Viral load at diagnosis and influenza A H1N1 (2009) disease severity in children

Cristian Launes,a Juan J. Garcia-Garcia,a Iolanda Jordan,b Laura Selva,c Jordi Rello,d Carmen Muñoz-Almagroe

aInfectious Diseases Unit, Department of Pediatrics, Hospital Sant Joan de Déu, University of Barcelona, Barcelona, Spain. bPediatric Intensive Care Unit, Hospital Sant Joan de Déu, University of Barcelona, Barcelona, Spain. cDepartment of Molecular Microbiology, Hospital Sant Joan de Déu, University of Barcelona, Barcelona, Spain. dCritical Care Department, Hospital Vall d’Hebron, Autonomous University of Barcelona, CIBERES, Barcelona, Spain. eDepartment of Molecular Microbiology, Hospital Sant Joan de Déu, University of Barcelona, Barcelona, Spain.

Correspondence: Cristian Launes, Pediatric Infectious Diseases Unit, Hospital Sant Joan de Déu, Passeig Sant Joan de Déu no2, ES-08150 Esplugues de Llobregat, Barcelona, Spain.
E-mail: claunes@hsjdbcn.org

Accepted 23 April 2012. Published Online 24 May 2012.

To assess viral load at diagnosis (VLAD) as a biomarker of novel influenza disease severity, epidemiologic and clinical data of admitted patients <18 years old with Influenza A H1N1 (2009) infection and respiratory symptoms were prospectively collected in a single pediatric tertiary hospital, from weeks 30–51 of 2009. Seventy patients were included. VLAD in children who had symptoms for \( \geq 5 \) days was an accurate parameter distinguishing the patients who required mechanical ventilation (MV) from those who did not require it (area under the ROC curve: 0.73; \( P = 0.03 \)). Having \( <4.5 \log_{10} \) copies/ml with \( \geq 5 \) days of symptoms was associated with a lower risk of requiring MV.

Keywords Influenza, mechanical ventilation, pediatrics, respiratory insufficiency, viral load.

Introduction

The Influenza A H1N1 (2009) virus affected thousands of children in Catalonia (Spain) in fall 2009 (1000–1200 infections per 100 000 inhabitants aged <15-year old between weeks 43 and 46 of 2009).\(^1\) The infection typically caused mild respiratory symptoms, but other less usual extrapulmonary manifestations and a severe clinical course were observed even in previously healthy patients.\(^2,3\) The attack rate of Influenza A H1N1 (2009) during the first postpandemic season has been much lower, nevertheless the virus has continued to cause serious illness in children.\(^4\)

Viral load may provide important information about the interaction between the infective agent and the host. The information about the possible correlation between viral load, epidemiological characteristics and the severity of disease in pediatric patients is scarce.

The aim of this study was to describe Influenza A H1N1 (2009) viral load at diagnosis (VLAD) and its relation to epidemiological, clinical characteristics and laboratory parameters in a series of hospitalized pediatric patients. We also wanted to assess viral load at diagnosis as a biomarker of disease severity.

Methods

The study was conducted in a 345-bed tertiary care pediatric hospital with a 18-bed pediatric intensive care unit, from weeks 30–51 of 2009. During the study period, patients with respiratory symptoms who required hospital admission were tested for Influenza A H1N1 (2009). Patients <18 years old with Influenza A H1N1 (2009) infection and hospitalized with respiratory symptoms were consecutively included. Epidemiologic and clinical data of them were prospectively collected. Severe cases were defined as those who required invasive or non-invasive mechanical ventilation (MV) because of respiratory failure or severe respiratory distress.

Diagnosis and quantification of viral load

Total nucleic acids were extracted from nasopharyngeal aspirate with the Roche MagNA Pure Compact System (Roche Molecular Diagnostics, Mannheim, Germany) according to manufacturer’s protocol. The Influenza A H1N1 (2009) infection was confirmed by detection of matrix protein 2 (M2) and pandemic Hemagglutinin H1 genes by using a real-time reverse transcriptase polymerase
chain reaction (Real-Time RT-PCR) (RealTime ready Inf A/H1N1 Detection Set; Roche Molecular Diagnostics). This test has a detection limit of 10 copies per PCR reaction. For the quantitative assay, 10 fold dilutions equivalent to $10^{-1}$–$10^0$ copies per reaction of a plasmid positive control for M2 gene positive control supplied by the manufacturer were prepared to generate a calibration curve. Viral load was retrospectively determined through cycle-threshold ($C_t$) values of M2 gene corrected according to the $C_t$ value of internal control (human myostatine gene). Nasopharyngeal aspirates with an internal control $C_t > 27$ were considered as poor quality samples for viral load determination. The reactions were performed and analyzed by using the LightCycler® 480 System (Roche Molecular Diagnostics).

Statistical analysis
Descriptive statistics of non-continuous variables were reported in terms of absolute frequencies and percentages, and data comparisons were performed using Pearson Chi-square test or Fisher exact test when the expected count in any category was <5. Continuous non-normally distributed variables were described in terms of median value with interquartile range (IQR, 25th percentile–75th percentile) and compared using Mann–Whitney U test. The receiver-operator characteristic (ROC) curve analysis was performed with VLAD distinguishing between patients who required MV and those who did not require MV.

spss® software V 19.0 for windows® was used to perform the statistical tests. For the analysis, the significance level considered was <0.05.

The study was approved by the institutional ethics board and informed consent was waived.

Results
During the study period, 93 children were admitted with respiratory distress and/or hypoxemia and Influenza A H1N1 (2009) infection. Twenty-three patients were excluded because either their nasopharyngeal aspirate sample had an internal control $C_t > 27$ (11/93) or they were diagnosed in other institutions (12/93) and were not retested. Thus, 70 children were included in the study. None had received a vaccine against pandemic virus. None had received antiviral treatment before admission.

Viral load was determined within a median of 3 days after onset of respiratory symptoms (IQR: 1–5). We established two different groups regarding to VLAD and duration of illness at the moment of sampling; those who had been with symptoms for four or less days and those who had symptoms for five or more days (median, $6 \log_{10}$ copies/ ml (IQR: 4.6–6.8) versus $4.7 \ (3.9–5.7); P = 0.02$) (Figure 1). The viral load was inversely correlated to the days after the onset of fever (Spearman rho = –0.36; P < 0.01).

Viral load according to demographic characteristics and comorbidity is shown in Table 1. Regarding to the association with laboratory parameters, VLAD in patients within first 4 days of symptoms was inversely correlated with the number of lymphocytes at diagnosis (Spearman rho = –0.47; P < 0.01), but there was no correlation in those diagnosed with ≥5 days of symptoms. Viral load was not correlated with values of C reactive protein. Neither was it with absolute neutrophil count or with hemoglobin (data not shown).

Twenty patients required MV because of respiratory failure or severe respiratory distress during the hospitalization. The ROC curve analysis was used to analyze the accuracy of VLAD as a biomarker of severity (need of MV). In patients within the first 4 days of symptoms, the area under the ROC curve was 0.55 (95% confidence interval, 0.39–0.69; P = 0.61). In children who had symptoms for ≥5 days, the area under the ROC curve of VLAD was 0.73 (95% confidence interval, 0.51–0.88; P = 0.03). The cutoff value of $4.5 \log_{10}$ copies/ml corresponded with the highest average of sensitivity (100%, 95% CI: 63–100) and specificity (65%, 95% CI: 38.3–85.8). The negative predictive value of a lower value of viral load for distinguishing patients who required MV was 100% (95% CI: 71.5–100). Having $<4.5 \log_{10}$ copies/ml with ≥5 days of clinical symptoms was associated with a lower risk of requiring MV [relative risk (RR) = 0.13 (95% CI: 0.02–0.92)]. Patients who were with symptoms for ≥5 days and with a VLAD ≥$4.5 \log_{10}$ copies/ml had an increased risk of requiring MV in comparison with those that exceeded that viral load and were still within the first 4 days of symptoms [RR = 2.08 (95% CI: 1.07–4.05)].
The number of deceased patients was too low (n = 4) to draw any conclusion regarding differences in VLAD. All of them had a VLAD > 4·5 log_{10} copies/ml.

Most of the patients (66/70, 94%) received oseltamivir during hospitalization immediately after collecting the sample for viral load determination.

**Discussion**

Children had been widely described as a population with an increased risk of severe disease by Influenza A H1N1 (2009). The immunopathology may play an important role in the severely affected children apart from the virus-mediated component of disease, which is the focus of this study. The literature published about these two components of the novel influenza disease in hospitalized children is scarce.

Viral load at diagnosis may be a biomarker of severity, as described previously for other respiratory virus, including H5N1 influenza virus. VLAD can be easily extrapolated with the same real-time RT-PCR used for the diagnosis of infection without almost adding any extra economic cost. In our study, VLAD within the first days of symptoms was not a good biomarker of severity. Nonetheless in patients with 5 or more days of symptoms, VLAD was an accurate parameter distinguishing the patients who required MV from those who did not require it. It is known that the lag time between the onset of symptoms and the initiation of antiviral treatment determines a different clinical response in Influenza A H1N1 (2009) infections. In patients within the first days of symptoms, early initiation of oseltamivir could have protected them from developing a more severe respiratory disease, and probably this may be one of the reasons for not finding the VLAD values as an accurate parameter in distinguishing the most severe cases in that group. On the other hand, the treatment has proved to be effective in decreasing viral load, suggesting that those patients with high viral loads after several days of symptoms could have benefited from an earlier treatment.

In this series, as reported previously for seasonal and pandemic influenza, VLAD was at high level in those patients in whom sample was collected during the first days of symptoms. Higher Influenza A H1N1 (2009) viral loads had been observed in patients with fever and in those with pneumonia in comparison with patients without systemic symptoms. Li et al. observed a lower clearance of viral load in patients younger than 13-year old, but they did not find in them a higher rate of severe disease, defined in that study as having chest X-ray opacities or meningoencephalitis. In our series, to have a ‘high’ VLAD with 5 days in comparison with previously healthy patients.

**Table 1.** Influenza A H1N1 (2009) viral load at diagnosis (VLAD) according to demographic characteristics and underlying comorbidity

| (No patients, %) | VLAD in children within first 4 days of symptoms (log_{10}copies/ml) (n = 45) | P-value | VLAD in children who had symptoms for ≥5 days (log_{10}copies/ml) (n = 25) | P-value |
|------------------|---------------------------------------------------------------------------------|--------|---------------------------------------------------------------------------------|--------|
| Sex              |                                                                                 |        |                                                                                 |        |
| Male (34, 48%)   | 6·0 (4·1–6·9)                                                                   | 0·8**  | 4·4 (3·6–5·1)                                                                   | 0·2**  |
| Female (36, 52%) | 5·9 (4·9–6·8)                                                                   |        | 5·0 (4·1–5·9)                                                                   |        |
| Age              |                                                                                 |        |                                                                                 |        |
| <2 year-old (23, 33%) | 5·9 (3·9–6·2)                                                                   | 0·01***| 5·0 (3·6–5·9)                                                                   | 0·6*** |
| 2–5 year-old (13, 19%) | 5·0 (3·3–6·5)                                                                   |        | 3·9 (2·2–6·8)                                                                   |        |
| 5–12 year-old (28, 39%) | 6·4 (5·1–6·4)                                                                   |        | 4·5 (4·0–5·1)                                                                   |        |
| ≥13 year-old (6, 9%) | 7·0 (5·1–7·9)                                                                   |        | 6·5 (5·7–7·4)                                                                   |        |
| Previously-known  |                                                                                 |        |                                                                                 |        |
| condition:       |                                                                                 |        |                                                                                 |        |
| Previously healthy (37, 53%) | 6·0 (4·4–6·5)                                                                   | 0·7†   | 4·6 (3·8–5·8)                                                                   | 0·9†   |
| Any 1 condition (33, 47%) | 6·0 (4·6–6·9)                                                                   |        |                                                                                 |        |

*Median (interquartile range).

**Male versus Female.

***<5-year old versus ≥5-year old.

†In comparison with previously healthy patients.
H1N1 (2009) disease. Further studies in different settings are needed to identify the weighted role of the direct viral damage and the immunopathology in pediatric patients with influenza A H1N1 (2009) disease.

Competing Interests
All authors declared no conflict of interest.

Acknowledgements
We thank Drs, Mariona F de Sevilla, Susanna Hernandez-Bou, Cristina Esteve, and Eva del Amo for their contribution in taking care of patients and/or microbiological studies and Pedro Brotons for the support in the statistical analysis. This study was partially supported by a grant from AGAUR (Agència de Gestió d’Ajuts Universitaris i de Recerca, Generalitat de Catalunya) SGR00136. AGAUR has not influenced in the design, analysis or has any role on preparation of the manuscript.

References
1 Subdirecció General de Vigilància i Resposta a Emergències en Salut Pública. (2010) Pla d’informació de les infeccions respiratòries agudes a Catalunya. Temporada gripal 2009–2010. Full informatiu núm.13. Generalitat de Catalunya. Departament de Salut; Available at http://www20.gencat.cat/docs/canalsalut/Home%20Canal%20SalutProfessionals/Temes_de_salut/Vigilancia_epidemiologica/documents/pidirac_2009_10.pdf. (Accessed 1 November 2011).
2 Larcombe PJ, Moloney SE, Schmidt PA. Pandemic (H1N1) 2009: a clinical spectrum in the general paediatric population. Arch Dis Child 2011; 96:96–98.
3 Launes C, García-García JJ, Jordán I, Martínez-Planas A, Selva L, Muñoz-Almagro C. 2009 Influenza A H1N1 infections: delays in starting treatment with Oseltamivir were associated with a more severe disease. Pediatr Infect Dis J 2011; 30:622–625.
4 Centers for Disease Control and Prevention. Weekly influenza surveillance report: 2010–2011 Season Week 7 ending February 19, 2011. Available at http://www.cdc.gov/flu/weekly/weeklyarchives2010-2011/weekly07.htm (Accessed 2 March 2011).
5 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.
6 Kawashima H, Go S, Kashiwagi Y et al. Cytokine profiles of suction pulmonary secretions from children infected with pandemic influenza A(H1N1) 2009. Crit Care 2010; 14:411.
7 de Jong MD, Simmons CP, Thanh TT et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytorkinemia. Nat Med 2006; 12:1203–1207.
8 Utokaparch S, Marchant D, Gosselink JV et al. The relationship between respiratory viral loads and diagnosis in children presenting to a pediatric hospital emergency department. Pediatr Infect Dis J 2011; 30:e18–23.
9 Jansen RR, Schinkel J, Dek I et al. Quantitation of respiratory viruses in relation to clinical course in children with acute respiratory tract infections. Pediatr Infect Dis J 2010; 29:82–84.
10 Rodríguez A, Díaz E, Martin-Loeches I et al. Impact of early oseltamivir treatment on outcome in critically ill patients with 2009 pandemic influenza A. J Antimicrob Chemother 2011; 66:1140–1149.
11 Li W, 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.
12 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.
13 Lau LL, Cowling BJ, Fang VJ et al. Viral shedding and clinical illness in naturally acquired influenza virus infections. J Infect Dis 2010; 201:1509–1516.
14 Li CC, Wang L, Eng HL et al. Correlation of pandemic (H1N1) 2009 viral load with disease severity and prolonged viral shedding in children. Emerg Infect Dis 2010; 16:1265–1272.
15 Giannella M, Alonso M, Viedma DG et al. Prolonged viral shedding in pandemic influenza A(H1N1): clinical significance and viral load analysis in hospitalized patients. Clin Microbiol Infect 2011; 17:1160–1165.
16 Wu UI, Wang JT, Chen YC, Chang SC. Severity of pandemic H1N1 2009 influenza virus infection may not be directly correlated with initial viral load in upper respiratory tract. Influenza Other Respir Viruses 2012; 6:367–373.