Antiviral Drug–Resistant Influenza B Viruses Carrying H134N Substitution in Neuraminidase, Laos, February 2016

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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 Response System, provided influenza A and B viruses to 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

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DOI: http://dx.doi.org/10.3201/eid2304.161876
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 Champasack (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 65 tested B-Victoria viruses. Analysis of NA sequences deposited to the GISAID database (http://www.gisaid.org) revealed that among 8,601 sequences of influenza B virus collected worldwide during October 2014–September 2016, only 3 other sequences contained a substitution at H134 (2 harbored H134Y and 1 H134L); the 3 sequences were for B-Victoria lineage viruses.

Epidemiologic data revealed that the NA-H134N viruses were collected from a young woman, a young man, and a 3-year-old girl residing in 2 distant provinces (Table 2). The 3 infections occurred 6–10 days apart in February 2016, and 1 of the patients received medical care for severe acute respiratory illness. No epidemiologic links were identified among the 3 patients infected with the drug-resistant viruses, and patients had no documented exposure to NAIs.

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

| Virus isolate          | NA amino acid change§ | Mean IC50 ± SD, nmol/L (fold change)†‡ | Date | GISAID accession no. |
|------------------------|------------------------|----------------------------------------|------|----------------------|
| B/Laos/0080/2016       | H134                   | 1.09 ± 0.16 (1)                        | 14 Jan | EPIISL 222862        |
| B/Laos/0406/2016       | H134N                  | 148.36 ± 14.40                        | 9 Feb | EPIISL 230596        |
| B/Laos/0525/2016       | H134N                  | 176.03 ± 11.14                        | 15 Feb | EPIISL 230599        |
| B/Laos/0654/2016       | H134N                  | 151.95 ± 16.30                        | 25 Feb | EPIISL 230600        |

*Viruses were isolated and propagated on MDCK cells. Susceptibility was determined using a fluorescence-based neuraminidase (NA) inhibition assay. †Fold change compared with the median IC50 value determined for influenza B-Victoria lineage viruses (n = 430) that were circulating worldwide during the 2015–2016 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 type B NA corresponds to residue Q136 in N1 and N2 NA amino acid numbering (5).

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′-CTGCTCCTTGTGTC ATTGTAG-3′; reverse biotinylated primer) were used in RT-PCR, essentially as described previously (10); primer NA-B-378Fs (5′-TGCAAAACTTGG CTTAAC-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; dATPdS 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-2016 viruses differed from each other by the following synonymous nucleotide mutations: B/Laos/0406/2016 possessed NS1-g345a, B/Laos/0525/2016 possessed NA-D390, 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, NS2-g186a, and B/Laos/0654/2016 possessed NS1-g345a, B/Laos/0525/2016 possessed NA-D390, and B/Laos/0654/2016 possessed NS1-V220I.

Results of the NA inhibition assay showed that NA-H134N viruses circulating in Laos communities.

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
caused 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
1. Burnham AJ, Baranovich T, Govorkova EA. Neuraminidase inhibitors for influenza B virus infection: efficacy and resistance. Antiviral Res. 2013;100:520–34. http://dx.doi.org/10.1016/j.antiviral.2013.08.023
2. Budd A, Blanton L, Kniss K, Smith S, Mustauqim D, Davlin SL, et al. Update: influenza activity—United States and worldwide, May 22–September 10, 2016. MMWR Morb Mortal Wkly Rep. 2016;65:1008–14. http://dx.doi.org/10.15585/mmwr.mm6537a5
3. Davlin SL, Blanton L, Kniss K, Mustauqim D, Smith S, Kramer N, et al. Influenza activity—United States, 2015–16 season and composition of the 2016–17 influenza vaccine. MMWR Morb Mortal Wkly Rep. 2016;65:767–75. http://dx.doi.org/10.15585/mmwr.mm6522a3
4. Okomo-Adhiambo M, Mishin VP, Sleeman K, Saguar E, Guevara H, Reisdorf E, et al. Standardizing the influenza neuraminidase inhibition assay among United States public health laboratories conducting virological surveillance. Antiviral Res. 2016;128:28–35. http://dx.doi.org/10.1016/j.antiviral.2016.01.009
5. World Health Organization. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility—Geneva, November 2011 and June 2012. Wkly Epidemiol Rec. 2012;87:369–74.
6. Colman PM, Hoyne PA, Lawrence MC. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J Virol. 1993;67:2972–80.
7. Kawai N, Ikematsu H, Iwaki N, Maeda T, Satoh I, Hirotsu N, et al. A comparison of the effectiveness of oseltamivir for the treatment of influenza A and influenza B: a Japanese multicenter study of the 2003–2004 and 2004–2005 influenza seasons. Clin Infect Dis. 2006;43:439–44. http://dx.doi.org/10.1086/505868
8. Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, et al. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother. 2006;50:2395–402. http://dx.doi.org/10.1128/AAC.01339-05
9. Sugaya N, Mitamura K, Yamazaki M, Tamura D, Ichikawa M, Kimura K, et al. Lower clinical effectiveness of oseltamivir against influenza B contrasted with influenza A infection in children. Clin Infect Dis. 2007;44:197–202. http://dx.doi.org/10.1086/509925
10. Sheu TG, Deyde VM, Garten RJ, Klimov AI, Gubareva LV. Detection of antiviral resistance and genetic lineage markers in influenza B virus neuraminidase using pyrosequencing. Antiviral Res. 2010;85:354–60. http://dx.doi.org/10.1016/j.antiviral.2009.10.022
11. Takashita E, Meijer A, Lackenby A, Gubareva L, Rebello-de-Andrade H, Besselaar T, et al. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013–2014. Antiviral Res. 2015;117:27–38. http://dx.doi.org/10.1016/j.antiviral.2015.02.003
12. Little K, Leang SK, Butler J, Baas C, Harrower B, Mosse J, et al. Zanamivir-resistant influenza viruses with Q136K or Q136R neuraminidase residue mutations can arise during MDCK cell culture creating challenges for antiviral susceptibility monitoring. Euro Surveill. 2015;20:30060. http://dx.doi.org/10.2807/1560-7917.ES.2015.20.45.30060
13. Centers for Disease Control and Prevention. Human influenza virus real-time RT-PCR detection and characterization panel. 510(k) summary. 2008. http://www.accessdata.fda.gov/cdrh_docs/pdf8/k080570.pdf.
14. Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, et al. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and swine origin human influenza a viruses. J Virol. 2009;83:10309–13. http://dx.doi.org/10.1128/JVI.01109-09
15. Ma LC, Guan R, Hamilton K, Aramini JM, Mao L, Wang S, et al. A second RNA-binding site in the NS1 protein of influenza B virus. Structure. 2016;24:1562–72. http://dx.doi.org/10.1016/j.str.2016.07.001

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Technical Appendix

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

| Virus name          | Collection Date | NA amino acid change | PB2     | PB1      | PA       | Genome Segment                      |
|---------------------|-----------------|----------------------|---------|----------|----------|-------------------------------------|
| B/Laos/0080/2016    | 2016-01-14      | None                 | EPI765461| EPI765462| EPI765460| EPI765464 | EPI765457 | EPI765463 | EPI765459 | EPI765458 |
| B/Laos/0406/2016    | 2016-02-09      | H134N                | EPI775445| EPI775446| EPI775444| EPI775448 | EPI775441 | EPI775447 | EPI775443 | EPI775442 |
| B/Laos/0525/2016    | 2016-02-15      | H134N                | EPI765556| EPI765557| EPI765555| EPI765559 | EPI765552 | EPI765558 | EPI765554 | EPI765553 |
| B/Laos/0654/2016    | 2016-02-25      | H134N                | EPI775437| EPI775438| EPI775436| EPI775440 | EPI775433 | EPI775439 | EPI775435 | EPI775434 |

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

| Virus              | NA amino acid | Mean (%) ± SD  |
|--------------------|---------------|----------------|
|                    |               | H134 | H134N |
| B/Laos/0080/2016   | H134          | 99.6 ± 0.5 | 0.4 ± 0.5 |
| B/Laos/0406/2016   | H134N         | 0.7 ± 0.6  | 99.3 ± 0.6 |
| B/Laos/0525/2016   | H134N         | 1.6 ± 0.7  | 98.4 ± 0.7 |

*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.