Longitudinal Study of Influenza Molecular Viral Shedding in Hutterite Communities

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Background. The nature of influenza viral shedding during naturally acquired infection is not well understood.

Methods. A cohort study was conducted in Hutterite colonies in Alberta, Canada. Flocked nasal swabs were collected during 3 influenza seasons (2007–2008 to 2009–2010) from both symptomatic and asymptomatic individuals infected with influenza. Samples were tested by real-time reverse-transcription polymerase chain reaction for influenza A and influenza B, and the viral load (VL) was determined for influenza A positive samples.

Results. Eight hundred thirty-nine participants were included in the cohort; 25% (208) tested positive for influenza viruses. They experienced 238 episodes of viral shedding, of which 23 (10%) were not accompanied by symptoms. For seasonal and pandemic H1N1, VL peaked at or before onset of acute respiratory infection. For H3N2, VL peaked 2 days after the onset of acute respiratory infection, which corresponded to peaks in systemic and respiratory symptom scores. Although the duration of shedding was shorter for asymptomatic participants, the peak level of VL shedding was similar to that of symptomatic participants. Viral loads for children and adults revealed similar patterns.

Conclusions. Molecular viral shedding values follow symptom scores, but timing of peak VL varies by subtype. Asymptomatic infections are infrequent.

Influenza virus causes annual epidemics of respiratory illness worldwide and is the most important cause of medically attended acute respiratory illness [1–3]. Achieving a better understanding of how influenza is transmitted is an important public health goal [4]. Most of what is known about influenza transmission has been derived from challenge studies [5–9], which provide an important but limited perspective on how natural infection evolves [10]. Such studies are limited because they select subjects with no preexisting immunity, have relatively small sample sizes, are limited largely to intranasal inoculation, rarely include children, and have a limited duration of follow-up [11–26]. In a recent meta-analysis of 56 challenge studies that included a total 1280 subjects, no studies included children, and only one study included studies outside of the age range of 18–50 years [10]. The majority (67%) of participants in the challenge studies showed symptoms and fever, and these varied depending on the infecting strain [10]. A recent community study from Hong Kong in which 59 participants in a household study were followed for 3 visits during the 2008–2009 season has helped to delineate viral shedding patterns [27].

The objective of this study was to describe patterns of molecular viral shedding in naturally infected children and adults over multiple seasons. We sought to determine ranges of duration of shedding and peak viral load (VL) by influenza type and subtype. We conducted this study in the Hutterite community of Alberta, Canada, during 3 influenza seasons.
METHODS

Study Population
The Hutterites, along with the Mennonites, are a Protestant sect that was founded during the 16th-century Anabaptist movement in Switzerland. The majority of Hutterites live in Alberta, Saskatchewan, and Manitoba, Canada, where they practice communal farming on small colonies relatively isolated from towns and cities. Within these homogeneous, moderately sized colonies (typically 80–120 people), regular influenza transmission is facilitated by a communal lifestyle. Outbreaks of influenza in Hutterite colonies occur regularly, with influenza introduced from exposure to outsiders. Because the colonies are not very large, it is possible to obtain detailed demographic, health, and immunization information from all members.

We enrolled participants from 10 Hutterite colonies in central Alberta from 2007 to 2010. The colonies were selected on the basis of location within 150 km from the city of Red Deer to allow feasibility of surveillance by research nurses based in Red Deer. Seven of the colonies had participated in a pilot study for a cluster randomized trial that began in 2008; 3 additional colonies were enrolled later. The children in 3 of the 7 colonies that participated in the pilot study had been offered inactivated influenza vaccine from the 2007-2008 to the 2008-2009 influenza season. All Hutterite colony members were eligible for this study because the goal was to describe viral shedding in different age groups.

Surveillance
Study participants, including study vaccine recipients and nonrecipients, were assessed for signs and symptoms of influenza over the follow-up period, defined by a start date of >1 lab-confirmed influenza case in 2 consecutive weeks from sentinel sites and a stop date of no lab-confirmed influenza cases for 2 consecutive weeks in colonies from the same geographic region. This period extended from 29 December 2007 (the first nurse visit) until 15 June 2010 (the last visit).

Research nurses assessed study participants at twice-weekly site visits using a standardized checklist of self-reported symptoms or signs from study participants or parents. One representative from each household would complete the checklist for their family members and provide this to the research nurse. If any new symptoms were reported, the nurse interviewed the study participant (adult or child) while onsite at the colony, confirmed the symptoms and their date of onset, and obtained 1 nasopharyngeal specimen and 1 flocked nasal specimen if ≥2 of the following were present: fever (≥38°C), cough, nasal congestion, sore throat, headache, sinus problems, muscle aches, fatigue, ear ache, and chills. We purchased identical thermometers for all study participants and provided instruction on thermometer use.

If the nasopharyngeal swab tested positive for influenza using reverse-transcription polymerase chain reaction (RT-PCR), flocked nasal swabs were obtained on a daily basis for 7 days, followed by specimen collection every 2 days for up to 8 weeks [28–30]. If 2 consecutive specimens tested negative, longitudinal follow-up was discontinued. In order to better define the nature of asymptomatic shedding, we enrolled colony members who were asymptomatic when it was established that the colony had an outbreak, which we defined as ≥2 positive specimens within any 48-hour period. In addition to obtaining a specimen upon enrollment, we obtained daily specimens from asymptomatic participants for the first week, followed by specimen collection every second day for up to 3 weeks. Surveillance was stopped when 2 consecutive negative specimens were detected. The research protocol was approved by McMaster University Research Ethics Review Board and by the Conjoint Health Research Ethics Board of the University of Calgary; written informed consent was obtained from all participants.

The nasopharyngeal and flocked nasal swabs were batched and tested by quantitative RT-PCR to detect influenza A and B and determine molecular VL. We used the Applied Biosystems One-Step RT-PCR kit; the amplification targets matrix gene and nonstructural gene for influenza A and B, respectively [31]. Analytic sensitivity was approximately 8 copies of target DNA in the 5-μL template added (approximately 400 copies/mL). In order to adjust for the effect of differences in VL due to differences in cellular content of samples, we measured the housekeeping gene beta-2-microglobulin (B2M) in each sample. We calculated the ratio of the target copy number (copies/mL) to those of B2M then multiplied this ratio by a standardized concentration of target VL, for which we selected the median VL for the particular influenza subtype.

Statistical Analysis
We defined acute respiratory infection (ARI) as ≥2 symptoms and plotted systemic and upper respiratory symptom scores by time with respect to ARI onset, defined as the first day that the ARI definition was met. We plotted quantitative VL by time since ARI onset using geometric means on logarithmic scales. Both target copy number and numbers normalized to the B2M gene (using the ratio of target primers to those of B2M multiplied by the median VL for the influenza A subtype) were plotted for influenza A. Daily symptom scores were grouped into 2 categories: systemic (fever, headache, myalgia, chills) and upper respiratory (cough, sore throat, runny nose, sinus). We summed the presence or absence of each symptom or sign (coded as 1 if present and 0 if absent) and divided by 4 to develop a systemic symptom score and an
upper respiratory symptom score. The symptom scores were plotted for comparison with quantitative VL. We used a Student t test to assess differences in length of shedding between symptomatic and asymptomatic participants.

RESULTS

There were a total of 839 participants in 194 households in 10 Hutterite colonies that took part in this longitudinal study. There were 163 participants aged <5 years, 237 aged 5–15 years, and 439 aged >15 years. The mean number of participants per colony was 84 (range, 41–121). A total of 208 participants (24.8%) were infected as confirmed by RT-PCR. Of these, 92 (44%) were vaccinated. The total number of episodes of viral shedding was 238, with 32 participants having >1 episode; each second episode occurred in a different season. Twenty-two percent (53) of episodes occurred in participants aged <5 years, 48% (113) occurred in participants aged 5–15 years, and 30% (72) occurred in participants aged >15 years. In the study colonies, there were 15 outbreaks, defined as ≥2 cases with ARI symptoms within a 48 hour period. An epidemic curve demonstrating the influenza clusters by type and subtype is shown in Figure 1. The first cluster was of seasonal H1N1, included 62 cases, and lasted almost 9 weeks, from January to March 2008. The second cluster was predominantly due to influenza B, included 43 cases, and lasted 6 weeks, from the beginning of February to mid-March 2009. The third cluster was due to H3N2, included 36 cases, and lasted 18 weeks, from mid-January to May 2009. The last cluster was pandemic H1N1, included 97 cases, and lasted 6 weeks, from the beginning of November to mid-December.

The frequency of symptoms by type or subtype of influenza is shown in Table 1. The frequency of fever, cough, or runny nose (58%, 89%, and 89%, respectively) appeared to be higher in episodes of infection due to H3N2 than in infection due to the other subtypes or influenza B. Notably, the frequency of symptoms due to influenza B was similar to that due to pandemic H1N1. Characteristics of viral shedding for influenza A are shown in Tables 2 and 3. As expected, the mean duration of shedding was greater for younger participants (<9 years). Of 238 episodes that occurred in 208 participants, 23 (10%) were not accompanied by symptoms. All 36 participants with H3N2 were symptomatic, differing significantly from other subtypes (P = .03). In contrast, 2 (5%) of those infected with influenza B, 9 (15%) of those infected with H1N1, and 12 (12%) of those infected with pandemic H1N1 were asymptomatic.

Plots of VL in relation to onset of symptoms are shown in Figure 2. As can be noted for both seasonal H1N1 and
pandemic H1N1, the VL peaked at or before onset of ARI, then declined gradually over the next 6–8 days, with continued shedding until 12–14 days after onset of ARI. The VL reflected trends in respiratory and systemic symptoms. In contrast, for H3N2, VL peaked 2 days after the onset of ARI, which corresponded to the peaks in systemic and respiratory symptom scores. Overall, for both H1N1 and H3N2, the standardized VL closely followed the trend in the target VL.

Given that there were no asymptomatic participants infected with H3N2, asymptomatic participants shedding virus for seasonal H1N1 and pandemic H1N1 were compared. Asymptomatic shedding was noted over a 10-day period for seasonal H1N1 and pandemic H1N1, compared with 8 days for pandemic H1N1, with no significant difference in mean duration of shedding (P = .11). The mean duration of viral shedding was 4.0 days (SD, 2.0) in asymptomatic participants and 4.9 days (SD, 2.6) in symptomatic participants (P < .0001). The peak level of viral shedding for asymptomatic participants appeared to be slightly lower than that for symptomatic participants (Figure 3). For pandemic H1N1, we plotted VL for children vs adults and found a high degree of overlap (Figure 4).

### Table 2. Influenza Viral Shedding by Age

<table>
<thead>
<tr>
<th>Age at Infection</th>
<th>No. of Participants</th>
<th>Mean No. of Days</th>
<th>SD (days)</th>
<th>Minimum No. of Days</th>
<th>Maximum No. of Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5 years</td>
<td>53</td>
<td>5.0</td>
<td>3.3</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>5–8 years</td>
<td>48</td>
<td>5.7</td>
<td>3.8</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>9–15 years</td>
<td>65</td>
<td>4.9</td>
<td>3.0</td>
<td>1</td>
<td>15</td>
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<tr>
<td>&gt;15 years</td>
<td>72</td>
<td>3.7</td>
<td>2.6</td>
<td>1</td>
<td>10</td>
</tr>
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</table>

Abbreviation: SD, standard deviation.

### DISCUSSION

Viral shedding of influenza in natural infection in the community is not well understood. Through prospective follow-up of 839 participants, we have summarized viral shedding in 208 individuals, including children, during 3 influenza seasons. Our main findings were that patterns of influenza A viral shedding appeared to vary by subtype and that the patterns of shedding were similar in both children and adults. Viral shedding without apparent symptoms was infrequent (occurring in 10% of episodes).

Patterns of VL differed by subtype, with pandemic and seasonal H1N1 having higher peak titers within 1 day of the initial rise in VL than H3N2, for which the VL increased over 2 days, peaked at a lower level, and diminished more gradually than for H1N1. We are unaware of other studies set in the community that have shown such differences, and 1 of the reports did not detect such differences in VL patterns [27]. Challenge studies of both H3N2 and H1N1 have shown sharp increases during the first day following inoculation, reaching maximum values on the second day [10]. We acknowledge that the difference we observed may be due to chance—that is, had the experiment been repeated we may have obtained curves similar to H1N1 for H3N2. Because there was only 1 outbreak of each virus type or subtype, more data are needed to confirm the generalizability of these patterns of viral shedding. Nevertheless, these data raise the possibility of a different pattern of viral growth dynamics with H3N2 compared with seasonal H1N1 or pandemic H1N1, which showed very similar dynamics of viral shedding.

The study indicates that a majority of viral shedding occurs within 2 days of ARI onset, peaking at about the same time as ARI onset. Thus, as has previously been suggested, this is an important time to apply isolation [27]. The greater frequency of symptoms with H3N2, including fever and cough, is in keeping with increased virulence of this subtype, as has previously been described [32]. We found that there was a rise in viral shedding just prior to ARI onset. We also found that
both respiratory and systemic symptoms correlated with the VL. This is in contrast with Lau et al [27], who found that systemic symptoms and signs subsided more rapidly than respiratory symptoms. The relatively large number of children in this study allowed for a comparison of viral shedding with adults. The length of shedding was greater in children (those aged <16 years) than in adults; this is in line with previous reports [33, 34].

Asymptomatic infections were infrequent, occurring in only 10% of episodes. This is similar to the findings of Lau et al, who reported a rate of 14% of inapparent symptoms [27]. Although the peak VLs were similar, the VLs of asymptomatic

Figure 2. Plot of H1N1, 2007–2008 (A); seasonal H3N2, 2008–2009 (B); and pandemic H1N1, 2009–2010 (C) molecular viral load by days with respect to onset of acute respiratory infection (ARI). The systemic and respiratory mean symptom score is plotted under the molecular viral load. The target lines represent the direct viral load in copy numbers/mL. For the standard lines, we computed the ratio of target copy number to B2M, a common housekeeping gene. This ratio was then multiplied by a standard amount of target material (the median concentration of viral load), and this result was plotted in copy numbers/mL.

Figure 3. Plot of seasonal H1N1, 2007–2008 (A) and pandemic H1N1, 2009–2010 (B) molecular viral load by days with respect to onset of acute respiratory infection (ARI) for symptomatic and asymptomatic participants. For asymptomatic shedders, we assigned day 0 as the last day of shedding. The target lines represent the direct viral load in copy numbers/mL. For the standard lines, we computed the ratio of target copy number to B2M, a common housekeeping gene. This ratio was then multiplied by a standard amount of target material (the median concentration of viral load), and this result was plotted in copy numbers/mL.
participants were lower and of shorter durations than those who were symptomatic, suggesting that asymptomatic transmission may be less frequent and less efficient than symptomatic transmission. We could not assess whether asymptomatic participants transmitted influenza to others. It should be noted that because we did not include serological evidence for infection, we could not provide an estimate of what proportion of those infected were asymptomatic shedders.

Use of the B2M housekeeping gene allowed us to assess the possible effect of sample quality variation on the results. That is, differences in the number of cells obtained with the nasal swabs may produce biased estimates of VL. Adjusting the results for the cellular content using B2M yielded similar results to the target copy number. This suggests that differences in sampling did not affect the results. Given that there are a number of housekeeping genes that can be used for target normalization and the copy number of these genes can vary, studies comparing different genes for normalization should be performed. One would expect to see similar trends in VL based on cellular content irrespective of the housekeeping gene used.

The strengths of this study are that the study was conducted over multiple seasons, included both adults and children, included multiple influenza A subtypes, included active follow-up by a research nurse for a relatively long period, and used a housekeeping gene for standardization. One limitation of this study is that we did not assess VL for influenza B; only duration of viral shedding was assessed. We also did not measure viral replication directly. Another limitation is that because of too few vaccinated participants, we could not assess the effect of vaccination on subtype. The study was conducted in a

Figure 4. Plot of H1N1, 2007–2008 (A); seasonal H3N2, 2008–2009 (B); and pandemic H1N1, 2009–2010 (C) molecular viral load, systemic mean symptom score, and respiratory mean symptom score by days with respect to onset of acute respiratory infection (ARI) for children and adults. The target lines represent the direct viral load in copy numbers/mL. For the standard lines, we computed the ratio of target copy number to B2M, a common housekeeping gene. This ratio was then multiplied by a standard amount of target material (the median concentration of viral load), and this result was plotted in copy numbers/mL.
Hutterite community, which has a different population structure. However, we do not believe that would have an effect on our findings.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. 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.

References