A Downward Trend of the Ratio of Influenza RNA Copy Number to Infectious Viral Titer in Hospitalized Influenza A-Infected Patients

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Background. Efficacy endpoints in influenza clinical trials may include clinical symptoms and virological measurements, although virology cannot serve as the primary endpoint. We investigated the relationship between influenza A RNA copy number and quantity of infectious viruses in hospitalized influenza patients.

Methods. One hundred fifty influenza-infected, hospitalized patients were included in this prospective cohort study spanning the 2012–2013 influenza season. Daily nasopharyngeal samples were collected during hospitalization, and influenza A RNA copy number and infectious viral titer were monitored.

Results. The decay rate for 50% tissue culture infectious dose (TCID50) was 0.51 ± 0.14 log10 TCID50/mL per day, whereas the RNA copy number decreased at a rate of 0.41 ± 0.04 log10 copies/mL per day (n = 433). The log ratio of the RNA copy number to the infectious viral titer within patient changes significantly with −0.25 ± 0.09 units per day (P = .0069). For a 12-day observation period, the decay corresponds to a decline of this ratio of 3 log influenza RNA copies.

Conclusions. Influenza RNA copy number in nasal swabs is co-linear with culture, although the rate of decay of cell culture-based viral titers was faster than that observed with molecular methods. The study documented a clear decreasing log ratio of the RNA copy number to the infectious viral titer of the patients over time.

Keywords. infectious viral titer; influenza A TCID50; influenza A viral load; influenza RNA copy number; nasopharyngeal swabs.

Although influenza frequently results in a self-limited respiratory illness, in most cases it can cause severe complications leading to hospitalization and death, especially in high-risk groups including elderly patients, immuno-compromised persons, pregnant women, very young children, and persons with underlying medical conditions [1]. Worldwide, the annual influenza epidemics are estimated to result in approximately 3 to 5 million cases of severe illness and approximately 250 000 to 500 000 deaths [2]. Among hospitalized patients, antiviral therapy is most efficacious if started within 48 hours of influenza illness onset [1, 3–5]. Nonetheless, some studies have demonstrated that antiviral treatment is still beneficial in hospitalized patients when started 4 and 5 days after illness onset [6–12]. Current available antiviral treatments include the neuraminidase inhibitors (NAIs) inhaled zanamivir, oral oseltamivir, and intravenous peramivir) and M2 ion channel blockers (amantadine and rimantadine). However, all currently circulating strains of influenza are adamantane-resistant, and therefore this class is not recommended for the prevention or treatment of influenza [13]. The NAI peramivir is given intravenously, and so it offers potential advantages in the management of severe hospitalized influenza. Indeed, NAI peramivir was used to
treat critically ill patients during the 2009 pandemic under emergency use authorization by the Food and Drug Administration (FDA) [14, 15] and, it received FDA approval to treat acute uncomplicated influenza patients [16–18].

Until now, little or no resistance to zanamivir has been observed [13], and only a small percentage of A(H1N1)pdm09 viruses had highly reduced peramivir inhibition [19]. Although oseltamivir resistance was found in virtually all former seasonal A(H1N1) viruses, the frequency of oseltamivir resistance in A(H1N1)pdm09 viruses has remained low on a global scale. However, with the appearance of permissive mutations in A(H1N1)pdm09 viruses, the risk that oseltamivir-resistant viruses may spread globally is increasing [13]. With the appearance of permissive mutations in A(H1N1)pdm09 viruses, the risk that oseltamivir-resistant or even multidrug-resistant viruses may spread globally is increasing [13, 20], and there is a need for new antivirals with other mechanisms of action.

One of the key challenges in designing influenza clinical trials is to define relevant efficacy endpoints. Although such efficacy endpoints are well established for acute uncomplicated influenza, there is a lack of consensus on the optimal endpoint to use for individuals hospitalized with influenza. Several antiviral drugs have been studied in hospitalized influenza patients, but none has clearly demonstrated to be universally effective in predicting how patients feel, function, and recover from the infection [6, 9, 11, 16, 17]. Proposed primary clinical endpoints in clinical trials for hospitalized influenza patients could include symptoms (e.g., fever, cough, sore throat), duration of hospitalization, time to normalization of vital signs and oxygenation, requirements for supplemental oxygen, need for admission to the intensive care unit (ICU), or assisted ventilation and mortality [21, 22]. Although antiviral drugs would be predicted to reduce viral shedding, and several studies have correlated viral load (VL) reduction with changes in chemokines and cytokines as well as clinical symptoms, the regulatory agencies do not permit virology to be a primary endpoint. Virological measurements include detection and quantification of shed virus by viral culture or quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Virologic endpoints that are typically assessed, as secondary endpoints, in clinical studies of novel antivirals include baseline viral RNA copy number or infectious viral titer during the baseline visit and change in viral RNA copy number or titer over the course of illness [16–18, 21]. Quantitation of viral RNA by molecular techniques is a standard and sensitive method for VL determination. Cell-based assays are more labor intensive but have the advantage of detecting the infectious virus particles. Regulators preclude virology from being a primary endpoint because they feel that there is insufficient correlation between viral titers and change in titers with clinical symptoms. Furthermore, there is significant variability in viral shedding from patient to patient. Lastly, there is a lack of standardization in the collection of samples and location (e.g., upper vs lower respiratory tract) and assays for virologic measurements [21].

In this study, we investigated the relationship between influenza A RNA copy numbers and infectious viral titer in laboratory-confirmed influenza A patients during hospitalization with the hope that this virological data will inform future study endpoints.

**MATERIALS AND METHODS**

**Study Design**

This is a prospective cohort study of laboratory-confirmed hospitalized and/or ICU adult influenza patients during the 2012–2013 influenza season. Five study centers were included: Northwestern University (Chicago, IL), Mount Sinai Hospital (Toronto, ON, Canada), North York General Hospital (Toronto, ON, Canada), Royal Adelaide Hospital (Adelaide, Australia), and Westmead Hospital (Westmead, Australia). Written informed consent was obtained from all patients. Hospitalized patients with any of the following conditions were screened: influenza-like illness (ILI), suspected respiratory infection (pneumonia and other respiratory infection), acute respiratory failure (e.g., chronic obstructive pulmonary disease exacerbation, asthma exacerbation), or fever. Patients with laboratory-confirmed influenza (using the study centers’ in-house RT-qPCR) were eligible for this study. Influenza-positive patients underwent daily nasopharyngeal (NP) sampling starting from enrollment (baseline = day 1 of enrollment) to day 7 (or until discharge or death). Hospitalized patients or patients in the ICU who developed ILI during their hospitalization, or patients who met other criteria for entry into the study, were screened with PCR. If these patients were positive for infection and consented to participate in the study, they were observed for 7 days or until discharge. For ICU patients, if still hospitalized, every-other-day sampling continued in the second week until day 15 or hospital discharge.

**Processing of Collected Nasopharyngeal Swabs**

Nasopharyngeal swabs were collected in 3 mL universal transport medium (UTM, Copan), which were divided across multiple aliquots. If aliquots could not be prepared immediately, specimens were kept refrigerated at 4°C for up to 72 hours. Specimens that could not be processed within 48 to 72 hours were frozen at −80°C, according to the manufacturer’s instructions. The collection vial containing the swab and the transport medium were vigorously agitated on a vortex mixer. The fluid was released by grabbing the end of the swab-stick and squeezing the tip against the inner wall of the vial or tube. After centrifuging at 1000 rpm for 5 minutes, the supernatant was aliquoted and stored at −80°C. The samples were sent to 1 location where all the subsequent analyses were performed.

In addition, the baseline samples were also analyzed with the FilmArray Respiratory Panel (BioFire Diagnostics), according
to the manufacturer’s protocol. The Respiratory Panel IVD version 1.6 (Northern hemisphere) and version 1.7 (Southern hemisphere) pouches were used.

**RNA Copy Number of the Influenza Viruses**

Nucleic acids were extracted from 100 µL NP sample using the EasyMAG extraction platform (BioMérieux). An internal extraction control (IEC) was added to all samples before the RNA extraction, as described previously [23, 24]. The RNA was eluted in 100 µL and stored at −80°C. Real-time PCR was performed according to the Centers for Disease Control and Prevention (CDC) protocol for influenza A virus (targeting the Matrix gene) with a panel of oligonucleotide primers and dually labeled hydrolysis (TaqMan) probes [25], as previously described [24, 26]. Amplification and detection were performed on a LightCycler 480 instrument (Roche Applied Science). Each sample was tested in duplicate. All quantification cycle values were corrected for the loss of RNA during extraction by use of the IEC [23]. A standard RNA dilution series (external quantification control [EQC]) was tested in duplicate in each RT-qPCR experiment [24]. The VLs were calculated as the \( \log_{10} \) copies/mL using the EQC standard curve, which has a linear range from 4.3 to 10.3 \( \log_{10} \) copies/mL (lower limit of quantification [LLOQ] and upper limit of quantification). Samples with an influenza VL below LLOQ, but with a detectable amount of influenza RNA, were extrapolated. The lowest extrapolated quantity that could be measured was 3.25 \( \log_{10} \) copies/mL. For quantification of influenza B viruses primers, targeting the NEP/NS1 gene (as described by the CDC), were used (InfB-FW: 5′-TCCTCAAYTCTACTTGCAGCG-3′; infB-RV: 5′-CGGTGCTCTTGACCAGATTG3′; InfB-probe: FAM-5′-CAATGCAGCAGCTGAAGACTGCGGTG-BHQ-1-3′ [Biotechnology]). The RNA extraction and PCR conditions were identical to the in

**50% Tissue Culture Infectious Dose Assay for Influenza A Viruses**

To measure the infectivity of the influenza viruses, 50% tissue culture infectious dose (TCID\(_{50}\)) assay on Madin-Darby canine kidney (MDCK) epithelial cells (obtained from ATCC) was performed. Growth medium included 1000 µL UltraMDCK serum-free medium (VWR), 10 mL L-glutamine (200 mM; Life Technologies), and 400 µL gentamicin (50 mg/mL; Life Technologies). Infection medium consisted of the growth medium supplemented with 80 µL 2.5% trypsin (Life Technologies) and 10 mL fungizone (amphotericin B 250 µg/mL; Life Technologies). Each sample was tested in 8 replicates and 11 dilutions (1 sample/96-well plate). The MDCK cells were harvested and counted with the Vi-CELL (Beckman Coulter), according to the manufacturer’s protocol. The MDCK cells were seeded (on day −1) at a concentration of 10 000 cells/100 µL growth medium per well in a 96-well plate (column 1–12). Plates were incubated overnight at 37°C in 5% CO\(_2\). On day 0, serial dilutions of the viruses (1:10 for column 1 and subsequently 1:4 dilutions for column 2–11) were made in infection medium. The growth medium was removed from the MDCK cells, and 100 µL virus dilutions were added to the MDCK cells (column 1–11). Only infection medium was added to column 12 (negative control column). The A/Aichi/2/1968 (H3N2) influenza strain (cultured in MDCK cells) was used as a positive control in every experiment (in 7 replicates). The empty wells (row H) on the control plate were filled with a dilution series (1:10 for column 1 and subsequently 1:4 dilutions for column 2–11) of UTM (negative control). Plates were incubated for 6 days at 37°C in 5% CO\(_2\). Subsequently, the cytopathogenic effect was scored with visual interpretation for the Northern hemisphere samples. A chemiluminescent readout (ATPlite 1Step kit; PerkinElmer) using the Viewlux equipment (PerkinElmer) was used for the Southern hemisphere samples. This kit is a high-sensitivity ATP-monitoring one-step addition assay kit for the quantification of viable cells. The wells were scored positive or negative for infection according to a cutoff of 3.490 \( \log_{10} \) relative luminescence units, defined by receiver operating characteristic analysis of positive control plates. The \( \log_{10} \) TCID\(_{50}/\)mL value was calculated in a template based upon the Reed-Muench method [27].

To determine the amount of infectious influenza A virus during hospitalization, TCID\(_{50}\) values were measured on the baseline and follow-up NP samples. Sixty-five influenza B samples (14 subjects [based on Filmarray and RT-qPCR data]) were excluded from further analysis. Although the antifungal fungizone was already added to the infection medium (end concentration, 2.50 µg/mL), 8 samples (2 subjects) still had fungi contamination and were therefore invalid.

**Statistical Analysis**

Linear trends of \( \log_{10} \) (VL), \( \log_{10} \) (TCID\(_{50}\)), and \( \log_{10} \) (TCID\(_{50}/\)VL) were calculated by way of a mixed-effect Tobit regression analysis (adapted from Thiebt and Jacqmin-Gadda [28]) using SAS/STAT software’s NLMIXED procedure (version 9.2). Applying a Tobit regression enabled accounting for left-censored TCID\(_{50}\) and VL values, each with their respective limit of detections (LODs). The mixed-effect analysis allowed for the patients to randomly affect both the trend slopes and intercepts. Pearson’s product-moment correlation coefficient and the sample means were estimated using a left-censored bivariate normal likelihood-based approach as proposed by Lyles et al [29]. Figures were generated using R (version 3.0.2). Confidence bands for linear trend were calculated by sampling the bivariate normal distribution and were based on the regression coefficients for intercept and slope as well as their covariance matrix (n = 5000).

Relative Infectivity of Influenza Viruses • OFID • 3
RESULTS

Collection of Influenza Samples From Northern and Southern Hemispheres

This study involved 3 study centers from the Northern hemisphere (Canada and United States) and 2 centers from the Southern hemisphere (Australia). A total of 150 patients, with laboratory-confirmed influenza (per the study centers’ in-house RT-qPCR), were included (Table 1). Follow-up samples were collected, and influenza RNA copy number and TCID<sub>50</sub> values were determined. Coinfection of influenza with another respiratory virus (parainfluenza type 4, coronavirus, bocavirus, rhinovirus/enterovirus) was detected in only 4% of the samples. Only influenza A-positive samples were included in analyses.

Monitoring Daily Influenza A RNA Copy Number and Infectious Viral Titer

A subset of 433 samples with paired influenza AVL and TCID<sub>50</sub> data was available and further analyzed (Figure 1). To avoid the upward bias caused by excluding data below the LOD (ie, “left-censored” data), statistics were calculated using maximum likelihood estimation. This procedure leads to a mean influenza A log<sub>10</sub> VL of 5.61 log<sub>10</sub> copies/mL (95% confidence interval [CI], 5.28–5.93) for the baseline samples (n = 134, 17 of which were left-censored) and of 4.70 log<sub>10</sub> copies/mL (95% CI, 4.50–4.90) for all samples (n = 433, 112 of which were left-censored). The highest observed VL was 9.82 log<sub>10</sub> copies/mL. The average (±standard error) number of days until the VL reached the detection limit (when >0) was 7.58 ± 0.35 days since enrollment.

Of the 433 samples, 325 were left-censored for TCID<sub>50</sub> with a detection limit of 2.2 log<sub>10</sub> TCID<sub>50</sub>/mL (range up to 7.5 log<sub>10</sub> TCID<sub>50</sub>/mL) (Figure 1). The mean log<sub>10</sub> TCID<sub>50</sub> for baseline samples was 1.80 (95% CI, 1.25–2.35; n = 134, 78 of which were left-censored) and for all samples was 0.70 log<sub>10</sub> TCID<sub>50</sub>/mL (95% CI, 0.30–1.10). The average (±standard error) number of days until TCID<sub>50</sub>/mL reached the detection limit (when >0) was 3.49 ± 0.38 days since enrollment. The correlation between log<sub>10</sub> VL and log<sub>10</sub> TCID<sub>50</sub> was estimated to be 0.87 (95% CI, .84–.90). Although for 106 samples infectious viral particles and viral RNA were both detected, variable levels of viral RNA in the absence of cultivable virus was obtained for 215 samples (Figure 1).

Influenza A RNA Copy Number and Infectious Viral Titer Trends

In our study, VL decreased significantly at a rate of 0.41 ± 0.04 log<sub>10</sub> copies per mL per day (P < .0001) (Figure 2A). In other words, a patient’s influenza A VL on average underwent a 10-fold drop every 2.5 days. The estimated decay rate for TCID<sub>50</sub> was 0.51 ± 0.14 log<sub>10</sub> TCID<sub>50</sub> per mL per day (P = .0004), corresponding with a 10-fold drop only every ± 2 days (Figure 2B).

The relative infectivity, here defined as the log ratio of TCID<sub>50</sub>/VL within the same patient, changed significantly with −0.25 ± 0.09 units per day (P = .0069) (Figure 2C). Because the ratio is negative, this means that for an average patient, the TCID<sub>50</sub> decays faster than the VL. For a 12-day observation period, the decay corresponds to a decline in relative infectivity of ±3 log<sub>10</sub> viral RNA copies. Thus, the data document a significant decline of the relative infectivity of influenza A virus particles. It is important to note that this result is observed irrespective of the investigational site, subtype, or the presence of a coinfection.

DISCUSSION

This is one of the largest multicenter studies to define the epidemiology of influenza in hospitalized patients. The study enrolled 150 influenza-infected, hospitalized patients during the

Table 1. Collection of Influenza Samples From Northern and Southern Hemispheres

| Study Center | Baseline Samples (n) | Follow-Up Samples (n) | Total (n) |
|--------------|----------------------|-----------------------|-----------|
| Northern Hemisphere | | | |
| Site 101 | 15 | 42 | 57 |
| Site 201 | 50 | 93 | 143 |
| Site 202 | 59 | 138 | 197 |
| Southern Hemisphere | | | |
| Site 301 | 22 | 79 | 101 |
| Site 302 | 4 | 4 | 8 |
| Total | 150 | 356 | 506 |

Figure 1. Influenza A (InfA) viral load (VL) versus 50% tissue culture infectious dose (TCID<sub>50</sub>) for 433 samples. Dashed lines represent the lower limit of detection for the VL assay (3.25 log<sub>10</sub> copies per mL) and the TCID<sub>50</sub> assay (2.2 log<sub>10</sub> TCID<sub>50</sub>/mL). Solid lines represent the maximum likelihood means (see text). The superposed rug plot extruding from both axes represent the results that fell below these limits. The insert table represents the amount of samples in the different categories.
2012–2013 influenza season. Serial assessment of viral shedding was studied with both molecular and cell culture methods. These 2 methods were highly co-linear, although the rate of decay of cell culture-based infectious viral titer was faster than that observed with molecular methods. This study documented a clear decreasing ratio of influenza RNA copy number to infectious viral titer of the patients over time.

Clinical diagnosis has limitations because ILI symptoms are not specific for influenza virus infection and can be caused by other respiratory pathogens. Influenza VL decay, which quantifies infectious and noninfectious viral particles over time, and TCID$_{50}$ decay, which quantifies only the infectious viral particles over time, could be used as virological (secondary) measurements [16–18, 30, 31]. In this study, paired influenza A VL decay and TCID$_{50}$ decay data points were available for 433 samples. The VL decay of $0.41 \pm 0.04$ log$_{10}$ copies per mL per day in this study is more pronounced but comparable with the respiratory tract A(H1N1)pdm09 decrease rates of $0.31$ log$_{10}$ VL units per day as reported by Lu et al [32]. Likewise, the TCID$_{50}$ decay ($-0.51$ TCID$_{50}$ [mL·day]$^{-1}$) was comparable with the median decay rate of $-0.39$ TCID$_{50}$ (mL·day)$^{-1}$ (corresponding to a 10-fold drop every 2.6 days over a period of 4.5 days) reported by de Jong et al [16]. The mechanism of prolonged viral shedding, ie, the detection of influenza viral RNA for a longer time period, has been described in many studies [30, 33, 34]. The clinical (and infection control) relevance of detecting low levels of viral RNA in the absence of cultivable virus can be questioned. In this context, it should be noted that the virus culture methods are less sensitive than the molecular methods and that improper sampling handling could also have an impact. In this study, a high percentage of samples ($n = 215$) have levels of viral RNA in the absence of cultivable virus in the NP swabs despite proper sampling handling. It is important to include the percentage of infectious viral particles in virological measurements used in clinical studies of antiviral agents.

Unlike assessment of VLs for human immunodeficiency virus and hepatitis, where plasma is used as input sample [35, 36], assessment of influenza VLs utilizes respiratory specimens. There is variability in the titer of virus in upper and lower airway; in addition, there can be significant variability due to sampling methods [26]. To keep variability related to sample collection, processing, and transport as low as possible, well defined collection and shipping instructions were provided to all clinical sites. We have shown that there is a significant decline of the log ratio of TCID$_{50}$/VL over time within the same patient. The absolute changes in VL due to variation in sampling methods could be captured by using this ratio as virological measurement (instead of using only the influenza A VL or TCID$_{50}$).

**CONCLUSIONS**

In conclusion, this epidemiological study contributes to our understanding of viral shedding patterns in influenza-infected hospitalized adult patients. The viral RNA copy numbers and viral infectious titer patterns in those patients demonstrated a downward trend of the log ratio of TCID$_{50}$/VL over time within the same patient. The absolute changes in VL due to variation in sampling methods could be captured by using this ratio as virological measurement (instead of using only the influenza A VL or TCID$_{50}$).

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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