Recovery of Influenza B Virus with the H273Y Point Mutation in the neuraminidase Active site from a Human Patient

Rachel R. Higgins, 1* Melissa Beniprashad, 1 Eddie Chong-King, 1 Yan Li, 5 Nathalie Bastien, 5
Donald E. Low 1,2,3 and Jonathan B. Gubbay 1,2,4

1 Public Health Ontario, 81 Resources Road, Toronto M9P 3T1, Canada
2 University of Toronto, Ontario, Canada
3 Mount Sinai Hospital, Toronto
4 The Hospital for Sick Children, Toronto, Ontario, Canada
5 National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba

*Corresponding author: Rachel R. Higgins, Public Health Ontario, 81 Resources Rd, Toronto, ON M9P 3T1, Canada; Phone: 416-235-6042; Fax: 416-235-6550; email: rachel.higgins@oahpp.ca

The H275Y oseltamivir resistance mutation confers high level resistance to oseltamivir in isolates of human A/H1N1 influenza. We report the recovery and identification of an influenza B virus with the H273Y neuraminidase point mutation directly from a human patient. The H273Y influenza B isolate is resistant to oseltamivir and peramivir but sensitive to zanamivir.

Resistance to oseltamivir is conferred on seasonal and pandemic A/H1N1 neuraminidase (NA) by a histidine-to-tyrosine mutation at position 275 (corresponds to 273 in B and 274 in N2 NA amino acid numbering) which causes a structural change in the protein active site that weakens the binding of oseltamivir (5). Although initially rare, this mutation became prevalent in clinical samples from treated patients during the 2007-2008 influenza season. Seasonal
influenza A/H1N1 viruses with this mutation reached a prevalence of nearly 11\% by the end of
the season in North American and began to spread globally to near fixation by the 2008-2009
season (2, 6, 12). Resistance to oseltamivir in the pandemic A/H1N1 virus subtype (A/H1N1-
pdm09) appears to be rare despite the widespread use of this drug during the 2009 influenza
A/H1N1 pandemic (10, 13, and manuscript in preparation). Although amino acid residues at
the neuraminidase active site are highly conserved in influenza A and B types, known
neuraminidase substitutions identified in resistant viruses from humans tend to be virus-type-
or subtype-specific. For example, E119V (Glu119Val), R292K (Arg292Lys), and N294S
(Asn294Ser) are specific to the A/H3N2 subtype; H275Y (His275Tyr) is specific to the
A/H1N1 and H5N1 subtypes and R152K (Arg152Lys), D198N (Asp198Asn), I222T
(Isoleucine222Threonine) and R371K (Arginine 371Lysine) are specific to the influenza B
type (1, 11, 12, 17). All these substitutions are associated with catalytic or frame work
residues in the active site of the neuraminidase protein (4). The H275Y specific to the N1
subtype was shown to arise in one influenza B (H273Y) isolate after 15 passages in cell culture
and in the presence of increasing concentrations of the neuraminidase inhibitor (NAI)
peramivir (3). Another study described the detection of a similar influenza B virus from a
clinical specimen with the H275Y point mutation and reduced sensitivity to oseltamivir (17).
Here we report the recovery and identification of an influenza B virus with the H273Y
oseltamivir mutation directly from a human patient with no known history of previous
oseltamivir treatment. We show that the H273Y influenza B isolate is resistant to oseltamivir
and peramivir but sensitive to zanamivir.

On April 10, 2011, a patient with fever and cough presented at an emergency room of a
regional hospital but was not admitted. The patient was male and age 33. No history of the
patient is available and it is not known if the patient was previously treated with antiviral or neuraminidase inhibitor. Nasopharyngeal swab was taken and tested for influenza by virus culture. Influenza B was detected and the sample designated B/Ontario/006876/2011 was submitted for further testing.

As part of Public Health Ontario pandemic surveillance program, the specimen was cultured in rhesus monkey kidney cells and subjected to NAI testing for resistance to oseltamivir carboxylate, peramivir and zanamivir. Specimens with elevated IC_{50} are further subjected to strain typing and DNA sequencing of NA or whole genome, using a modified World Health Organization protocol. Chemiluminescent NAI testing of B/Ontario/006876/2011 showed sensitivity to zanamivir (IC_{50} 0.93 ± 0.66), but elevated IC_{50} to both oseltamivir and peramivir.

The IC_{50} level for oseltamivir was 19.4 ± 0.65 nM and for peramivir 11.12 ± 0.23 nM or 11.4 fold and 34.8 fold increase respectively, relative to reference influenza B virus (Table 1). B/Ontario/006876/2011 isolate showed elevated oseltamivir and peramivir IC_{50} values compared with reference wild-type influenza B, wild-type pandemic A/H1N1-pdm09, and wild-type A/H3N2 viruses (Table 1). When comparing the pandemic A/H1N1-pdm09 virus with the oseltamivir-resistance conferring H275Y (N1 numbering) substitution and an influenza A/H3N2 virus with the oseltamivir-resistance conferring E119V substitution, the B/Ontario/006876/2011 B viruses showed intermediate susceptibility (Table 1). The reference influenza B virus carrying the R152K substitution was resistant to all NAIs compared with the influenza B viruses with H273Y (Table 1). Strain typing performed at Canada’s National Microbiology Laboratory (NML) identified the isolate as B/Wisconsin/01/2008-like strain. As well, H273Y resistance results, for oseltamivir and zanamivir, were independently confirmed by the NML.

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Sequence analysis for the NA gene of B/Ontario/006876/2011 showed a novel substitution for influenza B, present as Histidine to Tyrosine at position 273 (corresponds to position 275 in N1 and 274 in N2 NA amino acid numbering). A total of 251 influenza B virus isolates from patients (age 4 months to 92 years old) across Ontario submitted to the Public Health Ontario (PHO) from November 2010 through May 2011 for routine testing and surveillance were screened for susceptibility to the neuraminidase inhibitors oseltamivir and zanamivir. None of these isolates had the H273Y mutation. A substitution of H to Y at position 273 has previously been associated with reduced susceptibility to NAIs in one B virus isolate generated in tissue culture passage 15 and in response to increasing concentration of peramivir (3, 7, 11, 17). Moreover, reduced susceptibility to oseltamivir has been reported in viruses with variation at the corresponding residue (275, N1 NA numbering) in the pandemic A/H1N1-pdm09 virus (14, 18) and in influenza A/H5N1 (8). The sequence was deposited into GenBank under accession no JN601140. Furthermore, because some susceptibility-altering NA mutations have been shown to arise from virus propagation in tissue culture (15), sequencing of the neuraminidase gene was also performed on primary clinical specimens to rule out cell culture selection. The H273Y substitution was identified in the matching primary clinical specimen (Figure 1). Notably, the presence of the H273Y mutation in the primary clinical specimen, as well as, in the matching cultured virus isolates, may indicate a potential selective pressure for this mutation. In comparison with B/Wisconsin/01/2008 (influenza B, accession number ACT20880) four additional non-synonymous mutations were identified in the NA gene: T46P (Thr46Pro), L153M (Leu153Met), E288M (Glu288Gly) and T395M (Thr395Met), of which E288G appeared only in first passage culture and the remaining three mutations appeared in both first passage culture and primary specimen (Figure 1). In summary, sequencing the
neuraminidase gene of the influenza B isolate taken from this patient revealed the presence of
the H273Y oseltamivir resistance mutation in both primary and cultured specimens, as well as,
four additional mutations whose phenotypic and genotypic roles in the onset of resistance to
NAI is yet to be elucidated (Figure 1).

Although the NA change H275Y has been seen among the N1 NA subtype of influenza A
viruses (7, 16) and influenza B (3, 17) such a change has not been directly identified and
characterized in influenza B viruses isolated from human patients. Amino acid 273 is known to
be a highly conserved residue of the NA enzyme active site in influenza B. To date, all
influenza viruses with the H275Y substitution appear to be limited to the N1 type, H1N1 and
H5N1. Resistance to NAI in influenza is scored based on two criteria: a high IC50 (that is at
least ten fold greater than the mean) and the presence of a previously described mutation in the
NA, arising in drug treated patients (17). Accordingly, oseltamivir IC50 values obtained with
the influenza B viruses carrying the H273Y substitution are comparable to those seen with
influenza A/H1N1 viruses carrying the oseltamivir-resistance conferring substitution H275Y,
the clinical significance of the altered susceptibility associated with H273Y in influenza B
viruses is unknown at this time and warrants further investigation. Furthermore, such variant
with elevated IC50 values highlight the need for establishing a correlation between laboratory-
determined IC50 values and clinical resistance.

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FIG 1. Nucleic acid sequence of the neuraminidase gene from B/Ontario/006876/2011 virus isolated from matching primary (P) and culture (C) specimen. By comparison to wild type (WT) reference control (AC#: ACT2880), position 273 in the wild type virus contains a histidine (H) residue whereas in B/Ontario/006876/2011, tyrosine is present at this position. Note also the genetic alterations at positions 46 (T46P), 153 (L153M), 288 (E288G) and 395 (T395A).
TABLE 1. Antiviral susceptibility and genotype of an influenza B virus with H273Y substitution, recovered from a patient in Ontario, Canada during the 2010-2011 influenza season

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Type/subtype</th>
<th>Passage/Virus subset</th>
<th>NA Mutation</th>
<th>Oseltamivir carboxylate</th>
<th>Peramivir</th>
<th>Zanamivir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean IC&lt;sub&gt;50&lt;/sub&gt;±SD (nM)</td>
<td>x-fold difference</td>
<td>Mean IC&lt;sub&gt;50&lt;/sub&gt;±SD (nM)</td>
</tr>
<tr>
<td>B/Ontario/006876/2011</td>
<td>B</td>
<td>Primary</td>
<td>H273Y</td>
<td>not tested</td>
<td>not tested</td>
<td>not tested</td>
</tr>
<tr>
<td>B/Ontario/006876/2011</td>
<td>B</td>
<td>1</td>
<td>H273Y</td>
<td>19.4±0.65</td>
<td>11.4</td>
<td>11.12±0.23</td>
</tr>
<tr>
<td>B/Memphis/20/96</td>
<td>B</td>
<td>Reference</td>
<td>WT</td>
<td>1.32±0.85</td>
<td>1</td>
<td>0.32±0.23</td>
</tr>
<tr>
<td>B/Memphis/20/96</td>
<td>B</td>
<td>Reference</td>
<td>R152K</td>
<td>156.25±70.04</td>
<td>118</td>
<td>43.14±15.32</td>
</tr>
<tr>
<td>A/California/07/2009</td>
<td>A/H1N1-2009</td>
<td>Reference</td>
<td>WT</td>
<td>0.12±0.04</td>
<td>1</td>
<td>0.08±0.04</td>
</tr>
<tr>
<td>A/North Carolina/39/2009</td>
<td>A/H1N1-2009</td>
<td>Reference</td>
<td>H273Y</td>
<td>81.25±42.16</td>
<td>677</td>
<td>9.05±1.54</td>
</tr>
<tr>
<td>A/Wuhan/395/95</td>
<td>A/H3N2</td>
<td>Reference</td>
<td>WT</td>
<td>0.27±0.11</td>
<td>1</td>
<td>0.23±0.14</td>
</tr>
<tr>
<td>A/Wuhan/395/95</td>
<td>A/H3N2</td>
<td>Reference</td>
<td>E119V</td>
<td>5.01±2.18</td>
<td>18.5</td>
<td>0.19±0.09</td>
</tr>
</tbody>
</table>
Susceptibility to neuraminidase inhibitors, oseltamivir and zanamivir, was measured by a chemiluminescent assay, using the NA-Star® kit (Applied Biosystems Inc). NA inhibition was assayed with equivalent NA enzyme activity and incubated with increasing concentrations of NAI from 0.0316 to 1000 nM. Curve fitting and the 50% inhibitory concentration (IC₅₀) were calculated by plotting percent inhibition relative to NAI concentration using GraphPad Prism 4 software. NA: neuraminidase, NAI: neuraminidase inhibition, IC₅₀: 50% inhibitory concentration, WT: wild type, SD: standard deviation, I: isoleucine, V: valine, N: asparagine, D: aspartic acid, H: histidine, Y: tyrosine, R: arginine, K: lysine. Mean IC₅₀ values for wild-type reference control viruses for each type/subtype are in boldface and were used to calculate the X-fold differences for their respective type/subtypes as indicated. Reference, susceptible and resistant reference viruses used as controls in NAI assays. IC₅₀ values for reference viruses represent the average taken from at least 5 replicates.