Longitudinal Study of Influenza Molecular Viral Shedding in Hutterite Communities

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Abstract

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 where flocked nasal swabs were collected over three influenza seasons (2007/2008 to 2009/2010) from both symptomatic and asymptomatic individuals infected with influenza. Samples were tested by real-time RT-PCR for Influenza A and Influenza B and the viral load was determined for Influenza A positive samples.

Results: 839 participants were included in the cohort and 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, the viral load peaked at or before onset of acute respiratory infection. For H3N2, viral load peaked two days after the onset of acute respiratory infection, which corresponded to the peaks in systemic and respiratory symptom scores. Although the duration of shedding was shorter, the peak level of viral load shedding for asymptomatic participants was similar to those who were symptomatic. Viral loads for children and adults revealed similar patterns.

Conclusions: Molecular viral shedding values follow symptom scores but timing of peak viral load 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 through the selection of subjects with no pre-existing immunity, relatively small sample sizes, limited largely to intranasal inoculation, few studies that include children, and limited duration of follow up (11-26). A recent meta-analysis of 56 challenge studies including 1,280 subjects for example did not have studies that included children, and with one exception included studies limited to subjects ranging in age from 18 to 50 years (10). The majority (67%) of those included in the challenge studies showed that symptoms and fever tended to vary depending on the infecting strain (10). A recent community study from Hong Kong where 59 participants in a household study were followed over three 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 by influenza type and subtype as well as to assess the effect of pre-existing immunity on duration of shedding. We conducted this study in the Alberta Hutterite community over three influenza seasons.
Methods

Study Population

Hutterites, along with the Mennonites, were founded as Protestant sects in the 16th century Anabaptist movement of Switzerland. The majority of Hutterites live in Alberta, Saskatchewan, and Manitoba where they practice communal farming on small colonies relatively isolated from towns and cities. Within these homogeneous, moderately sized colonies, regular influenza transmission is facilitated by a communal lifestyle. Hutterite colonies, where members live communally in colonies of typically 80 to 120 people, are relatively isolated from towns and cities. However, outbreaks of influenza in Hutterite colonies occur regularly with influenza being introduced from exposure to outsiders. Since the colonies are not very large, it is possible to obtain detailed demographic, health, and immunization information from all members.

We enrolled subjects from 10 Hutterite colonies in central Alberta from 2007 to 2010. The colonies were selected on the basis of being within 150 km from the city of Red Deer to allow feasibility of surveillance by research nurses who were based in Red Deer. Seven of the colonies had participated in a pilot study for a cluster randomized trial that began in 2008, three additional colonies were enrolled later. The children in three of the seven colonies that participated in the pilot study had been offered inactivated influenza vaccine from 2007/2008 to 2009 seasons. All Hutterite colony members were eligible for this study since the goal was to describe viral shedding in different age groups.
Surveillance

Study participants, including study vaccine recipients and non-recipients were assessed for signs and symptoms of influenza over the follow up period, defined by the start date (> 1 lab-confirmed influenza case in 2 consecutive weeks from sentinel sites) and stop date (no lab-confirmed influenza cases for 2 consecutive weeks in colonies from the health region). This period extended from December 29, 2007 (the first nurse visit) until June 15, 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 on-site at the colony, confirmed the symptoms and their date of onset, and obtained one nasopharyngeal specimen and one flocked nasal specimen if two or more of the following were present: fever ($\geq 38^\circ$ Celsius), cough, nasal congestion, sore throat, headache, sinus problems, muscle aches, fatigue, ear ache, chills. We purchased identical thermometers for all study participants and provided instruction on thermometer use.

If the nasopharyngeal swab tested positive for influenza (using RT-PCR), flocked nasal swabs were obtained on a daily basis for seven days, followed by specimen collection every two days for up to eight weeks (28-30). If two 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 \( \geq 2 \) positive specimens within any 48 hour period. In addition to obtaining a specimen upon enrolment, we obtained daily specimens from asymptomatic participants daily for the first week followed by specimen collection every second day for up to 3 weeks. Surveillance was stopped when two 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 viral load. 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 about 8 copies of target DNA in the 5ul template added (approximately 400 copies/ml). In order to adjust for the effect of differences in viral load due to differences in cellular content of samples, we measured the housekeeping gene beta2-microglobulin (B2M) in each sample. We calculated the ratio of the target copy number (copies per ml) to those of B2M then multiplied this ratio by a standardized concentration of target viral load for which we selected the median viral load for the particular influenza subtype.
Statistical analysis

We defined acute respiratory infection (ARI) as $\geq 2$ symptoms and plotted systemic and upper respiratory symptom scores by time with respect to ARI onset defined as first day that ARI definition was met. We plotted quantitative viral load 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 viral load for the influenza A subtype) were plotted for influenza A. Daily symptom scores were grouped into two categories: systemic (fever, headache, myalgia, chills), upper respiratory (cough, sore throat, runny nose, sinus). We summed the presence or absence of each symptom or sign (coded as 1 is present and 0 if absent) and divided by four to develop a systemic symptom score and an upper respiratory symptom score. The symptom scores were plotted for comparison to quantitative viral load. We used a student’s 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 ten Hutterite colonies that took part in this longitudinal study. There were 163 participants under the age of 5 years, 237 aged from 5 years to 15 years, and 439 were older than 15 years. The mean number of participants per colony was 84 (range was from 41 to 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 those $< 5$ years, 48% (113)
in participants from 5 to 15 years, and 30% (72) in those > 15 years old. There were 15 outbreaks in the study colonies, as defined by two or more cases with ARI symptoms within a 48 hour period. An epidemic curve, demonstrating the influenza clusters by type and sub-type is shown in Figure 1. The first cluster was of seasonal H1N1, included 62 cases and lasted almost nine weeks from January to March 2008. The second cluster was predominantly due to influenza B, including 43 cases over six weeks from the beginning of February to the middle of March 2009. The third cluster, due to H3N2, lasted 18 weeks, from mid January to May 2009, and involved 36 cases. The last cluster was for pandemic H1N1 and lasted six weeks from the beginning of November to the middle of December and involved 97 cases.

The frequency of symptoms by type or subtype of influenza, are shown in Table 1. The frequency of fever, cough, or runny nose (58%, 89%, 89% respectively) appeared to be higher in episodes of infection due to H3N2 than with the other subtypes or with influenza B. Notably, the frequency of symptoms due to influenza B was similar to pH1N1. 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. 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 = 0.03). In contrast, 2 or 5% of those infected with influenza B, 9 or 15% infected with H1N1, 12 or 12% with pH1N1, were asymptomatic.

The plots of viral load with relation to onset of symptoms are shown in Figure 2. As can be noted for both seasonal H1N1 and pandemic H1N1, the viral load peaked at or before onset of
ARI, then declined gradually over the next 6 to 8 days, with continued shedding until 12 to 14 days. The viral load reflected trends in respiratory and systemic symptoms. In contrast, for H3N2, viral load peaked two 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 viral load closely followed the trend in the target viral load.

Given that there were no asymptomatic participants infected with H3N2, asymptomatic participants shedding virus for seasonal H1N1 and pandemic H1N1 was compared. For seasonal H1N1, asymptomatic shedding was noted over a 10 day period compared to 8 days for pandemic H1N1 (Figure 3). The mean duration of viral shedding for seasonal H1N1 was 5.0 days (standard deviation 3.0 days) compared to 3.2 days (standard deviation 2.2 days) for pandemic H1N1, with no significant difference in mean duration of shedding (P=0.11). The mean duration of viral shedding in asymptomatic participants was 4.0 days (standard deviation 2.0) and 4.9 days (standard deviation 2.6) for symptomatic participants (P <0.0001). The peak level of viral shedding for asymptomatic participants appeared to be slightly lower than those who were symptomatic (Figure 3). For pandemic H1N1, we plotted viral load for children versus adults and found a high degree of overlap (Figure 4).

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, over three influenza seasons. Our main findings were that patterns of influenza A viral shedding appear to vary by subtype and 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 viral load differed by subtype, with pandemic and seasonal H1N1 having higher peak titres within one day of the initial rise in viral load compared to H3N2 where the viral load increased over two days, peaked at a lower level, and diminished more gradually than H1N1. We are unaware of other studies set in the community that have shown such differences and one of the reports did not detect such differences in viral load 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 observe may be due to chance, that is, had the experiment been repeated we may have obtained curves similar to H1N1 as with H3N2. Since there was only one 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 to seasonal H1N1 or pandemic H1N1 which showed very similar dynamics of viral shedding.

The study indicates that a majority of viral shedding occurs within two 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 that 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 to the viral load. This is in contrast to Lau et al, 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 to adults. The length of shedding was greater in children (those aged < 16 years) than adults and this in keeping 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). Viral loads of asymptomatic 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 are 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 viral load. 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 viral load based on cellular content irrespective of the housekeeping gene used.
The strengths of this study were 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 use of a housekeeping gene for standardization. One limitation of this study was that we did not assess viral load for influenza B, only duration of viral shedding was assessed. Also, we 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 Hutterite community, which has a different population structure. However, we do not believe that would have an effect on our findings.
References:


Figure Legend

Figure 1.
Epidemic curve showing number of influenza cases detected by week over the study period.

Figure 2. Plot of influenza A molecular viral load by days with respect to onset of acute respiratory infection. 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 per 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 per ml.

Figure 3. Plot of influenza A molecular viral load by days with respect to onset of acute respiratory infection for symptomatic and asymptomatic participants. For asymptomatic shedders, we assigned day 0 as the last day of shedding for those who were asymptomatic. The target lines represent the direct viral load in copy numbers per 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 per ml.

Figure 4. Plot of influenza A molecular viral load by days with respect to onset of acute respiratory infection for children and adults. The target lines represent the direct viral load in copy numbers per 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 per ml.
Table 1. Symptom characterization of episodes by influenza type or subtype.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>H3N2 (n=36)</th>
<th>H1N1 (n=62)</th>
<th>B (n=43)</th>
<th>pH1N1 (n=97)</th>
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<tbody>
<tr>
<td>Fever (%)</td>
<td>21 (58)</td>
<td>15 (24)</td>
<td>17 (40)</td>
<td>33 (34)</td>
</tr>
<tr>
<td>Cough (%)</td>
<td>32 (89)</td>
<td>39 (63)</td>
<td>33 (77)</td>
<td>70 (72)</td>
</tr>
<tr>
<td>Runny Nose (%)</td>
<td>32 (89)</td>
<td>25 (40)</td>
<td>30 (70)</td>
<td>60 (62)</td>
</tr>
<tr>
<td>Sore Throat (%)</td>
<td>21 (58)</td>
<td>24 (39)</td>
<td>17 (40)</td>
<td>35 (36)</td>
</tr>
<tr>
<td>Headache (%)</td>
<td>12 (33)</td>
<td>13 (21)</td>
<td>21 (49)</td>
<td>47 (48)</td>
</tr>
<tr>
<td>Sinus Problems (%)</td>
<td>15 (42)</td>
<td>12 (19)</td>
<td>18 (42)</td>
<td>29 (30)</td>
</tr>
<tr>
<td>Muscle Aches</td>
<td>10 (28)</td>
<td>11 (18)</td>
<td>8 (19)</td>
<td>31 (32)</td>
</tr>
<tr>
<td>Chills</td>
<td>21 (58)</td>
<td>19 (31)</td>
<td>27 (63)</td>
<td>52 (54)</td>
</tr>
</tbody>
</table>
Table 2. Influenza viral shedding by age.

<table>
<thead>
<tr>
<th>Age at Infection</th>
<th>Number of participants</th>
<th>Mean number of days</th>
<th>SD (days)</th>
<th>Minimum n. of days</th>
<th>Maximum no. of days</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5</td>
<td>53</td>
<td>5.0</td>
<td>3.3</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>5 &lt; 9</td>
<td>48</td>
<td>5.7</td>
<td>3.8</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>9 &lt; 16</td>
<td>65</td>
<td>4.9</td>
<td>3.0</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>&gt; 16</td>
<td>72</td>
<td>3.7</td>
<td>2.6</td>
<td>1</td>
<td>10</td>
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