Pandemic A(H1N1)2009 influenza virus detection by real time RT-PCR: is viral quantification useful?

M. Bouscambert Duchamp1,2, J. S. Casalegno1,2, Y. Gillet3, E. Frobert1,2, E. Bernard1, V. Escuret1,2, G. Billaud1, M. Valette1,2, E. Javouhey3, B. Lina1,2, D. Floret3 and F. Morfin1,2
1) Hospices Civils de Lyon, National Influenza Centre (South of France), Laboratory of Virology, Bron, 2) Université de Lyon, Université Lyon 1 Virologie et Pathologie Humaine, CNRS FRE 3011, Lyon Cedex and 3) Hospices Civils de Lyon, Groupement Hospitalier Est, Hôpital femme-mère-enfant, Paediatric units, Bron, France

Abstract

The emergence of the influenza A(H1N1) 2009 virus prompted the development of sensitive RT-PCR detection methods. Most are real time RT-PCRs which can provide viral quantification. In this manuscript, we describe a universal influenza A RT-PCR targeting the matrix (M) gene, combined with an RNaseP RT-PCR. These PCRs allow the detection of all influenza A virus subtypes, including A(H1N1)2009, together with a real-time assessment of the quality of the specimens tested. These PCR procedures were evaluated on 209 samples collected from paediatric patients. Viral loads determined through Ct values were corrected according to the RNaseP Ct value. The mean viral load in the collected samples was estimated to be 6.84 log RNA copies/mL. For poor quality samples (RNaseP Ct > 27), corrections resulted in +3 to +8 Ct values for the M gene RT-PCR. Corrected influenza Ct values were lower in late samples. No correlation was established between viral loads and clinical severity or duration of disease. This study shows that real time RT-PCR targeting the matrix gene is a reliable tool for quantification of type A influenza virus but emphasises the need for sample quality control assessment through cellular gene quantification for reliable estimation of the viral load. This method would be useful for disease management when repeated specimens are collected from an infected individual.

Keywords: Clinical features, influenza A(H1N1)2009, matrix gene, paediatric, real time RT-PCR, RNaseP gene, viral load

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Introduction

The new variant A(H1N1) influenza virus was originally detected in April 2009 in Mexico and the USA. Subsequently, it disseminated worldwide. In France, this virus was first detected in May 2009 and the first epidemic was reported in October, with the proportion of severe cases and deaths increasing rapidly (http://www.invs.sante.fr/surveillance/grippe_dossier/index_H1N1.htm).

The RNA genome of A(H1N1)2009 virus comprises a specific combination of gene segments that had never previously been detected [1–3]. Moreover, specific clinical features have been reported for infections associated with the A(H1N1)2009 virus. Most hospitalized patients are under the age of 18 and very few are over 65 years [4]. Pneumonia is detected in 40% of cases [4]. Between 40% and 70% of patients with severe symptoms have an underlying medical history [2,4].

Since May 2009, the two French National Influenza Centres (NICs) have worked together to develop rapid and sensitive methods to detect the A(H1N1)2009 virus. The strategy chosen included two real-time RT-PCRs: one targeting the M gene (M RT-PCR) for sensitive detection of all subtypes of type-A influenza virus and the second, targeting the HA gene, for specific detection of A(H1N1)2009 virus. The M RT-PCR described here was actually developed for influenza A virus quantification before the emergence of the A(H1N1)2009 virus. This PCR strategy was supported by a ribonuclease P (RNase P) RT-PCR for assessment of the quality of the samples. In addition to real time evaluation of
the quality of specimens, we also looked for evidence of a relationship between Ct value (viral load) and disease severity during the A(H1N1)2009 pandemic.

**Materials and Methods**

**Patients**

Overall, 209 patients positive for A(H1N1)2009 were included in the study between the 1st of October 2009 and the 4th of November 2009. Clinical data were collected retrospectively. The mean age of the patients was 5.9 years (±3.8); 12.5% of the patients were below 1 year of age. The M:F sex ratio was 1.06. There was no health risk factor in 63.5% of the patients; reported health risk factors were asthma (24.5%), chronic cardiac disease (4.8%), prematurity (3.8%) and other (3.4%). The mean delay between onset of clinical symptoms and consultation was 2.07 days (±2 days). The most frequently reported symptoms were cough (87.9%), fever (86.2%), rhinitis (36.2%) and myalgia (22.2%). Nine percent of the patients were hospitalized, only one of whom required admission to the intensive care unit. Neuraminidase inhibitors were prescribed in 41.6% of the cases, with oseltamivir or zanamivir in 92.6% and 6.2% of the treated cases, respectively.

**Samples**

Nasal swabs were sent to the laboratory in <12 h after sampling. Upon arrival, they were placed in 3 mL of transport medium for viral culture; 200 lL of this medium was used for RNA purification on NucliSens easyMAG® instrument (bioMérieux). After extraction, RNA was eluted in 70 lL and stored at +4°C until RT-PCRs were performed the same day. Longer storage was carried out at -80°C.

To determine the specificity of the RT-PCRs, 16 samples from patients positive for different respiratory viruses were tested in M RT-PCR and H1 RT-PCR (developed by NIC, North of France, Institut Pasteur, Paris). These samples were known to be positive for seasonal influenza A(H1N1), A(H3N2), B viruses, respiratory syncytial virus A or B, bocavirus, parainfluenza 1–3, metapneumovirus, rhinovirus, enterovirus, adenovirus, cytomegalovirus, herpes simplex virus or varicella-zoster virus.

To validate the wide spectrum of influenza A subtypes detected by M RT-PCR, we tested 18 avian, swine and human A influenza viruses including A/Vietnam/1194/2004 (H5N1), A/Turkey/13/2006 (H5N1), A/Finch/England/2051/2002 (H5N2) and A/Swine/England/117316/86 (H1N1).

A calibrated synthetic RNA transcript was kindly provided by V. Enouf and S. van der Werf (NIC North of France, Institut Pasteur, Paris) and used for the assessment of the M RT-PCR. This transcript is the negative strand of the Open Reading Frame of the M gene (from the ATG to nucleotide 982). The transcript was diluted in distilled water to obtain a solution at a concentration of 10⁹ copies/µL and stored at -80°C. Two dilutions were used in each assay: 10⁴ copies/5 µL, corresponding to a Ct value of 30 and 10⁵ copies/5 µL, corresponding to a Ct value of 26.

**Influenza real time RT-PCR**

The primers and probe targeting the haemagglutinin gene and designed for the specific detection of A(H1N1)2009 are available upon request (grippe@pasteur.fr). The primers targeting the matrix gene and designed for universal detection of influenza A viruses were previously described for a classical RT-PCR [5] and the probe was designed on Primer Express version 3 software (Applied Biosystem, Foster City, CA, USA) (Table 1). Specificity of oligonucleotides was assessed by performing a local alignment search (blastn; http://blast.ncbi.nlm.nih.gov./Blast.cgi).

Reactions were performed in duplicate in 25 µL final volume reaction containing 5 µL of purified nucleic acid template, 12.5 µL of 2× reaction mix (3 mM Mg²⁺), 2 µL of each 10 µM primers (final concentration at 800 nM) and 1 µL of 5 µM probe (final concentration at 200 nM). Finally 0.5 µL of Rox reference dye (10-fold pre-diluted) was added and 0.5 µL of enzyme mix (Superscript III Platinum One-Step Quantitative RT-PCR System, Invitrogen, Carlsbad, CA, USA). Thermocycling reaction conditions were: reverse transcription at 50°C for 15 min; Taq activation and denaturation at 95°C for 2 min, followed by 50 PCR amplification cycles including one step at 95°C for 15 s and one step at 60°C for 40 s (hybridization-elongation). RT-PCR assays were performed on an ABI 7500 (Applied Biosystem).

Efficiency, reproducibility, repeatability, specificity and calculation of the limit of detection were determined according to guidelines proposed by Bustin et al. [6] and Rabenau et al. [7]. To validate the M RT-PCR as a quantitative RT-PCR for the determination of influenza A viral load, we prepared

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Viral target</th>
<th>PCR product size</th>
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<tbody>
<tr>
<td>M/Fw</td>
<td>Viral M gene</td>
<td>202 bp</td>
</tr>
<tr>
<td>M/Rv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M probe+</td>
<td></td>
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</tbody>
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10-fold dilution series of the calibrated synthetic RNA transcript. Six dilutions (10–10⁶ copies/5 μL of synthetic RNA transcript) were tested in duplicate within the same sample run and compared. Standard curves were produced with these six standard points.

**RNaseP real time RT-PCR and Influenza Ct correction**
The ribonuclease P (RNaseP) RT-PCR was an in-house multiplex method allowing the detection of rhinovirus and RNaseP gene, which is a human cellular gene. Primers and probe for RNaseP detection are available at the CDC (cdcinfo@cdc.gov). The detection of this gene was used both as an internal control and to check for the presence of human nucleic acids and indicates the presence of a sufficient amount of cells in the sample. RNaseP Ct values were all determined using the same fluorescence threshold (0.02 dRn). They were used to correct influenza A viral load for each sample, calculating a Ct value modified according to the ratio of sample RNaseP and mean RNaseP Ct values ([sample influenza A Ct value (M RT-PCR) x sample RNaseP Ct value]/mean RNaseP Ct value)).

**Statistical analysis**
Student’s t-test and Pearson chi square-test were used to assess intergroup differences. Statistical analyses were performed on EpiInfo software (V 3.5.1 CDC). A test was considered as significant when p-value was below 0.05.

**Results**

**Influenza A M gene RT-PCR assay performance**
Data on M RT-PCR performance were calculated from 28 assays (Table 2). The M RT-PCR quantification using 10-fold dilution series of the calibrated synthetic RNA transcript showed a strong correlation between viral load of the sample and Ct value (mean $R^2 = 0.99 \pm 0.01$). PCR reaction efficiency was between 86.6% and 98.6%. The detection limit was between 10¹ and 10² copies of synthetic RNA transcript. The lowest concentrations of the RNA transcript (10¹, 10² and 10³ copies/5 μL) were individually tested 20 times. The probability of detecting 10³ and 10² copies was 100%, compared with 35% for a concentration of ten copies. According to the RNA purification protocol used, a limit of detection between 10 and 10² copies/5 μL ensures detection of 700–7000 copies/mL of initial sample (Table 3).

The repeatability of the M RT-PCR, tested on six positive samples with a mean Ct value of 28.18 and three low positives samples (mean Ct value = 37.11), showed a standard deviation of Ct values of 0.07 ± 0.04 for positive samples and 0.79 ± 0.19 for low positive samples (Table 4).

For reproducibility, two positive samples with a mean Ct value of 29.92 and one low positive sample (Ct value = 36.43) tested three times within three different assays performed on a 3-days period, showed a standard deviation of Ct values at fluorescence threshold 0.2 dRn of 0.16 ± 0.02 for positive samples and 0.39 for the low positive sample (Table 4).

**Ct normalisation according to the quality of the samples**
The mean Ct value for influenza A was 26.4 ± 5.2 [16.4–39.9]. This is the same Ct value as the 10⁵ copies/5 μL RNA transcript standard point, corresponding approximately to a viral load of 6.84 log/mL of sample in viral transport medium. For RNaseP, the mean Ct value was 24.7 ± 1.9 [19.9–31.8]. Among the specimens tested, 52% had RNaseP Ct values below 25%, 21% between 25 and 27, and 9.6% over 27. This last group with high RNaseP Ct values represents swabs containing very few cells, as confirmed by immunofluorescence assays (data not shown). These correspond to virologically poor quality samples. According to the detection limit of our technique, 20% of samples with influenza A Ct values >35 had RNaseP Ct values >27, corresponding to poor quality specimens.

**TABLE 2. M gene RT-PCR performance**

<table>
<thead>
<tr>
<th>M gene RT-PCR performance</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Number of replicates</td>
<td>28</td>
</tr>
<tr>
<td>Slope (mean ± SD)</td>
<td>–3.359 ± 0.13</td>
</tr>
<tr>
<td>Coefficient of determination ($R^2$)</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>91.86 ± 3.44</td>
</tr>
</tbody>
</table>

**TABLE 3. M gene RT-PCR detection limit**

<table>
<thead>
<tr>
<th>No. of positive tests out of 20 tests (%)</th>
<th>Mean Ct ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 copies/5 μL</td>
<td>33.35 ± 0.40</td>
</tr>
<tr>
<td>100 copies/5 μL</td>
<td>36.70 ± 1.36</td>
</tr>
<tr>
<td>10 copies/5 μL</td>
<td>39.30 ± 1.09</td>
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**TABLE 4. M gene RT-PCR repeatability and reproducibility**

<table>
<thead>
<tr>
<th>No. of samples tested</th>
<th>Mean Ct ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>28.18 ± 0.07</td>
</tr>
<tr>
<td>Low positive</td>
<td>37.11 ± 0.79</td>
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<tr>
<td>Reproducibility</td>
<td></td>
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<tr>
<td>Positive</td>
<td>29.92 ± 0.16</td>
</tr>
<tr>
<td>Low positive</td>
<td>36.43 ± 0.39</td>
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</table>
Influenza A Ct values were normalized according to RNaseP Ct values in order to adjust the quantity of virus detected for the quality of the samples. This correction resulted in an increase of influenza Ct up to 6.5 for low quality samples (with high RNaseP Ct values) or a decrease of influenza Ct up to 8.4 for high quality samples (with low RNaseP Ct values). After correction, the influenza A mean Ct value was 26.3 ± 3 [16.7–43.4] and 31% of the samples had corrected influenza A Ct values below 23, 25% between 23 and 29, and 44% over 29, this last group corresponding to low viral loads.

**Ct value according to sampling time**
Corrected influenza A Ct values were analysed according to the period between the onset of the disease and sampling time. The mean value was 25.7 for a period <1 day, 27.1 between 2 and 3 days, and 28.7 over 3 days (differences were significant, p = 0.024). Most of the samples in this study were taken no more than 3 days after disease onset (Table 5); 26% were taken after this. Among this last group of late samples, 39% had high corrected influenza A Ct values >29. Nevertheless 46% of samples taken <1 day after disease onset also presented high influenza A Ct values, probably because of poor quality samples.

**Ct value according to clinical severity**
Corrected influenza A Ct values were analysed according to specific clinical features of influenza disease and to patient medical history (Table 6). Viral load, as estimated by corrected Ct values, never appeared to correlate with clinical history or specific clinical symptoms. However, we observed a trend for higher viral load in patients presenting with marked signs of upper respiratory infections, not related to severe cases.

**Discussion**
Here we report a real time RT-PCR protocol developed 2 years ago for research applications. This one-step RT-PCR targeting the matrix gene is able to detect all subtypes of influenza A viruses including A(H1N1)2009. This M RT-PCR is rapid (<1.5 h), specific and sensitive, with a limit of detection between 10 and 100 copies/reaction. Viral loads of 700 copies/mL of sample were frequently detected; this value is in accordance with other reports of detection limits between 570 and 900 copies/mL [8,9]. For the detection of A(H1N1)2009, this M RT-PCR was associated with a specific RT-PCR targeting the haemagglutinin gene. This strategy is pertinent in the context of a pandemic as the new virus may drift, mainly due to mutation in haemagglutinin and/or neuraminidase genes, but variants will still be detected by this universal M RT-PCR.

The relationship between viral quantification and clinical severity in viral respiratory diseases is an important issue. It has previously been reported for seasonal influenza that patients hospitalized with severe diseases have more active and prolonged viral replication [10]. Recent reports described a median of A(H1N1)2009 viral load of 4.6 log RNA copies/mL in respiratory samples [9]. The value reported here is higher (6.84 log RNA copies/mL), probably because it was measured in a paediatric population comprising mostly (91) non-hospitalized children presenting with mild influenza symptoms. However, a recent study reported even higher viral loads, reaching 8.26 log/mL [8]. In addition to developing a reliable quantification tool such as the one described here, the main difficulty in viral load measurement is that the usual nasal swab sample is often of poor quality. In this study, 10% of the samples received were associated with high RNaseP Ct value (>27). This reflects poor quality specimens with very few cells, as confirmed by immunofluorescence assays. The impact of the quality of the sample on influenza quantification may be very high as, for poor quality samples (RNaseP Ct > 27), the correction of influenza Ct is over 3 Ct (1 log) and may be up to 6–8 Ct (2–3 log). In order to attempt to establish influenza viral load for disease management or clinical severity correlation, it is very important to assess the quality of the sample and to correct viral load accordingly.
In addition to poor quality samples, we showed that sample delay resulted in an increase of Ct values. This has also been recently reported by To et al. [8] who described a 1 log decrease for samples taken 3 days after disease onset.

Finally, even though our study failed to correlate any specific clinical features with influenza viral load, probably because the cases were mostly non-hospitalized children with mild symptoms, we consider that this normalized technique is of interest for disease management. Our results provide a standard reference viral load method that can be used in similar studies analysing severe cases.

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Transparency Declaration

The authors declare no conflict of interest of any nature.

References