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Detection of antiviral resistance and genetic lineage markers in influenza B virus neuraminidase using pyrosequencing☆

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ABSTRACT

We report here the design of a pyrosequencing approach for the detection of molecular markers of resistance to the neuraminidase inhibitors zanamivir and oseltamivir in influenza viruses of type B. Primers were designed to analyze the sequences at eight amino acid positions E119, R152, D198, I222, S250, H274, R371, and G402 (universal A/N2 numbering) in the neuraminidase (NA) which have been previously found to be associated with resistance or reduced susceptibility to oseltamivir and/or zanamivir in the NA inhibition assay. In addition, the designed primers could be utilized to the distinguish between the NAs of influenza B viruses from the two major lineages (Victoria and Yamagata) that have co-circulated globally in recent years, thus providing a valuable tool for virus strain surveillance.

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1. Introduction

Vaccination is the primary strategy for the prevention of infections caused by influenza type A and B viruses. In contrast to influenza A viruses, which infect a wider range of species, influenza B viruses are primarily human pathogens. Antigenic and genetic analyses of the hemagglutinin (HA), a major surface antigen, are routinely performed for seasonal influenza A(H1N1), A(H3N2) and B viruses to update the components of the trivalent vaccine which includes both surface antigens, the HA and neuraminidase (NA), of epidemiologically relevant virus strains.

In some influenza seasons, type B viruses predominate globally while in other years they are less prevalent. Besides amino acid substitutions, insertions and deletions in the HA and/or the NA have been detected in influenza B viruses (McCullers et al., 1999; Nerome et al., 1998; Rota et al., 1992; Zou et al., 1997). Two lineages of influenza B viruses, designated by the HA and NA, Victoria-lineage (after B/Victoria/2/87) and Yamagata-lineage (after B/Yamagata/16/88) have been co-circulating since 1980s, until the mid-1990s when a new lineage with Victoria-like HA and Yamagata-like NA (VicHA–YamNA) emerged (Xu et al., 2004). The VicHA–VicNA lineage has since disappeared from circulation and the two lineages VicHA–YamNA and YamHA–YamNA have alternated in prevalence during the subsequent seasons. In recent years, the influenza B virus vaccine candidates for inclusion into seasonal vaccine have been selected either from the YamHA–YamNA (e.g., B/Florida/4/2006) or the VicHA–YamNA lineages (e.g., B/Brisbane/60/2008).

In addition to vaccination, antiviral drugs play an essential role in the prophylaxis and treatment of influenza infections. Currently, there are two FDA-approved classes of antiviral medications. Adamantane derivatives (rimantadine and amantadine) are the first and older class of anti-influenza drugs. This class, however, is not effective against the M2 of influenza B viruses (Hayden, 1996). Therefore, only the neuraminidase inhibitors (NAIs), comprising of the two licensed drugs, oseltamivir and zanamivir, are currently available for the control of influenza B virus infections. A third NAI, peramivir, is undergoing clinical evaluations. In the years prior to the 2007–2008 season, resistance to NAIs among influenza A and B viruses was low (<0.1%) among field isolates (McKimm-Breschkin et al., 2003; Monto et al., 2006; Mungall et al., 2004). From the 2007–2008 season to present, there has been an unprecedented increase of oseltamivir-resistance in influenza A(H1N1) viruses (Besselaar et al., 2009; Hauge et al., 2009; Lackenby et al., 2008b; Sheu et al., 2008). Influenza B viruses with reduced susceptibility to NAIs have also been detected through virus surveillance (Hurt et al., 2004, 2006; Sheu et al., 2008) and in clinical settings following drug treatment (Gubareva et al., 1998; Hatakeyama et al., 2007; Ison et al., 2006). Since molecular markers of resistance are not well characterized, the current method of choice for detecting resistance to NAIs is the NA enzyme inhibition assay. The NA genes of isolates with an elevated IC_{50} (concentration of the drug

☆ *Disclaimer*: The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Table 1
Influenza B reference viruses used for assay validation.

Virus	Lineage (HA–NA)[a]	Amino acid change in NA		Reference	Accession numbers
		B NA numbering	N2 NA numbering		
B/Illinois/03/2008	YamHA–YamNA	E117A	E119A	Present study	EU889022
B/Memphis/20/96	VicHA–VicNA	R150K	R152K	Gubareva et al. (1998)	GQ423424
B/Rochester/2/2001	VicHA–YamNA	D197N	D198N	Ison et al. (2006)	AY947471
B/Guatemala/558/2008	YamHA–YamNA	D197N	D198N	Present study	EPI163182
B/Canada/88/2007	YamHA–YamNA	D197N	D198N	Present study	GQ423426
B/Texas/38/2008	YamHA–YamNA	D197E	D198E	Present study	GQ340636
B/California/5/2005	VicHA–YamNA	I221T	I222T	Sheu et al. (2008)	EU879086
B/Michigan/20/2005	VicHA–YamNA	H273Y	H274Y	Sheu et al. (2008)	CY015375
B/Hong Kong/36/2005	VicHA–YamNA	R374K	R371K	Sheu et al. (2008)	EU879085

[a] Vic-like and Yam-like based on the antigenic and genetic analysis of the HA and the NA, respectively.

needed to reduce enzyme activity by 50%) determined by this assay are typically sequenced in order to determine molecular markers that could be responsible for reduced susceptibility to NAIs. However, the clinical relevance of some identified mutations remains unknown.

Pyrosequencing methods have been employed to detect molecular markers of resistance to adamantanes and NAIs for seasonal influenza A viruses as well as highly pathogenic avian influenza A(H5N1) viruses (Bright et al., 2006; Deyde et al., 2009a,b; Duwe and Schweiger, 2008; Higgins et al., 2009; Lackenby et al., 2008a) and the 2009 pandemic A(H1N1) viruses (Gubareva et al., 2009), however, a pyrosequencing approach for detection of molecular markers associated with resistance or reduced NAI susceptibility in influenza B viruses is not available. Although influenza A viruses typically cause more severe epidemics than influenza B viruses, influenza B viruses are still associated with annual outbreaks and contribute to morbidity (Hatakeyama et al., 2007) and mortality rates worldwide. Moreover, the dynamic changes in the prevalence of circulating lineages of B viruses as well as the drug and type/subtype specific nature of NAI-resistance emphasize the need for close and continued monitoring of resistance in influenza B viruses.

The goal of the present study was to develop a pyrosequencing assay for detection of signatory mutations previously associated with reduced susceptibility to NAIs in influenza B viruses.

2. Materials and methods

2.1. Viruses

Reference virus strains carrying mutations in the NA that confer resistance or reduced susceptibility to NAIs in the NA inhibition assays (Table 1), were used to validate the design of the primers and assays (Fig. 1). In addition, influenza B viruses isolated from 2001 to 2008 and belonging to VicHA–VicNA, YamHA–YamNA, or VicHA–YamNA lineages were tested (Fig. 2, Supplementary Figure 1). Six original clinical specimens containing influenza B viruses from either YamHA–YamNA or VicHA–YamNA lineages were also tested in the assay to assess sensitivity of the method (data not shown).

2.2. Primer design

NA sequences from over 1400 influenza B viruses available in public domain were aligned using BioEdit 7.0.9.0 software (Carlsbad, CA, USA). A consensus sequence was generated and used for primer design using the Pyrosequencing Assay Design software (Qiagen, Hilden, Germany).

2.3. RNA extraction and RT-PCR

Viral RNA was extracted from 100 μl of reference viruses and grown isolates from VicHA–VicNA, YamHA–YamNA, and VicHA–YamNA lineages using the MagNA pure LC (Roche, Indianapolis, IN, USA) as described elsewhere (Deyde et al., 2007). Viral RNA for clinical specimens was extracted using the MagNA pure Compact (Roche, Indianapolis, IN, USA). Reverse-transcription-PCR (RT-PCR) for cDNA synthesis and PCR for DNA amplification was done using a 96-well plate format with a one-step RT-PCR kit (Qiagen, Hilden, Germany) and 20 μM PCR amplification primers (Table 2) were used in a 50 μl volume containing 5 μl of total viral RNA for a total of 45 cycles. The PCR product (5 μl) was examined on 2% agarose E-gel (Invitrogen, Carlsbad, CA, USA) to confirm amplification of the appropriately sized fragment.

Table 2
Pyrosequencing (RT-PCR and sequencing) primers designed for detection of known markers of resistance or reduced susceptibility to NAIs in influenza B viruses.

RT-PCR primers	Sequence	Target region
BNA-F408	5′-AGGYCAACCAGGGGATACT-3′	BNA fragment 1
BNA-R848-biot	5′-CTRGCAAATCCGCATGTG-3′	
BNA-F962	5′-CTTATTTGGACACCCCCAGAC-3′	BNA fragment 2
BNA-1274-biot	5′-ATACAGGGGACATCRCATTT-3′	
BNA-F317	5′-CCAAAGGAAACTCAGCTCCC-3′	BNA fragment 3
BNA-F848-biot	5′-CTRGCAAATCCGCATGTG-3′	

Pyrosequencing primers	Sequence	NA target residue
BNA-F317	5′-CCAAAGGAAACTCAGCTCCC-3′	E119
BNA-F428	5′-ACAATGGAACAAGAGRAG-3′	R152
BNA-F563	5′-GGACATATATCGGAGTTG-3′	D198
BNA-F635	5′-ACACATACCATTCCTATGC-3′	I222
BNA-F719	5′-TAACTGATGGCYCAGCTTC-3′	S250
BNA-F787	5′-AAAGAAATATTTCCAACAGGAAG-3′	H274
BNA-F1099	5′-CGAACGATGTCTAAAACT-3′	R371
BNA-F1187	5′-TTAGYGGAGTAATGGTYTC-3′	G402

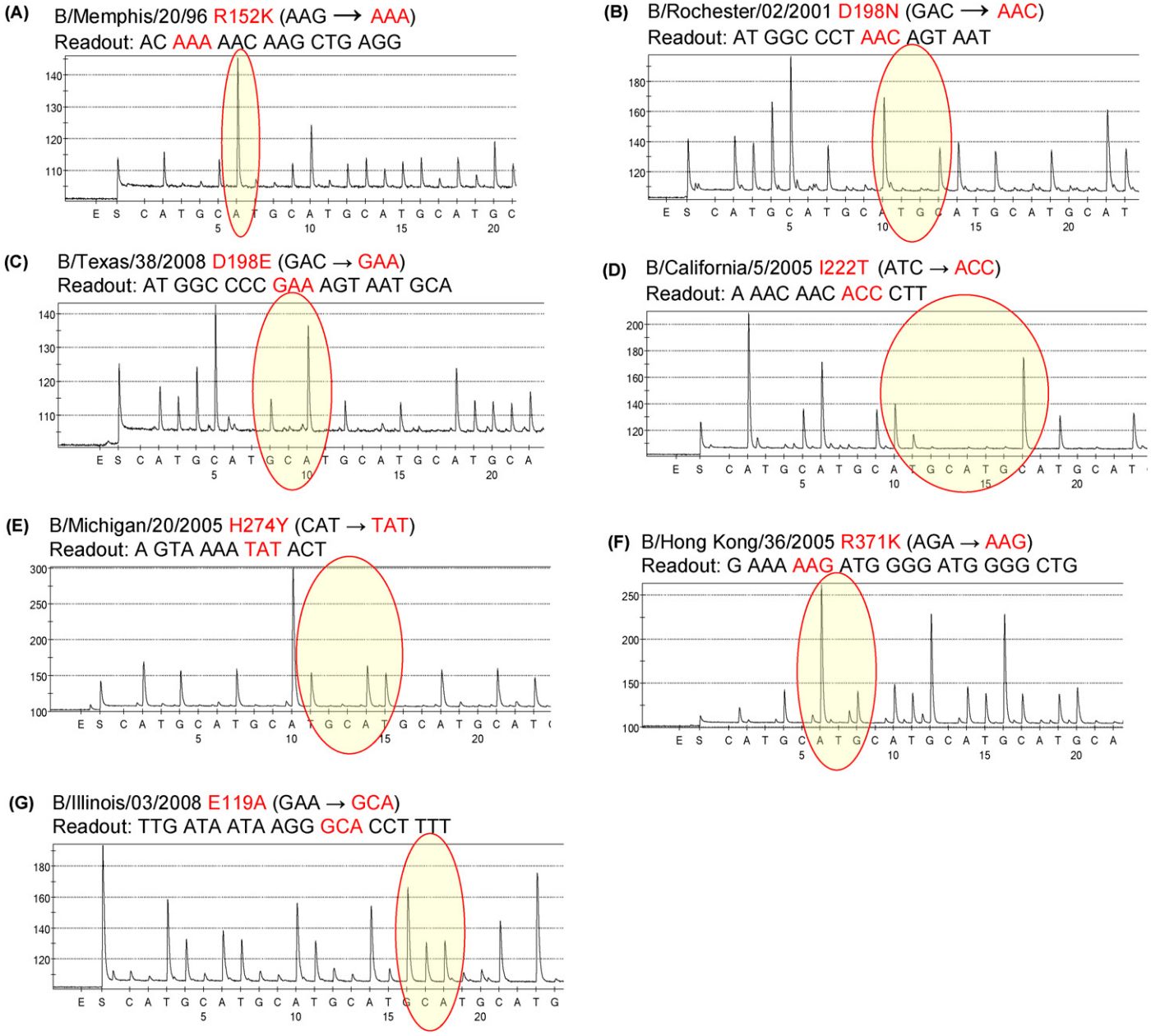

Fig. 1. Pyrograms and neuraminidase sequence readouts in the detection of molecular markers of drug resistance in influenza B viruses. Sequencing readouts and pyrograms are generated from the PyroMark ID and have been cropped to the regions of interest. Peaks corresponding to the mutations at the molecular markers of NAI-resistance are circled in red and highlighted in yellow. (*A*) The dually resistant viral isolate, B/Memphis/20/1996, shows presence of the change AAG to AAA (R → K) at residue 152. (*B*) B/Rochester/02/2001, isolated from a clinical specimen, with slightly reduced susceptibility to both NAIs, demonstrates the GAC to AAC (D → N) change at position 198. (*C*) B/Texas/38/2008, with slightly reduced susceptibility to zanamivir and oseltamivir, confirmed presence of the mutation GAC to GAA which corresponds to a D → E change at position 198. (*D*) The viral isolate B/California/05/2005, with slightly reduced susceptibility to oseltamivir, was used to detect the ATC to ACC (I → T) change in codon 222. (*E*) The pyrogram obtained from B/Michigan/20/2005, with mildly reduced susceptibility to oseltamivir, shows the CAT to TAT (H → Y) substitution at position 274. (*F*) The viral isolate B/Hong Kong/36/2005, with extremely reduced susceptibility to both NAIs, was used to detect the AGA to AAG (R → K) change at position 371. (*G*) The viral isolate B/Illinois/03/2008, with extremely reduced susceptibility to both zanamivir and oseltamivir, was used to analyze the sequence at residue 119. The pyrosequencing readout shows the mutation from GAA to GCA (E → A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.4. Pyrosequencing assay

The pyrosequencing assay was performed as previously described (Bright et al., 2006; Deyde et al., 2009a) essentially as described by the manufacturer's (Qiagen, Hilden, Germany) recommendations with the addition of the newly designed specific influenza B NA primers. Briefly, 20 μl of biotinylated PCR product was bound to 200 μg of streptavidin-coated beads (GE Biosciences, Uppsala, Sweden) by shaking at room temperature for 20 min. Single-stranded template was purified through a series of washes and hybridized with 100 μM of the appropriate B NA sequencing primer (Table 2). The pyrosequencing reaction was then performed using the PyroMark ID machine (Qiagen, Hilden, Germany).

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using the Genetic Algorithm for Rapid Likelihood Inference (GARLI 0.96b7) based on General Time Reversible (GTR) + I + γ4 substitution model (Zwickl, 2006). Phylogenetic trees were inferred using the maximum likelihood method in the GARLI 0.96b7 package and visualized in TreeView, version 1.6.6. Bootstrap values obtained from 1000 repli-

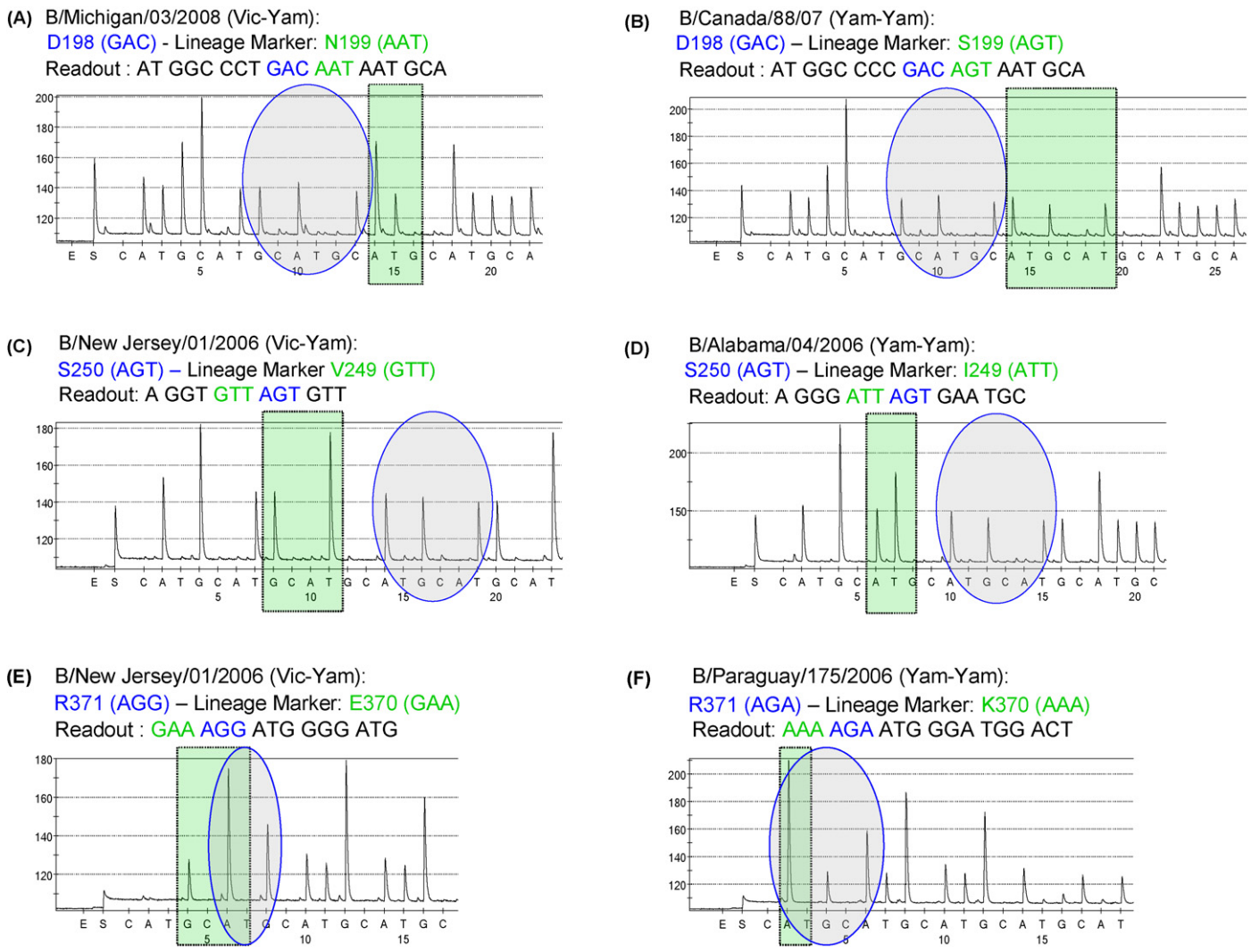

Fig. 2. Distinction between molecular markers of Victoria and Yamagata lineages as well as the analysis of NAI-resistance markers in B type viruses. Sequencing readouts and pyrograms are generated from the PyroMark ID and have been cropped to the regions of interest. The peaks that correspond to the wild type molecular markers of NAI-resistance are circled in blue and highlighted in grey. Lineage differences of between VicHA–YamNA and YamHA–YamNA lineages are boxed in green. (*A*, *B*) The sequences demonstrate the wild type GAC (D) at position 198 for two viruses: B/Michigan/03/2008 (VicHA–YamNA) and B/Canada/88/2007 (YamHA–YamNA). In addition to the NAI-resistance marker results, the sequence shows that the YamHA–YamNA virus contains AGT (S) at position 199 (panel A), while that from the VicHA–YamNA lineage have AAT (N) at the same position (panel B). (*C*, *D*) The sequences show the wild type AGT (S) at position 250 for two viruses B/New Jersey/01/2006 (VicHA–YamNA) and B/Alabama/04/2006 (YamHA–YamNA). Analysis of the sequence at codon 249 show a GTT (V) in the VicHA–YamNA virus (panel C) while the YamHA–YamNA contains an ATT (I) (panel D). (*E*, *F*) The sequences demonstrate the wild type arginine at position 371. Although sensitive viruses in all lineages contain an arginine at codon 371, at the nucleotide level they differ: VicHA–YamNA shows AGG (panel E) while the YamHA–YamNA lineage contains a AGA (panel F). Moreover, these lineages can be distinguished by amino acid change at position 370: GAA (E) for VicHA–YamNA (panel E) and AAA (K) for YamHA–YamNA viruses (panels F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cates are shown at major nodes. One hundred and seven B NA sequences (available in GenBank and/or other public domains) of viruses collected globally between 2000 and 2009 and are representative of the B lineages were analyzed using this method. The tree was outgrouped and rooted using B/Maryland/59 NA sequence.

3. Results

The NA sequences of over 1400 influenza B viruses isolated between 1940 and 2008 were analyzed. The consensus sequence derived from this alignment was used to design primers that allowed for the analysis of sequences at eight residues previously implicated in resistance or reduced susceptibility to NAIs based on the NA inhibition assay. Five of those residues, E119, R152, D198, I222, H274, and R371 (Ferraris and Lina, 2008), belong to the enzyme active site (Colman, 1989) whereas the two remaining residues, S250 and G402 (Hatakeyama et al., 2007), are located in the surrounding region. Three sets of PCR primers were designed (Table 2) to generate three amplicons that encompass all eight codons of interest.

Reference virus strains containing mutations at one of the five residues located in the NA active site (Table 1) were used to validate the design of the primers (Fig. 1). The designed sequencing primers for the pyrosequencing assay enabled detection of the following amino acid changes in the NA active site: R152K, D198N, D198E, I222T, H274Y and R371K (Fig. 1A–F, respectively) in the reference viruses (Table 1). Of note, the designed primers were able to detect the GAC to AAC (D → N) change at position 198 in three viruses that demonstrated slightly reduced susceptibility to both NAIs: one isolated from a clinical specimen (B/Rochester/02/2001) (Ison et al., 2006), another detected in routine seasonal surveillance (B/Guatemala/558/2008) (CDC unpublished data), and the last one as a community isolate (B/Canada/88/2007) (CDC unpublished data). The B/Hong Kong/36/2005 virus, which was reported

to exhibit extremely high IC_{50} values toward zanamivir and oseltamivir in the NA inhibition assay (Sheu et al., 2008), was used to detect the AGG to AAG change at residue 371, corresponding to a R → K amino acid change in the NA active site (Fig. 1F). To detect the amino acid change at 119, a specific forward PCR primer was designed to be used with the existing biotinylated reverse primer BNA-R848-biot (Table 2). The B/Illinois/03/2008 virus was used to confirm the presence of the GAA to GCA mutation that corresponds to the E → A change at position 119 (Fig. 1G). B/Illinois/03/2008 also demonstrated reduced susceptibility to both NAIs in the NA inhibition assay (CDC unpublished data).

A representative subset of influenza B viruses belonging to VicHA–VicNA, YamHA–YamNA, and VicHA–YamNA lineages were also tested to determine if the same primers could be used to detect mutations at these eight amino acid positions in the different lineages (Figs. 2A–F and 3, Supplementary Figure 1A–H), since variance among NA sequences of viruses from distinct genetic groups may necessitate a modification of primers.

Of particular note, the sequences generated by the primers used to analyze the three amino acid residues associated with drug resistance D198, S250, and R371 (Fig. 2A–F, indicated in blue) also included sequences of three residues S199N, I249V, and K370E (Fig. 2A–F, indicated in green) that distinguish the current VicHA–YamNA lineage. Therefore, these primers, designed to detect the molecular markers of NAI-resistance, can also differentiate between the NAs of viruses belonging to YamHA–YamNA or

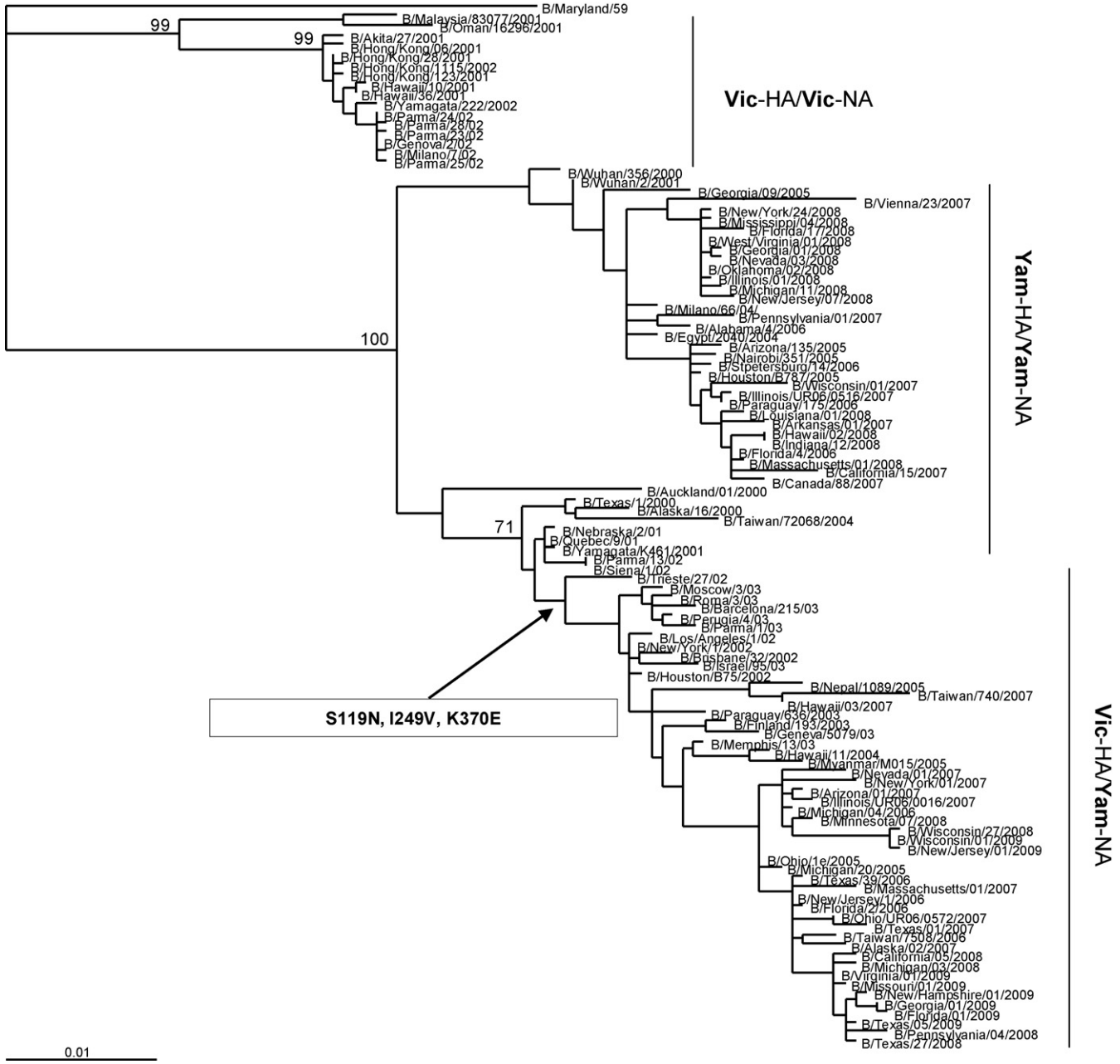

Fig. 3. Use of pyrosequencing for the detection of amino acid changes in the NA that discriminate between the Victoria and Yamagata lineages. This figure shows a phylogenetic tree generated using GARLI of one hundred and seven complete influenza B NA sequences (available in GenBank and/or other public domains) from viruses collected worldwide between 2000 and 2009. The ensure robustness of the analysis, 1000 replicates were analyzed and bootstrap values are shown at major nodes. The tree was rooted using B/Maryland/59 for clarity. Three amino acids (S199N, I249V, and K370E), shown in the tree, were detected in the regions analyzed by the pyrosequencing assay and are unique signature changes characteristic of B viruses from the VicHA and YamNA lineage.

VicHA–YamNA lineages (Fig. 3). The unique differences at position 199 between AAT (N) and AGT (S) between VicHA–YamNA and YamHA–YamNA lineages, respectively, were demonstrated as well as the wild type sequence GAC (D) at resistance marker 198 (Fig. 2A and B). The lineage marker at position 249 detected the GTT (V) for VicHA–YamNA and the ATT (I) for YamHA–YamNA while also including the wild type AGT (S) at position 250 for all lineages (Fig. 2C and D). This amino acid change at 250 was previously reported in B viruses recovered from oseltamivir-treated children (Hatakeyama et al., 2007). At the NAI-resistance marker 371, the wild type arginine was detected in all lineages; however, there was a difference at the nucleotide level between VicHA–YamNA and YamHA–YamNA (AGG → AGA). At amino acid position 370, VicHA–YamNA viruses showed GAA (E) while YamHA–YamNA viruses had AAA (K) (Fig. 2E and F).

Some variation was also observed between the VicHA–YamNA and historical YamHA–YamNA lineages based on the sequences generated using the primers for positions I222, H274 and G402. At position 222, the wild type ATC (I) was detected in both VicHA–YamNA and YamHA–YamNA lineages. The lineage marker at position 220 distinguishes between the AAC (N) in the VicHA–YamNA viruses and the AAA (K) in YamHA–YamNA viruses in most cases (Supplementary Figure 1A and B). However, many recent (2001–2007) YamHA–YamNA viruses also contained an asparagine at position 220. At position 274, there was a difference at the nucleotide level between viruses of different lineages (Supplementary Figure 1C–E), although all wild type viruses contain H274: viruses of the VicHA–YamNA lineage contained the wild type sequence CAC (Supplementary Figure 1C, blue) with CAT seen in viruses of either the VicHA–VicNA or the YamHA–YamNA lineage (Supplementary Figure 1D and E, blue). There was also a similarity at position 273 between VicHA–YamNA and the YamHA–YamNA lineages where the sequence AAA (K) was observed while viruses of the VicHA–VicNA lineage had GAG (E) at this position. Changes at amino acid position G402 (GGT) have been detected in B viruses recovered from oseltamivir-treated children (Hatakeyama et al., 2007). An additional difference, AAA (K) in VicHA–YamNA and GAA (E) for YamHA–YamNA, was seen at position 399 covered by the primer for the G402 region (Supplementary Figure 1F and G). A lysine at position 399 was observed in the VicHA–YamNA viruses tested in this study, which differs from the asparagine seen in VicHA–VicNA (data not shown) and YamHA–YamNA viruses, however, based on phylogenetic analysis both amino acids (K and N) are present among viruses belonging to the VicHA–YamNA lineage.

The primer used for analysis of sequence at position 152 generated the identical wild type sequence AGA (R) from viruses in each lineage: VicHA–YamNA (Supplementary Figure 1H), VicHA–VicNA and YamHA–YamNA (data not shown).

Six original clinical specimens collected in 2007–2008 were used to further validate the assay's design and to assess the sensitivity of the assay. Each clinical specimen was amplified with each set of primers and the correct wild type sequences were successfully detected for at the following positions: R152, D198, I222, S250, H274, R371 and G402 (data not shown).

4. Discussion

Following the successful application of pyrosequencing for the detection of adamantane resistance in influenza A viruses and because of its key role in the early detection of widespread resistance in 2005 (Bright et al., 2005), there has been an increased interest in applying this method to the detection of known and suspect molecular markers of NAI-resistance in seasonal influenza A(H1N1) and A(H3N2) as well as in highly pathogenic avian influenza A(H5N1) viruses (Bright et al., 2006; Deyde et al., 2009a,b, 2007; Duwe and Schweiger, 2008; Higgins et al., 2009; Lackenby et al., 2008a) and pandemic 2009 H1N1 viruses (http://www.who.int/csr/disease/swineflu/en/; Gubareva et al., 2009). The method proved readily adaptable and allowed for the timely detection of an unprecedented rise in oseltamivir-resistance among A(H1N1) viruses (with H274Y mutation) circulating globally (Dharan et al., 2009; Lackenby et al., 2008b).

Like influenza A viruses, influenza B viruses undergo continuous evolution, which allows them to evade human immune responses (e.g., changes in the surface antigens (HA and NA) and reassortment between co-circulating lineages). Furthermore, community isolates of influenza B viruses as well as viruses recovered from drug-treated patients that have displayed reduced NAI susceptibility in the NA inhibition assays have been reported (Gubareva et al., 1998; Hatakeyama et al., 2007; Hurt et al., 2004; Ison et al., 2006; Sheu et al., 2008). NAI-resistant viruses have also been generated in vitro by selection in the presence of drugs (Baum et al., 2003) or using reverse genetics. In the present study, we applied the pyrosequencing method to the analysis of influenza B NA sequences at residues potentially involved in resistance or susceptibility reduction to NAIs.

The amino acids E119, R152, H274, and R371 are associated with resistance to NAIs due to their positioning in the NA active site. The E119A mutation was detected in the B/Illinois/03/2008 virus that demonstrated reduced susceptibility to both oseltamivir and zanamivir in the chemiluminescent NA inhibition assay (CDC unpublished data). It is noteworthy that the RT-PCR and sequencing primers, used to analyze the sequences at the 119 residue, were not suitable for some strains tested in the present study due to substantial NA sequence variation resulting in primer mismatch within the targeted region. Efforts are underway to optimize the assay to detect changes at this position in all B viruses. It is also important to note that the oldest virus tested in this study was from 1996 (B/Memphis/20/96 with the R152K mutation). Additionally, only a representative sample of recent viruses of VicHA–VicNA, YamHA–YamNA, and VicHA–YamNA lineages circulating during 2001–2008 were tested in this study.

There is substantial variation in NA sequences due to the accumulation of point mutations and reassortment events between distinct, co-circulating lineages of influenza B viruses. Such variance in the NA sequences poses challenges in the design of universal primers for amplification and sequence analysis by pyrosequencing and may require frequent updates of the pyrosequencing primers to reflect evolutionary changes in the NA. Unlike the case of adamantane resistance, where the molecular markers are well established (Boivin et al., 2002; Hay et al., 1986; Klimov et al., 1995), analysis of NAI-resistance is rendered more complex due to the drug- as well as the NA type/subtype-specific nature of the resistance-conferring changes. It should be noted that the design of NAIs was based mainly on the crystal structures of A/N2 and A/N9 subtypes that differ from that of type B enzyme; wild type influenza B viruses appear to be less susceptible to NAIs compared to influenza A(H1N1) and A(H3N2) viruses (Kawai et al., 2007).

Due to the frequency at which viral quasispecies arise in patients following drug treatment (Gubareva et al., 1998; Ison et al., 2006), it was important to demonstrate that the designed assay is sufficiently sensitive to permit analyses of the sites associated with molecular markers of resistance directly in clinical specimens. While the clinical relevance of some of these mutations in the B NA remains uncertain, the development of this pyrosequencing assay provides another tool for the rapid detection and ability to analyze changes at these positions.

The assay allowed for the analysis of the markers defining viruses belonging to distinct influenza B lineages. This could be useful for screening purposes to identify potential HA and NA reassortment between co-circulating lineages. To our knowledge, this

is the first report of an application of the pyrosequencing approach to study influenza B virus genome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.10.022.

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