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

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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 ≥ 5 days was an accurate parameter distinguishing

the patients who required mechanical ventilation (MV) from those who did not required it (area under the ROC curve: 0.73; $P = 0.03$). Having <4.5 log₁₀ copies/ml with ≥ 5 days of symptoms was associated with a lower risk of requiring MV.

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

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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 $10\text{--}1.0 \times 10^5$ 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 determination⁵. 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 required 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, 6.0 log₁₀ 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

Figure 1. Viral load and duration of symptoms at the moment of sampling.

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 log₁₀ copies/ml corresponded with the highest average of sensitivity (100%, 95% CI: 63.1–100) 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 log₁₀ 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 log₁₀ 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)].

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)		4.8 (4.0–5.7)	

*Median (interquartile range).

**Male versus Female.

***<5-year old versus ≥5-year old.

†In comparison with previously healthy patients.

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 log₁₀ 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 children⁶ 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^{7–9} 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 required 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.^{3,10} 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,¹¹ 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,^{12–14} 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^{12,14} in comparison with patients without systemic symptoms. Li *et al.*¹⁴ 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 meningoen- cephalitis. In our series, to have a 'high' VLAD with ≥5 days of symptoms conferred a two-fold increase in the risk of MV. This result could be in relation with the observed lower viral load clearances in patients with more severe disease in other series.^{12,15,16}

The main limitation of the study is the small sample size. However, in this series, only pediatric subjects have been included. Detection of oseltamivir-resistant strains was not systematically performed, although resistant strains were a minority during the pandemic period.⁴ It would also be interesting to determine the clinical utility of serial analysis of viral load for each individual case, but it may be difficult to be introduced as a regular clinical practice in pediatrics.

Based on our observations, we conclude that VLAD in patients with several days of clinical symptoms (≥5 days in our study) was associated with the severity of influenza A

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.

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