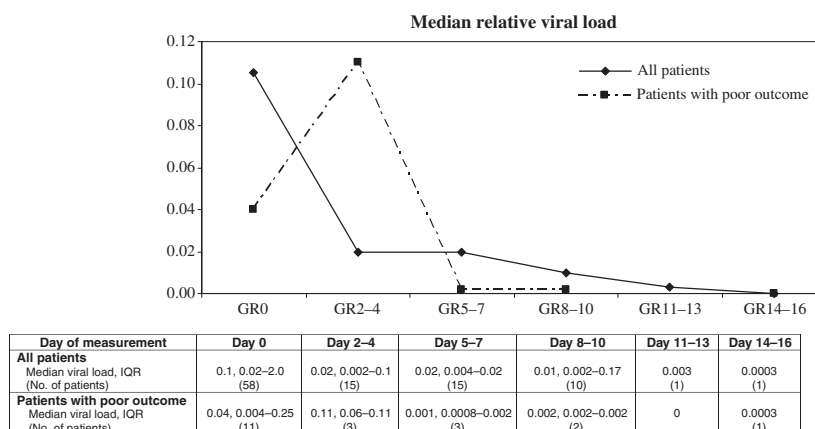Bold values indicate factors with p < 0.05.

Median relative viral load

Day of measurement	Day 0	Day 2–4	Day 5–7	Day 8–10	Day 11–13	Day 14–16
All patients						
Median viral load, IQR	0.1, 0.02–2.0	0.02, 0.002–0.1	0.02, 0.004–0.02	0.01, 0.002–0.17	0.003	0.0003
(No. of patients)	(58)	(15)	(15)	(10)	(1)	(1)
Patients with poor outcome						
Median viral load, IQR	0.04, 0.004–0.25	0.11, 0.06–0.11	0.001, 0.0008–0.002	0.002, 0.0002–0.002	0	0.0003
(No. of patients)	(11)	(3)	(3)	(2)		(1)

FIG. 1. Median relative viral load over the course of the infection for the groups (GR) of patients with specimens analyzed on days 0, 2–4, 5–7, 8–10, 11–13 and 14–16.

Relative VL was determined in 58 of the 64 patients (16 with PVS and 42 without), and ranged from 1.17×10^{-3} to 317 relative quantification units (median 0.1, IQR 0.02–2.02). We found a negative correlation between diagnostic VL and age ($r = -0.287$, p 0.03). There was no correlation between diagnostic VL and duration of symptoms ($r = -1.94$, p 0.14), time of oseltamivir initiation after illness onset ($r = -0.11$, p 0.4), duration of viral shedding ($r = -0.4$, p 0.09), or length of hospital stay ($r = -0.23$, p 0.09). We found a trend towards higher diagnostic VL in pregnant women (4.1 vs. 0.1, p 0.07) and in patients with PVS (0.56 vs. 0.08, p 0.08), although this was lower in patients with septic shock (0.006 vs. 0.14, p 0.03) and in-hospital mortality (0.02 vs. 0.12, p 0.06) (Table 2).

VL dynamics during hospitalization in all patients and in those with poor outcome (ARDS and/or death) are shown in Fig. 1. Viral clearance was different between these groups. A progressive decrease in VL was observed in all patients during the shedding period, whereas in those with poor out-come, a lower initial VL followed by a peak was observed at day 2–4 after diagnosis. Viral clearance occurred at day 5–7.

Discussion

We found that 25% of patients who were hospitalized with pandemic influenza A(H1N1) presented PVS after diagnosis. PVS was associated with major morbidity as a predisposing factor as well as complication of the influenza. We also observed that VL dynamics in patients with poor outcome were different from those of the group as a whole.

Current recommendations for respiratory isolation of patients hospitalized with influenza have established 7 days as a standard figure [12]. Data on PVS in both seasonal and pandemic influenza have shown that 22–57% of hospitalized patients continue to experience viral shedding 7 days after onset [6,7]. We are not sure whether data on persistence

mean that the virus is viable and can be transmitted. The frequency of positive viral culture in respiratory specimens collected at day 8 after onset has only been assessed in one study [13], in which a rate of 19% was observed. The potential for transmission in patients with PVS requires further study.

PVS as a marker of a severe underlying condition in patients with seasonal influenza has been suggested by Lee *et al.* [6]. The authors related PVS to older age, chronic comorbidity, and therapy with systemic corticosteroids. Little is known about the significance of PVS in patients with pandemic influenza. In a series of 22 patients with pandemic influenza A, younger age was the only factor associated with a longer period of viral shedding after onset [14]. In our series, we did not find a correlation between PVS and age or chronic comorbidity, although immunosuppression increased the risk of PVS five-fold (p 0.03). The other risk factor independently associated with PVS was the need for MV (p 0.002).

Most of our patients presented PVS for more than 7 days and <14 days. Only one patient with leukaemia presented PVS for more than 4 weeks. PVS for >4 weeks has been reported in patients with haematological conditions, particularly those with lymphocytopenia [15].

Given the heterogeneous quality of specimens obtained by nasopharyngeal swab, the need for relative quantification in influenza A(H1N1) has recently been reported [16,17]. We developed a reproducible assay to obtain relative quantification data at diagnosis and in consecutive samples.

We found that relative VL in the diagnostic specimen correlated negatively with age, septic shock, and in-hospital mortality. We did not find a correlation between VL at diagnosis and the presence of major comorbidity, duration of symptoms, or early onset of antiviral treatment, as reported by Lee *et al.*[6] in patients with seasonal influenza. Consistent with To *et al.*[9], we showed different viral dynamics in patients with poor outcome than in the study population as a whole. Patients with ARDS and/or who died in hospital had a lower initial VL, with a peak at day 2–4 and viral clearance at day 5–7 after diagnosis. The lower initial VL in this group suggests that replication of influenza A(H1N1) virus could be less efficient in the nasopharyngeal tract than in the lower respiratory tract in patients with a poorer prognosis.

Our study has several limitations. First, we did not perform a daily collection of nasopharyngeal specimens; consequently, we could not estimate the overall median duration of viral shedding. Second, because of the discomfort associated with collection of consecutive nasopharyngeal swabs, the number of patients is small, thus limiting conclusions about prognostic factors. Third, we analysed VL only in the samples obtained by nasopharyngeal swabbing; therefore, we could not compare VL in the upper and lower respiratory tract.

PVS is a common complication in hospitalized patients with pandemic influenza, and is associated with longer hospital stay and poorer outcome. Future recommendations regarding periods of respiratory isolation in hospitalized patients with influenza should consider the frequency and consequences of this phenomenon. The study of viral clearance can identify patients with a poorer prognosis.

Acknowledgements

Transparency Declaration

This study does not present any conflict of interest for the authors.

References

1. Hayden FG. Prevention and treatment of influenza in immunocompromised patients. *Am J Med* 1997; 102: 55–60.
2. Thompson WW, Shay DK, Weintraub E *et al.* Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* 2003; 289: 179–186.
3. Thompson WW, Shay DK, Weintraub E *et al.* Influenza-associated hospitalizations in the United States. *JAMA* 2004; 292: 1333–1340.
4. Chowell G, Bertozzi SM, Colchero MA *et al.* Severe respiratory disease concurrent with the circulation of H1N1 influenza. *N Engl J Med* 2009; 361: 674–679.
5. Jamieson DJ, Honein MA, Rasmussen SA *et al.* H1N1 2009 influenza virus infection during pregnancy in the USA. *Lancet* 2009; 374: 451–458.
6. Lee N, Chan PK, Hui DS *et al.* Viral loads and duration of viral shedding in adult patients hospitalized with influenza. *J Infect Dis* 2009; 200: 492–500.

7. Ling LM, Chow AL, Lye DC *et al.* Effects of early oseltamivir therapy on viral shedding in 2009 pandemic influenza A (H1N1) virus infection. *Clin Infect Dis* 2010; 50: 963–969.

8. Li IW, Hung IF, To KK *et al.* The natural viral load profile of patients with pandemic 2009 influenza A(H1N1) and the effect of oseltamivir treatment. *Chest* 2010; 137: 759–768.

9. To KK, Hung IF, Li IW *et al.* Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clin Infect Dis* 2010; 50: 850–859.

10. Bernard GR, Artigas A, Brigham KL *et al.* The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med* 1994; 149: 818–824.

11. Dellinger RP, Levy MM, Carlet JM *et al.* Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit Care Med* 2008; 36: 296–327.

12. Novel influenza A (H1N1) virus infections among health-care personnel—United States, April–May 2009. *MMWR* 2009; 58: 641–645.

13. De Serres G, Rouleau I, Hamelin ME *et al.* Contagious period for pandemic (H1N1) 2009. *Emerg Infect Dis* 2010; 16: 783–788.

14. To KK, Chan KH, Li IW *et al.* Viral load in patients infected with pandemic H1N1 2009 influenza A virus. *J Med Virol* 2010; 82: 1–7.

15. Gooskens J, Jonges M, Claas EC, Meijer A, Kroes AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. *J Infect Dis* 2009; 199: 1435–1441.

16. Duchamp MB, Casalegno JS, Gillet Y *et al.* Pandemic A(H1N1)2009 influenza virus detection by real time RT-PCR: is viral quantification useful? *Clin Microbiol Infect* 2010; 16: 317–321.

17. Ngaosuwankul N, Noisumdaeng P, Komolsiri P *et al.* Influenza A viral loads in respiratory samples collected from patients infected with pandemic H1N1, seasonal H1N1 and H3N2 viruses. *Virol J* 2010; 7: 75.