
Tatiana Baranovich, Phengta Vongphrachanh, Pakapak Ketmayoon, Thongchanh Sisouk, Khampheng Chomlasack, Viengphone Khanthamaly, Ha Thuy Nguyen, Vasilii P. Mishin, Henju Marjuki, John R. Barnes, Rebecca J. Garten, James Stevens, David E. Wentworth, Larisa V. Gubareva

In February 2016, three influenza B/Victoria/2/87 lineage viruses exhibiting 4- to 158-fold reduced inhibition by neuraminidase inhibitors were detected in Laos. These viruses had an H134N substitution in the neuraminidase and replicated efficiently in vitro and in ferrets. Current antiviral drugs may be ineffective in controlling infections caused by viruses harboring this mutation.

Influenza B viruses cause annual epidemics and contribute to ≥30% of influenza-associated deaths among children in the United States (1). Two lineages, B/Victoria/2/87 and B/Yamagata/16/88, have been co-circulating globally in recent years (2,3). Neuraminidase (NA) inhibitors (NAIs) are the only drugs available for treating influenza B virus infections, but NA mutations that emerge during treatment or due to natural variance can diminish the usefulness of NAIs.

The Study

For this study, the National Center for Laboratory and Epidemiology in Vientiane, Laos, a member of the World Health Organization Global Influenza Surveillance and Epidemiology in Vientiane, Laos, a member of the World Health Organization Collaborating Center at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA; the viruses had been collected during October 1, 2015–February 29, 2016. We propagated the viruses and then used the CDC standardized NA inhibition assay to assess their susceptibility to NAIs (4). Compared with the median 50% inhibitory concentration (IC\(_{50}\)) values for B-Victoria lineage viruses, IC\(_{50}\) values for 2 of the 24 B-Victoria lineage viruses, B/Laos/0406/2016 and B/Laos/0525/2016, were elevated for zanamivir (129- to 158-fold), oseltamivir (4-fold), peramivir (72- to 74-fold), and laninamivir (41- to 42-fold) (Table 1). These results were interpreted as highly reduced inhibition by zanamivir, normal inhibition by oseltamivir, and reduced inhibition by peramivir and laninamivir (Table 1) (5).

This interpretation is useful but obscures the higher median oseltamivir IC\(_{50}\) value (9.67 nmol/L vs. 0.42–1.47 nmol/L for other NAIs; Table 1) and the lower potency of oseltamivir in inhibiting NA activity of influenza B viruses (4,7). Moreover, reports from clinical studies indicate a lesser susceptibility of influenza B viruses to oseltamivir than to zanamivir (7–9). Although the laboratory criteria defining clinically relevant NAI resistance are not established, the inhibitory profiles of these 2 viruses suggest resistance to ≥1 antiviral drugs. NA sequence analysis revealed that both viruses had an amino acid substitution, histidine (H)→asparagine (N), at the highly conserved residue 134 (NA-H134N) (6); the presence of H134N in the respiratory specimens was confirmed by pyrosequencing (Figure 1) (10). NA-H134Y was previously reported in influenza B virus displaying reduced inhibition by peramivir (11). The inhibition profile of influenza B viruses bearing NA-H134N resembles that of influenza A(H1N1) viruses carrying NA-Q136R (residue 134 in influenza B NA corresponds to 136 in N1 numbering) (12). Residue 134 (136) has been implicated in the conformational change of the 150-loop, which may adversely affect the interaction between the NA active site and NAIs, especially those containing the guanidyl group (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/4/16-1876-Techapp1.pdf).

To expand testing, the Laos National Center for Laboratory and Epidemiology provided 40 additional specimens...
that were positive for B-Victoria lineage virus by real-time reverse transcription PCR (13), bringing the total number tested to 64. The specimens were collected during October 2015–April 2016 in Champassak (n = 41), Vientiane (n = 12), Luangprabang (n = 7), and Saravanh (n = 5) Provinces from 28 male and 37 female patients (median age 7 [range 0–67] years). Pyrosequencing revealed NA-H134N in 1 specimen; the respective isolate, B/Laos/0654/2016, displayed the expected NA inhibition profile (Table 1). In total, we found the NA-H134N substitution in 3 (4.6%) of the 8,601 sequences of influenza B virus deposited to the GISAID database (http://www.gisaid.org) that were positive for B-Victoria lineage virus by real-time reverse transcription PCR, essentially as described previously (10); primer NA-B-376Fs (5′-TGCAAAACACTTTG CTTTAACC-3′) was used for pyrosequencing. Underlining indicates nucleotide triplet encoding amino acid residue 134. Shading indicates the nucleotides used to determine the proportion of H134 and N134 neuraminidase variants. Pyrosequencing dispensation order: E-Enzyme mixture; S-substrate mixture; G, C, A and T – nucleotides dGTP, dCTP; dATPo and dTTP, correspondingly.

Table 1. Neuraminidase inhibitor susceptibility of influenza B viruses isolated from human respiratory specimens. Laos, 2016*

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>NA amino acid change§</th>
<th>Mean IC50 ± SD, nmol/L (fold change)††</th>
<th>Date specimen collected</th>
<th>GISAID accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Laos/0080/2016</td>
<td>H134</td>
<td>1.09 ± 0.16 (1)</td>
<td>14 Jan</td>
<td>EPIISL 222862</td>
</tr>
<tr>
<td>B/Laos/0406/2016</td>
<td>H134N</td>
<td>148.36 ± 14.40 (129)</td>
<td>9 Feb</td>
<td>EPIISL 230596</td>
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<tr>
<td>B/Laos/0525/2016</td>
<td>H134N</td>
<td>176.03 ± 11.14 (158)</td>
<td>15 Feb</td>
<td>EPIISL 230599</td>
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<tr>
<td>B/Laos/0654/2016</td>
<td>H134N</td>
<td>151.95 ± 16.30 (138)</td>
<td>25 Feb</td>
<td>EPIISL 230600</td>
</tr>
</tbody>
</table>

*Viruses were isolated and propagated on MDCK cells. Susceptibility was determined using a fluorescence-based neuraminidase (NA) inhibition assay. ††IC50 values (NA inhibitor concentration needed to reduce NA activity by 50%) represent mean ± SD from 3 independent experiments. §Fold change compared with the median IC50 value determined for influenza B-Victoria lineage viruses (n = 430) that were circulating worldwide during the 2015–16 influenza season. Median IC50 values are 1.11, 9.67, 0.42, and 1.47 nm for zanamivir, oseltamivir, peramivir, and laninamivir, respectively. Bold indicates fold increases that correspond to reduced inhibition (5- to 50-fold) or to highly reduced (>50-fold) inhibition by a NAI, as outlined by the World Health Organization Expert Working Group of the Global Influenza Surveillance and Response System for Surveillance on Antiviral Susceptibility (5). ¶Amino acid residue 134 in influenza A NA corresponds to residue Q136 in N1 and N2 NA amino acid numbering (6). ¶¶Oseltamivir carboxylate was used in NA inhibition assay.

Figure 1. Neuraminidase gene segment (nts 399–497) of influenza B/Laos/0080/2016 virus carrying NA-H134 (A) and B/Laos/0654/2016, NA-N134 (B). RNA extracted from respiratory specimens was used for reverse transcription PCR (RT-PCR) amplification. Two primers, NA-B-242F (5′-CATACCCGCGTTTAT CTTGC-3′, forward primer) and NA-B-426Rb (biotin-5′-CTGTCTCCTGTGCTTC ATTGTA-3′; reverse biotinylated primer) were used in RT-PCR, essentially as described previously (10); primer NA-B-376Fs (5′-TGCAAAACACTTTG CTTTAAC-3′) was used for pyrosequencing. Underlining indicates nucleotide triplet encoding amino acid residue 134. Shading indicates the nucleotides used to determine the proportion of H134 and N134 neuraminidase variants. Pyrosequencing dispensation order: E-Enzyme mixture; S-substrate mixture; G, C, A and T – nucleotides dGTP, dCTP; dATPo and dTTP, correspondingly.
The 3 drug-resistant viruses were genetically similar to other B-Victoria lineage viruses circulating in Laos during 2015–2016. Besides having the NA-H134N amino acid substitution, these viruses also shared the M1-H159Q amino acid substitution not identified in other virus sequences (Table 2). Also, these viruses have 3 synonymous nucleotide mutations: PB1-c93t, PB1-g1930a, and HA-g1520a. In addition, B/Laos-134N viruses differed from each other by the following synonymous nucleotide mutations: B/Laos/0406/2016 possessed NS1-g345a, B/Laos/0525/2016 possessed NA-D390D/E, B/Laos/0654/2016 possessed NS1-V225A, and B/Laos/0654/2016 possessed NS1-V220I. An analysis of influenza B NS1 sequences available in the GISAID database (as of September 12, 2016) indicated that NS1-V220I, NS1-V225A, and NS1-V220I, respectively (Table 2). Also, these viruses have 3 synonymous nucleotide mutations: PB1-c93t, PB1-g1930a, and HA-g1520a.

To determine whether this change also affects other properties (e.g., thermostability) of the enzyme, we incubated 3 H134N viruses at elevated temperatures for 15 min and then assessed their NA activity (Figure 2, panel A). The H134N substitution reduced the thermostability of the enzyme. This was evident from the undetectable activity levels starting at 47.5°C, which was 7.5°C lower than that for the control virus, B/Laos/0880/2016, with H134 (p<0.001) (Figure 2, panel A).

To assess the replicative fitness of NA-H134N viruses, we used primary human differentiated normal human bronchial epithelial (NHBE) cells, a cell culture system that morphologically and functionally recapitulates the human airway. The NA-H134N viruses displayed ≈1–2 log_{10} lower titers at 24–72 h after inoculation (Figure 2, panel B). Although, the virus yield reduction (area under the curve) was evident for 2 of the NA-H134N viruses (AUC_{72} (p<0.05)) (Figure 2, panel B), the difference was not statistically significant for B/Laos/0525/2016 (Figure 2, panel B). The growth kinetics data in differentiated NHBE cells indicate an attenuated phenotype for NA-H134N viruses in vitro. Unlike the other 2 drug-resistant viruses, B/Laos/0654/2016 harbored substitution NS1-V220I, which resides at the recently discovered second RNA binding site of the NS1 protein of influenza B viruses (15). This finding suggests a possible compensatory effect of NS1-V220I on the in vitro replicative capacity of B/Laos/0654/2016.

We assessed the replicative fitness of drug-resistant B/Laos/0654/2016 in three 4- to 6-month-old male ferrets (Mustela putorius furo) (Triple F Farms, Sayre, PA, USA) that were serologically negative by HI assay for currently circulating influenza A(H1N1)pdm09, A(H3N2), and B viruses. At 48 h after inoculation with virus (10^4 50% tissue culture infectious dose/mL), ferrets displayed fever (≥1.5°C above baseline) that lasted 21.8 ± 5.1 h on average. Virus shedding lasted 6 days; nasal wash virus titers, which were determined daily, were 4.2 ± 0.4; 6.0 ± 0.2; 4.8 ± 0.4, 4.7 ± 0.4, 4.7 ± 0.6, and 2.8 ± 0.4 log_{10} 50% tissue culture infectious doses/mL, respectively. These data suggest that the drug-resistant virus can replicate to high titers in the upper respiratory tract of ferrets and induce persistent fever.

Conclusions

In February 2016, we detected 3 influenza B viruses in Laos bearing a rare NA-H134N substitution. Current antiviral medications may not effectively control infections.
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casted by such viruses. Virus harboring NA-H134N and NS1-V220I replicated efficiently in NHBE cells and in the ferret upper respiratory tract. Studies to ascertain the effect of NA-H134N and NS1-V220I on influenza B virus virulence and transmissibility in a mammalian host are needed.

Acknowledgments
We thank the laboratories that and clinicians who submit specimens and isolates to the World Health Organization Collaborating Center for Influenza in Atlanta, Georgia, USA. We greatly value the technical assistance provided by Michelle Adamczyk, Lori Lollis, Juan De la Cruz, Anton Chesnokov, and members of Reference and Genomic Teams in the Virology, Surveillance and Diagnosis Branch, Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. We thank Hoffmann-La Roche Ltd, Switzerland, for providing oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir phosphate; GlaxoSmithKline, Australia, for providing zanamivir; BioCryst Pharmaceuticals, USA, for providing peramivir; and Biota, Australia, for providing laninamivir.

This study was funded by the Centers for Disease Control and Prevention.

Dr. Baranovich worked in the Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, during the conduct of this study. Her research interests include the molecular mechanisms of influenza virus resistance to antiviral medications and the effect of resistance mutations on viral fitness and evolution.

References

Figure 2. Characterization of influenza B viruses detected in Laos, February 2016. A) Thermostability of neuraminidase (NA) determined after viruses were incubated for 15 min at 4°C or at 30°C–57°C. NA enzyme activity was determined by a fluorescence-based assay (4). B) Replication kinetics of influenza B viruses in fully differentiated human primary NHBE cells that were inoculated with the designated viruses (multiplicity of infection 0.001). Apical washes were taken at indicated times after inoculation, and virus titers were determined on MDCK cells. The area under the virus titer curve from 2 to 72 h after inoculation (AUC<sub>2–72</sub>) was determined and compared with that of the control virus by repeated-measures analysis of variance with the Dunnett posttest, using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Dashed line represents the limit of detection of the assay (1.75 log<sub>10</sub> 50% tissue culture infective dose [TCID<sub>50</sub>/mL]). Values shown are means and SDs from 2 independent experiments performed in duplicates (n = 4). Error bars represent SDs. NS, not significant.

Address for correspondence: Larisa V. Gubareva, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G16, Atlanta, GA 30329-4027, USA; email: lgubareva@cdc.gov

Technical Appendix

**Technical Appendix Table 1.** Accession numbers for the genome sequences deposited into the GISAID database

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<th>Virus name</th>
<th>Collection Date</th>
<th>NA amino acid change</th>
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<th>HA</th>
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<td>None</td>
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<td>EPI765460</td>
<td>EPI765464</td>
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<tr>
<td>B/Laos/0406/2016</td>
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**Technical Appendix Table 2.** Quantification of a proportion of H134 and N134 neuraminidase variants in respiratory specimens harboring influenza B viruses collected in Laos, February 2016*

<table>
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<th>Virus</th>
<th>NA amino acid</th>
<th>Mean (%) ± SD</th>
</tr>
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<tbody>
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<td>H134</td>
<td>99.6 ± 0.5</td>
</tr>
<tr>
<td>B/Laos/0406/2016</td>
<td>H134N</td>
<td>0.7 ± 0.6</td>
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<tr>
<td>B/Laos/0525/2016</td>
<td>H134N</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>B/Laos/0654/2016</td>
<td>H134N</td>
<td>0.4 ± 0.5</td>
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*Pyrosequencing analysis in the allele quantification mode was conducted on the respective respiratory specimens in triplicate.
Technical Appendix Figure. Structure of the active site of B/Brisbane/60/2008 (Victoria lineage) neuraminidase with the bound neuraminidase inhibitor zanamivir; Protein Data Bank code 4cpn.