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The Dynamic Relationship Between Clinical Symptomatology and Viral Shedding in Naturally Acquired Seasonal and Pandemic Influenza Virus Infections

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(See the Editorial Commentary by Kwong on pages 438–9.)

Background. Although the pattern of viral shedding over time has been documented in volunteer challenge studies, understanding of the relationship between clinical symptomatology and viral shedding in naturally acquired influenza infections in humans remains limited.

Methods. In a community-based study in Hong Kong from 2008 to 2014, we followed up initially healthy individuals and identified 224 secondary cases of natural influenza virus infection in the household setting. We examined the dynamic relationship between patterns of clinical symptomatology and viral shedding as quantified using reverse transcription polymerase chain reaction and viral culture in 127 cases with a clinical picture of acute respiratory infection.

Results. Viral shedding in influenza A virus infections peaked on the first 1–2 days of clinical illness, and decreased gradually to undetectable levels by day 6–7, matching closely with the dynamics of clinical illness. Viral shedding in influenza B virus infections rose up to 2 days prior to symptom onset and persisted for 6–7 days after onset with a bimodal pattern.

Conclusions. Our results suggest that while clinical illness profiles may serve as a proxy for clinical infectiousness in influenza A virus infections, patients may potentially be infectious even before symptom onset or after clinical improvement in influenza B virus infections.

Keywords. influenza; viral shedding; symptoms.

Because influenza virus infections are associated with significant morbidity and mortality through regular epidemics and occasional pandemics, understanding the dynamics of infectiousness over the course of a typical infection is important to inform appropriate control strategies. The quantity of influenza virus shedding is generally considered a proxy for infectiousness [1–3]. Although the pattern of viral shedding over time has been documented in volunteer challenge studies [4], these experimental infections were mostly induced with high-dose viral challenge via artificial mode of inoculation on healthy adults [5, 6]. Studies on viral shedding pattern in naturally acquired influenza infection can therefore serve a theoretical linking role for assessing whether data from experimental infections can be generalized to the general population in the community setting, but remain limited [7, 8].

Although cases presenting for medical attention are not likely to be representative of all natural infections, because milder infections would tend not to require medical attention, we previously reported preliminary results on patterns in viral shedding and symptoms in confirmed influenza virus infections that were prospectively identified in households in a community-based study in 2008 [7]. Herein we report a more comprehensive analysis of the relationship between clinical symptomatology and viral shedding based on all such cases prospectively identified over a period of 5 years in our household study.

METHODS

Participants

A community-based randomized controlled trial investigating the efficacy of nonpharmaceutical interventions against influenza transmission in households was conducted in Hong Kong in 2008 [9]. An observational study with similar recruitment and follow-up protocols, but no randomly allocated interventions, was conducted in 2009 during the influenza A(H1N1) 2009 pandemic [10], and this protocol was continued in the influenza seasons of 2010–2014. In these studies, participants were recruited as household index cases from primary healthcare providers if they presented with acute respiratory signs or symptoms and were the first member within their household with recent acute respiratory signs or symptoms. Index cases who tested positive for influenza with the QuickVue Influenza A + B rapid diagnostic test (Quidel, San Diego, California) were

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invited to participate in the study. If recruited, the whole household was followed up with 3 home visits that were conducted on the day of recruitment, followed by additional visits 3 and 6 days later. Nose and throat swabs (NTSs) were collected from all household members at every home visit, regardless of the presence or absence of respiratory symptoms, allowing identification of symptomatic and asymptomatic infections. Daily symptom diaries were completed by all household members, and digital thermometers were distributed to each household for standardized recording of daily tympanic temperature. Specific symptoms and signs recorded in the symptom diaries included fever ≥37.8°C, headache, myalgia, cough, sore throat, runny nose, and sputum.

Household members excluding the index case were referred to as household contacts. A secondary case was defined as any household contact who tested negative for influenza virus on the first home visit and tested positive for influenza virus on a subsequent visit by laboratory testing. The analyses in this study will focus on these naturally acquired secondary influenza virus infections. Based on the symptoms recorded in the diaries, acute respiratory illness (ARI) was defined as ≥2 respiratory signs or symptoms from the 7 listed above, and influenza-like illness (ILI) was defined as the presence of fever (≥37.8°C) plus cough and/or sore throat [10].

Laboratory Methods

Detection of influenza A or B virus infection and quantification of viral shedding were done by standard reverse transcription polymerase chain reaction (RT-PCR) testing on collected NTS specimens. Nucleic acid was extracted using the NucliSens easyMAG extraction system (bioMérieux, Boxtel, the Netherlands) according to the manufacturer's instructions. Complementary DNA was prepared using extracted nucleic acid by using an Invitrogen Superscript III kit (Invitrogen) with random primer [11].

For detection of influenza A viruses, complementary DNA was amplified in a LightCycler 2.0 (Roche Diagnostics). The forward primer (5′-CTTCTAACCGAGGTCGAAACG-3′) and the reverse primer (5′-GGCATTTTGGACAAAKCGTCTA-3′) were used for amplification of the matrix gene [12]. At the end of the assay, PCR products were subjected to a melting curve analysis to determine the specificity of the assay. The lower limit of detection of the RT-PCR assay was 23 virus gene copies per reaction (ie, approximately 900 copies/mL).

For detection of influenza B viruses, the forward primer (5′-GCATCTTTTGTTTTTTATCCATTCC-3′) and the reverse primer (5′-CACAATTGCCTACCTGCTTTCA-3′) and 5′ nuclease probe (Fam-TGCTAGTTCTGCTTTGCCTTCTC CATCTTCT-TAMARA) were used for amplification of the matrix gene [13]. Testing was performed by using the TaqMan EZ RT-PCR system. Amplification and detection was performed on an ABI StepOne real-time PCR system (Applied Biosystems).

NTS specimens were additionally tested by quantitative viral dilutions to detect median tissue culture infectious dose ($TCID_{50}$) and determine replicating viral load. Madin-Darby canine kidney (MDCK) cells were rinsed in serum-free minimum essential medium (Gibco). The NTS specimen was diluted initially by 1 in 5 and then in 10-fold steps in serum-free minimum essential medium (Sigma Aldrich). One hundred microliters of the undiluted NTS specimen as well as each of the specimen dilutions was added in quadruplicate to the MDCK cell monolayers. Medium alone was added to control cells. An additional 100 μL of serum-free minimum essential medium with 2 μg/mL trypsin was added to each well, and the plates were incubated at 33°C for 7 days. The plates were evaluated for cytopathic effect daily. The $TCID_{50}$ was determined according to the Reed–Muench method. The lower limit of detection was approximately $10^{0.3}$ $TCID_{50}$.

Statistical Analysis

We grouped clinical symptoms and signs as systemic signs and symptoms (fever of ≥37.8°C, headache, and myalgia) and respiratory symptoms (sore throat, runny nose, cough, and sputum), and calculated daily scores by summing the presence vs absence of each symptom or sign and dividing by 3 and 4, respectively [2]. We plotted average symptom scores by time since ARI onset, which was defined as the first day when the subject reported ≥2 of the 7 symptoms.

Mean tympanic temperatures since ARI onset were also investigated by subtype of influenza. The dynamics of viral shedding were analyzed by plotting the daily geometric mean RT-PCR and mean $TCID_{50}$ levels by time since the day of ARI onset. For undetectable values, half of the lower limit of detection (ie, 450 copies/mL and 0.15 $\log_{10}$ $TCID_{50}$) was imputed when calculating daily geometric means. Plots were stratified according to type/subtype of influenza virus (pandemic A [H1N1]pdm09, seasonal A[H1N1], seasonal A[H3N2], and seasonal B). We also examined the correlation between viral shedding detected by RT-PCR and tympanic body temperature since ARI onset. All analyses were conducted with R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria) [14].

RESULTS

Over the study period of 7 years, we enrolled 824 households, each with an index case that tested positive for influenza on the rapid test, and obtained NTS specimens from 2494 household contacts in those 824 households. Two hundred thirty-five household contacts (9%) were identified as laboratory-confirmed secondary cases of influenza virus infection, with identification of influenza virus by either RT-PCR or virus culture. Of these 235 cases, 165 (70%) were identified in 2008 and 2009, and the remainder were identified in the subsequent years. These included 33 cases of pandemic A(H1N1)pdm09, 73 cases of seasonal A(H1N1), 69 cases of A(H3N2), and 49

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cases of influenza B virus infections. All 235 secondary cases were initially negative on both viral culture and PCR at day 0 but had a positive finding later, including 88 cases (37.4%) positive only at visit 2, 67 cases (28.5%) positive only at visit 3, and 80 cases (34%) positive at both visit 2 and 3. Among these, 11 positive cases of influenza A could not be subtyped due to insufficient quantity of biological material available, and were excluded from the subsequent analyses in this study, leaving 224 confirmed natural influenza virus infections for our analyses.

The age and sex distributions of the 224 cases were largely similar across all 4 types/subtypes (Table 1). Among the 224 secondary cases, 179 (76%) presented with a typical clinical picture of ARI, reporting 2 or more symptoms during their illness episode. Overall, 83 cases (35%) also reported an episode of febrile illness that met the definition of ILI, and this proportion ranged from 27% to 38% for different influenza types/subtypes (Table 1).

To assess the dynamic relationship between clinical symptoms and viral shedding, we focused on a subset of 127 secondary cases with a clinical picture of ARI, including 17 cases of pandemic A(H1N1), 43 seasonal A(H1N1), 43 A(H3N2), and 24 influenza B virus infections. A total of 97 cases were excluded from these particular analyses, including 25 asymptomatic cases and 30 cases that reported only 1 symptom and thus could not be included, and 42 cases that were already symptomatic in the first household visit and thus the time of illness onset could not be precisely determined. At the time of ARI onset (day 0), cough and runny nose were the 2 most common presenting symptoms for secondary cases with seasonal influenza infections, being reported in 70%–82% and 59%–81% of cases, respectively, of different influenza types/subtypes (Table 2). For influenza A(H1N1)pdm09 cases, cough (82%) and sore throat (59%) were the 2 most common presenting symptoms. On the other hand, as fever was a presenting symptom in less than half of these cases, a typical symptom complex fitting the definition of ILI only applied to 23%–42% of cases at the time of ARI onset. Over the whole course of the illness episode, cough (82%–95%) and runny nose (76%–93%) were the 2 most commonly reported symptoms for all 4 types/subtypes of influenza infections (Table 2), whereas fever (≥37.8°C) was only reported in around half (41%–51%) of both pandemic and seasonal influenza cases who met the ARI case definition.

Among the 127 cases who met the ARI case definition, we plotted patterns in viral shedding and signs/symptoms since ARI onset (Figure 1). The temporal trajectory of signs and symptoms followed a comparable pattern across infections by the types/subtypes studied (Figure 1). Mean symptom scores for systemic and respiratory symptoms were generally highest on the day of symptom onset (day 0), and gradually declined thereafter over a course of 6–8 days. Systemic symptoms subsided earlier than respiratory symptoms. Mean temperature was also highest around the day of symptom onset for all types/subtypes (Figure 1). Compared with infections by seasonal influenza viruses, A(H1N1)pdm09 infections were associated with fewer respiratory symptoms and a shorter mean duration of systemic symptoms on average.

In the 127 cases with ARI, viral shedding exhibited very different temporal relationships with clinical symptoms in infections with influenza A vs influenza B (Figure 1). For influenza A virus infections, mean viral shedding as quantified by

Table 1. Characteristics of 224 Naturally Acquired Influenza Virus Infections

Characteristic	Pandemic A (H1N1) (n = 33)	Seasonal A (H1N1) (n = 73)	Seasonal A (H3N2) (n = 69)	Seasonal B (n = 49)
Male sex	9 (27)	34 (47)	22 (32)	19 (39)
Age, y				
0–15	7 (21)	17 (24)	15 (22)	18 (38)
16–30	4 (12)	7 (10)	7 (10)	3 (6)
31–45	17 (52)	39 (54)	29 (43)	23 (48)
>45	5 (15)	9 (12)	17 (25)	4 (8)
Illness[a]				
ILI	9 (27)	28 (38)	24 (35)	15 (31)
ARI	24 (73)	61 (84)	53 (77)	31 (63)
Paucisymptomatic[b]	7 (21)	7 (10)	8 (12)	8 (16)
Asymptomatic[c]	2 (6)	5 (7)	8 (12)	10 (20)

Data are presented as No. (%).

Abbreviations: ARI, acute respiratory illness; ILI, influenza-like illness.

[a] Illness profile at any time during follow-up.

[b] Defined as the presence of, at most, 1 of 7 signs or symptoms on any given day of follow-up.

[c] Defined as the presence of 0 signs or symptoms during follow-up.

Table 2. Signs or Symptoms of 127 Naturally Acquired Cases of Influenza Virus Infection That Met the Case Definition of Acute Respiratory Illness[a]

Sign or Symptom	Pandemic A (H1N1) (n = 17)	Seasonal A (H1N1) (n = 43)	Seasonal A (H3N2) (n = 43)	Seasonal B (n = 24)
Reported at ARI onset				
Cough	14 (82)	35 (81)	30 (70)	19 (79)
Runny nose	10 (59)	27 (63)	35 (81)	15 (62)
Sore throat	9 (53)	23 (53)	18 (42)	12 (50)
Sputum	6 (35)	16 (37)	19 (44)	8 (33)
Fever (≥37.8°C)	5 (29)	13 (30)	13 (30)	11 (46)
Headache	3 (18)	10 (23)	19 (44)	6 (25)
Myalgia	4 (24)	9 (21)	15 (35)	7 (29)
ILI	5 (29)	12 (28)	10 (23)	10 (42)
Reported on at least 1 day of follow-up				
Cough	14 (82)	41 (95)	36 (84)	21 (88)
Runny nose	13 (76)	40 (93)	40 (93)	20 (83)
Sore throat	12 (71)	29 (67)	29 (67)	14 (58)
Sputum	9 (53)	26 (60)	30 (70)	18 (75)
Fever (≥37.8°C)	7 (41)	22 (51)	22 (51)	12 (50)
Headache	5 (29)	18 (42)	30 (70)	11 (46)
Myalgia	8 (47)	16 (37)	27 (63)	10 (42)
ILI	7 (41)	21 (49)	18 (42)	12 (50)

Data are presented as No. (%).

Abbreviations: ARI, acute respiratory illness; ILI, influenza-like illness.

[a] Reported at least 2 of 7 symptoms on 1 or more days of follow-up.

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Table 3. Geometric Mean of Viral Load According to Time in Relation to Acute Respiratory Illness Onset

Subtype	1 or 2 d Before ARI Onset	Day of ARI Onset	1 or 2 d After ARI Onset
Pandemic A(H1N1)	2.7	4.3	4.5
Seasonal A(H1N1)	3.0	5.1	5.2
Seasonal A(H3N2)	2.9	5.3	5.6
Seasonal B	3.5	5.8	4.4

Data are presented as geometric mean of viral shedding (mean $\log_{10}$ copies per mL).
Abbreviation: ARI, acute respiratory illness.

RT-PCR or $TCID_{50}$ could be detected in some cases before ARI onset, and peaked within 2 days of ARI onset (Figure 1 and Table 3). Thereafter, the amount of viral shedding as quantified by RT-PCR decreased gradually to an undetectable level by approximately day 6–7 of clinical illness, with a total shedding duration of 9–10 days in some cases. Whereas the patterns in viral shedding and symptoms matched quite closely for influenza A, this was not the case for influenza B (Figure 1). Instead of peaking at the day of symptom onset, influenza B viral shedding detected by RT-PCR started with a peak up to 2 days prior to symptom onset and persisted for 6–7 days thereafter, with evidence of a bimodal pattern (Figure 1 and Table 3). Duration of influenza B viral shedding as reflected by $TCID_{50}$ generally followed a largely similar trajectory, but with a shorter total shedding duration of 4–6 days than that reflected by RT-PCR (Figure 1). Patients with illness fitting the ILI definition had a similar viral shedding trajectory in terms of the duration and pattern of shedding compared with the ARI cases. There was insufficient sample size to explore the difference in viral shedding between adults and children.

The dynamics of viral shedding detected by quantitative culture revealed trends similar to those detected by RT-PCR. We did not observe any statistically significant trend on the ratio of RT-PCR vs $TCID_{50}$ over time since illness onset, and the ratios of RT-PCR to $TCID_{50}$ (both measured in the same volume of Viral Transport Medium) were 708, 547, and 185 matrix gene copies per $TCID_{50}$ for seasonal A(H1N1), A(H3N2), and influenza B, respectively.

We also investigated the associations between daily body temperature and levels of viral shedding since ARI onset. Among cases of seasonal influenza A, the strongest correlations were observed on the day of and the day immediately following ARI onset (Figure 2). No statistically significant correlations were found between body temperature and viral shedding among the cases with influenza B with a smaller sample size (Supplementary Appendix Figure 1).

DISCUSSION

This study described the clinical pattern of 224 cases of natural influenza virus infection, including seasonal and pandemic influenza cases, spanning 7 years, and examined the dynamic temporal relationship between clinical symptomatology and viral shedding in 127 cases having an ARI clinical picture. The duration and patterns of viral shedding observed in seasonal A (H1N1), A(H3N2), and pandemic A(H1N1)pdm09 influenza virus infections were generally comparable, rapidly rising and peaking on the day of or on day 1–2 following ARI onset before declining, whereas in cases of influenza B virus infection, viral shedding followed a more prolonged plateau pattern (Figure 1). This divergence between influenza A and B viral shedding patterns was largely compatible with previous observations from volunteer challenge studies [4]. The pattern in viral shedding by PCR was generally consistent with that observed by $TCID_{50}$, indicating that viral shedding by PCR does correlate with infectious virus.

Given that the patterns of symptoms were very comparable between influenza A and B virus infections (Figure 1), it is not clear why their viral shedding patterns are so different. Consistent with previous findings [7, 8], the close matching of the dynamics of clinical illness and viral shedding in influenza A infection allowed the use of the illness profiles as a proxy for clinical infectiousness, assuming that it is proportional to the amount of viral shedding. The high level of shedding during the initial 1–2 days of clinical illness may also explain our previous finding that nonpharmacological interventions, including hand hygiene and face masks, would be most effective in preventing transmission in a household setting if implemented within 36 hours of symptom onset in the index case [9]. Largely identical to findings in experimental studies, the bimodal course with early peak and prolonged period of viral shedding in influenza B infection might reflect a different temporal course of immune response for the less variable virus as compared to influenza A virus, and may imply the potential infectiousness before symptom onset and after clinical improvement.

The start of viral shedding before symptom onset, albeit at low levels as demonstrated by both RT-PCR and $TCID_{50}$ (Figure 1), indicates the potential for influenza virus transmission in the presymptomatic phase of the illness before it becomes clinically apparent [15]. As a consequence, symptom-based preventive measures may be inadequate, whereas more effective control of epidemics may be achieved through general preventive measures including hygiene campaigns, improvements in general immunity by healthier lifestyle, and the use of influenza vaccination, especially for individuals with high risk of severe disease.

Although the trajectory of viral shedding over time has been relatively better documented in volunteer challenge studies [4], there are very few studies with viral shedding data from naturally acquired infections [8, 16]. Cases infected by viral challenge were of doubtful generalizability to natural infections because of the route and high challenge doses involved in such an experimental setting, designed to lead to a mild to moderate illness, and the exclusive involvement of healthy adults [4]. The prospective household transmission study represents an ideal

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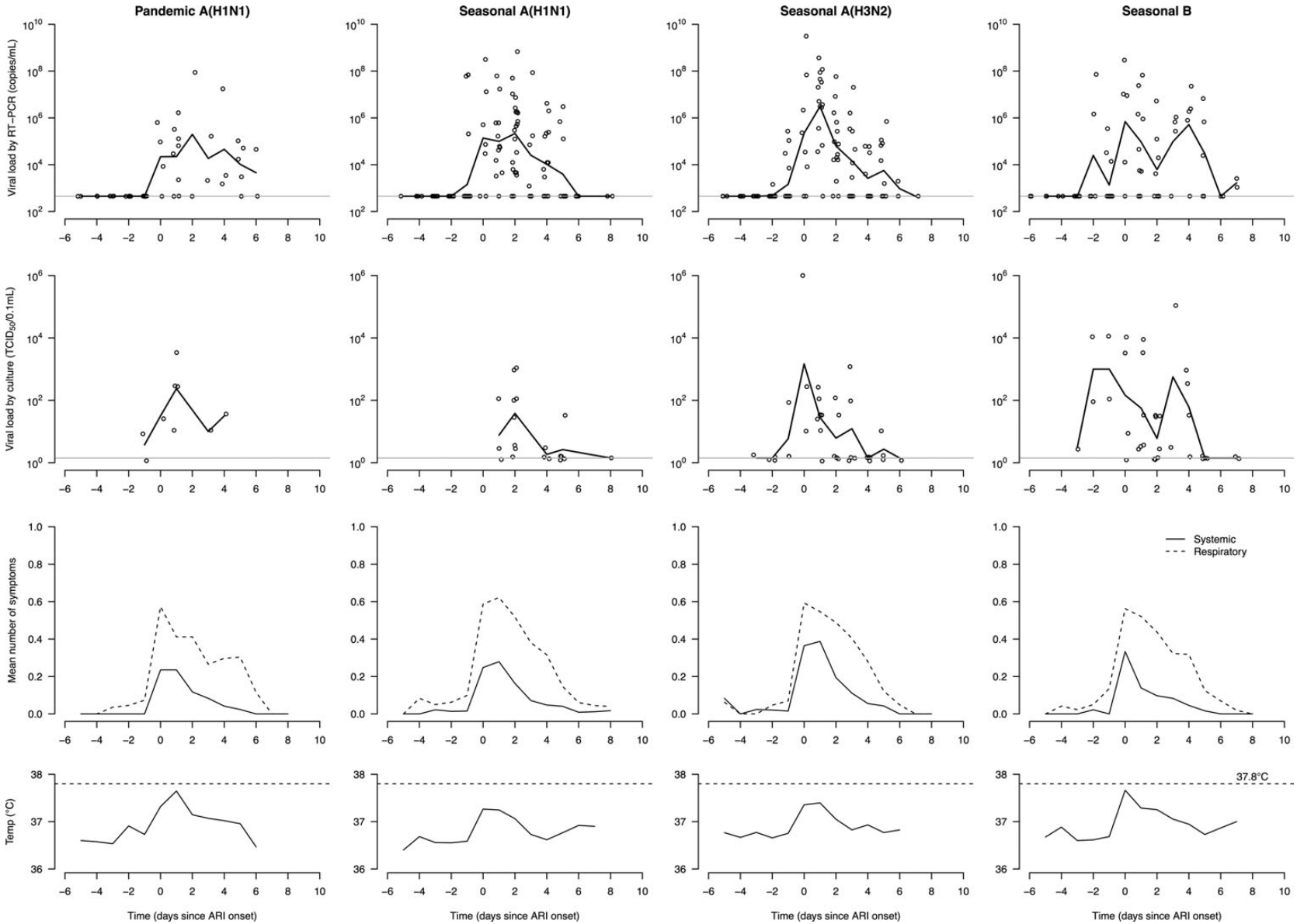

Figure 1. Patterns of viral shedding and clinical symptomatology in naturally acquired influenza A and B virus infections by day relative to acute respiratory illness (ARI) onset (day 0). First column: 33 cases with pandemic A(H1N1); second column: 73 cases with seasonal A(H1N1); third column: 69 cases with seasonal A(H3N2); and fourth column: 49 cases with seasonal B influenza B viral infections. First row: Viral shedding (circles) and the geometric mean viral shedding (solid lines) of collected nose and throat swab (NTS) specimen by reverse transcription polymerase chain reaction assay (RT-PCR). The lower limit of detection of RT-PCR was approximately 900 copies/mL (gray line). Second row: Median tissue culture infectious dose ($TCID_{50}$) (circles) and the geometric mean $TCID_{50}$ of collected NTS specimens. Third row: Mean number of symptoms and signs for subjects with influenza virus infection, splitting into respiratory (dotted line) and systemic (solid line). Bottom row: Mean tympanic temperature recorded. ARI onset is defined as at the first day with ≥2 of the 7 symptoms or signs listed in Table 2. Individuals with asymptomatic or subclinical infections were excluded.

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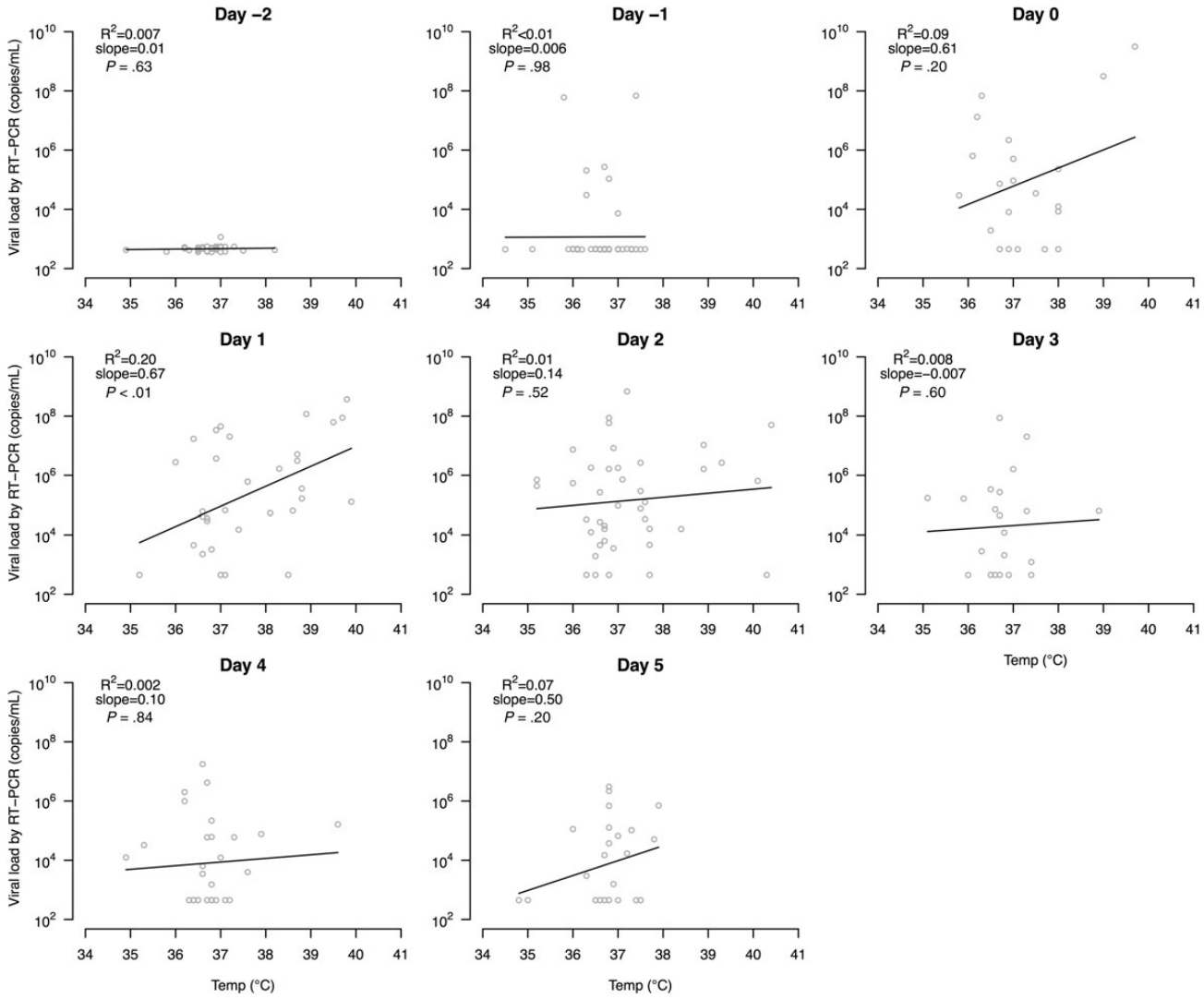

Figure 2. Scatterplot showing correlation (R^2) and line of best fit between tympanic temperature and influenza viral shedding by reverse transcription polymerase chain reaction (RT-PCR) by day since acute respiratory illness (ARI) onset in 175 cases with naturally acquired influenza A virus infections. ARI onset is defined as at the first day with ≥2 of the 7 symptoms or signs listed in Table 2.

setting for filling this important information gap as the study design allows both symptomatology and viral shedding to be measured prospectively on natural infections of any severity profile among exposed household contacts with a wide variety of demographic and medical backgrounds (eg, the ages ranged from 1 to 91 years). In this study, we refined our previous findings on 59 confirmed infections [7] with a much larger number of cases from 7 years of recruitment, including also cases infected with influenza A(H1N1)pdm09 virus. Although secondary cases in a household setting might not be representative of all natural influenza infections in other community settings because of the particular mechanisms of transmission in households [17], it has been estimated that a large proportion of community disease transmission does take place in the household setting [18]. In a subset of these households, we used viral genetic sequences to demonstrate that >95% of the infections among household contacts did occur within the household [19].

Although antiviral use has been reported to shorten the duration of viral shedding [20], these effects could not be assessed in this study because very few cases included here had sought medical attention and been prescribed antivirals for their illness. This study also faced other limitations. Short and mild infections could be missed because of the 1- to 2-day time gap of between NTS collection. Index case recruitment required subjects to seek outpatient care, possibly resulting in a bias toward subjects with more severe illness and health-seeking behavior. Additionally, when examining the interrelationship between dynamics of clinical symptoms and viral shedding, only secondary cases with typical ARI onset could be plotted, which did not account for the dynamics of virus shedding in asymptomatic

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infections. Finally, assessing the trajectories according to ARI onset is reliant upon the accuracy of self-reported respiratory signs and symptoms.

In conclusion, our results suggest that viral shedding in seasonal and pandemic influenza A virus infections matched closely with the dynamics of clinical illness, allowing clinical illness profiles to be used as a proxy for clinical infectiousness, whereas in influenza B virus infection, viral shedding followed a more prolonged plateau pattern, implying that patients may potentially be infectious even before symptom onset or upon clinical improvement.

Supplementary Data

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Disclaimer. The funding bodies were not involved in the collection, analysis, and interpretation of data; the writing of the article; or the decision to submit it for publication.

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