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Short Communication

Neuraminidase inhibitor susceptibility testing of influenza type B viruses in China during 2010 and 2011 identifies viruses with reduced susceptibility to oseltamivir and zanamivir

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ABSTRACT

Influenza type B viruses are responsible for substantial morbidity and mortality in humans. Antiviral drugs are an important supplement to vaccination for reducing the public health impact of influenza virus infections. Influenza B viruses are not sensitive to M2 inhibitors which limit the current therapeutic options to two neuraminidase inhibitors (NAIs), oseltamivir and zanamivir, which are licensed in many countries. Drug resistance is a public health concern which has necessitated monitoring of influenza virus drug susceptibilities through active global surveillance. Here, we report the results of drug susceptibility surveillance of influenza type B viruses ($n = 680$) collected in mainland China during two calendar years, 2010 and 2011, assessed using functional neuraminidase (NA) inhibition (NI) assays. Four influenza B viruses exhibited reduced susceptibilities to oseltamivir, but not zanamivir, and shared the amino acid substitution I221T (ATC → ACC), at this conserved residue in the NA active site (I222T in N2 numbering). Additionally, a single virus with reduced susceptibility to both oseltamivir and zanamivir was identified and contained an amino acid substitution D197N (GAC → AAC) at another conserved residue in the NA active site (D198N in N2 numbering). This report underlies the importance of continued influenza antiviral susceptibility surveillance globally, even in countries where the use of NAIs has been low or non-existing.

Influenza type B viruses cause epidemics and are responsible for substantial morbidity and mortality in humans (Influenza-associated Pediatric deaths, 2011). Two lineages of influenza B viruses currently co-circulate worldwide, the Victoria lineage (viruses derived from B/Victoria/02/87) and the Yamagata lineage (viruses derived from B/Yamagata/16/88) (Shaw et al., 2002). However, current annual trivalent influenza vaccines contain only antigens from a single lineage. The neuraminidase (NA) inhibitors (NAIs), oseltamivir and zanamivir, are currently the only antiviral drugs licensed to treat influenza type B infections in many countries, including China.

The emergence and spread of oseltamivir-resistant A(H1N1) viruses in countries where oseltamivir had not been used during the 2007–2008 and 2008–2009 influenza seasons (Meijer et al., 2009; Sheu et al., 2008) underscored the necessity for rigorous monitoring of the antiviral susceptibility of influenza viruses and the development of novel antiviral agents targeting influenza virus. Recent reports documenting the oseltamivir-resistant 2009 A(H1N1) pandemic influenza viruses (A(H1N1)pdm09) from patients with no known exposure to oseltamivir (Hurt et al., 2011; Lackenby et al., 2011; Storms et al., 2012) are worrisome and necessitate continuous surveillance globally.

In laboratory tests influenza B viruses have been shown to be less susceptible to oseltamivir than influenza A viruses (Escuret et al., 2008; Okomo-Adhiambo et al., 2010; Sheu et al., 2008). Although the clinical relevance of these findings is unknown at this time, oseltamivir has been shown to be less effective at treating influenza B infections in children than influenza A infections (Sato et al., 2008; Sugaya et al., 2007). Influenza B viruses with reduced susceptibilities to NAIs have been isolated either from patients following treatment with NAIs (Escuret et al., 2008; Gubareva et al., 1998; Hatakeyama et al., 2007; Ison et al., 2006) or from patients with no known NAI exposure at the time of specimen collection (Bastien et al., 2011; Hatakeyama et al., 2007; Hurt et al., 2006;

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Table 1
NAI susceptibility of influenza B isolates by chemiluminescent NI assay.

NA inhibitors	B lineage	IC_{50} (nM)						Outliers[e] [AA change (B numbering); IC_{50} (fold chage)]
		2010 (January–December)			2011 (January–December)			
		No. analyzed (n)[a]	Median[b] (range[c])	Mean ± SD[d]	No. analyzed (n)[a]	Median[b] (range[c])	Mean ± SD[d]	
Oseltamivir	All B viruses	90	2.72 (1.25–4.79)	2.74 ± 0.63	468	3.88 (0.20–45.59)	4.13 ± 1.62	
	B/Victoria/02/87	53	2.45 (1.25–4.05)	2.47 ± 0.54	299	3.42 (0.20–8.97)	3.64 ± 1.41	
	B/Yamagata/16/88	37	3.09 (1.88–4.79)	3.04 ± 0.61	169	4.83 (1.00–45.59)	5.02 ± 1.60	B/Beijing-Xicheng/11496/2011 [D197N; 26.78 nM (5)] B/Shanghai-Pudongxin/1125/2011 [I221T; 45.59 nM (9)]
Zanamivir	All B viruses	90	2.37 (0.91–6.97)	2.72 ± 1.21	468	0.96 (0.16–14.04)	1.26 ± 0.76	
	B/Victoria/02/87	53	1.93 (0.91–4.36)	2.11 ± 0.67	299	0.79 (0.16–2.84)	0.86 ± 0.35	
	B/Yamagata/16/88	37	3.34 (1.41–6.97)	3.59 ± 1.29	169	1.93 (0.25–14.04)	1.96 ± 0.83	B/Beijing-Xicheng/11496/2011 [D197N; 14.04 nM (7)]

AA, amino acid.
[a] Number of all tested isolates, including outliers, which were analyzed to determine statistical cutoff for outliers.
[b] Median of IC_{50} values for the drug among susceptible viruses (outliers excluded in calculation).
[c] Minimum to maximum IC_{50} values for all tested viruses (outliers included). Where IC_{50} is the inhibitory concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%. IC_{50} values were calculated using JASPR curve-fitting software.
[d] Mean and standard deviation (SD) of IC_{50} values for the drug among susceptible viruses (outliers excluded in calculation).
[e] Outliers with IC_{50} above the cutoff and >10 times the mean IC_{50} for each drug, were characterized as extreme outliers. Mild outliers were isolates with $IC_{50} > X_{0.75} + 3IQR$, but >2-fold and <10-fold that of the mean IC_{50} of the drug for the virus type/subtype.

Table 2
NAI susceptibility of influenza B isolates by fluorescent NI assay.

NA inhibitors	B lineage	IC_{50} (nM)						Outliers[e] [AA change (B numbering); IC_{50} (fold chage)]
		2010 (January–December)			2011 (January–December)			
		No. analyzed (n)[a]	Median[b] (range[c])	Mean ± SD[d]	No. analyzed (n)[a]	Median[b] (range[c])	Mean ± SD[d]	
Oseltamivir	All B viruses	34	11.43 (4.16–78.60)	16.44 ± 17.07	114	7.06 (2.22–19.35)	7.75 ± 3.52	B/Chongqing-Yuzhong/1476/2010B/
	B/Victoria/02/87	14	12.78 (4.16–26.83)	13.71 ± 6.69	70	7.48 (2.22–19.35)	8.45 ± 4.00	Chongqing-Yuzhong/1480/2010B/
	B/Yamagata/16/88	20	9.18 (4.17–78.60)	9.95 ± 5.84	44	6.18 (3.84–13.37)	6.66 ± 2.18	Chongqing-Banan/1304/2010 [I221T; 54.29nM-78.60 nM (5–8)]
Zanamivir	All B viruses	34	3.49 (0.93–7.34)	3.70 ± 1.52	114	1.09 (0.44–3.02)	1.24 ± 0.61	
	B/Victoria/02/87	14	3.89 (0.93–6.47)	3.90 ± 1.32	70	0.8(0.44–2.90)	0.90 ± 0.38	
	B/Yamagata/16/88	20	2.90 (2.00–7.34)	3.56 ± 1.66	44	1.65 (0.86–3.02)	1.71 ± 0.49	

AA, amino acid.
[a] Number of all tested isolates, including outliers, which were analyzed to determine statistical cutoff for outliers.
[b] Median of IC_{50} values for the drug among susceptible viruses (outliers excluded in calculation).
[c] Minimum to maximum IC_{50} values for all tested viruses (outliers included). Where IC_{50} is the inhibitory concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%. IC_{50} values were calculated using JASPR curve-fitting software.
[d] Mean and standard deviation (SD) of IC_{50} values for the drug among susceptible viruses (outliers excluded in calculation).
[e] Outliers with IC_{50} above the cutoff and >10 times the mean IC_{50} for each drug, were characterized as extreme outliers. Mild outliers were isolates with $IC_{50} > X_{0.75} + 3IQR$, but >2-fold and <10-fold that of the mean IC_{50} of the drug for the virus type/subtype.

Monto et al., 2006; Sleeman et al., 2011). The growing number of NA mutations (Nguyen et al., 2012), acquired as a result of drug–selection pressure or due to natural drift in influenza B viruses, presents a challenge for NAI-susceptibility testing by surveillance laboratories.

Antiviral susceptibility testing has become an integral facet of ongoing influenza surveillance activities and is performed worldwide by World Health Organization Collaborating Centers (WHO CC) as well as other public health laboratories. Antiviral susceptibility testing of influenza viruses using the functional phenotypic neuraminidase inhibition (NI) assay is a recently implemented initiative in mainland China. As part of influenza surveillance efforts and in collaboration with the WHO CC in Atlanta, GA, influenza type B virus isolates ($n = 680$) collected from the national influenza surveillance network in 31 provinces in mainland China during 2010 and 2011 were tested in the chemiluminescent (CL) and/or the fluorescent (FL) NI assays to assess their susceptibility to oseltamivir and zanamivir. Both NI assays were performed according to the US CDC assay protocols (Nguyen et al., 2010b; Okomo-Adhiambo et al., 2010) with the use of commercially available kits, the NA Star™ kit (Applied Biosystems) for the CL assay and the NA Fluor™ kit (Applied Biosystems) for the FL NI assay, which facilitates harmonization of data generated in different laboratories.

Influenza B viruses ($n = 98$) collected in mainland China in 2010 (1st January – 31st December) were assessed for drug susceptibility using either the CL ($n = 64$) or FL assay ($n = 8$), while a subset of viruses ($n = 26$) was tested using both assays. All viruses tested in the CL assay ($n = 90$) were susceptible to zanamivir and oseltamivir (Table 1) with IC_{50} values within previously reported ranges (Okomo-Adhiambo et al., 2010). The IC_{50} values were further analyzed according to antigenic lineage, with B/Victoria/02/87 lineage

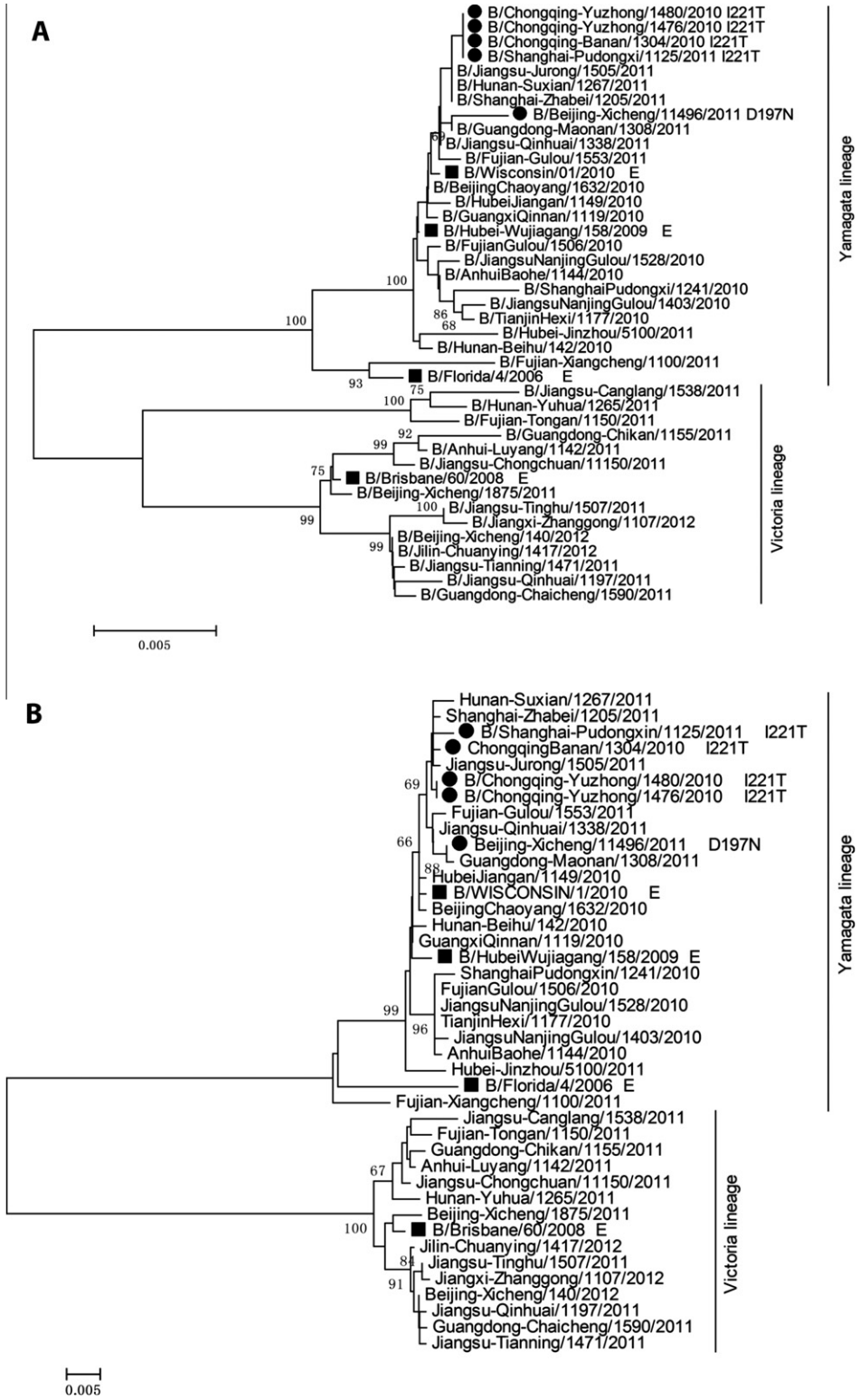

Fig. 1. Phylogenetic analysis of outliers from China in 2010 and 2011 with identified amino acid changes. Panel A: neuraminidase tree. Panel B: nemagglutinin tree. Black circle: outliers with identified amino acid changes from China in 2010 and 2011; black square: vaccine candidate strains (E, egg isolates); normal character: viruses with wild-type neuraminidase sequence and sensitive to both oseltamivir and zanamivir during 2010 and 2011 in China. Scale bars indicate number of base substitutions per site.

viruses ($n = 53$) exhibiting somewhat lower IC_{50} values to both zanamivir and oseltamivir compared to the B/Yamagata/16/88 lineage viruses ($n = 37$) (t test, $P < 0.0001$).

All viruses tested using the FL assay ($n = 34$) were susceptible to zanamivir and oseltamivir (Table 2), with the exception of three, B/Chongqing-Yuzhong/1476/2010, B/Chongqing-Yuzhong/1480/2010 and B/Chongqing-Banan/1304/2010, which demonstrated a 5 to 8-fold reduced susceptibility to oseltamivir compared to the mean IC_{50} for the B/Yamagata lineage viruses. These viruses were collected between 6th and 30th August 2010 and were isolated

from the same city, with two viruses originating from the same district. Whilst no epidemiological data is available for these patients, these findings highlight the importance of early detection of virus variants and the assessment of their prevalence in the community.

Pyrosequencing analysis (SQA) of the NA sequence of the three year 2010 viruses with elevated IC_{50} values for oseltamivir revealed a substitution of isoleucine (I) to threonine (T) at residue 221 (ATC → ACC, codon 222 in N2 numbering). Conventional sequence analysis of the NA gene confirmed the I221T substitution (GenBank accession numbers: KC244112, KC244113, KC244114). No additional changes, not seen in drug-susceptible viruses of B/Yamagata lineage, were found. The isoleucine at position 221 in the NA is conserved among all influenza A and B viruses and its replacement by other amino acids has been detected in influenza B viruses (Hatakeyama et al., 2007; Monto et al., 2006; Sheu et al., 2008; Sleeman et al., 2011) and influenza A viruses (Baz et al., 2006; Hurt et al., 2009; Nguyen et al., 2010a) with reduced susceptibility to one or more NAIs. Monitoring substitutions at this position is important since they have the potential to reduce the level of drug susceptibility (Hurt et al., 2009; Nguyen et al., 2010a; Van et al., 2010). The I221T substitution has previously been reported in influenza B viruses isolated from untreated patients (Hatakeyama et al., 2007), indicating that virus containing this substitution may be spreading in the absence of drug pressure. Furthermore, a cluster of influenza B viruses containing an I221V (valine) substitution isolated from patients ($n = 45$) in the U.S. with no documented exposure to NAIs, demonstrated an apparent reduced susceptibility to both oseltamivir and the investigational NAI, peramivir (Sleeman et al., 2011).

Viruses collected in 2011 ($n = 582$) were also tested in either the CL ($n = 468$) or FL ($n = 114$) NI assay. All tested viruses were susceptible to both zanamivir and oseltamivir in the CL NI assay, with the exception of five viruses which exhibited elevated IC_{50} for oseltamivir and/or zanamivir. Among them were two B/Yamagata lineage viruses, B/Shanghai-Pudongxin/1125/2011 and B/Beijing-Xicheng/11496/2011, which showed 5 to 9-fold reduced susceptibility to oseltamivir, and a 7-fold reduced susceptibility to zanamivir (Table 1). The virus B/Shanghai-Pudongxin/1125/2011 had the I221T (ATC → ACC) change in the NA, the same change identified in three viruses in 2010, while B/Beijing-Xicheng/11496/2011 (Table 1) had a D197N change (GAC → AAC) in the NA active site. The D197N substitution has previously been associated with reduced susceptibility to the two NAIs – oseltamivir and zanamivir in influenza B viruses (Hatakeyama et al., 2007; Ison et al., 2006; Sheu et al., 2008). No additional changes related to antiviral susceptibility were identified in these two viruses (GenBank accession numbers: KC244110, KC244111).

The other three outliers among year 2011 viruses were of B/Victoria lineage and demonstrated a 3-fold reduced susceptibility to zanamivir compared to the mean IC_{50} to zanamivir of the B/Victoria lineage viruses. No changes at positions previously associated with antiviral susceptibility were identified in the NA gene of these viruses.

All year 2011 viruses tested in the FL NI assay were susceptible to both NAIs and no apparent difference in IC_{50} values was seen between the two lineages (Table 2).

Phylogenetic analyses were performed for outliers with amino acid changes associated with altered drug susceptibility that were isolated during 2010 and 2011 (Fig. 1). The phylogenetic tree for NA sequences (Fig. 1Panel A) demonstrated that B viruses with the I221T and D197N change belong to the B/Yamagata lineage. The I221T viruses were clustered together due to the presence of this substitution, while the virus containing the D197N change was in a separate branch of the tree. A similar phylogeny was observed for the hemagglutinin (HA) sequences (Fig. 1Panel B) and the nucleic acid identity for the HA gene of the I221T viruses isolated in 2010 and 2011 was 99.6%.

This study identified influenza B viruses with reduced drug susceptibility to NAIs using either the CL or the FL NI assay. Four viruses with reduced susceptibilities to oseltamivir sharing the same amino acid change, I221T, were identified in two consecutive years, highlighting the active co-circulation of these viruses with wild-type influenza B viruses in China. In addition, a single B virus variant was detected which showed reduced susceptibility to oseltamivir and zanamivir caused by the D197N change in the NA. Both I221T and D197N changes have previously been identified in influenza B viruses collected from untreated and oseltamivir-treated patients in Japan (Hatakeyama et al., 2007), Australia (Hurt et al., 2006) and the United States (Sheu et al., 2008). Furthermore, the five virus variants detected in this study were each collected from patients who had no exposure to oseltamivir. These findings highlight the importance of sustained influenza antiviral susceptibility surveillance of community isolates.

Disclaimer

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the funding agency, the National Institute for Viral Disease Control and Prevention, Chinese Centre for Disease Control and Prevention (China CDC) and the U.S. Centers for Disease Control and Prevention (US CDC).

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