Quantification of the Influenza Virus Load by Real-Time Polymerase Chain Reaction in Nasopharyngeal Swabs of Patients Treated with Oseltamivir

Guy Boivin,1 Zoé Coulombe,1 and Cynthia Wat2
1Research Center in Infectious Diseases of CHUQ-CHUL and Laval University, Québec City, Quebec, Canada; 2Roche Global Development, Welwyn, United Kingdom

Influenza A virus load was assessed by real-time polymerase chain reaction (PCR) in nasopharyngeal swabs of infected patients treated with oseltamivir. The mean pretreatment virus load was significantly lower in the 24 patients (group A) who initiated treatment within 24 h of the onset of symptoms than it was in the 26 patients (group B) who initiated treatment between 24 and 48 h (1.6 × 10^8 vs. 8.4 × 10^7 copies/600 ng of total RNA, P = .04); after 48 h of treatment, twice as many patients in group B still had a positive PCR result, compared with patients in group A (42.3% vs. 20.7%). These virological results support the clinical benefit provided by early therapeutic intervention of influenza illness.

The recent introduction of 2 neuraminidase inhibitors (NAIs), zanamivir (Relenza; GlaxoSmithKline) and oseltamivir (Tamiflu; Hoffmann LaRoche), has facilitated the treatment of influenza A and B viral infections. Studies have shown that NAIs can decrease the median duration of symptoms by A and B viral infections. Studies have shown that NAIs can decrease the median duration of symptoms by 1–2 days when administered within the first 48 h of the onset of symptoms [1–5]. For both zanamivir and oseltamivir, it has been reported that early therapeutic intervention maximizes the clinical benefit, by shortening the duration of influenza illness [1, 6]. However, no study has assessed, by the use of sensitive and quantitative laboratory methods, the kinetics of the influenza virus load in respiratory tract samples of infected individuals receiving early treatment with NAIs versus those receiving deferred treatment. In the present study, we used a real-time polymerase chain reaction (PCR) assay to evaluate the kinetics of the influenza A virus load in samples from patients treated with oseltamivir.

Patients, materials, and methods. Nasal and pharyngeal swabs (combined in the same viral transportation medium [VTM]) (M4-RT; Micro Test) were collected from individuals enrolled in an oseltamivir-treatment study (protocol M76006) during winter of 1999–2000 in the Northern Hemisphere [6]. Informed consent was obtained from all participants at each study site. The trial included ambulatory patients (13–70 years) who sought care within 48 h of the onset of a flu syndrome, which consisted of fever (temperature ≥37.8°C) plus ≥2 of the following symptoms: cough, sore throat, coryza, myalgia, headache, fatigue, and chills/sweats. The total-symptom score at baseline was calculated by recording the presence and severity of all flu symptoms on a 4-point scale [6, 7]. All patients received 75 mg of oseltamivir twice a day for 5 days, with swabs collected before onset of treatment (day 1) and after 48 h of treatment with oseltamivir (day 3). VTM aliquots obtained from a nonselected subset of consecutive patients enrolled in Canada were frozen at −80°C, for subsequent PCR analysis of the influenza A virus load (H3N2 subtype).

Two plasmids were constructed and then were transcribed by use of the RNA transcription kit (Stratagene), for use in reverse transcription (RT)–PCR assay. The external standard (used to construct the standard curve of the assay) consisted of a 591-bp fragment of the influenza A:H3 gene cloned in the pCR 2.1 vector (Invitrogen). The internal standard (used to verify the presence of PCR-inhibitory substances) consisted of a 366-bp fragment of the pT713 Vector (Gibco BRL) flanked by influenza A:H3 sequences at both ends and cloned in the pCR 2.1 vector of the TA Cloning Kit (Invitrogen). Total RNA was extracted from 140 μL of VTM by use of the QIAamp Viral RNA mini kit (Qiagen) and was eluted in 40 μL of AVE buffer plus 1 μL of RNasin (Promega). Total RNA from each sample was quantified by spectrophotometry and then was diluted in diethylpyrocarbonate-treated water, to achieve a final concentration of 600 ng/10 μL. The RT step was performed by use of the SuperScript II RNase H– Reverse Transcriptase kit (Invitrogen), according to the manufacturer’s recommendation, with 10 μL (600 ng) of either sample RNA or external standard dilutions, 10 μL (500 copies) of the internal standard, and 2 μL (50 ng/μL) of random hexamer primers (Pharmacia Biotech).

Real-time PCR was performed in a volume of 20 μL by use of the LC DNA Master Hybridization Probes Kit (Roche Diagnostics) in the LightCycler instrument. Each reaction included 2.5 μL of the RT product, 2 μL of the LC DNA Master Hybrid-
Figure 1. Plots of influenza virus load for patients who initiated oseltamivir treatment within 24 h (group A) and between 24 and 48 h (group B) of the onset of symptoms. A, Virus loads at baseline (day 1). B, Virus loads after 48 h of treatment (day 3). The bars represent the median virus load for each group of patients (0 for both groups of patients in B).

Results. By testing serial dilutions of the influenza A:H3 external standard in a background of control RNA extracted from throat swabs, placed in a VTM, the sensitivity of the quantitative RT-PCR assay was estimated at 1000 copies/600 ng of input RNA, with a dynamic range encompassing 4 log₁₀ (10⁻⁴–10⁷ copies of influenza A:H3). Within that dynamic range, the means of intra-assay and interassay coefficients of variability were 9.7% and 20.3%, respectively.

The influenza A:H3 virus load was assessed at baseline (day 1) and after 48 h (day 3) of treatment with oseltamivir, for a subset of patients with confirmed influenza A:H3N2–positive cultures who began treatment within 24 h (group A; n = 24) and 24–48 h (group B; n = 26) of the onset of symptoms. Samples were tested at the end of the study with consecutive specimens from the same patients analyzed in the same PCR run. At baseline (figure 1A), the mean virus load for patients in group A (1.6 × 10⁵ copies/600 ng of total RNA) was significantly lower than that for patients in group B (8.4 × 10⁵ copies/600 ng of total RNA) (P = .038, Student’s t test). After 48 h of treatment, twice as many patients in group B still had a positive RT-PCR test for influenza, compared with patients in group A (11 of 26 [42%] in group B vs. 5 of 24 [21%] in group A).

On day 3 (figure 1B), the mean virus load in group A patients was also significantly lower than that in patients in group B (0.38 × 10⁴ and 2.2 × 10⁴ copies, respectively) (P = .044). The decrease in virus load (day 1–day 3) was more pronounced in patients in group B than in patients in group A (8.1 × 10³ vs. 1.6 × 10⁵ copies, P = .042). Overall, after 48 h of treatment, the decline in mean virus load was 4.4 × 10⁻² copies, with 34 (68%) of 50 patients having a negative PCR result at this time. The mean total-symptom score at baseline was similar—14.8 for patients in group A and 15.0 for patients in group B.

Discussion. It has been shown that influenza-virus titers significantly correlate with fever and systemic symptoms and that such titers peak rapidly (i.e., at ~48 h after inoculation in experimental human influenza viral infection) [9]. Viral replication declines somewhat slowly thereafter, with an insignificant amount of viral shedding occurring after 6–8 days [10].
In a clinical study of zanamivir, we previously showed that, in placebo recipients, peak levels of influenza titers were reached within 48–60 h of the onset of symptoms, a finding also suggesting that treatment of influenza should be initiated early, to gain maximal clinical benefit [11]. The benefit of early intervention was seen in a phase-2 clinical trial in which patients who initiated zanamivir within 30 h experienced greater clinical benefits than those who initiated treatment at later times [1], and this finding was confirmed in a large clinical trial of oseltamivir, in which the total duration of illness could be halved if patients were treated early instead of at 48 h [6]. In that instance, for every 6 h earlier that oseltamivir was administered, the predicted total duration of illness was shortened by 8%, corresponding to a duration of illness that is ∼10 h shorter [6].

In the present study, we developed a rapid, quantitative real-time PCR assay to more precisely evaluate the early viral-replication kinetics present while patients receive treatment with oseltamivir. We were able to demonstrate the important decline in influenza virus load (mean, copies, for all patients) after only 48 h of treatment, resulting in no detectable influenza RNA (limit of detection, 1000 copies/assay) in the nasopharynx of 34 (68%) of 50 patients. Although many groups have reported the superior sensitivity of RT-PCR for detection of influenza, compared with conventional culture, [8, 12, 13], as well as the longer persistence of viral RNA in the respiratory tract, compared with that of cultivable virus [14], there is a paucity of data regarding the use of quantitative PCR for influenza infection. Thus, additional longitudinal studies that include placebo recipients are needed to address the duration of viral shedding and the time of peak virus load, when such molecular assays are used.

Nevertheless, the main finding of our study concerns the more-rapid clearance of the virus load associated with earlier treatment with oseltamivir. Indeed, when the drug was initiated after 24 h, compared with when it was initiated within the first 24 h of the onset of symptoms, twice as many patients (11 of 26 [42%] vs. 5 of 24 [21%]) still had a positive PCR at 48 h after initiation of treatment. In other words, only 58% of patients in group B had a negative PCR result at 48 h, compared with 79% of patients in group A. Our data suggest that the more-rapid viral clearance in the early treatment group is due to a lower virus load at baseline for these patients, compared with that for the patients who received deferred treatment. It is noteworthy that our 2 groups of patients had symptoms of similar intensity at baseline (with the total-symptom score index used to evaluate clinical efficacy of NAIs), a finding eliminating the bias of having selected less-sick patients at earlier time. This finding is in agreement with that of a larger study [6], which indicates that influenza presents with a typical, sudden, and severe symptom onset, even in patients who seek early care.

In summary, our virological data are in agreement with a more-rapid resolution of influenza symptoms associated with early antiviral treatment, a more-rapid resolution due to a more-rapid viral clearance. However, before the influenza virus load can be used as a surrogate marker of either disease severity or antiviral activity, larger studies that include both untreated and treated patients and that have more frequent evaluations are required. Furthermore, other factors, such as levels of cytokines, should be evaluated in parallel to the virus load, for their potential to predict clinical outcome [9].

References