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Rapid detection of new B/Victoria-lineage haemagglutinin variants of influenza B viruses by pyrosequencing

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ABSTRACT

During 2016/2017, several antigenically and genetically distinct variant viruses of the influenza B/Victoria/2/87-lineage (B/Vic) viruses, which have either deletions or mutations in the haemagglutinin (HA) emerged and co-circulated with other influenza B viruses from both the B/Vic and B/Yamagata/16/88-lineages (B/Yam). In this study we developed a pyrosequencing assay that can detect and differentiate multiple influenza B virus variants currently in circulation. The assay targets a region of HA sequence that is unique for each of the B/Yam, B/Vic and B/Vic variant viruses. Our results demonstrated that it is a rapid, robust, high-throughput assay, highly sensitive and specific in differentiating among the B/Yam, B/Vic and B/Vic variant viruses, giving it an advantage over an existing rRT-PCR method. It works well for influenza virus isolates as well as original clinical respiratory specimens, and can therefore be used to provide important information for surveillance by closely monitoring the spread of these B/Vic variants.

1. Introduction

Each year, influenza viruses cause annual epidemics and while influenza A viruses often predominate, influenza B viruses make up a significant proportion of cases, on average around 22–24% (Ambrose and Levin, 2012; Barr and Jelley, 2012). Influenza B viruses often predominate for part of the season and occasionally are the predominant virus throughout the season, as was seen in Australia in 2015 (Barr et al., 2016). Influenza A and B viruses are both prone to antigenic drift due to their high mutation rates although influenza B viruses evolve more slowly than influenza A viruses (Vijaykrishna et al., 2015). Since the 1980s, 2 distinct lineages of the influenza B viruses have circulated, the B/Yamagata/16/88-like (B/Yam) and B/Victoria/2/87-like (B/Vic) viruses (Rota et al., 1990). Vaccination against influenza is still the best way to protect the population against infection and this can be achieved using either trivalent vaccines (containing 2 influenza A viruses – one A (H1N1)pdm09, one A(H3N2) virus and one influenza B lineage virus) or quadrivalent vaccines which contain both of the influenza B lineages. As there are clear antigenic and genetic differences between the lineages, vaccination with the trivalent vaccine may offer limited protection against the lineage that was not part of the vaccine. The major influenza B lineage circulating can vary between regions and from year to year, resulting in changes in influenza B vaccine strains over time. For example, from 2013 to 2015, the southern hemisphere vaccine included B/Yam strains, but in 2016, this was changed to a B/Vic strain (specifically B/Brisbane/60/2008) to reflect the change in the lineage of circulating viruses (World Health Organization (WHO), 2018a). It is important to identify the most prevalent B lineage virus through surveillance for inclusion in the trivalent vaccines, especially for children who may not have been exposed to that lineage of influenza B (Ambrose and Levin, 2012). Within one lineage, subgroups of low reacting viruses with poor reactivity to the ferret antisera raised against the vaccine strain may emerge, making the selection of the most appropriate virus within each lineage for inclusion in the influenza vaccines an important undertaking for the influenza surveillance network.

During the 2016/2017 season, the U.S. Centers for Disease Control and Prevention (CDC) reported the emergence of B/Vic lineage viruses with 2 amino acid deletions (at positions 162 and 163, corresponding to deletions at nucleotide positions 531–536) in the HA (Blanton et al., 2017). This variant has since been detected in several countries in America and Europe. Later, another group of B/Vic lineage with 3 amino acid deletions at positions 162–164 (nucleotide positions 531–539) also emerged in China (World Health Organization (WHO), 2018b). These 2 groups of HA deletion variant viruses are referred to as the B/Vic deletion variant subgroup B/Vic 2-Del and B/Vic 3-Del, respectively. In addition, other low reacting B/Vic variants have also emerged, for example viruses with K165 N and T221I mutations have

Abbreviations: HA, haemagglutinin; RT-PCR, reverse transcriptase polymerase chain reaction; rRT-PCR, real-time RT-PCR; MDCK, Madin-Darby canine kidney.

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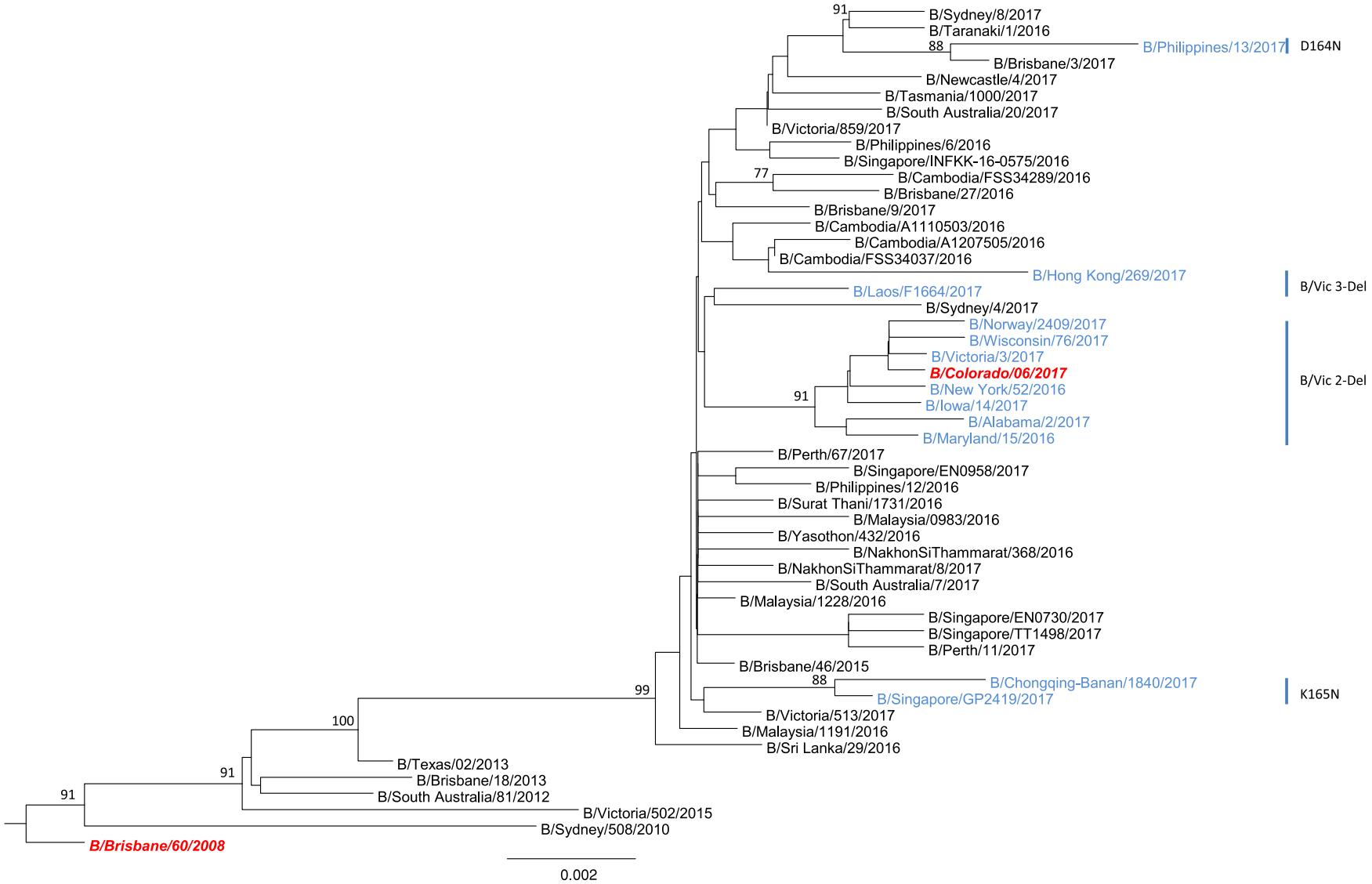

Fig. 1. Phylogenetic tree for the full HA gene of B/Vic-lineage viruses. The phylogenetic tree was created using Neighbor-joining method based on HKY Model in Geneious 9.0.4. Bootstrap value (n = 1000) of 70 or greater on the main branches are shown on the branches. B/Brisbane/60/2008 is the 2018 WHO southern hemisphere recommended vaccine strain for the quadrivalent vaccine; B/Colorado/06/2017 is the 2018–2019 WHO northern and 2019 southern hemisphere recommended vaccine strain for both trivalent and quadrivalent vaccines. Variant viruses that have a deletion or mutation in the HA and are low reactors to the ferret antisera against B/Brisbane/60/2008 are colored in blue.

been detected in China and Singapore since November 2017, and another variant with a D164N mutation was found in the Philippines. The positions and sequences of the affected nucleotides and amino acids of these B/Vic variants are summarized in Table 1. These B/Vic variant viruses are antigenically distinct, demonstrated by their poor response to ferret post-infection antisera against B/Brisbane/60/2008-like viruses (B/Vic lineage vaccine strain since 2010) as shown in Supplementary Table 1. The B/Vic 2-Del became the predominant B/Vic lineage viruses detected in the Americas and Europe in 2017/2018 influenza season, while B/Vic 3-Del and other variants were detected in Asia and Africa at low levels. The B/Vic component for both the trivalent and quadrivalent vaccine of the 2018/2019 northern and 2019 southern hemisphere influenza vaccines was changed to include a B/Vic 2-Del virus (B/Colorado/06/2017-like) (Fig. 1).

Rapid detection assays are widely used for influenza virus identification as they are relatively quick and easy to perform. They include molecular-based assays such as real-time RT-PCR (rRT-PCR) or immunoassays-based rapid influenza diagnostic tests (RIDTs), most of which can distinguish between influenza A and B, with a few also able to discriminate between influenza A subtypes (Centers for Disease Control and Prevention (CDC), 2016, 2018) or influenza B lineages (Biere et al., 2010; Wong et al., 2014). The rRT-PCR assay currently used by many National Influenza Centres in the WHO GISRS (Global Influenza Surveillance and Response System) is the rRT-PCR assay supplied by US CDC, which uses 3 separate rRT-PCR assays, one for influenza B detection and the other 2 for B/Yam and B/Vic lineage differentiation respectively. Although the new B/Vic 2-Del and B/Vic 3-Del viruses are still identified as B/Vic lineage with this assay, it does not specifically identify the 2 deletion variants, unless more primers/probes are used. This modification will require at least 2 additional reactions. Here we report a high-throughput pyrosequencing assay modified from our previously published method (Deng et al., 2013). The assay covers the important antigenic region of the HA from position 162–165, which can now distinguish the B/Yam, B/Vic, B/Vic 2-Del, B/Vic 3-Del, and other low reacting B/Vic viruses with mutations in this region of the HA, such as K165 N and D164N.

The objective of this study was to establish a rapid, robust, high-throughput pyrosequencing assay that detects signature sequences in the crucial HA antigenic region, that can be used to differentiate among circulating influenza B viruses: B/Yam, B/Vic and B/Vic variants, with similar or higher sensitivity and specificity as the rRT-PCR assay, and is suitable for analysis of both original clinical specimens and virus isolates.

2. Materials and methods

2.1. Viruses

Samples containing influenza B viruses of known lineage, including original clinical respiratory specimens and MDCK-derived virus isolates, were received as part of the influenza surveillance program conducted at the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne. These samples were either isolated or re-isolated in MDCK cells or embryonated hens' eggs, and viral growth determined by cytopathic effect (CPE) and haemagglutination activity using turkey red blood cells (RBCs) (World Health Organization (WHO), 2011).

2.2. RNA extraction

RNA extraction was performed using 140ul sample volume with the QIAamp Viral RNA kit (QIAGEN) according to the manufacturer's protocol, or 200ul sample when using the QIAxtractor (QIAGEN) for automated extraction as per manufacturer's protocol.

2.3. RT-PCR for influenza B HA gene

RT-PCR using the MyTaq One Step RT-PCR kit (Bioline) with primers specific for the HA gene of influenza B (forward primer: 5′-GATTTTTCGCAACAATGGCTTG-3′, biotinylated reverse primer: 5′Biotin-TTCCCCTTCTGTACAAATGTATGGT-3′) (Geneworks). Cycling conditions were: 45 °C for 40 min, 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 1 min, with a final hold of 72 °C for 2 min.

2.4. Pyrosequencing

Biotinylated PCR amplicons were processed using the PyroMark Q96 ID (QIAGEN) system according to manufacturer's protocol. Briefly, 20ul biotinylated amplicons were bound to streptavidin-coated Sepharose beads in binding buffer, put through 70% ethanol, denaturation solution and wash buffer to generate single stranded DNA (ssDNA), then transferred to 40ul annealing buffer containing 0.4uM pyrosequencing primer (5′-CTTGGGCTGTCCCRAA-3′) (Bioneer) using the following nucleotide dispensation order: TATACGTACACTA.

2.5. Real-time RT-PCR

The CDC Influenza B Lineage Genotyping Panel (RUO) (Catalog No. FluRUO-05), FR-1209 (available from: www.internationalreagentresource.org) was used for real-time RT-PCR with SensiFAST Probe Lo-ROX One-Step kit (Bioline) on a 7500 Real-Time PCR system (Applied Biosystems). Cycling conditions were: 45 °C for 10 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec.

3. Results

3.1. B-HA pyrosequencing assay validation

The B-HA pyrosequencing assay we previously published was designed to target B/Yam and B/Vic lineages only (Deng et al., 2013). In order to enable the detection of the newly emerged B/Vic deletion variants and other low reacting B/Vic variants with mutations in this HA region, we further modified the primer sequences and the pyrogram. To validate the new B-HA pyrosequencing assay, a selection of influenza B samples with known lineage (virus isolates collected during 2013–2017) confirmed either by real-time RT-PCR

Table 1
Differences in nucleotide and amino acid sequences for B/Vic variant viruses and B/Yam compared to current B/Vic lineage viruses.

Influenza B lineages	Variants	Differences to B/Vic lineage HA sequence			
		Nucleotide (from start codon ATG)		Amino acid	
		Position	Sequence*	Position	Sequence*
B/Vic		531-549	AAACGACAAAAACAAAACA	162–168	KNDKNKT
	B/Vic 2-Del	531–537	- - - - - - CAAAAACAAAACA	162–163	- - DKNKT
	B/Vic 3-Del	531–539	- - - - - - - - - AAACAAAACA	162–164	- - - KNKT
	B/Vic D164N	535	AAAC**A**ACAAAAACAAAACA	164	KN**N**KNKT
	B/Vic K165 N	540	AAACGACAA**T**AACAAAACA	165	KND**N**NKT
B/Yam		531–533	- - - CGACAAAAACAAAACA	162	- NDKNKT

* nucleotide/amino acid deletions; nucleotide/amino acid substitutions are in bold font.

or HA gene sequencing (data not shown) were tested. The group comprised of 65 B/Yam, 73 B/Vic, as well as 11 B/Vic 2-Del variants and 2 B/Vic 3-Del variant viruses (B/Laos/F1664/2017 and B/Hong Kong/269/2017). In addition, 3 other low reacting B/Vic variants with mutations D164N (B/Philippines/13/2017) and K165 N (B/Singapore/GP2419/2017 and B/Chongqing—Banan/1840/2017) were also included. These low reacting B/Vic variant viruses formed a distinct subgroup in the phylogenetic tree based on HA sequence (Fig. 1). Pyrosequencing clearly differentiated these 6 groups of influenza B viruses (Fig. 2).

3.2. Sensitivity comparison to real-time RT-PCR

Sensitivity of the B-HA pyrosequencing assay and the CDC real-time RT-PCR (rRT-PCR) assay was compared by using known copy numbers of HA RNA from one representative virus of the B/Yam lineage (B/Brisbane/9/2008), B/Vic lineage (B/Brisbane/60/2008) and the B/Vic deletion variants (2-Del: B/Maryland/15/2016, 3Del: B/Laos/F1664/2017). RNA copy numbers ranging from 10^{10} copies serially diluted 10-fold down to 1 copy were used as the starting material for both assays. Sensitivity of the pyrosequencing assay was determined to be

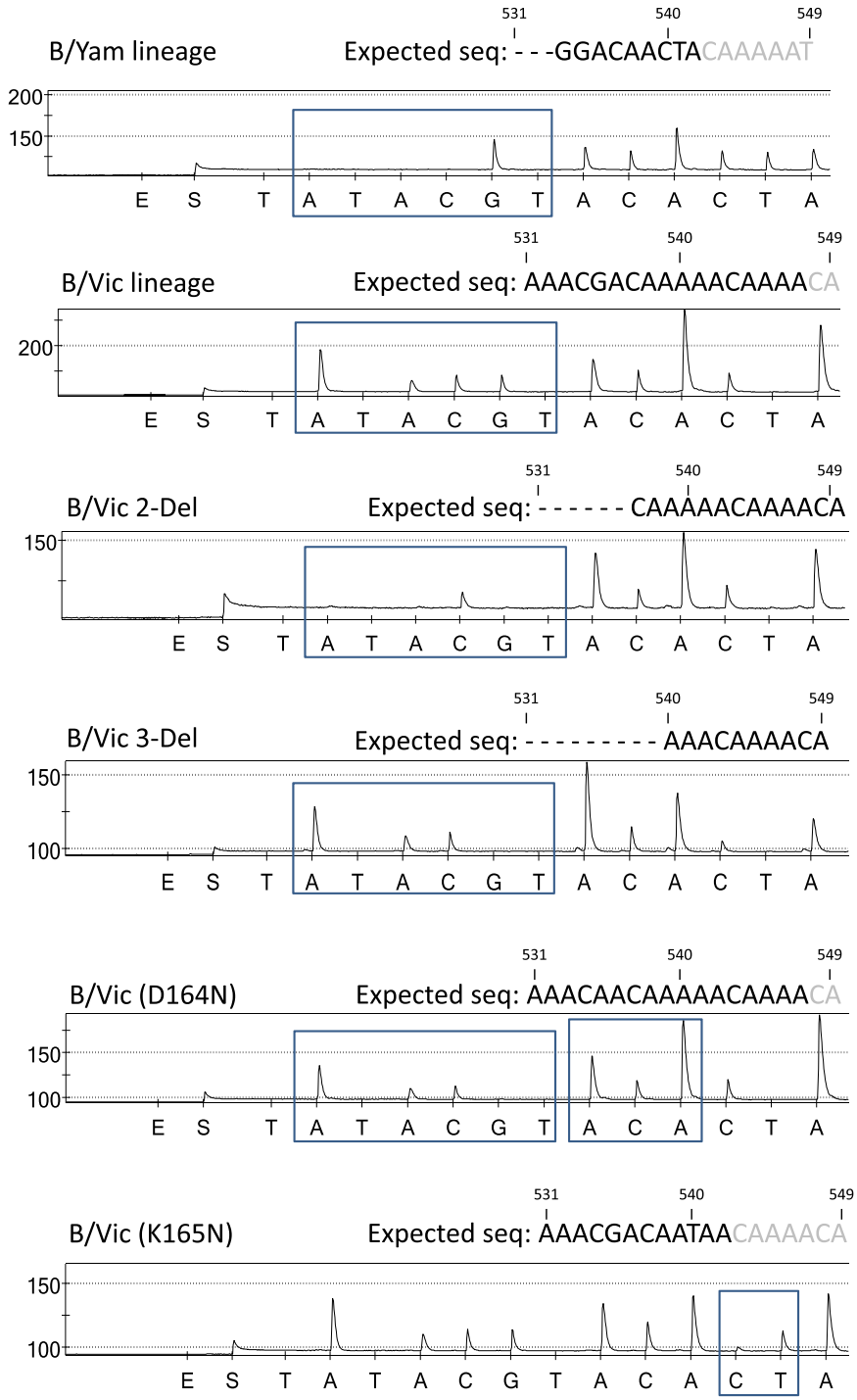

Fig. 2. Pyrosequencing results of B-HA on representatives from different groups of the B/Yam and B/Vic lineage viruses. The regions that clearly differentiate various B-HA groups are outlined. Using B/Vic sequence as a reference, B/Yam has a deletion of AAA at position 531–533, B/Vic 2-Del has a deletion of AAACGA at position 531–537, and B/Vic 3-Del has a deletion of AAACGACAA at position 531–539. The B/Vic variants with either the D164N or K165 N mutation has a single base change, from G to A at position 535, or A to T at position 540, respectively.

100 copies of RNA for B/Yam, 10 copies for B/Vic, 1000 copies for B/Vic 2-Del. In comparison, the sensitivity for the CDC rRT-PCR assay was 1000 copies of RNA for B/Yam, 100 copies for B/Vic, 1000 copies for B/Vic 2-Del (Fig. 3). B/Vic 3-Del gave similar results as B/Vic 2-Del (data not shown). In all cases, the sensitivity of B-HA pyrosequencing is either similar or greater than the CDC rRT-PCR assay.

3.3. Performance of B-HA pyrosequencing assay on original clinical specimens

To test if the B-HA pyrosequencing assay could be used on original clinical specimens with similar sensitivity and specificity, 105 influenza B positive original clinical specimens received by the Centre from late

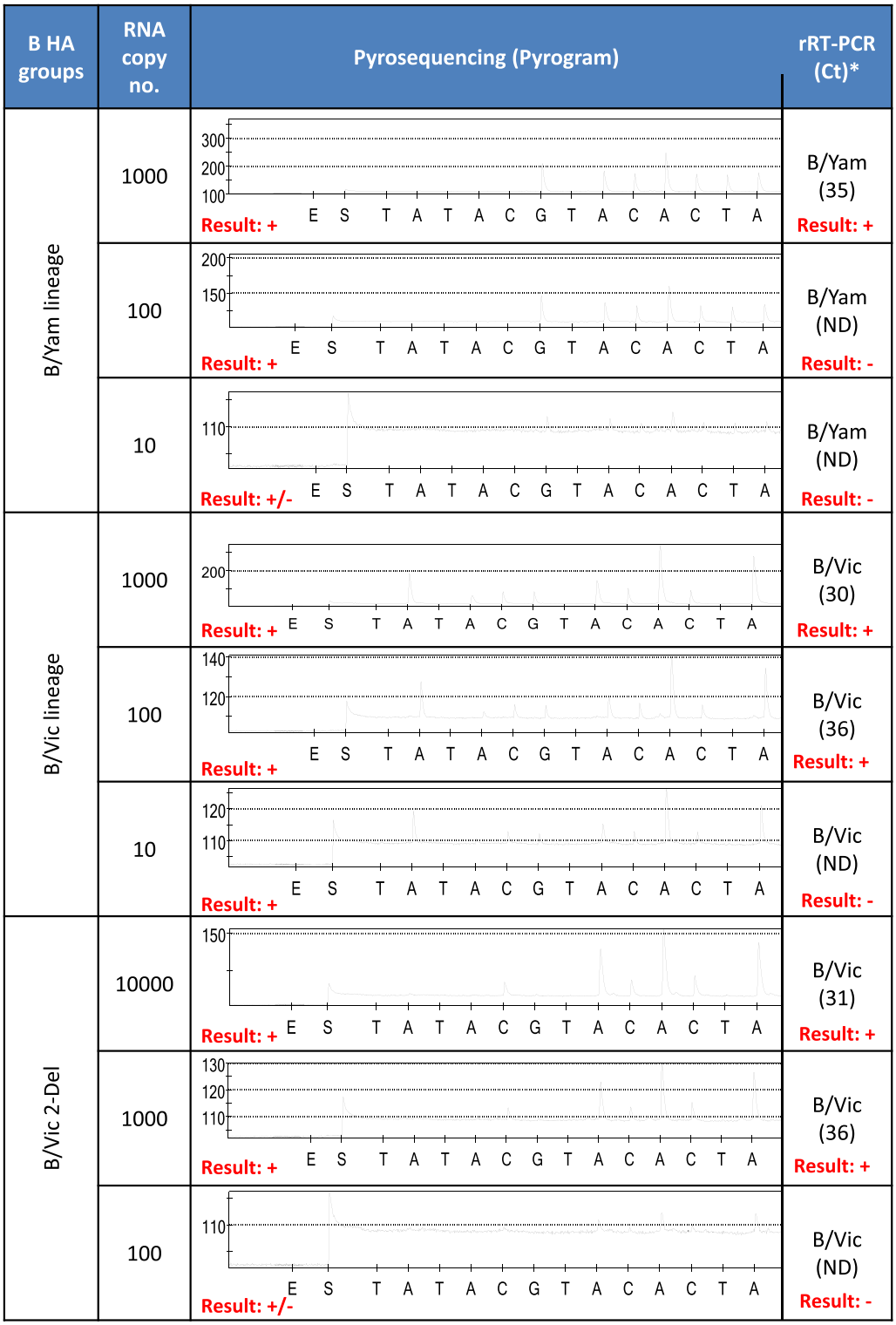

Fig. 3. Comparison of detection limit of B-HA pyrosequencing and the CDC rRT-PCR assay. Results are indicated as positives (+) for pyrograms that have clear peaks and rRT-PCR with Ct ≤ 36. Borderline (+/−) results are given for pyrograms with high background. Pyrograms with no peaks and rRT-PCR with non-detectable Ct values are called as negatives (−). Based on positive results, the detection limit of pyrosequencing is 100 copies for B/Yam, 10 copies for B/Vic, 1000 copies for B/Vic 2-Del (similar for 3-Del, data not shown). In comparison, the CDC rRT-PCR assay is similar or 10 fold less sensitive, depending on the influenza B lineage/variant tested.

Table 2
Comparison of CDC rRT-PCR and B-HA pyrosequencing assays using original clinical respiratory specimens.

Virus designation	rRT-PCR result (Ct)*		Pyrosequencing result
	B/Yam	B/Vic	
B/VICTORIA/511/2017	31	ND	B/Yam
B/NEWCASTLE/5/2017	29	ND	B/Yam
B/FIJI/9/2017	30	ND	B/Yam
B/SOUTH AUSTRALIA/13/2017	ND	29	B/Vic
B/VICTORIA/6/2017	ND	27	B/Vic
B/PERTH/3/2017	ND	29	B/Vic
B/VICTORIA/3/2017	ND	28	B/Vic 2-Del
B/SOUTH CAROLINA/5/2017	ND	30	B/Vic 2-Del
B/MARYLAND/14/2017	ND	23	B/Vic 2-Del
B/LOUISIANA/15/2017	ND	23	B/Vic 2-Del
B/MAINE/14/2017	ND	19	B/Vic 2-Del
B/NEW JERSEY/24/2017	ND	24	B/Vic 2-Del
B/ARIZONA/20/2017	ND	26	B/Vic 2-Del
B/SOUTH AUSTRALIA/9/2017	ND	ND	B/Vic
B/SOUTH AUSTRALIA/14/2017	ND	ND	B/Vic
B/SYDNEY/1008/2017	ND	ND	B/Vic

* Ct: cycle threshold; ND: not detected.

2016 to June 2017 from various geographic regions were tested. Supplementary Table 2 shows that all original specimens were successfully grouped into their respective B-HA groups using the new pyrosequencing assay. Of the 105 original specimens, there were 82 B/Yam, 16 B/Vic and 7 B/Vic 2-Del. The assay also identified the first B/Vic 2-Del virus in Australia, B/Victoria/3/2017, collected on 5th June 2017 in Melbourne; however, no further B/Vic deletion variant viruses were detected since. These original specimens were further tested by the CDC rRT-PCR methods to compare sensitivity and specificity (Table 2 shows a selection of the virus tested). Consistent results were obtained from all 82 B/Yam and 13 of the 16 B/Vic specimens, with Ct values from 17 to 33. In addition, the 7 B/Vic 2-Del were also confirmed as B/Vic lineage viruses. Pyrosequencing was found to be more informative, with the capability of identifying B/Vic deletion variant viruses, while the rRT-PCR assay that was used at the time only identified them as B/Vic. Pyrosequencing also had greater sensitivity as viruses could still be detected even when they were not detectable by rRT-PCR.

4. Discussion

Influenza B viruses are notorious for using an insertion–deletion strategy in the HA and NA gene segments during evolution. The divergence of the 2 B lineages (B/Vic and B/Yam) include amino acid insertion/deletion of the HA protein, among other mutations, relative to early influenza B viruses such as B/Lee/1940 (McCullers et al., 1999). Since 2016/2017, 2 B/Vic variants were derived from further deletions of 2 or 3 amino acids respectively, which were antigenically distinct from the circulating B/Brisbane/60/2008-like (B/Vic) viruses. Real-time RT-PCR (rRT-PCR)-based methods like the current singleplex CDC kit (CDC Influenza B Lineage Genotyping Panel) and other rRT-PCR multiplex tests (Biere et al., 2010; Wong et al., 2014) provide the ability to distinguish between B/Vic and B/Yam lineage viruses. Just recently, the CDC released a new set of rRT-PCR kit (CDC Influenza B/Victoria Lineage Deletion Panel (RUO) (Catalog No. FluRUO-10), FR-1597) (available from: www.internationalreagentresource.org) that should be able to identify the B/Vic 2-Del variants but this assay still does not cover the B/Vic 3-Del or other variants. The emergence of 2 additional B/Vic deletion variants made it technically challenging to develop a rRT-PCR method capable of differentiating all 4 possible B viruses because individual specific probes would be needed for each group. Additionally, while multiplex rRT-PCR can save reagents, reagents have to be optimized to minimize the interaction of different probes and primers and to eliminate false positive results. Further, all probes need to be labeled with suitable fluorophore dyes that will not interfere with each other. On the other hand, singleplex assays while easier to design, are more costly as they require the setup of multiple reactions and which may be difficult for clinical specimens with limited volumes.

In this study, we describe the use of a pyrosequencing assay that targets the influenza B HA to distinguish not only B/Yam and B/Vic, but also the B/Vic deletion variants (2-Del, 3-Del) and other potentially important emerging B/Vic variants, such as those with the D164N or K165 N mutation. Unlike rRT-PCR, only one set of PCR primers and one sequencing primer are required in the assay to generate distinctive signature sequences for each group. A range of respiratory sample types were successfully tested, as well as virus isolates obtained from propagation in MDCK cells or embryonated eggs. The assay was tested on 105 original clinical specimens and 154 virus isolates, and was 100% specific in distinguishing the different groups of human influenza B viruses that have circulated from 2013 to 2017.

The B-HA pyrosequencing assay described here offers 3 major advantages over the rRT-PCR assay. First, the assay has a detection limit of 100 copies of RNA for B/Yam, 10 copies for B/Vic, 1000 copies for B/Vic deletion variants, which is similar or more sensitive than the CDC rRT-PCR assay. The difference in sensitivity between the B lineages may be due to the slight sequence differences in the primer binding regions of the different B viruses, which can affect PCR sensitivity even when using the same primer set and PCR condition. As for differences between the pyrosequencing and rRT-PCR assays, these could be explained by the use of different primer sequences, PCR conditions and method of detection. The higher sensitivity of pyrosequencing in identifying B/Vic lineage viruses (Fig. 3) was further reflected in the testing of original specimens, where 3 of the original specimens were clearly identified as B/Vic by pyrosequencing, but failed to be detected by the rRT-PCR assay. Second, the assay has the ability to distinguish B/Vic deletion variants from the major B/Vic lineage viruses. Third, the assay is able to provide sequence information around the deletion site if further deletions occur at this site, and it can be adapted easily to other emerging variants such as B/Vic mutation variants, D164N and K165 N.

A possible limitation of the pyrosequencing assay is that it can be difficult to identify homopolymers accurately, whereby a sequence with the same nucleotide repeated consecutively tend to result in pyrogram peaks that does not reflect the correct number of nucleotides incorporated. Homopolymers can also cause a particular nucleotide to be exhausted in an initial dispensation reaction, which may cause a non-specific peak in later dispensations; however this can be solved by programming consecutive dispensations of same nucleotide. Despite these limitations, it is still obvious that we can use pyrogram patterns to differentiate all major variants of B/Vic and B/Yam lineage viruses based on the HA of this region with the aid of known reference sequences if required.

Although influenza B lineages can also be monitored for antigenic differences using ferret antisera generated to the different B lineages, this requires virus isolation which is often time-consuming, and does not necessarily distinguish deletion variants from other low reacting viruses (e.g. D164N and K165 N mutant viruses). Identification of these B/Vic deletion variants is also possible using next generation sequencing (NGS) and conventional Sanger sequencing. However, these methods are also time-consuming and costly, and are less sensitive compared to pyrosequencing. As new subgroups/variants of influenza viruses often emerge and co-circulate with the original lineage viruses, it is important to be able to determine the prevalence of new variants quickly, especially to aid in vaccine virus selection. Our study has shown that the B-HA pyrosequencing assay can rapidly differentiate between the currently circulating influenza B viruses and can be used for original clinical specimens and virus isolates. Therefore, this assay will be very useful for influenza virus surveillance in laboratories equipped with a pyrosequencer in the coming influenza seasons.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2018.11.003.

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Conflict of interest

None.

Ethical approval

Not required.

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